AHPATININS, NEW ACID PROTEASE INHIBITORS CONTAINING 4-AMINO-3-HYDROXY-5-PHENYLPENTANOIC ACID

SATOSHI ŌMURA*, NOBUTAKA IMAMURA, KAZUHITO KAWAKITA, YŌKO MORI, YUKIKO YAMAZAKI, ROKUROU MASUMA, YŌKO TAKAHASHI, HARUO TANAKA, LEE-YUAN HUANG† and H. BOYD WOODRUFF

The Kitasato Institute and School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan
†Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065, U.S.A.

(Received for publication April 11, 1986)

A soil isolate, *Streptomyces* sp. WK-142, was found to produce new acid protease inhibitors, ahpatinins A, B, D, E, F and G active against pepsin and renin. Ahpatinin C was found to be identical with pepstatin A. The structure determinations were based on mass spectral data. Four of the compounds contain the unusual amino acid, 4-amino-3-hydroxy-5-phenylpentanoic acid as a building component.

In the course of our screening work for new acid protease inhibitors from actinomycetes, a strain WK-142, obtained from the rhizosphere of a bayberry plant growing at Tsumekisaki, Izu was found to produce new inhibitors, ahpatinins A, B, D, E, F and G, active against acid proteases, pepsin and renin.

This paper describes the taxonomy of the producing organism, fermentation, isolation, structure determination and enzyme-inhibiting activities of ahpatinins.

Taxonomy of the Producing Strain WK-142

Morphology

The vegetative mycelia of strain WK-142 grow abundantly on both synthetic and complex agar media and do not show fragmentation into cocoid or bacillary elements. The aerial mycelia grow abundantly on yeast extract - malt extract agar and inorganic salts - starch agar.

The spore chains are of the spiral type and have more than ten spores per chain (Plate 1). The spores are cylindrical in shape, 0.5×0.9 μm in size and have a smooth surface (Plate 1).

Chemical Composition

The chemical analysis of 2,4-diaminopimelic acid (A_2pm) in the cell wall was carried out by the method of LECHEVALIER and LECHEVALIER[1]. Strain WK-142 showed the presence of LL-A_2pm in the cell wall.

Cultural and Physiological Characteristics

The International Streptomyces Project (ISP) media recommended by SHIRLING and GOTTLIEB[2] and those recommended by WAKSMAN[3] were used. Cultures were observed after incubation at 27°C for two weeks. Color names and hue numbers indicated in Table 1 are those of the Color Harmony Manual (4th Ed) published by Container Cooperation of America. The utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source at

Table 1. Cultural characteristics of strain WK-142.

Yeast extract - malt extract agar*	G:	Good, light wheat (2ea)
	R:	Bamboo (2gc)
	AM:	Abundant, silver gray (2fe)
	SP:	None
Oatmeal agar*	G:	Good, penetrant, pearl (3ba)
	R:	Pearl (3ba)
	AM:	Poor, mustard brown (2pl)
	SP:	None
Inorganic salts - starch agar*	G:	Good, light ivory (2ca)
	R:	Light ivory (2ca) and olive gray (1ig)
	AM:	Abundant, center, silver gray (2fe); outside, white (a)
	SP:	None
Glycerol - asparagine agar*	G:	Moderate, penetrant, pearl pink (3ca)
	R:	Pearl pink (3ca)
	AM:	Moderate, white (a)
	SP:	None
Glucose - asparagine agar	G:	Moderate, pearl pink (3ca)
	R:	Light ivory (2ca)
	AM:	Moderate, orchid tint (10ba)
	SP:	None
Peptone - yeast extract - iron agar*	G:	Moderate, pearl pink (3ca)
	R:	Colonial yellow (2ga)
	AM:	Poor, orchid tint (10ba)
	SP:	None
Tyrosine agar*	G:	Penetrant, pearl pink (3ca)
	R:	Light beige (3ec)
	AM:	Abundant, pale blue (13ca)
	SP:	None
Sucrose - nitrate agar**	G:	Moderate, light beige (3ec)
	R:	Pearl pink (3ca) or light tan (3gc)
	AM:	Moderate, white (a)
	SP:	None
Glucose - nitrate agar**	G:	Moderate, light beige (3ec)
	R:	Pearl pink (3ca)
	AM:	Moderate, white (a)
	SP:	None
Glycerol - calcium malate agar**	G:	Moderate, flesh pink (5ca)
	R:	Flesh pink (5ca)
	AM:	Very poor, white (a)
	SP:	None
Glucose - peptone agar**	G:	Moderate, light brown (4ng)
	R:	Oak brown (4pi)
	AM:	Poor, white (a)
	SP:	None
Nutrient agar**	G:	Moderate, light ivory (2ca)
	R:	Light ivory (2ca)
	AM:	Poor, pearl pink (3ca)
	SP:	None

* Medium recommended by ISP[2].

** Medium recommended by S. A. WAKSMAN[3].

Abbreviations: G; growth of vegetative mycelium, R; reverse, AM; aerial mycelium, SP; soluble pigment.

Plate 1. Scanning electron-micrograph of spore chains of strain WK-142 on oatmeal agar.
Bar represents 1 μm.

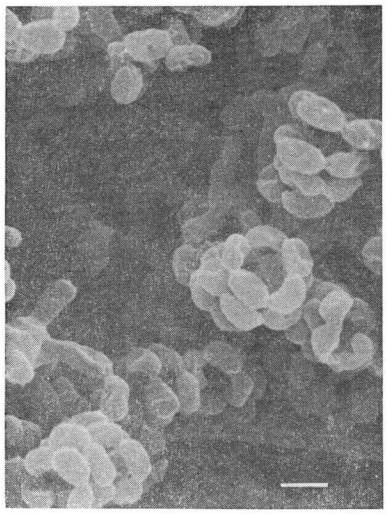


Table 2. Physiological properties of strain WK-142.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin	−
Peptonization of milk	−
Coagulation of milk	+
Cellulolytic activity	−
Hydrolysis of starch	+
Temperature range for growth	14~34°C

+; Active, −; not active.

Table 3. Utilization of carbon sources by strain WK-142.

D-Glucose	+
D-Fructose	+
L-Rhamnose	−
D-Mannitol	+
L-Arabinose	+
i-Inositol	+
Raffinose	+
D-Xylose	+
Sucrose	+
Melibiose	+

+; Utilized, −; not utilized.

27°C. The cultural and physiological characteristics, and the utilization of carbon sources of strain WK-142 are shown in Tables 1, 2 and 3, respectively.

Strain WK-142 exhibits the following properties. Spore chain spiral; spore, cylindrical and smooth surface; color of vegetative mycelia, pearl pink or light ivory; color of aerial mycelia, white or gray; soluble pigment, none; A_2pm in cell wall, LL type.

Based on the taxonomic properties described above, strain WK-142 is considered to belong to the genus *Streptomyces* and to be a strain of the white series or gray series of the PRIDHAM and TRESNER grouping[4]. Strain WK-142 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. WK-142 and the accession No. FERM P-8448.

Production and Isolation

The stock culture of *Streptomyces* sp. WK-142 was inoculated into 100 ml of a seed medium consisting of glucose 0.1%, starch 2.4%, peptone 0.3%, meat extract 0.3%, yeast extract 0.5% and $CaCO_3$ 0.4% in a 500-ml Erlenmeyer flask and incubated on a shaking machine at 27°C for 72 hours. Seven hundred milliliters of the seed culture was transferred into a 100-liter tank fermentor containing 70 liters of a production medium (pH 7.0) consisting of peptone 5.0%, NaCl 0.5%, $FeSO_4 \cdot 7H_2O$ 0.001%, $MnCl_2 \cdot 4H_2O$ 0.001%, $ZnSO_4 \cdot 7H_2O$ 0.001%, $CuSO_4 \cdot 5H_2O$ 0.001% and $CoCl_2 \cdot 6H_2O$ 0.001% and the aerobic fermentation was carried out at 27°C. Production of inhibitors started at 48 hours after the inoculation, then gradually increased and reached the maximum at 72 hours. The cultured broth (70 liters) was centrifuged to obtain about 65 liters of a supernatant fluid. The supernatant was adjusted to pH 3.0 with conc HCl and extracted with ethyl acetate (20 liters). The active principles were transferred from the organic layer into 0.1 N NH_4OH (5 liters), then the water layer was adjusted to pH 2.0 and re-extracted with ethyl acetate (1 liter). The extract was concentrated to a small volume

(50 ml) *in vacuo*. The concentrate was poured into *n*-hexane (500 ml) to yield active precipitate (dry weight, 840 mg). The crude material was chromatographed on a silica gel column with chloroform - methanol. The active fractions were combined and evaporated to dryness. The resulting brown material (410 mg) was dissolved in 50 ml of 30% aqueous acetonitrile and applied on 10 pieces of SEP-PAK (C_{18}) cartridges. The cartridges were washed with 30% and 50% acetonitrile, and then eluted with 80% acetonitrile. The eluate was evaporated to dryness, the resulting brown solid (29.1 mg) was dissolved in acetic acid (100 μl), and charged on a preparative HPLC (ODS) column and developed with 42.5% acetonitrile containing 0.2% acetic acid. Seven active compounds, A (1.39 mg), B (1.75 mg), C (5.17 mg), D (1.54 mg), E (3.45 mg), F (1.13 mg) and G (0.55 mg) were obtained. Component C was identified with pepstatin A[5] by co-HPLC with an authentic sample and through the EI-MS

Fig. 1. IR spectrum of ahpatinin E.

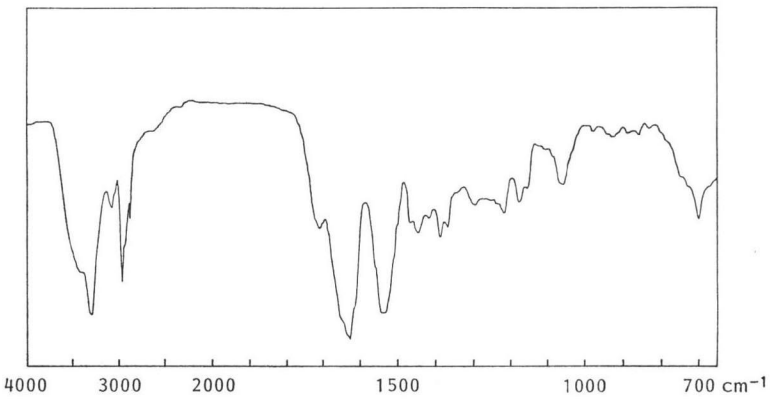
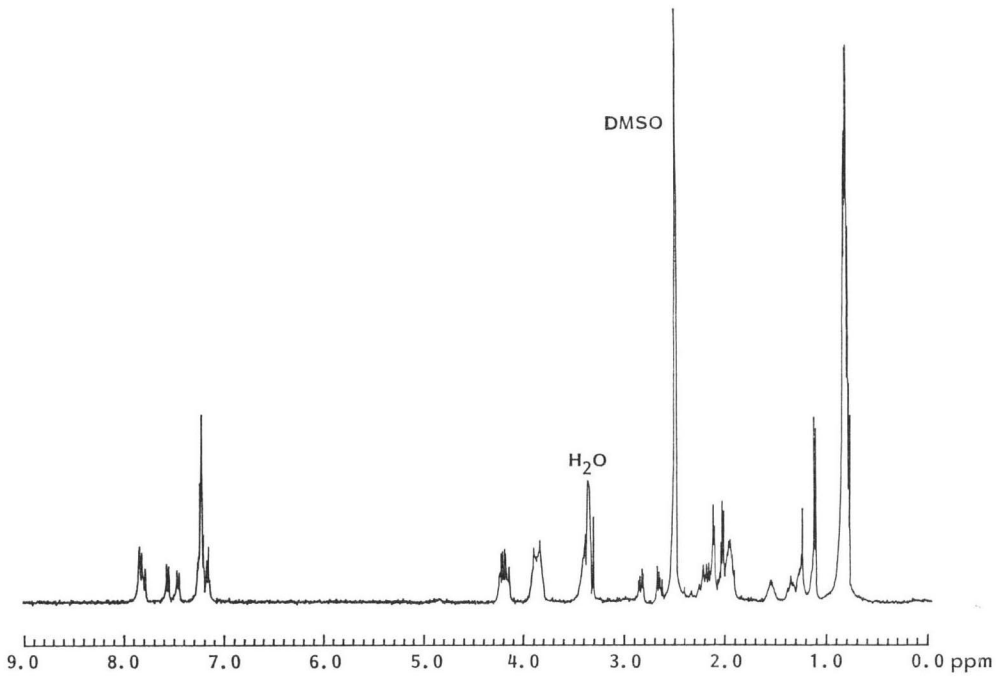


Fig. 2. ^{1}H NMR spectrum of ahpatinin E (400 MHz).



(m/z 699 (M^+)) pattern.

Physico-chemical Properties and Structures

The active components were soluble in dimethyl sulfoxide, slightly soluble in water and methanol. Component E crystallized from water - acetonitrile in the form of colorless needles, mp 312~313°C (dec). The IR spectrum (Fig. 1) showed peptide bond absorption. The UV (λ_{max}^{MeOH} nm (ε) 252 (648), 258 (648), 264 (583), 267 (540)) spectrum of component E suggested the presence of a mono-substituted benzene ring. The molecular formula was found to be $C_{37}H_{61}N_5O_9$ by the high-resolution mass spectrum data of its methyl ester (calcd for $C_{38}H_{61}N_5O_8$ 715.452 $(M-H_2O)^+$, found 715.451) and elemental analysis (calcd: C 61.73, H 8.54, N 9.73, found: C 62.02, H 8.65, N 9.65). A comparison of hydrolysates on TLC (silica gel, butanol - acetic acid - water, 3:1:1) of pepstatin A and component E indicated that the component E includes alanine, valine and statine moieties. The ^1H NMR spectrum in DMSO-d_6 (Fig. 2) indicated nine methyl groups (0.77~0.87 ppm, 24H and 1.11 ppm, Ala-methyl),

Fig. 3. EI-MS spectrum and fragmentation of ahpatinin E.

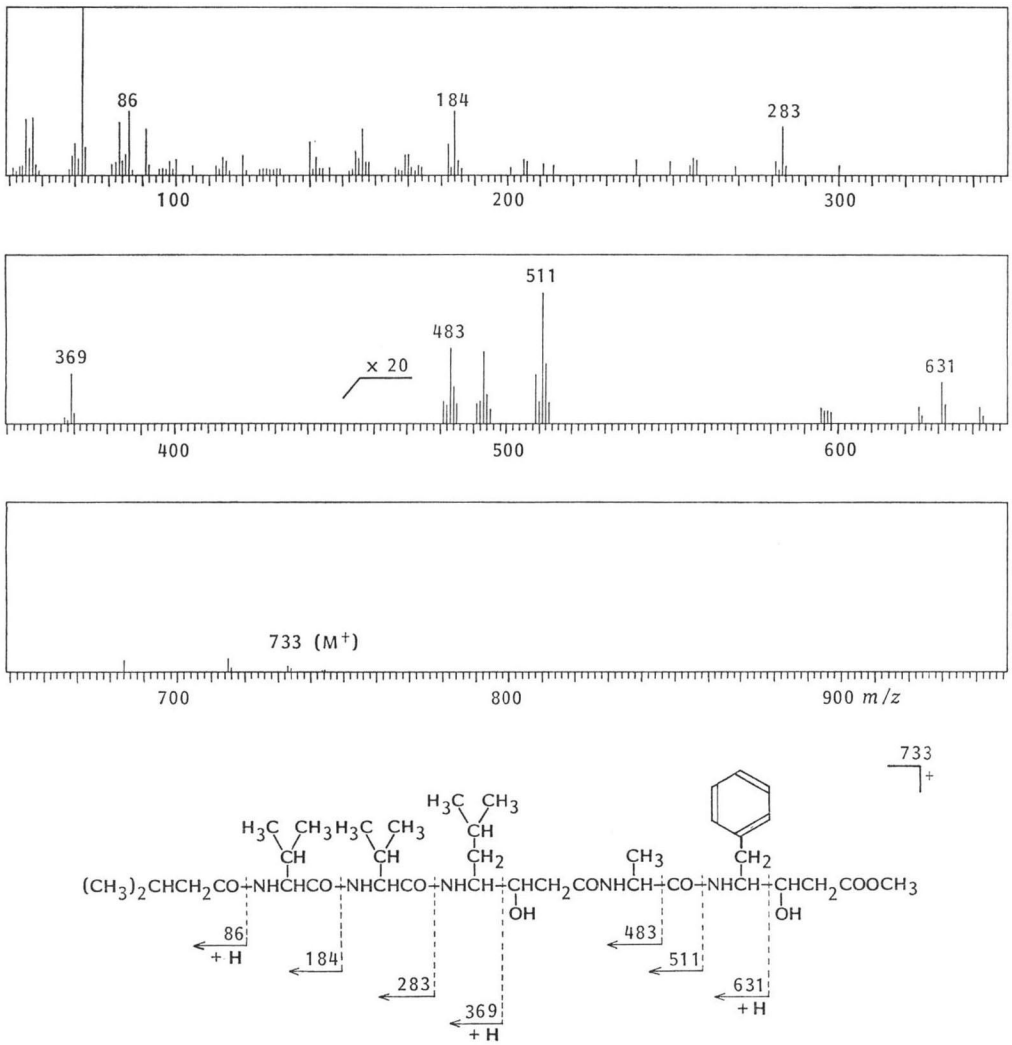
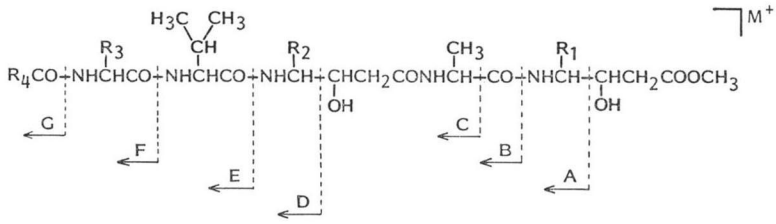


Table 4. Structures and MS fragment patterns of ahpatinins.
Component C is identical with pepstatin A.



Components	Fragment ion peaks (m/z)								
	M^+	$(A+H)^+$	B^+	C^+	$(D+H)^+$	E^+	F^+	$(G+H)^+$	Others
A	719	617	497	469	355	269	170	72	28 ($C_2H_4^+$), 91 (R_1^+)
B	719	617	497	469	355	269	170	72	91 (R_1^+)
C	699	597	511	483	369	283	184	86	—
D	697	595	509	481	367	281	182	84	—
E	733	631	511	483	369	283	184	86	91 (R_1^+)
F	733	631	545	517	403	317	218	86	190 (F^+-CO)
G	767	665	545	517	403	283	184	86	91 (R_1^+, R_2^+)

Components	R_1	R_2	R_3	R_4
A	$CH_2C_6H_5$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	C_3H_7
B	$CH_2C_6H_5$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	C_3H_7
C	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
D	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	C_4H_7
E	$CH_2C_6H_5$	$CH_2CH(CH_3)_2$	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
F	$CH_2CH(CH_3)_2$	$CH_2CH(CH_3)_2$	C_6H_5	$CH_2CH(CH_3)_2$
G	$CH_2C_6H_5$	$CH_2C_6H_5$	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$

one benzyl methylene (2.64 and 2.83 ppm), five aromatic protons (7.15~7.26 ppm), and five amide protons (7.46 (d) ppm, 7.56 (d), 7.80 (d), 7.84 (d) and 7.85 (d)). These data suggested that component E was an *N*-acyl-pentapeptide.

The MS spectrum of pepstatin A (component C) methyl ester indicated significant fragment ion peaks, *i.e.* m/z 699 (M^+), 597, 511, 483, 369, 283, 184 and 86. The components of ahpatinins also demonstrated the similar MS fragment patterns, for example, the MS spectrum of component E methyl ester is shown in Fig. 3. The ion peak m/z 631 was attributable to the $(M-C_4H_6O_3)^+$ ion peak and the other peaks, m/z 511, 483, 369, 283, 184 and 86 were identical to those of pepstatin A. The difference of m/z 631 and m/z 597 in pepstatin A methyl ester clearly indicated the structural difference between component E and pepstatin A. Since component E contained a benzene ring as mentioned above, it must have a benzyl group instead of the isobutyl group in the *C*-terminal statine of pepstatin A. The structures of the other components were similarly determined through the MS data as demonstrated in Table 4. The components A and B seem to be geometrical isomers of the acyl group. The fragment pattern was confirmed by high-resolution MS data. The unusual amino acid, 4-amino-3-hydroxy-5-phenylpentanoic acid (AHPPA) is the first finding from a natural source.

Acid Protease Inhibiting Activities

It was reported that the acid protease inhibitors such as pepstatins exhibited potent inhibiting

activities against pepsin and cathepsin D but very weak activity against renin[6]. The inhibiting activities of ahpatinins were measured against pepsin and renin compared with pepstatin A.

The assay method of pepsin or renin was essentially that of ANSON[7] or IKEDA *et al.*[8] but modified slightly to give more sensitivity and reproducibility. Each component inhibited not only pepsin but also renin activity. The components showed similar pepsin-inhibiting activities, while they were different in their ability to inhibit renin. When the IC_{50} ratios against both enzymes were measured, that of pepstatin A was 3,000 while the IC_{50} ratios of the other components were in the range of 100 to 1,000, component G showing the lowest value. Thus, the renin-inhibiting activities of these components are higher than that of pepstatin A.

Discussion

Ahpatinins A, B, E and G contain the unusual amino acid, AHPPA, and components D and F include an unsaturated acyl group and phenylglycine, respectively. Although several compounds of the pepstatin group were described[9,10], no compound containing AHPPA, phenylglycine or an unsaturated acyl group has been reported. Thus ahpatinins A, B, D, E, F and G are new acid protease inhibitors. Ahpatinin G containing two AHPPA moieties is the most potent inhibitor of renin among ahpatinins and pepstatin A.

The *C*-terminal statine of pepstatin is considered to be not essential for the pepsin inhibitory activity. The central one should be the most important binding site to pepsin[6]. Numerous model compounds containing a statine moiety or its analogs were synthesized[11]. Among these compounds, the tetrapeptide containing AHPPA instead of statine was as active against pepsin as the statine-containing one. Since component E which contains AHPPA instead of the *C*-terminal statine of pepstatin A showed higher renin-inhibiting activity than pepstatin A, the *C*-terminal AHPPA seems to be an important binding site to renin.

Acknowledgment

We wish to thank Miss MAHO NIWA for the enzyme assays.

References

1) LECHEVALIER, M. P. & H. A. LECHEVALIER: The chemotaxonomy of actinomycetes. Proc. of Papers of Actinomycete Taxonomy Workshop. pp. 1~49, Soc. Ind. Microbiol., Aug. 13, Texas, 1978
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) WAKSMAN, S. A.: The Actinomycetes. Vol. 2. Williams & Wilkins Co., Baltimore, 1961
4) PRIDHAM, T. G. & H. D. TRESNER: *Genus* I. *Streptomyces* Waksman and Henrici 1943, 339. *In* BERGEY's Manual of Determinative Bacteriology. 8th Ed., *Eds.*, R. E. BUCHANAN & N. E. GIBBONS, pp. 748~829, Williams & Wilkins Co., Baltimore, 1974
5) MORISHIMA, H.; T. TAKITA, T. AOYAGI, T. TAKEUCHI & H. UMEZAWA: The structure of pepstatin. J. Antibiotics 23: 263~265, 1970
6) AOYAGI, T.; H. MORISHIMA, R. NISHIZAWA, S. KUNIMOTO, T. TAKEUCHI & H. UMEZAWA: Biological activity of pepstatins, pepstanone A and partial peptides on pepsin, cathepsin D and renin. J. Antibiotics 25: 689~694, 1972
7) ANSON, M. L.: The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J. Gen. Physiol. 22: 79~89, 1938
8) IKEDA, I.; K. IINUMA, M. TAKAI, Y. YANAGAWA, K. KURATA, T. OGIHARA & Y. KUMAHARA: Measurement of plasma renin activity by a simple solid phase radioimmunoassay. J. Clin. Endocrinol. Metab. 54: 423~428, 1982
9) MIYANO, T.; M. TOMIYASU, H. IIZUKA, S. TOMISAKA, T. TAKITA, T. AOYAGI & H. UMEZAWA: New pepstatins, pepstatins B and C, and pepstanone A, produced by *Streptomyces*. J. Antibiotics 25: 489~491, 1972
10) AOYAGI, T.; Y. YAGISAWA, M. KUMAGAI, M. HAMADA, H. MORISHIMA, T. TAKEUCHI & H. UMEZAWA: New pepstatins, pepstatins Bu, Pr and Ac produced by *Streptomyces*. J. Antibiotics 26: 539~541, 1973
11) RICH, D. H.: Pepstatin-derived inhibitors of aspartic proteinases. A close look at an apparent transition-state analogue inhibitor. J. Med. Chem. 28: 263~278, 1985