Propeptin, a New Inhibitor of Prolyl Endopeptidase Produced by *Microbispora*

I. Fermentation, Isolation and Biological Properties

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Propeptin, an inhibitor of the prolyl endopeptidase isolated from the mycelium of *Microbispora* sp. SNA-115, is an atypical cyclic peptide antibiotic. It was purified by column chromatographies on silica gel and Sephadex LH-20 and high performance liquid chromatography using an ODS column. Propeptin has the molecular formula of $C_{113}H_{142}N_{26}O_{27}$ and consists of nineteen amino acids. Propeptin inhibited prolyl endopeptidase of the genus *Flavobacterium* competitively when *Z*-Gly-Pro-*p*NA was used as a substrate. The inhibitor constant (*Ki*) was 0.70 μM.

Prolyl endopeptidase (PEP: post-proline cleaving enzyme, the latest name is prolyl oligopeptidase: EC 3.4.21.26) was first isolated from human uterus as an oxytocin-inactivating enzyme and cleaves peptide bonds at the carboxyl side of proline residues[1~3]. It is distributed in a wide range of species, especially reported to be in human brain[4] and prolyl endopeptidase-like immunoreactivity has been detected in the mouse hippocampus[5]. Proline is an imino acid, considered to play an important role in conformation of many peptides. PEP, a new type of serine proteinase, is proposed to hydrolyze biologically active peptides containing proline such as oxytocin, vasopressin, substance P, bradykinin, LH-RH, neurotensin and angiotensins[6,7]. Therefore, it is suggested that it regulates the biological action of these peptides. Vasopressin has been suggested to be involved with learning and memory processes[8,9]. PEP activity in ALZHEIMER's patients was significantly higher than normal[10]. A putative amyloid A4-generating enzyme in ALZHEIMER's disease was identified as a PEP[11]. Moreover it was reported that the neurodegenerative effects of β amyloid could be prevented by intracerebral or systematic administration of substance P[12]. Thus, specific inhibitors for PEP may be expected to have anti-amnesic effects and were synthesized as anti-amnesic drugs[13,14]. Many of the synthetic inhibitors have an aldehyde moiety at the *C*-terminus and the structure-activity relationships were examined[15,16].

In the course of screening for a new type of PEP inhibitor from Actinomycetes, staurosporine, a known antifungal antibiotic and protein kinase inhibitor, was found to inhibit PEP activity[17~19]. During further screening, it was found that *Microbispora* sp. SNA-115 isolated from a soil sample produced a new PEP inhibitor designated as propeptin. In this communication, we report the fermentation, isolation, physico-chemical and biological properties of propeptin.

Materials and Methods

Materials

Prolyl endopeptidase (*Flavobacterium*) and substrate (*Z*-Gly-Pro-*p*NA) were purchased from Seikagaku Kogyo Co., Ltd.

Fermentation

A loopful of the producing strain SNA-115 from a slant culture was inoculated into a 500-ml volume Erlenmeyer flask containing 70 ml of medium composed of glucose 2%, soluble starch 1%, meat extract 0.1%, dried yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K_2HPO_4 0.005%, (pH 6.7), and the flask was incubated for 10 days at 27°C on a rotary shaker (200 rpm). Afterward two milliliters of the cultures was transferred into a flask containing the same medium and incubated for 3 day at 27°C, 140 ml of the seed culture were

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transferred into a 30-liter jar fermenter containing 18 liters of the medium. It was fermented for 212 hours at 27°C with agitation at 180 rpm and aeration of 18 liters/minute. The pH value of the culture broth reached 7.0.

Measurement of Enzyme Activities

PEP inhibition assay using cultured broth of Actinomycetes and the inhibition activity of propeptin were measured as described previously[19].

Instrumental Analyses

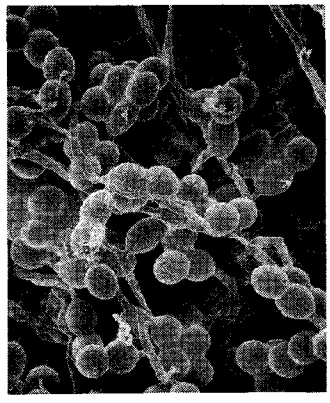
The mp was measured with a Yanagimoto micro melting point apparatus and was uncorrected. FAB and HR-FAB mass spectra were obtained on a JEOL JMS HX-110 mass spectrometer. Sample was dissolved in hydrochloric acid and glycerol, and ionized by FAB using Xe atoms. The optical rotation was determined with a Perkin-Elmer 241 polarimeter using a micro-cell. The UV spectrum was measured with a HITACHI U-3210 spectrophotometer. The IR spectrum was recorded on a JASCO DIP-181 digital polarimeter.

Results

Producing Strain

Strain SNA-115, which was isolated from a soil sample collected in Misakiguchi, Miura-city, Kanagawa Prefecture, Japan, produces characteristic paired spores on the aerial hyphae on tyrosine agar medium (ISP No. 7). The spores are $1.1 \sim 1.3 \times 1.3 \sim 1.7\,\mu m$ in size with smooth surface (Fig. 1). Aerial mass color of the colony is pink. The whole-cell hydrolysate of the strain showed that it contained *meso*-diaminopimelic acid. Based on its characteristics, strain SNA-115 is considered to belong to the genus *Microbispora*[20]. *Microbispora* sp. SNA-115 has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan under the accession number FERM-P12094. Type strains such as *Microbispora rosea* (IFO 14044), *Microbispora rosea* subsp. *nonnitritogenes* (IFO 14045) and *Microbispora indica* (IFO 14879) were also found to produce propetin (data not shown).

Fig. 1. Cryo scanning electron micrograph of *Microbispora* sp. SNA-115.



Medium: Tyrosine agar (ISP No. 7). Cultivation : 27°C for 21 days.

Production and Isolation of Propeptin

The time course of the production in a 30-liter jar fermenter is shown in Fig. 2. The maximum peak of propeptin production was obtained at 8~9 days, thereafter the amount slowly decreased. The production

Fig. 2. A typical time course of fermentation by *Microbispora* sp. SNA-115 (30-liter jar fermenter).

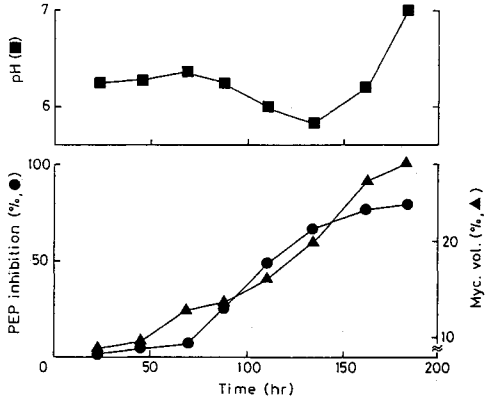


Fig. 3. Isolation procedure of propeptin.

Culture broth (18 liters)
↓ centrifugation
Mycelium (1.75 kg)
↓ extracted with acetone
↓ evaporation
Crude powder (34.8 g)
↓ Silica gel column (4 x 36 cm)
↓ eluted with $CHCl_3$-CH_3OH (1 : 1, 1 : 2)
↓ evaporation
Crude powder (3.9 g)
↓ Sephadex LH-20 column (3 x 73 cm)
↓ eluted with CH_3OH
↓ evaporation
Crude powder (3.1 g)
↓ Preparative HPLC (2 times)
↓ Nucleosil $5C_{18}$, 40 % CH_3CN-0.1 % TFA
↓ evaporation
↓ lyophilization
Propeptin (SNA-115, 0.6 g)

of propeptin in the mycelium is 4 times more than in the supernatant.

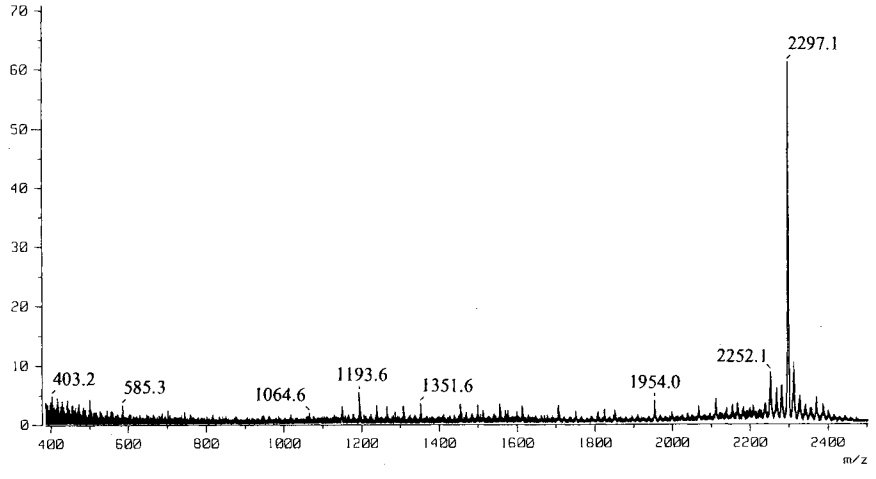
The flow diagram for the isolation of the inhibitor is shown in Fig. 3. The cultured broth was centrifuged to obtain 1.75 kg of the mycelium. It was extracted with acetone and evaporated to crude complex (34.8 g), which was suspended in chloroform-methanol (1:1) and chromatographed on a silica gel column (4 × 36 cm) with chloroform-methanol (1:1 and 1:2). Active fraction was concentrated *in vacuo* and lyophilized. The crude powder (3.9 g) was dissolved in a small amount of methanol and loaded onto a Sephadex LH-20 column (3 × 73 cm) using methanol as the eluent. Active fraction was concentrated *in vacuo* and lyophilized (3.1 g). Final purification was carried out by preparative HPLC using Nucleosil $5C_{18}$ (20 × 250 mm) with CH_3CN-0.1% TFA (40:60). After concentration and lyophilization, propeptin was obtained as a white powder. The yield of pure propeptin was 600 mg from 18 liters of the culture.

Table 1. Physico-chemical properties of propeptin.

Nature	White amorphous powder
Melting point	230 ~240 °C (dec)
Molecular formula	$C_{113}H_{142}N_{26}O_{27}$
FAB-MS (m/z)	2296 $(M+H)^+$
HRFAB-MS (m/z)	Found : 2296.0627
	Calcd.: 2296.0616 for $C_{113}H_{143}N_{26}O_{27}$
$[\alpha]_D^{24}$	-87 ° (c 0.1, MeOH)
UV λ_{max}^{MeOH} nm (ε)	280 (11934)
IR ν_{max} (KBr) cm^{-1}	3350, 1670, 1520, 1450, 1205, 1135
Solubility	
Soluble	MeOH, EtOH, DMSO
Insoluble	CH_3CN, $CHCl_3$, H_2O
TLC, Rf value *	0.36

* Silica gel 60 F_{254} (Merck), BuOH : MeOH : H_2O (4 : 1 : 2)

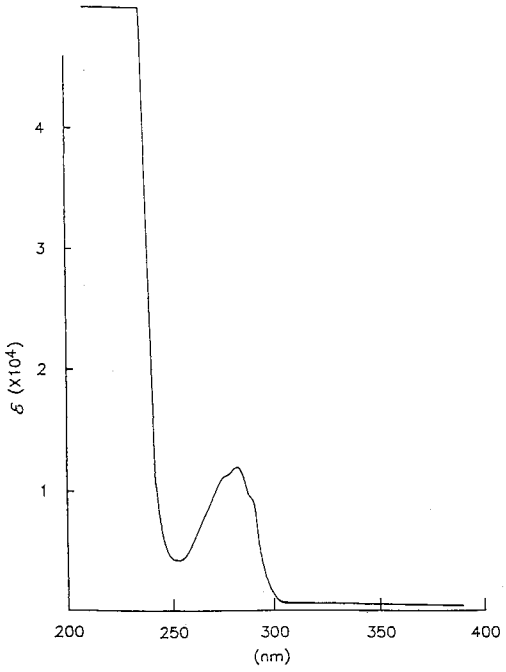
Fig. 4. FAB-MS spectrum of propeptin.



Physico-chemical Properties of Propeptin

The physico-chemical properties of propeptin are summarized in Table 1. The molecular weight and formula of propeptin were determined by FAB-MS and HRFAB-MS. FAB-mass spectrum of propeptin is shown in Fig. 4. The UV and IR spectra of propeptin are shown in Figs. 5 and 6. The HPLC profile of propeptin is shown in Fig. 7. Though two peaks were observed in 30% CH_3CN-0.1% TFA~60% CH_3CN-0.1% TFA (linear gradient), the latter peak changed to the former peak when the sample was prepared immediately. The eluted solution from one peak was collected and the concentrate was re-injected into the ODS column under the original conditions, the HPLC profile was almost identical to that shown in Fig. 7. The presence of conformational isomers of propeptin could explain this phenomenon. The broadening of the proton signals in the ^1H NMR spectrum (data not shown) supports this explanation. Propeptin was hydrolyzed with 4 N methane sulfonic acid at 90°C for 24 hours and amino acid analysis of the hydrolyzate showed the presence of 19 amino acids (molar ratio): Asp (1.94), Thr (0.85), Ser (0.98), Gly (3.09), Ile (0.91), Leu (1.18), Tyr (1.85), Phe (1.71), His (1.05), Arg (0.85), Trp (1.72), Pro (1.85). The difference of 18 in the molecular weight of propeptin by FAB-MS and that indicated by amino acid composition supports a cyclic structure for propeptin.

Fig. 5. UV spectrum of propeptin (in MeOH).



Biological Properties of Propeptin

Propeptin potently inhibited PEP in a dose dependent manner. The concentration of propeptin causing 50% inhibition of PEP was 1.1 μM. As shown in Fig. 8, propeptin inhibited PEP competitively. The inhibitor constant (*Ki*) was 0.70 μM. The potential of propeptin for inhibiting PEP was 500 times more than that of *Z*-Pro-prolinol and it was almost the same as staurosporine[19]. It also inhibited mammalian PEP from human placenta and bovine brain at equivalent concentrations as for *Flavobacterium*. Inhibition activity of various compounds against bacterial and mammalian PEP will be reported elsewhere. Otherwise propeptin did not inhibit other serine proteinases such as trypsin, chymotrypsin, plasmin, pancreatic kallikrein, thrombin and elastase at 10 μM. Propeptin showed no toxicity against tumor cell lines such as KB and L1210 cells at

Fig. 6. IR spectrum of propeptin (KBr).

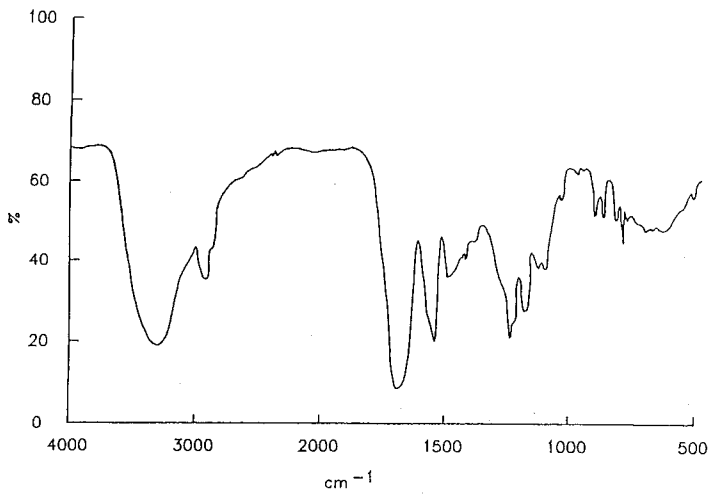
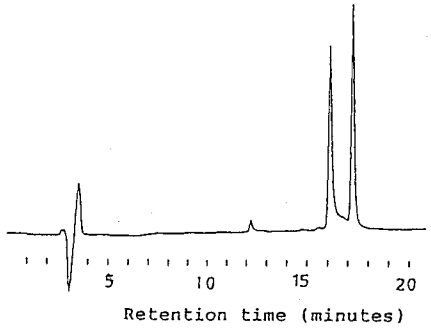
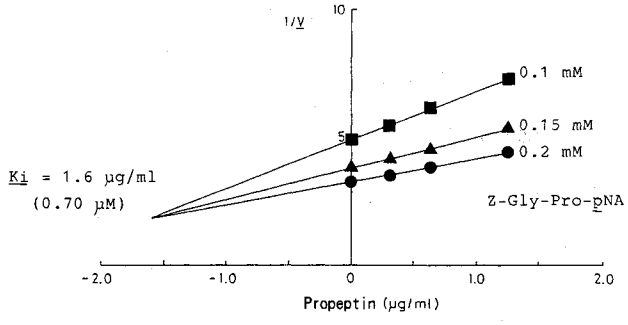


Fig. 7. HPLC chromatogram of propeptin.



Column: Senshu Pak ODS-1251-N (4.6 i.d. × 250 mm).
Solvent: 30~60% CH_3CN containing 0.1% TFA (linear gradient, 20 minutes).
Flow rate: 1 ml/minute, detector: UV (220 nm).

Fig. 8. Dixon plot of the inhibition of prolyl endopeptidase by propeptin.

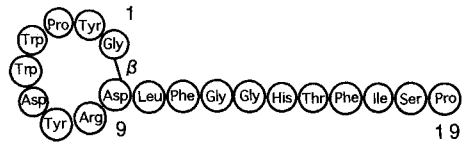


1000 μg/ml and weak antimicrobial activity against *Pseudomonas aeruginosa* N-10 L-form (10.6 mm), *Mycobacterium phlei* IFO 3158 (14.5 mm) and *Xanthomonas oryzae* IFO 3312 (11.5 mm) at 40 μg/disc.

Discussion

Many inhibitors of PEP such as *Z*-Pro-prolinol, *Z*-Pro-prolinal, *Z*-thiopro-thioprolinal and *Z*-thioprothiazolidine were chemically synthesized as anti-amnesic drug candidates[13~16,21,22]. Structure activity relationships for inhibitors specific to PEP were also investigated. On the other hand, the inhibitors isolated from natural sources are only bacitracin[23], staurosporine[19], postatin[24] and eurystatin[25]. Propeptin reported in this paper was found to be produced by a strain of Actinomycetes in our screening program for new PEP inhibitors. It was confirmed by the molecular weight, molecular formula and amino acid composition to be a new compound. Propeptin is a unique cyclic peptide containing nineteen amino acids which is composed of

Fig. 9. Structure of propeptin.



a ring region with nine amino acids and a side chain region with ten amino acids (Fig. 9.). Such structure was also elucidated by the results of Edman degardation, trypsin digestion, FAB/linked scan spectrum, calculated and observed molecular weight. All amino acids except glycine are L-form (data not shown). An ANF antagonist, anantin[26] and an endothelin antagonist, RES-701-1[27], both compounds produced by *Streptomyces* sp. have similar cyclic structure to propeptin. That is, all compoumds have an amide bound between the α-amino group of the amino-terminal Gly^1 and the β-carboxyl group of Asp^8 or Asp^9.

However, propeptin did not inhibit [^{125}I]ANF binding to the ANF receptor and [^{125}I]endothelin binding to the endothelin receptors A and B even at 10 μM. Thus biological activities were different from each other in this structurally related series, the activities might depend on the amino acid sequences. This is the first report that *Microbispora* sp. produces a compound having such unique peptide structure. Determination of the chemical structure of propeptin on detail will be reported elsewhere.

Acknowledgments

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References

1) WALTER, R.; H. SHLANK, J. D. GLASS, I.L. SCHWARTZ & T. D. KERENYI: Leucylglycinamide released from oxytocin by human uterine enzyme. Science 173: 827~829, 1971
2) KOIDA, M. & R. WALTER: Post-proline cleaving enzyme. J. Biol. Chem. 251: 7593~7599, 1976
3) YOSHIMOTO, T.; R. C. ORLOWSKI & R. WALTER: Post-proline cleaving enzyme: Identification as serine protease using active site specific inhibitors. Biochemistry 16: 2942~2948, 1977
4) KALWANT, S. & A. G. PORTER: Purification and characterization of human brain prolyl endopeptidase. Biochem. J. 276: 237~244, 1991
5) FUKUNARI, A.; A. KATO, Y. SAKAI, T. YOSHIMOTO, S. ISHIURA, K. SUZUKI & T. NAKAJIMA: Colocalization of prolyl endopeptidase and amyloid β-peptide in brains of

senescence-accelerated mouse. Neurosci. Lett. 176: 201 ~204, 1994

6) RENNEX, D.; B. A. HEMMINGS, J. HOFSTEENGE & S. R. STONE: cDNA cloning of porcine brain prolyl endopeptidase and identification of the active-site seryl residue. Biochemistry 30: 2195~2203, 1991

7) YOSHIMOTO, T.; T. NISHIMURA, T. KITA & D. TSURU: Post-proline cleaving enzyme (prolyl endopeptidase) from bovine brain. J. Biochem. 94: 1179~1190, 1983

8) BURBACH, J. P. H.; G. L. KOVÁCS, D. DE WIED, J. W. VAN NISPEN & H. M. GREVEN: A major metabolite of arginine vasopressin in the brain is a highly potent neuropeptide. Science 221: 1310~1312, 1983

9) DE WIED, D.; O. GAFFORI, J. M. VAN REE & W. DE JONG: Central target for the behavioural effects of vasopressin neuropeptides. Nature 308: 276~278, 1984

10) AOYAGI, T.; T. WADA, M. NAGAI, F. KOJIMA, S. HARADA, T. TAKEUCHI, H. TAKAHASHI, K. HIROKAWA & T. TSUMITA: Deficiency of kallikrein-like enzyme activities in cerebral tissue of patients with Alzheimer's disease. Experientia 46: 94~97, 1990

11) ISHIURA, S.; T. TSUKAHARA, T. TABIRA, T. SHIMIZU, K. ARAHATA & H. SUGITA: Identification of a putative amyloid A4-generating enzyme as a prolyl endopeptidase. FEBS Letters 260: 131~134, 1990

12) KOWALL, N. W.; M. F. BEAL, J. BUSCIGLIO, L. K. DUFFY & B. A. YANKNER: An *in vivo* model for the neurodegenerative effects of β amyloid and protection by substance P. Proc. Natl. Acad. Sci. U.S.A. 88: 7247~7251, 1991

13) YOSHIMOTO, T.; K. KADO, F. MATSUBARA, N. KORIYAMA, H. KANETO & D. TSURU: Specific inhibitors for prolyl endopeptidase and their anti-amnesic effect. J. Pharmacobio-Dyn. 10: 730~735, 1987

14) SAITO, M.; M. HASHIMOTO, N. KAWAGUCHI, H. FUKAMI, T. TANAKA & N. HIGUCHI: Synthesis and inhibitory activity of acylpeptidyl-prolinal derivatives toward post-proline cleaving enzyme as nootropic agents. J. Enzyme Inhibition 3: 163~178, 1990

15) TSURU, D.; T. YOSHIMOTO, N. KORIYAMA & S. FURUKAWA: Thiazolidine derivatives as potent inhibitors specific for prolyl endopeptidase. J. Biochem. 104: 580~586, 1988

16) YOSHIMOTO, T.; D. TSURU, N. YAMAMOTO, R. IKEZAWA & S. FURUKAWA: Structure activity relationship of inhibitors specific for prolyl endopeptidase. Agric. Biol. Chem. 55: 37~43, 1991

17) OMURA, S.; Y. IWAI, A. HIRANO, A. NAKAGAWA, J. AWAYA, H. TSUCHIYA, Y. TAKAHASHI & R. MASUMA: A new alkaloid AM-2282 of *Streptomyces* origin. Taxonomy, fermentation, isolation and preliminary characterization. J. Antibiotics 30: 275~282 , 1977

18) TAMAOKI, T.; H. NOMOTO, I. TAKAHASHI, Y. KATO, M. MORIMOTO & F. TOMITA: Staurosporine, a potent inhibitor of phospholipid/Ca^{++} dependent protein kinase. Biochem. Biophys. Res. Commun. 135: 397~402, 1986

19) KIMURA, K.; N. KAWAGUCHI, M. YOSHIHAMA & G. KAWANISHI: Staurosporine, a prolyl endopeptidase inhibitor. Agric. Biol. Chem. 54: 3021~3022, 1990

20) BERGEY's Mannual of Systematic Bacteriology, Volume 4, *Eds.*, S. T. WILLIAMS, *et al.*, The Williams and Wilkins Co., Baltimore, U.S.A., 1989

21) WILK, S. & M. ORLOWSKI: Inhibition of rabbit brain prolyl endopeptidase by *N*-benzyloxycarbonyl-prolyl-prolinal, a transition state aldehyde inhibitor. J. Neurochem. 41: 69~75, 1983

22) FRIEDMAN, T. C.; M. ORLOWSKI & S. WILK: Prolyl endopeptidase: Inhibition *in vivo* by *N*-benzyloxycarbonyl-prolyl-prolinal. J. Neurochem. 42: 237~241, 1984

23) KATO, T.; T. NAKANO, K. KOJIMA, T. NAGATSU & S. SAKAKIBARA: Changes in prolyl endopeptidase during maturation of rat brain and hydrolysis of substance P by the purified enzyme. J. Neurochem. 35: 527~535, 1980

24) AOYAGI, T.; M. NAGAI, K. OGAWA, F. KOJIMA, M. OKADA, T. IKEDA, M. HAMADA & T. TAKEUCHI: Poststatin, a new inhibitor of prolyl endopeptidase, produced by *Streptomyces* viridochromogenes MH534-30F3. I. Taxonomy, production, isolation, physico-chemical properties and biological activities. J. Antibiotics 44: 949~955, 1991

25) TODA, S.; Y. OBI, K. NUMATA, Y. HAMAGISHI, K. TOMITA, N. KOMIYAMA, C. KOTAKE, T. FURUMAI & T. OKI: Eurystatins A and B, new prolyl endopeptidase inhibitors. I. Taxonomy, production, isolation and biological activities. J. Antibiotics 45: 1573 ~1579, 1992

26) WYSS, D. F.; H.-W. LAHM, M. MANNEBERG & A. M. LABHARDT: Anantin-A peptide antagonist of the atrial natriuretic factor (ANF). II. Determination of the primary sequence by NMR on the basis of proton assignments. J. Antibiotics 44: 172 ~180, 1991

27) MORISHITA, Y.; S. CHIBA, E. TSUKUDA, T. TANAKA, T. OGAWA, M. YAMASAKI, M. YOSHIDA, I. KAWAMOTO & Y. MATSUDA: RES-701-1, A novel and selective endothelin type B receptor antagonist produced by *Streptomyces* sp. RE-701. I. Characterization of producing strain, fermentation, isolation, physico-chemical and biological properties. J. Antibiotics 47: 269~275, 1994