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PIPERASTATIN A, A NEW SELECTIVE SERINE CARBOXYPEPTIDASE INHIBITOR PRODUCED BY ACTINOMYCETE II. PHYSICO-CHEMICAL PROPERTIES AND STRUCTURE DETERMINATION

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Piperastatin A, a new inhibitor of serine carboxypeptidase, was discovered in the culture broth of *Streptomyces lavendofoliae* MJ908-WF13. The molecular formula of piperastatin A was established as $C_{38}H_{67}N_9C_{10}$ by HRFAB-MS and elemental analysis. The structure was determined to be N-formyl-*allo* Ile-Thr-Leu-Val-Pip-Leu-Pip (Pip = hexahydropyridadine-3-carboxylic acid) by various spectral analyses of piperastatin A and piperastatin A methylester.

KEY WORDS: Serine carboxypeptidase, carboxypeptidase Y, enzyme inhibitor, peptide, structure determination, natural product

INTRODUCTION

In the preceding paper, we have described the taxonomy and fermentation of the producing strain, as well as the purification and biological properties of piperastatin A.[1] In this paper, we describe the physico-chemical properties and structure determination of piperastatin A, a major component of inhibitors from *Streptomyces lavendofoliae* MJ908-WF13.

MATERIALS AND METHODS

Chemicals

Chemicals employed were as follows: TLC-plate Silica gel F254 (0.25 mm thickness) from E. Merck, Darmstadt, FRG; acetonitrile (HPLC grade) and 0.25 M lithium citrate buffer (pH 2.2) from Wako Pure Chemical Industries, Ltd., Osaka, Japan; reversed phase column

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Abbreviations: CPC, centrifugal partition chromatography; FAB-MS, fast atom bombardment mass spectrometry; HRFAB-MS, high resolution FAB-MS; DEPT, distortionless enhancement by polarization transfer; COSY, correlated spectroscopy; HSQC, heteronuclear single quantum coherence; HMBC, heteronuclear multiple bond correlation.

of Capcellpak C18 SG120 ϕ 4.6 × 150 mm from Shiseido Co., Tokyo, Japan. All other chemicals were of analytical grade.

Analytical Instruments

Amino acid analysis was performed on a Hitachi 850 automatic amino acid analyzer. Amino acid sequence was determined by a ABI 477A/120A peptide sequencer system. NMR spectra were recorded on a JEOL JNM-A500 NMR spectrometer and mass spectra were obtained using a JEOL JMS-SX102 spectrometer. UV spectrum was recorded on a Hitachi U-3210 spectrometer. IR spectrum was measured on a Hitachi Model 260–10 spectrometer. Optical rotation was determined using a Perkin-Elmer 241 polarimeter. Melting point was measured on a Yanaco melting point apparatus MP500D.

Hydrolysis of Piperastatin A

Piperastatin A (0.05 mg) was hydrolyzed at 110°C for 20 h with 6 N HCl (0.15 ml) using a vacuum hydrolysis tube (Pierce, Illinois, USA). After removal of the solvents, the hydrolysate was dissolved in 0.25 M lithium citrate buffer (pH 2.2) and subjected to the amino acid analyzer. The amino acids of the hydrolysate were determined as 1 mole of valine, threonine, *allo*-isoleucine and 2 moles of leucine.

Preparation of Deformylpiperastatin A

Piperastatin A (3.0 mg) was treated with 2N HCl (0.05 ml) in MeOH (0.05 ml) at room temperature for 48 h. After neutralization by 1 M $NaHCO_3$, the product was purified by HPLC: a Gilson's HPLC system consisting of model 303 pumps and a model 201 fraction collector, equipped with a Waters 991J photodiode array detector. The chromatography was performed using a Shiseido capcellpak C18 S-5 μm reversed-phase column (4.6 × 150 mm) by a linear gradient of aqueous acetonitrile solution (20–60% in 30 min) at a flow rate of 1.0 ml/min to give deformylpiperastatin A (1.8 mg). The Rf value was 0.33: silica gel TLC ($CHCl_3$-MeOH-H_2O, 65:25:2). The molecular weight was 781 by FAB-MS spectrum. The amino acid composition (determined as described in the hydrolysis of piperastatin A) was the same as that of piperastatin A.

Preparation of Piperastatin A methylester

Piperastatin A (10.0 mg) in 0.8 ml MeOH was treated with 0.08 ml of 10% trimethylsilyl-diazomethane solution in hexane for 30 min at room temperature. The reaction mixtures were evaporated under reduced pressure and separated by preparative silica gel TLC plate ($CHCl_3$-MeOH, 20:1). The crude product was further purified by centrifugal partition chromatography (CPC) to give piperastatin A methylester (9.1 mg). The CPC apparatus was model NMF (Sanki Engineering Ltd., Japan) and the chromatography was performed under the following conditions: solvent system, $CHCl_3$-MeOH-H_2O (2:2:1); ascending mode; flow rate, 5 ml/min; centrifugation, 700 rpm; temperature, 20°C. The Rf value of piperastatin A methylester was 0.56 : silica gel TLC ($CHCl_3$-MeOH, 10:1). The molecular weight was 823 by FAB-MS spectrum.

TABLE 1
Physico-chemical properties of piperastatin A.

Appearance	colourless needles
Melting point	225~228°C (dec.)
FAB-MS (positive, m/z)	810 $(M+H)^+$
Molecular weight	809
Molecular formula	$C_{38}H_{67}N_9O_{10}$
Elemental analysis	
Found	C56.01 H8.46 N15.55%
Calc for $C_{38}H_{67}N_9O_{10}$	
	C56.35 H8.34 N15.56%
HRFAB-MS (positive, m/z)	
Found	810.5067 $(M+H)^+$
Calc for $C_{38}H_{68}N_9O_{10}$	
	810.5089
$[\alpha]_D^{27.5}$ (*C* 0.35, MeOH	−38.6°
UV λ max, MeOH nm	end absorption
IR ν max cm^{-1}	3400, 3310, 2960, 2950, 2880, 1650, 1530, 1480,
	1470, 1410, 1390, 1360, 1240, 1160, 920
Rf value on TLC	0.64 (1-BuOH-MeOH-H_2O, 4:1:2, Silica gel)
Colour reaction	Mo-H_2SO_4, Greig-Leaback
Solubility	soluble in MeOH, EtOH, DMSO

RESULTS AND DISCUSSION

Physico-chemical Properties of Piperastatin A

The physico-chemical properties of piperastatin A were summarized in Table 1. The molecular formula was determined to be $C_{38}H_{67}N_9O_{10}$ by HRFAB-MS, ^{13}C NMR and elemental analysis. The IR spectrum indicated the presence of NH or OH groups ($3300 \sim 3400\ cm^{-1}$) and peptide bonds (1650 and $1530\ cm^{-1}$). The UV spectrum showed end adsorption in MeOH. Piperastatin A is soluble in MeOH, EtOH and DMSO. It gives positive colour reaction with Greig-Leaback and Phosphomolybdate-H_2SO_4, (Mo-H_2SO_4).

Structure Determination of Piperastatin A

The molecular formula of piperastatin A was established as $C_{38}H_{67}N_9O_{10}$ by HRFAB-MS and elemental analysis. All 38 carbons were detected in the ^{13}C NMR spectrum measured in DMSO-d_6 (Table 2). The DEPT spectra revealed the presence of 31 carbons: 9 methyls, 9 methylenes, 13 methines, all of which were confirmed by the HSQC spectrum. The ^1H NMR spectrum showed 7 exchangeable proton signals (Another two exchangeable proton signal were not observed.).

The amino acid analysis of piperastatin A gave 1 mole of valine, threonine, *allo*-isoleucine and 2 moles of leucine, and the presence of these amino acid residues was supported by

TABLE 2
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) chemical shifts of piperastatin A in DMSO-d_6.

	δ_c ppm	δ_H ppm (J in Hz)
Formyl		
CHO	161.1	8.07 d(J=1.0)
allo-Isoleucine		
NH		8.24 dd(J=9.3,1.0)
CO	170.7	
α CH	54.4	4.46 dd(J=9.3,3.9)
β CH	36.5	1.85 m
γ CH_3	14.1	0.78 d(J=6.4)
γ CH_2	25.8	1.10,1.31 m
δ CH_3	11.5	0.85 t(J=7.1)
Threonine		
NH		7.84 d(J=8.8)
CO	169.9	
α CH	58.3	4.23 dd(8.8,3.9)
β CH	66.7	3.95 dq(J=6.4,3.9)
γ CH_3	19.6	1.00 d(J=6.4)
Leucine-1		
NH		7.80 d(J=7.8)
CO	171.5	
α CH	51.1	4.34 m
β CH_2	40.3	1.45 m
γ CH	23.8	1.61 m
δ CH_3	21.2	0.81 d(J=6.4)
δ CH_3	23.1	0.85 d(J=6.4)
Valine		
NH		7.61 d(J=9.3)
CO	172.0	
α CH	53.5	5.10 dd(9.3,6.0)
β CH	30.1	2.01m
γ CH_3	17.3	0.78 d(J=6.8)
γ CH_3	19.5	0.81 d(J=6.8)
Piperazic acid-1		
CO	173.2	
α CH	49.9	4.94 m
β CH_2	26.5	1.57, 2.11 m
γ CH_2	20.7	1.43 m

TABLE 2
Continued.

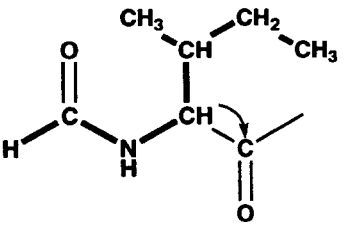
	δ_c ppm	δ_H ppm (J in Hz)
δ CH_2	46.6	2.53,3.00 m
NH		4.93 m
Leucine-2		
NH		7.98 d(J=8.3)
CO	173.2	
α CH	47.1	5.22 m
β CH_2	39.6	1.40 m
γ CH	24.4	1.62 m
δ CH_3	21.3	0.84 d(J=6.4)
δ CH_3	23.2	0.88 d(J=6.4)
Piperazic acid-2		
CO	172.3	
α CH	50.8	4.93 m
β CH_2	25.1	1.61, 2.11 m
γ CH_2	21.4	1.50 m
δ CH_2	46.5	2.68,2.94 m
NH		4.59 br d(J=12.2)

the ^1H-^1H COSY and HMBC spectra (Figure 1). The ^1H-^1H COSY spectrum showed that a formyl group masked the amino group of *allo*-isoleucine. The presence of the other components, 2 moles of piperazic acid (hexahydropyridadine-3-carboxylic acid) was also suggested by these spectra.

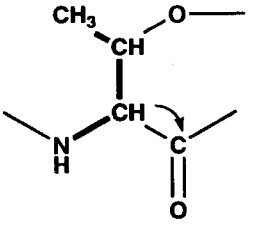
In order to determine the sequence of amino acid residues, deformylpiperastatin A was prepared and subjected to an amino acid sequence analyzer. As a result, the partial sequence, *allo*-isoleucyl-threonyl-leucyl-valine was established, but no more amino acid residues after the 4th Edman degradation cycles were detected.

The complete sequence was determined by the HMBC spectra of piperastatin A and piperastatin A methylester (Table 3). The 5 amide protons in piperastatin A were coupled respectively with 5 amide carbonyl carbons as follows: δ_H 8.24 (*allo*-isoleucine) to δ_c 161.1 (formyl), δ_H 7.84 (threonine) to δ_c 170.7 (*allo*-isoleucine), δ_H 7.80 (leucine-1) to δ_c 169.9 (threonine), δ_H 7.61 (valine) to δ_c 171.5 (leucine-1) and δ_H 7.98 (leucine-2) to δ_c 173.2 (piperazic acid-1) and the proton at δ_H 8.07 (formyl) was also coupled to the α carbon at δ_c 54.4 (*allo*-isoleucine). From these connectivities three partial structures, as shown in Figure 2(a) were established. The carboxyterminal amino acid was determined by the HMBC spectrum of piperastatin A methylester. The ^1H-^{13}C long range coupling from the methyl protons at δ_H 3.75 (methylester) to the carbonyl carbon at δ_c 171.2 (piperazic acid-2) revealed that piperazic acid-2 was the carboxyterminal (Figure 2(b)). Thus, the complete sequence of piperastatin A was determined as shown in Figure 2(c).

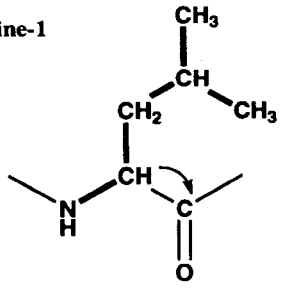
Formyl *allo*-isoleucine



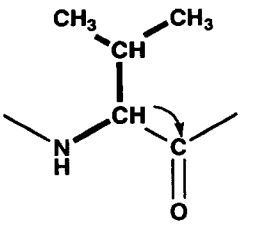
Threonine



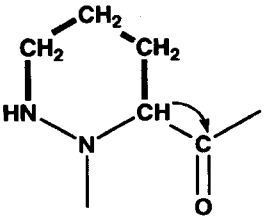
Leucine-1



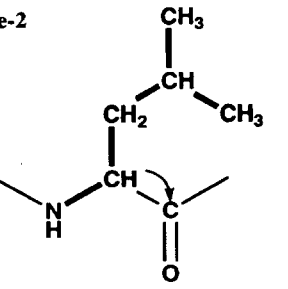
Valine



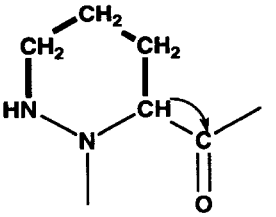
Piperazic acid-1



Leucine-2



Piperazic acid-2



Connectivities observed in the ^{1}H-^{1}H COSY spectrum

^{1}H-^{13}C long-range correlations observed in the HMBC spectrum

FIGURE 1 Structure of amino acids of piperastatin A elucidated by ^{1}H-^{1}H COSY and HMBC.

TABLE 3
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) chemical shifts of piperastatin A methylester in acetone-d_6.

	δ_c ppm	δ_H ppm (J in Hz)
Formyl		
CHO	163.3	8.32 s
allo-Isoleucine		
NH		7.80 br d(7.0)
CO	171.7	
α CH	57.2	4.46 m
β CH	37.3	2.07 m
γ CH_3	14.8	0.96 d(J=6.9)
γ CH_2	27.1	1.29,1.50 m
δ CH_3	12.1	0.93 t(J=7.8)
Threonine		
NH		7.43 br d(J=6.5)
CO	171.7	
α CH	59.9	4.26 m
β CH	67.6	4.30 m
γ CH_3	20.3	1.33 d(J=6.3)
Leucine-1		
NH		7.44 br d(J=7.9)
CO	172.8	
α CH	53.4	4.43 m
β CH_2	41.3	1.68 m
γ CH	25.4	1.76 m
δ CH_3	23.7	0.92 d(J=6.6)
δ CH_3	21.7	0.87 d(J=6.6)
Valine		
NH		7.25 br d(J=8.9)
CO	174.5	
α CH	54.5	5.31 m
β CH	31.5	2.10 m
γ CH_3	20.2	0.90 d(J=6.9)
γ CH_3	17.7	0.85 d(J=6.9)
Piperazic acid-1		
CO	172.3	
α CH	52.0	5.14 m
β CH_2	26.8	1.76,2.29 m
γ CH_2	22.1	1.54,1.61 m

TABLE 3
Continued.

	δ_c ppm	δ_H ppm (J in Hz)
δ CH_2	48.0	2.74,3.09 m
NH		4.80 br d (J=10.4)
Leucine-2		
NH		7.32 br d(J=8.3)
CO	174.8	
α CH	48.9	5.42 m
β CH_2	41.4	1.53 m
γ CH	25.8	1.73 m
δ CH_3	23.8	0.92 d(J=6.5)
δ CH_3	22.0	0.92 d(J=6.5)
Piperazic acid-2		
CO	171.2	
α CH	52.7	5.20 m
β CH_2	26.2	1.87, 2.22 m
γ CH_2	22.1	1.52, 1.59 m
δ CH_2	47.7	2.91, 3.04 m
NH		4.51 dd(J=12.3, 1.8)
OCH_3	52.7	3.75 s

The amino acid sequence was confirmed by FAB-MS fragments analyses of piperastatin A and piperastatin A methylester (Figure 3). The fragment ion peaks appeared at m/z 243, 356, 455, 567, 680 (N^+, N-terminal fragments) and 131 ($C^+ + 2$, C-terminal fragment) for piperastatin A, m/z 243, 356, 455, 567, 680 (N^+, N-terminal fragments) and 145 ($C^+ + 2$, C-terminal fragment) for piperastatin A methylester.

We discovered piperastatin A, a selective serine carboxypeptidase inhibitor, in the culture broth of *Streptomyces lavendofoliae* MJ908-WF13 and determined its structure to be N-formyl-*allo* Ile-Thr-Leu-Val-Pip-Leu-Pip (Pip = hexahydropyridadine-3-carboxylic acid) by various spectral analyses of piperastatin A and piperastatin A methylester. We have already discovered and characterized β-lactone-containing serine carboxypeptidase inhibitors, belactins A and B.[2,3] While, piperastatin A is a linear heptapeptide of which N-terminal is masked by a formyl group and contains 2 moles of the unusual amino acid, piperazic acid in the molecule. These structural characteristics show that piperastatin A is a new type of serine carboxypeptidase inhibitor. Among the structural characteristics of this compound, the N-formyl group is not important for the inhibitory activity because deformylpiperastatin A is found to retain its inhibitory activity against CP-Y (data not shown). Piperazic acid has been found in azinothricin,[4] A83586,[5] variapeptin or citrupeptin,[6] but the role of piperazic acid moiety to exhibit biological activity has not been clarified. On this aspect, the studies on piperastatin A may be interested in establishing the structure-activity relationship of these compounds. Being a linear analog of an antimicrobial

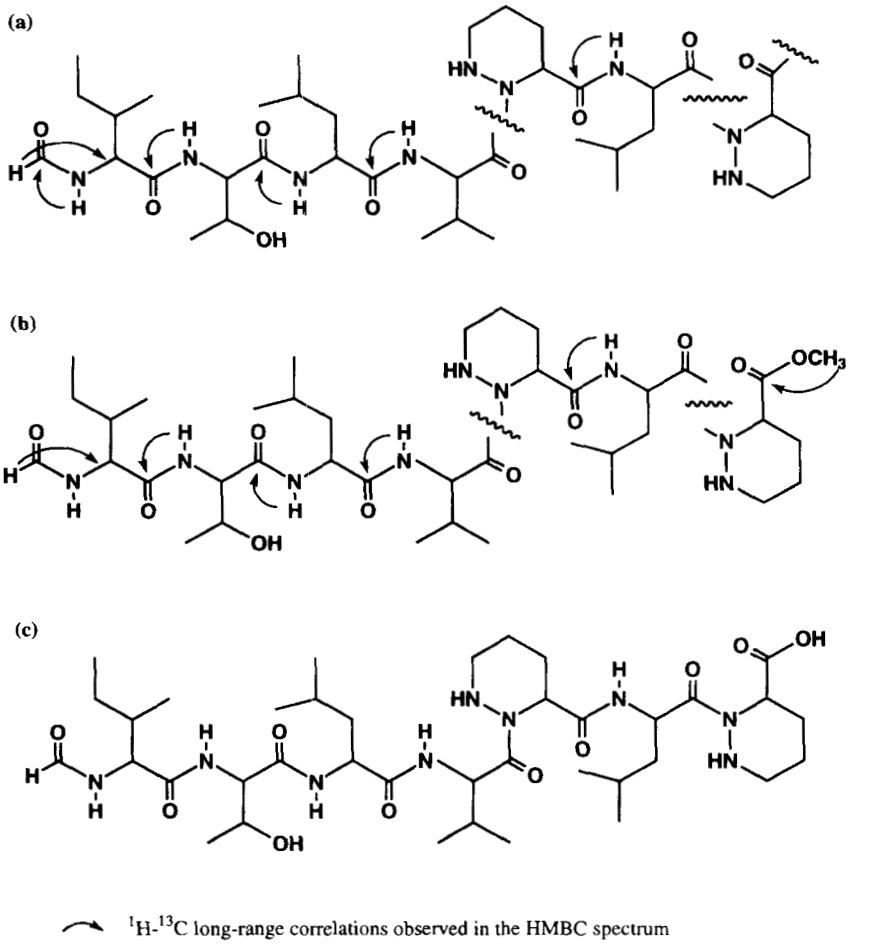


FIGURE 2 Complete structure of piperastatin A elucidated by HMBC spectra of piperastatin A and piperastatin A methylester.
(a) partial structures of piperastatin A revealed by HMBC experiment, (b) partial structures of piperastatin A methylester revealed by HMBC experiment, (c) complete structure of piperastatin A.

cyclic depsipeptide, depsidomycin (the absolute configuration has not been reported yet),[7] piperastatin A does not have antimicrobial activity but has highly selective inhibitory activity against serine carboxypeptidases as described in the preceding paper.[1] The structure-activity relationship of piperastatin A and depsidomycin including the absolute configuration of the amino acid residues remains to be elucidated.

In order to obtain more information about the physiological roles of serine carboxypeptidases, it would be advantageous for us to apply selective inhibitors such as piperastatin A, belactins A and B in biochemical, cell biological or pharmaceutical studies.

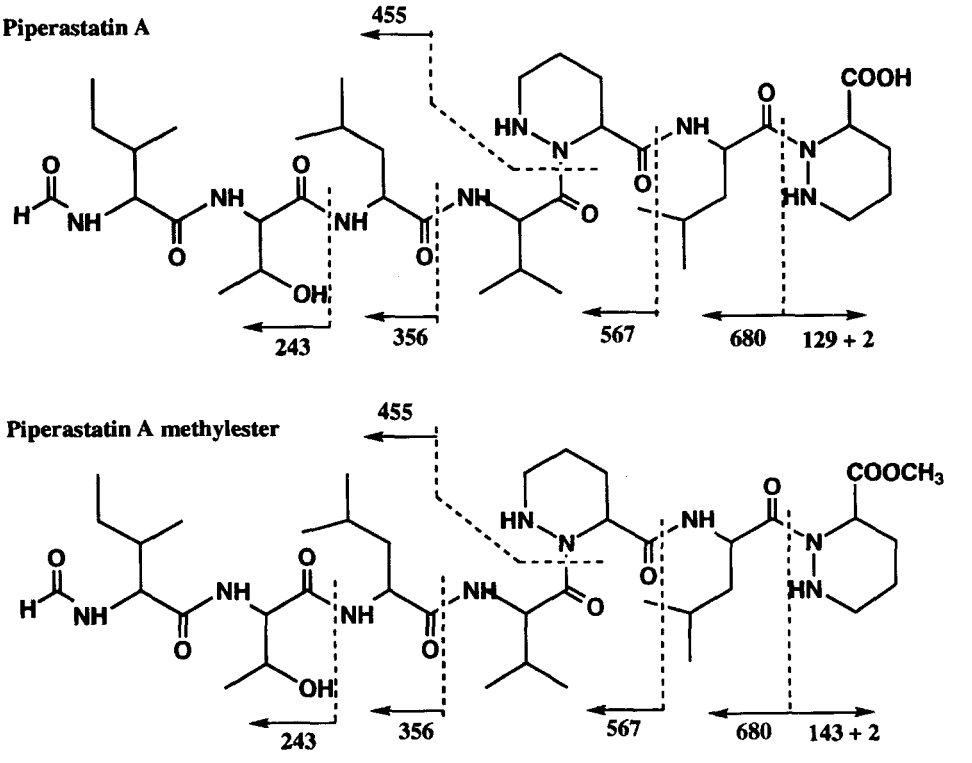


FIGURE 3 FAB-MS fragment analyses of piperastatin A and piperastatin A methylester.

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