Fluostatins A and B, New Inhibitors of Dipeptidyl Peptidase III, Produced by *Streptomyces* sp. TA-3391

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

TETSUO AKIYAMA*, SHIGEKO HARADA, FUKIKO KOJIMA, YOSHIKAZU TAKAHASHI, CHIAKI IMADA, YOSHIRO OKAMI, YASUHIKO MURAOKA, TAKAAKI AOYAGI and TOMIO TAKEUCHI

Institute of Microbial Chemistry,
3-14-23 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan

(Received for publication January 30, 1998)

New inhibitors of dipeptidyl peptidaseIII (EC 3.4.14.4) from human placenta, designated as fluostatins A and B, were discovered in the fermentation broth of a strain isolated in our institute. The strain has been identified as *Streptomyces* sp. TA-3391 on the basis of taxonomic studies. Fluostatins A and B were purified by Diaion HP-20 chromatography, ethyl acetate extraction, silica gel chromatography and reverse phase preparative HPLC.

With the synthetic substrate, arginyl-arginine-2-naphthylamide, the IC_{50} values of fluostatins A and B were 0.44 and 24.0 μg/ml, respectively. Fluostatins A and B were slightly inhibitory against other dipeptidyl peptidases. Fluostatin A showed mixed-type (competitive and noncompeptitive) inhibition with human leucine-enkephalin as a substrate, and the inhibition constant (*Ki*) was 14.2 μM.

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 III (EC 3.4.14.4, DPP-III), classified by the preferential cleavage of L-arginyl-L-arginine, has been purified from the cytosolic fractions of bovine pituitary gland[2], rat skin[3], and other organs[4~7].

Human placental DPP-III seems to require both cysteine residue(s) and metal ion(s) in its catalytic process because of the sensitivity to various peptidase inhibitors[7]. Its proteolytic activity was stimulated by Co^{2+}, and the stimulant ratio was dependent on the Co^{2+} concentration in a saturable manner[8]. The physiological functions of DPP-III are still unclear, but it has been proposed to play a role in the regulation of hormonal peptides such as angiotensin[6] and/or enkephalin[6,8] because of the ability to degrade these peptides *in vitro*.

In the course of screening for an inhibitor of human placental DPP-III, we discovered fluostatins A and B in the culture broth of *Streptomyces* sp.TA-3391. In this paper, we report the taxonomy of the strain, production, isolation, physico-chemical properties and biological activities of these inhibitors.

Materials and Methods

Chemicals

Chemicals employed were as follows: Arginyl-arginine-2-naphthylamide, glycyl-arginine-2-naphthylamide, lysyl-alanine-2-naphthylamide, glycyl-proline-2-naphthylamide and human leucine-enkephalin from Bachem Feinchemikalian AG, Bundendorf, Switzerland; fast garnet GBC, Sigma Chemical Co., Missouri, USA; DEAE-Sephadex A-50 and Sephadex G-200 from Pharmacia Fine Chemicals AB, Uppsala, Sweden; HPTLC-plate silica gel F_{254} (0.25 mm thickness, Art. 1.05628.) from E. Merck, Darmstadt, Germany; Diaion HP-20 from Nippon Rensui Co., Tokyo, Japan; Wakogel C-200 for silica gel from Wako Pure Chemical Industries,

Ltd., Osaka, Japan; packed column of Capcell Pak C_{18} from Shiseido Co., Tokyo, Japan. All other chemicals were of analytical grade.

Analytical Instruments

Inhibitory activities of fluostatins were measured with a BIO-RAD microplate reader model 3550. HPLC was performed with a Gilson system. MPs were taken using a Yanaco MP-500D apparatus and were uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter using micro-cell (light path 10 cm). UV spectra were recorded on a Hitachi U-3210 spectrophotometer, and IR spectra on a Hitachi I-5020 FT-IR spectrophotometer. MS spectra were obtained on a JEOL JMS-SX 102 mass spectrometer.

Enzymes

DPP-III was prepared from human placenta as described by SHIMAMORI *et al.*[7], with some modifications. Briefly, to human placental post-microsomal extract was added solid ammonium sulfate and the precipitate obtained with between 40 and 65% saturation was collected by centrifugation. The fraction containing DPP-III was applied sequentially to DEAE-Sephadex A-50, and Sephadex G-200. The enzyme was purified 127-fold from the post-microsomal extract.

DPP-I and DPP-II were prepared from rat spleen homogenate as described by METRIONE *et al.*[9] and MCDONALD *et al.*[10], respectively. DPP-IV was prepared from rat kidney homogenate as described by ÔYA *et al.*[11].

Assay for Enzyme and Inhibitory Activity

With synthetic substrates, DPP-I, -II, -III, and -IV activities were measured by minor modifications of the methods of METRIONE *et al.*[11], MCDONALD *et al.*[12], SHIMAMORI *et al.*[7] and ÔYA *et al.*[11], respectively. The principle of these assays for DPPs is based on the absorbance at 525 nm of a chromophoric complex of the 2-naphthylamine hydrolysed from these synthetic substrates. This is 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 percent inhibition was calculated by the formula $100 \times (A-B)/A$, where A is the 2-naphthylamine 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 at 50% inhibition of the enzyme activity.

With human leucine-enkephalin (Tyr-Gly-Gly-Phe-Leu) as substrate, DPP-III activity was assayed by the method of C.-M. LEE *et al.*[6]. The activity was detected as a peak of glycyl-phenyl-leucine at 210 nm with a Gilson HPLC system on a packed column of Capcell Pak C_{18}.

Microorganism

Strain TA-3391 was isolated from a soil sample collected in Kobuchizawa, Yamanashi Prefecture, Japan.

Taxonomic Characterization

Morphological and physiological characteristics of strain TA-3391 were examined according to the methods described by SHIRLING and GOTTLIEB[12] after cultivation at 27°C for 14 days. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Illinois) was used to identify the color of the mycelium and soluble pigment.

Results and Discussion

Cultural and Taxonomic Characterization of the Producing Strain

Cultural characteristics of strain TA-3391 with various agar media are summarized in Table 1. The strain produced white to light pink color aerial mycelia. Soluble pigments were tinged with light pink to light reddish brown. The form of mature sporophores were *Retinaculiaperti*. The spores were cylindrical in shape with smooth surface. The hydrolzed cell wall of the strain contained L,L-diaminopimelic acid but no *meso*-diaminopimeric acid. Glycine was detected in the cell wall. Table 2 summarizes the taxonomic characteristics of the strain TA-3391. Based on these characteristics, strain TA-3391 was designated as *Streptomyces* sp. TA-3391. A subculture of this strain has been deposited in the National Fermentation Institute of Bioscience and Human-Technology, The Agency of Industrial Science and Technology, Tsukuba-shi, Japan under the accession No. FERM P-13048.

Production of Fluostatins A (1) and B (2)

The strain TA-3391 was inoculated into 110 ml of a seed medium consisting of glycerol 2.0%, Bacto-soytone (Difco) 2.0%, and $CaCO_3$ (pH adjusted to 7.4 with 2 N NaOH before sterilization) in a 500-ml baffled Erlenmeyer flask, and cultured at 27°C for 3 days on a rotary shaker (180 rpm). After incubation, two-ml of this seed medium was transferred to 110 ml of the fresh medium in the same type of flask and cultured under the same

Table 1. Cultural characteristics of strain TA-3391.

Agar medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose - nitrate agar	Poor	White	White	None
Glucose - aspargine agar	Moderate	None	Light yellowish brown	None
Yeast extract - malt extract agar (ISP No. 2)	Abundant	Light pink	Reddish gray	Light reddish brown
Oatmeal agar (ISP No. 3)	Good	Light pink	Light orange	None
Inorganic salts - starch agar (ISP No. 4)	Good	Light pink	Dark reddish orange	Light pink
Glycerol - asparagine agar (ISP No. 5)	Moderate	None	Dark reddish orange	Light pink
Tyrosine agar (ISP No. 7)	Good	Light pink	Light pink	None
Nutrient agar	Moderate	None	Light brown	None

The color names used in this table were based on the Color Harmony Manual (Container Corporation of America, Illinois).

Table 2. Taxonomic characterization of strain TA-3391.

Spore chain morphology	*Retinaculiaperti*
Spore surface	Smooth
Cell wall type	I
Aerial mass color	White to light pink
Reverse side color	White to reddish gray
Soluble pigment	Light pink to light reddish brown
NaCl concentration range for growth	0∼6%
Temperature range for growth (Optimum)	9.7∼32.6°C (21.2∼27.0°C)
Coagulation of skim milk	Positive
Peptonization of skim milk	Positive
Liquefaction of gelatin	Negative
Nitrate reduction	Positive
Hydrolysis of:	
starch	Positive
urea	Negative
chitin	Negative
casein	Positive
Carbon utilization:	
D-Glucose	Positive
D-Arabinose	Positive
D-Xylose	Positive
D-Fructose	Positive
D-Mannitol	Negative
Inositol	Negative
D-Rhamnose	Positive (doubtful)
Raffinose	Negative
Sucrose	Positive (doubtful)

condition as above until the production reached maximum.

The time course of the production in the broth was measured (Fig. 2). Residual glycerol in the cultivated broth were measured according to D. J. HANAHAN *et al.*[13]. The maximum level of fluostatins was obtained at 40 hours and was maitained thereafter. The amount of residual glycerol was decreased as the production of the inhibitors increased.

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

Isolation of Fluostatins A (1) and B (2)

The isolation scheme of **1** and **2** is summarized in Fig. 3.

The culture broth (6.0 liters) was filtered and separated into the mycelial cake and culture filtrate (5.8 liters). The broth filtrate was adsorbed onto a Diaion HP-20 column. The column was washed with water and eluted with 50% aqueous acetone. The active fractions were pooled and concentrated *in vacuo* to remove acetone. The solution was adjusted to pH 3.0 with concentrated HCl and extracted twice with an equal volume of ethyl acetate (500 ml × 2). The active extract was dried under reduced pressure to give a brown residue (1.25 g). The residue was suspended in chloroform - acetic acid (100 : 1) and was charged on a silica gel column (Wakogel C-200, 50 g), washed with the same solution, and developed with chloroform - methanol - acetic acid (98 : 2 : 1) to give first

Fig. 1. Structures of fluostatins A (**1**) and B (**2**).

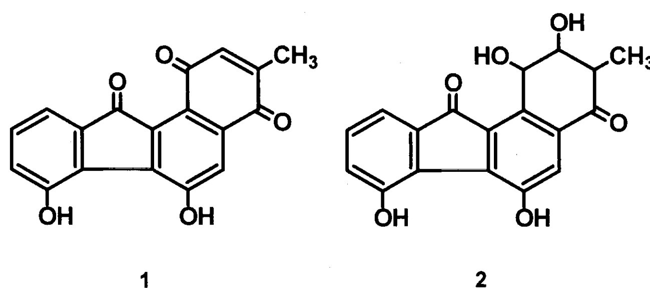
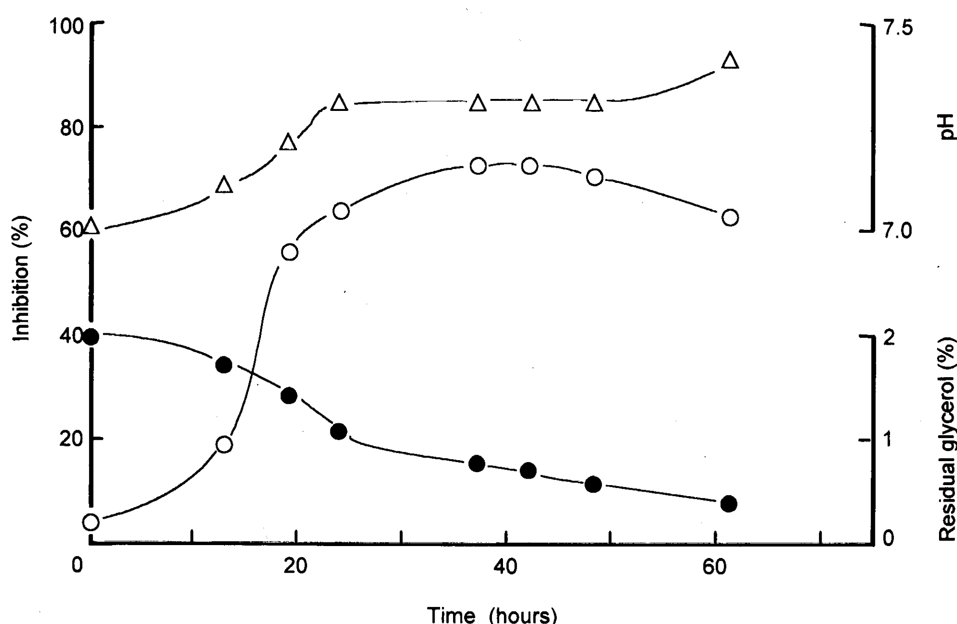


Fig. 2. Time course of fluostatins A (**1**) and B (**2**) production by *Streptomyces* sp. TA-3391.

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



active fraction (fraction 1), and then developed with chloroform-methanol-acetic acid (95:5:1) to give second active fraction (fraction 2).

The fraction 1 was evaporated to dryness to give a brown powder (102 mg), which was applied to a reverse phase HPLC (Capcell Pak C_{18} column, 2.0 × 25 cm, flow rate 8 ml/minute, Gilson system), and eluted with a solvent mixture of aqueous 27% acetonitrile containing 1% acetic acid. The fractions containing **1** (Fig. 1-1) were concentrated under reduced pressure to give a dark brown solid (1.8 mg). This solid was not soluble in various solvents, even dimethyl sulfoxide. Therefore, further purification was done by converting the free acid to the sodium salt. The solid was suspended in methanol (0.9 ml) and was added to saturated aqueous sodium hydrogen carbonate (0.1 ml). The resulting clear solution was diluted immediately with deionized water (25 ml) and was adsorbed onto Diaion HP-20 culumn (5 ml), washed with 10% aqueous acetone, and then eluted with 30% aqueous acetone. These operations were done at 4°C. The eluate was concentrated to dryness under reduced pressure, and then the residue was crystallized from methanol (5 ml) to give the sodium salt of pure **1** (1.1 mg, **1**-Na) as purple needles.

The fraction 2 containing **2** (Fig. 1-2) was evaporated to dryness to give a orange powder (288 mg). After crystallization from methanol (30 ml), pure **2** (237 mg) was obtained as orange rods.

Physico-chemical Properties of Fluostains A (**1**-Na) and B (**2**)

Both **1**-Na and **2** are soluble in dimethyl sulfoxide, but

Fig. 3. Isolation of fluostatins A (**1**, sodium salt) and B (**2**).

Whole broth (6.0 liters)

Culture filtrate (5.8 liters)

- Diaion HP-20 (0.4 liters)
- washed with water
- eluted with 50% aq. Me_2CO
- concentrated
- adjusted to pH 3.0
- extracted with EtOAc (0.5 liters × 2)

Organic layer (1 liter)

- dried over Na_2SO_4
- evaporated *in vacuo*

Crude powder (1.25 g)

- Silica gel chromatography (50 g)
- developed with $CHCl_3$ - MeOH - AcOH

(98 : 2 : 1) Fraction 1

- evaporated *in vacuo*

Brown powder (102 mg)

- HPLC (Capcell pak C_{18}, 20 × 250mm)
- MeCN - H_2O- AcOH (27 : 73 : 1)
- evaporated *in vacuo*

Dark brown solid (1.8 mg)

- MeOH (0.9 ml)
- satd. aq. $NaHCO_3$ (0.1 ml)
- diluted with H_2O (25 ml)
- Diaion HP-20 (5 ml)
- washed with 10% aq. Me_2CO
- eluted with 30% aq. Me_2CO
- evaporated *in vacuo*
- crystallized from MeOH

Fluostatin A (Na salt)
(1.1 mg, IC_{50} = 0.44 μg/ml)

(95 : 5 : 1) Fraction 2

- evaporated *in vacuo*

Orange powder (288 mg)

- crystallized from MeOH

Fluostatin B
(237 mg, IC_{50} = 24.0 μg/ml)

insoluble in chloroform and water. **2** is partially soluble in acetone and ethyl acetate, and **1**-Na is insoluble in these solvents. On the basis of HRFAB-MSs and elemental analyses, the molecular formulae of **1** and **2** were determined to be $C_{18}H_{10}O_5$ and $C_{18}H_{14}O_6$, respectively. Physico-chemical properties of these compounds are summarized in Table 3. Both structures of these compounds (Fig. 1) contain a fluorenone moiety. Structure determination of **1** and **2** will be described in the following paper[14].

Biological Activities of Fluostatins A (1-Na) and B (2)

The inhibitory activities of **1**-Na and **2** to DPPs are shown in Table 4. The IC_{50} values of **1**-Na and **2** to DPP-III were 0.44 and 24.0 μg/ml respectively, and these compounds were only slightly inhibitory to other DPPs (data not shown).

We examined whether or not **1**-Na inhibited the hydrolysis of human leucine-enkephalin by DPP-III *in*

Table 3. Physico-chemical properties of fluostatins A (**1**, sodium salt) and B (**2**).

	1, sodium salt	**2**
Appearance	Purple needles	Orange rods
MP (°C)	324～327 (dec.)	266～269 (dec.)
$[\alpha]_D^{26}$ (*c* 0.25, MeOH)	—	−6.4°
Molecular formula	$C_{18}H_9O_5Na$	$C_{18}H_{13}O_6$
HRFAB-MS (*m/z*, negative)		
Found	307.0605	325.0715
Calcd.	307.0607 for $C_{18}H_{11}O_5$	325.0712 for $C_{18}H_{13}O_6$
Elemental analysis		
Found	C: 64.48 H: 2.85 Na: 6.52	C: 64.99 H: 4.59
Calcd.	C: 64.10 H: 2.98 Na: 6.62 for $C_{18}H_9O_5Na \cdot 1/2H_2O$	C: 64.48 H: 4.51 for $C_{18}H_{14}O_6 \cdot 1/2H_2O$
UV nm (log ε) λ_{max}^{MeOH}	215 (4.76), 230 (sh, 4.65), 262 (4.42), 284 (4.39), 365 (4.19), 504 (3.86)	205 (4.78), 264 (4.62), 291 (sh, 4.37), 332 (sh, 4.11), 475 (3.79), 526 (sh, 3.69)
$\lambda_{max}^{MeOH-HCl}$	210 (4.73), 255 (4.56), 282 (sh, 4.22), 319 (4.23), 351 (sh, 4.12), 418 (3.78)	204 (4.79), 237 (4.42), 262 (4.66), 301 (4.43), 340 (sh, 3.88), 446 (3.90)
$\lambda_{max}^{MeOH-NaOH}$	229 (sh, 4.69), 262 (4.47), 284 (4.43), 367 (4.19), 505 (3.90)	267 (4.61), 337 (4.23), 521 (3.93)
IR cm^{-1} ν_{max}^{KBr}	3441, 1691, 1647, 1612, 1550, 1475, 1398, 1296, 956, 887	3451, 3260, 1687, 1599, 1460, 1385, 1280, 1188, 1035, 974
Rf values on TLC[a]	0.27[b], 0.55[c]	0.56[b], 0.32[c]
Solubility Soluble	DMSO	DMSO
Insoluble	$CHCl_3$, Me_2CO, H_2O	$CHCl_3$, H_2O

[a] Silica gel $60F_{254}$ (Merck, Art. 1.05628.), [b] $CHCl_3$ - MeOH (4 : 1), [c] $CHCl_3$ - MeOH - AcOH (90 : 5 : 5).

Table 4. Inhibitory activities of fluostatins A (**1**, sodium salt) and B (**2**) to dipeptidyl peptidases.

Enzyme	Inhibitory activity IC_{50} (μg/ml)	
	1 (sodium salt)	**2**
DPP-I	>100	>100
DPP-II	>100	>100
DPP-III	0.44	24.0
DPP-IV	>100	>100

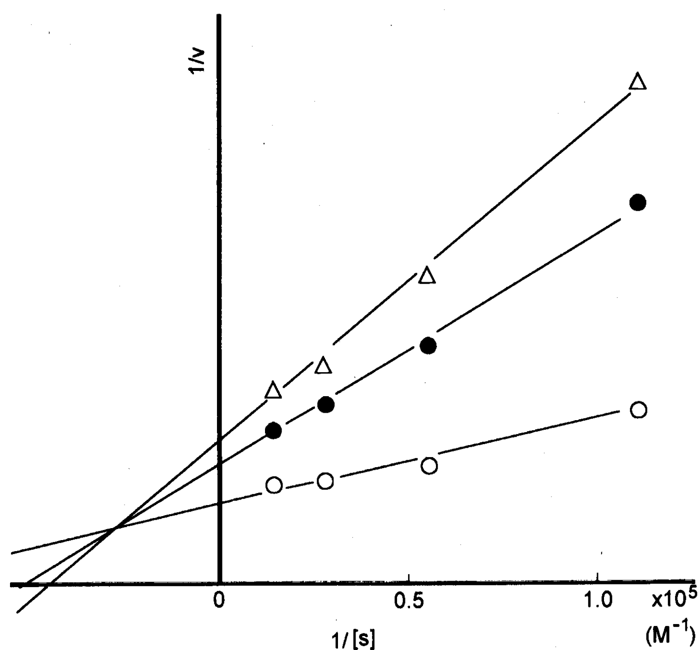
vitro. At 20 μg/ml **1**-Na inhibited DPP-III activity more than 80%. And as shown in Fig. 4, the inhibition by this compound of DPP-III was mixed-type (competitive and noncompetitive), when leucine-enkephalin was the substrate, and the *Ki* value was 14.2 μM (*Km* value was 10.5 μM).

1-Na and **2** showed very weak antimicrobial activities against bacteria and fungi (Data not shown). And both compounds had low toxicity; there were no deaths after an ip injection in mice of 100 mg/kg.

These data suggested that **1** is a potent and selective inhibitor of DPP-III *in vitro*. Both **1** and **2**, contained a fluorenone skelton and are unique non-peptide in-

Fig. 4. The Lineweaver-Burk reciprocal plot of the substrate (leucine-enkephalin) concentration against the rate of hydrolysis by DPP-III.

Without (○), and with the inhibitor (**1**, sodium salt) (●) 10 μg/ml or (△) 20 μg/ml.



hibitors. This paper is the first report that a non-peptide inhibitor of microbial origin of this enzyme. *In vivo* studies should help to elucidate the physiological role of this enzyme.

References

1) McDonald, J. K. & C. Schwade: Proteases in Mammalian Cells and Tissues. *Ed.*, A. J. Barrett, pp. 311 ~ 391, Elsevier/North-Holland Biomedical press, Amsterdam, 1977
2) Ellis, S & J. M. Nuenke: Dipeptidyl arylamidase III of the pituitary. Purification and characterization. J. Biol. Chem. 242: 4623 ~ 4629, 1967
3) Hopse-Havu, H. K.; C. T. Jansen & M. Jarvinen: Partial purification and characterization of an alkaline dipeptide naphthylamidase (Arg-Arg-NAase) of the rat skin. Arch. Klin. Exp. Dermatol. 236: 267 ~ 281, 1970
4) Swanson, A. A.; B. Albers-Jackson & J. K. McDonald: Mammalian lens dipeptidyl aminopeptidase III. Biochem. Biophys. Res. Commun. 84: 1151 ~ 1159, 1978
5) Pontremoli, S.; E. Melloni, F. Salamino, B. Sparatore, M. Michetti, U. Benatti, A. Morelli & A. De Flora: Identification of proteolytic activities in the cytosolic compartment of mature human erythrocytes. Eur. J. Biochem. 110: 421 ~ 430, 1980
6) Lee, C.-M. & S. H. Snyder: Dipeptidyl-aminopeptidase III of rat brain. J. Biol. Chem. 257: 12043 ~ 12050, 1982
7) Shimamori, Y.; Y. Watanabe & Y. Fujimoto: Purification and characterization of dipeptidyl aminopeptidase III from human placenta. Chem. Pharm. Bull. (Tokyo) 34: 3333 ~ 3340, 1986
8) Shimamori, Y.; Y. watanabe & Y. Fujimoto: Human placental dipeptidyl aminopeptidase III: Hydrolysis of enkephalins and its stimulation by cobaltous ion. Biochem. Med. Metab. Biol. 40: 305 ~ 310, 1988
9) Metrione, R. M.; A. G. Neves & J. S. Fruton: Purification and properties of dipeptidyl transferase (cathepsin C). Biochemistry 5: 1597 ~ 1604, 1966
10) McDonald, J. K.; F. H. Leibach, R. E. Grindeland & S. Ellis: Purification of dipeptidyl aminopeptidase II (dipeptidyl arylamidase II) of the anterior pituitary gland. J. Biol. Chem. 243: 4143 ~ 4150, 1968
11) Ôya, H.; I. Nagatsu & T. Nagatsu: Purification and properties of glycylprolyl β-naphthylamidase in human submaxillary gland. Biochem. Biophys. Acta 258: 591 ~ 599, 1972
12) Shirling, E. B. & D. Gottlieb: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313 ~ 440, 1966
13) Hanahan, D. J. & J. N. Olley: Chemical nature of monophosphoinositides. J. Biol. Chem. 231: 813 ~ 828, 1958
14) Akiyama, T.; K. T. Nakamura, Y. Takahashi, H. Naganawa, Y. Muraoka, T. Aoyagi & T. Takeuchi: Fluostatins A and B, new inhibitors of dipeptidyl peptidase III, produced by *Streptomyces* sp. TA-3391. II. Structure determination. J. Antibiotics 51: 586 ~ 588, 1998