Stachybocins, Novel Endothelin Receptor Antagonists, Produced by *Stachybotrys* sp. M6222

I. Taxonomy, Fermentation, Isolation and Characterization

MASATO NAKAMURA, YASUNOBU ITO, KIYOSHI OGAWA, YUJI MICHISUJI, SHIN-ICHI SATO, MASAKI TAKADA, MITSUO HAYASHI, SATOSHI YAGINUMA and SYUJI YAMAMOTO

Institute for Life Science Research, Asahi Chemical Industry Co., Ltd., Ohito-cho, Shizuoka 410-23, Japan

(Received for publication July 5, 1995)

Stachybocins A, B and C, novel endothelin (ET) receptor antagonists, were isolated from the culture filtrate of *Stachybotrys* sp. M6222. They were extracted with ethyl acetate and then purified by alumina and silica gel column chromatographies. The molecular formulae of stachybocins were determined to be $C_{52}H_{70}N_2O_{10}$ (stachybocin A) and $C_{52}H_{70}N_2O_{11}$ (stachybocins B and C). It was supposed that they consisted of spirobenzofuran and terpene units from NMR spectra. They showed the inhibitory activity of ^{125}I-ET-1 binding to rat ET_A, human ET_A and human ET_B receptors.

Endothelin (ET) is a potent and long-lasting vasoconstrictor peptide that is secreted by endothelial cells[1] and three isopeptides of ET (ET-1, 2 and 3) have been characterized[2]. At least two receptor subtypes are known that are classified according to their ligand selectivity into ET_A (ET-1 = ET-2 > ET-3) and ET_B (ET-1 = ET-2 = ET-3)[3,4]. Because of its activity, ET seems to be involved in some cardiovascular diseases. Therefore ET receptor antagonists can be potential candidates for treatment of some cardiovascular diseases caused by ET.

In the course of our screening for ET antagonists from the culture broths of microorganisms, we found new compounds stachybocins (STBs) A, B and C from the fermentation broth of *Stachybotrys* sp. M6222.

In this paper, we describe the taxonomy of the producing organism and its fermentation, as well as the isolation, physico-chemical properties and biological properties of STBs A, B and C. The structural elucidation studies of these compounds are described in the succeeding paper[5].

Materials and Methods

Microorganism

Strain M6222 was isolated from a soil sample collected in Sakaigawa-mura, Yamanashi Prefecture, Japan. The soil was dried, suspended in water, diluted and plated on a potato dextrose agar medium. The plates were incubated for 2 weeks at 24°C. The isolated strain was stored as freeze dried sample at 4°C or as freezed sample with potato dextrose agar medium containing 10% glycerol at −80°C.

Taxonomic Characterization

The methods described in JONG and DAVIS[6] were employed principally for this taxonomic study. Morphological observation of this strain M6222 were carried out by a light and microscopies using cultures grown at 25°C for 7~14 days on potato dextrose agar.

Fermentation

A loopful of *Stachybotrys* sp. M6222 was inoculated into four 500-ml erlenmeyer flasks containing a seed medium (400 ml) consisting of glucose 1%, dextrine 1%, yeast extract 0.5%, casein hydrolysate 0.5%, celite 1% and $CaCO_3$ 0.1% (pH 6.5), the flasks were incubated at 26°C for 3 days on a rotary shaker (200 rpm, 6.8 cm-throw).

The seed culture (4 ml) was inoculated into one hundred 500-ml Erlenmeyer flasks each containing a production medium consisting of glucose 2%, peptone 1%, corn steep liquor 1%, KH_2PO_4 0.2%, $MgSO_4 \cdot 7H_2O$ 0.1% and celite 1% (pH 6.5), and the cultivation was carried out at 26°C for 3 days with agitation at 200 rpm.

The potency of STBs was monitored by HPLC using a JASCO 880-PU pump and a JASCO 875-UV UV detector. Shodex C18 column (4.6 × 150 mm) was used with flow rate of 1 ml/minute at 30°C. The mobile phase used was CH_3CN-0.01 M (pH 7.6) phosphate buffer (50 : 50), and the detector wavelength was set at 218 nm.

Mycelial growth was expressed as packed cell volume obtained after centrifugation of 10 ml of the culture broth at 3,000 rpm for 10 minutes.

Physico-chemical Properties

UV spectra were recorded with a Shimadzu UV-365 spectrometer. IR spectra were measured on a JEOL Diamond-20 FT-IR spectrometer. Optical rotations were measured on a Horiba SEPA-200 polarimeter. High resolution FAB-MS spectra were measured on a JEOL JMS-SX102 mass spectrometer. ^{1}H and ^{13}C NMR spectra were measured on a JEOL JNM-GSX-400 NMR spectrometer at 400 and 100 MHz, respectively, using TMS as an internal standard.

ET Receptor Assay

(1) Crude Receptor Membrane Preparation

(i) Rat ET_A

Rat fetal aortic smooth muscle cells (A-10, ATCC # CRT 1476) were cultured at 37°C in T-75 flasks under humidified 95% air / 5% CO_2 in DULBECCO's modified EAGLE's medium (DME) containing 10% fetal calf serum (Gibco Labs), 100 U/ml penicillin G and 100 μg/ml streptomycin. Following the attainment of confluence (5 days), the cells were washed with phosphate buffered saline (PBS) buffer (0.02% KCl, 0.02% KH_2PO_4, 0.29% $Na_2HPO_4 \cdot 12H_2O$ and 0.8% NaCl; pH 7.4), scrapped and collected into assay buffer (50 mM Tris-HCl, 10 μM $CaCl_2$, 10 μM $MgCl_2$, 100 μM phenylmethylsulfonyl fluoride, 2 μM leupeptin, 1 μM phosphoramidon and 0.1% bovine serum albumin; pH 7.4). After storage at −80°C for 20 ~ 24 hours, the cells were thawed, homogenized with a biotron homogenizer and centrifuged at 31,000 rpm for one hour at 4°C. The supernatant was discarded, and the membranes were resuspended in the above assay buffer and stored at −80°C until use.

(ii) Human ET_A and ET_B

Human ET_A (h-ET_A) and ET_B (h-ET_B) receptors were cloned and expressed in COS-1 cells as described[7,8]. Human ET_A and ET_B receptor genes were obtained by reverse transcription and polymerase chain reaction (PCR) methods[7]. These genes were subcloned into pUC118 or pUC119, and these genes were cloned into pSVMTU-BHX vector for transient expression in COS-1 cells by DEAE-dextran method[8]. COS-1 cells culture and membranes preparation were carried out by the same method as rat membranes.

(2) [^{125}I]-ET-1 binding assay

1.35×10^{-11} M of [^{125}I]-ET-1 (Amersham, specific activity 74 TBq/mmol) was incubated with 100 μl of A-10 membrane preparation in assay buffer at 37°C for 3 hours in a final volume of 200 μl. After incubation, the mixtures were filtered with a cell harvester (Skatron, Mode 7020) using a filtermat (Skatron, Mode 11731) and the filtermat was washed five times with a total 3 ml of 0.9% NaCl. The filters were counted in a gamma counter (Aloka, ARC-600). Specific binding was determined as total binding minus non-specific binding in the presence of 10^{-7} M unlabeled ET-1 (Peptide Institute Inc.).

Rabbit Aorta Constriction *In Vitro*

Thoracic aortae were isolated from male albino rabbits (11 weeks old) and cut into spiral strips (2 mm width and 25 mm length). The tissues were suspended in 30 ml organ chambers filled with Krebs-Henseleit solution being maintained at 37°C and gassed with 95%O_2/5%CO_2. The contractile activity was measured isometrically *via* a strain gauge transducer (Orientec, T7-8-240) by maintaining the preparation under a resting tension of 1 g. STBs A, B and C (3×10^{-5} M) added after the full contractile induced by ET-1 were tested against the contractile response of ET-1 (1×10^{-8} M).

Antimicrobial Activity

Antimicrobial activity was tested against several microorganisms at concentrations up to 5,000 μg/ml by the agar diffusion test using paper disks (6 mm i.d. Toyo Roshi Co.) on nutrient agar (Eiken) plates for bacteria, agar (Taiyo) plate containing 2% glucose, 1% poly pepton for fungus and potato dextrose agar (Eiken) plate containing 1% peptone, 0.01% chloramphenicol for *Trichophyton*.

Acute Toxicity

The acute toxicity of STBs was determined in ICR mice (SLC, male, 4-weeks weighing 23 to 26 g) by a single intraperitoneal injection of graded doses of STBs.

Results

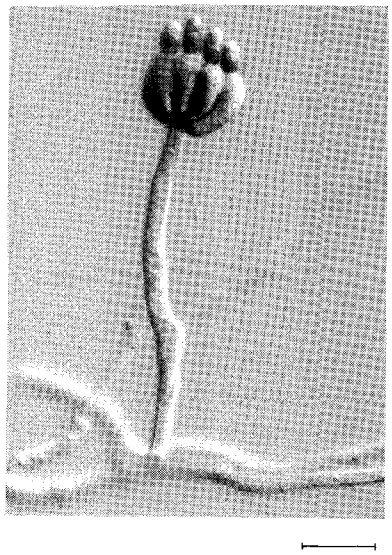
Taxonomy

Morphological Characteristics

Colonies on potato dextrose agar for 14 days at 25°C reached 22 ~ 26 mm in diameter, appeared humped in the center and velvety to floccose. At first, colony color was

Fig. 1. Micrograph of phialoconidia of strain M6222 grown on potato dextrose agar 25°C for 14 days.

Bar represents 10 μm.



white, later becoming pale grey to grey on the surface, brown to greyish orange on the reverse. Greyish orange soluble pigment was produced in the medium.

Conidiophores were macronematous, mononematus, unbranched, 2~3 septate, 38~100 μm in length, 3~6 μm thick, smooth or sometimes minutely rough at the upper parts.

Phialides formed in groups of 6~12 were clavate, somewhat curbed, uncolored smooth, 8~11×4.5~5.0 μm (Fig. 1). Conidia were ovoid, ellipsoidal or cymbiform, smooth or sometimes verruculose, olive brown one-celled with two oil globules, 7.5~10×3.5~4.5 μm.

Physiological Characteristics

Strain M6222 showed good growth at 13.0~37.0°C on potato dextrose agar. The optimum temperature for growth was 25.0~32.7°C. pH range for growth was 3.4~11.2. The optimum pH for growth was 4.4~10.4.

Identification

M6222 was considered to belong to the genus *Stachybotrys* from those characteristics described above. Especially, the taxonomic characteristics of strain M6222 were very similar to those of *Stachybotrys chartarum*. But it is little different from *S. chartarum* in the formation of phialoconidia and the surface appearance of conidia. We therefore identified this strain as *Stachybotrys* sp. M6222. A lyophilized sample of this strain has been deposited at the National Institute of Bioscience and Human Technology, Agency of Industrial Science and Technology, Japan, under the accession No. FERM P-13344.

Fermentation

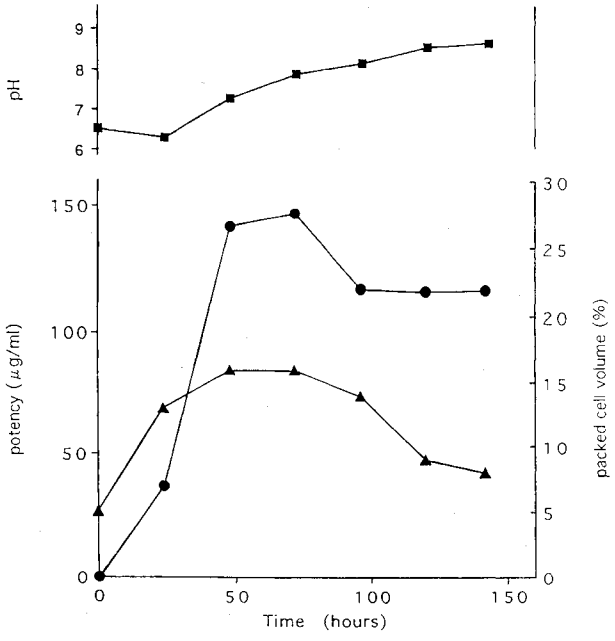
Fig. 2 presents the time course of the fermentation including the production of STB A, pH and growth. The production of STB A paralleled growth. The production of STBs B and C were similar pattern to STB A. The maximum production titer of STBs A, B and C reached about 147 μg/ml, 41 μg/ml and 34μg/ml, respectively, after 3 days of fermentation.

Isolation

The isolation scheme for STBs is shown in Fig. 3. The culture broth (10 liters) was filtered using diatomaceous earth and the filtrate (8 liters) was extracted with EtOAc (5 liters) at pH 2. The solvent layer was back-extracted with water at pH 9 and the aqueous layer was extracted with EtOAc (1.3 liters) at pH 2. Then the extract was concentrated *in vacuo* to give a crude powder (9 g). It was dissolved in MeOH and absorbed on an alumina column (400 ml). After washing with 1.6 liters of 80% MeOH, the column was eluted with 80% to 50% MeOH to give two active fractions.

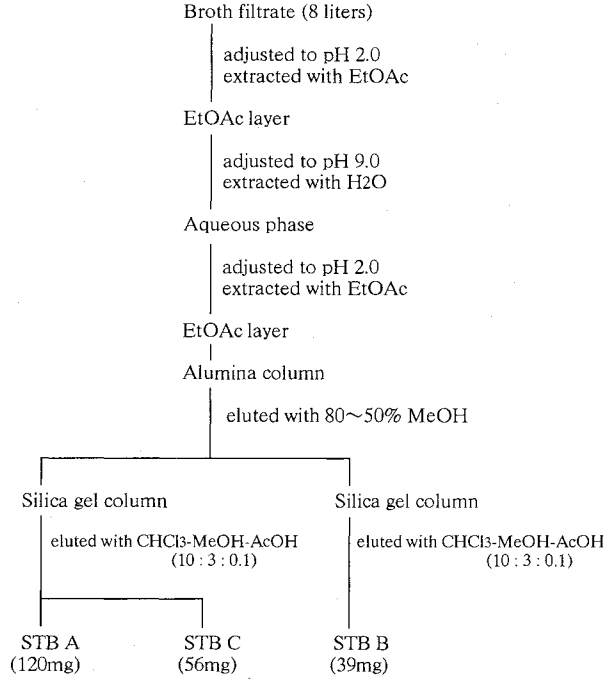
Fig. 2. Time course of fermentation for STB A.

● Potency, ▲ packed cell volume, ■ pH.



Mycelial growth was expressed as packed cell volume obtained after centrifugation of 10 ml of the culture broth at 3,000 rpm for 10 minutes.

Fig. 3. Isolation procedure of STBs A, B and C.



The early fraction containing STBs A and C was concentrated and extracted with EtOAc at pH 2. After concentration *in vacuo*, the extract was applied to a silica gel column (400 ml), and developed with $CHCl_3$-MeOH-AcOH (10:3:0.1). Two active fractions were separately concentrated under a reduced pressure to give STB A (early fraction) and STB C (later fraction) as white powder.

Combined later fraction containing STB B as concentrated and extracted with EtOAc at pH 2. The solvent layer was concentrated and applied to a silica gel column (150 ml) developed with $CHCl_3$-MeOH-AcOH (10:3:0.1). The combined active fraction as concentrated under a reduced pressure to give STB B as white powder.

Physico-chemical Properties

STBs A, B and C were soluble in MeOH, DMSO and alkaline water, and insoluble in water, benzene and hexane. They gave positive color reactions to potassium permanganate, iodine, but negative to ninhydrin, Molisch and ferric chloride reactions. The other physico-chemical properties of STBs A, B and C are summarized in Table 1. Their molecular formulae were found to be $C_{52}H_{70}N_2O_{10}$ (STB A) and $C_{52}H_{70}N_2O_{11}$ (STBs B and C) by high resolution FAB-MS measurements and elemental analysis. The IR spectrum of STB A in KBr (Fig. 4) showed the presence of hydroxyl (3400 cm^{-1}) and amide (1670 cm^{-1}) functions. IR spectra of STBs B and C showed the similar absorptions to STB A. The ^1H NMR spectrum of STB A (Fig. 5) showed

Table 1. Physico chemical properties of STBs A, B and C.

	STB A	STB B	STB C
Appearance	White powder	White powder	White powder
$[\alpha]_D^{23}$ ($c=0.4$, MeOH)	$-57°$	$-64°$	$-65°$
UV λ_{max}^{MeOH} (ε)	218 (70500), 266 (15400), 300~310 (5300)	218 (70500), 265 (15700), 300~310 (5400)	218 (69100), 265 (15300), 300~310 (5400)
UV $\lambda_{max}^{MeOH+HCl}$ (ε)	218 (70600), 266 (15400), 300~310 (5300)	218 (70500), 265 (15700), 300~310 (5400)	218 (69100), 265 (15300), 300~310 (5400)
UV $\lambda_{max}^{MeOH+NaOH}$ (ε)	222 (61600), 274 (13200), 310~325 (5300)	222 (62000), 274 (13500), 310~325 (5400)	222 (59100), 275 (12800), 310~325 (5200)
Molecular formula	$C_{52}H_{70}N_2O_{10}$	$C_{52}H_{70}N_2O_{11}$	$C_{52}H_{70}N_2O_{11}$
HRFAB-MS (m/z)			
Calcd $(M+H)^+$	883.5019	899.5059	899.5059
Found $(M+H)^+$	883.5066	899.5026	899.5067
Elemental analysis			
Calcd:	C 70.72, H 7.99, N 3.17	C 69.46, H 7.85, N 3.12	C 69.46, H 7.85, N 3.12
Found:	C 70.81, H 8.02, N 3.16	C 69.22, H 7.76, N 2.97	C 69.22, H 7.62, N 3.02
TLC (Rf)[a]	0.60	0.30	0.43
HPLC Rt (minutes)	10.0	5.5	4.8

[a] Merck Kieselgel $60F_{254}$, $CHCl_3$-MeOH-AcOH (10:3:0.1).

Fig. 4. IR spectrum of STB A (KBr).

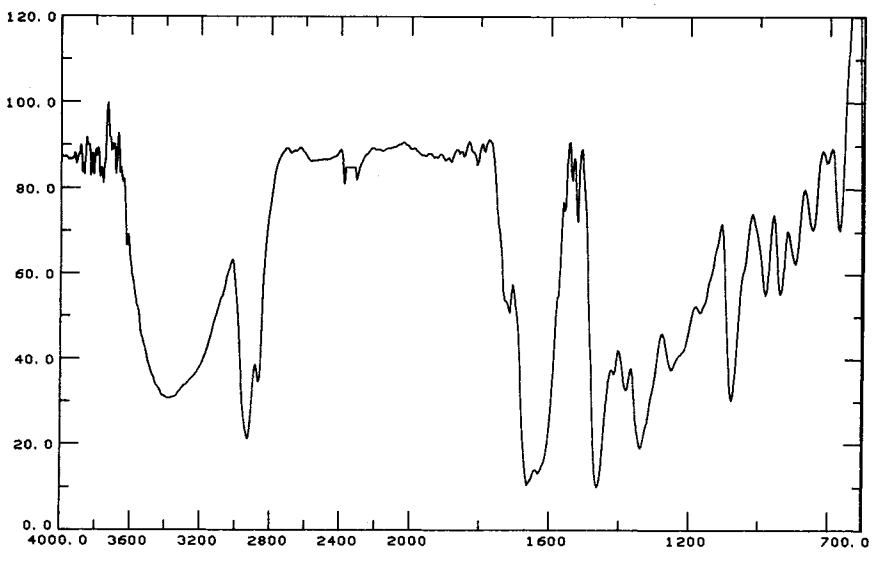


Fig. 5. ^{1}H NMR spectrum of STB A in DMSO-d_6 (400 MHz).

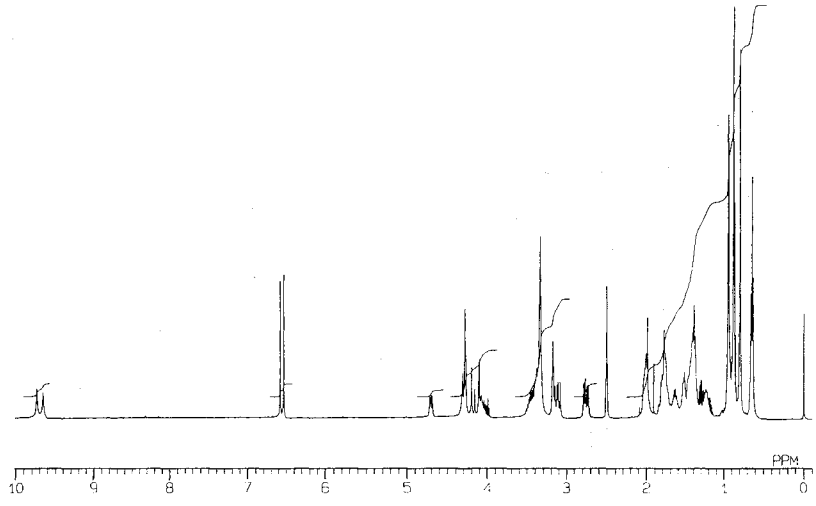
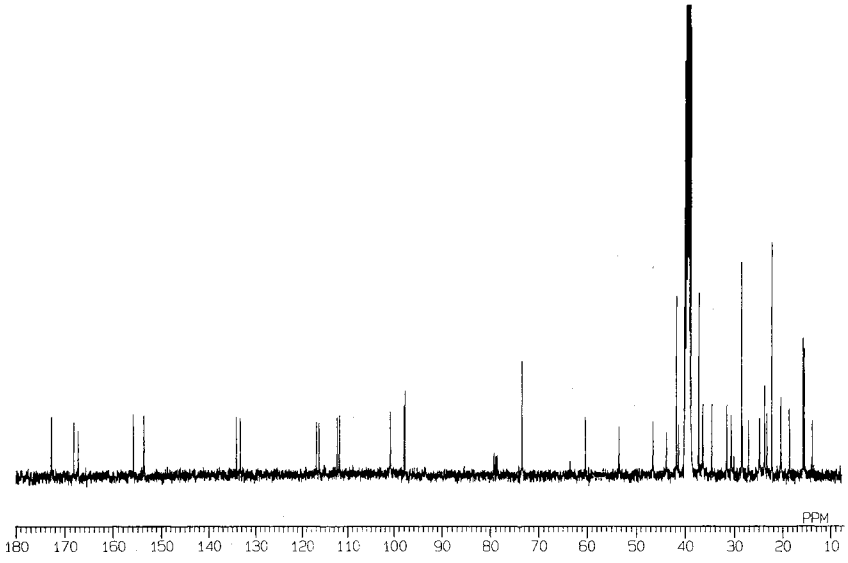


Fig. 6. ^{13}C NMR spectrum of STB A in DMSO-d_6 (100 MHz).



two aromatic singlet protons and two phenolic hydroxy protons. In the ^{1}H NMR of STBs B and C, one proton peak was observed at δ 3.8 ppm that was not observed in STB A. The ^{13}C NMR spectrum of STB A (Fig. 6) confirmed the presence of 52 carbons including 46 signals of the pairs or equivalent carbons. The detail of structural elucidation of STBs will be pubulished in the succeeding paper[5].

Biological Properties

Effect of STBs A, B and C on the Binding of ^{125}I-ET-1 to Rat ET_A Receptors

The inhibition of ^{125}I-ET-1 binding to rat ET_A receptor for STBs A, B and C are shown in Fig. 7. The IC_{50} values of STBs A, B and C against rat ET_A were 2.3×10^{-5}, 2.8×10^{-5} and 2.9×10^{-5} M respectivly. STB A showed slightly higher activity than STBs B and C.

Effect of STBs A, B and C on the Binding of ^{125}I-ET-1 to Human ET_A and Human ET_B Receptors

The inhibition of ^{125}I-ET-1 binding to human ET_A and human ET_B receptors for STBs A, B and C are shown in Table 2. STBs A, B and C showed the almost same activity to human ET_A. To human ET_B, STB A showed more higher activity than STB B and C. STB A, B and C showed the slightly higher activity to human ET_B than human ET_A.

Fig. 7. Inhibitory activities of STBs A, B and C for ^{125}I-ET-1 binding to rat ET_A receptor.

● STB A, ▲ STB B, ■ STB C.

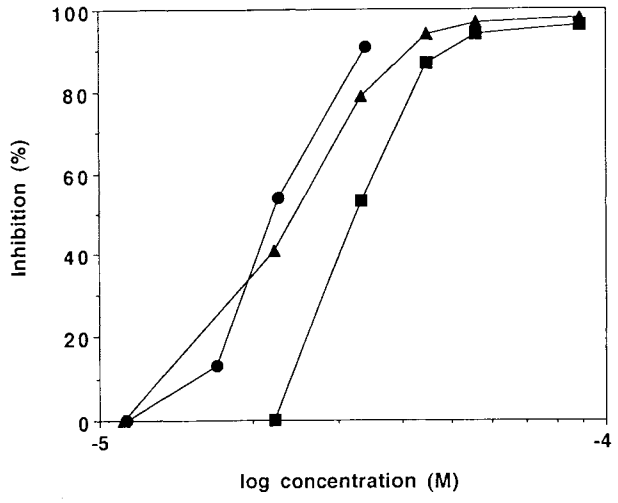


Table 2. The IC_{50} values of STBs A, B and C for ^{125}I-ET-1 binding to human ET_A and human ET_B receptors.

Compounds	IC_{50} (M)	
	h-ET_A	h-ET_B
STB A	1.3×10^{-5}	7.9×10^{-6}
STB B	1.2×10^{-5}	9.5×10^{-6}
STB C	1.5×10^{-5}	9.4×10^{-6}
BE-18257B*	9.1×10^{-6}	No activity

* We found this compound from the culture broth of some *Streptomyces*.

Relaxation Effect

The relaxation rates by STBs A, B and C (3×10^{-5} M) against the rabbit aortae contractile response induced by ET-1 (1×10^{-8} M) were 86%, 70% and 68%, respectively. STB A showed the most strong relaxation effect in the 3 compounds. STBs had no relaxation activities. These result suggest that STBs are ET receptor antagonists, not agonists.

Antimicrobial Activity

No antimicrobial activities were found against *Staphylococcus aureus* FDA-209P, *Bacillus subtilus* PCI-219, *Bacillus stearothermophylus* ATCC 12978, *Micrococcus luteus* ATCC-9341 (Gram-positive bacteria), *Pseudomonas aeruginosa* ML-4262R-, *Alcaligenes faecalis* ATCC-8750, *Escherichia coli* NIHJ No. 34, *Comanonas terrigena* ATCC-8461 (Gram-negative bacteria), *Candida albicans* NI-7491 (fungus) and *Trichophyton rubrum* M-5153 at 5,000 μg/ml by the agar diffusion test using paper disks.

Acute Toxicity

No acute toxicity of STBs A, B and C was observed in mice at the level of 200 mg/kg, ip.

Discussion

From the taxonomic studies performed, it is evident that strain M6222 shoud be classified in the genus *Stachybotrys*. As the bioactive compounds produced by *Stachybotrys*, K-76[9], Q-11270[10] (complement inhibitor) and FR901459[11] (immunosuppressant) *etc.* were reported. However, ET antagonist produced by *Stachybotrys* have never been reported. Therefore STBs are first compounds as ET antagonist produced by *Stachybotrys*.

STBs inhibited the ^{125}I-ET-1 binding to both ET_A and ET_B. Recently, many ET receptor antagonists were reported. In them, BE-18257B[12], WS009[13], 50-235[14], asterric acid[15] are ET_A specific antagonists, so they are different from STBs. As ET_A and ET_B non specific antagonists, cochinmicins[16], PD 145065[17] and Ro 46-2005[18] are reported. Cochinmicins are depsipeptides, PD 145065 is a peptide and Ro 46-2005 is a derivative of pyrimidinil sulphonamide, therefore they are different from STBs. So STBs are novel structural ET antagonists which are non specific to ET_A and ET_B receptors.

As a result of examining the effect of STB A for the binding of other G-protein coupled receptors and its ligands (for example, histamine, opiate, adrenalin, dopamine, serotonin), STB A showed no activity (data not shown). So, it was supposed that STBs were ET specific antagonists. And STBs didn't show either enzyme inhibitory activity (phospholipase C, phospholipase A_2 and cathepsin L) or other bioactivities (immunosupression, nerve growth factor promotor *etc.*).

STBs are ET_A and ET_B non-specific antagonists, and have almost no toxicity both *in vitro* and *in vivo*. Therefore these compounds may be applicable to treating cardiovascular diseases.

References

1) Yanagisawa, M.; H. Kurihara, Y. Tomobe, M. Kobayashi, Y. Mitsui, T. Yazaki, K. Goto & T. Masaki: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. Nature 332: 411~415, 1988
2) Inoue, A.; M. Yanagisawa, S. Kimura, Y. Kasuya, T. Miyauchi, T. Goto & T. Masaki: The human endothelin family : Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc. Natl. Acad. Sci. U.S.A. 86: 2863~2867, 1989
3) Arai, H.; S. Hori, I. Aramori, H. Ohkubo & S. Nakamichi: Cloning and expression of a cDNA encoding an endothelin receptor. Nature 348: 730~732, 1990
4) Sakurai, T.; M. Yanagisawa, Y. Takuwa, H. Miyazaki, S. Kimura, K. Goto & T. Masaki: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. Nature 348: 732~735, 1990
5) Ogawa, K.; M. Nakamura, M. Hayashi, S. Yaginuma, S. Yamamoto, K. Furihata, K. Shin-Ya & H. Seto:

Stachybocins, novel endothelin receptor antagonists, produced by *Stachybotrys* sp. M6222. II. Structure determination of stachybocins A, B and C. J. Antibiotics 48: 1396~1400, 1995

6) JONG, S. C. & E. E. DAVIS: Contribution to the knowledge of *Stachybotrys* and *Memnoniella* in culture. Mycotaxon. 3: 409~485, 1976

7) WILLIAMS, Jr. D. L.; K. L. JONES, K. ALVES, C. P. CHAN, G. F. HOLLIS & J.-S. TUNG: Characterization of cloned human endothelin receptors. Life Sci. 53: 407~414, 1993

8) ADACHI, M.; Y.-Y. YANG, Y. FURUICHI & C. MIYAMOTO: Cloning and characterization of cDNA encoding human A-type endothelin receptor. Biochem. Biophys. Res. Commun. 180: 1265~1272, 1991

9) KAISE, H.; M. SHINOHARA, W. MIYAZAKI, T. IZAWA, Y. NAKANO, M. SUGAWARA & K. SASAKI: Structure of K-76, a complement inhibitor produced by *Stachybotrys complementi nov.* sp. K-76. J. Chem. Soc. Chem. Commun. 726~729, 1979

10) SATO, T.; K. NAGAI, Y. FUKUNAGA & Y. SUGITA: Jpn. Kokai 170769 (1993), July 9, 1993

11) SAKAMOTO, K.; E. TSUJII, M. MIYAUCHI, T. NAKANISHI, M. YAMASHITA, N. SHIGEMATSU & M. OKUHARA: FR901459, novel immunosuppressant isolated from *Stachybotrys chartarum* No. 19392. J. Antibiotics 46: 1788~1798, 1993

12) IHARA, M.; T. FUKURODA, T. SAEKI, M. NISHIKIBE, K. KOJIRI, H. SUDA & M. YANO: An endothelin receptor (ET_A) antagonist isolated from *Streptomyces misakiensis*. Biochem. Biophys. Res. Commun. 178: 132~137, 1991

13) MIYATA, S.; N. OHHATA, H. MURAI, Y. MASUI, M. EZAKI, S. TAKASE, M. NISHIKAWA, S. KIYOTA, M. OKUHARA & M. KOHSAKA: WS009 A and B, new endothelin receptor antagonists isolated from *Streptomyces* sp. No. 89009. J. Antibiotics 45: 1029~1040, 1992

14) FUJIMOTO, M.; S. MIHARA, S. NAKAJIMA, M. UEDA, M. NAKAMURA & K. SAKURAI: A novel non-peptide antagonist isolated from bayberry, Myrica cerifica. FEBS Lett. 305: 41~44, 1992

15) OHASHI, H.; H. AKITAMA, K. NISHIKORI & J. MOCHIZUKI: Asterric acid, a new endothelin binding inhibitor. J. Antibiotics 45: 1684~1685, 1992

16) LAM, Y. K. T.; D. L. WILLIAMS, Jr., J. M. SIGUMUND, M. SANCHEZ, O. GENILLOUD, Y. L. KONG, S. STEVENS-MILES, L. HUANG & G. M. GARRITY: Cochinmicins, novel and potent cyclodepsipeptide endothelin antagonists from a *Microbispora* sp. Production, isolation, and characterization. J. Antibiotics 45: 1709~1716, 1992

17) WELLINGS, R. P.; T. D. WARNER, C. THIEMERMANN, J. P. CRISTOL, R. CORDER & J. R. VANE: Vasoconstriction in the rat kidney induced by endothelin-1 is blocked by PD-145065. J. Cardiovasc. Pharmacol. 22: S103~S106, 1993

18) CLOZEL, M.; V. BREU, K. BURRI, J. M. CASSAL, W. FISCHLI, G. A. GRAY, G. HIRTH, B. M. LOFFLER, M. MULLER, W. NEIPHART & H. RAMUZ: Pathophysiological role of endothelin revealed by the first orally active endothelin receptor antagonist. Nature 365: 759~761, 1993