

B-90063, a Novel Endothelin Converting Enzyme Inhibitor Isolated from a New Marine Bacterium, *Blastobacter* sp. SANK 71894

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A novel endothelin-converting enzyme (ECE) inhibitor, B-90063, was isolated from the culture supernatant of the newly discovered marine bacterium *Blastobacter* sp. SANK 71894. Based on spectral analyses and chemical reactions, the structure of B-90063 was determined to be bis[6-formyl-4-hydroxy-2-(2'-*n*-pentylloxazol-4'-yl)-4-pyridon-3-yl]-disulfide (**1a**). Human and rat ECEs were inhibited more potently by B-90063, with respective IC₅₀ values of 1.0 and 3.2 μM, than were other neutral endopeptidases such as NEP and type-I and -IV collagenases. B-90063 also inhibited the binding of ET-1 to rat ET_A and bovine ET_B receptors, though its antagonistic activities were weak. B-90063, thus, may abolish the physiological actions of endothelins through the ECE inhibitory and receptor antagonistic mechanisms.

Endothelin (ET-1)¹⁾ is an endothelium-derived 21-amino acid peptide. It has a very potent vasoconstrictor activity in isolated blood vessels and constitutes an ET family with its two isoforms, ET-2 and ET-3, and its four highly homologous, cardiotoxic peptides, sarafotoxin S6a-d. ET-1 is biosynthesized from big ET-1, a 203-amino acid peptide by a phosphoramidon-sensitive neutral metalloprotease, endothelin converting enzyme (ECE)^{2~5)}; ECE mediates the selective hydrolysis between ²¹Trp and ²²Val, resulting in the regulation of ET-1 level in many organs. Since the discovery of the ET family and their receptors, a variety of natural and synthetic ET receptor antagonists⁶⁾ have been found; some of these antagonists have been used in clinical trials as drugs for the prevention and treatment of various diseases such as acute renal insufficiency, acute myocardial infarction, hypertension, arteriosclerosis, and so on.

In addition to these antagonists, selective ECE inhibitors may also be useful for the prevention and treatment of the above-named diseases. Phosphoramidon,⁶⁾ a natural ECE inhibitor, abolishes pressor responses induced by big endothelins in animal models

and also mitigates diseases such as myocardial ischemia, hypertension, and renal failure in animal models. Phosphoramidon also inhibits neutral endopeptidase (NEP),⁶⁾ a key enzyme in the ET degradation system, more potently than ECE. This inhibition of NEP in particular can result in complicated reactions such as the ET-induced contraction of airway smooth muscles. Selective ECE inhibitors are therefore the preferred drugs for the treatment of such diseases. Recently, some natural ECE inhibitors, such as WS75624 A~B,^{7,8)} WS79089A~C,⁹⁾ and aspergillosamines A~B^{10,11)} have been isolated from microbial sources.

In the course of a program designed to discover new selective ECE inhibitors, we extensively screened the extracts of marine organisms, especially rare marine bacteria, and found that the fermentation supernatant of one marine bacterium, *Blastobacter* sp. SANK 71894, showed potent inhibitory activity against human ECE (h-ECE). We herein report on the fermentation, isolation and structure elucidation of B-90063 (**1a**) and on the activities of this compound against h-ECE and other endopeptidases.

Taxonomy of the Producing Strain

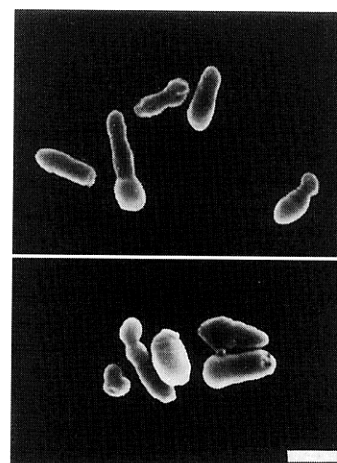
The producing strain SANK 71894 was isolated from sea water collected on the coast of Ojika Peninsula, Miyagi Pref., Japan. The bacterium is non-motile, non-spore forming, and Gram-negative and morphologically is either cylindrical, spherical, or oval shaped (Table 1). Multiplication by budding occurs at cell poles, rarely laterally (Fig. 1); sea water is required for growth. Based on the morphological and physiological properties, the GC content, and the quinone system, strain SANK 71894 was considered to belong to the genus *Blastobacter*. As the chemotaxonomical properties of bacterium SANK 71894 were different from all known species belonging to the genus *Blastobacter* and, as it was of marine origin, produced dark green water-insoluble pigments, and required sea water for growth, it was assigned to a new species of the genus *Blastobacter*.^{12~14)}

Fermentation and Isolation

Blastobacter sp. SANK 71894 was cultured at 23°C for 3~4 days. The culture supernatant, obtained by centrifugation at 8,000 rpm for 15 minutes, was extracted with EtOAc to remove inactive neutral materials. The aq layer, after being adjusted to pH 3.0, was reextracted with EtOAc. The h-ECE inhibitory activity remained in the acidic EtOAc extract, which was then subjected to Lobar column chromatography [RP-18, MeOH-H₂O, 80:20 (v/v)] and HPLC [Pegasil ODS, MeOH-H₂O-TFA, 75:25:0.2 (v/v)] to give the desired product, B-90063 (**1a**) as light yellow crystals (Table 2). During the above EtOAc extraction of the fermentation product, a dark green material often precipitated concurrently with **1a**, thus lowering the yield of the desired product. Hence, we searched for a more effective recovery method

Fig. 1. Scanning electron micrograph of the producing strain SANK 71894.

Bar represents 1 μm .



of **1a** and found that separation could be effectively accomplished by subjecting the acidified and centrifuged supernatant to Diaion HP-20 chromatography. Elution with either 80~100% acetone or 80~100% MeOH gave nearly complete recovery of **1a**. This separation method was especially convenient for large scale preparation of the desired compound. Separation was monitored by inhibition against h-ECE obtained from human umbilical vein endothelial cells.

Structure Elucidation

Mass spectra and elemental analysis of **1a** showed it to have the chemical formula C₂₈H₃₀N₄O₆S₂ (Table 2). ¹³C NMR (Table 3) indicated the presence of 14 carbons, including one *n*-pentyl, 3 isolated *sp*² methine and 6 fully substituted *sp*² carbons; suggesting that B-90063 (**1a**) is dimeric through a disulfide linkage (C) (Fig. 3). The

Table 1. Morphological, physiological, and chemotaxonomical properties of the strain SANK 71894.

Gram stain: —	Catalase: +
Multiplication: Budding	Oxidase: +
Cell shape: Cylindrical, spherical, or oval	OF test: —
Cell size: (0.3 ~ 0.8) x (0.5 ~ 1.3) μm	Seawater requirement: +
Motility: —	Utilization of MeOH: —
Endospore: —	G + C M% in DNA: 57.3 %
Colony pigmentation: Dark green	Quinone system: Ubiquinone Q-10
Chlorophyll: +	

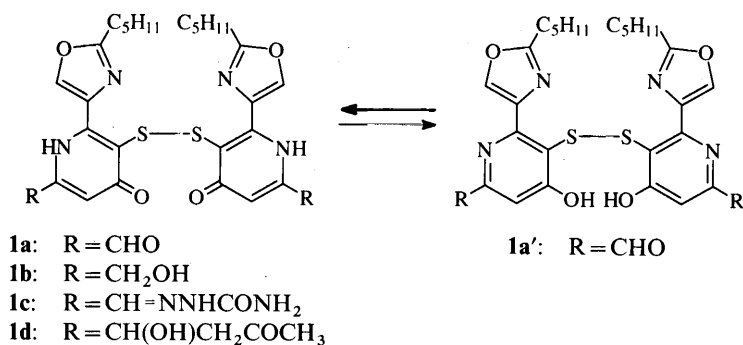
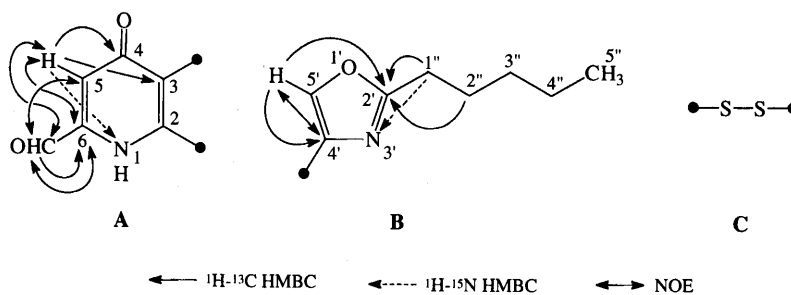
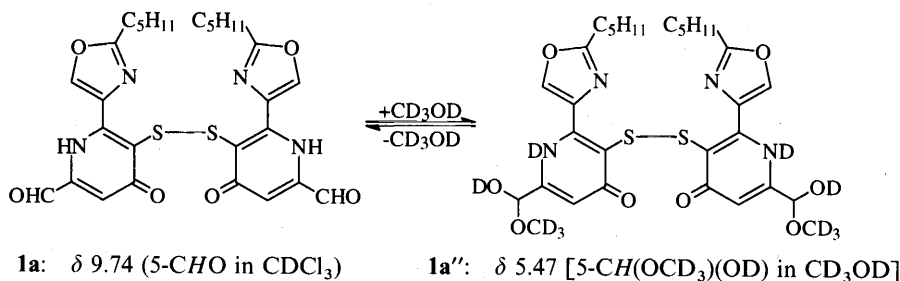
Table 2. Physico-chemical properties of B-90063 (**1a**).

Appearance	Yellow crystalline powder
Melting point	73 ~ 74°C
FAB-MS [QM ⁺ /QM ⁻] (3-NBA)	<i>m/z</i> 583/581
FAB-MS [QM ⁺ /QM ⁻] (Glycerol)	<i>m/z</i> 293/291
HR-EI-MS [M/2 +H] ⁺	<i>m/z</i> 292.0887 (C ₁₄ H ₁₆ N ₂ O ₃ S, Δ -0.6 mmu)
Molecular formula	C ₂₈ H ₃₀ N ₄ O ₆ S ₂
IR [ν _{max} cm ⁻¹ (KBr)]	3302, 1706, 1674, 1629, 1602, 1550, 1467, 1178, 1113, 1046, 1007.
UV [λ _{max} (ε, CH ₃ CN)]	214 (47,400), 261 (37,200), 340 (sh, 8,100), 360 (sh, 4,100).
Elemental analysis	
Calcd:	C 55.99, H 5.37, N 9.33, S 10.68 (C ₂₈ H ₃₀ N ₄ O ₆ S ₂ ·H ₂ O)
Found:	C 55.75, H 5.60, N 9.35, S 10.44.

Table 3. ¹H and ¹³C NMR data of **1a** and **3a** in CDCl₃.

C#	1a		3a	
	δ _H (Multi., <i>J</i>)	δ _C (Multi.)	δ _H (Multi., <i>J</i>)	δ _C (Multi.)
1 (N)		130.0*		-
2		144.9 (s)		143.7 (s)
3		123.8 (s)		126.3 (s)
4		179.6 (s)		167.9 (s)
5	7.15 (1H, s)	121.2 (d)	7.45 (1H, s)	102.8 (d)
6		141.2 (s)		153.5 (s)
3-SCH ₃			2.41 (3H, s)	18.7 (q)
4-OCH ₃			4.08 (3H, s)	56.9 (q)
6-CHO	9.73 (1H, s)	186.3 (d)	10.16 (1H, s)	194.9 (d)
2'		166.3 (s)		165.9 (s)
3' (N)		225.0*		-
4'		132.5 (s)		138.0 (s)
5'	8.81 (1H, s)	143.4 (d)	8.45 (s)	141.6 (d)
1''	2.81 (2H, t, <i>J</i> = 7.6 Hz)	28.4 (t)	2.92 (2H, t, <i>J</i> = 7.2 Hz)	28.8 (t)
2''	1.79 (2H, m)	27.0 (t)	1.86 (2H, m)	27.4 (t)
3''	1.37 (2H, m)	31.9 (t)	1.40 (2H, m)	32.1 (t)
4''	1.37 (2H, m)	22.9 (t)	1.40 (2H, m)	23.0 (t)
5''	0.93 (3H, t, <i>J</i> = 6.6 Hz)	14.5 (q)	0.92 (3H, t, <i>J</i> = 7.2 Hz)	14.6 (q)

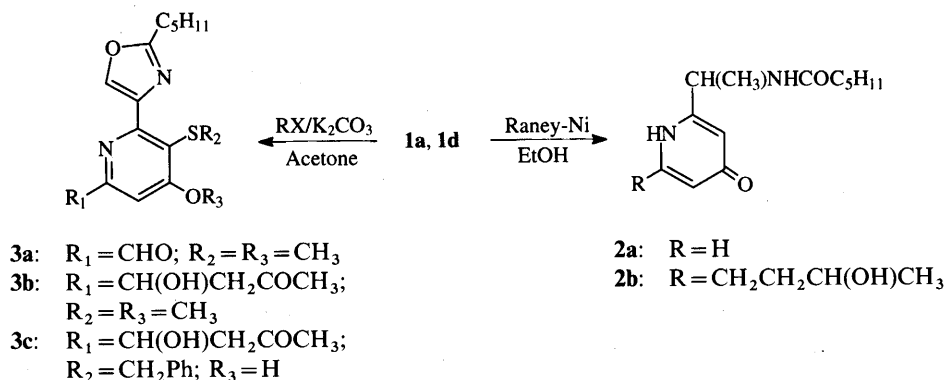
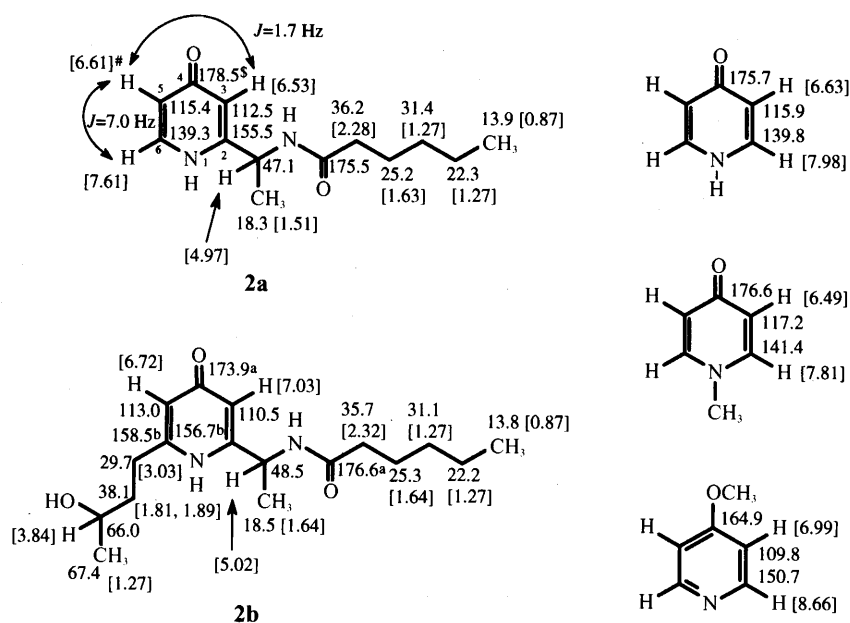
* ¹⁵N Chemical shifts with liq. NH₃ as an internal standard.

Fig. 2. Structures of B-90063 (**1a**) and its derivatives (**1b~d**).Fig. 3. Partial structures of **1a** by HMBC and NOE.Fig. 4. Interconversion between an aldehyde and acetal forms of **1a**.

presence of the dimeric structure was supported by the formation of a monomeric thioether (**3a~c**) described later. A signal of a formyl group (δ_{H} 9.74, δ_{C} 186.3) in CDCl₃, the presence of this group was later confirmed by the formation of its benzyl alcohol derivative (**1b**) and of semicarbazone (**1c**), was shifted up-field (δ_{H} 5.47, δ_{C} 96.2) in CD₃OD. Furthermore, the UV spectrum in CH₃CN underwent a gradual blue shift with the addition of a small amount of EtOH, finally leading to a spectrum superimposed to that obtained in EtOH. This evidence was compatible with the fact that B-90063 (**1a**) exists as a methyl acetal (**1a''**) in MeOH (Fig. 4). ¹³C{¹H} NOE and ¹H-¹⁵N HMBC techniques were used to elucidate the structure of **1a**. CD₃OD was used as a solvent in

these NMR correlation experiments, because it dissolved **1a** sufficiently and acetal formation had little influence on chemical shifts of other protons and carbons. Cross peaks of H-5' (δ_{H} 8.81) both with C-2' (δ_{C} 166.3) and with C-4' (δ_{C} 132.5) in HMBC were observed in addition to the NOE between H-5' and C-4'. H-1'' (δ 2.81) of the *n*-pentyl group correlated with C-2' by long-range coupling in HMBC and displayed a cross peak with N-3' (δ 225) in ¹H-¹⁵N HMBC. This constituted proof of the presence of a 2-*n*-pentyloxazole moiety (**B**) (Fig. 3).

The only further information provided by other HMBC and NOE features of **1a** was that the formyl group is located *ortho* to H-5 and correlated to N-1 (δ 130)¹⁴⁾ through long-range coupling in ¹H-¹⁵N HMBC.

Fig. 5. Reactions of **1a** and **1d**.Fig. 6. ¹H and ¹³C NMR data of **2a**, **2b** and model compounds.

No other information could be obtained from the spectra, because line broadening of ¹H NMR caused by tautomerism between the 4-pyridone and 4-hydroxypyridine forms of (**1a**) made it impossible to identify any other long-range couplings, and thus another partial structure (A) (Fig. 3) could not be elucidated. Therefore, chemical conversions of **1a** to its derivatives to be used for spectral analyses were attempted.

On desulfurization with Raney-Ni in boiling EtOH, B-90063 (**1a**) gave compound **2a**, identified with the UV (λ_{\max} 258 nm) and NMR (Fig. 6)¹⁴⁻¹⁶ spectral analyses which correlated either with 4-pyridone (λ_{\max} 253 nm) or *N*-methyl-4-pyridone (λ_{\max} 260 nm). Moreover, HMBC and NOE features also showed that a 1-(*N*-*n*-hexanoyl-

amino)ethyl group derived from the oxazole moiety¹⁷ is located at C-2 of the 4-pyridone moiety as shown in Fig. 3. The 4-pyridone moiety may have been produced through formyl decarbonylation. Compound **1d**, which was obtained by aldol condensation of **1a** with acetone, underwent desulfurization with Raney-Ni to give **2b** as well as **1a**. The four-carbon chain at C-6 was retained in **2b**, indicating that the decarbonylation of **1a** was a special case for formyl groups. Decarbonylation of formyl groups has already been observed in the reduction with Pd-C¹⁸ or RhCl(Ph₃P)₃.¹⁹ Thus, NMR data for **2a**~**2b** revealed the presence of the partial structure (A) in **1a**; this was supported by a comparison of EI-MS fragmentation patterns among **1a**, **3a**, and a 2-phenyl-

Table 4. Inhibitory activities of B-90063 (**1a**) and model compounds against ECEs and other neutral endopeptidases.

Compound	h-ECE	r-ECE	IC ₅₀ (μM)		
			NEP	Collagenase I	Collagenase IV
1a	1.0	3.2	66.0	11.0	9.9
Phosphoramidon	0.9	-	0.0045	>100	>100
Thiorphan	92.0	-	0.0021	-	-

oxazole derivative.²⁰⁾ B-90063 could therefore be depicted as bis [6-formyl-2-(2'-*n*-pentyloxazol-4'-yl)-4-pyridon-3-yl]disulfide (**1a**). B-90063 adopted the 4-pyridone structure (**1a**), a major tautomer, rather than the alternative 4-hydroxypyridine one (**1a'**). Methylation of **1a** under basic conditions described later, however, generated the 4-methoxypyridine derivative (**3a**); thus indicating that these two structures are mutually interconvertible depending on the pH. It is not, therefore, surprising that compound **2a** slowly changed to its 4-hydroxypyridine tautomer [δ 7.24 (H-3, d, $J=2.0$ Hz); 7.20 (H-5, dd, $J=2.0, 5.5$ Hz); 8.60 (H-6, d, $J=5.5$ Hz)] during the NMR measurement in CDCl₃.

In addition to the reactions above, further derivatizations of **1a** were carried out to investigate structure-activity relationships; methylation with CH₃I-K₂CO₃ in boiling acetone afforded a monomeric ether (**3a**), albeit in low yield, together with an acetone adduct (**3b**). This reaction, preceded or followed by methylation of hydroxyl groups, may have been triggered by CH₃⁺-mediated electrophilic fission of the disulfide bond.²¹⁾ In order to obtain **3a** in higher yield, the disulfide bond was cleaved with K₂S and the resulting mercaptide was trapped with CH₃I, resulting in a complex mixture. Reduction reactions with the aim of preparing thiols or desulfured compounds were carried out. However, reductions with Zn-AcOH and Al (Hg)-EtOH, and with H₂/Pd-C under medium or atmospheric pressure did not proceed, and oxidation with MnO₂, Ag₂O, PCC, or PDC yielded complex mixtures, without producing the desired carboxylic acid.

Biological Activities

The abilities of B-90063 (**1a**) and its derivatives (**1b~d**, **2a**, **3a~c**) to inhibit the neutral endopeptidases ECE, NEP,^{22,23)} and type-I and -IV collagenases²⁴⁾ were compared to those of phosphoramidon and thiorphan,

Table 5. Inhibitory activities of B-90063 (**1a**) and its derivatives (**1b~d**, **2a**, **3a~c**) against h-ECE.

Compounds	h-ECE
	IC ₅₀ (μM)
1a	1.0
1b	5.6
1c	5.6
1d	5.3
2a	>100
3a	18.0
3b	59.0
3c	>100

typical NEP inhibitors (Table 4, 5). B-90063 was also tested for rat ET_A and bovine ET_B antagonism.²⁵⁾

Discussion

The new marine bacterium *Blastobacter* sp. SANK 71894 produced B-90063 (**1a**) which has the structurally unique 4-pyridone and oxazole skeletons. The inhibitory activity of **1a** (IC₅₀ 1.0 μM) on h-ECE was nearly as potent as that of phosphoramidon (IC₅₀ 0.9 μM). **1a** inhibited rat ECE (r-ECE) with an IC₅₀ of 3.2 μM. In contrast to phosphoramidon and thiorphan, well-known potent NEP inhibitors, **1a** inhibited h-ECE more strongly than it did NEP and type-I and type-IV collagenases (respective IC₅₀s: 66.0, 11.0, and 9.9 μM). The formyl group of **1a** may potentiate its h-ECE inhibitory activity, because a chemical modification of this inhibitor resulted in a lowering of h-ECE inhibitory activity, as shown in

1b~c. Monomeric compounds (**3a~b**) were rather weaker than their progenitors, and **2a** and **3c** lost activity at $>100 \mu\text{g/ml}$. The dimeric structure may also play an important role in the expression of ECE inhibitory activity. In addition, B-90063 weakly inhibited the binding of ET-1 to rat ET_A and bovine ET_B receptors, with respective IC_{50} values of $43.7 \mu\text{M}$ and $27.2 \mu\text{M}$, interestingly suggesting that B-90063 may effectively abolish the physiological actions of endothelins through the ECE inhibitory and receptor antagonistic mechanisms.

Experimental

General Procedures

Melting points were measured on a Yanaco melting point apparatus. ^1H , ^{13}C , and ^{15}N NMR spectra were recorded on JEOL GX270, GSX 500, and A500 spectrometers with TMS as an internal standard. IR spectra were recorded on Nicole 5SXC and JASCO Valor-III spectrometers. UV spectra were recorded on a Shimadzu UV-265FW spectrometer. Mass spectra were recorded on a JEOL JMS-D300 spectrometer. Diaion HP-20 (Nihon Rensui) and RP-18 (reverse-phase Lobar column, Merck) were used for column chromatography. Silica gel plates ($20 \times 20 \times 0.2 \text{ cm}^3$, Merck) were employed for TLC. Packed ODS columns (Pegasil ODS & ODS-H, 6 i.d., 10 i.d., 20 i.d. $\times 250 \text{ mm}$, Senshu) were used for HPLC. Cell homogenization was done with a Kinematica polytron PTA-10S homogenizer unless otherwise mentioned. All enzyme assays were monitored by a Corona MTP 32 for fluorescence assay and Bio-Tek EL 340 microplate readers.

Fermentation and Isolation

a) *Blastobacter* sp. SANK 71894 was inoculated into 100 ml of medium, composed of marine broth (3.74 g, Difco), phytone (1.0 g, BBL), and glucose (1.0 g) in 100 ml of distilled water and contained in a 500 ml-Erlenmyer flask, and was then pre-cultured at 23°C for 48 hours with 200 rpm rotary shaking. The pre-cultured mixture was introduced into thirty 500 ml-Erlenmyer flasks, each containing 100 ml of the above medium, and cultured at 23°C for 48 hours with 200 rpm rotary shaking. The combined supernatant (pH 7.5) obtained by centrifugation at 8,000 rpm was twice extracted with an equal volume of EtOAc. The aq layer, after being adjusted to pH 3.0, was twice extracted with an equal volume of EtOAc. The combined EtOAc layer, after the usual work-up, gave a green oily material (362 mg), which was

fractionated on a RP-18 column. On elution with $\text{MeOH} - \text{H}_2\text{O}$, 8:2 (v/v) at a flow rate of 9.0 ml/minute, combined fractions eluted between 8~11 minutes were concentrated under reduced pressure to give a dark brown oil (42.1 mg), which was further subjected to HPLC (Pegasil ODS, 20 i.d. $\times 250 \text{ mm}$). On elution with a solvent mixture [$\text{MeOH} - \text{H}_2\text{O} - \text{TFA}$, 75:25:0.2 (v/v)] at a flow rate of 10.0 ml/minute, the combined fractions eluted between 19~20 minutes were concentrated under reduced pressure to give a light yellow oily substance (**1a**) (30.0 mg), which crystallized under storage in a refrigerator. **1a**: mp $73 \sim 74^\circ\text{C}$; HR-EI-MS m/z 292.0871 $\{[\text{M}/2 + \text{H}]^+$, $\text{C}_{14}\text{H}_{16}\text{N}_2\text{O}_3\text{S}$, $\Delta -0.6 \text{ mmu}$ (32.3%)}, 236.0216 [$\text{C}_{10}\text{H}_8\text{N}_2\text{O}_3\text{S}$, $\Delta -4.0 \text{ mmu}$ (22.4%)], 221.0011 [$\text{C}_9\text{H}_5\text{N}_2\text{O}_3\text{S}$, $\Delta -1.0 \text{ mmu}$ (29.6%)], 194.0148 [$\text{C}_8\text{H}_6\text{N}_2\text{O}_2\text{S}$, $\Delta -0.2 \text{ mmu}$ (100%)]; *Anal.* Calcd for $\text{C}_{28}\text{H}_{30}\text{N}_4\text{O}_6\text{S}_2 \cdot \text{H}_2\text{O}$: C 55.99, H 5.37, N 9.33, S 10.68. Found: C 55.75, H 5.60, N 9.35, S 10.44. Recrystallization of **1a** from aq MeOH gave yellow plates, containing 2 molecules of MeOH as a crystal solvent. mp $116 \sim 118^\circ\text{C}$; HR-FAB-MS $[\text{M} + \text{H}]^+ m/z$ 647.2216 ($\text{C}_{28}\text{H}_{31}\text{N}_4\text{O}_6\text{S}_2 \cdot 2\text{CH}_3\text{OH}$, $\Delta +0.1 \text{ mmu}$); IR $\nu_{\text{max}} \text{ cm}^{-1}$ (KBr) 3315, 3177, 1708, 1631, 1599, 1554, 1482, 1175, 1098, 1048; ^1H NMR (δ , CDCl_3) 0.92 (6H, t, $J=6.8 \text{ Hz}$), 1.35 (8H, m), 1.75 (4H, m), 2.75 (4H, t, $J=7.8 \text{ Hz}$), 3.49 (6H, s), 6.80 (2H, s), 8.45 (2H, s), 9.66 (2H, s), 10.16 (2H, br s); ^{13}C NMR (δ , CDCl_3) 13.9 (2 \times q), 22.0 (2 \times t), 26.4 (2 \times t), 27.7 (2 \times t), 31.2 (2 \times t), 50.8 (2 \times q), 121.7 (2 \times d), 123.2 (2 \times s), 131.8 (2 \times s), 140.0 (2 \times d), 142.6 (2 \times d), 143.1 (2 \times s), 165.1 (2 \times s), 178.2 (2 \times s), 186.1 (2 \times d); *Anal.* Calcd for $\text{C}_{28}\text{H}_{30}\text{N}_4\text{O}_6\text{S}_2 \cdot 2\text{CH}_3\text{OH}$: C 55.27, H 5.88, N 8.76, S 9.91. Found: C 55.31, H 5.75, N 8.64, S 9.87.

b) The centrifuged supernatant (3 liters) obtained in a) was subjected to Diaion HP-20 column chromatography (HP-20, 500 ml). After elution with 60~70% aq acetone, elution with 80~100% aq acetone gave a green oily substance, which gave **1a** (45.0 mg) by chromatography with RP-18 and Pegasil ODS followed by recrystallization from aq MeOH as well.

Sodium Borohydride Reduction of **1a**

To a solution of **1a** (27.5 mg) in 4 ml of EtOH was added NaBH_4 (3.4 mg). After the reduction was complete, EtOAc and 4N-HCl were added to the reaction mixture under ice-cooling. The EtOAc layer, after the usual work-up, was concentrated under reduced pressure to a residue (22.5 mg), which was purified by HPLC [Pegasil ODS, 20 i.d. $\times 250 \text{ mm}$, $\text{MeOH} - \text{H}_2\text{O}$, 75:25 (v/v), 10.0 ml/minute]. The combined fractions eluted

between 13~15 minutes were concentrated to give **1b** (15.4 mg) as an oily substance. HR-FAB-MS m/z 587.2001 ($[M+H]^+$, $C_{28}H_{35}N_4O_6S_2$, $\Delta +0.6$ mmu); IR ν_{\max} cm^{-1} (liquid) 3286, 2960, 2933, 2873, 1679, 1626, 1601, 1557, 1463, 1201, 1141, 1115, 1076, 1051, 1016, 991, 936, 841, 799, 758, 721; 1H NMR (δ , $CDCl_3$) 0.93 (6H, t, $J=7.2$ Hz), 1.26 (8H, br s), 1.84 (4H, br s), 2.87 (4H, t, $J=7.2$ Hz), 4.70 (4H, br s), 6.58 (2H, br s), 8.77 (2H, s); ^{13}C NMR (δ , $CDCl_3$) 14.3 (2 \times q), 23.4 (2 \times t), 27.5 (2 \times t), 28.5 (2 \times t), 32.4 (2 \times t), 60.2 (2 \times t), 111.7 (2 \times d), 119.9 (2 \times s), 133.4 (2 \times s), 143.6 (2 \times d), 145.9 (2 \times s), 154.2 (2 \times s), 166.6 (2 \times s), 179.0 (2 \times s); UV λ_{\max} nm (ϵ , EtOH) 360 (sh, 1,600), 315 (sh, 4,800), 253 (20,800), 208 (29,700).

Semicarbazone (**1c**)

To a solution of semicarbazide hydrochloride (11.8 mg) and crystalline sodium acetate (14.0 mg) in 1 ml of distilled water was added a solution of **1a** (20.1 mg) in 3 ml of MeOH, and the mixture was kept at room temperature overnight. After drying under reduced pressure, the residue in a small amount of DMSO was subjected to RP-18 column chromatography, eluted with MeOH-H₂O, 60:40 (v/v) at a flow rate of 4.5 ml/minute. The combined fractions eluted between 25~27 minutes, after concentration under reduced pressure, gave **1c** (5.0 mg), as yellow crystals, which was recrystallized from aq MeOH. mp >200°C (dec.); IR ν_{\max} cm^{-1} (KBr) 3482, 3313, 3152, 2956, 2932, 2870, 1698, 1630, 1592, 1561, 1479, 1414, 1151, 1100, 1047, 935, 850; UV λ_{\max} nm (ϵ , EtOH) 375 (1,400), 286 (40,800); 1H NMR (δ , d_6 -DMSO) 0.88 (6H, t, $J=6.6$ Hz), 1.30 (8H, m), 1.62 (4H, m), 2.65 (4H, t, $J=7.7$ Hz), 6.40 (2H, br s), 6.77 (4H, br s), 7.66 (2H, s), 8.25 (2H, br s), 10.80 (4H, br s).

Methylation of **1a**

A solution of **1a** (40.3 mg) and CH₃I (1.0 ml) in 10 ml of dry acetone was stirred in the presence of K₂CO₃ (40.0 mg) at room temperature for 6 hours. The EtOAc extract (29.0 mg) obtained by the usual work-up, was subjected to HPLC [Pegasil ODS+ODS-H, MeOH-H₂O-TFA, 75:25:0.2 (v/v), 7.5 ml/minute]. The fractions of 37~40 minutes gave **1d** (6.8 mg) as a light yellow amorphous powder. The fractions of 42~43 minutes gave **3a** (1.8 mg) as a light yellow oil. **3a**: HR-EI-MS m/z 320.1181 ($[M]^+$, $C_{16}H_{20}N_2O_3S$, $\Delta -1.4$ mmu (30.1%)), 264.0558 ($C_{12}H_{12}N_2O_3S$, $\Delta -1.1$ mmu (6.5%)), 249.0346 ($C_{11}H_9N_2O_3S$, $\Delta +1.2$ mmu (14.3%)), 221.036 ($C_{10}H_9N_2O_2S$, $\Delta -2.0$ mmu (100%)); IR ν_{\max} cm^{-1} (CHCl₃) 1705, 1587, 1560, 1460, 1198, 1170, 1160, 1115,

1075, 1043, 950, 975, 960, 938, 860, 810; UV λ_{\max} nm (ϵ , EtOH) 330 (sh, 1,200), 300 (sh, 2,500), 247 (11,500), 223 (18,000), 209 (19,500); 1H NMR (δ , $CDCl_3$) 0.92 (3H, t, $J=7.2$ Hz), 1.40 (4H, m), 1.86 (2H, m), 2.40 (3H, s), 2.89 (2H, t, $J=7.2$ Hz), 4.08 (3H, s), 7.30 (1H, s), 8.43 (1H, s), 10.14 (1H, s). **1d**: HR-FAB-MS $[M+H]^+$ m/z 699.2522 ($C_{34}H_{43}N_4O_8S_2$, $\Delta +1.1$ mmu); IR ν_{\max} cm^{-1} (CHCl₃) 3270, 1710 (1723, 1675, 1605, 1465, 1365, 1170, 1115); UV λ_{\max} nm (ϵ , EtOH) 360 (sh, 1,500), 315 (sh, 2,900), 250 (15,000), 210 (19,200); 1H NMR (δ , $CDCl_3$) 0.91 (6H, t, $J=6.9$ Hz), 1.36 (8H, m), 1.78 (4H, m), 2.20 (6H, s), 2.79 (4H, t, $J=7.7$ Hz), 2.95 (4H, d, $J=5.7$ Hz), 5.15 (2H, m), 6.55 (2H, s), 8.61 (2H, s); ^{13}C NMR (δ , $CDCl_3$) 14.6 (2 \times q), 22.9 (2 \times t), 27.0 (2 \times t), 28.5 (2 \times t), 31.3 (2 \times q), 31.9 (2 \times t), 50.3 (2 \times t), 66.1 (2 \times d), 111.0 (2 \times d), 119.3 (2 \times s), 132.9 (2 \times s), 142.3 (2 \times d), 144.6 (2 \times s), 154.8 (2 \times s), 165.9 (2 \times s), 178.1 (2 \times s), 207.9 (2 \times s).

According to the method for preparation of **3a**, **1d** (47.0 mg) gave **3b** (8.3 mg) by HPLC [Pegasil ODS, 20 i.d. \times 250 mm, MeOH-H₂O-TFA, 75:25:0.2 (v/v), 10 ml/minute, 24~25 minutes]. EI-MS m/z 378 $[M]^+$, 360, 279, 261, 221; 1H NMR (δ , CD_3OD) 0.93 (3H, t, $J=7.2$ Hz), 1.39 (4H, m), 1.83 (2H, m), 2.22 (3H, s), 2.29 (3H, s), 2.85 (1H, dd, $J=9.3$, 16.5 Hz), 2.87 (2H, t, $J=7.3$ Hz), 3.07 (1H, dd, $J=3.9$, 16.5 Hz), 4.02 (3H, s), 5.19 (1H, dd, $J=3.9$, 9.3 Hz), 7.17 (1H, s), 8.51 (1H, s).

Desulfurization of **1a**

Raney-Ni (5 ml, Aldrich) was washed with EtOH, and was suspended in 4 ml of EtOH. To this suspension, was added a solution of **1a** (10 mg) in 1 ml of EtOH, and the mixture was heated under reflux for 2 hours. The filtrate obtained by filtration under N₂ was dried under reduced pressure. The residue (9.0 mg) was purified to **2a** (4.2 mg) as a colorless amorphous powder by TLC [MeOH-CHCl₃, 15:85 (v/v)]. IR ν_{\max} cm^{-1} (CHCl₃) 3258, 2962, 2930, 2874, 2858, 1642, 1600, 1539, 1505, 1464, 1383, 1261; UV λ_{\max} nm (ϵ , EtOH) 258 (9,900).

Desulfurization of **1d**

According to the reaction of **1a**, desulfurization was carried out using **1d** (66.9 mg) and Raney-Ni (8 ml). The residue was purified to **2b** (1.3 mg) as a colorless oil by TLC [MeOH-CHCl₃, 15:85 (v/v)] followed by HPLC [Pegasil ODS, 10 i.d. \times 250 mm, MeOH-H₂O-TFA, 60:40:0.2 (v/v), 2 ml/minute]. IR ν_{\max} cm^{-1} (CHCl₃) 3258, 2962, 2930, 2858, 1641, 1600, 1539, 1505, 1464, 1383, 1347, 1261.

Benzoylation of **1d**

A mixture of **1d** (48 mg), benzyl chloride (0.5 ml), and K_2CO_3 (48 mg) in 20 ml of dry acetone was heated under reflux for 2 hours. The reaction mixture (28 mg), after the usual treatment, was subjected to HPLC [Pegasil ODS, 20 i.d. \times 250 mm, MeOH-H₂O-TFA, 75:25:0.2 (v/v), 10 ml/minute] to give **5c** (5.9 mg) as a colorless oil. HR-EI-MS m/z 440.1172 {[M]⁺, C₂₄H₂₈N₂O₄S, Δ +0.2 mmu (40%)}, 422.1651 [C₂₄H₂₆N₂O₃S, Δ -1.3 mmu (60%)], 382.1332 [C₂₁H₂₂N₂O₃S, Δ -1.9 mmu (35.6%)], 341.0953 [C₁₈H₁₇N₂O₃S, Δ -0.7 mmu (39.5%)], 323.0855 [C₁₈H₁₅N₂O₂S, Δ +0.1 mmu (100%)], 283.0562 [C₁₅H₁₁N₂O₂S, Δ +2.1 mmu (66.7%)]; IR ν_{max} cm⁻¹ (liquid) 3311, 1716, 1620, 1602, 1494, 1467, 1428, 1360, 1169, 1109, 1054, 989, 856, 754, 701; ¹H NMR (δ , CD₃OD) 0.93 (3H, t, $J=7.1$ Hz), 1.35 (4H, m), 1.76 (2H, m), 2.02 (3H, s), 2.78 (2H, t, $J=7.4$ Hz), 2.96 (2H, d, $J=5.2$ Hz), 4.05 (1H, d, $J=12.8$ Hz), 4.07 (1H, d, $J=12.8$ Hz), 5.15 (1H, t, $J=5.2$ Hz), 6.38 (1H, s), 7.07 (5H, m), 8.72 (1H, s).

Preparation of h-ECE

The preparation and the inhibition assays of all enzymes were conducted at 4°C unless otherwise mentioned. h-ECE was prepared according to Okada's method.²⁶ Human umbilical vein endothelial cells were obtained from Kurashiki Bouseki (Tokyo, Japan). They were grown in M-199 medium supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 mM streptomycin, 2.5 μ g/ml fungizone, 10 U/ml heparin, and 20 μ g/ml endothelial mitogen. Cells were incubated at 37°C in 5% CO₂ and 95% air. Confluent cells were washed with phosphate-buffered saline (PBS) after removal of the medium, and were scraped by cell scrapers. The cell suspension was centrifuged at 3,000 rpm for 10 minutes at 4°C, and the pellets were homogenized by a polytron homogenizer in 30 ml of 50 mM phosphate buffer containing 1 mM E-64, 0.4 mM leupeptin, and 10 μ M pepstaine A. The homogenates were centrifuged at 1,600 $\times g$ for 10 minutes, and the supernatant was centrifuged at 105,000 $\times g$ for 30 minutes. The pellets were suspended in 5 ml of the above buffer solution containing 0.5% Triton X-100, and the suspension was then centrifuged at 105,000 $\times g$ for 40 minutes. The supernatant thus obtained was stored at -80°C and used as a h-ECE fraction.

Inhibition of h-ECE

Test samples, prepared by dissolving MeOH or DMSO solutions of inhibitors into 130 μ l of PBS, were added to

each well of 96-well microplates fixed with anti-ET-1¹⁵⁻²¹ rabbit IgG. To each well were added 20 nM big ET-1 and 10 μ l of the ECE fraction, and microplates containing the mixture were incubated at 37°C for 2 hours. The plates were next cooled to 4°C to avoid the proteolysis of big ET-1 and were then left at 4°C for 30 minutes to stabilize the binding of ET-1 with anti-ET-1¹⁵⁻²¹ rabbit IgG. After washing the microplates with PBS, 100 μ l (0.2 μ g/well) of peroxidase-labeled anti-ET-1¹⁵⁻²¹ rabbit IgG (Fab')-HRP was added to each well, and was incubated at 37°C for 30 minutes. After removal of unreacted anti-ET-1¹⁵⁻²¹ rabbit IgG (Fab')-HRP by washing with PBS, the formed IgG-(ET-1)-IgG (Fab')-HRP complexes were quantified by photometric analysis of its peroxidase activity as follows:

The peroxidase enzyme reaction was started by mixing IgG-(ET-1)-IgG (Fab')-HRP complex with 100 μ l of a solution prepared from 7 μ l of 30% hydrogen peroxide and *o*-phenylenediamine (13 mg) in 7 ml of 0.2 M potassium phosphate-citric acid buffer (pH 5.1), followed by incubation at room temperature for 20 minutes, and was then quenched by addition of 100 μ l of 1 N H₂SO₄ to each reaction mixture. The optical density of each well at λ 490 nm was measured by a microplate reader. Each amount of ET-1 was calculated according to the standard curve. Each ECE inhibition (%) was calculated according to the equation: ECE inhibition (%) = [1 - (A/B)] \times 100, where A = an amount of ET-1 in the presence of inhibitors and B = an amount of ET-1 in the absence of inhibitors as a control.

Preparation of Neutral Endopeptidase (NEP)

According to the method of MALFROY *et al.*,²¹ spleens extracted from 7 SD rats were homogenized (Polytron) in 200 ml of 5 mM Tris-HCl buffer (pH 7.4) containing 125 mM D-mannitol and 12 mM MgCl₂. The supernatant obtained by centrifugation of the homogenates at 1,000 $\times g$ for 15 minutes, was recentrifuged at 7,000 $\times g$ for 2 hours. The suspension of the pellets in 2.5 mM Tris-HCl buffer was homogenized, and then incubated at 40°C for 30 minutes. The supernatant, obtained by centrifugation of the homogenates at 1,000 $\times g$ for 15 minutes, was recentrifuged at 2 hours to give pellets as a crude membrane fraction. The crude membrane fraction was stirred in 40 ml of 5 mM HEPES buffer (pH 7.4) containing 1% Triton X-100 for 16 hours, and the soluble fraction was obtained as pellets by centrifugation at 80,000 $\times g$ for 2 hours. They were stirred in 15 ml of 5 mM HEPES buffer containing 5% Triton X-100 for 16 hours followed by centrifugation at 80,000 $\times g$ for 2 hours

to give the supernatant as a soluble NEP fraction.

Inhibition of Neutral Endopeptidase

According to the method of FRENCH *et al.*,²⁴⁾ the mixture of 20 μ l of a DMSO solution of an inhibitor and 40 μ l of the NEP fraction was incubated at 25°C for 6 minutes in 1.9 ml of 5 mM HEPES buffer containing 20 μ l of 4 mM *N*-dansyl-D-Ala-Gly-(*p*-nitrophenyl)-Gly-OH (Sigma) and 20 μ l of 50 mM ascorbic acid. The amount of each enzymatically digested peptide was measured as a concentration of corresponding dansyl chloride by a fluorescence emission spectrophotometer. The increased fluorescence was calculated as its reaction rate. The NEP inhibitory activities were calculated according to the equation below.

NEP inhibition (%) = $[1 - A/B] \times 100$, where A = reaction rate in the presence of an inhibitor and B = reaction rate in the absence of an inhibitor.

Inhibition of Type-I Collagenase

Assay was carried out using a commercially available collagen I measuring kit (Yagai), measuring a decomposition product from FITC-labeled collagen I.

Inhibition of Type-IV Collagenase

Assay was carried out using a commercially available collagen IV measuring kit (Yagai), measuring a decomposition product from FITC-labeled collagen IV.

ET_A Receptor Binding Assay

ET_A receptor binding activity was measured using a commercially available ET_A receptor binding assay kit (NENQUEST Drug Discovery System: Endothelin-1 Receptor, Daiichi Pure Chemicals), in which membrane fractions of A10 cell, derived from the thoracic aorta of embryonic rat, and [¹²⁵I]ET-1 were contained. The binding inhibitory activities were calculated as usual.

Preparation of ET_B Receptor

ET_B receptor-containing membrane fractions were prepared from bovine cerebellum homogenates according to the slightly modified Miyata's method.²⁵⁾ A bovine brain was purchased from a local slaughter house and cerebellum was obtained. It was homogenized in 100 ml of 10 mM Tris-HCl buffer containing 0.25 M sucrose and 0.1 mM EDTA (pH 7.5) and the homogenate was centrifuged at 10,000 $\times g$ at 4°C for 20 minutes. The supernatant was recentrifuged at 100,000 $\times g$ at 4°C for 1 hour. The pellets were suspended in 25 ml of 50 mM Tris-HCl buffer containing 100 mM NaCl, 5 mM MgCl₂, 1.5 μ g/ml

PMSF, 120 μ g/ml bacitracin, 12 μ g/ml leupeptin, 6 μ g/ml chymostatin, and 0.1% BSA (pH 7.5) with a glass-glass homogenizer. The suspension was used as an ET_B receptor-containing membrane fraction.

ET_B Receptor Binding Assay

ET_B receptor binding assay was thus carried out using [¹²⁵I]ET-1 and test samples according to basically the same protocol as ET_A receptor binding assay. The binding inhibitory activities were calculated as usual.

References

- 1) RUBANY, G. M. & M. A. POLOKOFF: Endothelin: Molecular biology, biochemistry, pharmacology, physiology, and pathophysiology. *Pharmacol. Rev.* 46: 325~415, 1994
- 2) TAKAHASHI, M.; Y. MATSUSHITA, Y. IJIMA & K. TANZAWA: Purification and characterization of endothelin-converting enzyme from rat lung. *J. Biol. Chem.* 268: 21394~21398, 1993
- 3) SHIMADA, K.; M. TAKAHASHI & K. TANZAWA: Cloning and functional expression of endothelin-converting enzyme from rat endothelial cells. *J. Biol. Chem.* 269: 18275~18278, 1994
- 4) OHNAKA, K.; R. TAHASHI, M. NISHIKAWA, M. HAJI & H. NUMATA: Purification and characterization of a phosphoramidon-sensitive endothelin-converting enzyme in porcine aorta endothelium. *J. Biol. Chem.* 268: 26759~26766, 1993
- 5) MATSUMURA, Y.; Y. TSUKAHARA, K. KUNINOBU, M. TAKAOKA & S. MORIMOTO: Phosphoramidon-sensitive endothelin-converting enzyme in vascular endothelial cells converts big endothelin-1 and big endothelin-3 to their mature forms. *FEBS.* 305: 86~90, 1992
- 6) CHENG, X.-M.; S. S. NIKAM & A. M. DOHERTY: Development of agents to modulate the effects of endothelin. *Current Med. Chem.* 1: 271~312, 1994
- 7) TSURUMI, Y.; H. UEDA, K. HAYASHI, S. TAKASE, M. NISHIKAWA, S. KIYOTO & M. OKUHARA: 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. *J. Antibiotics* 48: 1066~1072, 1995
- 8) YOSHIMURA, S.; Y. TSURUMI, S. TAKASE & M. OKUHARA: WS75624 A and B, new endothelin converting enzyme inhibitors isolated from *Saccharothrix* sp. No. 75624 II. Structure elucidation of WS75624 A and B. *J. Antibiotics* 48: 1073~1075, 1995
- 9) TSURUMI, Y.; N. OHHATA, T. IWAMOTO, N. SHIGEMATSU, K. SAKAMOTO, M. NISHIKAWA, S. KIYOTO & M. OKUHARA: WS79089A, B, and C, new endothelin converting enzyme inhibitors isolated from *Streptosporangium roseum*. No. 79089 Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *J. Antibiotics* 47: 619~629, 1994
- 10) MIKAMI, Y. & T. SUZUKI: Novel microbial inhibitors of angiotensin-converting enzyme, aspergillomarasmies A and B. *Agri. Biol. Chem.* 47: 2693~2695, 1983
- 11) MATSUURA, A.; H. OKUMURA, R. ASAKURA, M. ASHIZAWA, M. TAKAHASHI, F. KOBAYASHI, N. ASHIKAWA

- & K. ARAI: Pharmacological profiles of aspergillomarasmies as endothelin converting enzyme inhibitors. *Jpn. J. Pharmacol.* 63: 187~193, 1993
- 12) TROTSENKO, Y. A.; N. V. DORONIA & P. HIRSCH: Genus *Blastobacter* Zavarzin 1961 in BERGEY's Manual of Systematic Bacteriology. 3: 1963~1968, The Williams & Wilkins Co., Baltimore, 1989
- 13) YOKOTA, A.; M. AKAGAWA-MATSUSHITA, A. HIRAISHI, Y. KATAYAMA, T. URAKAMI & K. YAMASATO: Distribution of quinone system in microorganisms: Gram-negative eubacteria. *Bull. Jpn. Fed. Cult. Coll.* 8: 136~171, 1992
- 14) WITANOWSKI, M.; L. STEFANIAK & G. A. WEBB: Nitrogen NMR Spectroscopy. *Ann. Rept. on NMR Spectroscopy*. Vol. 7, Ed., G. A. WEBB, pp. 193~195, Academic Press, 1977
- 15) BATTERHAM, T. J.: NMR Spectra of simple heterocycles. pp. 47~52, John Wiley & Sons, New York, 1973
- 16) SHAMA, M. & D. M. HINDENLAG: Carbon-13 NMR Shift Assignments of Amines and Alkaloids. pp. 18~19, Plenum Press, New York, 1979
- 17) KOZIKOWSKI, A. P. & A. AMES: Oxazoles in organic chemistry. 2. Application to the syntheses of benzylquinoline alkaloids. *J. Org. Chem.* 45: 2548~2550, 1980
- 18) HAWTHORNE, J. O. & H. W. MYRON: Decarbonylation of aromatic aldehydes. *J. Org. Chem.* 25: 2215~2216, 1960
- 19) OHNO, K. & T. TSUJI: Organic syntheses by means of novel metal compounds XXXV. Novel decarbonylation reactions of aldehydes and acyl halides using rhodium complexes. *J. Am. Chem. Soc.* 90: 99~107, 1968
- 20) MARYANOFF, C. A.: Heterocyclic compounds, oxazoles, spectroscopic properties of oxazoles, 45: pp. 343~360, Interscience Publication, John Wiley & Sons, 1986
- 21) FIELD, L.: Disulfides and polysulfides. Organic chemistry of sulfur. Ed., S. OAE, pp. 362~364, Plenum Press, New York and London, 1977
- 22) MAFROY, B. P. & J.-C. SCHWARTZ: Enkephalinase from rat kidney: Purification, characterization, and study of substrate specificity. *J. Biol. Chem.* 259: 14365~14370, 1984
- 23) FRENCH, J. F.; G. A. FLYNN, E. L. GIROUX, S. MEDHI, B. ANDERSON, D. C. BEACH, J. R. KOEHL & R. C. DAGE: Characterization of a dual inhibitor of angiotensin I-converting enzyme and neutral endopeptidase. *J. Pharmacol. Exp. Ther.* 268: 180~186, 1994
- 24) NAGAI, H.; H. HORI, S. HATTORI, Y. SUNADA, K. TERATO. R. HASHIDA & K. MIYAMOTO: A micro-assay method of collagenase activity and its application in the study of collagen metabolism in pathological tissues. Part I Assay of collagenase activity using FITC-labeled collagen as substrate. *Jpn. J. Inflammation* 4: 123~130, 1984
- 25) MIYATA, S.; N. OHHATA, H. MURAI, Y. MASUI, M. EZAKI, S. TAKASE, M. NISHIKAWA, S. KIYOTO, M. OKUHARA & M. KOHSAKA: WS009 A and B, new endothelin receptor antagonists isolated from *Streptomyces* sp. No. 89009. I. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *J. Antibiotics* 45: 1029~1040, 1992
- 26) OKADA, K.; Y. MIYAZAKI, J. TANAKA, K. MIYASHITA, T. YAMAKI & M. YANO: Conversion of big endothelin-1 by membrane-bound metalloendopeptidase in cultured bovine endothelial cells. *Biol. Biophys. Res. Comm.* 171: 1192~1198, 1990