Journal of Microbiology and Biotechnology
Vol. 6. No. 6, 445 - 450, 1996

Pyridoxatin, an Inhibitor of Gelatinase A with Cytotoxic Activity

LEE, HO-JAE, MYUNG-CHUL CHUNG, CHOONG-HWAN LEE, HYO-KON CHUN, HWAN-MOOK KIM[1], AND YUNG-HEE KHO*

Enzyme Inhibition Research Unit, Biomolecule Research Division,
[1]Bioresources Program, Genetic Resources Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), KIST, P.O. Box 115, Yusong, Taejon 305-600, Korea

Gelatinase A is a member of the matrix metalloproteinases that play an important role in cancer invasion and metastasis. In the course of screening gelatinase A inhibitors from microbial sources, a fungal strain PT-262 showed a strong inhibitory activity. The strain was identified as *Chaunopycnis alba* on the basis of its morphological characteristics. The inhibitor was isolated from acetone extract of mycelial cake by sequential chromatographies on MCI-gel, Sephadex LH-20, and a reverse-phase HPLC column. The purified inhibitor was identified as pyridoxatin by its physico-chemical properties and spectroscopic analysis. Pyridoxatin is not a peptide analog and has cyclic hydroxamic acid moiety. It inhibited activated gelatinase A with an IC_{50} value of 15.2 μM using fluorescent synthetic peptide. It also had a strong cytotoxicity against human cancer cell lines *in vitro*. Furthermore, this compound inhibited DNA synthesis with an IC_{50} value of 2.92 μM in PC-3 prostate cancer cells by [^{3}H]thymidine incorporation assay.

Gelatinase A (72-kDa type IV collagenase, EC 3.4.24. 24) is a member of the family of matrix metalloproteinases (MMPs), which also include collagenases and stromelysins. MMPs are involved in various physiological and pathological processes, such as tissue remodeling, reproduction, various connective tissue diseases, angiogenesis, cancer cell invasion, and metastasis (18). Gelatinase A hydrolyzes denatured collagens, but also type IV collagen, a principle structural component of the basement membrane. Gelatinase A is synthesized and secreted at low levels by a variety of normal cells. In contrast, this enzyme is produced at high levels in many invasive and malignant cancers (13). Consequently gelatinase inhibitors may be of value in the therapy of cancers as well as other disease states involving tissue remodeling.

Recently a number of groups have reported their efforts in the development of inhibitors. For example, matlystatins, natural low molecular weight inhibitors isolated from an actinomycete strain, inhibit type IV collagenases (15). Batimastat (BB-94), a synthetic MMP inhibitor, decreased tumor burden and prolonged the survival of mice bearing ovarian carcinoma xenografts (3). To date, most inhibitors of MMPs are peptides or pseudo-peptides and contain zinc coordinating ligands such as thiol, phosphorous, carboxylic acid, and hydroxamic acid (11). Hydroxamic acids are known to be potent inhibitors of several other metalloproteinases including thermolysins, collagenases, and angiotensin-converting enzymes (1).

During the screening for inhibitors of gelatinase A, we found that pyridoxatin, known as a free radical scavenger, has a gelatinase A inhibitory activity and a strong inhibitory effect on the growth of cancer cells. Pyridoxatin is a non-peptidic compound and contains cyclic hydroxamic acid for a zinc coordinating ligand. Pyridoxatin is unique in its structure to inhibit gelatinase. In this report the isolation and identification of pyridoxatin and its inhibitory activity on gelatinase A and its cytotoxicity against cancer cell lines are discussed.

MATERIALS AND METHODS

Chemicals

All chemicals were of the highest purity commercially available. Tissue culture plastics were purchased from Falcon, and media and additives were from Gibco. Protease inhibitors, hydroxyurea, and Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 were from Sigma.

Instrumental Analyses

UV spectra were recorded on a Shimadzu UV-260 spectrometer. IR spectra were acquired by diffuse reflectrance (KBr) on a Laser Precision Analytical IFX-

*Corresponding author
Key words: pyridoxatin, gelatinase A inhibitor, cytotoxicity, DNA synthesis inhibitor, *Chaunopycnis alba* PT-262

65S spectrometer. Mass spectra were obtained with a JEOL JMS-HX 110A spectrometer. NMR spectra were recorded on a Bruker AMX-FT 500MHz NMR spectrometer. HPLC analysis used a Hitachi L-6200 intelligent pump with a L-4000 UV detector system.

Preparation of Test Materials

Culture broths of various fungal strains were extracted with *n*-butanol at neutral pH. The resulting organic phase was evaporated to dryness. The residual material was suspended into distilled water containing 1 mM phenylmethyl sulfonyl fluride and 10 mM N-methylmaleimide to inactivate the proteinases derived from the fungal culture. This solution was added to a gelatinase A assay.

Fungal Strain and Cultivation

Fungal strain PT-262 was isolated from soil. A loopful mycelium of potato dextrose agar slant of PT-262 was inoculated into a baffled 500-ml Erlenmeyer flask containing 100 ml of seed medium composed of 1% glucose, 0.5% tryptone, 0.3% yeast extract, and 0.3% malt extract. The flask was shaken on a rotary shaker at 180 rpm for 3 days at 25°C. This seed culture was inoculated into a 5-liter fermentor containing 3 liters of production medium composed of 2% soluble starch, 1% lactose, 2.5% soybean meal, 0.1% malt extract, 0.1% yeast extract, 0.1% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.5% glycerol. Fermentation was carried out for 6 days at 25°C with an agitation rate of 200 rpm and air flow rate of 1.0 vvm.

Taxonomic Studies

For the identification of the fungus PT-262, oat meal agar (OA), corn meal agar (CMA), and potato-carrot agar (PCA) were used. Morphological observation was performed under a microscope (Nikon Microphot FXA, Japan) and photographed with a scanning electron microscope (Philips SEM 515, Netherlands).

Isolation of Inhibitor

The culture broth fermented in the production medium was used for the isolation of the inhibitor. The 6 liters of culture broth were centrifuged to obtain mycelial cake. The mycelial cake was extracted with 80% aqueous acetone. The extracted materials were dissolved in 500 ml of distilled water and extracted twice with equal volumes of *n*-butanol. The organic layer was dried, dissolved in 100 ml of 20% methanol and applied to a MCI-gel (CHP-20P) column equilibrated with 20% methanol. The column was eluted with a linear gradient of 20 to 100% methanol. Fractions were assayed for enzyme inhibitory activity and active effluent was combined and evaporated. The active materials were dissolved in 100% methanol, applied to a Sephadex LH-20 column and eluted with 100% methanol. The active fractions were evaporated and applied to a HPLC column (YMC-Pack ODA AQ) equilibrated with 60% methanol. The column was eluted by isocratic mode at a flow rate of 1.2 ml/min and absorption at 290 nm was detected. The effluent was fractioned according to the UV peaks and each fraction was assayed for enzyme inhibitory activity.

Preparation of Progelatinase A and Activity Assay

Human progelatinase A was purified from T98G glioblastoma cells to a nearly homogeneous form as described previously (9). Screening for the inhibitor of gelatinase A was carried out by examining the inhibitory activity with a test sample and gelatinase A. Gelatinase A activity was assayed according to Knight *et al.* (8) with some modifications. Briefly, 1 nM of purified TIMP-2 free progelatinase A was incubated with 0.5 mM *p*-aminophenylmercuric acetate (APMA) in a buffer containing 20 mM Tris-HCl, 5 mM $CaCl_2$, and 0.15 N NaCl (pH 7.5) for 15 min at 37°C. Test sample and substrate, Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (10 μM), were then added to the reaction mixture. Incubation was carried out for another 30 min. The reaction was terminated by the addition of 0.1 N sodium acetate buffer (pH 4.0). The hydrolysis of the substrate was assessed by fluorescence with a luminescence spectrophotometer (Perkin Elmer LS-50, U.K.) with excitation at 328 nm and emission at 393 nm.

Other Enzyme Assay

The activity of aminopeptidase M was determined colorimetrically by measuring the amount of nitroaniline that was liberated when L-leucine-*p*-nitroanilide was used as a substrate (17). A reaction mixture containing 0.4 mM substrate, 1 mU aminopeptidase M (from porcine kidney, Sigma L6032) and 0.1 M Tris-HCl buffer (pH 7.0) was incubated at 37°C for 30 min. The absorbance of the liberated nitroanilline was measured by a microplate reader at 405 nm. The activity of thermolysin was determined by the casein hydrolysis method (14). A reaction mixture containing 0.3% casein, 0.5 μg/ml of thermolysin (from *Bacillus thermoproteolyticus*, Sigma P 1512), and 0.05 M Tris-HCl (pH 7.5) was incubated at 37°C for 30 min. After incubation, trichloroacetic acid (final 10%) was added and the mixture was kept for 30 min at room temperature. After centrifugation, the absorbance of the acid soluble fraction was read at 280 nm.

***In vitro* Cytotoxicity Assay**

The *in vitro* cytotoxicity assay against the panels of human cancer cell lines was conducted according to a National Cancer Institute protocol (12). Briefly, various kinds of disease oriented human cancer cells were precultured in 96-well plates (3×10^3 cells/well) with 180 ml of RPMI 1640 containing 5% fetal bovine serum (FBS) for 24 h. This was then added to diluted sample solution to a final volume of 200 μl and cultured under 5% CO_2 at 37°C for 48 h. After fixing with 10% trichloroacetic acid, the cells were stained with 0.4% sulforhodamine after which the dye was extracted from the stained cells with 10 mM Tris (hydroxymethyl)-aminomethan solution. The absorbance of the extract was read

at 570 nm.

[^{3}H]Thymidine Incorporation Assay

The effect of pyridoxatin on DNA synthesis was determined by incorporation of [^{3}H]thymidine. Human PC-3 prostate cancer cells in 96-well plates (3×10^3 cells/well) were incubated for 24 h. Cells were exposed to [^{3}H] thymidine (1 μCi/well) for 4 h at 37°C in the presence of graded concentrations of pyridoxatin. The cells were trypsinized and harvested on glass fiber filter paper using a cell harvester (Inotech, Switzerland). The glass fiber filter was dried and put into the vial containing 5 ml of scintillation cocktail solution. The radioactivity was counted for 1 min in a liquid scintillation counter. DNA synthesis was expressed as mean dpm per well ±SD for quadruplicate determinations.

RESULTS AND DISCUSSION

Taxonomy of the Inhibitor-producing Organism

Fungal strain PT-262 was originally isolated from a soil sample collected in the province of Pyunsan, Chullapuk-Do, Korea in 1995.

This strain grew slowly to form white, thinly, floccose, and eventually granular colonies with a diameter of 2.0-2.8 cm after incubation at 25°C for 10 days. When strain PT-262 was grown on PCA at 25°C for 7 days, conidiomata of irregular roundish shape formed in the hyaline aerial mycelium. Conidiomata surrounded by a thin loose weft of hyphae inwardly formed branched conidiophores as shown in Fig. 1. The phalides were cylindrical or had an inflated base, slightly tapering in the distal region and were 3.5-10 μm long. The conidia were globose, hyaline, smooth-walled, 1.5-2.0 μm diameter, and aggregated in slimy heads. From the above morphological characteristics, strain PT-262 was identified as *Chaunopycnis alba* (6) and named *Chaunopycnis alba* PT-262. This strain was deposited at the Korean Collection for Type Cultures (KCTC), Korea Research Institute of Bioscience and Biotechnology, KIST, Taejon, Korea, under accession No. KCTC 0209BP.

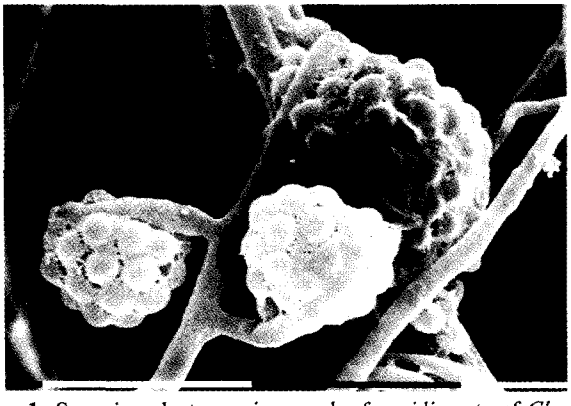

Fig. 1. Scanning electron micrograph of conidiomata of *Chaunopycnis alba* PT-262 grown on oat meal agar for 10 days at 25°C (Bar=10 μm).

Production and Isolation of Inhibitor

Gelatinase A inhibitor was obtained by the fermentation in production medium as described in materials and methods. The production of inhibitor began after 2 days and reached a maxium after 6 days. The mycelial cake collected by centrifugation from the fermentation broth of 6 liters was used for the isolation of the inhibitor. Inhibitor was isolated from the mycelial cake by acetone extraction, butanol extraction, column chromatographies using MCI-gel and Sephadex LH-20. Finally, inhibitor was obtained as a white powder (8.5 mg) by reverse-phase HPLC using a YMC-Pack ODS column.

Structural Identification of Inhibitor

The physico-chemical properties of the purified inhibitor are summarized in Table 1. Its molecular formula was determined as $C_{15}H_{21}NO_3$ by high resolution electron impact mass spectrometry (HREI-MS). The ^{13}C and ^{1}H NMR spectral data for the inhibitor are summarized in Table 2. These NMR spectra indicated that the inhibitor existed as a mixture of two isomers A and B. Their ratio was estimated to be approximately 1:1 in methanol-d_4 and 2:1 in acetone-d_6 by ^{1}H NMR spectral analysis. Two isomers of inhibitor are known as rotamers (16). From physicochemical properties and spectroscopic analysis the inhibitor was identified as pyridoxatin.

Inhibitory Activity against Metalloproteinases

Table 3 shows the concentrations of pyridoxatin and other metalloproteinase inhibitors required for 50% inhibition (IC_{50}) of gelatinase A, aminopeptidase M, and thermolysin. Pyridoxatin inhibited gelatinase A with an IC_{50} value of 15.2 μM but did not inhibit aminopeptidase M and thermolysin with 250 μM. Actinonin, a pseudopeptide hydroxamic acid of microbial origin (17), was more potent against gelatinase A than pyridoxatin. Tripeptide hydroxamate (CBZ-PLG-NHOH), a synthetic collagenase inhibitor, specifically inhibited gelatinase A and weakly inhibited aminopeptidase M and thermolysin. While pyridoxatin has been isolated from *Acremonium*

Table 1. Physico-chemical properties of pyridoxatin.

Appearance	White powder
Molecular formula	$C_{15}H_{21}NO_3$
HREI-MS (*m/z*)	Calculated; 263.1521
	Found: 263.1528 (M^+)
Color reaction	$FeCl_3$
UV λ_{max} nm in MeOH	217, 289
in MeOH+HCl	214, 247, 267
in MeOH+NaOH	231, 266, 295
IR (cm^{-1}, KBr)	3436, 3106, 2950, 2919
	1633, 1544, 1444, 1251

Table 2. ^{13}C and ^{1}H NMR chemical shifts of pyridoxatin in CD_3OD.

Carbon No.	Isomer A		Isomer B	
	^{13}C-NMR[a]	^{1}H-NMR[b]	^{13}C-NMR	^{1}H-NMR
2	160.4		162.7	
3	115.0		115.3	
4	163.9		163.2	
5	99.0	5.92	99.9	5.92
6	132.8	7.52	132.8	7.50
7	47.5	2.46	48.1	2.62
8	44.1	3.01	45.2	2.84
9	43.8	0.92, 1.70	43.6	0.97, 1.70
10	33.1	1.62	33.0	1.64
11	45.9	0.77, 1.73	45.9	0.81, 1.73
12	33.1	2.37	34.1	2.24
13	144.7	5.53	144.7	5.57
14	113.0	4.60, 4.78	112.9	0.60, 4.72
15	23.2	0.91	23.1	0.91
16	21.0	0.68	20.9	0.71

[a]500 MHz; δ in ppm, [b]300 MHz; δ in ppm.

Table 3. Inhibitory activity of several metalloproteinase inhibitors against gelatinase A, aminopeptidase M, and thermolysin.

Compounds	IC_{50} (μM)		
	Gelatinase A	Aminopeptidase M[a]	Thermolysin[b]
Pyridoxatin	15.2	>250	> 250
Actinonin	0.18	0.44	2.71
CBZ-PLG-NHOH[c]	1.52	20.5	54.2
1, 10-Phenanthrolin	38.3	58.8	56.0

[a]Substrate (L-leucine-*p*-nitroanilide, 0.4 mM) was reacted with porcine kidney aminopeptidase M (1 mU) in 0.1 M Tris-HCl buffer, pH 7.0 at 37°C. [b]Casein (0.3%) was reacted with thermolysin (0.5 μg/ml) in 0.05 M Tris-HCl buffer, pH 7.5 at 37°C. [c]N-Carbobenzoxy-Pro-Leu-Gly hydroxamate.

sp. as a free radical scavenger (16), this is the first description of pyridoxatin as a gelatinase A inhibitor from *Chaunopycnis alba* PT-262. Most inhibitors of MMPs including gelatinase are peptides or peptide-like structures and contain zinc-coordinating ligands. Hydroxamic acid is a strong metal chelator and some hydroxamic acid-containing compounds are known to be potent inhibitors of metalloproteinases. Pyridoxatin is a non-peptidic compound and has a cyclic hydroxamic acid moiety. It specifically inhibited gelatinase A and was found to be unique in its structure to inhibit gelatinase A.

***In vitro* Cytotoxicity**

During our evaluation of the other biological activities of pyridoxatin, we found that pyridoxatin has a strong cytotoxicity against human cancer cell lines. As shown in Table 4, pyridoxatin exhibited strong cytotoxicity against the growth of NCI-H226, SW620, ACHN, UO-31, MCF7, G-361, M14, UACC-62, PC-3, and K562 with ED_{50} values ranging from 0.10 to 0.30 μg/ml. Pyridoxatin was more potent against ACHN, UO-31, M14, and K-526/ADR cells than adriamycin.

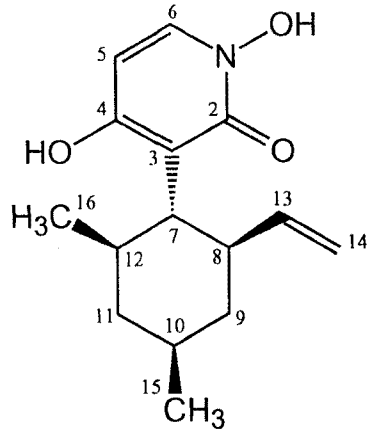

Fig. 2. Structure of pyridoxatin.

Table 4. Cytotoxicity of pyridoxatin and adriamycin against the panels of hyman cancer cell lines.

Disease type	Cell lines	ED_{50} (μg/ml)*	
		Pyridoxatin	Adriamycin
Non-small cell lung cancer	A549	0.57	0.11
	NCI-H23	0.52	0.22
	NCI-H226	0.10	0.04
Colon cancer	HCC2998	5.03	0.24
	HCT-15	5.56	0.43
	HCT116	0.88	0.08
	HT-29	7.04	0.34
	SW620	0.15	0.05
Renal cancer	ACHN	0.12	0.26
	KM12	5.70	0.14
	UO-31	0.10	0.19
Breast cancer	MCF7	0.28	0.11
	MCF7/ADR	4.30	>3.00
Melanoma	C32TG	0.79	0.05
	G-361	0.21	0.03
	M14	0.18	0.38
	UACC-62	0.27	0.16
Prostate cancer	PC-3	0.26	0.23
Leukemia	K562	0.28	0.18
	K562/ADR	1.22	>3.00
Sarcoma	HT-1080	1.20	0.10

*ED_{50} value against each tumor cell lines, which was defined as a concentration (μg/ml) that caused 50% inhibition of the cell growth *in vitro*.

Inhibition of DNA Synthesis

Various naturally occurring hydroxamic acids act as

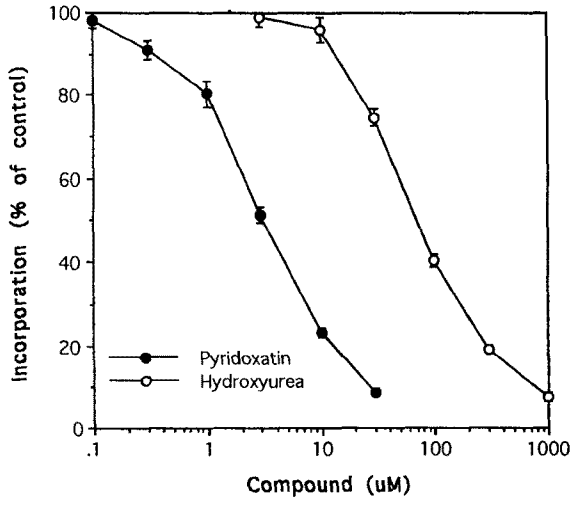

Fig. 3. Effects of pyridoxatin and hydroxyurea on the incorporation of [^{3}H]thymidine into DNA of PC-3 cells.

antibiotics, metalloproteinase inhibitors, or tumor inhibitors (16, 10). It has been shown that some hydroxamic acids may act as inhibitors of ribonucleotide reductase (5). To investigate whether pyridoxatin has an inhibitory activity against ribonucleotide reductase, we determined the effect of pyridoxatin on DNA synthesis by the incorporation of [^{3}H]thymidine of PC-3 prostate cancer cells. As shown in Fig. 3, [^{3}H]thymidine uptake was decreased by pyridoxatin and this inhibition of DNA synthesis was dose dependent. The concentration of pyridoxatin required for 50% inhibition of DNA synthesis of PC-3 cells was 2.92 μM. Pyridoxatin was about 25-fold more potent than hydroxyurea, a well known ribonucleotide reductase inhibitor (4). Ribonucleotide reductase is a rate-limiting enzyme in the biosynthetic pathway for DNA, and needs iron and free radicals for its activity (2). Pyridoxatin contains a cyclic hydroxamic acid moiety capable of chelating metal ions and is also known as a free radical scavenger. This might be a possible mechanism for the inhibition of DNA synthesis by pyridoxatin in prostate cancer cell line but its demonstration of the inhibition of ribonucleotide reductase requires further study.

REFERENCES

1. Bihovsky, R., B. L. Levinson, R. C. Loewi, P. W. Erhardt, and M. A. Polokoff. 1995. Hydroxamic acids as potent inhibitors of endothelin-converting enzyme from human bronchiolar smooth muscle. *J. Med. Chem.* **38**: 2199-2129.
2. Chitambar, C. R. and J. P. Wereley. 1995. Effect of hydroxyurea on cellular iron metabolism human leukemic CCRF-CEM cells: changes in iron uptake and the regulation of transferrin receptor and ferritin gene expression following inhibition of DNA synthesis. *Cancer Res.* **55**: 4361-4366.
3. Davies, B., P. D. Brown, N. East, M. J. Crimmin, and F. R. Balkwill. 1993. A synthetic matrix metalloproteinase inhibitor decreases tumor burden and prolongs survival of mice bearing human ovarian carcinoma xenografts. *Cancer Res.* **53**: 2087-2091.
4. Elford, H. 1968. Effect of hydroxyurea on ribonucleotide reducatase. *Biochem. Biophys. Res. Commun.* **33**: 129-135.
5. Elford, H. L., G. L. Wampler, and B. V. Riet. 1979. New ribonucleotide reductase inhibitors with antineoplastic activity. *Cancer Res.* **39**: 844-851.
6. Gams, W. 1980. *Chaunopycnis alba*, gen. et. sp. nov., a soil fungus intermediate between moniliales and sphaeropsidales. *Persoonia.* **11**: 75-79.
7. Gordon, J. J., B. K. Kelly, and M. A. Miller. 1962. Actinonin: an antibiotic substance produced by an actinomycete. *Nature* **195**: 701-702.
8. Knight, C. G., F. Willenbrock, and G. Murphy. 1992. A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. *FEBS Lett.* **296**: 263-266.
9. Lee, H. J., M. C. Chung, C. H. Lee, H. K. Chun, and Y. H. Kho. 1995. Purification of progelatinase A (matrix metalloproteinase 2) and a tissue inhibitor of metalloproteinase-2 (TIMP-2) from T98G human glioblastoma cells. *J. Biochem. Mol. Biol.* **28**: 33-39.
10. Neilands, J. B. 1967. Hydroxamic acids in nature. *Science* **156**: 1443-1447.
11. Porter, J. R., N. R. A. Beeley, B. A. Boyce, B. Mason, A. Millican, K. Millar, J. Leonard, J. R. Morphy, and J. P. O'Connel. 1994. Potent and selective inhibitors of gelatinase A. 1. Hydroxamic acid derivatives. *Bioorg. Med. Chem. Lett.* **4**: 2741-2746.
12. Skehan, P., R. Storeng, D. Scudiero, A. Monks, J. Mcmahon, D. Vostica, J. T. Warren, H. Bokesch, S. Kenney, and M. R. Boyd. 1990. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.* **82**: 1107-1112.
13. Stetler-Stevenson, W. G., S. Aznavoorian, and L. A. Liotta. 1993. Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Ann. Rev. Cell Biol.* **9**: 541-573.
14. Suda, H., T. Aoyagi, T. Takeuchi, H. Umezawa.1973. A thermolysin inhibitor produced by actinomycetes: phosphoramidon. *J. Antibiot.* **26**: 621-623.
15. Tanzawa, K., M. Ishii, T. Ogita, and K. Shimada. 1992. Matlystatins, new inhibitors of typeIV collagenases from *Actinomadura altramentaria*. II. Biological activities. *J. Antibiot.* **45**: 1733-1737.
16. Teshima, Y., K. Shin-ya, A. Shimazu, K. Furihata, S. C. Ha, K. Furihata, Y. Hayakawa, K. Nagai, and H. Seto. 1991. Isolation and structural elucidation of pyridoxatin, a free radical scavenger of microbial origin. *J. Antibiot.* **44**: 685-687.
17. Umezawa, H., T. Aoyagi, T. Yanaka, H. Suda, A. Okuyama, H. Naganawa, M. Hamada, and T. Takeuchi. 1985.

Production of actinonin, an inhibitor of aminopeptidase M, by actinomycetes. *J. Antibiot.* **38**: 1629-1630.
18. Woessner, J. F. Jr. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* **5**: 2145-2154.

(Received July 25, 1996)