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Valistatin (3-Amino-2-Hydroxy-4-Phenylbutanoyl-Valyl-Valine), a New Aminopeptidase M Inhibitor, Produced by *Streptomyces* sp. SL20209

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Valistatin, a new inhibitor of aminopeptidase M(AP-M) was discovered in the culture broth of *Streptomyces* sp. SL20209 isolated from a soil sample. The inhibitor was purified by extraction with *n*-butanol and the various column chromatographies, and then isolated as whitish powder. The ^{1}H- and ^{1}H, ^{1}H-COSY NMR studies, amino acid analysis, and fragmentation patterns by FAB-MS suggested the presence of one 3-amino-2-hydroxy-4-phenylbutanoic acid and two valine residues in the inhibitor. Thus, the structure of valistatin was determined as 3-amino-2-hydroxy-4-phenylbutanoyl-valyl-valine. Valistatin has the molecular formular $C_{20}H_{31}N_3O_5$ (MW 394), and its IC_{50} value against hog kidney AP-M was determined to be 3.12 μg/ml.

Aminopeptidase M (AP-M; EC 3.4.11.2) is a membrane-bound metallo exopeptidase which can hydrolyze N-terminal peptide bonds on the surface of various cells and tissues (9). It is stalked into integral membranes and mainly located in the small intestinal and kidney brush borders but also found in brain, lung, liver and fibroblasts (8, 9). It has been speculated that this enzyme might participate in the various cellular phenomena including the metabolism of regulatory molecules in mammals. Especially, in the brain AP-M has been found to be involved in the degradation of neuropeptides such as enkephalins, the endogenous opioid peptides with the morphine-like action (5, 7). It has also been found that the aminopeptidase inhibitors block the *in vivo* metabolism of enkephalins leading to analgesic effects (6, 10).

On the other hand, Umezawa *et al.* started the screening of aminopeptidase (AP) inhibitors to elucidate the role of aminopeptidases (APs) in 1976 and thereafter discovered a number of AP inhibitors such as bestatin (13), amastatin (1), arphamenines (12), actinonin (14), probestin (3), leuhistin (2) and so on. Among these inhibitors, bestatin, an inhibitor against aminopeptidase B (AP-B, EC 3.4.11.6) which hydrolyses an N-terminal peptide bond containing L-arginine or L-lysine has the structure of L(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine (11) and has been reported to have therapeutically useful effects including immunopotentiation, antitumor activity and analgesia (4). In practice bestatin has been used as a drug for the treatment of nonlymphocytic leukemia in adults since 1987 in Japan. The structure of probestin discovered as an inhibitor against AP-M was reported to be (2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucyl-L-prolyl-L-proline.

In order to search new AP-M inhibitor useful as drug from the secondary metabolites of microorganims, we screened the inhibitory activities of microbial cultures against AP-M and isolated an inhibitor, named as valistatin from *Streptomyces* sp. SL20209. In this paper we report the isolation and determination of the structure of valistatin.

MATERIALS AND METHODS

Chemicals

Chemicals employed were as follows: Amberlite XAD-4 and Dowex-50W from Sigma Co. (USA); MCI gel CHP-20P from Mitsubishi Co.(Japan); DEAE-Sephadex A-25 and Sephadex LH-20 from Pharmacia Co. (Sweden); Packed column of Maxil 5C18 from Phenomenex Co. (USA); L-Leucine-p-nitroanilide from Sigma Co.(USA). All other chemicals were of analytical grade.

Enzyme

AP-M (EC 3.4.11.2) of hog kidney was purchased from

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Sigma Co. (USA).

Microorganism

Strain SL20209 was isolated from a soil sample in the Screening Room, Genetic Engineering Research Institute, KIST. This strain was identified as *Streptomyces* sp. SL 20209, and its taxonomic studies will be published elsewhere.

Assay for AP-M Inhibitory Activity

The principle of the assay for AP-M was based on the absorbance at 405 nm of p-nitroanilide measured by microplate reader (BioRad 3550). The reaction mixture contained 160 μl of L-leucine-p-nitroanilide (0.125 mg/ml in a 0.1 M Tris-HCl buffer, pH 7.0) and 20 μl of water or aqueous solution containing the test compound. After the reaction mixture was incubated at 37℃ for 3 minutes, 20 μl of AP-M solution (1 mU in 0.1 M Tris-HCl buffer, pH 7.0) was added. After allowing the mixture to incubate at 37℃ for 30 minutes, the absorbancy was read at 405 nm. The percent inhibition was calculated by the formula (D-C)-(B-A)/(D-C)×100, where B and D are the measured values by the enzymatic reaction with (B) and without (D) an inhibitor, respectively, A and C are those by non-enzymatic reaction with (A) and without (C) an inhibitor, respectively.

Production of AP-M Inhibitor

Streptomyces sp. SL20209 was inoculated into the 100 ml of a production medium (pH 7.0 before sterilization) consisting of glucose 1.0%, soluble starch 2.0%, soybean meal 2.5%, beef extract 0.1%, yeast extract 0.4%, NaCl 0.2% and K_2HPO_4 0.025% in a 500 ml baffled Erlenmeyer flask and cultured at 28℃ for 3 days on a rotary shaker (150 rpm) to obtain a seed culture. Three ml of this seed culture were inoculated into 100 ml of the same medium in 500 ml baffled Erlenmeyer flasks and cultured for 4 days under the same conditions. The production of inhibitor was examined as an inhibitory activity of 10 μl of the culture filtrate against AP-M.

Isolation of AP-M Inhibitor

The culture filtrate of *Streptomyces* sp. SL20209 was adsorbed on a Amberlite XAD-4 column, which was washed with water and eluted with 60% methanol to give active fractions. The active eluate was dissolved in water and adjusted to pH 3.0 with HCl followed by extraction with *n*-butanol. The butanol extract was suspended in a solvent mixture of butyl acetate-butanol-acetic acid-water (3:4:1:1). The suspension was passed through a column of silica gel which had been packed with the same solvent mixture. The active fraction was chromatographed on Dowex-50W equilibrated with water and eluted by a linear gradient between water and 0.5 N NH_4OH. The active eluate was applied to a DEAE-Sephadex A-25 column previously equilibrated with water and the active fraction was obtained by the elution with water. The eluate was dissolved in a small volume of 50% methanol, and the solution was applied to a MCI gel CHP-20P column and eluted with 50% methanol. The active eluate was subjected to Sephadex LH-20 column chromatography and developed with methanol to give a yellowish powder. This powder was further purified by Lobar RP-18 column chromatography with 50% methanol and a reversed phase HPLC with 35% methanol. The active compound was concentrated and isolated as whitish powder.

Amino Acids Analysis

Valistatin (100 μg in 200 μl of D.W) was hydrolyzed at 105℃ for 20 hours with hydrochloric acid (200 μl) in a sealed tube. The hydrolysate was evaporated to remove HCl. Amino acids analysis was carried out on a Pharmacia LKB 4151α Amino Acid Autoanalyzer.

Spectral analysis

The UV spectrum was recorded on a Shimazu UV-260 spectrophotometer, and the IR spectrum on a FT-IR (Laser precision analytical IFX-65S). The ^1H- and ^1H, ^1H-COSY NMR spectra were recorded on a Varian UNITY 300 at 300 MHz. FAB-MS was measured by a Kratose Concept-1S mass spectrometer.

RESULTS AND DISCUSSION

Isolation of AP-M Inhibitor, Valistatin

The inhibitor, valistatin, was produced by the cultivation of *Streptomyces* sp. SL20209 at 28℃ for 4 days on a rotary shaker using an 500 ml baffled Erlenmeyer flasks. The inhibitor was purified from the culture filtrate by extraction with *n*-butanol and a number of column

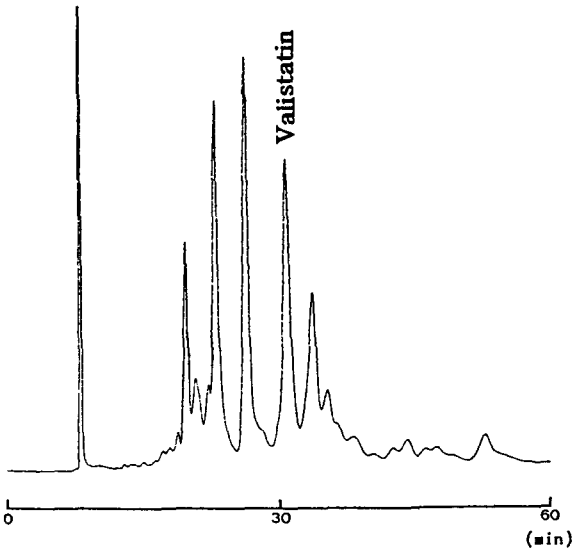

Fig. 1. HPLC profile of valistatin. Column; Phenomenex Maxil 5C18 (250×10.6 mm, 5 μm), Wavelength; 210 nm, Flow rate; 2.0 ml/min, Eluant; 35% MeOH.

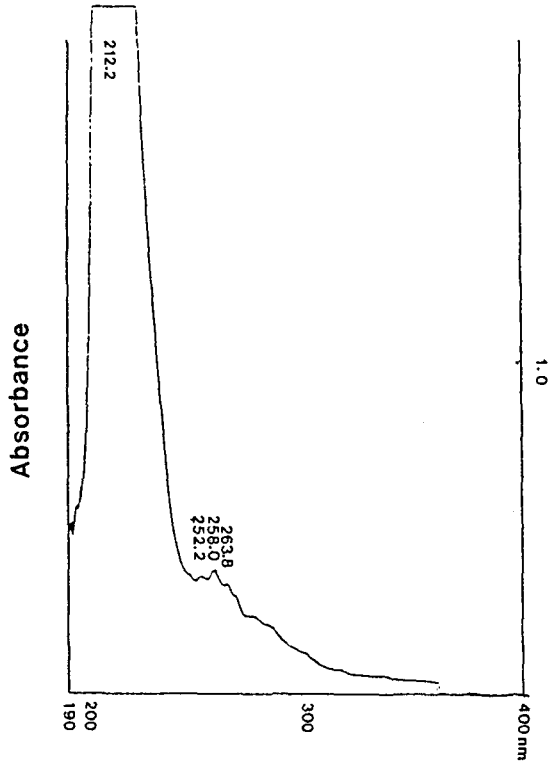

Fig. 2. UV spectrum of valistatin.

chromatographies. Fig. 1 showed a reversed phase HPLC profile of the active eluate passed through Lobar RP-18 column. One peak with 31 minutes of retention time was active against AP-M. Through preparative HPLC under the same conditions, the active compound named as valistatin was isolated as a whitish powder.

Determination of the Structure of Valistatin

The UV spectrum of valistatin showed the maxima at 252, 258, and 264 nm in methanol indicating the presence of aromatic ring in the molecule (Fig. 2). The IR (KBr) spectrum showed absorption at 1650 and 32 00-3400 cm^{-1} suggesting the presence of peptide bonds (Fig. 3). From the above results it was anticipated that valistatin contained amino acids, therefore amino acid analysis of HCl-hydrolysate of valistatin was performed. As shown in Fig. 4, amino acid analysis suggested that valine and an unknown amino acid existed in molar ratio 2:1. Accordingly, there was the possibility that valistatin consisted of one molecule of unknown amino acid and two of valines. The assignment of proton was determined by the ^{1}H-NMR data including ^{1}H, ^{1}H-COSY (Table 1 and Fig. 5). The ^{1}H-NMR data indicated the presence of five proton signals at 7.3 ppm, which appeared to be a phenyl group, and proton signals of four methyl groups (at 0.89, 0.92, 0.97, and 0.99 ppm, respectively), those of one methylene group (at 2.14 and 3.03 ppm) and those of six methine groups (at 2.14, 2.22, 3.59, 4.05, 4.11 and 4.18 ppm, respectively). It also confirmed

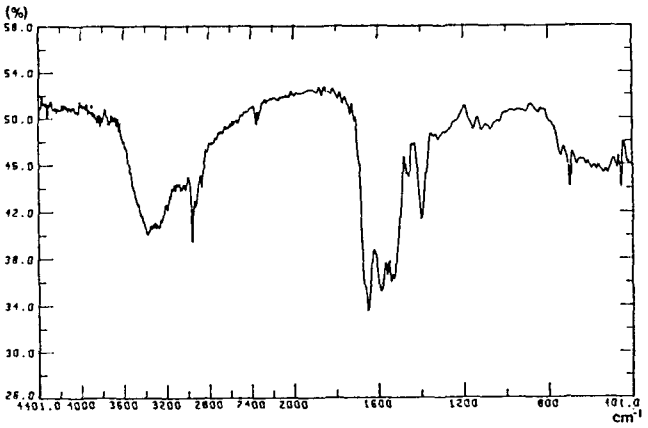

Fig. 3. IR spectrum of valistatin.

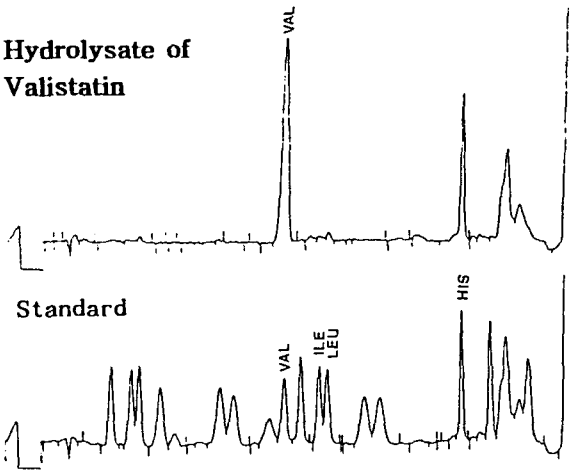

Fig. 4. Amino acids analysis of the acid-hydrolysate of valistatin.

the presence of one -CH_2-CH-CH- and two of $(CH_3)_2$ -CH-CH- by ^{1}H, ^{1}H-COSY NMR spectrum (data not shown). In the fragmentation patterns of valistatin by FAB-MS (Fig. 6), the parent peak (*m/z* 394) was recognized and the major ion peaks of *m/z* 91, 121($^+$H), 150 and 249 were found. Based on these results, the unknown amino acid detected in amino acid analysis (in Fig. 4) was logically thought to be AHPA (3-amino-2-hydroxy-4-phenylbutanoic acid), N-terminal amino acid of bestatin (11) and probestin (15), because of the existence of five protons at 7.3 ppm in ^{1}H-NMR and ion peaks at *m/z* 91, 121($^+$H) and 150 and FAB-MS spectra. Thus the amino acids obtained by acid hydrolysate of valistatin were compared with reference compounds by amino acid analysis, HPLC and TLC. Amino acid and HPLC analytical peaks of authentic (2*S*, 3*R*)-AHPA hydrolyzed under the same conditions were almost identical with those of the unknown amino acid in valistatin, respectively (Fig. 7 and Table 2). In addition Rf values of the two spots of valistatin hydrolysate de-

Table 1. ^{1}H- and ^{1}H, ^{1}H-COSY NMR[a] data for valistatin in CD_3OD.

Assignment		Valistatin
AHPA	2-CH	4.05[b](d, J=3.0[c])
	3-CH	3.59(m)
	4-CH_2	2.81(dd, J=7.8, 13.5)
		3.03(dd, J=7.8, 13.5)
	Ph-*p, o, m*	7.30(m)
Val_1	α-CH	4.18(d, J=4.8)
	β-CH	2.14(m)
	CH_3	0.92(d, J=6.9)
	CH_3	0.89(d, J=6.9)
Val_2	α-CH	4.11(d, J=7.8)
	β-CH	2.22(m)
	CH_3	0.99(d, J=6.9)
	CH_3	0.97(d, J=6.9)

[a]300 MHz, [b]in ppm, [c]Hz.

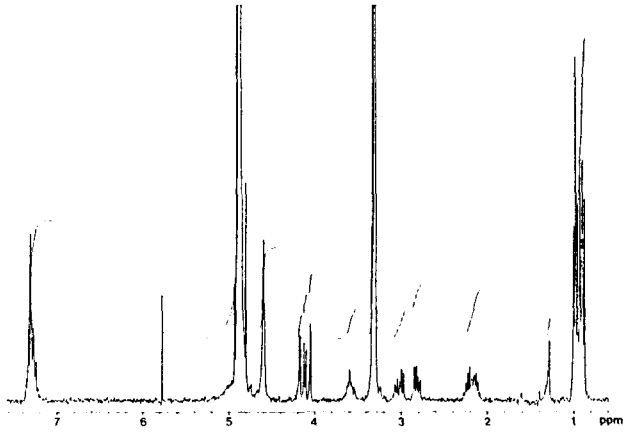

Fig. 5. ^{1}H-NMR spectrum of valistatin in CD_3OD.

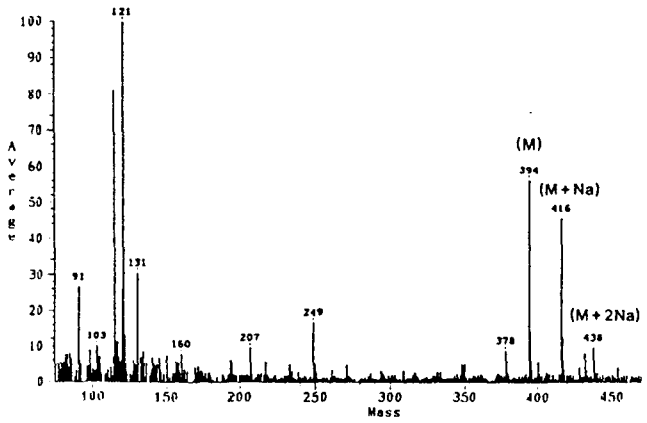

Fig. 6. FAB-MS spectrum of valistatin.

veloped on TLC plate were also identical with those of (2*S*, 3*R*)-AHPA and valine, respectively (Table 2).

Therefore the peaks at *m/z* 249, 150 and 121 were derived from the succesive elimination of Val, Val-Val and Val-Val-CH-OH from the parent peak(*m/z* 394),

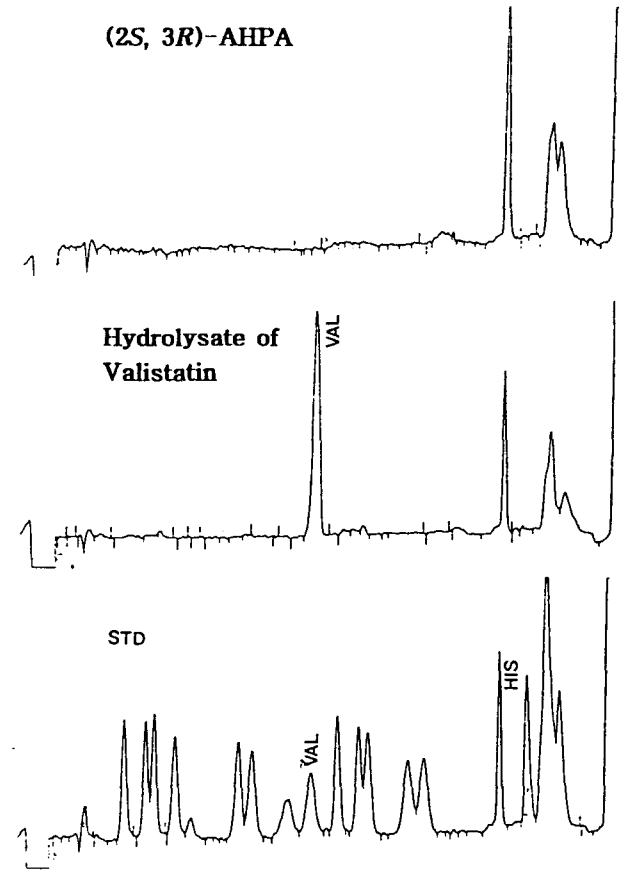

Fig. 7. Amino acids analysis of the hydrolysates of valistatin and authentic (2*S*, 3*R*)-AHPA.

Table 2. Identification data for (2*S*, 3*R*)-AHPA and L-valine composed valistatin by HPLC and TLC analysis.

Compounds	Retention time (min) on HPLC[a]	Rf value on TLC[b]
(2*S*, 3*R*)-AHPA	14.64	0.46
L-Valine	16.22	0.39
Hydrolysate of Valistatin	14.70	0.46

[a]On the reverse column of Lichrosorb RP-18 (250×4.6 mm), flow rate 1.0 ml/min, eluant MeCN-0.1% TFA (12:88). [b]On the silica gel TLC plate 60 F-254 (Merck), mobile phase BuOH-AcOH-Water (4:1:1).

respectively. The peak at *m/z* 91 can be regarded as being derived from the elimination of H_2N-CH-CH-OH from AHPA(*m/z* 150). These fragmentation patterns can arise only from the amino acid sequence of AHPA-Val-Val. Thus, the structure of valistatin was determined to be 3-amino-2-hydroxy-4-phenylbutanoyl-valyl-valine (Fig. 8).

Suda *et al.* (11) reported that the structure of bestatin, an aminopeptidase B inhibitor produced by an actinomycetes, was [(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl]-L-leucine. Thereafter Yoshida *et al.* (15) reported probestin, an inhibitor of aminopeptidase M, having the structure of bestatin-L-prolyl-L-proline. Therefore, valis-

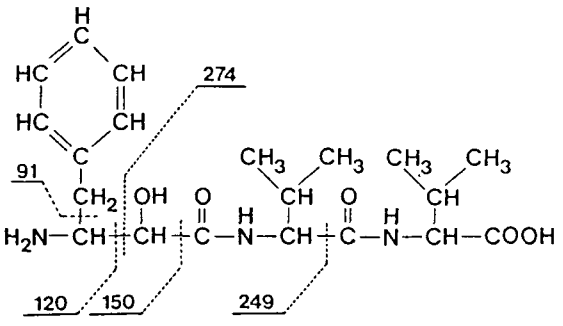

Fig. 8. Structure of valistatin.

tatin, an inhibitor of aminopeptidase M produced by *Streptomyces* sp. SL20209, is a new inhibitor, although AHPA, N-terminal amino acid of valistatin, was identical with those of bestatin (11) and probestin (15).

Physico-Chemical and Biological Properties of Valistatin

Valistatin was soluble in methanol and water, but not in chloroform, ethyl acetate and *n*-hexane. The molecular weight and formular were proposed as $C_{20}H_{31}N_3O_5$ (MW 394) from FAB-MS and ^{1}H-NMR spectra. The spot of valistatin on silica gel TLC plate was visualized by ninhydrin. The IC_{50} value of valistatin against hog kidney AP-M was 3.12 μg/ml. Its value was about 20-fold lower than that of bestatin against AP-M, but about 100-fold higher than that of probestin.

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