# Lipohexin, a New Inhibitor of Prolyl Endopeptidase from *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096)

## II. Inhibitory Activities and Specificity

CLAUDIA CHRISTNER, MARION ZERLIN[†], UDO GRÄFE[††], STEPHAN HEINZE[††],
GERHARD KÜLLERTZ and GUNTER FISCHER*

Max-Planck-Arbeitsgruppe "Enzymologie der Peptidbindung"
Kurt-Mothes-Straße 3, D-06120 Halle/Saale, Germany
[†] Max-Planck-Arbeitsgruppe "Molekulare Zellbiologie"
Drackendorfer Straße 1, D-07747 Jena, Germany
[††] Hans-Knöll-Institut für Naturstoff-Forschung e.V.
Beutenbergstraße 11; D-07745 Jena, Germany

The new proline-containing lipohexapeptide lipohexin (I) isolated from three fungal strains, *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055 and HKI-0096) is a competitive inhibitor of prolyl endopeptidase (PEP) from human placenta with $IC_{50}$ of 3.5 $\mu$M. Specificity of lipohexin (I) is indicated by the much weaker inhibitory activity against bacterial prolyl endopeptidase from *Flavobacterium meningosepticum* ($IC_{50}$ 25 $\mu$M). No effect of lipohexin (I) was found on the activity of mechanistically related proteases such as proline specific proteases and other serine proteases.

The prolyl endopeptidase family (PEP) [EC 3.4.21.26] of serine proteases[1], degrades proline-containing peptides by hydrolysis of the peptide bond at the carboxyl side of proline residues. Proteins with molecular weights in the range of 60 ~ 120 kDa showing this enzyme activity were isolated from various microorganisms, plants and invertebrates. In animal tissues, the highest activities were detected in the CNS[2]. In contrast to aminopeptidases, PEP possesses a high degree of substrate specificity, which is caused by the stereospecific requirements of the amino acid proline[3]. *In vivo* as well as *in vitro* investigations indicate that PEP plays an important role in the processing and degradation of prolyl-containing biologically active peptides such as angiotensin I and II, oxytocin, vasopressin, neurotensin or angiotensin[4]. Clinical studies suggest PEP involved in the *in vivo* regulation of essential biological functions, *e.g.* inflammation, homeostasis as well as learning and memory processes[3]. Furthermore, there is striking evidence that region-specific alteration in the enzyme activity seems to be associated with the pathophysiology of certain infections and diseases like HIV, malaria and several tumors[5]. Abnormal PEP levels may be related to neuropathological disorders, for instance major depression, mania, schizophrenia[6] and senile dementia of the ALZHEIMER's type[7].

As specific inhibitors of PEP are expected to have anti-amnesic effects[8], the chemical synthesis of inhibitors as well as the search for natural products as possible anti-amnesic drugs is of interest. Recently we isolated the new lipopeptide antibiotic lipohexin (I, Fig. 1)[9]. In the preliminary PEP assay lipohexin (I) was shown to have a significant inhibitory effect. In this paper the inhibition kinetics as well as the specificity of lipohexin (I) towards 18 members of 8 different protease classes will be reported.

## Materials and Methods

### General Experimental Procedures

Enzyme activities of the different proteases were measured with different methods and devices: Spectrophotometric investigations were performed with an Uvicon 940 (Kontron), a Specord M 500 (Zeiss), or a microplate reader (MR7000, Dynatech). Capillary electrophoresis was carried out with 270A-HT capillary electrophoresis system (Applied Biosystems) and FPLC with L-6210 (Hitachi), column superformance 300-10, BioGPC-Diol 250, 5 $\mu$m (Merck). Analytical HPLC was performed using the column Spherisorb RP18, 4.3 × 250 mm, 5 $\mu$m.

### Chemicals

Chemicals employed were as follows: $N$-succinyl-Ala-Phe-Pro-Phe-$p$-nitroanilide (Suc-Ala-Phe-Pro-Phe-$p$NA), Suc-Ala-Ala-Pro-Arg-$p$NA, Suc-Ala-Pro-$p$NA,

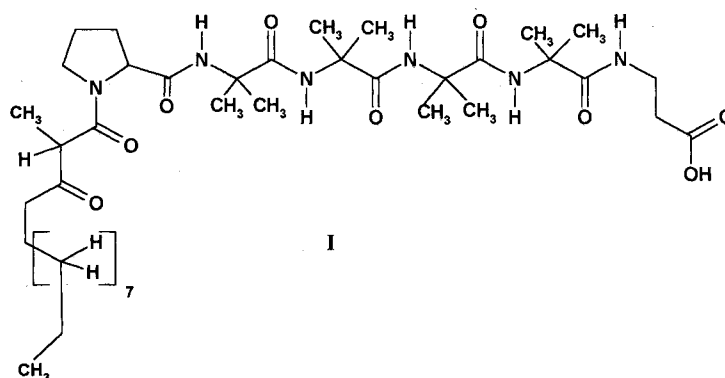Fig. 1. Chemical structure of lipohexin (I).



I

Table 1. Proteinases and proteases tested for the specificity of lipohexin (I).

| Enzyme class | Enzyme (EC number) | Source |
|---|---|---|
| Aminopeptidases | Prolyliminopeptidase (3.4.11.5)[3,13] | *Bacillus coagulans* |
| Dipeptidases | Prolidase (3.4.13.9)[3,13] | Pig kidney |
| Dipeptidyl peptidases | Dipeptidyl peptidase II (3.4.14.2)[3,10] | Pig kidney |
| | Dipeptidly peptidase IV (3.4.14.5)[3,10] | Pig kidney |
| Metallo carboxypeptidases | Carboxypeptidase A (3.4.17.1)[14] | Bovine pancreas |
| Omega peptidases | Pyroglutamate-aminopeptidase (3.4.19.3)[13] | *Bacillus amyloliquefaciens* |
| Serine proteases | α-Chymotrypsin (3.4.21.1)[14] | Bovine serum |
| | Nargarse (3.4.21.14)[13] | *Bacillus amyloliquefaciens* |
| | Pronase (3.4.21.14)[13] | *Streptomyces griseus* |
| | Proteinase K (3.4.21.14)[13] | *Tritirachium album* |
| | Subtilisin (3.4.21.14)[13] | *Bacillus licheniformis* |
| | Thermitase (3.4.21.14)[13] | *Thermoactinomyces vulgaris* |
| | Trypsin (3.4.21.4)[14] | Bovine pancreas |
| | Thrombin (3.4.21.5)[14] | Bovine plasma |
| | Prolyl endopeptidase (3.4.21.26)[10] | Human placenta |
| | Prolyl endopeptidase (3.4.21.26)[11] | *Flavobacterium meningosepticum* |
| Cysteine proteinases | Papain (3.4.22.2)[13] | *Carica papaya* |
| Aspartic proteinases | HIV-protease (3.4.23.-)[12] | HIV |

Gly-Pro-pNA, Pro-pNA, Ala-Pro-pNA, and Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH$_2$ from Bachem, Bubendorf, Switzerland; Hippuryl-Phe, Pyroglutamic acid-pNA from Sigma, Deisenhofen, Germany. All other chemicals were of analytical grade.

Lipohexin

The purity of lipohexin (I), isolated from the culture broth of *Moeszia lindtneri* (HKI-0054)[9] was controlled by analytical HPLC. For inhibitor studies I was dissolved in methanol in a stock solution of 1.3 mM and diluted to concentrations between 100 and 0.05 μM in the reaction mixture. Inhibition experiments were performed after preincubation of lipohexin (I) with the corresponding enzyme, for at least 10 minutes.

Enzymes

PEP [EC 3.4.21.26] was prepared from human placenta[10] as a trimeric complex of PEP, α$_1$-antitrypsin and human albumin. This trimeric complex, which is not separable under native conditions, was used for the inhibitor studies of lipohexin (I). PEP from *Flavobacterium meningosepticum*[11] was obtained from the Weissheimer Research Laboratory, Andernach. Dipeptidyl peptidases II and IV were purified from pig kidney, as previously described[10]. HIV-protease was prepared according to the method by IDO *et al.*[12]. The following enzymes were purchased commercially: trypsin (Boehringer, Mannheim, Germany), pronase (Ferak, Berlin, Germany), carboxypeptidase A (Reanal, Budapest, Hungary), papain and thrombin (Serva, Heidelberg, Germany), prolidase, nagarse, subtilisin, thermitase and pyroglutamate aminopeptidase (Sigma, Deisenhofen, Germany), α-chymotrypsin and proteinase K (Merck, Darmstadt, Germany). Characteristics of the used enzymes were compiled in Table 1.

## Prolyl Endopeptidase Assay

PEP activity was determined by the cleavage of the chromogenic substrate Suc-Ala-Pro-$p$NA. The increase of absorbance of 4-nitroaniline was measured at 390 nm using the microplate reader. The assay was performed at 30°C. The reaction solution contained enzyme (5.3 nM PEP from human placenta or 51 nM PEP from *Flavobacterium meningosepticum* with specific activities of 9.8 and 3.3 U/ml, respectively), 233 $\mu$M substrate, 100～0.05 $\mu$M inhibitor, 10 mM HEPES, 1.5 mM MgCl$_2$· 6H$_2$O, 150 mM KCl and 0.5 mM DTT, pH 7.8. PEP activity was calculated using an extinction coefficient ($\varepsilon_{390}$) of 11500 M$^{-1}$cm$^{-1}$ for 4-nitroaniline[10]. The kinetic constants Vmax, Km, and Ki were determined applying the Lineweaver-Burk transformation.

## Assays for Other Enzymes

HIV protease activity was measured spectrophotometrically at 300 nm and 30°C with the Specord M500, using Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH$_2$ as substrate. The reaction mixture containing 23 $\mu$M HIV protease, 75～20 $\mu$M lipohexin, 100 mM sodium acetate, 1 M NaCl, 5 mM mercaptoethanol, 4 mM EDTA, pH 4.7 was preincubated for 20 minutes at 30°C and the reaction was started by addition of 0.28 mM substrate.

The carboxypeptidase A assay was performed at 228 nm and 37°C in a reaction mixture composed of 2.42 $\mu$M Hippuryl-Phe, 11.3 $\mu$M caboxypeptidase, 75～20 $\mu$M lipohexin, 50 mM Tris-HCl, 1 mM NaCl, pH 7.5.

The activity of all other enzymes was determined analogous to the PEP assay by measuring the concentration of 4-nitroaniline according to the literature cited in Table 1. In the prolidase assay the release of 4-nitroaniline from the substrate cleavage product Pro-4-nitroanilide was reached by the addition of 0.15 $\mu$M prolyliminopeptidase to the reaction mixture[3]. In general, the reactions were started by addition of 25 $\mu$M of 1 mM substrate stock solution. Substrates used are given in Table 2.

## Gelpermeation

100 $\mu$l of a mixture consisting of 8 $\mu$g PEP and 0.4 mg lipohexin were applied to a gel filtration experiment. This lipohexin (I) concentration was sufficient to inhibit PEP completely. Separation of PEP from lipohexin (I) was accomplished by a GPC-Diol column (250, 5 $\mu$m, MERCK) eluted with a buffer solution consisting of 10 mM HEPES, 1.5 mM MgCl$_2$·6H$_2$O, 150 mM KCl and 0.5 mM DTT (pH 7.8) with a flow rate of 0.5 ml/minute. The enzyme activity was measured by the PEP assay,

described above, with 20 $\mu$l of the protein-containing fractions and 0.37 mM substrate in 100 ml buffer. Analogous, lipohexin (I) was detected by measuring the inhibition in a reaction mixture of 5.3 nM PEP, 20 $\mu$l of the FPLC fractions, and 0.37 mM substrate in 100 ml buffer.

## Capillary Electrophoresis

Capillary electrophoresis to prove the substrate character of lipohexin (I) was performed in 100 mM citrate-NaOH buffer (pH 2.4). A mixture consisting of 30 $\mu$M lipohexin (I) and 86 $\mu$M PEP in 10 mM HEPES, 1.5 mM MgCl$_2$·6H$_2$O, 150 mM KCl and 0.5 mM DTT (pH 7.8) was analysed in intervals of 120 minutes over a period of 24 hours.

# Results

Because lipohexin (I) showed an inhibitory effect on prolyl endopeptidase from human placenta (hPEP) as well as from *Flavobacterium meningosepticum* (bPEP), the following studies were carried out in parallel with both enzymes.

## Inhibition Studies

The Lineweaver-Burk plot (Fig. 2A) reveals a competitive inhibition mode of lipohexin (I). The inhibitor constants for hPEP and bPEP were graphically determined to 3.6 and 27 $\mu$M (Fig. 2B), indicating the nearly ten-fold weaker inhibitory activity of lipohexin (I) on the bacterial prolyl endopeptidase. Using the formula for a competitive inhibitor model the calculated Ki values of 3.8±0.7 $\mu$M (hPEP) and 20.0±2 $\mu$M (bPEP) confirm the graphically determined results.

Additionally, the reversibility of the lipohexin-PEP interaction was investigated using gel chromatography. A mixture of lipohexin (I) and hPEP, without detectable enzymatic activity in the PEP activity test was separated in fractions containing I (66～100) and fractions containing hPEP (45～62). Measurements of the hPEP activity after column passage showed that the enzyme could be reactivated to 79% of the activity compared to the positive control (fractions 45～62). Consequently, lipohexin (I) seems to be a reversible inhibitor of PEP.

## Lipohexin Stability

Considering the chemical structure of lipohexin (I)[9], which includes a $N$-terminally blocked proline residue as well as a potential cleavage site $C$-terminally to proline, the prolyl-containing lipopeptide could embody a substrate for prolylendopeptidases. This hypothesis was

examined by controlling the enzyme activity in the presence of lipohexin (I) during a reaction time of 24 hours. The initial enzyme activity was adjusted to 30% of the positive control by addition of 7.5 μM lipohexin (I). However, a time-dependent increase of the PEP activity, which should be the consequence of a slow cleavage of lipohexin (I), was not observed. In parallel, a possible enzymatic consumption of lipohexin (I) could not be detected using capillary electrophoresis as a sensitive analytic tool. Consequently, the potential cleavage site for PEP of lipohexin (I) is completely inert even if the competitive inhibition may indicate proper binding to a Michaelis complex.

### Specificity of Lipohexin Mediated Inhibition

To characterize the specificity of lipohexin (I), representatives of several proteases, which differ with regard to the structure and amino acid sequence of their catalytic centres, were tested in presence of lipohexin (I). Except for an inhibitory effect on the cystein protease papain (IC$_{50}$ = 34 μM), no further inhibition of the activity of the tested serine proteases, carboxypeptidases, aminopeptidases, dipeptidases, dipeptidyl peptidases, omega peptidases or aspartic proteinases (IC$_{50}$ ≫ 75 μM) by lipohexin (I) was detected (Table 2). Interestingly, no inhibitory effects could be observed on the activity of other proline-specific proteases, like HIV-protease, prolidase, prolyliminopeptidase, dipeptidyl peptidase II and dipeptidyl peptidase IV.

Fig. 2.  Inhibition kinetics of lipohexin (I) on the activity of prolyl endopeptidase from human placenta.

(A)  Lineweaver-Burk plot.

(□) 0 μM, (▲) 4 μM, (△) 6 μM (●) 7 μM and (○) 9 μM of lipohexin (I).
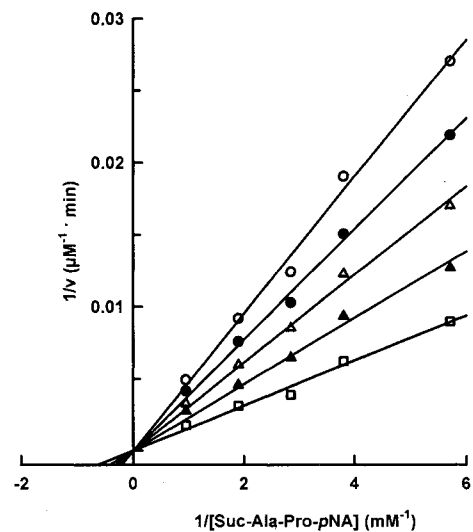


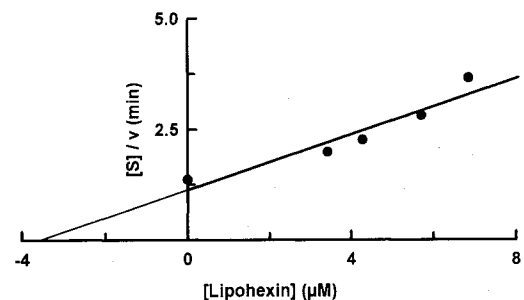(B)  Determination of Ki value for lipohexin (I).



Table 2.  Specificity of lipohexin (I) towards members of aminopeptidases, dipeptidases, dipeptidyl peptidases, carboxypeptidases, omega peptidases, serine proteases, cysteine proteinases and aspartic proteases.

| Enzyme | Substrate | IC$_{50}$ (μM) |
|---|---|---|
| Prolyliminopeptidase | Pro-pNA | ≫ 75 |
| Prolidase | Suc-Ala-Pro-pNA | ≫ 75 |
| Dipeptidyl peptidase II | Gly-Pro-pNA | ≫ 75 |
| Dipeptidlyl peptidase IV | Gly-Pro-pNA | ≫ 75 |
| Carboxypeptidase A | Hippuryl-Phe | ≫ 75 |
| Pyroglutamate-aminopeptidase | Pyroglutamic acid-pNA | ≫ 75 |
| α-Chymotrypsin | Suc-Ala-Phe-Pro-Phe-pNA | ≫ 75 |
| Nargarse | Suc-Ala-Phe-Pro-Phe-pNA | ≫ 75 |
| Pronase | Suc-Ala-Ala-Pro-Arg-pNA | ≫ 75 |
| Proteinase K | Suc-Ala-Phe-Pro-Phe-pNA | ≫ 75 |
| Subtilisin | Suc-Ala-Phe-Pro-Phe-pNA | ≫ 75 |
| Thermitase | Suc-Ala-Phe-Pro-Phe-pNA | ≫ 75 |
| Trypsin | Suc-Ala-Ala-Pro-Arg-pNA | ≫ 75 |
| Thrombin | Suc-Ala-Ala-Pro-Arg-pNA | ≫ 75 |
| hPEP | Suc-Ala-Pro-pNA | 3.5 |
| bPEP | Suc-Ala-Pro-pNA | 25.0 |
| Papain | Suc-Ala-Ala-Pro-Arg-pNA | 34.0 |
| HIV-protease | Lys-Ala-Arg-Val-Nle-Nph-Glu-Ala-Nle-NH$_2$ | ≫ 75 |

## Discussion

The majority of the PEP inhibitors described so far are synthetic pyrrolidine derivatives including the especially potent Z-Pro-Prolinal[3] and JTP-4819[15]. A number of high molecular weight endogenous PEP inhibitors have been purified from different animal tissues[3]. However, only a few PEP inhibitors from microbial sources have been reported. The linear peptide poststatin[16] from *Streptomyces viridochromogenes* and the acyl-cyclopeptides eurystatins A and B[17] which were isolated from the culture broth of *Streptomyces eurythermus* show potent and specific PEP inhibitory activity.

With lipohexin (I) a new member of the group of microbial PEP inhibitors embodying the essential α-keto-β-amide functionality[17] has been found. In contrast to the previously detected inhibitors, lipohexin (I) represents a combination of a prolyl containing peptide sequence with a long chain fatty acid which may contribute to the characteristic properties of the compound. In general, the fatty acid component of a lipopeptide provides a means of enhancing the lipophilicity and interaction with lipophilic surface structures of peptides. Besides, it may significantly account for the biological stability of the compound[18].

Peptide bonds involving the amino acid proline manifest distinguishing conformational characteristics: the restriction of the $\phi$ torsion angle to values around 65° and the occurrence of relatively stable *cis* and *trans* conformers[19]. As a result these linkages are fairly resistant to the cleavage by broad specificity aminopeptidases. However, a few sets of prolylspecific peptidases are able to hydrolyze peptide bonds located *N*- or *C*-terminally to proline[3]. As the prolyl-containing lipohexin (I) contains many features of a substrate or inhibitor, ready to be bound by other proline-specific proteases in addition to bacterial and human PEP, various related peptidases were included in the determination of the specificity of the inhibitory effect.

Surprisingly, lipohexin (I) specifically inhibited PEP whereby an about ten-fold better inhibition of the enzyme isolated from human placenta could be observed, compared to the PEP from *Flavobacterium meningosepticum*. Specificity differences of inhibitors of mammalian and bacterial PEPs were also seen with alkylacyl-prolinal derivatives which strongly inhibited only the bacterial enzyme[20]. Whether or not the differential inhibition of lipohexin (I) can be influenced by varying the long chain fatty acid will be shown by further investigations.

## References

1)  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

2)  WILK, S.: Minireview: Prolyl endopeptidase, Life Sci. 33: 2149~2157, 1983

3)  YARON, A. & F. NAIDER: Proline-dependent structural and biological properties of peptides and proteins. Crit. Rev. Biochem. Molec. Biol. 28: 31~81, 1993

4)  WELCHES, R.; K. B. BROSNIHAN & C. M. FERRARIO: Minireview: A comparison of the properties and enzymatic activities of three angiotensin processing enzymes: Angiotensin converting enzyme, prolyl endopeptidase and neutral endopeptidase 24.11. Life Sci. 52: 1461~1480, 1993

5)  GOOSSENS, F.; I. DEMEESTER, G. VANHOOF & S. SCHARPE: Distribution of prolyl oligopeptidase in human peripherial tissues and body fluids. Europ. J. Clin. Chem. Clin. Biochem. 34: 17~22, 1996

6)  MAES, M.; F. GOOSSENS, S. SCHARPE, J. CALABRESE, R. DESNYDER & H. Y. MELTZER: Alterations in plasma prolyl endopeptidase activity in depression, mania, and schizophrenia: effects of antidepressants, mood stabilizers, and antipsychotic drugs. Psych. Res. 58: 217~225, 1995

7)  ICHAI, C.; N. CHEVALLIER, P. DELAERE, P. DOURNAUD, J. EPELBAUM, J.-J. HAUW, J.-P. VINCENT & F. CHECLER: Influence of region-specific alterations of neuropeptidase content on the catabolic fates of neuropeptides in Alzheimer's disease. J. Neurochem. 62: 645~655, 1994

8)  KAMEI, H.; T. UEKI, Y. OBI, Y. FUKAGAWA & T. OKI: Protective effect of eurystatins A and B, new prolyl endopeptidase inhibitors, on scopolamine-induced amnesia in rats. J. Pharmacol. 60: 377~380, 1992

9)  HEINZE, S.; M. RITZAU, W. IHN, H. HÜLSMANN, B. SCHLEGEL, K. DORNBERGER, W. F. FLECK, M. ZERLIN, C. CHRISTNER, U. GRÄFE, G. KÜLLERTZ & G. FISCHER: Lipohexin, a new inhibitor of prolyl endopeptidase produced by *Moeszia lindtneri* (HKI-0054) and *Paecilomyces* sp. (HKI-0055; HKI-0096). I. Screening, isolation and structure elucidation. J. Antibiotics 50: 379~383, 1997

10) DEMUTH, H. U.; D. SCHLENZIG, A. SCHIERHORN, G. GROSCHE, M. P. CHAPOT-CHARTIR & J. C. Gripon: Design of (omega-*N*-(*O*-acyl) hydroxy amid) aminodicarboxylic acid pyrrolidides as potent inhibitors of proline-specific peptidases. FEBS Lett. 320: 23~27, 1993

11) DIEFENTHAL, T.; H. DARGATZ, V. WITTE, G. REIPEN & I. SVENDSEN: Cloning of prolinespecific gene from *Flavo-*

*bacterium meningosepticum*: expression in *Escherichia coli* and purification of the protein. Appl. Microbiol. Biotechnol. 40: 90~97, 1993

12) IDO, E.; H. HAN, F. J. KEZDY & J. TANG: Kinetic studies of human immunodeficiency virus type I protease and its active-site hydrogen bond mutant A28S. J. Biol. Chem. 266: 24359~24366, 1991

13) SCHOMBURG, B. & M. SALZMANN, GBF: Enzyme Handbook. Springer Verlag, Berlin Heidelberg, 1991

14) BERGMEYER, H. U.; J. BERGMEYER & M. GRAßL: Methods of Enzymatic Analysis, Vol. V Enzymes 3: Peptides, Proteinases and Their Inhibitors. pp. 55~371, VCH, Weinheim, 1988

15) TOIDE, K.; K. OKAMIYA, Y. IWAMOTO & T. KATO: Effect of a novel prolyl endopeptidase inhibitor, JTP-4819, on prolyl endopeptidase activity and substance P- and arginine-vasopressin-like immunoreactivity in the brains of aged rats. J. Neurochem. 65: 234~240, 1995

16) NAGAI, M.; K. OWAGA, Y. MURAOKA, H. NAGANAWA, T. AOYAGI & T. TAKEUCHI: Poststatin, a new inhibitor of prolyl endopeptidase, produced by *Streptomyces*

*viridochromogenes* MH534-30F3. II. Structure determination and inhibitory activities. J. Antibiotics 44: 956~961, 1991

17) TODA, S.; Y. OBI, K. NUMATA, Y. HAMAGASHI, 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

18) FINN, N.; S. COPPARD & I. TOTH: Oral absorption studies of lipidic conjugates of thyrotropin releasing hormone (TRH) and luteinizing hormone-releasing hormone (LHRH). Intern. J. Pharmazeutics 137: 33~39, 1996

19) VANHOF, G.; F. GOOSSENS, I. DE MEESTER, D. HENDRIKS & S. SCHARPE: Proline motifs in peptides and their biological processing. FASEB J. 9: 736~744, 1995

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