# WS79089A, B AND C, NEW ENDOTHELIN CONVERTING ENZYME INHIBITORS ISOLATED FROM *Streptosporangium roseum*. NO. 79089

## TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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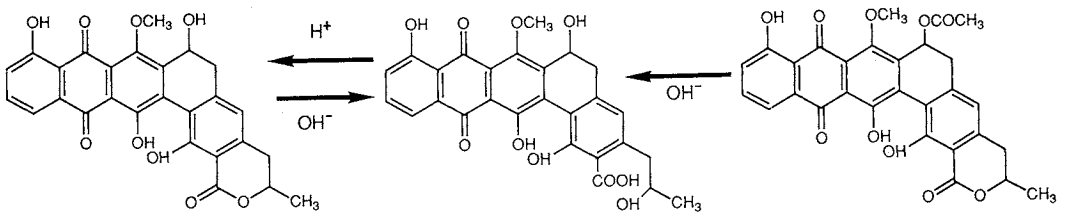
WS79089A, B and C†, which are novel endothelin converting enzyme (ECE) inhibitors have been isolated from the fermentation broth of *Streptosporangium roseum* No. 79089. These inhibitors were purified from an acetone extract of whole culture broth followed by Silicar CC-4 column chromatography and HPLC. WS79089A, B and C showed highly selective ECE inhibition activity with $IC_{50}$ values of 0.73 $\mu$M 0.14 $\mu$M and 3.42 $\mu$M, respectively. On the basis of spectroscopic and chemical evidence, the tentative structures of WS79089A, B and C have been proposed, they have benzo[*a*]naphtacen chromophores.

The 21-amino acid peptide endothelin 1 (ET-1), initially isolated from endothelial cells in culture, is a potent vasoconstrictor *in vitro* and *in vivo*[1]. It is synthesized as a precursor peptide of 203 amino acids, which is proteolytically cleaved to produce either 38 (human) or 39 (porcine) amino acid intermediate big endothelin-1 (big ET-1). The big ET-1 is converted to ET-1, a mature form, through an unusual cleavage between $Trp^{21}$ and $Val^{22}$ by endothelin converting enzyme (ECE)[1]. The normal human plasma concentration of big ET-1 is about twice that of ET-1[2], and various endothelial cell lines secrete both big ET-1 and ET-1 into culture medium[3~5], indicating that ECE does not perfectly process the available big ET-1 intracellularly. Recent studies suggest that a phosphoramidon-sensitive neutral metalloprotease is a likely candidate for the physiological ECE functioning in vascular endothelial cells[5,6]. It has been suggested that the precursor of ET-1, big ET-1, induces various pharmacological effects *in vivo* and *in vitro* following an active conversion by phosphoramidon-sensitive ECE[7,8]. Phosphoramidon also blocks the *in vivo* effects of exogenously added big ET-1, suggesting that ECE may be involved in both intracellular and extracellular

Fig. 1. Proposed chemical structures of WS79089A, B and C.




WS79089A WS79089B WS79089C

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processing of big ET-1[9,10]. Phosphoramidon is not a selective inhibitor, rather it is more of a neutral endopeptidase than an ECE inhibitor. Our goal is to discover more potent and more selective ECE inhibitors which may be therapeutically useful *in vivo* experimental models.

During the course of a screening program for selective ECE inhibitors, we isolated three novel ECE inhibitors WS79089A, B and C (Fig. 1) from the culture broth of *Streptosporangium roseum* No. 79089. In this paper, we report the taxonomy of the producing strain, production, isolation, physico-chemical properties and biological activities of WS79089A, B and C.

## Materials and Methods

### Taxonomic Studies

Strain No. 79089 was isolated from a soil sample obtained from Tateyama city, Chiba Prefecture, Japan. The taxonomic analysis was performed as described by SHIRLING and GOTTLIEB[11] and WAKSMAN[12]. Observations were made after 14 to 21 day's cultivation at 30°C. The morphological observations were made on cultures grown on yeast extract-malt extract agar and oatmeal agar using an optical microscope and a scanning electron microscope. The color names used in this study were taken from the Methuen Handbook of Color[13]. The temperature range for growth and NaCl tolerance were determined on yeast extract-malt extract agar. The preparation of cell walls and detection of the isomer of diaminopimelic acid was performed by procedure of BECKER *et al.*[14]. Detection of whole cell sugar was performed by the methods of LECHEVALIR and LECHEVALIR[15]. Phospholipid composition was determined by the method of LECHEVALIER *et al.*[16].

### Fermentation

A loopful of strain No. 79089 from a mature slant culture was transferred into seven 225-ml Erlenmeyer flasks containing 60 ml of sterile seed medium composed of corn starch 1% (w/v), glycerin 1% (w/v), glucose 0.5%, Pharmamedia (Traders Protein Co., Ltd.) 1% (w/v), dried yeast 1.5%, corn steep liquor 0.5% (w/v) and $CaCO_3$ 0.2% (w/v). The medium was adjusted to pH 6.5 prior to addition of $CaCO_3$. These flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 4 days at 30°C. The resultant seed culture (400 ml) was transferred into a 30-liter fermentor containing sterilized 20 liters of production medium which contained starch 5% (w/v), potato protein 1% (w/v), wheat germ 1% (w/v), soy bean powder 0.5% (w/v), $ZnCl_2$ 0.01% (w/v) and $CaCO_3$ 0.2% (w/v) supplemented with Adekanol LG-109 (defoaming agent, Asahi Denka Co., Ltd.) 0.05% (w/v) and Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% (w/v). The medium was adjusted to pH 6.5 prior to addition of $CaCO_3$. The fermentation ran for 7 days at 30°C with aeration at 20 liters per minute, back pressure of 1.0 kg/cm$^2$ and agitation speed of 200 rpm. The progress of the fermentation was monitored by an HPLC assay. Packed mycelial volume was determined after centrifugation of 10-ml of culture broth at 2,000 rpm for 10 minutes.

### General Procedures

Melting points were determined on a Yanagimoto micro melting point apparatus and are reported uncorrected. Optical rotations were measured on a Jasco DIP-140 polarimeter in a 10 cm microcell. IR spectra were recorded on a Jasco A-102 infrared spectrometer. Low-resolution FAB-MS and HRFAB-MS spectra were measured on a VG ZAB-SE mass spectrometer. NMR spectra were acquired on a Bruker AM400wb spectrometer. The standard Bruker software library was employed for a series of 2D NMR experiments.

### Endothelin Converting Enzyme (ECE) Inhibition Assay

#### Preparation of Cell Homogenate

The enzyme was obtained from bovine carotid endothelial cells. Bovine carotid endothelial cells were the kind gift of Dr. HAGIHARA from Tokyo Institute of Technology. Bovine carotid endothelial cells were grown in DULBECCO's modified EAGLE's minimum essential medium supplemented with 20% (v/v) fetal

bovine serum and 50 U/ml penicillin, 50 $\mu$g/ml streptomycin at 37°C in a humid atomosphere (5% $CO_2$-95% air). Cells were subcultured by trypsinization and used between passage 6 to 8. The confluent cultures cells (about $1 \times 10^9$ cells) were scraped off, washed with calcium, magnesium PBS (phosphate buffered saline) and homogenized with a polytron (Brinkman Instrument) at a maximal speed setting for $3 \times 10$ seconds on ice in 10 ml of 0.25 M sucrose containing 25 mM HEPES buffer (pH 7.4).

Measurement of ECE Activity

Human big ET-1 (50 ng) was incubated with the cell homogenate preparation (20~30 $\mu$g protein) for 16 hours at 37°C in 250 $\mu$l of 50 mM Tris-HCl buffer (pH 7.0) containing 0.5 mM *p*-chloromercuriphenylsulfonic acid and 0.05 mM diisopropylfluorophosphate. Twenty microliters of the reaction mixture was diluted with 80 $\mu$l of RIA buffer (50 mM Tris-HCl buffer containing 0.1% BSA, 0.1% Triton X-100 and 1 mM EDTA). Fifty microliters of anti-ET-1 serum (antisera against the C-terminal peptide of ET-1 (16-21), final dilution, 1:20,000) and 50 $\mu$l of $^{125}$I-ET-1 (Amersham Japan, 37 KBq/10 ml) were added and incubated for 2 hours at 4°C. After 2 hours, 0.5 ml of Amerlex-M donkey anti-rabbit (Amersham Japan, 1/4 conc.) was added, the tubes were mixed and incubated for 10 minutes at room temperature. After magnetic separation (Amerlex-M system) the supernatant was removed and the immunocomplex was counted in a gamma counter (Packard Auto Gamma Model 5650). Antisera against the C-terminal peptide of ET-1 (16-21) was the kind gift of Dr. R. TAKAYANAGI and Dr. K. OHNAKA from the third department of internal medicine, Kyushu University. Less than 10% of the substrate is consumed during the assay incubation. Also, the assay is linear with respect to incubation time and enzyme.

Collagenase Inhibition Assay

Human Skin Fibroblast

Cells were obtained from explants of skin of a normal healthy volunteer, grown in DULBECCO's modified minimum essential medium (DMEM) supplemented with 10% (v/v) fetal calf serum, penicillin (50 U/ml) and streptomycin (50 $\mu$/ml) at 37°C in a humid atomosphere (5% $CO_2$-95% air). Cells were subcultured by trypsinization and used between passage 5 to 15.

Collagenase Induction

Fibroblasts ($1.5 \times 10^6$ cells) were seeded and cultured in 75 $cm^2$ plastic flask. After 3 days, medium was removed, cells were washed with 20 ml DMEM, and then 11.25 ml DMEM supplemented with bovine serum albumin (3 mg/ml) and Interleukin 1$\beta$ (Genzyme Co., U.S.A., 100 U/ml) was added and cultured for 3 days. The medium was collected, centrifuged at 1,500 rpm for 5 minutes to eliminate cell fragments and stored at −20°C until use. Prior to use, one milliliter of cultured supernatant was activated with 200 $\mu$l of trypsin (Sigma, 100 $\mu$g/ml) for 40 minutes at room temperature. The reaction was terminated by addition of 200 $\mu$l of soybean trypsin inhibitor (Sigma, 400 $\mu$g/ml).

Assay

All reactions were performed in U-shaped microtiter plates. Twenty-five microliters of inhibitor were serially diluted in 0.1 M Tris-HCl buffer (pH 7.5) containing 0.4 M NaCl and 10 mM $CaCl_2$. Fifty microliters of FITC (Fluorescein isothiocyanate)-collagen (Collagen Gijutsu Kenshukai, Tokyo, Japan, 1 mg/ml) solution and 25 $\mu$l of 10-fold diluted collagenase (trypsin-activated fibroblast supernatant) solution were added and incubated at 37°C for 2 hours. After the incubation, 5 $\mu$l of 80 mM *o*-phenanthrolin was added, followed by 1 hour incubation. To the reaction mixture, 70 $\mu$l of ethanol and 30 $\mu$l of Tris-HCl buffer were added, mixed and centrifuged at 3,000 rpm for 10 minutes. Fluorescene (excitation 485 nm, emission 538 nm), derived from the eliminated FITC by collagenase, in the supernatant was measured and inhibitory percent by test sample was calculated.

Neutral Endopeptidase (NEP) Inhibition Assay

Preparation of Neutral Endopeptidase (NEP)

Forty Sprague-Dawley male rats (6 week old) were sacrificed by $CO_2$ gas generated from dry ice. Eighty kidneys (600 g) were dissected to remove the capsules and their combined volume was measured

by displacement in 150 ml of ice-cold 10 mM phosphate buffer (pH 7.0). Unless specified, all the following procedures were carried out at 0~4°C. The kidneys were minced with scissors and then homogenized with a polytron (Brinkman PT-20, maximal speed for 6 × 20 seconds) in 100 ml of 10 mM phosphate buffer (pH 7.0). The homogenate was centrifuged at 20,000 × *g* for 60 minutes at 4°C. The pellet was resuspended in 250 ml buffer (10 mM Tris-HCl, 50 mM NaCl, pH 7.0) and then 250 ml of 10% Triton X-100 was added and the suspension stirred for 3 hours at room temperature before centrifugation at 100,000 × *g* for 60 minutes. The supernatant, containing the NEP was dialyzed with two changes against 50 liters of 5 mM Tris-HCl pH 7.5, 0.05% Triton X-100 at 4°C. The dialyzed enzyme was chromatographed at 4°C on a column (26 × 2.6 cm) of DEAE Toyopearl 650 M equilibrated in 5 mM Tris-HCl pH 7.5, 0.05% Triton X-100. The column was washed with 2,000 ml of the same buffer. The column was then developed with 900 ml of linear gradient of 0~0.3 M NaCl in 10 mM Tris-HCl, 0.05% Triton X-100 buffer (pH 7.5) at a flow rate of 1 ml/minute. The fractions containing the NEP activity were pooled and stored at 4°C.

Measurement of NEP Activity Using Microplates for Enzyme Inhibitor Detection

Reaction solution contains 50 μl of 50 mM HEPES pH 6.5, 50 μl of leucine aminopeptidase (Sigma Type III-CP, porcine kidney cytosol) diluted 1/100 in water, 50 μl of NEP prepared as described above, 50 μl of 20 μM Glt-Ala-Ala-Phe-MCA (made by Peptide Institute, Osaka), 50 μl of water and 10 μl of sample solution in a total volume of 210 μl. Blank values were obtained by using water (100 μl) instead of NEP and leucine aminopeptidase under identical experimental conditions. The reaction was stopped by the addition of 10 μl of 20% acetic acid. MCA fluorescence was measured with Titertek Fluoroskan II (excitation 355 nm, emission 460 nm).

Antimicrobial Activity

The antimicrobial activity of WS79089A, B and C were determined by a serial broth dilution method in bouillon medium for bacteria and in SABOURAUD's medium for fungi and yeasts. The antimicrobial activity was observed after overnight incubation at 37°C for bacteria and 48~72 hours incubation at 28°C for a filamentous fungus and a yeast.

## Results

### Taxonomy of Strain No. 79089

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched and produced sporangia at the tips of short lateral branches. The sporangia were globose and 5.5~11 μm (usually 7~9 μm) in diameter. Sporangiophores were up to 10 μm in length. Sporangiospores were formed by septation of a coiled hyphae within the sporangium. They were spherical to oval, 0.6~1.0 × 0.8~1.4 μm, 20 to 50 spores per sporangium and nonmotile (Fig. 2).

The results of cultural characteristics are shown in Table 1. Strain No. 79089 required B vitamins for growth, so that it grew poorly on some synthetic agar media. The strain formed white to pale red aerial mycelium on yeast extract-malt extract agar and oatmeal agar. Reverse side color was yellowish white, light orange, red and reddish brown. Reddish soluble pigments were produced in yeast extract-malt extract agar. Both reverse mycelial pigments and soluble pigments were pH sensitive, changing from red to yellowish orange with addition of 0.05 N HCl. Melanoid pigments were not produced in

Fig. 2. Scanning electron micrograph of sporangium and sporangiospores of strain No. 79089 (on ISP2 medium, 30°C, 14 days).

Bar represents 5 μm.

Table 1. Cultural characteristics of strain No. 79089.

| Medium | Cultural characteristics | Medium | Cultural characteristics |
|---|---|---|---|
| Yeast extract - malt extract agar | G: Good<br>A: Abundant, pale red (9A3)<br>R: Reddish brown (9D6)<br>S: Reddish | Tyrosine agar | G: Poor<br>A: None<br>R: White to yellowish white (4A2)<br>S: None |
| Oatmeal agar | G: Moderate<br>A: Abundant, white to pale red (7A3)<br>R: Pastel red (7A4)<br>S: Pale orange | Nutrient agar | G: Moderate<br>A: None<br>R: Pale yellow (3A3)<br>S: None |
| Inorganic salts - starch agar | G: Poor<br>A: None<br>R: White to yellowish white (4A2)<br>S: None | Starch - yeast extract agar[a] | G: Good<br>A: Moderate, white to reddish white (8A2)<br>R: Reddish red (8C6)<br>S: Pale orange |
| Glycerol - asparagine agar | G: Poor<br>A: None<br>R: White to yellowish white (4A2)<br>S: None | Sucrose - nitrate agar | G: Poor<br>A: Thin, white<br>R: White to yellowish white (4A2)<br>S: None |
| Peptone - yeast extract - iron agar | G: Moderate<br>A: None<br>R: Light orange (5A4)<br>S: None | Glucose - asparagine agar | G: Poor<br>A: Thin, white<br>R: White to yellowish white (4A2)<br>S: None |

Abbreviation: G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.
[a] Starch - yeast extract agar comprises soluble starch 1%, yeast extract 0.2% and agar 1.5% (pH 7.3).

Table 2. Characteristics of strain No. 79089 and IFO 3776.

| Conditions | Strain No. 79089 | IFO 3776 |
|---|---|---|
| Diameter of sporangium | 5.5～11 μm | 6～10 μm[a] |
| Size of sporangiophore | Up to 10 μm | Up to 10 μm |
| Spore shape | Spherical to oval | Spherical to oval |
| Color of spore mass | Pale red, reddish white | Reddish white |
| Color of substrate mycelium | Reddish brown, light orange, yellowish white | Reddish brown, grayish orange, yellowish white |
| Soluble pigments | + (Reddish) | + (Pink to reddish) |
| Melanoid pigments | − | − |
| Starch hydrolysis | + | + |
| Nitrate reduction | + | + |
| Gelatin liquefaction | + | + |
| Milk coagulation | − | − |
| Milk peptonization | − | + |
| Decomposition of cellulose | − | − |
| B vitamins requirement | + | + |
| Temperature range for growth | 13～33°C | 16～32°C |
| NaCl tolerance (%) | 3≤, <4 | 3≤, <4 |
| Carbon utilization[b] | | |
| D-Glucose | + | + |
| L-Arabinose | + | + |
| D-Xylose | + | + |
| Inositol | ± | ± |
| Sucrose | − | + |
| D-Fructose | ± | ± |
| D-Mannitol | − | − |
| L-Rhamnose | + | + |
| Raffinose | − | − |

+: positive, ±: weakly positive, −: negative.
[a] Inviced from p. 2549～2550 in BERGEY's manual[8].
[b] Basal medium was PRIDHAM-GOTTLIEB's inorganic medium (ISP medium No. 9)[1], containing HAYAKAWA's B-vitamins (10).

tryptone-yeast extract broth, peptone-yeast extract-iron agar and tyrosine agar.

DL-Diaminopimelic acid was detected in the whole-cell hydrolysates of strain No. 79089. Rhamnose, ribose, madurose, mannose, glucose and galactose were detected as whole-cell sugar, placing this strain in the cell wall type 3B[15]. The detected phospholipids included glucosamine, phosphatidylethanolamine, phosphatidylinositol and diphosphatidylglycerol, placing it in the type PIV phospholipid group[16].

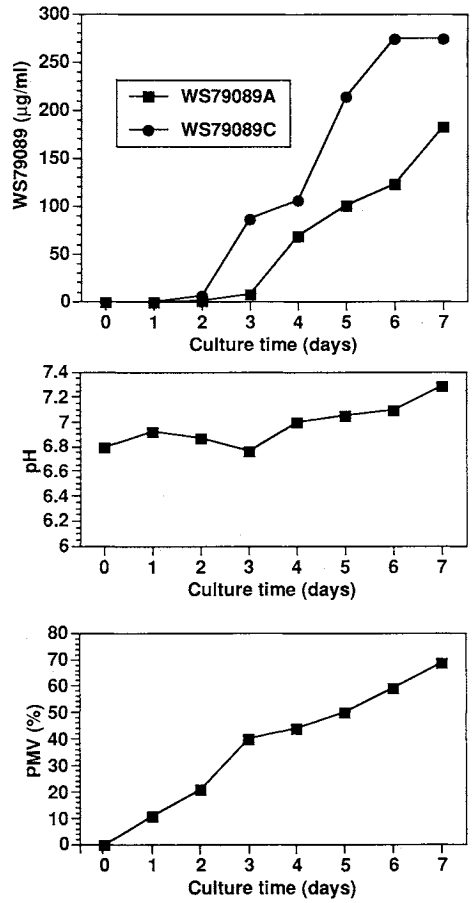
From the morphological and chemical characteristics described above, strain No. 79089 is considered to belong to the genus *Streptosporangium* Couch[17,18]. The strain was then compared with *Streptosporangium* species in the Approved lists of bacterial names[19]. As the characteristics of strain No. 79089 resembled them of *Streptosporangium roseum*, the strain was compared with *Streptosporangium roseum* IFO 3776 in detail. The results were summerized in Table 2. Both strains have almost the same characteristics, but different capability of utilizing sucrose and peptonization of milk. It is considered that these differences were not sufficient to separate the two strains as different species. Therefore, strain No. 79089 was identified as *Streptosporangium roseum*, and has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4009 (deposited date: 11 September 1992).

## Fermentation

The amount of WS79089A and C produced by *Streptosporangium roseum* No. 79089 in a 30-liter jar fermentor reached its maximum after 7 days of cultivation.

Fig. 3 presents the data from a typical 30-liter fermentation and gives information regarding WS79089A and C production, pH and packed mycelial volume (PMV). WS79089A and C production began at day 2, and the maximum production was observed after 7 days of cultivation and the yields of WS79089A and C were 183 $\mu$g/ml and 275 $\mu$g/ml, respectively. The production level of WS79089B was very low, but WS79089B can be produced easily from WS79089A or C by hydrolysis with alkali.

Fig. 3. Time course of fermentation.




## Isolation

The whole cultured broth (15 liters) was extracted with an equal volume of acetone at neutral pH, and filtered through diatomaceous earth. The filtrate was concentrated *in vacuo* to give an aqueous solution (1 liter). Following adjustment of the pH to 2.0 with 6N HCl, the aqueous solution was extracted twice with 1 liter of ethyl acetate. Two liters of ethyl acetate extract was concentrated under reduced pressure after dehydration over anhydrous

Table 3. Physico-chemical properties of WS79089A, B and C.

| | WS79089A | WS79089B | WS79089C |
|---|---|---|---|
| Appearance | Deep red powder | Deep red powder | Deep red powder |
| MP | 186~193°C | 190~195°C | Above 260°C |
| UV $\lambda_{max}^{MeOH}$ nm (ε) | 220 (36,800), 245 (38,600), 266 (sh) (34,100), 300 (sh) (13,200), 345 (sh) (5,400), 477 (16,600) | 224 (39,300), 245 (31,200), 266 (sh) (26,600), 313 (14,200), 486 (14,500) | 222 (sh) (29,300), 245 (31,300), 266 (sh) (25,000), 300 (sh) (10,100), 345 (sh) (4,300), 479 (12,100) |
| Mass spectrum | | | |
| HRFAB-MS Calcd: | 489.1185 $(M+H)^+$ | | |
| Found: | 489.1204 $(M+H)^+$ | | |
| FAB-MS (*m/z*) | 489 $(M+H)^+$ | 507 $(M+H)^+$ | 531 $(M+H)^+$ |
| IR $\nu_{max}$ (KBr) $cm^{-1}$ | 3457, 1656, 1616 | 3212, 1716, 1615 | 1744, 1663, 1617 |
| Molecular formula | $C_{27}H_{20}O_9$ | $C_{27}H_{22}O_{10}$ | $C_{29}H_{22}O_{10}$ |
| Elemental analysis | Calcd for $C_{27}H_{20}O_9 \cdot \frac{1}{2}H_2O$: C 62.91, H 4.50 | | |
| | Found: C 62.21, H 4.20 | | |
| Solubility | | | |
| Soluble: | Chloroform, dichloromethane, ethyl acetate | Methanol | Chloroform, dichloromethane |
| Slightly soluble: | *n*-Hexane | Chloroform | *n*-Hexane, methanol |
| Insoluble: | Water | *n*-Hexane | Water |
| TLC (Rf) | | | |
| Silica gel $CHCl_3$-MeOH (100:1) | 0.13 | 0 | 0.55 |
| RP-18 85% MeOH | 0.26 | 0.48 | 0.21 |

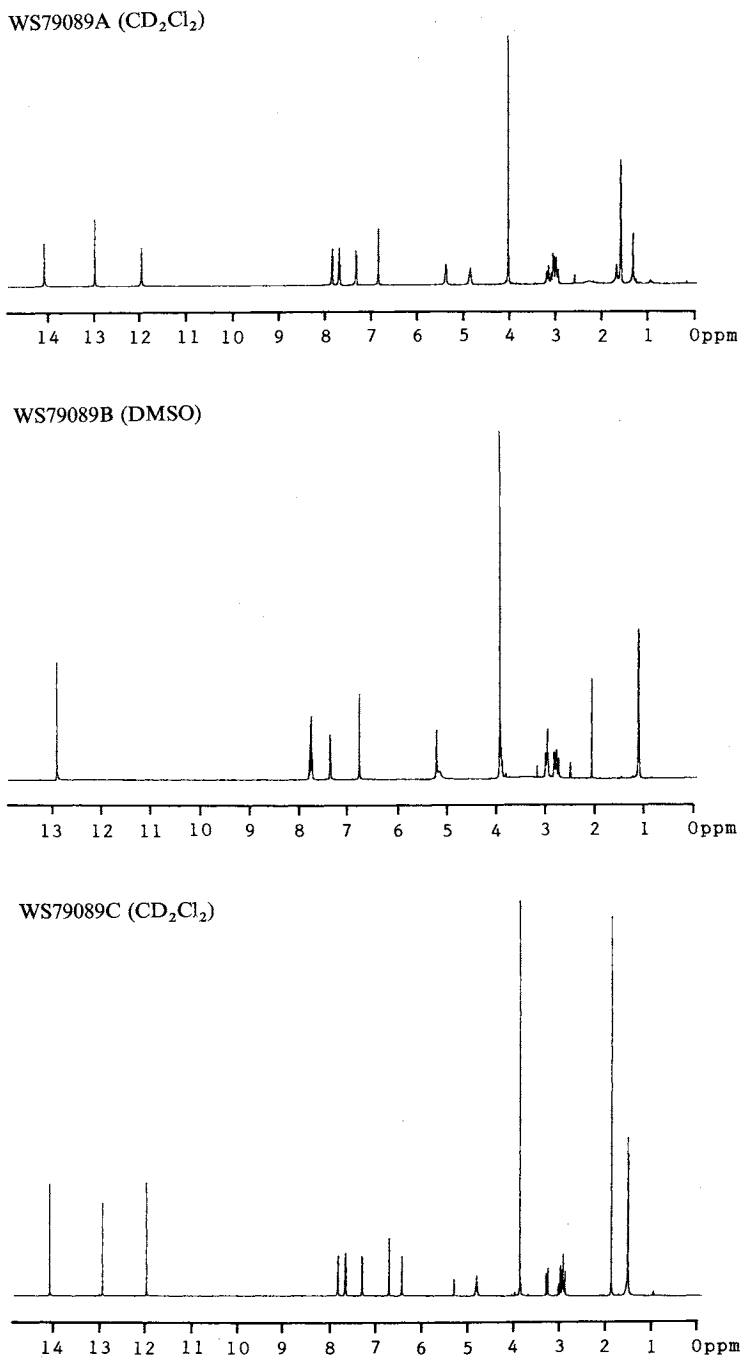
$Na_2SO_4$. The resultant oily material was applied to a Silicar CC-4 (Mallinckrodt) column chromatography (2 liters). The column was washed with 1 liter of chloroform and the first active fractions were eluted with 2 liters of chloroform and the second active fractions were eluted with 2 liters of chloroform-methanol (100:1). The first active fractions containing WS79089C were pooled and concentrated *in vacuo* to give a deep red powder. To remove impurities the deep red powder was washed with 5 ml of methanol, and methanol insoluble material was evaporated to dryness *in vacuo*. WS79089C was obtained as a deep red pure powder (36 mg). The second active fractions containing WS79089A were pooled and concentrated under reduced pressure to give a red oily residue. The oily residue was applied to a Lobar pre-packed LiChroprep Si 60 (40~63 μm) column (E. Merck, size C; 440-37). The column was washed with 400 ml of chloroform-methanol (100:1) and WS79089A was eluted with 700 ml of chloroform-methanol (100:1). The active fractions containing WS79089A were pooled and concentrated *in vacuo* to give a deep red powder containing WS79089A (125 mg).

WS79089B may be produced by subjecting WS79089A or WS79089C to hydrolysis with alkali. By such hydrolysis with alkali, corresponding salt may be obtained according to the alkali used in the process. Thus obtained salt may be converted to free type, WS79089B, *per se* by a conventional manner.

## Physico-chemical Properties

The physico-chemical properties of WS79089A, B and C are summarized in Table 3. WS79089A and C are soluble in chloroform, ethyl acetate, and slightly soluble in *n*-hexane and insoluble in water. WS79089B is soluble in methanol, slightly soluble in chloroform and insoluble in *n*-hexane. The sodium salt of WS79089B is soluble in water. The color reactions of WS79089A, B and C are as follows: positive to

Fig. 4. $^{1}H$ NMR spectra of WS79089A, B and C.

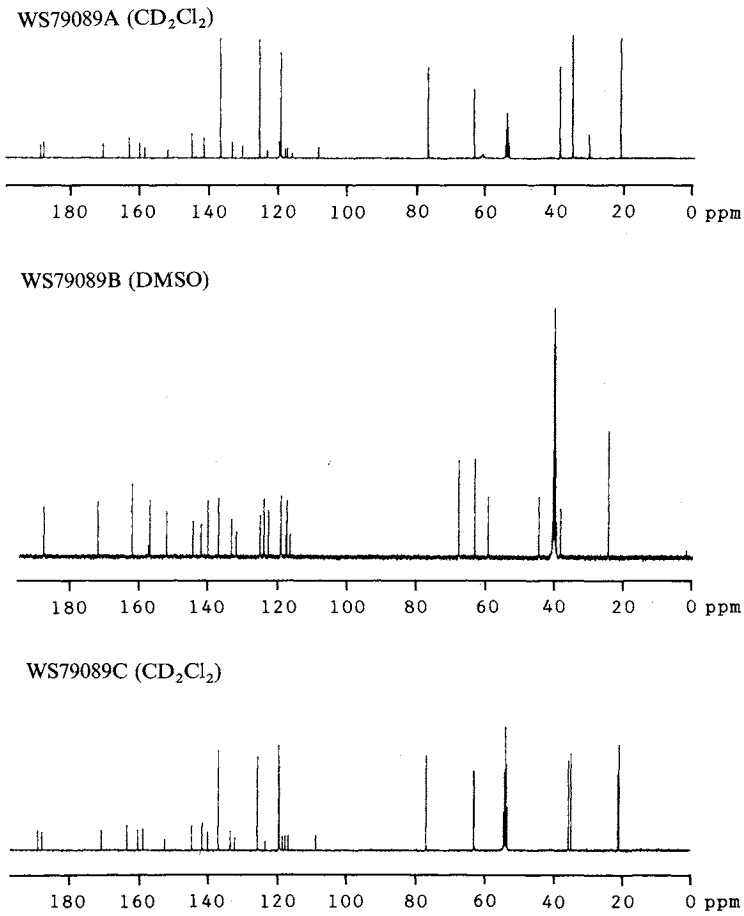



iodine vapor and cerium sulfate though negative to ninhydrin.

The $^{1}H$ NMR spectra of WS79089A, B and C are shown in Fig. 4. $^{13}C$ NMR spectra of WS79089A, B and C are shown in Fig. 5. The IR spectra of WS79089A, B and C are shown in Fig. 6.

Structural elucidation of WS79089A, B and C will be published elsewhere.

Fig. 5. $^{13}C$ NMR spectra of WS79089A, B and C.



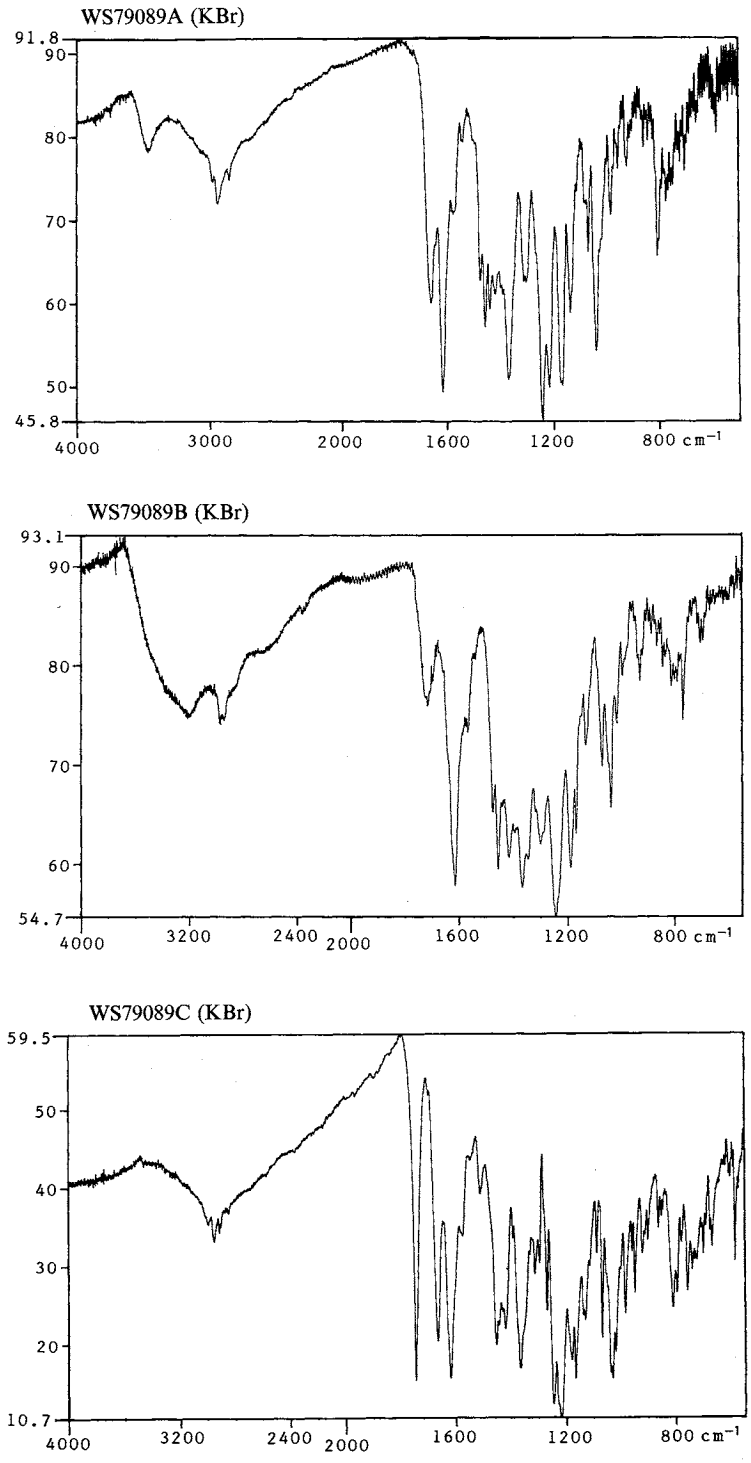

## Biological Activity

Antimicrobial activities of WS79089A, B and C were evaluated by the serial broth dilution method. WS79089A, B and C have no antibiotic activity against *Escherichia coli* NIHJ JC-2, *Staphylococcus aureus* 209P JC-1, *Pseudomonas aeruginosa* NCTC 10490, *Bacillus subtilis* ATCC 6633, *Proteus vulgaris*, *Candida albicans*, *Cryptococcus neoformans* YC203 or *Aspergillus fumigatus* IFO 5840 at 1,000 $\mu$g/ml.

The inhibitory activities of WS79089A, B, C and phosphoramidon against ECE, collagenase and NEP are summarized in Table 4. WS79089B markedly inhibited ECE activity with an $IC_{50}$ value of $1.4 \times 10^{-7}$ M, while it did not inhibit collagenase and NEP activities below $4.9 \times 10^{-5}$ M. It has been suggested that ECE is a phosphoramidon sensitive neutral metalloprotease[21~23]. Here we described the inhibitory activities of WS79089B compared to phosphoramidon for the metalloprotease listed in Table 4. Inhibitory activity of WS79089B is selective for ECE among the metalloprotease. Phosphoramidon is not a selective ECE inhibitor. Phosphoramidon is about 50 times more active against NEP than ECE. WS79089A and C were less active against ECE than WS79089B, and exhibited $IC_{50}$ values of 0.73 and 3.42 $\mu$M, respectively.

WS79089A, B and C showed no cytotoxic activity at 100 $\mu$g/ml against mouse bone marrow cells and bovine carotid endothelial cells. Furthermore, WS79089A, B and C showed negative mutagenic

Fig. 6. IR spectra of WS79089A, B and C (KBr).



activity at 5,000 μg/ml in Ames test (*Salmonella typhimum* strains TA98 and TA100)[24].

Acute toxicity studies with WS79089A, B and C were conducted in ICR mice and its $LD_{50}$ value given intravenously was greater than 100 mg/kg and did not show either toxicity or abnormal movements until day 14.

Table 4. The $IC_{50}$ values (μM) of ECE inhibitors to various metalloproteinases.

| Inhibitors | ECE | Collagenase (μM) | NEP |
|---|---|---|---|
| WS79089A | 0.73 | 102 | >100 |
| WS79089B | 0.14 | 49.4 | >100 |
| WS79089C | 3.42 | 61.3 | >100 |
| Phosphoramidon | 0.49 | 92.4 | 0.0092 |

## Discussion

In the course of our screening program for ECE inhibitors from microbial products, we isolated the highly selective ECE inhibitors WS79089A, B and C from the culture of *Streptosporangium* sp. No. 79089. IKEGAWA *et al.* have reported phosphoramidon as being an ECE inhibitor[2]. Convertion of big ET-1 to ET-1 is essential for the full expression of biological activity since phosphoramidon inhibits both the *in vivo* pressor effect and *in vitro* contractile action of big ET-1 without affecting the vasoconstrictor properties of ET-1[3]. Phosphoramidon is a potent NEP inhibitor and is less active against ECE. Among the WS79089A, B and C, WS79089B exhibit the most potent ECE inhibition activity ($IC_{50}$: 0.075 μg/ml; 0.14 μM). Therefore WS79089B may be expected to be useful for the treatment and prevention of acute renal failure, myocardial infarction, subarachnoid hemorrhage, asthma, hypertension and some other peripheral circulatory failure, and the like. WS79089B has the benzo[*a*]naphtacen quinone structure like as pradimicin[25]. Pradimicin is a potent antifungal antibiotics and which has very weak ECE inhibition activity in our assay systems ($IC_{50}$: 50~100 μg/ml), furthermore WS79089B has no antifungal activity.

## Experimental

Conversion of WS79089A to WS79089B

WS79089A (50 mg) was solubilized with 5 ml of aqueous 1 N NaOH solution at room temperature for 30 minutes for subjecting hydrolysis reaction. The hydrolysed product was diluted with 50 ml of water and desalted on a Diaion HP-20 column (100 ml, Mitsubishi Chemical Ind.). The column was washed with 200 ml of water, then a sodium salt of WS79089B was eluted with 100 ml of methanol. The active fraction was concentrated *in vacuo* to give a sodium salt of WS79089B (32 mg) as deep red powders (Fig. 1).

Conversion of WS79089C to WS79089B

WS79089C (20 mg) was solubilized with 2 ml of aqueous 1 N NaOH solution at room temperature for 30 minutes for subjecting hydrolysis reaction. The conversion product was diluted with 20 ml of water and desalted on a Diaion HP-20 column (40 ml, Mitsubishi Chemical Ind.). The column was washed with 80 ml of water, then a sodium salt of WS79089B was eluted with 40 ml of methanol. The active fraction was concentrated *in vacuo* to give a sodium salt of WS79089B (14 mg) as deep red powder (Fig. 1).

Conversion of a Sodium Salt of WS79089B to a Free Type of WS79089B

A sodium salt of WS79089B (70 mg) was dissolved in 50 ml of water. The solution was adjusted to pH 3.5 with 1 N HCl, from which WS79089B, *per se*, free type was extracted with 50 ml of ethyl acetate. This procedure was repeated. The ethyl acetate layers were accumulated, dried over anhydrous $Na_2SO_4$, and concentrated *in vacuo* to give WS79089B, *per se*, free type (50 mg).

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