RES-1149-1 and -2, Novel Non-peptidic Endothelin Type B Receptor Antagonists Produced by *Aspergillus* sp.

I. Taxonomy of Producing Strain, Fermentation, Isolation, and Physico-chemical and Biological Properties

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RES-1149-1 and -2, novel and non-peptidic endothelin antagonists, were isolated from the cultured broth of a fungus, *Aspergillus* sp. RE-1149. RES-1149-1 and -2 selectively inhibited the ET-1 binding to endothelin type B receptor (ET_B receptor) with IC_{50} values of 1.5 μM and 20 μM, respectively. Taxonomy of producing strains, fermentation, isolation, and physico-chemical properties of RES-1149-1 and -2 are described.

Endothelins (ETs) are a family of potent vasoactive peptides termed ET-1, ET-2 and ET-3. Their receptors are classified into at least two subtypes, ET_A and ET_B, depending on their affinity for isopeptides[1]. The ET_A receptor has a high affinity for ET-1 but not for ET-3, while the ET_B receptor is non-selective for ET isopeptides. The ET_A receptors are distributed predominantly in vascular smooth muscle to mediate vasoconstriction. The ET_B receptors are present on endothelial cells and mediate endothelium-dependent relaxation. We previously reported four peptidic antagonists, RES-701-1 , -2, -3 and -4 isolated from the cultured broths of *Streptomyces* sp.[2~4] These peptides selectively recognize the ET_B receptor and inhibit the increase in intracellular Ca^{2+} concentration elicited by 1 nM ET-1 in COS-7 cells expressing human ET_B receptor. The ET_B receptor-selective antagonists reported so far are BQ-788 and IRL-1038[5,6]. The BQ-788 is tripeptide synthesized by chemical modification of the cyclic pentapeptide BE-18257B, isolated from cultured broth of *Actinomycete*[7]. IRL-1038 is an analog of ET-1, [Cys^{11}-Cys^{15}]-ET-1(11~21). However, non-peptidic antagonists selective for ET_B receptor have not been reported. Only one compound, haloemodin, has been reported to have weak inhibitory activity against ET-1 binding to ET_B receptor[8].

In the course of our screening for endothelin antagonists, we have found that *Aspergillus* sp. RE-1149 produced non-peptidic antagonists for ET_B receptor, designated RES-1149-1 and -2. In this article, we report taxonomy of the producing strains, fermentation, isolation, physico-chemical properties, and biological properties of RES-1149-1 and -2.

Materials and Methods

Materials

(3-[^{125}I]iodotyrosyl^{13})Endothelin-1 was purchased from Du Pont-New England Nuclear. Other radioligands used for binding assays were purchased from Du Pont-New England Nuclear and Amersham. Endothelin-1 (ET-1) was purchased from Peptide Institute, Inc., Osaka, Japan. BQ-123 was purchased from American Peptide Co., Santa Clara, CA. RES-701-1 was purified from the culture broth of *Streptomyces* sp. RE-701 in our laboratories. Bovine cerebellum and lung were obtained from a local slaughterhouse. All other chemicals were of analytical grade.

Culture and Medium Conditions

A loopful of spores of the microorganism, grown on an agar slant, was inoculated into 10 ml of seed medium composed of V8 vegetable juice (Campbell) 20% and dextrin 3% (pH 6.5 before sterilization) in a test tube (21 i.d. × 200 mm). The agar slant medium consisted of malt extract 2%, glucose 2%, peptone (Kyokuto) 0.1% and agar 2% (pH 6.5 before sterilization). The inoculated tube was incubated at 25°C for 4 days. A 10%-inoculum from the above seed medium was added to a 300-ml Erlenmeyer flask containing 50 ml of the same medium. After incubation for 1 day on a rotary shaker (200 rpm) at 25°C, 50 ml of the second seed culture was transferred to a 2-liter Erlenmeyer flask containing 400 ml of the fermentation medium composed of V8 vegetable juice 20%, sucrose 3%, soluble starch 2%, malt extract (Difco) 1%, corn steep liquor 0.5%, dry yeast (Asahi Brewery)

0.5% and $CaCO_3$ 0.5% (pH 6.5 before sterilization). The fermentation was carried out for 5 days on a rotary shaker (200 rpm) at 25°C. The production of RES-1149-1 and -2 was traced by HPLC. For this measurement, 2 ml of the culture broth was sampled and extracted with 2 ml of methyl ethyl ketone, and the extract ($2 \sim 10\,\mu l$) was provided for HPLC analysis.

Determination of RES-1149-1 and -2 by HPLC

HPLC analysis was performed on a Develosil, ODS HG (4.6 mm i.d. × 250 mm, Nomura Chemical Co., Ltd.). The column was eluted with 72% CH_3CN, at a flow rate of 1.0 ml per minute. The effluent was monitored at a wavelength of 300 nm. The retention times of RES-1149-1 and -2 were 13.3 minutes and 11.8 minutes, respectively.

Receptor Binding Assay

ET-1 binding assays were performed as described previously[4]. Briefly, bovine cerebellum membranes were used for a source of ET_B receptor. Bovine lung membranes, on which are expressed both ET_A and ET_B receptors were used for a source of ET_A receptor in the presence of 5 μM RES-701-1 (ET_B receptor selective antagonist). The reaction mixtures (1 ml) containing 0.74 kBq/ml ^{125}I-ET-1, 50 mM Tris-HCl buffer (pH 7.6), 1 mM EDTA, 0.2% bovine serum albumin (BSA), 0.02% bacitracin, 14 μg of lung membrane protein or 14 μg of cerebellum membrane protein, and various concentration of samples were incubated at room temperature for 2 hours and then filtered through GF/B glass filters. The glass filters were washed three times with cold 50 mM Tris-HCl buffer (pH 7.6), containing 1 mM EDTA, using a Brandel M-24R cell harvester. The radioactivity on the washed filters was measured by a Packard γ counter. Nonspecific binding was measured in the presence of 0.1 mM unlabeled ET-1.

Results

Characterization of the Producing Strains

The fungal strain RE-1149 was isolated from a soil sample. Colonies on 2% malt extract agar are 63 mm to 69 mm in diameter after culturing at 25°C for 2 weeks. The surface of a colony is dark brown. The color of the reverse of the colony is yellowish brown at the center and dark greenish brown at the marginal area. A soluble pale yellowish pigment is produced in the medium. Colonies on potato-glucose agar are 49 to 53 mm in diameter after culturing at 25°C for 2 weeks. The surface of a colony is dark brown. The color of the reverse of the colony is dark brown at the center and yellowish deep green at the marginal area. A soluble pale yellowish pigment is produced in the medium.

Smooth and colorless hyphae are developed on 2% malt extract agar medium. The hyphae are septate and well-branched, but not synnematous. The smooth and colorless or pale brown conidiophores are formed on hyphae, and are aseptate, 130 to 360 μm in length and 4 to 8 μm in width. A vesicle produced at the top of the conidiophore, is spherical or subspherical and 9.5 to 18 μm in diameter. The upper three quarters of the vesicle bears cylindrical metulae abundantly which are smooth and colorless, and 6 to 8 μm in length and 3 to 4 μm in width. Phialides are formed the upper part of the metulae, and are flask-shaped, smooth, and 6 to 8 μm in length and 3 to 3.5 μm in width at the widest part. The conidial ontogeny is enteroblastic. The phialidic conidia, formed in chains on the top of the phialides, are single-celled and spherical or subspherical and 4 to 5.5 μm in diameter. The conidia are echinulate, and pale brown or are brown when aggregated. No teleomorph was observed in this strain.

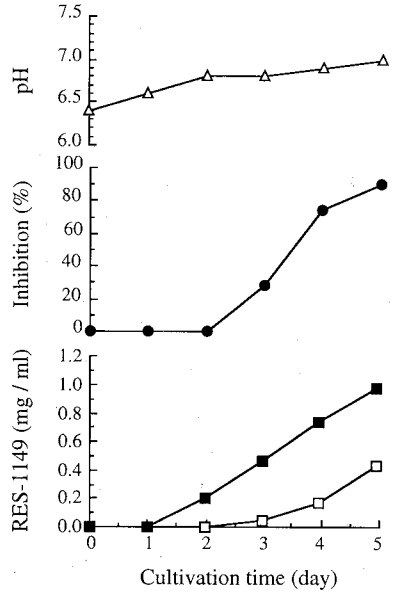
From the characteristics mentioned above, the fungal strain RE-1149 was identified as *Aspergillus* sp.[9]. The fungus has been deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan, as FERM BP-4243.

Production of RES-1149-1 and -2 by Fermentation

The time course of the production of RES-1149-1 and -2 in 2-liter Erlenemeyer flask is shown in Fig. 1. The inhibitory activity against ET-1 binding to bovine

Fig. 1. Time course of RES-1149 production in a 2-liter Erlenmeyer flask.

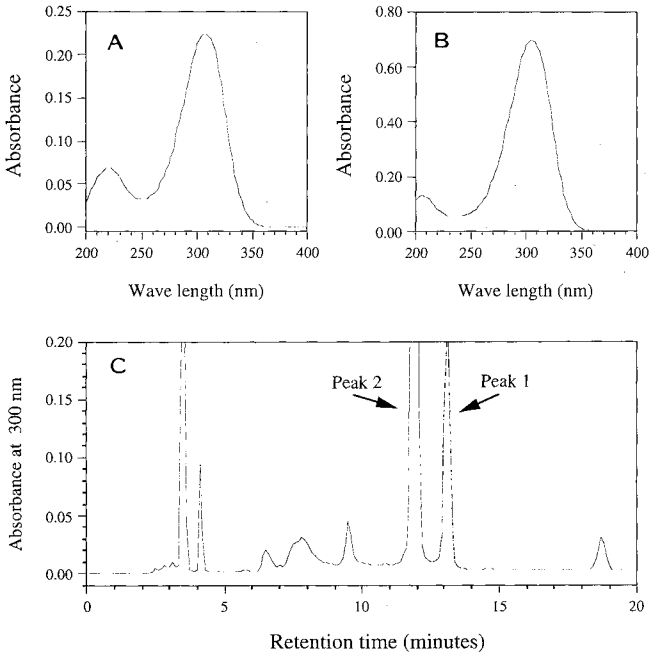
□ RES-1149-1, ■ RES-1149-2, ● inhibition of ET-1 binding (%), △ pH.



cerebellum membranes in the culture broth was seen on day 3. The production of RES-1149-1 and -2 was parallel to the production of the inhibitory activity against ET-1 binding, and the amounts of RES-1149-1 and -2 reached a maximum on day 5. HPLC profile of the extract of cultured broth sampled on day 5, is shown in Fig. 2.

Fig. 2. HPLC profile of an extract from fermentation broth on day 5.

A, UV spectra of peak 1 (RES-1149-1); B, UV spectra of peak 2 (RES-1149-2); C, HPLC chromatogram of the extract monitored at 300 nm.



The extract of cultured broth was performed as described in materials and methods. The extract (10 μl) was analyzed by HPLC.

There were two major peaks showing similar UV spectrum. One of the major peaks eluted at 13.2 minutes in HPLC corresponded to RES-1149-1, and the other major peak at 11.8 minutes corresponded to RES-1149-2. Calculated from the HPLC analysis, the yields of RES-1149-1 and -2 were 0.3 mg/ml and 1.2 mg/ml, respectively. The RES-1149-1 and -2 produced were present in mycelia as well as in broth filtrate.

Isolation and Purification

The isolation procedure for RES-1149-1 and -2 is shown in Fig. 3. The cultured broth was extracted with methyl ethyl ketone (5 liters). The organic layer was concentrated *in vacuo* to give an aqueous solution which

Fig. 3. Purification procedure of RES-1149-1 and -2.

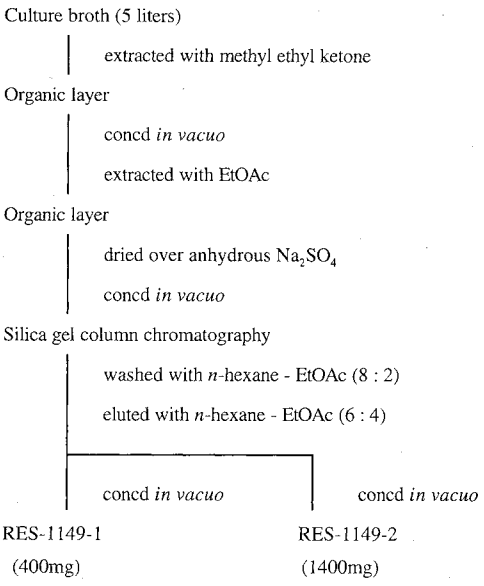
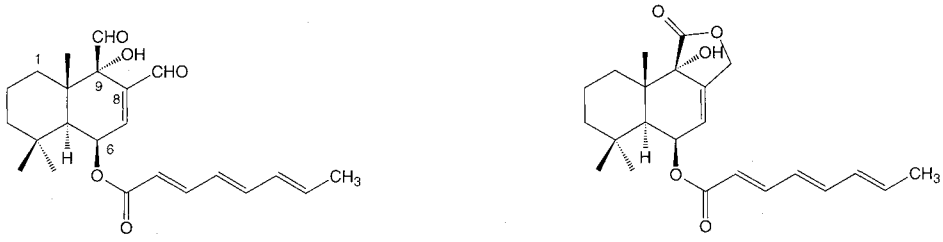


Table 1. Physico-chemical properties of RES-1149-1 and -2.

	RES-1149-1	RES-1149-2
Appearance	Colorless powder	Colorless powder
Molecular formula	$C_{23}H_{30}O_5$	$C_{23}H_{30}O_5$
High resolution FAB-MS		
Observed	387.2190 $(M+H)^+$	387.2173 $(M+H)^+$
Calculated	387.2172 (as $C_{23}H_{31}O_5$)	387.2172 (as $C_{23}H_{31}O_5$)
MP (°C)	51.5 ~ 52	131 ~ 134
IR (KBr) cm^{-1}	3452, 2947, 1707, 1614, 1352, 1267, 1124, 1041, 1005	3435, 2949, 1778,1701, 1616, 1269, 1132, 1007
UV λ_{max} nm (ε) (CH_3CN)	218 (sh. 14,800), 304 (24,400)	302 (22,900)
TLC (Rf)		
CH_2Cl_2 - Et_2O (9 : 1)	0.6	0.5
Solubility		
Soluble	MeOH, acetone, EtOAc, $CHCl_3$	MeOH, acetone, EtOAc, $CHCl_3$
Insoluble	H_2O	H_2O

Fig. 4. Chemical structures of RES-1149-1 and -2.



RES-1149-1 RES-1149-2

Table 2. The antibiotic activities of RES-1149-1 and -2.

Test microorganisms	MIC (μg / ml)	
	RES-1149-1	RES-1149-2
Bacillus subtilis No.10707	2.6	0.82
Staphylococcus aureus subsp. *aureus* ATCC6538P	10.4	13
Enterococcus hirae ATCC10541	5.2	13
Escherichia coli ATCC26	>83	>830
Klebsiella pneumoniae subsp. *pneumoniae* ATCC10031	>83	>830
Pseudomonas aeruginosa BMH No.1	>83	>830
Proteus vulgaris ATCC6897	>83	>830
Shigella sonnei ATCC9290	>83	>830
Salmonella choleraesuis subsp. *choleraesuis* ATCC9992	>83	>830
Candida albicans ATCC10231	>83	>830

was then extracted with ethyl acetate. The organic layer was dried over anhydrous sodium sulfate and then concentrated *in vacuo*. The concentrated solution was added to *n*-hexane and applied to a silica gel column (500 ml). The column was washed with *n*-hexane - ethyl acetate (8 : 2), and eluted with *n*-hexane - ethyl acetate (6 : 4). RES-1149-2 was eluted followed by RES-1149-1. Each fraction were concentrated *in vacuo* to dryness. RES-1149-1 and -2 were obtained as colorless powders.

Physico-chemical Properties of RES-1149-1 and -2

The physico-chemical properties of RES-1149-1 and -2 are summarized in Table 1. RES-1149-1 and -2 are readily soluble in methanol, acetone and dimethylsulfoxide, slightly soluble in *n*-hexane, and insoluble in water. The structures of RES-1149-1 and -2 were determined as shown in Fig. 4 on the basis of HRFAB-MS, ^{1}H and ^{13}C NMR spectral data. Details of structural elucidation studies are described in the succeeding paper[10].

Biological Properties

The antibacterial activities of RES-1149-1 and -2 are summarized in Table 2. RES-1149-1 and -2 inhibited growth of Gram-positive bacteria, but not that of Gram-negative bacteria and *C. albicans*.

Discussion

We have isolated two novel compounds, RES-1149-1 and -2, from the cultured broth of *Aspergillus* sp., and demonstrate that these compounds have inhibitory activities on ET-1 binding to the ET_B receptor. The structures of RES-1149-1 and -2 are close to that of cinnamodial. Cinnamodial has been reported as an African worm antifeedant isolated from the stem bark of a South American arboreal species[10]. The structural difference between RES-1149-1 and cinnamodial is an acyl group bound to the C-6 oxygen. The inhibitory activities of cinnamodial on ET receptor binding has not yet been reported. To investigate its inhibitory activity against ET-1 binding, we synthesized cinnamodial from RES-1149-1. Details of its synthesis and activity are described in the succeeding paper.

To date, many structurally diverse antagonists that have differing selectivity for ET receptor subtypes have been reported. Although haloemodin is a non-peptidic compound that has been reported as an ET_B receptor binding inhibitor[11], its biological activity is weak. Other

ET_B receptor binding inhibitors reported are peptidic compounds: RES-701-1, BQ-788 and IRL-1038[3,5,6]. RES-1149-1 and -2 are structurally different from those of the authentic compounds. RES-1149-1 can be a useful tool to elucidate the physiological and pathological roles of the ET_B receptor and a lead compound to synthesize divergent ET_B receptor antagonists.

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