Pergamon

Tetrahedron Vol. 50, No. 33, pp. 9975-9982, 1994

0040-4020/94 $7.00+0.00

0040-4020(94)00571-0

Pseudostellarins D - F, New Tyrosinase Inhibitory Cyclic Peptides from *Pseudostellaria heterophylla* [1)]

Hiroshi Morita, Takashi Kayashita, Hideyuki Kobata, Akira Gonda, Koichi Takeya and Hideji Itokawa*

Department of Pharmacognosy, Tokyo College of Pharmacy,
Horinouchi 1432-1, Hachioji, Tokyo 192-03, Japan

Abstract: New potent tyrosinase inhibitory cyclic peptides, pseudostellarins D - F, have been isolated from the roots of *Pseudostellaria heterophylla* and the structures were elucidated by extensive 2D NMR methods, chemical and enzymatic degradation and ESI tandem MS spectroscopic analysis.

The roots of *Pseudostellaria heterophylla* are well known Chinese traditional medicine which are used as a lung and spleen tonic.[2)] During a survey of novel bioactive cyclic peptides from higher plants,[3)] we have already isolated several peptidic compounds, showing tyrosinase inhibitory activity and melanin production inhibitory activities, from the roots of *P. heterophylla* (Caryophyllaceae), and reported the structure of novel cyclic peptides, named pseudostellarins A, B, C and G.[1,4)] As a result of our further bioassay guided fractionation efforts, three novel tyrosinase inhibitory cyclic peptides, named pseudostellarins D - F (**1** - **3**) have been isolated from the roots of *P. heterophylla*. We report herein, the isolation, structure elucidation and the evaluation of tyrosinase inhibitory activities of analogous cyclic peptidic compounds (**1** - **3**).

The methanolic extract of the roots of *P. heterophylla* was partitioned between *n*-butanol and water. The *n*-butanol soluble material was subjected to Diaion HP-20 column (water - methanol), and 80% and 100% methanol eluted fractions were chromatographed on a silica gel column, followed by HPLC on ODS to yield three peptidic compounds as colorless needles, named pseudostellarins D - F (**1** - **3**).

Pseudostellarin D (**1**), colourless needles, mp. 177 - 179 °C, $[\alpha]_D$ -64.8° (c 0.54, MeOH), showed a high-resolution FAB-MS spectral quasimolecular ion peak at *m/z* 714.4184 (M^++H, Δ +0.5 mmu), corresponding to molecular formula, $C_{36}H_{55}N_7O_8$. The IR absorptions at 3300 and

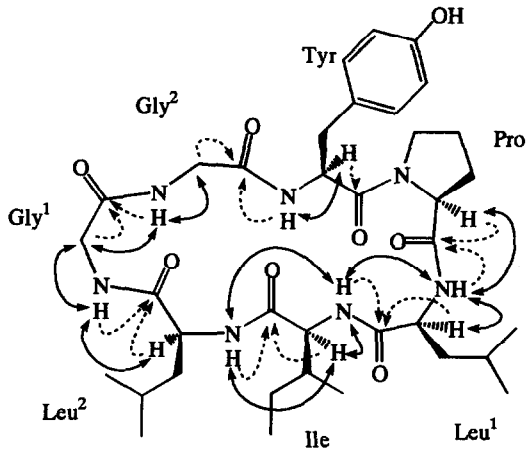

Fig. 1 Structure of pseudostellarin D (**1**). Arrows show NOE relationship and dashed arrows show HMBC correlations.

1650 cm^{-1} were attributed to amino and amide carbonyl groups, respectively. The heptapeptide nature of **1** was evident from its ^1H and ^{13}C NMR spectra, showing six amide NH and seven amide carbonyl groups, as shown in Table 1. Further, the relatively high intensity of the molecular ion and the lack of terminal amino group protons in the ^1H NMR suggested **1** to be a cyclic heptapeptide. Amino acid analysis of the acid hydrolysate of **1** revealed the presence of proline

Table 1. ^1H and ^{13}C NMR Signal Assignments of Pseudostellarin D (**1**) in [$^2H_6$]DMSO.

assignment		^1H NMR δ_H (int, mult, J(Hz))	^{13}C NMR δ_C
Gly[1]			
	α	3.18 (1H, dd, 4.0, 16.7)	42.37
		3.99 (1H, dd, 8.5, 16.7)	
	NH	8.86 (1H, dd, 4.0, 8.5)	
	C=O		168.63
Gly[2]			
	α	3.90 (1H, dd, 3.1, 17.7)	42.87
		4.15 (1H, dd, 3.1, 17.7)	
	NH	8.14 (1H, br s)	
	C=O		166.99
Tyr			
	α	4.49 (1H, dt, 4.0, 10.5)	56.09
	β	2.50 (1H, dd, 10.5, 13.5)	39.29
		2.84 (1H, dd, 4.0, 13.5)	
	γ		127.66
	δ	6.98 (2H, d, 8.5)	129.93
	ε	6.60 (2H, d, 8.5)	114.72
	ζ		155.68
	NH	8.15 (1H, d, 10.5)	
	C=O		170.68
Pro			
	α	4.08 (1H, dd, 6.6, 8.1)	61.40
	β	1.68 (1H, m)	29.49
		2.23 (1H, m)	
	γ	1.90 (2H, m)	24.43
	δ	3.50 (1H, m)	45.80
		3.64 (1H, m)	
	C=O		171.08
Leu[1]			
	α	4.35 (1H, ddd,3.2,9.0,9.4)	51.15
	β	1.56 (2H, m)	40.71
	γ	1.51 (1H, m)[b]	23.96[c]
	δ	0.80 (3H, d, 6.0)[a]	20.49[d]
		0.88 (3H, d, 6.0)[a]	22.19[d]
	NH	7.88 (1H, d, 9.4)	
	C=O		171.68
Ile			
	α	4.36 (1H, t, 9.0)	55.80
	β	1.58 (1H, m)	38.08
	γ	1.00 (1H, m)	23.96
		1.39 (1H, m)	
	Meγ	0.85 (3H, d, 6.7)	14.59
	Meδ	0.74 (3H, t, 7.3)	10.58
	NH	6.82 (1H, d, 9.0)	
	C=O		171.26
Leu[2]			
	α	3.89 (1H, dt, 2.1, 7.1)	54.00
	β	1.51 (2H, m)	39.49
	γ	1.39 (1H, m)[b]	24.43[c]
	δ	0.89 (3H, d, 6.0)[a]	22.75[d]
		0.94 (3H, d, 6.0)[a]	23.02[d]
	NH	8.79 (1H, d, 2.1)	
	C=O		171.77

a-d) Assignments may be interchanged.

(Pro), glycine (Gly) × 2, isoleucine (Ile), leucine (Leu) × 2 and tyrosine (Tyr). The absolute stereochemistry of each amino acid in **1** was determined to be L-configuration by derivatization of the acid hydrolysate with Marfey's reagent,[5] followed by HPLC analysis.

NMR signals for individual amino acids, Pro, Gly, Ile, Leu and Tyr were readily assigned by extensive analysis of ^{1}H-^{1}H COSY and HMQC spectra.[6] 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 (Figure 1).[7] From the HMBC experimental results, the sequence was identified as cyclo[Gly-Gly-Tyr-Pro-Leu-Ile-Leu]. The deduced structure of pseudostellarin D (**1**) was also in good agreement with the result of the NOE correlation in NOESYPH spectrum.[8]

Pseudostellarin E (**2**), colourless needles, mp. 168 - 170 ℃, $[\alpha]_D$ -112.1° (c 0.33, MeOH), showed a molecular formula, $C_{45}H_{67}N_9O_9$, which was permitted by HR-FAB-MS spectrum, indicating 17 degrees of unsaturation. Amino acid analysis of **2** showed the presence of Pro × 3, Phe, Gly × 2, Val, Leu and Ile, which were confirmed to be all L-configuration by Marfey's derivatization, followed by HPLC analysis.

In the NMR spectra, most of ^{1}H and ^{13}C signals were broadened in both apolar and polar solvents, *e.g.* in $CDCl_3$, [$^{2}H_5$]pyridine, [$^{2}H_3$]MeOD and [$^{2}H_6$]DMSO. This phenomenon might have resulted from the conformational change due to the isomerization about the proline amide bond. Since sequence analysis by the NMR method was disturbed by the signal broadening, electrospray ionization (ESI)[9] tandem mass spectrometry (MS/MS) method[10] was applied to determine the primary structure as follows.

ESI is a very soft ionization technique, which generates chiefly ions related to the molecular weight, such as $(M+Na)^+$, or $(M+K)^+$ etc. These rather stable ions undergo little fragmentation and, therefore, contain little sequence information. However, the $(M+H)^+$ ions can be fragmented upon collision with neutral atoms, such as He, to produce relatively abundant daughter ions, which are

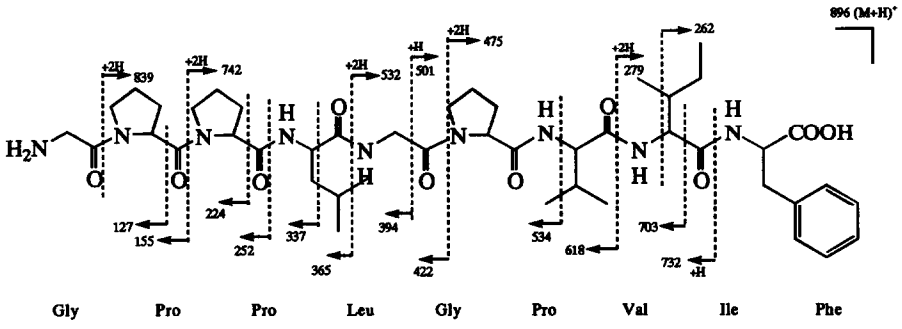

Figure 2 ESI MS/MS fragmentation of m/z 896 $(M+H)^+$ ion of compound **4**

then mass analyzed in a second mass spectrometer. Fragmentation at peptide bonds generates ions, the masses of which can be correlated with the sequence of the amino acids in the peptide. Though ESI MS spectrum of **2** in 50% methanol solution produced only rather stable ions such as $(M+Na)^+$ and $(M+K)^+$, that of a corresponding linear peptide (**4**) generated by digestion of **2** with α-chymotrypsin produced $(M+H)^+$ ion, which was then analyzed in a second mass spectrometer. As can be seen from fragmented ions of **4** (Figure 2), the sequence of **4** was easily determined to be Gly-Pro-Pro-Leu-Gly-Pro-Val-Ile-Phe. Therefore, the structure of **2** was established to be Cyclo[Gly-Pro-Pro-Leu-Gly-Pro-Val-Ile-Phe].

Pseudostellarin F (**3**), colourless needles, mp. 169 - 171 °C, $[\alpha]_D$ -58.9° (c 0.98, MeOH), showed a molecular formula, $C_{38}H_{56}N_8O_{10}$, which was permitted by HR-FAB-MS spectrum, indicating 15 degrees of unsaturation. Amino acid analysis of **3** showed the presence of Pro × 2, Tyr, Ser, Gly × 2 and Leu × 2, which were confirmed to be all L-configuration by Marfey's derivatization, followed by HPLC analysis.

In the NMR spectra of **3** (Table 2), six amide protons and eight amide carbonyl carbons were observed, corresponding to eight amino acids with two prolines as indicated above. The sequence analysis was conducted, in a similar way to those in **1**, by the 2D NMR analyses, *e.g.* HMBC and NOESYPH spectra. From the results of the important HMBC correlation as shown in Figure 3, the sequence constituted with seven amino acids was deduced to be Pro-Leu-Ser-Gly-Gly-Tyr-Leu . This sequence was also supported by the NOE correlation between each α-proton and amide proton. The residual amino acid, Pro, must be connected between Pro and Leu. Therefore, the whole structure was established to be cyclo[Gly-Gly-Tyr-Leu-Pro-Pro-Leu-Ser], whose structure was also satisfied with 15 degrees of unsaturation.

Tyrosinase, which is distributed in animals and plants, can convert tyrosine into melanin via a series of intermediate quinone and indolequinone compounds.[11] The melanin product formed is a complex heterogeneous biopolymer with many unusual properties; it is responsible for most of the pigmentation visible in the skin, hair and eyes of mammals. Tyrosinase in mammals is found exclusively within specialized dendritic cells called melanocytes. The initial reaction in the pathway of melanin formation from tyrosine through the catalytic action of tyrosinase involves the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA). Tyrosinase inhibitory activities (IC_{50}), showing the ratio of the production of DOPA, are 100 μM for **1**, 175 μM for **2** and 50 μM for **3** (Table 3). This inhibitory concentration was more potent than that of arbutin (1.2 mM), well known as a potent inhibitor of tyrosinase, and also cyclo(Pro-Tyr-Pro-Val) (1.5 mM), which was recently isolated from the lactic bacterium *Lactobacillus helveticus*.[12] Furthermore, pseudostellarin D showed potent inhibitory effect on the melanogenesis using cultured B16 melanoma cells (IC_{50} 49 μM).[13]

Table 2. ^{1}H and ^{13}C NMR Signal Assignments of Pseudostellarin F (3) in [$^{2}H_6$]DMSO.

assignment	^{1}H NMR δ_H (int, mult, J(Hz))	^{13}C NMR δ_C	assignment	δ_H	δ_C
Gly^1			Pro^1		
α	3.55 (1H, dd, 5.5, 16.2)	43.04	α	4.50 (1H, d, 7.4)	58.68
	3.72 (1H, dd, 5.5, 16.2)		β	1.68 (1H, m)	27.91
NH	8.40 (1H, t, 5.5)			2.23 (1H, m)	
C=O		169.34	γ	1.89 (2H, m)	24.58
			δ	3.49 (1H, m)	46.94
Gly^2				3.60 (1H, m)	
α	3.48 (1H, dd, 5.7, 16.6)	42.04	C=O		170.13
	3.68 (1H, dd, 5.7, 16.6)		Pro^2		
NH	8.12 (1H, t, 5.7)		α	4.50 (1H, d, 7.4)	60.16
C=O		168.83	β	1.96 (1H, m)	30.24
				2.30 (1H, m)	
Tyr			γ	1.41 (1H, m)	21.68
α	4.27 (1H,ddd,4.4,8.3,10.6)	55.47		1.79 (1H, m)	
β	2.73 (1H, dd, 10.6 14.1)	36.55	δ	3.29 (1H, m)	45.93
	2.96 (1H, dd, 4.4, 14.1)			3.41 (1H, m)	
γ		127.91	C=O		170.77
δ	7.04 (2H, d, 8.4)	129.84	Leu^2		
ε	6.64 (2H, d, 8.4)	114.91	α	4.20 (1H, m)	52.65
ζ		155.75	β	1.52 (1H, m)	39.95[a]
NH	7.64 (1H, d, 8.3)			1.79 (1H, m)	
C=O		170.42	γ	1.56 (1H, m)	24.44
			δ	0.87 (3H, d, 6.5)	21.80
Leu^1				0.89 (3H, d, 6.0)	23.00
α	4.49 (1H, m)	48.75	NH	8.09 (1H, d, 7.6)	
β	1.42 (1H, m)	39.74[a]	C=O		172.00
	1.56 (1H, m)		Ser		
γ	1.89 (1H, m)	23.88	α	4.23 (1H, dt, 7.0, 12.5)	55.17
	1.96 (1H, m)		β	3.64 (2H, m)	60.84
δ	0.79 (3H, d, 6.4)	21.10	OH	5.04 (1H, t, 5.5)	
	0.88 (3H, d, 6.1)	22.85	NH	7.65 (1H, d, 7.0)	
NH	7.45 (1H, d, 7.8)		C=O		171.38
C=O		169.70			

a) Assignments may be interchanged.

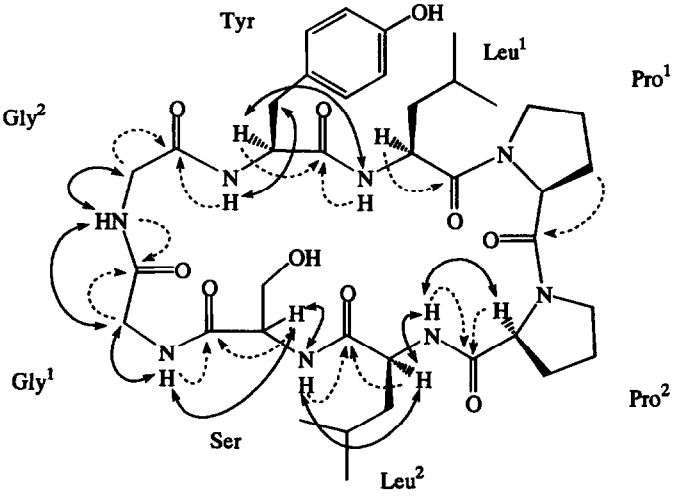

Fig. 3 Structure of pseudostellarin F (3). Arrows show NOE relationship and dashed arrows show HMBC correlations in [$^{2}H_6$]DMSO.

A series of pseudostellarins A - G showed potent inhibitory activities against tyrosinase from mushroom as listed in Table 3. It is interesting that, however, constituted amino acids, amino acid sequence and ring size of these cyclic peptides were different from each other. Efforts are currently underway to determine the precise backbone conformation, their homogeneity and biological activity relationship.

Table 3. Structures of pseudostellarins and their inhibitory activities against tyrosinase

Pseudostellarins	Structures	IC_{50} (μM)
A	Cyclo[Gly-Pro-Tyr-Leu-Ala]	131
B	Cyclo[Gly-Ile-Gly-Gly-Gly-Pro-Pro-Phe]	187
C	Cyclo[Gly-Thr-Leu-Pro-Ser-Pro-Phe-Leu]	63
D	Cyclo[Gly-Gly-Tyr-Pro-Leu-Ile-Leu]	100
E	Cyclo[Gly-Pro-Pro-Leu-Gly-Pro-Val-Ile-Phe]	175
F	Cyclo[Gly-Gly-Tyr-Leu-Pro-Pro-Leu-Ser]	50
G	Cyclo[Pro-Phe-Ser-Phe-Gly-Pro-Leu-Ala]	75

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 and ESI MS/MS spectrum with TSQ-700 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 pseudostellarins D, E and F in a 5mm tube (0.5ml [$^2H_5$]pyridine, [$^2H_3$]MeOD and [$^2H_6$]DMSO, degassed) was used for the homonuclear and heteronuclear measurements. The spectra were recorded at 303K. NOESYPH experiments were acquired with mixing times of 0.6s. The value of the delay to optimize one-bond correlations in the HMQC spectrum and suppress them in the HMBC spectrum was 3.2 msec and the evolution delay for long-range couplings in the HMBC spectrum was set to 50 msec.

Extraction and Isolation

The roots of *Pseudostellaria heterophylla* (10.0 Kg) were extracted with hot MeOH at three times to give a MeOH extract which was treated with *n*-BuOH and H_2O. The *n*-BuOH soluble fraction (167 g), showing tyrosinase inhibitory activity, was subjected to Diaion HP-20 column chromatography using a water - MeOH gradient system (1:0 - 0:1). The fractions eluted by 80% and 100% MeOH were further subjected to silica gel column chromatography using a CH_2Cl_2 - MeOH gradient system (1:0 - 0:1). The fraction eluted by 10% MeOH was finally recrystallized and subjected to ODS HPLC with an 30% CH_3CN / 0.05% TFA solvent system to give pseudostellarin D (400 mg), E(850 mg) and F (125 mg) as colourless needles.

Pseudostellarin D (1). - Colourless needles, m.p. 177-179°C (from MeOH), $[\alpha]_D$ -64.8° (c 0.54, MeOH); *m/z* 714 (Found: M^++H, 714.4184. $C_{36}H_{56}N_7O_8$ requires, 714.4190); ν_{max} (KBr)/cm^{-1} 3300 (NH) and 1650 (amide C=O); λ_{max} (MeOH) / nm 276 (ε 1800).

Pseudostellarin E (2).- Colourless needles, m.p. 168-170°C (from MeOH), $[\alpha]_D$ -112.1° (c 0.33, MeOH); *m/z* 878 (Found: M^++H, 878.5154. $C_{45}H_{68}N_9O_9$ requires, 878.5140); ν_{max} (KBr)/cm^{-1} 3350 (NH) and 1650 (amide C=O); λ_{max} (MeOH) / nm 257 (ε 230).

Pseudostellarin F (3). - Colourless needles, m.p. 169-171°C (from MeOH), $[\alpha]_D$ -58.9° (c 0.98, MeOH); *m/z* 785 (Found: M^++H, 785.4181. $C_{38}H_{57}N_8O_{10}$ requires, 785.4197); ν_{max}(KBr)/cm^{-1} 3350 (NH) and 1650 (amide C=O); λ_{max} (MeOH) / nm 276 (ε 1660).

Acid Hydrolysis of **1** - **3**

Solutions of **1** - **3** (each containing 1 mg of peptide) in 6N HCl were heated at 110°C for 24h. 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.

Absolute Configuration of Amino Acids[5]

Solutions of **1** - **3** (each containing 1 mg of peptides) in 6N HCl were heated at 110° for 12h. 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. The t_R values were L-Ser 20.25, L-Pro 28.04, L-Tyr 31.63, L-Val 33.75, L-Ile 40.46, L-Phe 40.79 and L-Leu 41.08 min, respectively.

Enzymatic Hydrolysis of **2**

α-Chymotrypsin (500 μg dissolved in 50 μl of 0.001% HCl, Wako Pure Chemical Industries, substrate-enzyme ratio, 400:1) was added to **2** (10 mg) in NH_4HCO_3 solution (1%, 0.9 ml) and the digestion was performed at 35 °C with the pH maintained at 8.0 by the manual addition of 0.1N HCl. After 24 h the reaction was stopped by adjusting the solution to pH 2.2 with 1N HCl. The digestion mixture was lyophilized to dryness and hydrolysates were subjected to HPLC (Inertsil PREP-ODS column, 20mm i.d.×250mm, GL Science Inc., packed with 10μm ODS, eluted with 30% CH_3CN / 0.05%TFA) to give compound **4** (8 mg), amorphous powder, $[\alpha]_D$ -134.1° (c 0.08, MeOH).

Enzyme Assay of Tyrosinase Inhibitory Activity

Tyrosinase inhibitory activity was assayed by the dopachrome method[11] with slight modification. Twenty five μl of mushroom tyrosinase solution (1000 U/ml, Sigma Chemical Co.), 37.5 μl of L-tyrosine (2.5 mM), 37.5 μl of 0.4 M HEPES buffer (pH 6.8) and 50 μl of ethanol with or without the test specimen, were mixed in a 96-well plate, incubated at 37°C for 15 minutes, and the absorbance of each well measured at 475 nm with a BIO-RAD plate reader (Model 3550), before and after incubation. The percentage inhibition of tyrosinase was calculated as follows:

$$\text{Tyrosinase inhibition (\%)} = \{(D-C)-(B-A)\}/(D-C) \times 100$$

A and B represent the absorbance in the presence of the test specimen before and after incubation, respectively. C and D represent the absorbance without the test specimen, before and after incubation, respectively.

Inhibitory Effect on Melanogenesis using Cultured B16 Melanoma Cells

Inhibitory effect on melanogenesis was performed in a 6-well plate.[13] Mouse B16 melanoma cells (2.4×10^4 cells/well) were inoculated in each well containing Dulbecco modified MEM medium supplemented with 10% fetal calf serum. After 2 d incubation (37°C, 5% CO_2), the medium was exchanged and the samples at various concentrations were added to each well and the plates were incubated for 4 d. Then after trypsin treatment, melanin and protein contents were measured. Melanin inhibitory activity (IC_{50}) was calculated by comparison of melanin and protein contents in control groups.

Acknowledgments: We are grateful to Miss Sachiko Shirota, Dr. Ritsuo Aiyama and Mr. Minoru Ichioka of Yakult Central Institute for Microbiological Research for the biological evaluations.

References and Notes

1) Cyclic Peptides from Higher Plants. Part 7., Part 6, H. Morita, T. Kayashita, H. Kobata, A. Gonda, K. Takeya and H. Itokawa, *Tetrahedron*, **1994**, *50*, 6797.
2) Chinese Academy of Medical Sciences, *Zhong yao zi*, **1985**, *2*, 266. The People's Health Publishing House, Beijing.
3) H. Itokawa, H. Morita, K. Takeya, N. Tomioka, A. Itai and Y. Iitaka, *Tetrahedron*, **1991**, *47*, 7007; H. Itokawa, H. Morita, K. Takeya, N. Tomioka and A. Itai, *Chem. Lett.*, **1991**, 2217; H. Itokawa, T. Yamamiya, H. Morita and K. Takeya, *J. Chem. Soc. Perkin Trans. 1*, **1992**, 455; H. Morita, T. Yamamiya, K. Takeya and H. Itokawa, *Chem. Pharm. Bull.*, **1992**, *40*, 1352; H. Itokawa and K. Takeya, *Heterocycles*, **1993**, *35*, 1467; K. Takeya, T. Yamamiya, H. Morita and H. Itokawa, *Phytochemistry*, **1993**, *33*, 613; H. Morita, S. Nagashima, K. Takeya and H. Itokawa, *Chem. Pharm. Bull.*, **1993**, *41*, 992; H, Morita, S. Nagashima, O. Shirota, K. Takeya and H. Itokawa, *Chem. Lett.*, **1993**, 1877; H. Morita, S. Nagashima, K. Takeya and H. Itokawa, *Heterocycles*, submitted.; H. Morita, S. Nagashima, K. Takeya, H. Itokawa and Y. Iitaka, *J. Chem. Soc. Perkin Trans. 1*, submitted.
4) H. Morita, H. Kobata, K. Takeya and H. Itokawa, *Tetrahedron Lett.*, **1994**, *35*, 3563.
5) P. Marfey, *Carlsberg Res. Commun.*, **1984**, *49*, 591.
6) A. Bax and S. Subramanian, *J. Magn. Reson.*, **1986**, *67*, 565 .
7) A. Bax and M. F. Summers, *J. Am. Chem. Soc.*, **1986**, *108*, 2093 .
8) G. Bodenhauser, H. Koger and R. R. Ernst, *J. Magn. Reson.*, **1984**, *58*, 370.
9) A. P. Bruins, T. R. Corey and J. D. Henion, *Anal. Chem.*, **1987**, *59*, 2642.
10) "Tandem Mass Spectrometry," ed. by F. W. McLafferty, Wiley, New York (1983).
11) V. J. Hearing, Methods in Enzymology, **1987**, *142*, 154.
12) H. Kawagishi, A. Somoto, J. Kuranari, A. Kimura and S. Chiba, *Tetrahedron Lett.*, **1993**, *34*, 3439.
13) S. Akiu, Y. Suzuki, T. Asahara, Y. Fujinuma and M. Fukuda, *Jpn. J. Dermatol.*, **1991**, *101*, 609.

(*Received in Japan* 23 *May* 1994; *accepted* 22 *June* 1994)