ENDOTHELIN-BINDING INHIBITORS, BE-18257A AND BE-18257B

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

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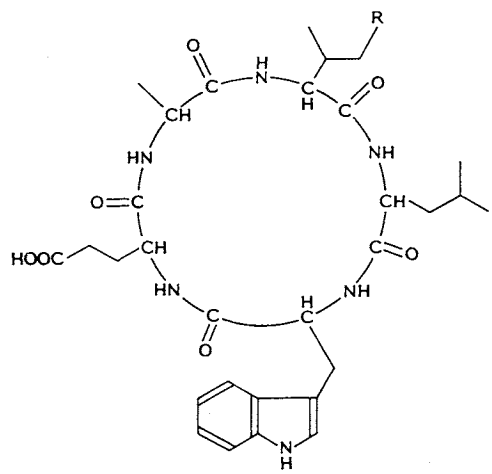
Two endothelin (ET)-binding inhibitors, BE-18257A and BE-18257B, which antagonized ^{125}I-ET-1 binding to a porcine aortic smooth muscle membrane, were isolated from the mycelium of a strain of *Streptomyces misakiensis*. These binding inhibitors were extracted with methanol from mycelium and purified by HPLC.

In the course of our screening program for endothelin (ET)-binding inhibitors from the culture broths of microorganisms, strain BA18257 was found to produce two active components, designated BE-18257A and BE-18257B. BE-18257A and B were found to be a novel cyclic pentapeptides as shown in Fig. 1. This paper describes the taxonomy of the producing strain and the fermentation, isolation, physico-chemical and biological properties of BE-18257A and B. The structural elucidation studies of these compounds are described in an accompanying paper[1].

Taxonomy of the Producing Organism

The producing organism, strain BA18257, was isolated from a soil sample collected in Nukada-cho, Aichi Prefecture, Japan. For the taxonomic characterization of BA18257, the methods and media recommended by the International Streptomyces Project (ISP)[2] and WAKSMAN[3] were used. The vegetative mycelium developed well without fragmentation. The strain formed straight spore chains in monopodial branching on the aerial mycelium. The spore chains have more than fifty spores per chain. The spores are cylindrical in shape, ranging from 0.5×0.8 to $0.5\times1.0\,\mu$m in size and have smooth surfaces. The cultural characteristics on various agar media are shown in Table 1. The strain formed an abundant amount of pale brownish gray powdery mycelium. The physiological properties and the utilization of carbon sources of strain BA18257 are shown in Tables 2 and 3,

Fig. 1. Structures of BE-18257A (**1**) and B (**2**).



BE-18257A (**1**) R=H
BE-18257B (**2**) R=CH_3

Table 1. Cultural characteristics of strain BA18257.

Agar medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP-2)	Good	Abundant Pale brownish gray Powdery	Reddish brown	Yellow orange (faint)
Oatmeal agar (ISP-3)	Good	Abundant Pale brownish gray Powdery	Reddish brown	Yellowish brown
Inorganic salts-starch agar (ISP-4)	Good	Abundant Pale brownish gray Powdery	Brown	None
Glycerol-asparagine agar (ISP-5)	Moderate	Scant	Brown	None
Peptone-yeast extract-iron agar (ISP-6)	Moderate	None	Grayish brown	Yellowish brown
Tyrosine agar (ISP-7)	Good	Abundant Pale brownish gray Powdery	Blackish brown	None
Nutrient agar	Good	None	Pale yellowish white	None
Sucrose-nitrate agar	Poor	Scant	Colorless	None
Glucose-asparagine agar	Moderate	Scant	Brown	None

respectively. The hydrolyzed cell wall contained L,L-diaminopimelic acid and glycine. Accordingly, the cell wall of the strain was classified as type I.

Table 2. Physiological properties of strain BA18257.

Coagulation of milk	Negative
Peptonization of milk	Positive
Liquefaction of gelatin	Negative
Melanin formation	Negative
Hydrolysis of starch	Positive
NaCl tolerance	4%
Temperature range for growth	12~33°C

Based on the taxonomic properties described above, strain BA18257 belongs to the genus *Streptomyces*. The strain was compared with the published descriptions of various *Streptomyces* species, and was similar to *Streptomyces aburaviensis* and *Streptomyces misakiensis*. The results of comparison of the strain BA18257 and the type strains of these two species (JCM 4170 and JCM 4062) are summarized in Table 3. As a result, good agreement was obtained between the strain BA18257 and *S. misakiensis* except for utilization of sucrose and D-fructose. Therefore, strain BA18257 was identified as a strain of *S. misakiensis*, and was named *S. misakiensis* BA18257.

This strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession No. FERM P-10751.

Fermentation

Spores of strain BA18257 were inoculated into 100 ml of a medium (pH 6.7) composed of glycerol 2.0%, maltose syrup 1.0%, meat extract 0.2%, cotton seed meal 0.5%, yeast extract 0.1%, wheat germ meal 0.5%, $MgSO_4 \cdot 7H_2O$ 0.2%, NaCl 0.2%, $CaCO_3$ 0.2%, KH_2PO_4 0.1%, $(NH_4)_2SO_4$ 0.1%, $Fe_2SO_4 \cdot 7H_2O$ 0.001%, and $ZnSO_4 \cdot 7H_2O$ 0.001% in a 500-ml Erlenmeyer flask, and cultured at 28°C for 3 days on a rotary shaker. Twenty ml of the seed culture were transferred into each of five 5-liter Erlenmeyer flasks containing 1 liter of the above medium and the fermentation was conducted at 28°C for 4 days on a rotary shaker.

Isolation

The mycelium was obtained by filtration from the whole broth (*ca.* 5 liters) and washed with 500 ml of water. This mycelium was extracted with 2.5 liters of methanol and the extract (*ca.* 2.4 liters) was obtained

Table 3. Comparison of taxonomic characteristics of strain BA18257 with *Streptomyces aburaviensis* and *Streptomyces misakiensis*.

	BA18257	*S. aburaviensis* (JCM 4170)	*S. misakiensis* (JCM 4062)
Spore chain morphology	*Rectiflexibiles*	*Rectiflexibiles*	*Rectiflexibiles*
Spore surface	Smooth	Smooth	Smooth
Aerial mass color	Pale brownish gray to gray	Grayish white to gray	Light gray to gray
Color of reverse	Reddish brown to blackish brown	Pale yellow to yellow orange	Pale yellowish brown to blackish brown
Soluble pigment	None to yellowish brown	None	None to brown
Melanoid formation (ISP-7)	Negative	Negative	Negative
Growth at 45°C	Negative	Negative	Negative
Tolerance to NaCl, 7%	Negative	Positive	Negative
Hydrolysis of starch	Positive	Positive	Positive
Carbon source utilization:			
D-Glucose	+	+	+
D-Xylose	−	−	−
L-Arabinose	−	−	−
L-Rhamnose	−	−	−
D-Fructose	−	+	+
D-Galactose	+	−	+
Raffinose	+	+	+
D-Mannitol	−	−	−
Inositol	−	−	−
Salicin	+	−	+
Sucrose	−	+	+

JCM: Japan Collection of Microorganisms, RIKEN.
+: Utilized.

by filtration. The filtrate was concentrated *in vacuo* to about 300 ml and 300 ml of water was added. After adjusting the pH to 8.5 with 1 N NaOH, this solution was extracted with 600 ml of ethyl acetate. The pH of the aqueous layer was adjusted to 3.0 with 1 N HCl and the aqueous layer was extracted with 700 ml of ethyl acetate. The ethyl acetate extract was evaporated *in vacuo* and 730 mg of crude material was obtained. Sixty mg of the crude material was dissolved in 2 ml of *N*,*N*'-dimethylformamide and subjected to HPLC (Inertsil ODS, 16.7 × 300 mm, mobile phase: 42% acetonitrile containing 0.2% trifluoroacetic acid). BE-18257A (15 mg) and BE-18257B (34 mg) were obtained from two separate fractions.

Physico-chemical Properties

BE-18257A and B were obtained as white powder. These inhibitors are soluble in dimethyl sulfoxide, sparingly soluble in methanol and alkaline water and hardly soluble in neutral water. The IR spectra of BE-18257A and B showing the characteristic two amide bands are shown in Figs. 2 and 3, respectively. ^{1}H NMR spectra of BE-18257A and B are shown in Figs. 4 and 5, respectively. BE-18257A and B are stable in the range pH 2.0 to 9.0.

BE-18257A possessed the following physical constants: $[\alpha]_D^{20}$ −10.0° (*c* 1.0, DMSO); UV λ_{max}^{MeOH} nm (ε) 222 (37,300), 223 (5,800), 282 (6,200), 290 (5,400); FAB-MS *m/z* 599 $(M+H)^+$.

BE-18257B possessed the following physical constants: $[\alpha]_D^{20}$ −6.9° (*c* 1.0, DMSO); UV λ_{max}^{MeOH} nm (ε) 222 (40,600), 273 (6,100), 282 (6,500), 290 (5,700); FAB-MS *m/z* 613 $(M+H)^+$.

Biological Properties

ET-binding inhibitory activities of BE-18257A and B were determined as follows. The smooth muscle

Fig. 2. IR spectrum of BE-18257A in KBr.

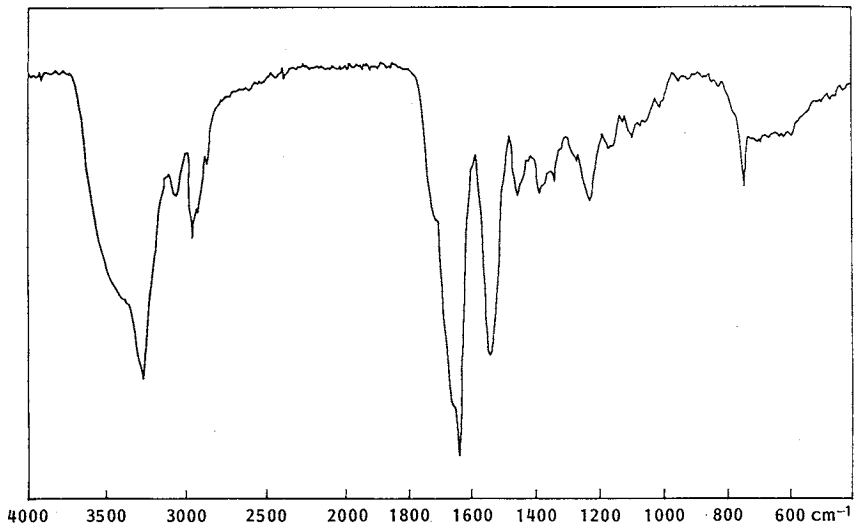
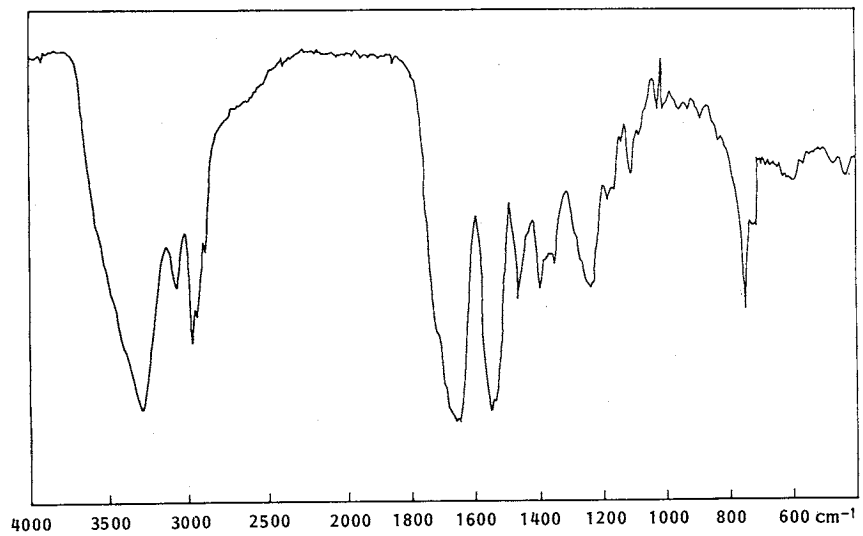


Fig. 3. IR spectrum of BE-18257B in KBr.



layer of the porcine aorta was homogenized in 10 volumes of 10 mM MOPS (pH 7.4) containing 20% sucrose with a Polytron (setting 7 for 30 seconds × 2) at 4°C. The homogenates were centrifuged at 1,000 × g for 15 minutes, and the resulting supernatants were centrifuged at 10,000 × g for 15 minutes. The supernatants were then centrifuged at 90,000 × g for 40 minutes. The pellets were homogenized in 5 mM HEPES-Tris (pH 7.4). The resulting homogenates were incubated at 25°C with ^{125}I-ET-1 in the presence and absence of BE-18257A and B in 50 mM Tris-HCl buffer (pH 7.4) containing $CaCl_2$ 10 μM, $MgCl_2$ 10 μM, phenylmethylsulfonyl fluoride (PMSF) 0.1 mM, pepstatin A 1 μM, leupeptin 2 μM, 1,10-phenanthroline 1 mM and bovine serum albumin (BSA) 0.1%. After 4 hours of incubation, 2 ml of cold 5 mM HEPES-Tris

Fig. 4. ^{1}H NMR spectrum of BE-18257A in DMSO-d_6 (300 MHz).

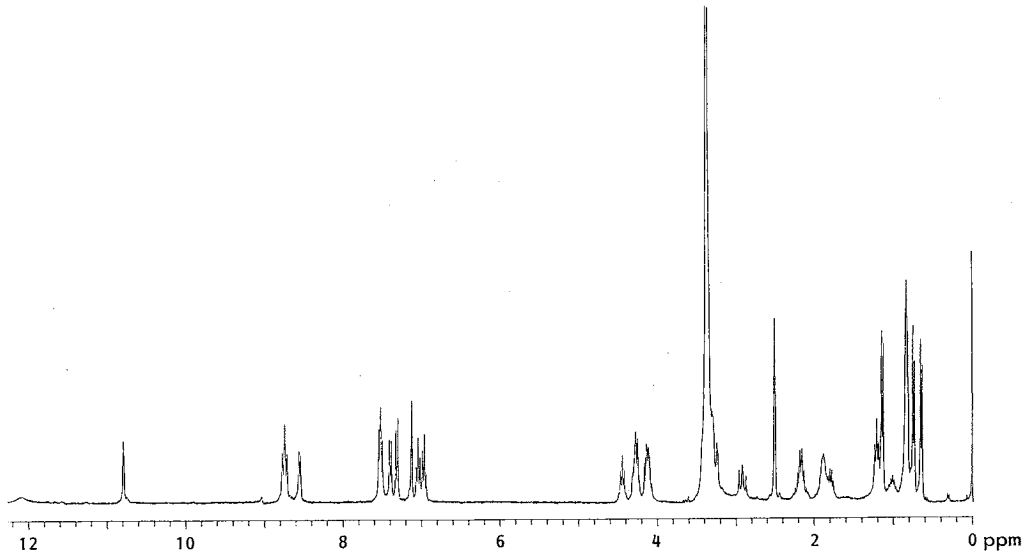
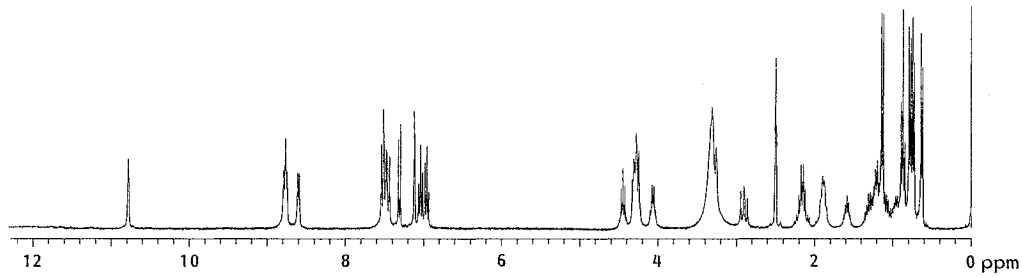


Fig. 5. ^{1}H NMR spectrum of BE-18257B in DMSO-d_6 (300 MHz).



(pH 7.4) containing 0.3% BSA (Buffer A) was added to the mixture, which was then rapidly filtered through Whatman GF/C glass fiber filters. After washing the filters with buffer A, the radioactivity on the filter was determined by a gamma counter. Nonspecific binding was defined by adding 200 nM ET-1 to the assay mixture. BE-18257A and B inhibited ^{125}I-ET-1 binding in a concentration-related manner. The concentrations required for IC_{50} were 3.0 μM for BE-18257A and 1.4 μM for BE-18257B. In addition, no acute toxicity (100 mg/kg, ip) of BE-18257A and B was observed in mice.

Discussion

The endothelium is one of the important regulators of vascular tone. Endothelium-derived relaxing factor (EDRF)[4] and prostacycline[5] released from endothelial cells had been known to induce vasodilation. Recently, an endothelium-derived vasoconstrictor factor (ET) was discovered[6]. The exact physiological and pathophysiological roles of ET are as yet unclear. In order to elucidate the precise function of ET, antagonists of ET are desired. Thus, we started an ET-binding inhibitor screening program and discovered novel cyclic pentapeptides BE-18257A and B which inhibit ^{125}I-ET-1 binding in porcine aortic smooth muscle membrane. These compounds are probably useful tools to clarify ET biology. Furthermore, the chemical structures elucidated in an accompanying paper may give us valuable information for the design of new ET-antagonist leads.

References

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