PROBESTIN, A NEW INHIBITOR OF AMINOPEPTIDASE M, PRODUCED BY *STREPTOMYCES AZUREUS* MH663-2F6

II. STRUCTURE DETERMINATION OF PROBESTIN

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Probestin, a new inhibitor of aminopeptidase M, has been isolated from the culture broth of *Streptomyces azureus* MH663-2F6. The ^{1}H and ^{13}C NMR studies and amino acid analysis confirmed the presence of one 3-amino-2-hydroxy-phenylbutanoic acid, leucine and two proline residues in the molecule. Stereochemistries of these amino acids were determined by HPLC analysis. The fragmentation pattern shown in the mass spectrum and the chemical analysis on probestin clarified the amino acid sequence. Thus the structure of probestin was defined as (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucyl-L-prolyl-L-proline.

In the preceding paper[1], we have described the taxonomy, the isolation, the physico-chemical properties and the biological properties of probestin, a novel inhibitor of aminopeptidase M (AP-M). In this paper, we describe the structure determination of probestin. The molecular formula of probestin was elucidated as $C_{26}H_{38}N_4O_6$ by the MS spectrum, the elemental analysis and the ^{13}C NMR study. The UV absorption spectrum showed the maxima at 252 (ε 210), 258 (230), 264 (200) and 280 nm (sh, 110) in MeOH. The IR (KBr) spectrum of probestin is shown in Fig. 1. The IR spectrum showed the absorption of peptide bond (1640 and 1530 cm^{-1}). Amino acid analysis of the acid hydrolysate of probestin suggested the amino acid constitution is Leu(1), Pro(2) and *threo*-3-amino-2-hydroxy-phenylbutanoic acid

Fig. 1. IR spectrum of probestin.

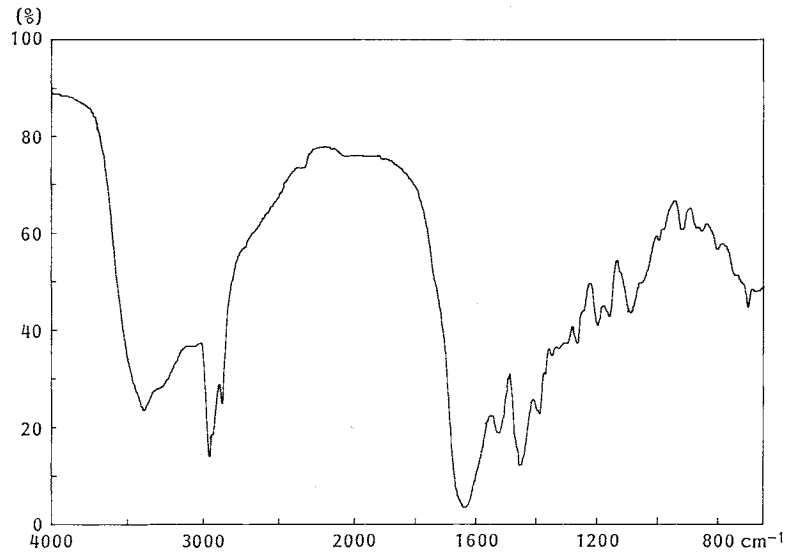


Table 1. ^{1}H and ^{13}C NMR data for probestin in CD_3OD.

Assignment		^{1}H[a]	^{13}C[b]
AHPA	CO		172.3 (s)[c]
	2-CH	4.23 d (J=3.7)	69.8 (d)
	3-CH	3.76 m	56.4 (d)
	4-CH_2	2.93 dd (J=8.0, 13.7), 3.10 dd (J=8.0, 13.7)	36.4 (t)
	Ph-*i*		136.7 (s)
	Ph-*o*	7.32 m	130.0 (d)[d]
	Ph-*m*		130.5 (d)[d]
	Ph-*p*		128.5 (d)
Leu	CO		172.7 (s)[c]
	α-CH	4.63 m	51.4 (d)
	β-CH_2	1.61 m, 1.73 m	40.9 (t)
	γ-CH	1.75 m	26.0 (d)
	CH_3	0.98 d (J=6.5)	23.6 (q)
	CH_3	0.98 d (J=6.5)	22.1 (q)
Pro[1]	CO		173.3 (s)[c]
	α-CH	4.66 m	59.7 (d)
	β-CH_2	1.97 m, 2.25 m	29.1 (t)
	γ-CH_2	*ca.* 2.00 m, 2.10 m	26.0 (t)
	δ-CH_2	*ca.* 3.62 m, *ca.* 3.87 m	48.6 (t)
Pro[2]	CO		175.2 (s)
	α-CH	4.41 m	60.1 (d)
	β-CH_2	*ca.* 2.00 m, 2.20 m	30.0 (t)
	γ-CH_2	*ca.* 2.00 m, 2.03 m	26.0 (t)
	δ-CH_2	*ca.* 3.62 m, *ca.* 3.87 m	48.6 (t)

[a] 400 MHz; δ in ppm, J in Hz.
[b] 100 MHz; δ in ppm.
[c,d] Assignment could be interchanged.

Table 2. HPLC data for [L-Phe]amino acids and Pro composed probestin on reverse phase column (a) and Chiralpak (b).

(a)

Amino acids	Elution time of [L-Phe]dipeptide (minutes)	
	Authentic amino acids	Amino acids composed probestin
(2*S*,3*R*)-AHPA	23.07	23.12
(2*R*,3*S*)-AHPA	21.52	—
L-Leu	15.21	15.16
D-Leu	19.83	—
L-Pro	14.53	14.49
D-Pro	14.71	

(b)

Pro	Elution time (minutes)	
	Authentic Pro	Pro composed probestin
L-Pro	16.87	16.86
D-Pro	27.49	—

—: Not detected.

(AHPA)[2] (1). The ^{1}H and ^{13}C NMR data on probestin are presented in Table 1. The assignment of proton and carbon signals were determined by the ^{1}H-^{1}H COSY and the ^{1}H-^{13}C COSY. These data supported the presence of the amino acids which were detected by the amino acid analysis.

The stereochemistry of the each amino acid residues was examined as follows. The amino acid mixture obtained by acid hydrolysis of probestin was L-phenylalanylated and the resulting peptides were compared by HPLC (Nucleosil $5C_{18}$) with reference compounds. The HPLC elution times of the [L-Phe]dipeptides of amino acids contained in probestin are listed in Table 2(a). As a result, the existence of L-Leu and (2*S*,3*R*)-AHPA were confirmed, but the peaks of the dipeptides of L- and D-Pro were not distinguished by this HPLC analysis. The absolute configurations of two Pro residues in probestin were determined by HPLC analysis using Chiralpak. As shown in Table 2(b), the elution time of Pro, obtained by hydrolysis of probestin, identified the configurations of the two Pro residues as L.

The amino acid sequence of probestin was determined by the SI-MS and the chemical analysis. In the spectrum of probestin, as shown in Fig. 2, the parent peak (m/z 503, M+H) is recognized. The mass difference of 114 between m/z 502 and 388 corresponds to the loss of one Pro from the *C*-terminus of probestin, indicating that the *C*-terminal amino acid is Pro. This was supported by hydrazinolysis to give Pro. The peak at m/z 291 can be regarded as being derived from the elimination of Pro-Pro. The major fragment ion at m/z 213 supported the presence of Pro-Pro in probestin as its *C*-terminal sequence. The peak at m/z 120 suggested that *N*-terminal amino acid is AHPA[2]. From these results, the amino acid sequence of probestin was determined to AHPA-Leu-Pro-Pro. This was supported by the acid hydrolysis of dinitrophenylated (DNP) probestin to give *N*-dinitrophenyl-AHPA (*N*-DNP-AHPA) and *N*-DNP-

Fig. 2. Mass spectrum of probestin.

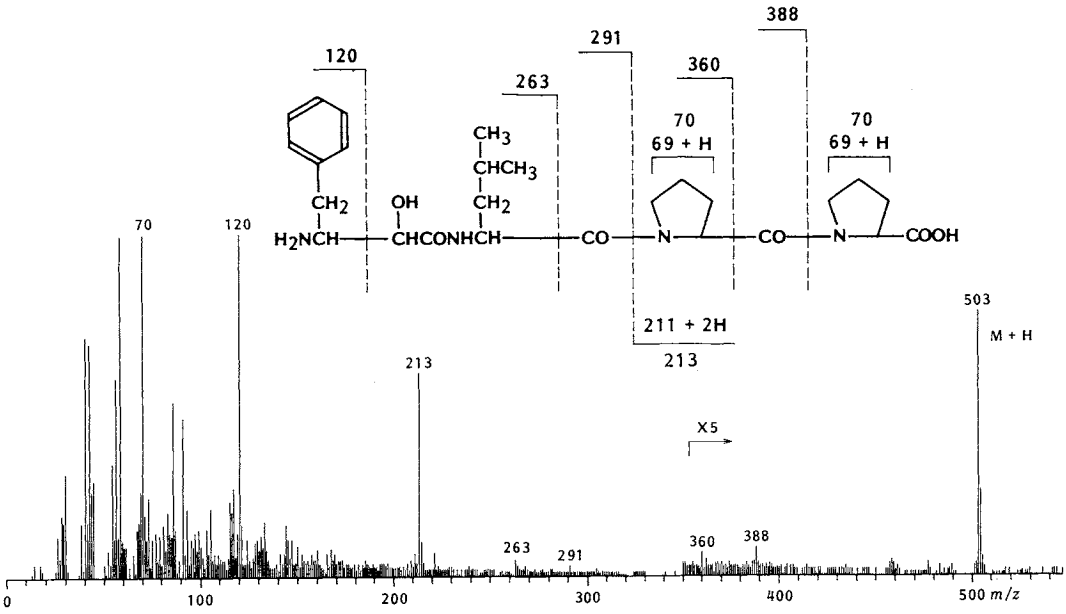
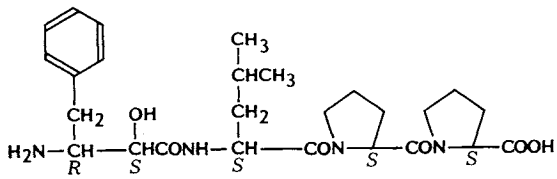


Fig. 3. Structure of probestin.



AHPA-Leu (*N*-DNP-bestatin). Furthermore, this structure was confirmed by the synthesis of probestin from bestatin and L-proline. Synthesis of probestin will be reported in the next paper.

Thus the structure of probestin was determined to be (2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucyl-L-prolyl-L-proline (Fig. 3).

Experimental

Mass spectra were carried out on a Hitachi M-80H spectrometer. NMR spectra were recorded on a Jeol JNM-GX400 NMR spectrometer with ^{1}H NMR at 400 MHz and ^{13}C NMR at 100 MHz. UV spectra were recorded on a Beckman DU-8 spectrophotometer, and IR spectra on a Hitachi 260-10 spectrophotometer. The amino acid analysis was carried out on a Hitachi 835 automatic amino acid analyzer. MP's were measured with a micro mp apparatus MP-S3 (Yanagimoto Seisakusyo Co., Japan) and were uncorrected. HPLC was on a Waters and Spectra-Physics SP8700 systems.

Chemicals

tert-Butyloxycarbonyl-L-phenylalanine-*N*-hydroxysuccinimide ester (Boc·L-Phe·OSu) was purchased from Kokusan Chemical Works, Tokyo, Japan. Packed column of Nucleosil $5C_{18}$ (0.46 × 15 cm) was from Senshu Scientific Co., Tokyo, Japan. Packed column of Chiralpak WH (0.46 × 15 cm) was from Daicel Chemical Ind., Tokyo, Japan.

Hydrolysis of Probestin

HCl solution (6 N, 200 μl) was added to a solution of probestin (3 mg) and heated at 105°C for 20

hours in a sealed tube. The solution was concentrated to dryness. The evaporation was carried out for several times with H_2O to remove HCl.

Preparation of [L-Phe]Dipeptide

The procedure by NISHIKIORI *et al.*[3] was carried out as follows. A sodium bicarbonate solution (100 μl, 23 μmol) was added to a reaction tube containing the hydrolysate (*ca.* 2 mg) or known free amino acid (1 mg). The stock solution (100 μl, 22 μmol) of Boc·L-Phe·OSu in dioxane was added to the amino acid solution. The mixture was allowed to stand at the room temperature for 16 hours and concentrated to dryness. The residue was dissolved in TFA (10 μl) and allowed to stand at the room temperature for an hour to remove the Boc group. The TFA was removed by evapolation under reduced pressure. The final residue was extracted with H_2O (200 μl) and filtered through a sintered glass funnel to give dipeptide solutions.

Determination of Absolute Configuration for AHPA and Leu

The reverse phase column of a Nucleosil $5C_{18}$ (0.46 × 15 cm) was operated at a flow rate of 1 ml/minute with a linear gradient from the solvent mixtures A to B per 30 minute (A: 15% CH_3COONH_4 - AcOH - H_2O - MeCN, 80 : 1 : 1,600 : 0, B: 80 : 1 : 1,000 : 720) using a Waters Spectra-physics SP8700 system. The detection was carried out on OD at 254 nm.

Isolation of Pro

The hydrolysate of probestin was subjected to silica gel TLC with BuOH - AcOH - H_2O (4 : 1 : 1). The extract from Rf 0.18 fraction was concentration to give Pro as colorless powder.

Determination of Absolute Configuration for Pro

The Chiralpak WH (0.46 × 15 cm) was operated (1 ml/minute) with 0.25 mM $CuSO_4$ at 50°C. The detection was carried out on OD at 210 nm.

Dinitrophenylation of Probestin and Hydrolysis of Dinitrophenylprobestin

One ml of 2,4-dinitrofluorobenzene (5%, EtOH) was added to a solution (0.5 ml) containing 10 mg of probestin and 10 mg of $NaHCO_3$. The solution was allowed to stand at the room temperature for 6 hours. The reaction mixture was diluted with 4 ml of water and shaken with diethyl ether. The aqueous layer was extracted with EtOAc under acidic condition (pH 1.0). The EtOAc layer was concentrated to dryness and heated at 105°C for 16 hours in a sealed tube. The residue was subjected to silica gel TLC with $CHCl_3$ - MeOH - AcOH (100 : 30 : 1). The extract from Rf 0.30 was concentrated to dryness. It was applied to Sephadex LH-20 eluting with MeOH and concentrated to give yellowish oil. The oil was crystallized from hexane - CH_2Cl_2 to yield yellowish crystals of *N*-DNP-AHPA. Rf 0.30 ($CHCl_3$ - MeOH - AcOH, 100 : 30 : 1); mp 73~76°C; SI-MS *m/z* 362 (M+H); $[\alpha]_D^{20}$ (*c* 0.3, MeOH) +295.9°C; UV λ_{max}^{MeOH} nm (ε) 260 (7,200), 349 (14,000); IR (KBr) cm^{-1} 3310, 1730, 1610, 1580, 1520, 1490, 1420, 1340, 1300, 1260, 1140, 920, 740, 700; ^1H NMR (400 MHz, CD_3OD) δ 3.03 (1H, dd, J=9.2 and 13.2 Hz), 3.20 (1H, dd, J=6.8 and 13.2 Hz), 4.11 (1H, d, J=2.4 Hz), 4.61 (1H, m), 6.94 (1H, d, J=10.0 Hz), 7.20 (1H, t-like, J=7.6 Hz), 7.28 (2H, t, J=10.0 Hz), 7.36 (2H, d, J=7.6 Hz), 8.04 (1H, dd, J=3.0 and 10.0 Hz), 8.97 (1H, d, J=3.0 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 40.1, 59.0, 73.3, 116.5, 124.5, 127.6, 129.6, 130.3, 130.7, 131.2, 136.5, 139.3, 149.7, 178.1. The extract from Rf 0.47 was concentrated to dryness. It was applied to Sephadex LH-20 eluting with MeOH and concentrated to give yellowish oil. The oil was crystallized from hexane - Me_2CO to yield yellowish crystals of *N*-DNP-AHPA-Leu (*N*-DNP-bestatin). Rf 0.47 ($CHCl_3$ - MeOH - AcOH, 100 : 30 : 1); mp 185~187°C; SI-MS *m/z* 475 (M+H); $[\alpha]_D^{20}$ (*c* 0.3, MeOH) +150.1°; UV λ_{max}^{MeOH} nm (ε) 259 (7,200), 348 (14,100); IR (KBr) cm^{-1} 3350, 1740, 1630, 1620, 1580, 1520, 1340, 1310, 1260, 1190, 1140, 1080, 920, 750, 710; ^1H NMR (400 MHz, CD_3OD) δ 0.53 (3H, d, J=6.9 Hz), 0.74 (3H, d, J=6.9 Hz), 1.12 (1H, m), 1.45 (2H, m), 3.09 (1H, dd, J=7.6 and 13.8 Hz), 3.18 (1H, dd, J=6.9 and 13.8 Hz), 4.30 (1H, m), 4.34 (1H, d, J=2.1 Hz), 4.71 (1H, m), 6.92 (1H, d, J=9.0 Hz), 7.22 (1H, t-like, J=6.9 Hz), 7.30 (1H, d, J=6.9 Hz), 7.38 (1H, d, J=6.9 Hz), 8.09 (1H, dd, J=3.5 and 9.0 Hz), 9.00 (1H, d, J=3.5 Hz); ^{13}C NMR (100 MHz, CD_3OD) δ 23.5, 24.2, 25.9, 39.9, 43.4, 54.2, 58.4, 72.9, 116.3, 124.4, 127.8, 129.7, 130.6, 131.1, 135.9, 138.8, 149.7, 173.3, 180.4.

Hydrazinolysis of Probestin

Hydrazine anhydrous (500 μl) was added to probestin (2 mg) and heated at 105°C for 16 hours in a sealed tube. The solution was evaporated in a vacuum desiccator over sulfuric acid. A solution (0.7 ml) of the residue in water was shaken with equal volume of *n*-heptyl aldehyde for 30 minutes to remove the hydrazide compound. Extraction with *n*-heptyl aldehyde was carried out two times. The aqueous layer was shaken with equal volume of EtOAc two times. The aqueous layer was used for identification of amino acid by TLC experiment.

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