Epostatin, New Inhibitor of Dipeptidyl Peptidase II, Produced by *Streptomyces* sp. MJ995-OF5

I. Taxonomy of Producing Strain, Fermentation, Isolation, Physico-chemical Properties and Biological Properties

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A new inhibitor of dipeptidyl peptidase II (DPP-II, EC 3.4.14.2), designated as epostatin, was discovered in the fermentation broth of a strain isolated in our institute. The strain has been identified as *Streptomyces* sp. MJ995-OF5 on the basis of taxonomic studies. Epostatin was obtained as a yellow powder after seqential purification by chromatography on Diaion HP-20, *n*-butanol extraction, Sephadex LH-20 column chromatography and centrifugal partition chromatography (CPC). Epostatin inhibited DPP-II competitively in a dose dependent manner. The compound was slightly inhibitory against other dipeptidyl peptidases.

Dipeptidyl peptidases[1] (DPPs), which catalyze the sequential release of dipeptides from the NH_2 terminus of peptide substrates, have been identified as at least 4 distinct enzyme proteins from various mammalian tissues. Dipeptidyl peptidase II (DPP-II, EC 3.4.14.2), a serine proteinase classified by the the preferential cleavage of L-lysyl-L-alanine, has been identified in the lysosomal fractions of the anterior pituitory gland[2], the urogenital system[3] and so on.

The physiological functions of DPP-II are still unclear, but have been proposed to involve the breakdown of some oligopeptides or their fragments[3]. This enzyme may have some measurable relation to the development of immunological abnormarities because the activity of this enzyme is increased in serum from rheumatoid arthritis and systemic lupus erythematosus patients in contrast to decreased DPP-IV activity[4].

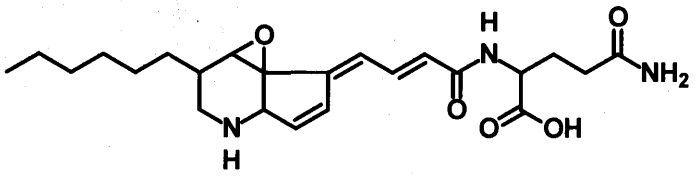
In the course of screening for an inhibitor of DPP-II, we discovered epostatin (Fig. 1) in the culture broth of *Streptomyces* sp. MJ995-OF5. In this paper, we report the taxonomy of the strain, fermentation, isolation, physico-chemical properties and biological activities of the compound.

Materials and Methods

Chemicals

Chemicals employed were as follows: Lysyl-alanine-β-naphtylamide, glycyl-arginine-β-naphtylamide, arginyl-arginine-β-naphtylamide and glycyl-proline-β-naphtyl-amide from Bachem Feinchemikalian AG, Bundendorf, Switzerland; fast garnet GBC, Sigma Chemical Co.,

Fig. 1. Structure of epostatin.



Missouri, USA; Diaion HP-20 from Nippon Rensui Co., Tokyo, Japan; Sephadex LH-20 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; TLC-plate silica gel F_{254} (0.25 mm thickness, Art. 1.05715.) from E. Merck, Darmstadt, Germany. All other chemicals were of analytical grade.

Analytical Instruments

Inhibitory activity of epostatin was measured with BIO-RAD's microplate reader model 3550. CPC was performed with a model LLB-M system (Sanki). The MP was taken using a Yanaco MP-500D apparatus and was uncorrected. The optical rotation was measured on a Perkin-Elmer 241 polarimeter using micro-cell (light path 10 cm). UV spectra were recorded on a Hitachi U-3210 spectrophotometer. The IR spectrum was recorded on a Hitachi I-5020 FT-IR spectrophotometer. MS spectra were obtained on a JEOL JMS-SX102 mass spectrometer.

Enzymes

DPP-II was prepared as a precipitated fraction between 0 and 1.7 M $(NH_4)_2SO_4$ from rat spleen homogenate described by McDONALD *et al.*[2].

DPP-I was prepared from rat spleen as described by METRIONE *et al.*[5]. DPP-III was prepared from human placenta as described by SHIMAMORI *et al.*[6]. DPP-IV was prepared from rat kidney as described by ÔYA *et al.*[7].

Assay for Enzyme and Inhibitory Activity

With synthetic substrates, DPP-I, -II, -III, and -IV activities are measured by minor modifications of the methods of METRIONE *et al.*[5], McDONALD *et al.*[2], SHIMAMORI *et al.*[6] and ÔYA *et al.*[7], respectively. The principle of these assays for DPPs is based on the absorbance at 525 nm of a chromophore complex of β-naphtylamine hydrolysed from these synthetic substrates, measured with a microplate reader. The assay mixtures for each DPP are preincubated at 37°C for 10 minutes and then incubated with substrate (3.2 mM) for 1 hour.

The ratio of inhibition (%) is calculated by the formula $100 \times (A - B)/A$, where A is the β-naphtylamine content in the assay system without an inhibitor and B is that with an inhibitor. The IC_{50} value is the concentration of an inhibitor that gives 50% inhibition of the enzyme activity.

Microorganism

Strain MJ995-OF5 was isolated from a soil sample collected in Ichihara-shi, Chiba Prefecture, Japan.

Taxonomic Characterization

Morphological observations were made with a scanning electron microscope (model Hitachi S-570). Cultural and physiological characteristics of the strain MJ995-OF5 were determined by the methods of SHIRLING and GOTTLIEB[8] and by the methods of WAKSMAN[9]. Permissive temperatures for growth were determined on yeast-starch agar (soluble starch 1.0%, yeast extract 0.2%, agar 2.0%, pH adjusted to 7.0 before sterilization). Color codes were assigned to the substrate and aerial mass color including soluble pigments according to the Color Harmony Manual, 1958 (Container Corporation of America, Chicago, Illinois). Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[10]. Diaminopimelic acid isomers in the cell wall were analyzed from the hydrolysate of the grown culture according to the method of BECKER *et al.*[11]. Menaquinones were extracted and purified by the method of COLLINS *et al.*[12] and analyzed using atmospheric pressure chemical ionization mass spectrometry with a Hitachi M-1200H mass spectrometer.

Fig. 2. Scanning electron micrograph of strain MJ995-OF5 grown on sucrose-nitrate agar for 8 days.

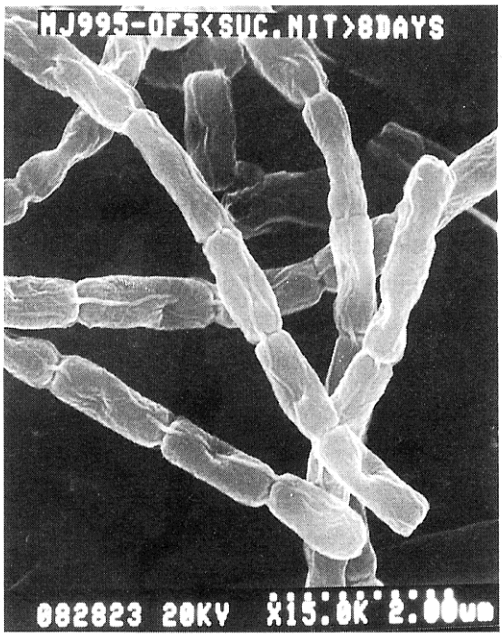


Table 1. Cultural characteristics of strain MJ995-OF5.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Bamboo [2 gc]~Lt Mustard Tan [2 ie]	None	None
Yeast extract-malt extract agar (ISP No. 2)	Yellow Maple [3 ng]~Lt Brown [4 ng]	None	Brownish
Oatmeal agar (ISP No. 3)	Cinnamon [3 le]	Thin, white	Brownish
Inorganic salts-starch agar (ISP No. 4)	Cinnamon [3 le]~Yellow Maple [3 ng]	Thin, white ~Sand [3 cb]	Faint, brownish
Glycerol-asparagine agar (ISP No. 5)	Cinnamon [3 le]~Golden Brown [3 pi]	None	Brownish
Tyrosine agar (ISP No. 7)	Camel [3 ie]~Clove Brown [3 pl]	Thin, white	Brown
Glucose-asparagine agar	Cinnamon [3 le]~Yellow Maple [3 ng]	Scant, white	Faint, brownish
Nutrient agar	Lt Ivory [2 ca]	None	None
Starch agar	Bamboo [2 gc]~Mustard Gold [2 pg]	None	Faint, yellowish

Observation after incubation at 30° for 21 days.
Color names and numbers from Color Harmony Manual, Container Corporation of America.

Table 2. Comparison of strain MJ995-OF5, *Streptomyces atroaurantiacus* (*Kitasatosporia melanogera*), *S. mediocidicus* (*K. mediocidica*) and *S. azaticus* (*K. azatica*).

	Strain MJ995-OF5	*S. atroaurantiacus* IMC A-0125^T	*S. mediocidicus* IMC S-0828^T (IFO 14789)	*S. azaticus* IMC S-0829^T (IFO 13803)
Spore chain	Straight	Straight	Straight	Straight
Spore surface	Smooth	Smooth	Smooth	Smooth
Aerial mycelium	White~brownish white	Brownish white ~light gray	Brownish white ~light gray	White~grayish white
Substrate mycelium	Pale yellowish brown ~yellowish brown	Yellow orange ~light brown	Pale yellow	Pale yellow ~pale yellow orange
Soluble pigment	Brownish	Brownish~yellowish	None~faint, brownish~yellowish	None~faint, brownish~pale pink
Formation of melanoid pigment				
ISP No. 1	Negative	Positive	Negative	Negative
ISP No. 6	Negative	Negative	Negative	Negative
ISP No. 7	Positive	Positive	Negative	Negative
Liquefaction of gelatin	Negative	Positive (weak)	Negative	Negative
Coagulation of milk	Positive	Positive	Positive	Positive
Peptonization of milk	Negative	Positive (weak)	Negative	Negative
Hydrolysis of starch	Positive	Positive	Positive	Positive
Reduction of nitrate	Positive	Positive	Positive	Positive
Utilization of				
L-Arabinose	+	+	+	+
D-Xylose	+	+	±	±
D-Glucose	+	+	+	+
D-Fructose	−	+	−	+
Sucrose	±	+	−	−
Inositol	−	−	−	−
Rhamnose	−	−	−	−
Raffinose	(−)	−	−	±
D-Mannitol	−	−	−	−

+: Utilization, ±: doubtful, (−): probably no utilization, −: no utilization.

Results and Discussion

Cultural and Taxonomic Characterization of the Producing Strain

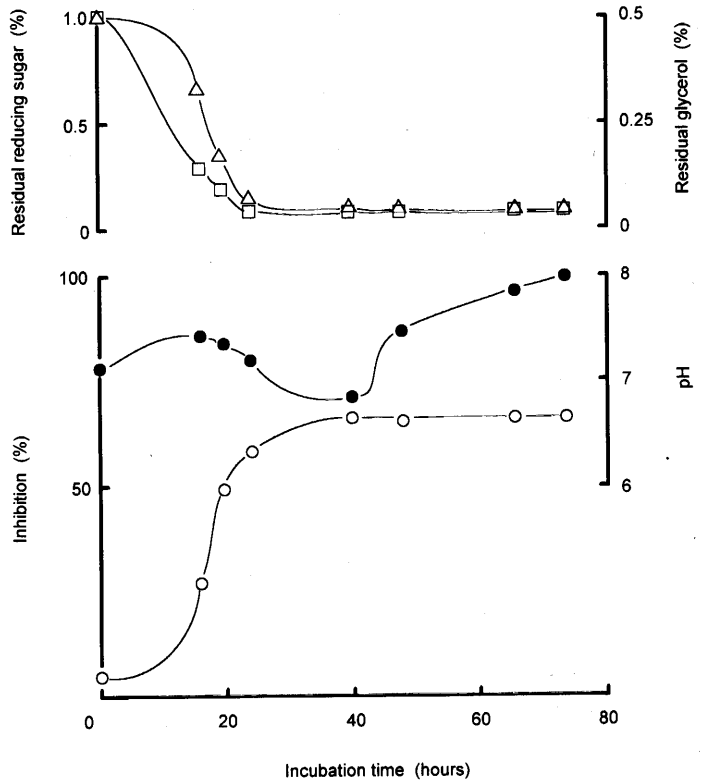
Strain MJ995-OF5 had branched substrate mycelia, on which developed aerial hyphae formed into straight spore-chains, of which the whirl-formation or spiral-formation was not observed. Each matured spore-chain had more than 10 spores, which were cylindrical (0.5 to 0.7 by 1.0 to 1.5 μm), and the spore surface was smooth. No synnemata or sporangia were observed (Fig. 2). Many submerged spores were observed on cultivation in yeast extract-glucose broth (yeast extract 1.0%, glucose 1.0%, pH 7.2) at 27°C for 2 days on a rotary shaker (180 rpm). The cultural characteristics of strain MJ995-OF5 are summarized in Table 1. On various agar media, the substrate mycelia of this strain were pale yellowish brown to yellowish brown. Aerial mycelia were thin and white to brownish white, soluble pigment was brownish. Physiological characteristics and carbohydrate utilization of this strain are summarized in Table 2. The permissive temperature range for growth was 10°C to 30°C, with the optimal temperature for growth between 24°C and 30°C. Formation of melanoid pigment was positive on ISP No. 7 medium, while negative on ISP No. 1 and No. 6 media. This strain hydrolyzed starch strongly. As for the chemotaxonomic characteristics of strain MJ995-OF5, whole-cell hydrolysates contained both *meso*- and L,L-diaminopimelic acid. Major menaquinones were MK-9 (H_6) and MK-9 (H_8).

The taxonomic properties given above suggested that this strain belonged to the genus *Streptomyces* 'setae group'[13] (*Kitasatosporia*[14], *Kitasatospora*[15]). Among the known species of this taxon, *S. atroaurantiacus*[13] ('*K. melanogena*'[15]), *S. azaticus*[16] (*K. azatica*[15]) and *S. mediocidicus*[17] (*K. mediocidica*[15,18]) were closely related to strain MJ995-OF5. Accordingly, strain MJ995-OF5 was compared with *S. atroaurantiacus* IMC A-0125^T, *S. azaticus* IMC S-0829^T (IFO 13803) and *S. mediocidicus* IMC S-0828^T (IFO 14789) in more detail. As shown in Table 2, strain MJ995-OF5 was considered not to correspond to these strains on the basis of the results. It is still a matter of controversy how to classify this taxon. Therefore, the strain MJ995-OF5 was designated as *Streptomyces* sp. MJ995-OF5 for the present.

A subculture of this strain has been deposited in the National Institute of Bioscience and Human-Technol-

Fig. 3. Time course of epostatin production by *Streptomyces* sp. MJ995-OF5.

○ Inhibition (%, 10 μl/ml), ● pH, □ glycerol, △ reducing sugar.



ogy, The Agency of Industrial Science and Technology, Tsukuba-shi, Ibaragi Prefecture, Japan, under the accession No. FERM P-14608.

Production of Epostatin

The strain MJ995-OF5 was inoculated into 110ml of a seed medium consisting of galactose 2.0%, dextrin 2.0%, glycerol 1.0%, Bacto-soytone (Difco) 1.0%, corn steep liquor (Iwaki) 0.5%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2% and antifoaming agent 1 drop (pH adjusted to 7.4 with 2N NaOH before sterilization) in a 500ml baffled Erlenmeyer flask and cultured at 30°C for 6 days on a rotary shaker (180 rpm). The content of the antifoaming agent was soybean oil: silicone oil KM-70 (Shin-etsu Chemical Industry) = 1 : 1. After incubation, 3-ml of this seed medium was transferred into the same type flask containing 110ml of production medium consisting of galactose 1.0%, dextrin 1.0%, glycerol 0.5%, Bacto-soytone (Difco) 0.5%, corn steep liquor (Iwaki) 0.3%, $(NH_4)_2SO_4$ 0.1%, $CaCO_3$ 0.2% and the same antifoaming agent 1 drop (pH adjusted to 7.4 with 2N NaOH before sterilization) and cultured at 27°C on a rotary shaker (180 rpm) as above until the production reached maximum. The time course of the production in the broth was measured (Fig. 3). Residual glycerol and reducing sugar in the cultivated broth were measured according to D. J. HANAHAN *et al.*[19] and J. H. ROE[20], respectively. The maximum level of epostatin was obtained at 40 hours and was maintained thereafter. The amount of residual glycerol and reducing sugar decreased as the production of the inhibitor increased.

Thirty-ml portions of seed medium were inoculated into ten 500-ml Erlenmeyer flasks containing 110ml of the same fresh medium and cultured for 40 hours as described above.

Isolation of Epostatin

The isolation scheme of epostatin (Fig. 1) is summarized in Fig. 4. The culture broth (1.1 liters) was filtered and separated into the mycerial cake and culture filtrate (1.0 liter). The mycelial cake was extracted with methanol (300ml) and evaporated under reduced

Fig. 4. Isolation of epostatin.

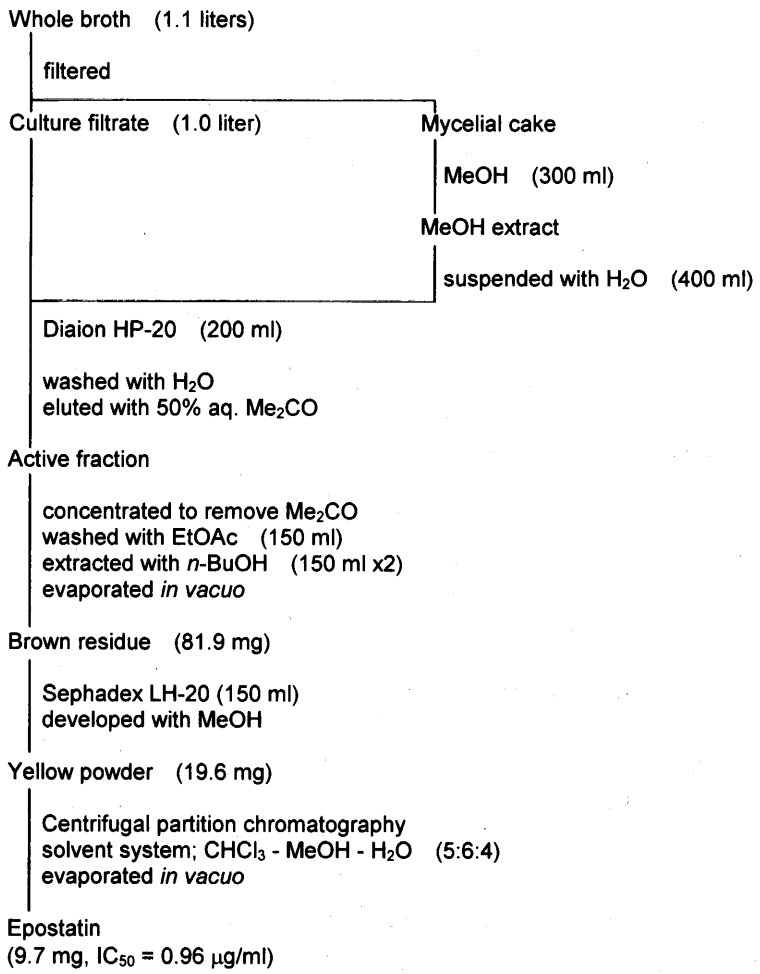


Table 3. Physico-chemical properties of epostatin.

Appearance	Pale yellow powder
MP (°C)	157~159 (dec.)
$[\alpha]_D^{23}$ (c 0.1, MeOH)	−174°
Rf value on TLC[a]	0.16
Rm value on HVPE[b] (L-Alanine=1.0)	0.21
UV λ_{max}^{MeOH} nm (log ε)	218 (sh, 3.54), 305 (4.43)
$\lambda_{max}^{MeOH-HCl}$	222 (3.24), 291 (4.44)
$\lambda_{max}^{MeOH-NaOH}$	305 (4.42)
IR ν_{max}^{KBr} (cm^{-1})	3370, 2930, 2860, 1660, 1600, 1290, 980, 900, 810, 690, 650
FAB-MS (*m/z*)	
Positive	432 $(M+H)^+$
Negative	430 $(M-H)^-$
HRFAB-MS (*m/z*, Positive)	
Found	432.2506
Calcd. for $C_{23}H_{34}N_3O_5$	432.2498
Elemental analysis	
Found	C: 60.33 H: 7.98 N: 9.26
Calcd. for $C_{23}H_{33}N_3O_5 \cdot 3/2H_2O$	C: 60.24 H: 7.91 N: 9.16
Molecular formula	$C_{23}H_{33}N_3O_5$

[a] Silica gel $60F_{254}$ (Merck, Art. 1.05715) with $CHCl_3$-MeOH-H_2O (65:25:4) as eluent.
[b] HVPE in HCOOH-AcOH-H_2O (25:75:900) under 3000 V for 20 minutes.

pressure. After the extract was suspended in deionized water (400 ml) and added to the broth filtrate, the inhibitor was adsorbed onto a Diaion HP-20 column (200 ml). The column was washed with water (400 ml) and eluted with 50% aqueous acetone (600 ml). The active fractions were pooled and concentrated *in vacuo* to remove acetone. The solution (150 ml) was washed with ethyl acetate (150 ml) and extracted twice with an equal volume of *n*-butanol (150 ml × 2). The active extract was dried under reduced pressure to give a brown residue (81.9 mg). The residue was applied on a Sephadex LH-20 column (150 ml) with methanol as eluant. The active fractions were pooled and concentrated *in vacuo* to give a yellow powder (19.6 mg). The powder was applied to CPC (Sanki systems) employing the following conditions: chloroform-methanol-distilled water (5:6:4), 8 ml/minute, at 700 rpm and at room temperature. After washing with the lower phase (750 ml), the active fractions containing epostatin were eluted with the upper phase and concentrated under reduced pressure to give pure epostatin as a pale yellow powder (9.7 mg).

Physico-chemical Properties of Epostatin

Epostatin is soluble in dimethyl sulfoxide and methanol, partially soluble in acetone and water, and insoluble in chloroform and ethyl acetate. The compound appeares to decompose gradually with darkening. The molecular formula of epostatin was determined to be $C_{23}H_{33}N_3O_5$ on the basis of FAB-MS, HRFAB-MS, NMR spectra and elemental analysis. Physico-chemical properties of the compound are summarized in Table 3. The sturcture of epostatin (Fig. 1) contains a dihydroabikoviromycin[21] (dihydrolatumcidin) moiety and a glutamine residue. Structure determination of the compound will be reported in the following paper[22].

Table 4. Inhibitory activities of epostatin to dipeptidyl peptidases.

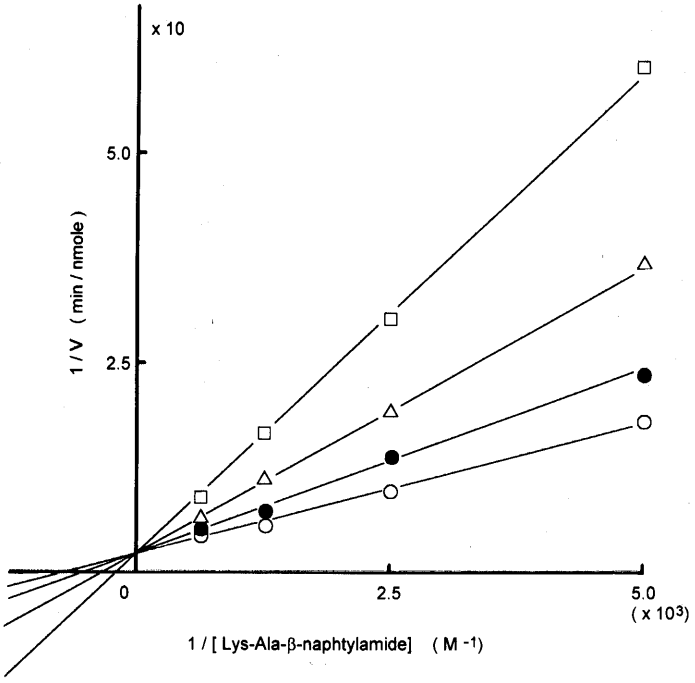
Enzyme	IC_{50} (μg/ml)
DPP-I (rat spleen)	>100
DPP-II (rat spleen)	0.96
DPP-III (human placenta)	>100
DPP-IV (rat kidney)	>100

Biological Activities

The inhibitory activities of epostatin to DPPs are shown in Table 4. Epostatin inhibited DPP-II in a dose

Fig. 5. Lineweaver-Burk reciprocal plot of the substrate concentration against the rate of hydrolysis by DDP-II.

Epostatin was added at the following concentrations (μg/ml): ○, 0; ●, 0.5; △, 1.0; □, 2.0.



dependent manner. The IC_{50} value of the compound to DPP-II was 0.96 μg/ml, whereas the compound was only slightly inhibitory against other DPPs (data not shown). These data suggested that epostatin is potent and selective inhibitor of DPP-II *in vitro*. As shown in Fig. 5, the inhibition of this compound to DPP-II was competitive, and the *Ki* value was 1.44 μM (*Km* value was 0.708 μM). Epostain seemed to be an unique peptide analog having a dihydroabikoviromycin[21] moiety.

Epostatin showed very weak antimicrobial activities against bacteria and fungi (data not shown). This compound had low toxicity; there were no deaths after iv injection in mice at 62.5 mg/kg.

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