Pergamon

Tetrahedron, Vol. 52, No. 4, pp. 1165-1176, 1996

0040-4020/96 $15.00 + 0.00

0040-4020(95)00974-4

Dichotomins A - E, New Cyclic Peptides from *Stellaria dichotoma* L. var. *lanceolata* Bge. [1)]

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Abstract : New cyclic penta or hexapeptides, dichotomins A - E [A: *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Val-); B: *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Thr-); C: *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Ala-); D: *cyclo*(-Gly-Val-Gly-Phe-Tyr-Ile-); E: *cyclo*(-Gly-Tyr-Ala-Phe-Ala-)] , have been isolated from the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge. Their structures were elucidated on the basis of extensive 2D NMR, chemical degradation and X-ray crystallographic analysis.

Cyclic peptides are frequently encountered natural products exhibiting a wide variety of biological activities. Despite their importance, surprisingly few studies of higher plants occurring cyclic peptides exist in the literature.[2)] As a part of our continuing studies in search of new bioactive cyclic peptides from higher plants,[3)] we have already isolated a novel cyclic peptide, named dichotomin A, showing cell growth inhibitory activity, from the roots of *Stellaria dichotoma* L. var. *lanceolata* Bge. (Caryophyllaceae),[4)] which was used as folk medicines for antifebrile and so on. Continuous fractionation effort of the methanolic extract led to a several peptidic compounds, named dichotomins B - E. Here, we report a full account of the structure elucidation of dichotomins A - E (**1** - **5**) by extensive 2D NMR, chemical degradation and X-ray analysis. The cell growth and cyclooxygenase inhibitory activities were also discussed by using the structures and conformations of dichotomins, analyzed by X-ray and extensive NMR methods.

The methanolic 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), and 80% MeOH eluted fractions were chromatographed on a silica gel column, followed by HPLC on ODS to yield five peptidic compounds, named dichotomins A - E (**1**: 0.007 %, **2**: 0.0004 %, **3**: 0.003 %, **4**: 0.0012 %, **5**: 0.0002 %).

Dichotomins A -D were cyclic hexapeptides with the very similar amino acids and sequencing as follows. Dichotomin A (**1**), colorless needles, mp. 179 - 180 °C, $[\alpha]_D$ +14.0° (c 0.10, MeOH),

showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m/z* 681.3652 (MH^+, Δ -4.0 mmu), corresponding to molecular formula, $C_{35}H_{48}N_6O_8$. The IR absorptions at 3309 and 1649 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The hexapeptide nature of **1** was evident from its ^1H and ^{13}C NMR spectra, showing six amide NH and six amide carbonyl groups, as shown in Table 3. Further, the relatively high intensity of the molecular ion (base peak) and the lack of terminal amino group protons in the ^1H NMR suggested that **1** might be cyclic peptide. In order to elucidate the amino acid composition, **1** was subjected to complete hydrolysis with 6N HCl by heating at 110 °C for 24 h in a sealed tube. The hydrolysate was then analyzed by HPLC and the amino acid composition was shown to be one glycine (Gly), one threonine (Thr), one valine (Val), one leucine (Leu), one phenylalanine (Phe) and one tyrosine (Tyr) per molecule of **1**. These six amino acid units accounted for the observed mass molecular weight and 15 degrees of unsaturation. The absolute stereochemistry of the component amino acids in **1** was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,[5] followed by HPLC analysis.

The NMR spectra of **1** were taken at 500 MHz in pyridine-d_5 which gave the best dispersion for analysis. The ^1H NMR spectrum showed five doublet methyl signals (δ 0.78, 0.78, 1.09, 1.13 and 1.49) ascribable to Leu, Val and Thr residues. The ^1H-^1H COSY spectrum allowed the coupling sequence of each amino acid resonance and the corresponding carbon resonances were elucidated on the basis of HMQC spectra[6] as shown in Table 3. The gross structure including the sequence of the amino acids for **1** was assembled by connecting the individual amino acids on the basis of connectivities observed in the HMBC experiment (Fig. 1).[7] From the HMBC experimental results, the sequence was deduced to be *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Val-). As the amide carbonyl signals of Phe^3 and Val^6 showed the same chemical shift at δ 172.72, the deduced structure of dichotomin A (**1**) was confirmed by the NOE correlations (Fig. 1) in ROESY spectrum[8] and in good agreement with the above result.

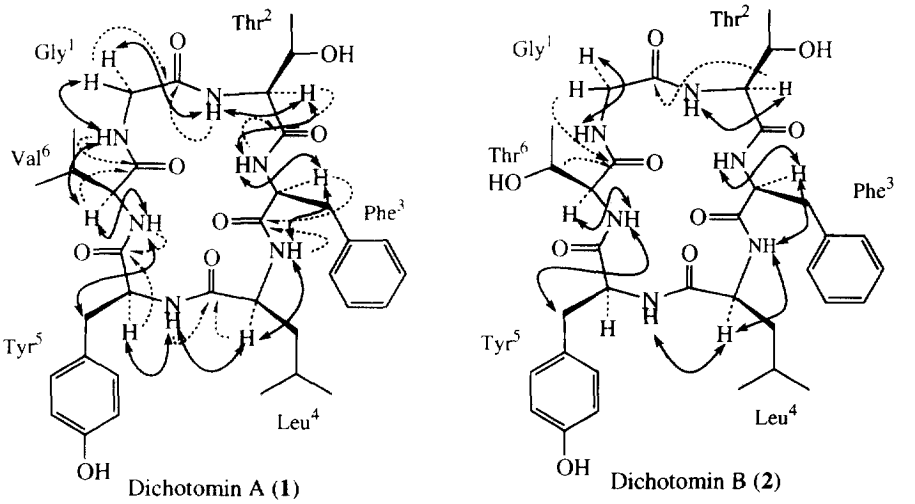


Fig. 1. Structures of Dichotomins A (**1**) and B(**2**). Dashed arrows show HMBC relationship and arrows show NOE relationship.

Dichotomins A showed cell growth inhibitory activity against p-388 lymphocytic leukemia cells. Since chemical and biological activity are intimately related to the conformation of a peptide, it is essential to establish the principles governing stable conformation. For that purpose and confirmation of the structure of **1**, a single crystal X ray analysis was conducted.

Dichotomin A was crystallized from MeOH-H_2O mixtures in monoclinic crystals of space group $P2_1$ (Z=2). The crystallographic data were collected from one single crystal sealed in a thin-walled glass capillary with a small amount of mother liquor. Three solvent molecules ($3H_2O$), which were assigned full occupancy, have been found in the crystallographic asymmetric unit. A summary of the final crystallographic data is presented in Table 1. The X-ray crystal structure determination gave exactly the same amino acid sequence and absolute configuration originally assigned dichotomin A. Figure 2 shows a ORTEP perspective view of the backbone of **1**.

Intramolecular hydrogen bonds and intramolecular very short contacts are summarized in Table 2. In the crystal form of **1**, the β-turns and system of intramolecular hydrogen bonds restrain the 18-membered cyclic frame in **1**. The end of the molecule is constrained to two β-turns formed by the residues from Val^6 to Gly^1, and from Phe^3 to Leu^4. The former is denoted as type II [Val^6 φ, ψ (-57.9, 130.5); Gly^1 φ, ψ (90, -9.6)] with the intramolecular hydrogen bond between Thr^2-NH and Tyr^5-CO, and the other type I [Phe^3 φ, ψ (-76.7, -11.8); Leu^4 φ, ψ (-114.8, -12.6)] without a transannular intramolecular hydrogen bond. Additionally, a side chain-main chain interaction has been observed between the backbone NH group of Leu^4 and the side chain oxygen of Thr^2. It is interesting that the side chain of Thr^2 directs toward the interior and a significant intramolecular NH···O hydrogen bonding contact between Thr^2-O and Leu^4-NH exists. Obviously, due to geometrical constraints, an intramolecular hydrogen bond between Thr^2-CO and Tyr^5-NH cannot occur simultaneously in this system.

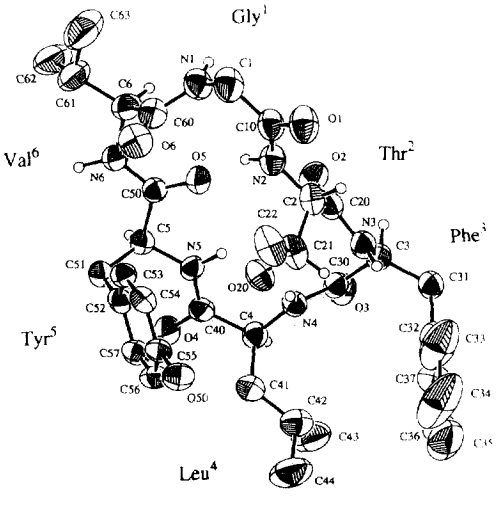


Fig. 2. A ORTEP perspective view of the crystal structure of **1**.

Table 1. Crystal data of dichotomin A (**1**).

Empirical Formula	$C_{35}H_{54}N_6O_{11}$(734.85)
Color, Habit	colorless, prismatic
Dimensions	0.60 × 0.50 × 0.25 mm
System	monoclinic
Lattice Type	Primitive
Dcalc	1.134 g/cm^3
Lattice Parameters (Å)	a=13.176(3) b=11.450(3) c=15.166(3) β=109.83(1)° V=2152.3(8)Å^3
Space Group	$P2_1$
Z value	2
Final R value (R_w)	0.070 (0.105)

Table 2. Hydrogen bonds and intramolecular very short contacts of **1**.

From	To	Distance (Å)	Angle (°)*
O2	N4	3.13	99
O5	N1	3.25	73
O5	N2	1.95	162
O6	N2	3.09	112
O20	N2	3.02	81
O20	N3	3.06	81
O20	N4	2.11	164
O20	N5	2.60	132

* Angle for N-H ··· O

Table 3. ^{1}H and ^{13}C NMR Signal Assignments of Dichotomin A (**1**) in pyridine-d_5.

assignment	^{1}H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C		δ_H	δ_C
Gly[1]			Leu[4]		
α	4.83 (1H, dd, 6.6, 15.6)	44.24	α	4.40 (1H, dt, 7.5, 15.0)	55.08
	3.88 (1H, dd, 5.0, 15.6)		β	1.97 (2H, br t, 7.5)	40.04
NH	9.97 (1H, br t, 5.8)		γ	1.51 (1H, m)	24.86
C=O		170.69	δ	0.78 (3H, d, 6.5)	21.48
				0.78 (3H, d, 6.6)	23.08
Thr[2]			NH	9.07 (1H, d, 7.5)	
α	4.99 (1H, dd, 3.2, 7.9)	59.49	C=O		172.39
β	4.91 (1H, dq, 3.2, 6.4)	66.97	Tyr[5]		
γ	1.49 (3H, d, 6.4)	20.18	α	4.82 (1H, m)	56.34
NH	9.21 (1H, d, 7.9)		β	3.56 (1H, dd, 6.7, 14.0)	37.12
C=O		171.29		3.51 (1H, dd, 6.8, 14.0)	
			γ		128.57
Phe[3]			δ	7.42 (2H, d, 8.4)	131.21
α	5.19 (1H, br dd,7.6, 7.8)	56.34	ε	7.14 (2H, d, 8.4)	116.29
β	3.57 (1H, dd, 8.2, 13.9)	38.06	ζ		157.73
	3.37 (1H, dd, 8.6, 13.9)		NH	8.60 (1H, d, 6.9)	
γ		138.16	C=O		172.33
δ	7.20 (2H, d, 7.4)	128.73	Val[6]		
ε	7.35 (2H, t, 7.4)	129.56	α	4.53 (1H, br t, 6.0)	61.25
ζ	7.17 (1H, m)	126.84	β	2.46 (1H, m)	30.22
NH	8.79 (1H, d, 7.5)		γ	1.13 (3H, d, 6.7)	19.39[a]
C=O		172.72		1.09 (3H, d, 6.8)	19.23[a]
			NH	8.35 (1H, d, 5.8)	
			C=O		172.72

a) Assignment may be interchanged.

Table 4. ^{1}H and ^{13}C NMR Signal Assignments of Dichotomin B (**2**) in pyridine-d_5.

assignment	^{1}H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C		δ_H	δ_C
			Leu[4]		
Gly[1]			α	4.48 (1H, m)	54.55
α	4.81 (1H, m)	44.11	β	1.96 (1H, m)	40.44
	4.00 (1H, dd, 4.8, 15.9)			1.86 (1H, m)	
NH	9.74(1H, br s)		γ	1.52 (1H, m)	24.78
C=O		170.70	δ	0.78 (3H, d, 6.5)	23.14
				0.76 (3H, d, 6.5)	21.39
Thr[2]			NH	9.19 (1H, d, 6.3)	
α	4.95 (1H, dd, 4.0, 8.0)	60.22	C=O		173.07
β	4.79 (1H, m)	67.05	Tyr[5]		
γ	1.42 (3H, d, 6.3)	20.46	α	4.93 (1H, m)	56.71
NH	9.11 (1H, d, 7.8)		β	3.57 (2H, d, 6.7)	36.93
C=O		171.42			
			γ		128.33
Phe[3]			δ	7.43 (2H, d, 8.4)	131.16
α	5.19 (1H, br dd, 7.8, 7.8)	56.25	ε	7.15 (2H, d, 8.4)	116.33
β	3.54 (1H, dd, 6.5, 14.0)	38.03	ζ		157.79
	3.42 (1H, dd, 8.5, 13.8)		NH	8.79 (1H, br d, 5.7)	
γ		138.21	C=O		172.59
δ	7.35 (2H, d, 6.6)	129.60	Thr[6]		
ε	7.19 (2H, m)	128.74	α	4.84 (1H, m)	61.71
ζ	7.16 (1H, m)	126.86	β	4.81 (1H, m)	67.35
NH	8.79 (1H, br d, 5.7)		γ	1.53 (3H, d, 5.8)	20.64
C=O		172.87	NH	8.51 (1H, br d, 4.5)	
			C=O		172.03

Dichotomin B (**2**), obtained as colorless powder, $[\alpha]_D$ +16.0° (c 0.10, MeOH), had the molecular formula, $C_{34}H_{46}N_6O_9$ established by high-resolution FAB-MS spectrometry (*m/z* 683.3414). The IR spectrum (3325 and 1649 cm^{-1}) exhibited bands characteristic of amino and amide carbonyl groups, respectively. The ^1H and ^{13}C NMR spectra of **2**, closely resembled those of **1**, showed the presence of six amide NH and six amide carbonyl groups, indicating the hexapeptide nature. The amino acid composition of **2** was revealed to be one Gly, two Thr, one Leu, one Phe and one Tyr per molecule after complete hydrolysis with 6N HCl. In addition, the lack of terminal amino group protons in the ^1H NMR suggested that **2** might be cyclic hexapeptide.

A close inspection of the NMR spectra by ^1H-^1H COSY and HMQC experiments led to complete ^1H and ^{13}C assignments of individual amino acid shown in Table 4. 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 (Fig. 1). The ROE correlations between Hα of Phe^3 and NH of Leu^4, between Hα of Leu^4 and NH of Tyr^5, and between Hβ of Tyr^5 and NH of Thr^6 indicate the tetrapeptide sequence of Phe-Leu-Tyr-Thr. In addition, the connectivity between Thr^6 and Gly^1 was revealed by $^3J_{H\text{-}C}$ ones between Hα of Gly^1 and carbonyl carbon of Thr^6 at δ 172.03, and between Hβ of Thr^6 and the same carbon. Therefore, the remaining Thr residue must be sited between Gly and Phe.

From these evidences, the structure of **2** was elucidated to be *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Thr-). The absolute configuration of the component amino acids was confirmed to be all L-configuration by Marfey's method.[5)]

Table 5. ^1H and ^{13}C NMR Signal Assignments of Dichotomin C (**3**) in pyridine-*d*5.

assignment	^1H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C			δ_H	δ_C
Gly^1			Leu^4			
α	4.74 (1H, dd, 6.7, 15.7)	44.48		α	4.48 (1H, br q, 7.4)	54.86
	3.95 (1H, dd, 5.2, 15.7)			β	1.89 (2H, br t, 7.3)	40.51
NH	9.77 (1H, br t, 5.8)			γ	1.52 (1H, m)	24.85
C=O		170.71		δ	0.76 (3H, d, 6.3)	23.03
					0.77 (3H, d, 6.2)	21.60
Thr^2				NH	9.01 (1H, d, 7.0)	
α	5.02 (1H, dd, 3.4, 8.4)	59.59		C=O		172.63
β	4.84 (1H, dq, 3.5, 6.4)	67.22	Tyr^5			
γ	1.45 (3H, d, 6.4)	20.33		α	4.76 (1H, m)	56.29
NH	9.07 (1H, d, 8.4)			β	3.56 (1H, dd, 6.7, 14.0)	37.06
C=O		171.57			3.50 (1H, m)	
				γ		128.41
Phe^3				δ	7.38 (2H, d, 8.5)	131.27
α	5.12 (1H, q, 7.4)	56.45		ε	7.12 (2H, d, 8.5)	116.25
β	3.46 (1H, m)	38.00		ζ		157.75
	3.42 (1H, dd, 8.7, 13.9)			NH	8.55 (1H, d, 6.6)	
γ		138.13		C=O		172.13
δ	7.34 (2H, m)	129.64	Ala^6			
ε	7.20 (2H, m)	128.75		α	4.62 (1H, dq, 5.3, 6.9)	51.25
ζ	7.17 (1H, m)	126.88		β	1.62 (3H, d, 6.9)	17.14
NH	8.69 (1H, d, 7.5)			NH	9.05 (1H,d,5.3)	
C=O		172.65		C=O		173.87

Dichotomin C (**3**), colorless powder, $[\alpha]_D$ +34.0° (c 0.10, MeOH), $C_{33}H_{44}N_6O_8$, is also a peptidic compound as shown from the IR absorption bands at 3314 and 1649 cm^{-1}. As the same as **1** and **2**, hexapeptide nature was suggested by the presence of six amide protons and six amide carbonyl carbons in the NMR spectra. A different point from **1** and **2** is the presence of Ala residue in place of Val in **1** and Thr in **2**, which was corresponding with the amino acid compositions (each one Gly, Thr, Ala, Leu, Phe and Tyr) revealed by acid hydrolysis, followed by amino acid analysis. After complete ^1H and ^{13}C assignments of individual amino acid using ^1H-^1H COSY and C-H COSY spectra (Table 5), sequencing was elucidated by a combination of COLOC and NOE correlations in neighboring amino acid residues (Fig. 3). From these results, the whole structure of **3** was clarified as *cyclo*(-Gly-Thr-Phe-Leu-Tyr-Ala-) and the composed amino acids were confirmed to be all L-configuration by Marfey's method.[5]

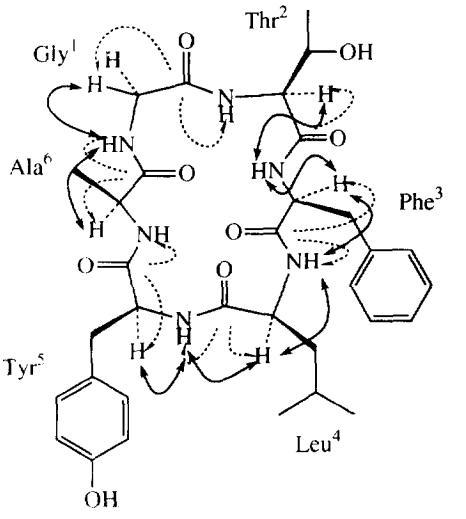


Fig. 3. Structure of Dichotomin C (**3**). Dashed arrows show COLOC relationship and arrows show NOE relationship.

Dichotomin D (**4**) was obtained as colorless needles, mp 156-158° (from MeOH), $[\alpha]_D$ -21.4° (c 0.10, MeOH). The molecular formula, $C_{33}H_{44}N_6O_7$, was consistent with the quasimolecular ion at *m/z* 637.3349 (HR-FAB MS, MH^+, Δ +0.1 mmu). The ^1H and ^{13}C NMR spectra in DMSO-d_6 of **4**, showing six amide NH and six amide carbonyl groups, as shown in Table 6, indicated the hexapeptide nature, together with the IR absorption bands at 3303 and 1653 cm^{-1}, corresponding to amino and amide carbonyl groups, respectively. In addition, the relatively high intensity of the molecular ion and the lack of terminal amino group protons in the ^1H NMR suggested **4** to be a cyclic peptide. Amino acid analysis of the acid hydrolysate of **4** revealed the presence of one residue each on Val, Ile, Tyr and Phe, and two residues of Gly. The absolute stereochemistry of each amino acid in **4** was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,[5] followed by HPLC analysis.

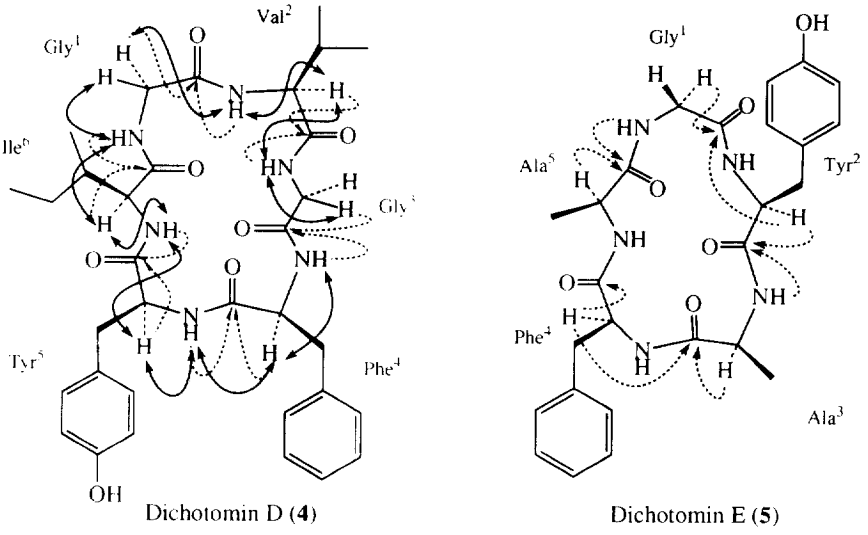
Structure elucidation of each NMR spin system, Val, Ile, Tyr, Phe and Gly was conducted by extensive analysis of ^1H-^1H COSY and HMQC spectra (Table 6). As in the case of **1**, the sequencing of the amino acids for **4** was elucidated by connecting the individual amino acids on the basis of HMBC correlations (Fig. 4). From the HMBC correlations among each Hα, NH and the intermediate carbonyl carbon, the sequence was identified as *cyclo*(-Gly-Val-Gly-Phe-Tyr-Ile-). The deduced structure of dichotomin D was also in good agreement with the result of the NOE correlations (Fig. 4) in a phase sensitive NOESY spectrum.[9]

Table 6. ^1H and ^{13}C NMR Signal Assignments of Dichotomin D (**4**) in DMSO-d_6.

assignment	^1H NMR δ_H (int. mult, J(Hz))	^{13}C NMR δ_C	assignment	δ_H	δ_C
Gly[1]			Phe[4]		
α	3.40 (1H, dd, 5.6, 15.2)	43.04	α	4.07 (1H,ddd,5.2,7.0,9.7)	55.91
	3.98 (1H, dd, 5.6, 15.2)		β	2.64 (1H, dd, 9.7, 14.1)	36.59
NH	8.66 (1H, t, 5.6)			2.76 (1H, dd, 5.2, 14.1)	
C=O		169.26	γ		137.78
			δ	7.01 (2H, d, 7.2)	128.72
			ε	7.21 (2H, t, 7.2)	128.05
Val[2]			ζ	7.16 (1H, d, 7.2)	126.15
α	4.17 (1H, dd, 5.3, 8.5)	57.68	NH	8.18 (1H, d, 7.0)	
β	2.27 (1H, m)	28.50	C=O		171.05
γ	0.83 (3H, d, 6.9)	17.65	Tyr[5]		
	0.86 (3H, d, 6.9)	18.70	α	4.39 (1H,ddd,4.1,9.0,11.3)	54.93
NH	7.89 (1H, d, 8.5)		β	2.87 (1H, dd, 11.3, 14.0)	36.52
C=O		170.88		2.98 (1H, dd, 4.1, 14.0)	24.38
			γ		127.99
			δ	7.06 (2H, d, 8.5)	129.84
			ε	6.70 (2H, d, 8.5)	114.89
Gly[3]			ζ		155.81
α	3.71 (2H, br d, 5.7)	42.27	NH	8.02 (1H, d, 9.0)	
NH	8.19 (1H, t, 5.9)		C=O		171.32
C=O		168.47	Ile[6]		
			α	4.05 (1H, t, 6.8)	57.42
			β	1.73 (1H, m)	36.70
			γ	1.08 (1H, m)	24.78
				1.51 (1H, m)	
			Me(γ)	0.83 (3H, d, 6.9)	14.99
			Me(δ)	0.86 (3H, t, 7.6)	11.10
			NH	7.66 (1H, d, 6.8)	
			C=O		171.40

Table 7. ^1H and ^{13}C NMR Signal Assignments of Dichotomin E (**5**) 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	4.49 (1H, dd, 6.5, 14.5)	44.51	α	4.87 (1H, m)	57.76
	3.82 (1H, dd, 5.1, 14.5)		β	3.53 (2H, m)	37.63
NH	9.74 (1H, br t)		γ		138.42
C=O		171.02	δ	7.39 (2H, d, 7.3)	129.7[1]
Tyr[2]			ε	7.28 (2H, t, 7.3)	128.76
α	5.15 (1H, dt, 6.6,8.6)	56.83	ζ	7.23 (1H, m)	126.95
β	3.23 (1H, dd, 8.6, 14.0)	37.36	NH	9.30 (1H, br s)	
	3.43 (1H, dd, 6.6, 14.0)		C=O		173.92
γ		128.30			
δ	7.28 (2H, d, 8.4)	130.73			
ε	7.07 (2H, d, 8.4)	116.21	Ala[5]		
ζ		157.67	α	4.89 (1H, m)	50.16
NH	9.01 (1H, d, 8.6)		β	1.61 (3H, d, 6.9)	17.34
C=O		172.65	NH	9.30 (1H, br s)	
Ala[3]			C=O		173.92
α	4.58 (1H, br t, 7.0)	51.75			
β	1.57 (3H, d, 7.1)	17.34			
NH	9.47 (1H, d, 8.1)				
C=O		173.23			



Dichotomin D (**4**) Dichotomin E (**5**)

Fig. 4. Structures of Dichotomins D (**4**) and E (**5**). Dashed arrows show HMBC relationship and arrows show NOE relationship.

Dichotomin E (**5**), colorless powder, $[\alpha]_D$ -66.7° (c 0.11, MeOH) had a molecular formula, $C_{26}H_{31}N_5O_6$, which was consistent with the quasimolecular ion at *m/z* 510.2328 (HR-FAB MS, MH^+, Δ +2.5 mmu). The pentapeptide nature of **5** was deduced from the presence of five amide protons in the ^1H NMR spectrum, agreeing with five amide carbonyl carbons in the ^{13}C NMR spectrum. The amino acid composition (Gly, Ala × 2, Phe and Tyr) of **5**, determined by amino acid analysis after complete acid hydrolysis with 6N HCl, was compatible with the molecular formula, if **5** was a cyclic pentapeptide. Five independent ^1H coupling network of the above amino acids was clarified using ^1H-^1H COSY spectrum, and the corresponding ^{13}C signal was assignable by HMQC spectrum except for amide carbonyl carbons (Table 7). The two and three bond long range correlation observed in HMBC spectrum (pyridine-d_5) enabled the assignment of amide carbonyl carbons and indicated the peptide sequence of **5** to be Gly-Tyr-Ala-Phe-Ala as shown in Fig. 4. The composed amino acids were determined to be all L-configuration by Marfey's method.[5]

Dichotomins A, B, C and E showed cell growth inhibitory activities against p-388 lymphocytic leukemia cells (IC_{50} A: 2.5 μg/ml; B: 3.5 μg/ml, C: 5.0 μg/ml; E: 2.0 μg/ml). Whereas dichotomin D did not show. Dichotomins A, B and C possess the same sequencing except for one amino acid at residue 5 (dichotomins A: Val; B: Thr; C: Ala). In the structure of cyclic pentapeptide, dichotomin E, the similar sequence (Tyr-Ala-Phe) to Phe-Leu-Tyr in dichotomins A, B and C, whose central aliphatic amino acid is sandwiched among two aromatic amino acids, is contained. The activity may be responsible for the sequencing and/or their conformation.

In addition, dichotomin D showed potent cyclooxygenase inhibitory activity (72.6 % inhibition at 100 μM). It is interesting that, however, dichotomin A did not show any activity. It also may be based on the different sequencing and/or conformation of them.

Studies on the detail conformational analyses, and biological evaluations of a series of dichotomins are in progress.

Experimental

M.p.s were determined on a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-4 spectrometer and the $[\alpha]_D$ values are given in 10^{-1}deg cm^2 g^{-1}. FAB and high resolution mass spectra were taken with a VG Autospec spectrometer. IR spectrum was recorded on a JASCO A-302 spectrophotometer. High-pressure liquid chromatography (HPLC) was performed with an Inertsil PREP-ODS column (20mm i.d. ×250mm and 30mm i.d. ×250mm, GL Science Inc.) packed with 10μm ODS. TLC was conducted on precoated Kieselgel 60 F_{254} (Art. 5715; Merck) and the spots were detected by spraying Dragendorff reagent. Proton and carbon spectra were recorded on Bruker spectrometers (AM400 and AM500) and processed on a Bruker data station with an Aspect 3000 computer. The 15 mg each sample of dichotomins A - E in a 5mm tube (0.5ml pyridine-d_5 or DMSO-d_6, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. Phase sensitive NOESY experiments were acquired with mixing times of 0.6 sec and ROESY experiments 90 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 msec.

Extraction and Isolation

The dry roots of *Stellaria dichotoma* L. var. *lanceolata* Bge. (8.5 Kg) were extracted with hot methanol at three times to give a methanol extract (2.4 kg) which was treated with *n*-butanol and water. The *n*-butanol soluble fraction (294 g) was subjected to Diaion HP-20 column chromatography using a water - methanol gradient system (1:0 - 0:1). The fractions eluted by 80% methanol were further subjected to silica gel column chromatography using a methylene chloride - methanol gradient system (1:0 - 0:1). The fraction eluted by 10% methanol was subjected to ODS HPLC with 45 - 65 % MeOH, and 30 - 40 % CH_3CN solvent systems to give dichotomin A (0.007%), B (0.0004%), C (0.003%), D (0.0012%) and E (0.0002%) as colorless needles and colorless powder.

Dichotomin A (1). - Colorless needles, m.p. 179-180°C, $[\alpha]_D$ +14.0° (c 0.10, MeOH); *m/z* 681 (Found: MH$^+$, 681.3652. $C_{35}H_{49}N_6O_8$ requires, 681.3612); ν_{max} (KBr)/cm^{-1} 3309 (NH) and 1649 (amide C=O); λ_{max} (MeOH) / nm 276 (ε 1800).

Dichotomin B (2).- Colorless powder, $[\alpha]_D$ +16.0° (c 0.10, MeOH); *m/z* 683 (Found: MH$^+$, 683.3414. $C_{34}H_{47}N_6O_9$ requires M+1, 683.3405); ν_{max} (KBr)/cm^{-1} 3325 (NH) and 1649 (amide C=O); λ_{max} (MeOH) / nm 277 (ε 1960).

Dichotomin C (3). - Colorless powder, $[\alpha]_D$ +34.0° (c 0.10, MeOH); *m/z* 653 (Found: MH$^+$, 653.3305. $C_{33}H_{45}N_6O_8$ requires, 653.3299); ν_{max} (KBr)/cm^{-1} 3314 (NH) and 1649 (amide C=O); λ_{max} (MeOH) / nm 276 (ε 2240).

Dichotomin D (4).- Colorless needles, m.p. 156-158°C, $[\alpha]_D$ -21.4° (c 0.10, MeOH); *m/z* 637 (Found: MH$^+$, 637.3349. $C_{33}H_{45}N_6O_7$ requires M+1, 637.3350); ν_{max} (KBr)/cm^{-1} 3303 (NH) and 1653 (amide C=O); λ_{max} (MeOH) / nm 277 (ε 2000).

Dichotomin E (5). - Colorless powder, $[\alpha]_D$ -66.7° (c 0.11, MeOH); *m/z* 510 (Found: MH^+, 510.2328. $C_{26}H_{32}N_5O_6$ requires, 510.2353); ν_{max} (KBr)/cm^{-1} 3307 (NH) and 1654 (amide C=O); λ_{max} (MeOH) / nm 270 (ε 4350).

Acid Hydrolysis of **1** - **5**

Solutions of **1** - **5** (each containing 1 mg of peptide) in 6N HCl were heated at 110°C for 24h in a sealed tube. After cooling, each solution was concentrated to dryness. The hydrolysates were soluble in 0.02N HCl and applied to the analysis by an amino acid analyzer (Hitachi L-8500 Amino acid Analyzer).

Absolute Configuration of Amino Acids[5)]

Solutions of **1**- **5** (each containing 1 mg of peptides) in 6N HCl were heated at 110° for 12h in a sealed tube. After being cooled, each solution was concentrated to dryness. The residue was soluble in water and treated with 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide (Marfey's reagent) and 1M $NaHCO_3$ at 35° for 1h. After being cooled, 2M HCl was added and then concentrated to dryness. This residue was subjected to HPLC (Lichrospher 100, RP-18 (10μm), Merck), flow rate 1 ml/min, detection 340nm, solvent : 10 - 50% CH_3CN / 50mM triethylamine phosphate (TEAP) buffer.

X-ray analysis of Dichotomin A

A colorless prismatic crystal of $C_{35}H_{48}N_6O_8$ having trihydrates and approximate dimensions of 0.60 × 0.50 × 0.25 mm was sealed in a glass capillary. All measurements were made on a Rigaku AFC7R diffractometer with graphite monochromated Cu-Kα radiation and a 12kW rotating anode generator. Cell constants and an orientation matrix for data collection, obtained from a least-squares refinement using the setting angles of 25 carefully centered reflections in the range $58.93<2\theta<59.91°$ corresponded to a primitive orthorhombic cell with dimensions: a=13.176(3), b=11.450(3), c=15.166(3) Å, β=109.83(1)°, V=2152.3(8) $Å^3$. For Z=2 and F.W.=734.85, the calculated density is 1.13 g/cm^3. Based on the systematic absences of: 0k0: $k \neq 2n$, packing considerations, a statistical analysis of intensity distribution, and the successful solution and refinement of the structure, the space group was determined to be: $P2_1$(#4). The data were collected at a temperature of 23 ± 1°C using the ω-2θ scan technique to a maximum 2θ value of 120.0°. Omega scans of several intense reflections, made prior to data collection, had an average width at half-height of 0.23° with a take-off angle of 6.0°. Scans of $(1.63 + 0.30 \tan \theta)°$ were made at a speed of 16.0° / min (in omega). The weak reflections ($I < 10.0\sigma(I)$) were rescanned (maximum of 12 scans) and the counts were accumulated to ensure good counting statistics. Stationary background counts were recorded on each side of the reflection. The ratio of peak counting time to background counting time was 2:1. The diameter of the incident beam collimator was 1.0 mm, the crystal to detector distance was 235 mm, and the computer controlled detector aperture was set to 3.0 × 6.5 mm (horizontal × vertical).

Of the 3550 reflections which were collected, 3377 were unique (R_{int}=0.014). The intensities of three representative reflection were measured after every 100 reflections. Over the course of data collection, the standards increased by 10.4 %. A linear correction factor was applied to the data to account for this phenomenon. The linear absorption coefficient, μ, for Cu-Kα radiation is 7.0 cm^{-1}. An empirical absorption correction using the program DIFABS[10)] was applied which resulted in

transmission factors ranging from 0.89 to 1.16. The data were corrected for Lorentz and polarization effects. A correction for secondary extinction was appled (coefficient = 7.51044e-06).

The structure was solved by direct methods[11] and expanded by using Fourier techniques.[12] The non-hydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full-matrix least-squares refinement was based on 2603 observed reflection ($I>3.00\sigma(I)$) and 469 variable parameters and converged (largest parameter was 0.08 times its esd) with unweighted and weighted agreement factors of : R=0.070, R_w=0.105. The standard deviation of an observation of unit weight was 1.32. The weighting scheme was based on counting statistics and included a factor (p=0.154) to downweight the intense reflections. Plots of $\Sigma\omega(|Fo|-|Fc|)^2$ versus $|Fo|$, reflection order in data collection, sin θ/λ and various classes of indices showed no unusual trends. The maximum and minimum peaks on the final difference Fourier map corresponded to 0.45 and -0.19 $e^-/Å^3$, respectively. Neutral atom scattering factors were taken from Cromer and Waber.[13] Anomalous dispersion effects were included in Fcalc[14]; the values for $\Delta f'$ and $\Delta f''$ were those of Creagh and McAuley.[15] The values for the mass attenuation coefficients are those of Creagh and Hubbel.[16] All calculations were performed using the teXsan[17] crystallographic software package of Molecular Structure Corporation.

The refined fractional atomic coordinates, the bond lengths, the bond angles, the hydrogen-atom coordinates and the thermal parameters have been deposited at the Cambridge Crystallographic Data Centre (CCDC).

Cytotoxic Activity on P388 cells

The MTT(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium 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 of kanamycine and containing mouse P388 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 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.

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 microsomes solutions (0.2 mg/ml) which were dissolved in 50 mM Tris-HCl buffer solution. Their mixture solutions were pre-incubated with shaking for 3 min at 25 °C. After pre-incubation, 2 ml of arachidonic acid solution (1 mg/ml) was added to above enzymatic solutions and incubated continually for further 3 min. At the end of reaction was added 10 ml of $FeCl_3$ solution (25 mM) to the reaction mixtures. After completion of the reactions, they 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 company, USA).

Acknowledgments: We are grateful to Dr. Ken Matsuzaki and Dr. Shusuke Hashimoto of Yakult Central Institute for Microbiological Research for the biological evaluations and thank the Ministry of Education, Science and Culture, Japan, for financial support through Grant-in-Aid for General Scientific Research.

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(*Received in Japan* 4 *September* 1995; *accepted* 1 *November* 1995)