WS75624 A and B, New Endothelin Converting Enzyme Inhibitors Isolated from *Saccharothrix* sp. No. 75624

I. Taxonomy, Fermentation, Isolation, Physico-chemical Properties and Biological Activities

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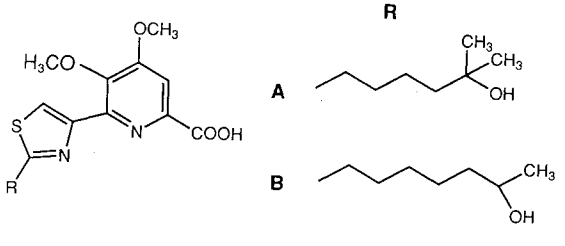
Novel endothelin converting enzyme (ECE) inhibitors, WS75624 A and B†, have been isolated from the fermentation broth of *Saccharothrix* sp. No. 75624. These inhibitors were purified from an acetone extract of whole culture broth followed by HP-20 column chromatography, silica gel column chromatography and HPLC. WS75624 A and B showed highly potent ECE inhibitory activity, and both had IC_{50} values of 0.03 μg/ml. WS75624 A and B also showed other metalloprotease (collagenase and neutral endopeptidase) inhibitory activity with IC_{50} values of 1 μg/ml.

Since large amount of WS75624 B was isolated, we tried *in vivo* evaluation using WS75624 B. WS75624 B inhibited big endothelin-induced pressor effect when administered to SD rat intravenously with big ET-1.

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].

During the course of a screening program for selective ECE inhibitors, we isolated two novel ECE inhibitors WS75624 A and B (Fig. 1) from the culture broth of *Saccharothrix* sp. No. 75624. In this paper, we report the taxonomy of the producing strain, production, isolation, physico-chemical properties and biological activities of WS75624 A and B.

Fig. 1. Chemical structures of WS75624 A and B.



Materials and Methods

Taxonomic Studies

Strain No. 75624 was isolated from a soil sample collected in Tsukuba city. For the taxonomic study of strain No. 75624, the methods and media described by SHIRLING and GOTTLIEB[9], and by WAKSMAN[10] were employed. The observations were made after 21 days cultivation at 30°C. The morphological observations were made on the cultures grown on inorganic salts-starch agar and glycerol-asparagine agar, using an optical microscope and a scanning electron microscope. The color name used in this study were taken from

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Methuen Handbook of Colour[11]. Growth range of temperature and NaCl tolerance were determined on yeast extract-malt extract agar. Cell wall type and whole-cell sugar and phospholipid compositions and menaquinones were determined by previously described methods[12~16].

Fermentation

A loopful of *Saccharothrix* sp. No. 75624 on mature slant culture was transferred into twenty five 500-ml Erlenmeyer flasks each containing 160 ml of sterile seed medium composed of corn starch 1% (w/v), glycerin 1% (w/v), glucose 0.5% (w/v), Pharmamedia (Traders Protein) 1% (w/v), dried yeast 0.5% (w/v), 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 3 days at 30°C. The resultant seed culture was used to inoculate 20 liters of production medium containing starch 3% (w/v), glycerin 1% (w/v), corn steep liquor 2% (w/v), potato protein 1% (w/v), $CaCO_3$ 0.2% (w/v) and Adekanol LG-109 (deforming agent, Asahi Denka Co.) 0.07% (w/v) and Silicone KM-70 (deforming agent, Shin-etsu Chemical Co.) 0.05% (w/v) in five 30-liter jar fermenters and those were cultured at 30°C for 5 days under aeration of 20 liters/minute, back pressure of 1.0 kg/cm^2 and agitation speed of 200 rpm. The cultivation was carried out two times.

The progress of fermentation was monitored by high performance liquid chromatography (HPLC) using an Hitachi Model 655 pump. A steel column (4.00 mm diameter, 250 mm length) packed with YMC-Pack ODS-AM 303 (250 × 4.5 mm i.d.) (YMC Co., Ltd.) was used at a flow rate of 1.0 ml/minute. The mobile phase was an aqueous solution of 40% acetonitrile with 0.5% ammonium dihydrogen phosphate and 0.1% phosphoric acid. The sample for the HPLC assay was prepared as follows; an equal volume of acetone was added to a broth with vigorous stirring and the mixture was allowed to stand for one hour and then centrifuged. Ten μl of the supernatant was injected *via* the injector of the Hitachi Model 655 HPLC. 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 were 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

ECE activity was measured as described previously[17]. Briefly, human big ET-1 (50 ng) was incubated with the cell homogenate preparation (20~30 μg protein) for 16 hours at 37°C in 250 μl 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 μl 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 μl 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).

Collagenase Inhibition Assay

Collagenase activity was measured as described previously[17]. Briefly, 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 μl of 10-fold diluted collagenase (trypsin-activated fibroblast supernatant) solution were added and incubated at 37°C for 2 hours. After the incubation, 5 μl of 80 mM *o*-phenanthrolin was added, followed by 1 hour incubation. To the reaction mixture, 70 μl of ethanol and 30 μl 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

NEP activity was measured as described previously[17]. Briefly, 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 WS75624 A and B 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.

Vasoconstrictor Effect of Big ET-1 in Rats

Male Sprague-Dawley rats (250~300 g) were anesthetized with pentobarbital sodium (Nembutal-Abbott, 50 mg/kg of boby weight). A femoral artery catheter was implanted for monitoring arterial pressure and heart rate, and a femoral vein catheter was used for injection of saline or drugs. Blood pressure and heart rate was recorded from the femoral artery through a cannula (PE-50) connected to a pressure transducer (Nihon Koden, MPU-0.5A) coupled to a Biophysiograph RM 6000 system. Two groups of rats were injected with the following: Big ET-1 (1 nmol/kg) alone (n=7), big ET-1 (1 nmol/kg) with WS75624 B (30 mg/kg) iv bolus injection (n=7).

Statistical Analysis

Results are expressed as means±S.E. of number of experiments as indicated. Statistical analysis was done by means of Student's *t*-test.

Results

Taxonomic Characteristics of *Saccharothrix* sp. No. 75624

(1) Morphological characteristics

The substrate mycelium developed well and branched irregularly. This strain produced long and straight to flexuous aerial mycelia which were divided into cylindrical fragments with irregular size. These fragments were often intercalated with empty hyphae. Long chain of fragments comprised more than 20 fragments per chain. The shape of the fragments were cylindrical and 0.3~0.5×0.7~1.7 μm in size, and the surface of them was smooth (Fig. 2). These fragments were not motile.

(2) Cultural and physiological characteristics

The results of cultural and physiological characteristics are shown in Tables 1 and 2 respectively. Growth was moderate on all of the media tested. Aerial mass color was white or yellowish white. Reverse side color of growth was light orange or light brown. Melanoid pigments and other soluble pigments were not produced.

(3) Chemotaxonomic characteristics

meso-Diaminopimelic acid was detected in the whole-cell hydrolysates of this strain. Rhamnose and galactose, in addition to ribose, mannose and glucose, were detected as whole-cell sugar, placing this strain in the cell-wall type III C[14]. The diagnostic phospholipid detected was phosphatidyl-ethanolamine, placing it in the type PII phospholipid group[15]. The major menaquinone was MK-9 (H_4).

Fig. 2. Scanning electron micrograph of aerial haphae and its fragments of strain No. 75624 on inorganic salts-starch agar.

Bar represents 5 μm.

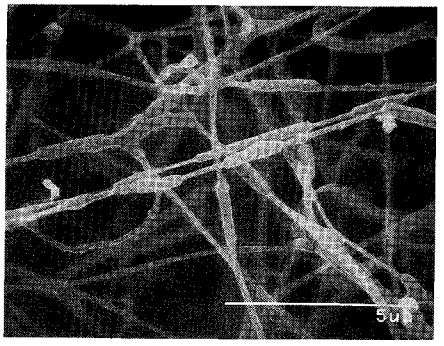


Table 1. Cultural characteristics of strain No. 75624.

Medium	Cultural characteristics
Yeast extract-malt extract agar	G: Moderate A: Moderate, white R: Light orange (5A4) S: None
Oatmeal agar	G: Moderate A: Poor, white R: Light brown (6D7) S: None
Inorganic salts-starch agar	G: Moderate A: Moderate, white R: Light brown (6D6) S: None
Glycerol-asparagine agar	G: Moderate A: Moderate, white R: Brownish orange (6C7) S: None
Peptone-yeast extract-iron agar	G: Moderate A: None R: Brownish orange (5C6) S: None
Tyrosine agar	G: Moderate A: Poor, white to pale yellow (4A2) R: Light brown (5C2) S: None
Starch-yeast extract agar*	G: Moderate A: Moderate, white R: Light brown (6D6) S: None
Sucrose-nitrate agar	G: Moderate A: None R: Dark brown (8F5) S: None

Abbreviation: G: growth, A: aerial mycelium, R: reverse side color S: soluble pigment.

* Soluble starch 1%, yeast extract 0.2%, agar 1.5%, pH 7.3.

Table 2. Physiological characteristics of strain No. 75624.

Conditions	Characteristics
Temperature range for growth (°C)	12~40
(optimum)	(35)
NaCl tolerance (%)	>4, <5
Gelatin liquefaction	+
Milk coagulation	+
Milk peptonization	−
Starch hydrolysis	+
Production of melanoid pigments	−
Decomposition of cellulose	−
Carbon utilization	
D-Glucose	+
L-Arabinose	+
D-Xylose	+
Inositol	+
Sucrose	+
D-Fructose	+
D-Mannitol	+
L-Rhamnose	+
Raffinose	−

+: Positive, −: negative.

(4) Classification

Based on the morphological and chemical characteristics described above, strain No. 75624 is considered to belong to the genus *Saccharothrix*[18,19]. Therefore, this strain was designated as *Saccharothrix* sp. No. 75624.

Fermentation

The amount of WS75624 A and B produced by *Saccharothrix* sp. No. 75624 in a 30-liter jar fermentor reached its maximum after 5 days of cultivation.

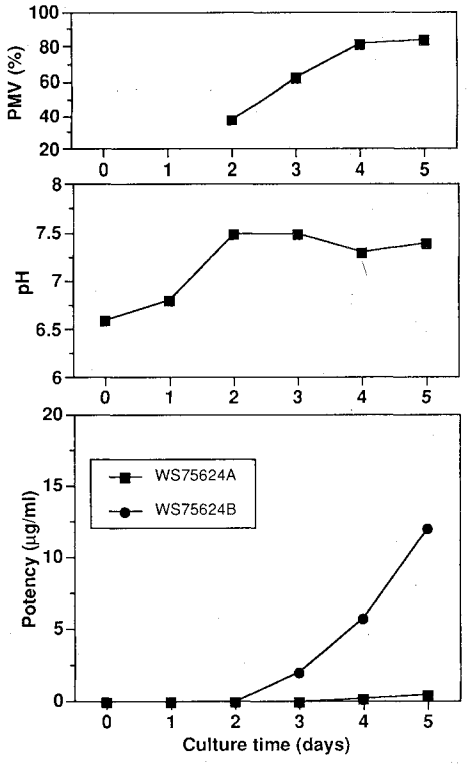
Fig. 3 presents the data from a typical 30-liter fermentation and gives information regarding WS75624 A and B production, pH and packed mycelial volume (PMV). WS75624 B production began at day 2, and the maximum production was observed after 5 days of cultivation and the yields of WS75624 A and B were 0.4 μg/ml and 12 μg/ml, respectively.

Isolation and Purification

The cultured broth (75 liters) was filtered with the aid of diatomaceous earth (Showa Kagaku; Radiolite #600) (1 kg). The cultured broth was treated with 75 liters of acetone and stirred for 60 minutes and then after filtrated extract was concentrated to 72 liters.

The broth extract (72 liters) was absorbed on an Diaion HP-20 (Mitsubishi Kasei) column (4.5 liters). After washing with 15 liters of water and 13 liter of 40% methanol and then the active fractions were eluted with 18 liters of 100% methanol. The elute was concentrated to 63.1 g dry powder under reduced pressure. The powder (63.1 g) was dissolved to small amount of ethyl acetate

Fig. 3. Time course of fermentation.



and then absorbed to 0.4 liters of silica gel (Kieselgel 60, 70~230 mesh). The resultant silica powder (0.4 liters) was applied to a column of silica gel (Kieselgel 60, 70~230 mesh, 0.8 liters). The column was washed with 3.0 liters of ethyl acetate, 3 liters of ethyl acetate - acetone (1 : 1) and then the column was developed with 2 liters of acetone - methanol (3 : 1) and 3 liters of acetone - methanol (2 : 1). The combined elute was concentrated to dry powder (26.5 g) under reduced pressure. Five grams of the residue was dissolved with methanol (6 ml) and solution was diluted by adding 18 ml of water. The solution was applied to a preparative HPLC column (YMC pack ODS-AM: 384 ml; flow rate: 25 ml/minute). The column was preequilibrated with 30% acetonitrile - 0.1% TFA, then the active fractions were eluted with 27.5% acetonitrile - 0.1% TFA (fraction A and B). Each of the active fractions were concentrated under reduced pressure to give crude crystals.

Each active fractions were dissolved in chloroform - methanol (3 : 1) and recrystallization from methanol gave WS75624 A and B as colorless pure amorphous powder. The yields of pure WS75624 A and B from 75 liters of whole broth were 37 mg and 216 mg, respectively.

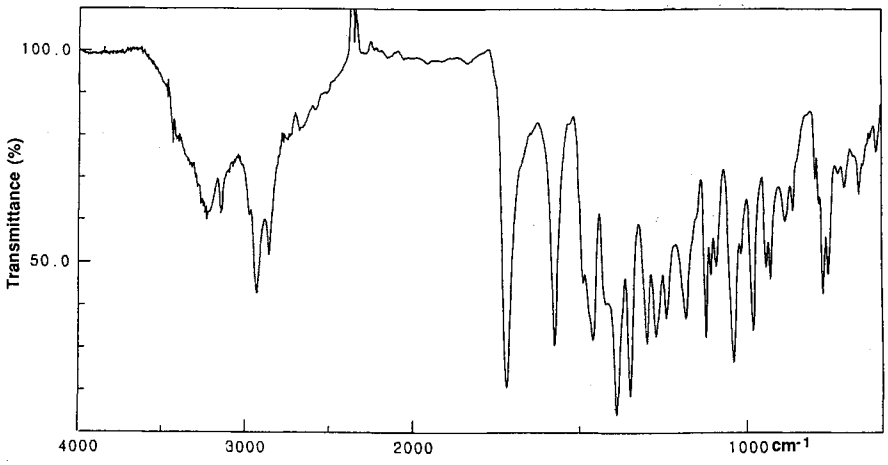
Physico-chemical Properties

The physico-chemical properties of WS75624 A and B are summarized in Table 3. WS75624 A and B are soluble

Table 3. Physico-chemical properties of WS75624 A and B.

	WS75624 A	WS75624 B
Appearance	Colorless powder	Colorless powder
Melting point	53~54°C	139~140°C
$[\alpha]_D^{23}$ (MeOH)	0° (*c* 0.5)	+3° (*c* 1.0)
UV λ_{max}^{MeOH} nm (MeOH):	215, 245, 290~305	215, 245, 290~305
(MeOH-HCl):	215, 242, 305~320	215, 242, 305~320
(MeOH-NaOH):	215, 238, 290, 295~300	215, 238, 285, 295~300
FAB-MS (*m/z*)	381 $(M+H)^+$	381 $(M+H)^+$
HRFAB-MS Found:		381.1484
Calcd:		381.1482
IR ν_{max} (KBr) cm^{-1}	3200, 2940, 1720, 1580	3200, 2930, 1720, 1580
Molecular formula	$C_{18}H_{24}N_2O_5S$	$C_{18}H_{24}N_2O_5S$
Anal (%) Found:	C 55.51, H 6.47, N 7.19, S 8.23	C 56.82, H 6.36, N 7.36, S 8.43
Calcd:	C 55.78, H 6.56, N 7.13, S 8.55	C 56.81, H 6.50, N 7.31, S 8.57
Solubility		
Soluble:	Methanol, chloroform, acetone	Methanol, chloroform
Slight soluble:	Ethyl acetate	Acetone
Insoluble:	Water	Ethyl acetate, water

Fig. 4. IR spectrum of WS75624 B (KBr).



in methanol and insoluble in water. The color reactions of WS75624 A and B are as follows: positive to iodine vapor, cerium sulfate and Dragendorff reagents though negative to $FeCl_3$, ninhydrin, Erhlich and Molisch reagents. The IR spectrum of the most abundant component, WS75624 B is shown in Fig. 4 and the ^1H NMR spectra and ^{13}C NMR spectra are reproduced in Figs. 5 and 6, respectively. WS75624 A and B possess the same chromophore, 4,5-dimethoxy-6-(2-alkyl-4-thiazolyl)-2-pyridinecarboxylic acid, in their molecules and are differentiated by the alkyl chains. A detailed account of the structure elucidation will be reported in this journal[20].

Biological Activity (*In Vitro*)

Antimicrobial activities of WS75624 A and B were evaluated by the serial broth dilution method. WS75624 A and B have no antibiotic activity against *Escherichia coli* NIHJ JC-2, *Staphylococcus aureus* 209P JC-1, *Pseudomonas aeruginosa* NCTC 10490, *Bacillus subtilis* ATCC 6633 and *Proteus vulgaris* at 100 μg/ml. WS75624A and B have weakly antimicrobial activity at 100 μ/ml against *Cryptococcus neoformans* YC203 or *Aspergillus fumigatus* IFO 5840 and 50 μg/ml against *Candida albicans*, and have no antibiotic activity against the same microbial strains at 10 μg/ml.

The inhibitory activities of WS75624 A, B and phosphoramidon against ECE, collagenase and NEP are summarized in Table 4.

WS75624 B markedly inhibited ECE activity with an IC_{50} value of 0.03 μg/ml, while it weakly inhibit collagenase and NEP activities with IC_{50} values of 1.0 μg/ml and 1.25 μg/ml, respectively. It has been suggested that ECE is a phosphoramidon sensitive

Fig. 5. ^{1}H NMR spectrum of WS75624 B in CD_3OD.

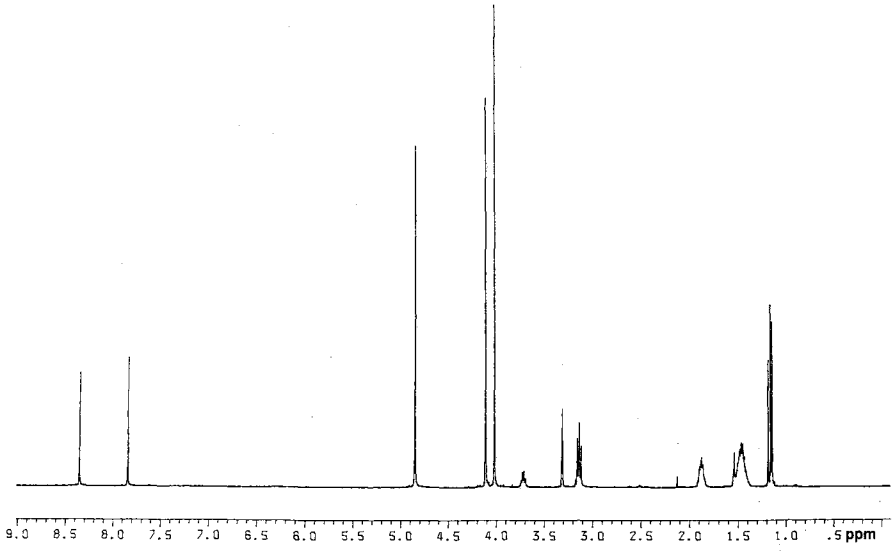


Fig. 6. ^{13}C NMR spectrum of WS75624 B in CD_3OD.

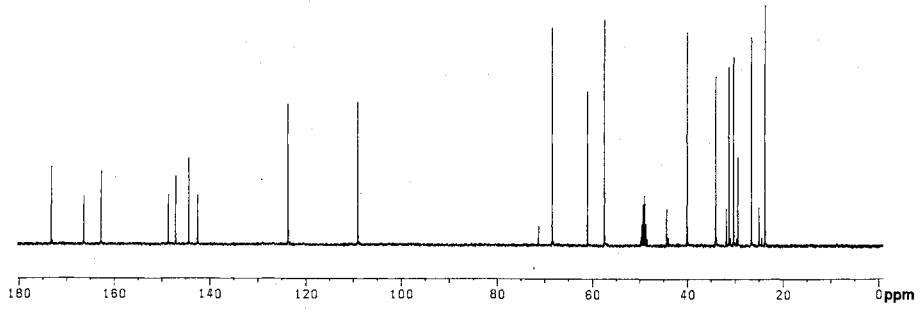


Table 4. The IC_{50} values (μg/ml) of ECE inhibitors to various metalloproteases.

Inhibitors	ECE	Collagenase (μg/ml)	NEP
WS75624 A	0.03	1.0	1.25
WS75624 B	0.03	1.0	1.25
Phosphoramidon	0.1	>50.0	0.002

neutral metalloprotease[7,8]. Here we described the inhibitory activities of WS75624 B compared to phosphoramidon for the metalloprotease listed in Table 4. Inhibitory activity of WS75624 B is selective for ECE among the metalloprotease.

WS75624 A and B showed cytotoxic activity at 10 μg/ml against mouse bone marrow cells and bovine carotid endothelial cells.

The Effect of WS75624 on the Pressor Activity of Porcine Big ET-1 *In Vivo*

Since we obtained larger amount of WS75624 B than WS75624 A so we examined the *in vivo* evaluation of WS75624 B.

In the present study, a bolus of 1 nmol/kg of big ET-1 was used. WS75624 B (30 mg/kg) and phosphoramidon (30 mg/kg; data not shown) were markedly attenuated the hypertensive effect of big ET-1 (1 nmol/kg, iv) the inhibiting the conversion of big ET-1 to ET-1 (Fig. 7).

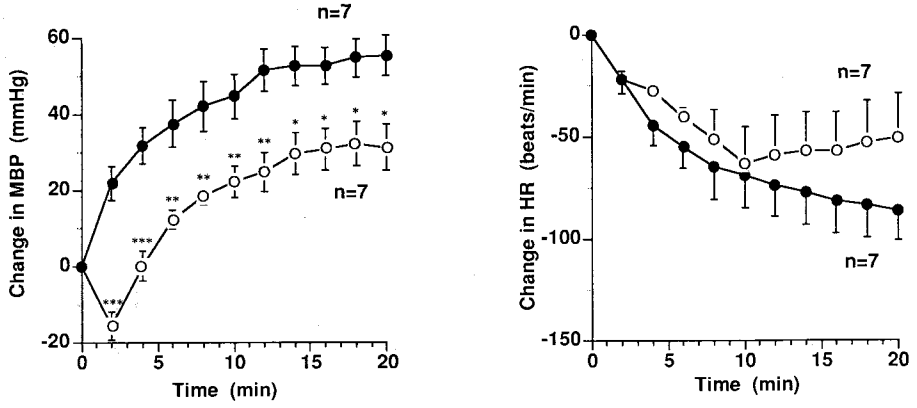
WS75624 B and phosphoramidon did not affect the ET-1 induced hypertensive effect.

Discussion

In the present study we have demonstrated that the intravenously bolus injection of WS75624 B has similar protective effect to the same dose of phosphoramidon in antagonizing the pressor effect induced by big ET-1 without affecting the pressor effects produced by ET-1. Recently structurally resemble karnamycin were reported

Fig. 7. Effects of WS75624 on big ET-1-induced changes in MBP and HR in rats.

● Control, ○ WS-75624 B; 30 mg/kg i.v.



Data are means ± S.E.M. Significant differences between the groups are indicated; * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

as antifungal substance. WS75624 A and B have also weak antifungal activity against *Candida albicans* at 50 μg/ml.

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