POSTSTATIN, A NEW INHIBITOR OF PROLYL ENDOPEPTIDASE, PRODUCED BY *Streptomyces viridochromogenes* MH534-30F3

II. STRUCTURE DETERMINATION AND INHIBITORY ACTIVITIES

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Poststatin, a new inhibitor of prolyl endopeptidase, has been isolated from the culture broth of *Streptomyces viridochromogenes* MH534-30F3. The structure of poststatin was defined as L-valyl-L-valyl-3-amino-2-oxovaleryl-D-leucyl-L-valine by analysis of spectral properties and chemical studies of poststatin and its derivatives. The α-keto group of postine in poststatin plays the most important role on the inhibitory mechanism.

In the preceding paper[1], we have described the taxonomy of the producing strain, fermentation, purification and the biological properties of poststatin, a novel inhibitor of prolyl endopeptidase (PEP). In this paper, we wish to describe the structure of poststatin, and the inhibitory activities of poststatin and its derivatives.

Materials and Methods

Production and Isolation

Poststatin was prepared from fermentation broths of *Streptomyces viridochromogenes* MH534-30F3 as described in the previous paper[1].

Chemicals

Boc-L-Phe-OSu and CBZ-S were purchased from Kokusan Chemical Work; cathepsin B from bovine spleen was from Sigma Chemical Company. All other chemicals were of analytical grade. The packed column of Nucleosil $5C_{18}$ (0.46 × 15 cm) was from Senshu Scientific Co., Tokyo, Japan. The packed column of Capcell Pak C18 SG120 (1 × 25 cm) was from Shiseido Company, Ltd., Tokyo, Japan.

Analytical Instruments

The UV spectrum was recorded on a Hitachi 220S spectrophotometer, and the IR spectrum on a Hitachi 260-10 spectrophotometer. Amino acid analysis was carried out on a Hitachi 835 automatic amino acid analyzer. SI-MS were measured by a Hitachi M-80H mass spectrometer. NMR spectra were recorded on a Jeol JNM-GX400 NMR spectrometer with ^1H NMR at 400 MHz and ^{13}C NMR at 100 MHz. HPLC was performed by a Spectra-physics sp 8700 system. The Edman degradation was carried out using an Applied Biosystem 470A sequence analyzer.

Hydrolysis of Poststatin and Analysis of Amino Acids

Poststatin (1 mg) was hydrolyzed at 150°C for 22 hours with constant boiling hydrochloric acid (200 μl) in a sealed tube. The hydrolysate was evaporated to remove HCl.

Preparation of L-Phenylalanyl Amino Acid

A modification of the procedure described by MITCHELL *et al.*[2], and NISHIKIORI *et al.*[3] was carried

out with L-phenylalanine. A sodium bicarbonate stock solution (100 μl, 23 μmol) was added to a 1 ml reaction tube containing the hydrolysate (2 mg) or known free amino acid (1 mg). The stock solution (100 μl, 22 μmol) of Boc-L-phenylalanine-*N*-hydroxysuccinimide ester (Boc-L-Phe-OSu) in dioxane was added to the amino acid solution. The mixture was allowed to stand at room temperature for 18 hours and evaporated to dryness *in vacuo*. The residue was dissolved in TFA (10 μl) and allowed to stand at room temperature for 30 minutes to remove the Boc group. The TFA was removed by evaporation *in vacuo*. The final residue was extracted with H_2O (200 μl) and filtered through a Millipore filter (SJHV 004 NS). An aliquot of the dipeptide solution thus prepared was injected onto the reverse phase HPLC column.

Determination of L- and D-Amino Acids by HPLC for Diastereometric Dipeptide

The reverse phase column (Nucleosil $5C_{18}$, 0.46 × 15 cm) was operated at a flow rate of 1 ml/minute with a linear gradient from the solvent mixture of A to B over 60 minutes (A: 15% ammonium acetate - acetic acid - H_2O - MeCN, 80 : 1 : 1,600 : 0; B: 80 : 1 : 880 : 720) using a Spectra-physics sp 8700 system. The detection was carried out by monitoring absorbance at 254 nm.

Reduction of Poststatin

To the solution of poststatin (27 mg) in 2 ml of H_2O on an ice water bath under stirring, 56 mg of $NaBH_3CN$ in 1 ml of cold water was added dropwise for 10 minutes. After stirring for 1 hour, the reaction mixture was adsorbed on a Diaion HP-20 column, which was washed with H_2O and eluted with 80% MeCN. Dihydropoststatin (**2**) was further purified by chromatography on a Sephadex LH-20 column (1.5 × 28 cm).

Isolation of 3-Amino-2-hydroxyvaleric Acid (**1**)

2 (25 mg) was hydrolyzed at 150°C for 22 hours with constant boiling hydrochloric acid (4 ml). The hydrolysate was evaporated to remove HCl. The residue was dissolved in H_2O (1 ml) and was subjected to silica gel TLC with BuOH - AcOH - H_2O (4 : 1 : 1). The extract from Rf 0.24 fraction with H_2O, was evaporated to dryness *in vacuo* and further purified by chromatography on a Sephadex LH-20 column (1.5 × 28 cm).

Acetylation of Poststatin

To the solution of poststatin (20 mg) in 2.5 ml of MeOH on an ice water bath under stirring, acetic anhydride was added dropwise. After stirring for 2 hours, the mixture was allowed to stir for 17 hours at room temperature, and evaporated to remove AcOH and MeOH *in vacuo*. *N*-Acetylpoststatin (**3**) was separated by HPLC (Capcell Pak C18, 1 × 25 cm) with a 1 : 4 mixture of MeCN and buffer A (1% citric acid mono hydrate plus 5% potassium acetate pH 5.4). **3** was finally purified by chromatography on a Sephadex LH-20 column (1.5 × 28 cm).

Methylation of Poststatin

The solution of poststatin (30 mg) in 5% anhydrous HCl - MeOH (5 ml) was stirred for 16 hours at room temperature, and evaporated to remove HCl and MeOH *in vacuo*. Poststatin methyl ester (**4**) was separated by HPLC (Capcell Pak C18, 1 × 25 cm) with a 3 : 10 mixture of MeCN and buffer A, and further purified by chromatography on a Sephadex LH-20 column (1.5 × 28 cm).

Methylation of **3**

The methylation of **3** and the purification of *N*-acetylpoststatin methyl ester (**5**) were carried out with the same method described above except that the solvent used for HPLC was a 3 : 7 mixture of MeCN and buffer A.

Benzyloxycarbonylation of Poststatin

To the solution of poststatin (15 mg) in MeOH (1.5 ml) on an ice water bath under stirring, triethylamine (10 μl) and CBZ-S (15 mg) were added. After stirring for 22 hours, AcOH (20 μl) was added. *N*-Benzyloxycarbonylpoststatin (**6**) was purified by chromatography on a Sephadex LH-20 column (1.5 × 28 cm).

Enzyme Assay

PEP was purified from pig kidney by the procedure described by KOIDA and WALTER[4]. The activities of PEP and cathepsin B were measured as described in the previous paper[1].

Results and Discussion

Structure of Poststatin

The UV spectrum of poststatin showed a weak shoulder around 250 nm (ε 649) in MeOH. The IR spectrum of poststatin suggested the presence of peptide bonds (1660 and 1520 cm^{-1}), (Fig. 1). Acid hydrolysis of poststatin gave two ninhydrin positive products on TLC chromatography. The result of amino acid analysis of poststatin indicated th presence of valine and leucine in molar ratio 3:1.

The ^{1}H and ^{13}C NMR data on poststatin are shown in Table 1. The assignments were defined by the confirmation of ^{1}H and ^{13}C NMR data on the intact molecule, **3** and **5**. ^{1}H and ^{13}C NMR data including ^{1}H-^{1}H COSY, ^{13}C-^{1}H COSY and DEPT spectra confirmed the result of amino acid analysis. These NMR spectra indicated the presence of the carbon chain, $CH_3-CH_2-\underset{\underset{NH}{|}}{CH}-$, and the signal attributable to ketone at δ 196.19 and the characteristic signal at δ 160.53, which appeared to be due to an amide function of a α-keto acid. **2** gave a negative reaction to 2,3,5-triphenyltetrazolium chloride reagent. The SI-MS spectrum of **2** showed a molecular ion peak at m/z 544 $(M+H)^+$, which was increased by two mass units in comparison with that of intact poststatin. The ^{13}C NMR spectrum of **2** indicated that two carbonyl carbon signals at δ 196.19 and 160.53 in the spectrum of poststatin changed to methine signals at δ 71.60 and 73.49, and carbonyl signals at δ 170.51 ~ 173.08 in the region of usual amide carbonyl signal. Hydrolysis of **2** gave one ninhydrin positive product (**1**) except for valine and leucine. **1** was isolated by preparative TLC and purified by Sephadex LH-20 column chromatography. The SI-MS spectrum of **1** showed the molecular ion peak at m/z 134 $(M+H)^+$. The ^{1}H and ^{13}C NMR data on **1** are presented in Table 2. The

Fig. 1. IR spectrum of poststatin.

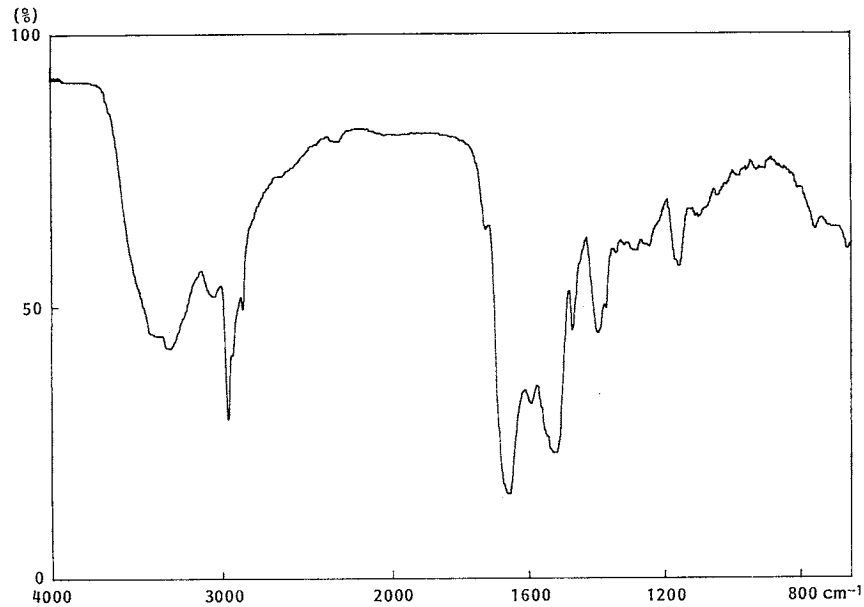


Table 1. ^1H and ^{13}C NMR data for poststatin in DMSO-d_6.

Assignment	^1H (ppm)[a]	^{13}C (ppm)[b]
Valine (*N*-terminal)		
CO	—	170.95 (s)[c]
α-CH	3.17 d (J=4.4)	59.03 (d)
β-CH	1.98 m	30.78 (d)
CH_3	0.78 m	16.82 (q)
CH_3	0.78 m	19.17 (q)[c]
NH_2	—	—
Valine		
CO	—	170.88 (s)[c]
α-CH	4.28 m	56.64 (d)
β-CH	1.98 m	31.01 (d)
CH_3	0.84 m	17.98 (q)[d]
CH_3	0.84 m	19.22 (q)[c]
NH	8.09 d (J=9.0)	—
Valine (*C*-terminal)		
CO	—	172.68 (s)
α-CH	4.04 m	57.45 (d)
β-CH	2.04 m	30.23 (d)
CH_3	0.80 m	17.85 (q)[d]
CH_3	0.80 m	19.22 (q)[c]
NH	7.98 d (J=9.0)	—
Leucine		
CO	—	171.03 (s)[c]
α-CH	4.42 m	51.45 (d)
β-CH	1.49 m, 1.60 m	40.75 (t)
γ-CH	1.53 m	24.32 (d)
CH_3	0.90 m	22.94 (q)
CH_3	0.86 m	21.44 (q)
NH	8.55 d (J=9.0)	—
3-Amino-2-oxovaleric acid (postine)		
CO	—	160.53 (s)
α-CO	—	196.19 (s)
β-CH	4.94 m	54.79 (d)
γ-CH_2	1.51 m, 1.77 m	22.66 (t)
δ-CH_3	0.87 m	10.36 (q)
NH	8.37 d (J=7.0)	—

[a] 400 MHz; J in Hz.
[b] 100 MHz.
[c~e] May be interchanged in the column where they appear.

Table 2. ^1H and ^{13}C NMR data for 3-amino-2-hydroxyvaleric acid[a] in D_2O[b].

$$CH_3-CH_2-\underset{NH_2}{CH}-\underset{OH}{CH}-COOH$$

1

Assignment	^1H (ppm)[c]	^{13}C (ppm)[d]
CO	—	177.67
	—	178.23[e]
α-CH	4.19 d (J=3.8)	72.41
	4.10 d (J=3.8)	71.50[e]
β-CH	3.46 ddd (J=3.8, 7.2, 7.2)	56.32
	3.35 ddd (J=3.8, 7.2, 7.2)	56.17[e]
γ-CH_2	1.63 m	21.32
	ca. 1.65 m, *ca.* 1.76 m	23.35[e]
δ-CH_3	0.98 t (J=7.2)	10.21
	1.02 t (J=7.2)	10.00[e]

[a] Mixture of diastereomer ([e] *threo*).
[b] Refer to the following paper[5].
[c] 400 MHz; J in Hz.
[d] 100 MHz.

Table 3. HPLC data for L-[Phe]-amino acids derived from poststatin.

Amino acids	Elution time of L-[Phe]-amino acid (minutes)	
	Authentic amino acids	Amino acids from poststatin hydrolysate
L-Val	13.16	13.15
D-Val	25.18	—
L-Leu	21.38	—
D-Leu	30.55	30.37

—: Not detected.

^1H NMR spectrum of **1** including its ^1H-^1H COSY indicated the presence of the carbon chain, $CH_3-CH_2-\underset{N}{CH}-\underset{O}{CH}-$. The ^{13}C NMR data indicated that **1** was diastereomeric mixture of 3-amino-2-hydroxyvaleric acid. Thus, the structure of the new amino acid in poststatin was determined to be 3-amino-2-oxovaleric acid. This amino acid was named postine.

To determine the configuration of the free amino acids contained in poststatin, the L-[Phe]dipeptides were prepared using Boc-L-Phe-OSu from the hydrolysate of poststatin and analyzed by HPLC. Table 3 shows the elution time of the L-[Phe]dipeptides of amino acids contained in poststatin. Judging from the elution time observed, the existence of L-Val and D-Leu was confirmed. However, the configuration of postine was not determined because of its degradation during acid hydrolysis.

The amino acid sequence of poststatin was determined in the following way. The *C*-terminal amino

Fig. 2. Mass spectrum of dihydropoststatin.

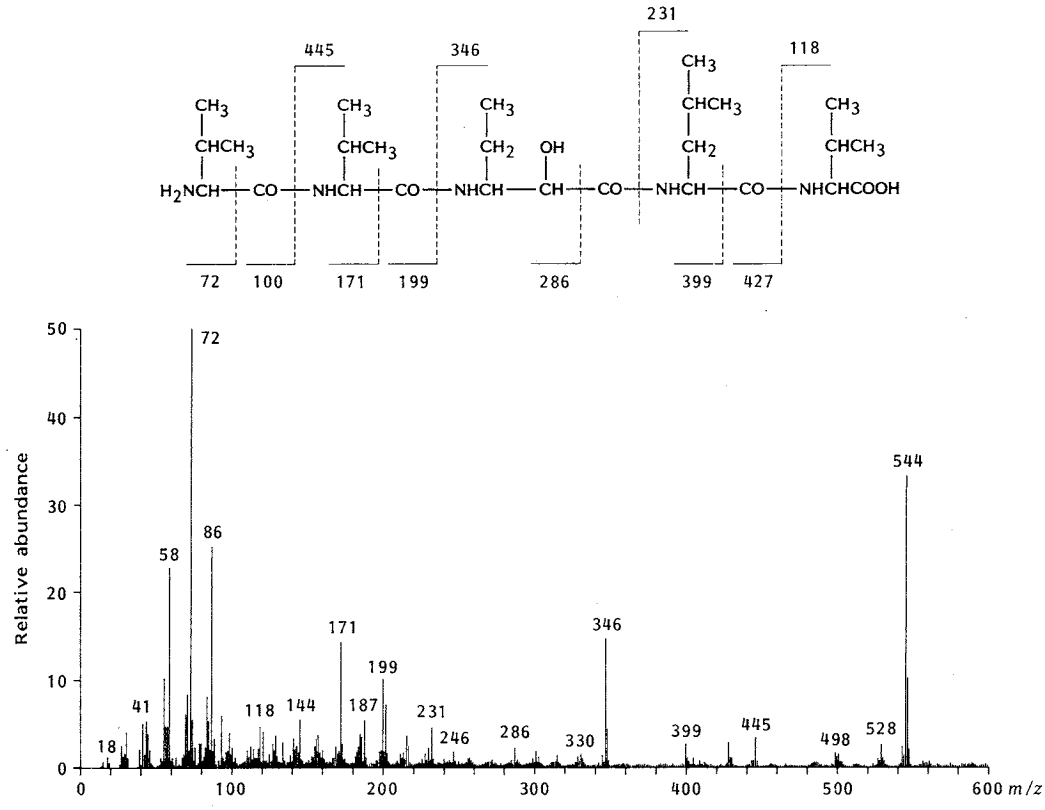
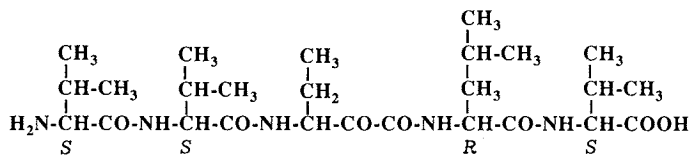


Fig. 3. Structure of poststatin.



acid of poststatin was determined to be valine by the usual hydrazinolysis. The Edman degradation of **2** gave valine at the first and the second stages, respectively, but no amino acid at the third stage. Finally, the amino acid sequence of poststatin was determined by mass spectrometric analysis of **2** as shown in Fig. 2. The peaks at *m/z* 445, 346, 231 and 118 were derived from the successive elimination of Val, Val, 3-amino-2-hydroxyvaleric acid, Leu, Val from *m/z* 544 (the parent peak), respectively. Thus, the structure of poststatin is determined to be L-valyl-L-valyl-3-amino-2-oxovaleryl-D-leucyl-L-valine (Fig. 3). The absolute configuration of the

Table 4 Inhibitory activities of poststatin and its derivatives against PEP and cathepsin B.

Compound	IC_{50} (μg/ml)	
	PEP	Cathepsin B
Val-Val-Pos-D-Leu-Val	0.030	2.1
Val·Val-(2*RS*)H_2Pos-D-Leu-Val (**2**)	>100	>100
Z·Val-Val-Pos-D-Leu-Val (**6**)	0.010	0.84
Ac·Val-Val-Pos-D-Leu-Val (**3**)	0.070	0.84
Val-Val-Pos-D-Leu-Val·OMe (**4**)	0.50	18
Ac·Val-Val-Pos-D-Leu-Val·OMe (**5**)	1.4	32

Pos: Postine, 3-amino-2-oxovaleric acid, H_2Pos: 3-amino-2-hydroxyvaleric acid, Z: benzyloxycarbonyl.

new amino acid, postine will be described in the following papers[5,6].

Inhibitory Activities

Table 4 shows the inhibitory activities of poststatin and its derivatives. The structure of **2**, **3**, **4**, **5** and **6** were established by NMR and MS spectra. Among them, **6** most strongly inhibited PEP, and both **3** and **6** also inhibited cathepsin B. **3** was less active than poststatin against PEP. **4** and **5** were less active than poststatin against PEP and cathepsin B. However, **2** at 100 μg/ml showed no inhibitory activity against either enzyme. Thus, it is proposed that the α-keto group of postine in poststatin plays the most important role in the inhibitory mechanism.

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