Azaphilones with Endothelin Receptor Binding Activity Produced by *Penicillium sclerotiorum*: Taxonomy, Fermentation, Isolation, Structure Elucidation and Biological Activity

L. PAIRET, S. K. WRIGLEY*, I. CHETLAND, E. E. REYNOLDS†, M. A. HAYES, J. HOLLOWAY, A. M. AINSWORTH, W. KATZER, X-M. CHENG†, D. J. HUPE†, P. CHARLTON and A. M. DOHERTY†

Xenova Ltd,
240 Bath Road, Slough, SL1 4EQ, UK
†Parke-Davis Pharmaceutical Research, Division of Warner Lambert Company,
Ann Arbor, Michigan 48105, U.S.A.

(Received for publication March 13, 1995)

A series of azaphilones produced by *Penicillium sclerotiorum* (Xenova culture collection number X11853) active in assays for the detection of antagonists of the endothelin-A (ET_A) and endothelin-B (ET_B) receptors has been identified. The series includes two novel sclerotiorin analogues, (8*S*,8*a*-*R*)-7-deacetyl-1,O^8,8,8a-tetrahydro-7-*epi*-sclerotiorin, **1**, and its 5-dechloro analogue, **2**. It also includes 5-chloroisorotiorin, **6**, previously unreported as a natural product, in addition to the major product of these fermentations, (+)-sclerotiorin, **5**. Data for the inhibition of endothelin-1 (ET-1) and endothelin-3 (ET-3) binding in the ET_A and ET_B receptor assays respectively are reported for this series. Compounds **1** and **2** were more selective for the rabbit ET_A receptor than for the rat ET_B receptor. The IC_{50} values for **1** and **2** were 9 and 28 μM respectively in an assay based on binding of ET-1 to rabbit ET_A receptors. In an assay based on the binding of ET-3 to the rat ET_B receptor compounds **1** and **2** exhibited IC_{50}'s of 77 and 172 μM. Members of this series of compounds demonstrated antagonist behavior in a secondary assay based on blockade of ET-1 stimulated arachidonic acid release from rabbit renal artery smooth muscle cells, when present at concentrations of $\geq 30\,\mu$M.

The endothelins (which exist as three isoforms: ET-1, ET-2 and ET-3) are a family of potent vasoconstricting peptides with a variety of biological activities including bronchoconstriction, positive inotropic and chronotropic effects, mitogenesis and potent renal effects. Endothelins are implicated in several human disease states including hypertension, congestive heart failure, renal failure, pulmonary hypertension, ischemia and cerebral vasospasm[1~4]. Recent results obtained with mice deficient in endothelin-1 suggest that it is essential for normal mouse development and may also play a physiological role in cardiovascular homeostasis[5].

Two subtypes of endothelin receptors, classified as ET_A and ET_B receptors, have been cloned and characterised in mammalian systems[6~9]. Both receptor subtypes are rhodopsin-like in structure and are coupled to G-proteins. A third endothelin receptor subtype has been cloned from *Xenopus* dermal melanophores and heart[10~11] although this subtype has not yet been described in mammalian tissues.

Vasoconstriction in a wide variety of animal tissues can clearly occur *via* activation of ET_A and/or ET_B receptors, depending upon the species and vascular bed under study[12~16]. The ET_A receptor mediates vasoconstriction and mitogenic responses and is widely localised in vascular smooth muscle in most tissues. The ET_B, or non-selective receptor, recognising the ET isopeptides with equal affinity, was originally identified as the non-vascular smooth muscle receptor. This receptor is localised on endothelial cells in certain tissues and has been associated with vasodilatory activity, perhaps through the release of the endothelin-derived relaxing factor (EDRF). It has been reported, however, that the ET_B receptor is also localised on vascular smooth muscle and mediates a vasoconstrictor response in certain tissues/species.

The use of endothelin receptor antagonists is furthering the understanding of the pathophysiological role of the endothelins[17~22]. The endothelin antagonists discovered to date from microbial sources are predominantly actinomycete metabolites. These include the ET_A receptor specific cyclic pentapeptide BE-18257B from *Streptomyces misakiensis*[23], the benzanthraquinones WS009A and WS009B from a *Streptomyces* sp.[24], and the depsipeptide cochinmicins from a *Microbispora* sp.[25] There have been fewer reports of endothelin receptor

antagonists from fungal sources but these include asteric acid from an *Aspergillus* sp.[26] and the recently published aselacins from an *Acremonium* sp.[27] Reports of natural product endothelin receptor antagonists from non-microbial sources are sparse but notably include the ET_A selective myriceron caffeoyl ester from *Myrica cerifera* (bayberry)[28,29].

We have developed a high-throughput screen for ET_A receptor binding for use in conducting a microbial screening programme concentrated on fungal samples. This programme has been successful in identifying several new series of endothelin receptor antagonists, the first of which, a family of azaphilones produced by a naturally occuring microfungus, are reported herein. The structures of the azaphilones, two of which (**1** and **2**) are novel fungal metabolites, are shown in Figure 1.

Materials and Methods

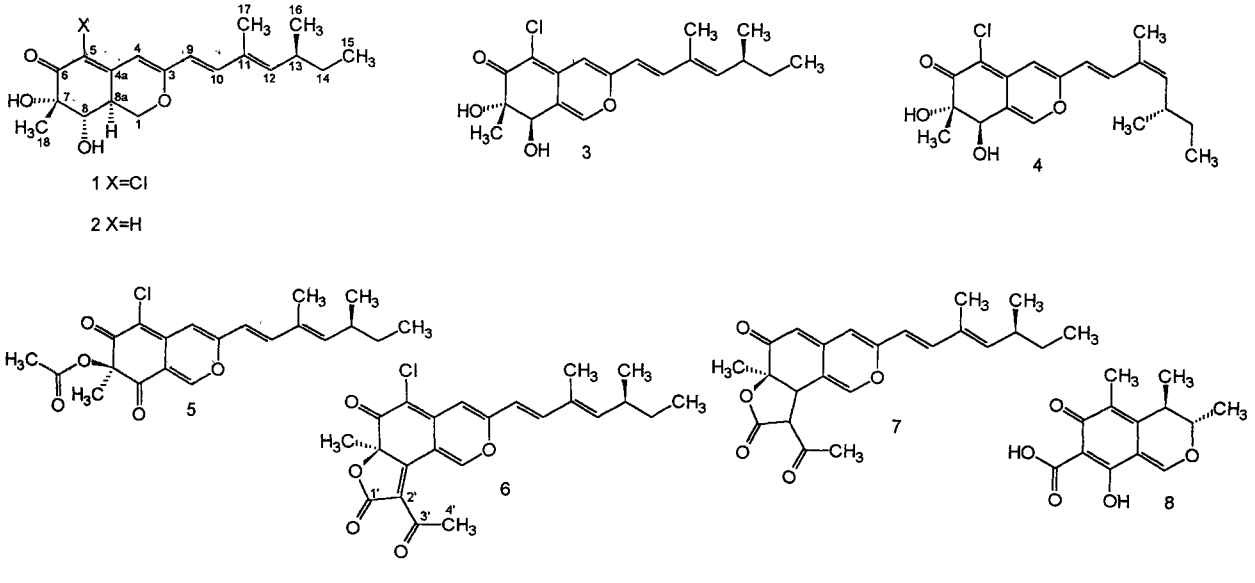
Preparation of Membranes for ET_A Receptor Binding Assay

ET_A subtype receptors were prepared from cultured rabbit renal artery vascular smooth muscle (RRA-VSM) cell membranes. The culture medium consisted of DME/Ham's F12 (Dulbeccos Modifed Eagle medium) (1 : 1) and 10% FBS (fetal bovine serum). The cells were sub-cultured at confluency by washing with CMF-PBS (calcium and magnesium free phosphate buffered saline) and incubating for 5 minutes with CMF-PBS containing 0.05% trypsin and 0.02% EDTA (Ethylenediaminetetraacetic acid) at 37°C. The cells were then split at a dilution of 1 in 10. Cells were grown in 150 mm dishes. Dishes containing confluent RRA-VSM cells were washed twice with cold buffer (5 mM Tris, 2 mM EDTA, pH 7.4). The cells were lysed by addition of cold buffer and incubation at 4°C for 15 minutes. The lysed cells were scraped off the dishes and broken up with a large pre-cooled Dounce glass homogenizer by hand homogenizing on ice. The lysate was centrifuged at 30,000 × *g* for 20 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in freezing buffer (20 mM Tris, 2 mM EDTA, 100 μM bacitracin, 100 μM PMSF (Phenyl methyl sulphonyl fluoride), pH 7.4) at approximately 1 mg/ml, membranes were then aliquoted and stored at −80°C until required.

ET_A Receptor Binding Assay

The [^{125}I]-ET-1 binding assay was performed as follows. The assay buffer consisted of phosphate buffer (40 mM Na_2HPO_4, 10 mM KH_2PO_4, pH ~7.2), 5 mM EDTA, 0.1 mM PMSF, 1% BSA (bovine serum albumin). RRA-VSM membranes (8 ~ 10 μg) were pre-incubated in polypropylene microtitre plates with antagonist (highest final concentration of 0.1 mg/ml) or ET-1 (final concentration of 100 nM) to determine non-specific binding for 30 minutes prior to addition of [^{125}I]-ET-1 (final concentration of 56 pM). The assay mixture was incubated at room temperature for 2 hours with shaking. "Bound" was separated from "Free" by filtration through Wallac 1205-404 (GF/B) filtermats pre-treated with phosphate buffer containing 0.2% BSA for 30 minutes at 4°C. The filtermats were placed in a Skatron 96-well harvester and washed with phosphate buffer at room temperature for 10 seconds. After drying liquid scintillant (Wallac β-plate Scint) was added and the filtermats counted in a Wallac β-plate Reader. Total binding of [^{125}I]-ET-1 in the absence of cold ET-1 was typically 9000 cpm. Non-specific binding as measured in the presence of cold ET-1 was typically 1000 cpm.

Fig. 1. Structures of azaphilones (**1**~**7**) and citrinin (**8**).



Preparation of Membranes for ET_B Receptor Binding Assay

ET_B subtype receptors were prepared from rat brain cerebellum. Dura and blood vessels were removed from rat brain cerebella and the tissue stored at −80°C until required. When required the tissue was re-suspended in 2.5 ml ice-cold homogenization buffer (250 mM sucrose, 20 mM Tris, 2 mM EDTA, pH 7.4) and homogenized in a pre-cooled motor-driven Teflon pestle using slow then high speed. The homogenate was centrifuged at 30,000 × *g* for 20 minutes at 4°C. The supernatant was discarded and the pellet re-suspended in 2 ml ice-cold buffer (0.9% NaCl, 20 mM Tris, 5 mM EDTA, pH 7.4) per cerebellum. Membrane preparations were aliquoted and stored at −80°C until required.

ET_B Receptor Binding Assay

The [^{125}I]-ET-1 binding assay was performed as follows. A 100 μl aliquot of frozen rat cerebellar membrane was diluted with ice-cold buffer (20 mM Tris, 2 mM EDTA, 100 μM PMSF, 100 μM Bacitracin, pH 7.4). For binding experiments the assay mixture (120 μl) contained 90 μl diluted membrane, [^{125}I]-ET-1 (67 pM final concentration) and either 30 μl compound (highest final concentration of 0.1 mg/ml) or 100 nM ET-3 (final concentration) to determine non-specific binding. The assay mixture was incubated in polypropylene microtitre plates (Greiner) at 37°C for 2 hours. "Bound" was separated from "Free" as described earlier. The filtermats were pretreated with 50 mM Tris (pH 7.4), 0.2% BSA for 60 minutes at 4°C. The wash buffer was ice cold 50 mM Tris (pH 7.4), washing for 15 seconds. Counting was performed as described earlier. Total binding of [^{125}I]-ET-1 in the absence of cold ET-3 was typically 12400 cpm. Non-specific binding as measured in the presence of cold ET-3 was typically 1500 cpm.

Preparation of Recombinant Human ET_A and ET_B Receptors

Recombinant DNA techniques were performed essentially as described in SAMBROOK *et al.*[30] and according to manufacturer's instructions. Rat brain ET_B receptor cDNA was obtained from Dr F. CHUNG at Parke Davis.[31] A human placenta cDNA library was constructed in bacteriophage lambda gt11 and approximately 10^6 plaques were screened with a ^{32}P labelled 1.3 kilobase HindIII/Xbal restriction fragment ET_B receptor cDNA as a probe. Plaque hybridisation was carried out for 16 hours at 42°C in a solution containing 100 μg/ml calf thymus DNA, 1 × Denhardts solution, 5 × SSC, 50 mM sodium phosphate, and 0.1% SDS (sodium dodecylsulphate). The membranes were then washed twice for 30 minutes each in 2 × SSC with 0.1% SDS at 55°C. The positive clones were purified and sub-cloned into PUC19 plasmid. DNA sequencing was performed by the dideoxynucleotide chain termination method and was identified as human ET_B receptor by reading both DNA strands.

The 1.35 kb HindIII/Xbal restriction fragment of clone 12 of the Human ET_B receptor was inserted into the eucaryotic expression vector pRc-CMV (pRcCMV-hET_BR). CHO-K1 cells were transfected with 20 μg of pRcCMV-hET_B receptor by electroporation at 300 V, 800 μF, low ohms for 1 second. Cell populations expressing human ET_B receptor were selected with geneticin (0.5 mg/ml) and from these selected cell populations clonal cell lines were isolated by single cell cloning. Expression levels of human ET_B receptor were determined by the receptor binding assay described below using [^{125}I]-ET-1 as the radioligand. CHO-K1 cells expressing recombinant human ET_BR were grown in DME/F12 (1 : 1) supplemented with 10% fetal bovine serum and 0.5 mg/ml geniticin.

Human ET_A receptor expressed in mouse Ltk-cells were a gift of Dr M. YANAGISAWA, and were transfected as described by SAKAMOTO *et al.*[32]

Human ET_A and ET_B Receptor Binding Assays

The following cultured cells were used in binding experiments: CHO-K1 cells expressing recombinant human ET_BR, Ltk-cells expressing human ET_A receptor, and RRA-VSM cells (prepared as described by REYNOLDS *et al.*)[33] expressing rabbit ET_A receptor. Membranes were prepared from cultured cells by lysing cells in cold lysis buffer (5 mM HEPES, 2 mM EDTA, pH 7.4) and homogenising with a Dounce "A" homogeniser.

Homogenates were centrifuged at 30,000 × *g* for 20 minutes at 4°C. Membrane pellets were resuspended in cold buffer containing 20 mM Tris, 2 mM EDTA, 200 μM Pefabloc, 10 μM phosphoramidon, 10 μM leupeptin, 1 μM pepstatin, pH 7.4, and frozen at −80°C until use. Membranes were thawed and homogenised with a Brinkmann Polytron (Westbury, NY), then diluted in tissue buffer containing 20 mM Tris, 2 mM EDTA, 200 μM Pefabloc and 100 μM bacitracin (pH 7.4). Radioligand and competing ligands were prepared in binding buffer containing 20 mM Tris, 2 mM EDTA and 0.1% BSA. Competition binding assays were initiated by combining membranes, [^{125}I]-ET-1 (30,000 cpm) and competing ligand in a final volume of 250 μl and incubating for 2 hours at 37°C. The assay was terminated by filtration over Whatman GF/B filters which were presoaked with 50 mM Tris, pH 7.4, containing 0.2% BSA and 100 μM bacitracin. Non-specific binding was defined as binding in the presence of 100 nM unlabelled ET-1, specific binding was defined as total binding minus non-specific binding. Specific binding was analysed by non-linear least squares curve fitting (InPlot, GraphPad Software, San Diego, CA). Kd values for the binding of [^{125}I]-ET-1 to each of the membrane preparations was determined in separate experiments by saturation binding and Scatchard analysis.

Functional Activity—Arachidonic Acid Release Assay (AAR_A)

The effects of the isolated compounds on ET-1-

stimulated [^{3}H]-arachidonate release from cultured rabbit renal artery vascular smooth muscle cells were studied as follows: rabbit renal artery vascular smooth muscle cells (RRA-VSMC) were grown in 12-well cluster dishes (Costar; Cambridge, MA) in DME/F12 media (DME/F12, 1 : 1) containing 10% FBS in an atmosphere of 5% CO_2/95% air. Confluent cells were incubated with 0.5 ml of loading medium (DME/F12) containing 0.5% FBS and 0.25 μCi/ml of [^{3}H]-arachidonic acid for 24 hours. The loading media was aspirated, the cells washed twice with assay buffer (Hank's BSS, 10 mM HEPES, 0.1% fatty-acid free BSA, pH 7.4). The buffer was replaced with 1 ml fresh assay buffer containing agonist (0.3 nM ET-1) and competing ligand, and the incubation continued for 30 minutes. [^{3}H]-arachidonic acid released into the assay buffer was determined by collecting the assay buffer and counting in a liquid scintillation counter. Under basal conditions the release of AA is negligible, *i.e.* in the absence of either endothelin or the compounds. For example, in the presence of 10 nM ET-1, incubated for 10 minutes, the [^{3}H]-arachidonic acid release minus the basal levels (basal levels are $<$ 50 cpm) is measured as approximately 1400 cpm.

Source of Organism

The microfungus designated Xenova culture collection number X11853 was isolated from foam in a tropical forest stream during 1989. It was deposited at the Centraalbureau Voor Schimmelcultures, Baarn, The Netherlands on 15 February 1994 under accession number CBS123.94.

Fermentation of Organism

A suspension from a mature slant culture, grown on MEA (2% malt extract, 1.5% agar) was transferred into a 250-ml Erlenmeyer flask containing 40 ml seed medium. The seed medium consisted of glycerol, D-glucose, malt extract, soybean peptone, NaCl and $CaCO_3$, adjusted to pH 6 with hydrochloric acid before sterilisation. The flask was shaken at 240 rpm and 25°C for 3 days and transferred into a 2-liter Erlenmeyer flask containing 250 ml seed medium. This flask was shaken for another 3 days under the same conditions. The contents of the flask were transferred to a 14-liter fermenter containing 11 liters of the following medium: beet molasses, casein, phytic acid, $CaCO_3$ and antifoam A (Sigma), adjusted to pH 6 with hydrochloric acid before sterilisation. The vessel was agitated for 150 hours at 450 rpm and aerated at 0.5 vvm. The temperature was controlled at 25°C.

Dissolved oxygen tension (DOT) and pH were monitored *via* Ingold probes and dry cell weight (DCW) was determined by conventional methods. Production of compounds of interest was monitored by extracting biomass from 10 ml aliquots of culture with methanol (3 x 10 ml). The extracts were analysed by reverse phase HPLC on a Waters Novapak C-18 column with photo-diode array detection, eluting with a water - acetonitrile gradient (0→100% over 13 minutes).

Isolation of Azaphilones **1** to **7**

Harvested cells obtained from 10 litres of broth by centrifugation were extracted with methanol (8 litres). The methanol extract was concentrated, diluted with water and extracted with dichloromethane. The dichloromethane was concentrated to dryness to yield 27.2 g of brown residue. This residue was redissolved in methanol and purified by semi-preparative reversed phase HPLC on two Prep Nova-Pak HR C_{18} radial compression cartridge columns (40 mm x 10 cm, 6 μm particle size, 60 Å pore size, Waters, WAT037704) connected in series in a PrepPak Holder Assembly with Extension (Waters) along with a Prep Nova-Pak HR C_{18} Guard-Pak Insert (Waters, WAT037854). The elution method consisted of a linear water - acetonitrile gradient increasing, after an initial delay of 2 minutes, from 60 to 100% acetonitrile over a period of twenty minutes at a flow rate of 50 ml/minute. The eluate was monitored by its absorbance at 340 nm and fractions were tested for activity in the rabbit ET_A receptor binding assay. Six peaks with endothelin binding activity were obtained.

The materials concentrated from the first three peaks (with retention times of 8, 10 and 11 minutes respectively) were separately purified by preparative TLC (Merck 5717 plates, 20 cm x 20 cm, 2 mm thickness of Kieselgel $60F_{254}$). Development of the first peak material with dichloromethane - methanol (9 : 1) produced a yellow band with an Rf of 0.4. Elution and concentration of this band yielded **2** (8 mg). Similarly, purification of the second and third peak materials using dichloromethane - methanol (19 : 1) generated bands with Rf values of 0.32 and 0.21 respectively. Elution and concentration from these bands yielded **3** (90 mg) and **1** (100 mg).

The fourth, fifth and sixth HPLC peaks (with retention times of 12, 15 and 17 minutes respectively) were concentrated to dryness to obtain orange powders of **7** (35 mg), **5** (1 g) and **6** (190 mg).

Determination of Physico-chemical Properties

UV/visible spectra were measured on a Perkin-Elmer Lambda 17 UV/visible spectrometer. IR spectra were recorded on a Nicolet 5PC FTIR spectrometer using a Spectra Tech "Collector" diffuse reflectance accessory. Low resolution EI-MS and DCI-MS were obtained on a VG Trio 3 triple quadrupole mass spectrometer. High resolution EI-MS were obtained on a Finnigan Mat 95 mass spectrometer. ^{1}H and ^{13}C NMR spectra were recorded at 308 K on a Bruker ACF400 spectrometer at 400 MHz and 100 MHz respectively. All chemical shifts (δ) are quoted in ppm. Standard techniques were used to obtain the DEPT, COSY-45, HMQC, HMBC and NOESY spectra. In HMQC experiments the J_{CH} was optimised for 145 Hz. In HMBC experiments the long range coupling constant $^{3\sim5}J_{CH}$ was optimised for 5 Hz. Mixing times from 570 to 1200 ms were used in the NOESY experiments.

Chemicals

Chemicals were obtained as follows: Endothelin-1 (ET-1) from Novabiochem, Nottingham, England (Catalogue No. 05-23-3800) or from American Peptide Co., Sunnyville, Ca; [^{125}I]-ET-1 from Amersham International, Amersham, England (Catalogue No. IM223) or from New England Nuclear, Beverly, MA; Endothelin-3 (ET-3) from Peptides International, Louisville, KY; Human placenta cDNA library from Clontech Lab Inc. Palo Alto, CA; ^{32}P-dCTP from Amersham, Arlington Heights, IL; Calf thymus DNA, bacitracin, Denhardt's solution, SSC buffer and SDS from Sigma, St Louis, MO; BSA from Miles, Kankakee, IL; Sequenase kit from United States Biochemical Corp., Cleveland, OH; DME/F12 media, F12 media and G418 from Gibco/BRL, Gathersburg, MD; fetal bovine serum from HyClone, Logan, UT; CHO-K1 cells from American Type Culture Collection, Rockville, MD; pRc-CMV from Invitrogen, San Diego, CA; Pefabloc from Boehringer-Mannheim, Indianapolis, IN; Citrinin from Sigma Chemical Co. Ltd., Poole, Dorset, England.

Asterric acid and BE18257-B were isolated from in house fermentations of *Penicillium aragonense* (Xenova culture collection number X3911) and a *Streptomyces* sp. (X19254) respectively. Purification procedures for these compounds were similar to reported methods[23,26]. The ^{1}H, ^{13}C NMR, UV, IR and mass spectra of purified asterric acid and BE-18257B were identical to published data.

Biological Activity

The concentration of the isolated compounds **1**~**3**, **5**~**7** which resulted in a 50% inhibition of the endothelin binding (IC_{50}) to the relevant receptor membrane preparation was determined in the assay described.

Results

Taxonomy of Fungus X11853

This fungus was identified as a strain of *Penicillium sclerotiorum* on the basis of its morphological characteristics following inoculation at the centre of Petri dishes containing two different media, Czapek Dox (Unipath Ltd) agar (CZ) and 2% malt extract (Difco Laboratories) agar (2% MEA), using conidia transferred on a sterile agar-coated needle. Over a period of ten days, the mycelial diameter extension rate at 25°C was within the range 1.7~2.3 mm d^{-1} on 2% MEA and 2.2~3.7 mm d^{-1} on CZ. No mycelial extension occurred at 5°C or 37°C. However, mycelial margins could be rendered non-uniform by the emergence of faster-extending appressed sectors. Mycelia on 2% MEA and CZ were radially sulcate or wrinkled, often with orange droplet exudation. Aerial mycelium ranged from white or buff at the margin to saffron or deeper orange red nearer the inoculum, overlain by scattered penicilli. Conidial heads were sparse and greyish turquoise or greenish grey. A yellowish or pale orange soluble pigment was often produced in the medium and the reverse mycelial pigmentation ranged from yellow, through vivid orange red, to sienna. The penicillate conidiophores were monoverticillate with smooth to finely roughened stipes, 70~250 μm long, terminating in a 3~5 μm diameter vesicle. The projecting phialides were ampulliform, 8~11 μm long, producing ellipsoidal conidia, 2.5~3.0 × 1.9 μm, with smooth to finely roughened walls.

These morphological characteristics are in accordance with the variation described for *Penicillium sclerotiorum* van Beyma, although the values for mycelial extension are lower than stated as typical for the species[34].

Fermentation

The changes in pH, DOT, DCW, and concentration of **1** that occured during the fermentation are shown in Figure 2. The dry cell weight reached a maximum value of 22 g/liter after approximately 48 hours and then remained constant. The compounds of interest were first detected after 53 hours and their concentrations increased linearly with time until harvest.

Identification of Known Azaphilones **3** to **7**

The major components of the cell extracts of fermentations of *Penicillium sclerotiorum* X11853 were identified as (+)-sclerotiorin, **5**, and 5-chloroisorotiorin, **6** by their UV, IR, mass, ^{1}H and ^{13}C NMR spectra and their optical rotations. The UV, mass and ^{1}H NMR spectra and optical rotation of **5** were identical to those reported for (+)-sclerotiorin[35,36]. The UV and ^{1}H NMR spectra and optical rotation of **6** were identical to those reported for (+)-5-chloroisorotiorin obtained by a synthetic route[37]. 5-chloroisorotiorin has not previously been reported as a natural product. The ^{1}H and ^{13}C NMR assignments for **5** and **6** are given in Table 1.

Compound **3** was found to be identical with the recently reported monoamine oxidase inhibitor (8*R*)-7-deacetyl-*O*^{8},8-dihydro-7-*epi*-sclerotiorin from *Talaromyces luteus*[38,39]. Its UV, mass, ^{1}H and ^{13}C NMR spectra were as reported. When **3** was purified from previous fermentations of *P. sclerotiorum* X11853 it was occasionally contaminated with small quantities of **4**, its 11-(*Z*) isomer, which was also reported as a *Talaromyces luteus* metabolite[38].

Compound **7** was identified as ochrephilone, previously reported as a metabolite of *Penicillium multicolor*[40], by its UV, IR, mass, ^{1}H and ^{13}C NMR spectra.

Fig. 2. (a) Changes in dry cell weight (DCW) and production of compound **1**; and (b) changes in dissolved oxygen tension (DOT) and pH during fermentation of *Penicillium sclerotiorum* X11853.

Figure 2(a)

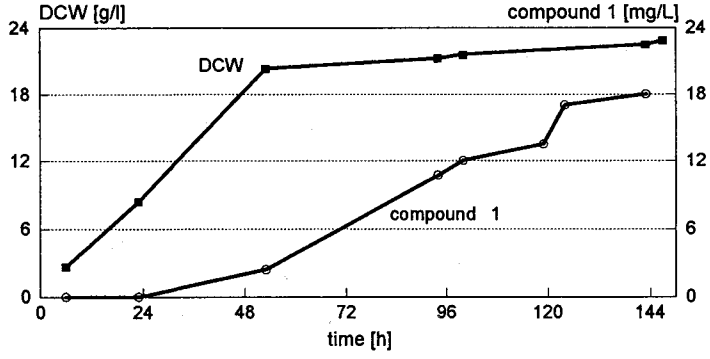


Figure 2(b)

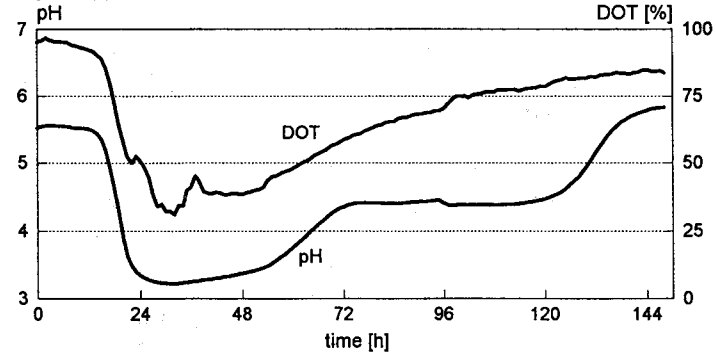


Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data for **1**, **2**, **5** and **6** in $CDCl_3$.

Position	1		2		5		6	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	68.8	4.86 (dd, 10.9, 5.2) 3.82 (dd, 13.6, 10.9)	68.8	4.82 (dd, 10.7, 5.4) 3.80 (dd, 13.5, 10.8)	152.6	7.92 (s)	151.9	8.82 (s)
3	164.1		161.8		158.1		158.0	
4	101.6	6.10 (s)	104.6	5.65 (s)	106.4	6.64 (s)	105.5	6.61 (s)
4a	146.6		151.8		138.6		139.4	
5	118.2		115.2	5.72 (d, 2.0)	114.7		110.2*	
6	189.1		196.1		191.7		194.1	
7	75.1		73.2		84.6		87.4	
8	73.6	3.47 (d, 9.5)	74.3	3.46 (d, 9.5)	185.9		163.6	
8a	37.7	3.14 (dddd, 13.6, 9.5, 5.0, 0.6)	36.7	(dddd, 13.5, 9.5, 5.5, 1.3)	111.0		108.7*	
9	119.1	6.00 (d, 15.6)	118.9	5.90 (d, 15.6)	115.8	6.08 (d, 15.6)	115.6	6.09 (d, 15.7)
10	142.1	7.02 (d, 15.6)	140.5	6.92 (d, 15.6)	142.8	7.06 (d, 15.6)	143.5	7.12 (d, 15.7)
11	132.4		132.0		132.0 d		131.9	
12	147.2	5.63 (d, 9.6)	146.0	5.58 (d, 9.7)	148.7	5.71 (d, 9.8)	149.2	5.73 (d, 9.7)
13	35.0	2.46 (m)	34.8	2.45 (m)	35.1	2.48 (m)	35.1	2.48 (m)
14	30.2	1.42 (m) 1.32 (m)	30.0	1.40 (m) 1.30 (m)	30.1	1.43 (m) 1.31 (m)	29.8	1.44 (m) 1.35 (m)
15	11.9	0.87 (t, 7.4)	11.7	0.82 (t, 7.4)	11.9	0.86 (t, 7.4)	11.8	0.85 (t, 7.4)
16	12.4	1.81 (d, 1.2)	12.2	1.80 (d, 1.2)	12.3	1.85 (d, 1.1)	12.2	1.86 (d, 1.2)
17	20.2	0.99 (d, 6.6)	20.2	1.00 (d, 6.6)	20.1	1.02 (d, 6.6)	20.0	1.02 (d, 6.6)
18	21.1	1.54 (s)	20.6	1.49 (s)	22.5	1.56 (s)	26.1	1.71 (s)
7-$OCOCH_3$					170.0			
7-$OCOCH_3$					20.0	2.16 (s)		
1^1							167.7	
2^1							123.7	
3^1							182.9	
4^1							29.9	2.59 (s)

Spectra were measured at 25°C. TMS was used as an internal reference (δ 0.00).
Chemical shifts are expressed in ppm and coupling constants in Hz, *These assignments are interchangeable.

Table 2. Physico-chemical properties of **1** and **2**.

	1	**2**
Appearance	Yellow powder	Yellow powder
Molecular formula	$C_{19}H_{25}O_4Cl$	$C_{19}H_{26}O_4$
HREI-MS (*m/z*) for M^+ Found:	352.1439	318.1827
Calculated:	352.1434	318.1824
UV* λ_{max} (MeOH) nm (ε)	211 (5,700), 265 (4,400), 389 (21,100)	208 (8,300), 265 (5,500), 374 (19,300)
IR ν_{max} KBr cm^{-1}	3600~3200, 2926, 1663, 1551, 1372 1267, 1140, 1062	3600~3200, 1575, 1400, 1030
Solubility Soluble:	MeOH, $CHCl_3$, CH_2Cl_2	MeOH, $CHCl_3$, CH_2Cl_2
Insoluble:	H_2O, hexane	H_2O, hexane
TLC, Rf on Silica gel $60F_{254}$ (Merck No 5554) developed with CH_2Cl_2-MeOH (19:1)	0.21	0.23

* No changes in UV/Vis spectra of **1** and **2** were observed on addition of either 0.1 M NaOH or 0.1 M HCl.

Structure Elucidation of **1** and **2**

The physico-chemical properties of **1** and **2** are shown in Table 2. The ^1H and ^{13}C NMR spectra of **1** were very similar to those of sclerotiorin, the main differences being the absence of the olefinic proton and carbon signals for C-1, the absence of signals for an acetyl group at C-7 and the presence of four new, highly coupled proton signals at δ 4.86, 3.82, 3.47 and 3.14. The HMQC NMR spectrum showed that the proton signals at δ 4.86 and 3.47 were due to a non-equivalent pair of methylene protons attached to a carbon with a shift of δ 68.8. The proton signals at δ 3.47 and 3.14 were likewise due to methine protons attached to carbons with shifts of δ 73.6 and 37.7 respectively. Consideration of these chemical shift data and inspection of the COSY-45 NMR spectrum suggested the partial structure shown in Figure 3.

These NMR data and the molecular formula of $C_{19}H_{25}O_4Cl$ indicated by HR-EI-MS suggested that **1** was a reduced 7-deacetylsclerotiorin. Comparison of the NMR data for **1** and **5** required **1** to be 7-deacetyl-1,O^8,8,8a-tetrahydrosclerotiorin. This was confirmed by correlations observed in the HMBC NMR spectrum of **1**, these correlations are shown in Figure 4.

The scalar coupling constants for the proton spin system comprising the methylene protons at C-1 and the methine protons at C-8 and C-8a are almost identical to those reported for the very similar system present in the recently reported azaphilone, falconensin A[41]. Cross peaks observed in the NOESY NMR spectrum of **1** suggested that these four protons have the same spatial arrangement as their counterparts in falconensin A. In contrast to the NOE results observed for falconensin A, however, there was no cross peak observed for the protons of the methyl group attached to C-7 and the proton at C-8a; the only cross peak observed for these methyl protons was with the proton at C-8. These

Fig. 3. Partial structure of **1**.

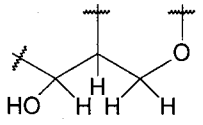
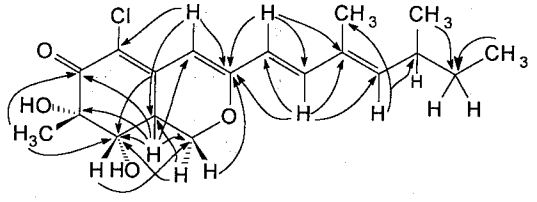
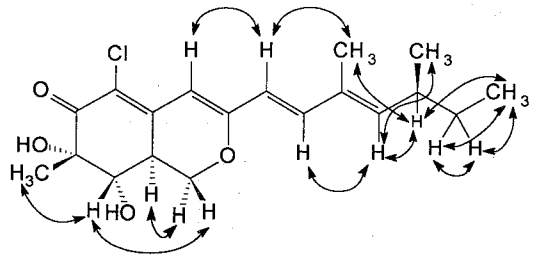


Fig.4. Long range ^1H-^{13}C correlations observed in the HMBC NMR spectrum of **1**.



A similar set of correlations was observed in the HMBC spectrum of **2**.

Fig. 5. NOE's observed in the NOESY NMR spectra of **1**.



Identical NOE's were observed for **2**, with an additional interaction for the proton at C-5.

observations are best accomodated by the relative stereochemistry indicated for **1**, the configuration at C-7 being the opposite of that reported for falconensin A. The NOE enhancements observed in the NOESY NMR spectrum of **1** are shown in Figure 5.

The ^1H and ^{13}C NMR spectra of **2** were very similar to those of **1**, the main difference being the presence of an extra proton singlet at δ 5.72. The molecular formula

of $C_{19}H_{26}O_4$ suggested by HR-EI-MS indicated the replacement of the chlorine in the structure of **1** by a hydrogen to generate the 5-dechloro-analogue of **1**. This was confirmed by correlations observed in the HMBC NMR spectrum of **2**.

The ^1H and ^{13}C NMR assignments for **1** and **2** are given in Table 1.

Biological Properties

Endothelin Receptor Assays

The IC_{50} values of compounds **1**~**3** and **5**~**7** and the azaphilone-related compound citrinin **8**, for inhibition of endothelin binding in the rabbit ET_A and rat ET_B receptor assays are shown in Table 3. The IC_{50} curve for **1** in the rabbit ET_A receptor assay is shown in Figure 6. The IC_{50} curve for **1** in the rat ET_B receptor assay is shown in Figure 7. The IC_{50} values for compounds **1**~**3** and **5**~**7** in the human ET_A and ET_B receptor assays are shown in Table 3.

Citrinin **8** was not active in either the rabbit ET_A and rat ET_B receptor assays at any of the concentrations tested. The known microbial ET_A antagonists asterric acid and BE-18257B were also tested in the rabbit ET_A assay and their IC_{50} values, also shown in Table 3, agree with those reported[23,26].

Arachidonic Acid Release Assay (AAR_A)

Compounds **1**, **3**, **5**, **6** and **7** were active at 30 μM in

Fig. 6. IC_{50} curve for compound **1** in rabbit ET_A assay.

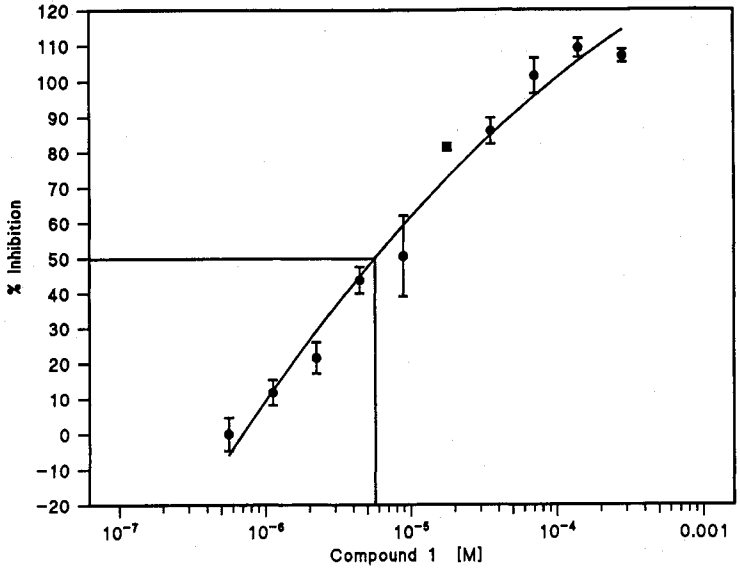


Fig. 7. IC_{50} curve for compound **1** in rat ET_B assay.

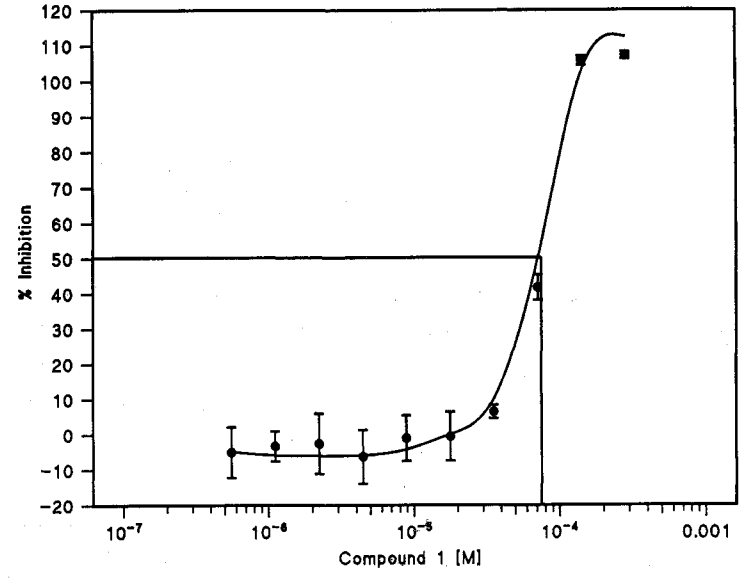


Table 3. Data determined for compounds **1**~**3**, **5**~**8**, asteric acid and BE18257B in ET_A, ET_B and AAR_A assays.

	Rabbit ET_A (μM)	Rat ET_B (μM)	Human ET_A (μM)	Human ET_B (μM)	AAR_A (μM)
1	9±3	77±3	205	104±8	>30
2	28±2	172±2	>250	>250	nt
3	5±2	50±1	140	127±18	33
5	75±17	12±1	152	114±37	>30
6	35±12	8±1	>250	56±16	>30
7	26±2	85±3	197	148±10	>30
8	>300	>300	nt	nt	nt
Asterric acid	20±1	nt	nt	nt	nt
BE-18257B	0.6±0.1	nt	nt	nt	nt

IC_{50}'s in rabbit ET_A and rat ET_B assays are shown as the mean of ≥4 competition experiments±SE. IC_{50}'s in human ET_A assay represent data from a single competition experiment. IC_{50}'s in human ET_B receptor assay represent 3 competition experiments±SE. IC_{50}'s in AAR_A represent data obtained from one experiment. Binding data was computer-analysed by non-linear least squares analysis giving the best fit for a one site model, nt=not tested.

the AAR_A assay although the IC_{50} values determined were in excess of 30 μM. This data is shown in Table 3. The most active was compound **3** with an IC_{50} of 33 μM. Compound **2** was not tested in the AAR_A assay due to scarcity of material.

Discussion

We have described a series of azaphilones produced by *Penicillium sclerotiorum* X11853, including two novel sclerotiorin analogues, which inhibit the binding of endothelin-1 and endothelin-3 in ET_A and ET_B receptor assays. The new sclerotiorin analogues, **1** and **2**, are the most fully reduced analogues of the sclerotiorins yet reported. These compounds bear some structural similarities to the recently reported falconensins[41]. The few reports of the biological activities of the azaphilones include monoamine oxidase inhibition by **3**[33,34], inhibition of phospholipase A_2 by **5**[42] and inhibition of the gp120-CD4 interaction by the recently reported isochromophilones[43].

(+)-Sclerotiorin has also been reported to induce morphological changes in fungi.[35] This activity was correlated with the reactivity of (+)-sclerotiorin with methylamine. Ochrephilone, however, did not react with methylamine. The most active compound with respect to reactivity with methylamine and induction of changes in fungal morphology was the azaphilone-related compound citrinin. Citrinin showed no activity in our rabbit ET_A and rat ET_B receptor assays when tested at concentrations up to 300 μM. The azaphilones discussed here thus appear to act as endothelin receptor antagonists by a mechanism not involving simple reactivity with amine functionalities.

The IC_{50} values of compounds **1**, **2** and **3** (9, 28, 5 μM) compare well with IC_{50} values for inhibition of ET-1 binding to the ET_A receptor reported for other fungal metabolites such as asteric acid (10 μM) and aselacin A (22 μM)[26,27]. In contrast compounds isolated from actinomycetes are reported to have somewhat more potent inhibitory properties as exemplified by cochinmycin A (0.24 μM) and the benzanthraquinone WS009A (0.67 μM)[24,25]. The IC_{50} values for asteric acid and BE-18257B in the rabbit ET_A assay were in good agreement with those reported[23,26].

Compounds **1**, **2** and **3** were more selective for rabbit ET_A receptors whilst **5** and **6** are more selective for rat ET_B receptors. In general all the compounds were less active when tested against the human receptors. A trend towards higher IC_{50} values was observed when the compounds were tested in rabbit ET_A and rat ET_B receptor assays when the pre-incubation step was omitted (data not shown).

Considerable experimental data reveals that endothelin stimulates phospholipase A2 to produce arachidonate-derived secondary messengers such as prostaglandins, thromboxanes and leukotrienes[2]. Activity was observed for the most potent compounds in the AAR_A assay, demonstrating their ability to block ET-1 mediated arachidonic acid release in a complex physiologically relevant assay.

Addendum in Proof

Compound **1** appears to be identical to the recently published isochromophilone III (ARAI, N.; K. SHIOMI, H. TOMODA, N. TABATA, D. J. YANG, R. MASUMA, T. KAWAKUBO & S. ŌMURA: Isochromophilones III~VI, inhibitors of Acyl-CoA : cholesterol acyltransferase produced by *Penicillium multicolor* FO-3216. J. Antibiotics 48: 696~702, 1995).

Acknowledgements

The authors would like to acknowledge MO LATIF for his assistance with the NMR studies, TREVOR GIBSON and STEVE HOARE for the MS measurements and JACKIE JOHNSON for her help with the early purification studies.

References

1) INOUE, A.; M. YANAGISAWA, S. KIMURA, Y. KASUYA, T. MIYAUCHI, K. 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
2) DOHERTY, A. M.: Endothelin: a new challenge. J. Med. Chem 35: 1493~1508, 1992
3) WATANABE, T.; N. SUZUKI, N. SHIMAMOTO, M. FUJINO & A. IMADA: Endothelin in myocardial infarction. Nature 344: 114, 1990
4) SAITO, Y.; K. NAKAO, M. MUKOYAMA & H. IMUR: Increased plasma endothelin level in patients with essential hypertension. New Engl. J. Med. 322: 205, 1989
5) KURIHARA, Y.; H. KURIHARA, H. SUZUKI, T. KODAMA,

K. MAEMURA, R. NAGAI, H. ODA, T. KUWAKI, W.-H. CAO, N. KAMADA, K. JISHAGE, Y. OUCHI, S. AZUMA, Y. TOYODA, T. ISHIKAWA, M. KUMADA & Y. YAZAKI: Elevated blood pressure and cranofacial abnormalities in mice deficient in endothelin-1. Nature 368: 703~710, 1994

6) ARAI, H.; S. HORI, I. ARAMORI, H. OHKUBO & S. NAKAHISHI: Cloning and expression of a cDNA encoding on endothelin receptor. Nature 348: 730~732, 1990

7) 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

8) SAKAMOTO, A.; M. YANAGISAWA, T. SAKURAI, Y. TAKUWA, H. YANAGISAWA & T. MASAKI: Cloning and functional expression of human cDNA for the ET_B endothelin receptor. Biochem. Biophys. Res. Commun. 178: 656~663, 1991

9) HOSADA, K.; K. NAKAO, H. ARAI, S. SUGA, Y. OGAWA, M. MUKOYAMA, G. SHIRAKAMAI, Y. SAITO, S. NAKANISHI & H. IMURA: Cloning and expression of human endothelin-1 receptor cDNA. FEBS Lett. 287: 23~26, 1991

10) KARNE, S.; C. K. JAYAWICKREME & M. R. LERNER: Cloning and characterisation of an endothelin-3 specific receptor (ET_C receptor) from *Xenopus laevis* dermal melanophores. J. Biol. Chem. 268: 19126~19133, 1993

11) KUMAR, C.; V. MWANGI, P. NUTHULAGANTI, H.-L. WU, M. PULLEN, K. BRUN, H. AIYER, R. A. MORRIS, R. NAUGHTON & P. NAMBI: Cloning and characterisation of a novel endothelin receptor from *Xenopus* heart. J. Biol. Chem. 269: 13414~13420, 1994

12) CLOZEL, M.; G. A. GRAY, V. BREU, B.-M. LOFFLER & R. OSTERWALDER: The endothelin ET_B receptor mediates both vasodilation and vasoconstriction *in vivo*. Biochem. Biophys. Res. Commun. 186: 867~873, 1992

13) SUMNER, M. J.; T. R. CANNON, J. W. MUNDIN, D. G. WHITE & I. S. WATTS: Endothelin ET_A and ET_B receptors mediate vascular smooth muscle contraction. Br. J. Pharmacol. 107: 858~860, 1992

14) MORELAND, S.; D. M. MCMULLEN, C. L. DELANEY, V. G. LEE & J. T. HUNT: Venous smooth muscle contains vasoconstrictor ET_B-like receptors. Biochem. Biophys. Res. Commun. 184: 100~106, 1992

15) SUDJARWO, S. A.; M. HORI, Y. TANAKA, T. OKADA & H. KARAKI: Subtypes of endothelin ET_A and ET_B receptors mediating venous smooth muscle contraction. Biochem. Biophys. Res. Commun. 200: 627, 1994

16) FUKURODA, T.; M. KOBAYASHI, S. OZAKI, M. YANO, T. MIYAUCHI, M. ONIZUKA, Y. SUGISHITA, K. GOTO & M. NISHIKIBE: Endothelin receptor subtypes in human versus rabbit pulmonary arteries. J. Appl. Physiol. 76: 12994, 1986

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

18) ISHIKAWA, K.; T. FUKAMI, T. NAGASE, K. FUJITA, T. HAYAMA, K. NIYAMA, T. MASE, M. IHARA & M. YANO: Cyclic pentapeptide endothelin antagonists with ETA selectivity. Potency and solubility enhancing modifications. J. Med. Chem. 35: 2139~2142, 1992

19) IHARA, M.; I. NOGUCHI, T. SAEKI, T. FUKURODA, S. TSUCHIDA, S. KIMURA, T. FUKAMI, K. ISHIKAWA, M. NISHIKIBE & M. YANO: Biological profiles of highly potent novel endothelin antagonists selective for ET_A receptor. Life Sci. 50: 257~255, 1992

20) SOGABE, K.; H. NIREI, M. SHOUBO, A. NOMOTO, S. AO, T. NOTSU & T. ONO: Pharmacological profile of FR 139317, a novel potent endothelin ET_A receptor antagonist. J. Pharmacol. Exp. Ther. 264: 1040~1046, 1993

21) CODY, W. L.; A. M. DOHERTY, J. X. HE, P. M. DEPUE, S. T. RAPUNDALO, G. A. HINGORANI, T. C. MAJOR, R. L. PANEK, S. HALEEN, D. LADONCEUR, E. E. REYNOLDS, K. E. HILL & M. A. FLYNN: Design of a functional antagonist of endothelin. J. Med. Chem. 35: 3301~3303, 1992

22) CODY, W. L.; A. M. DOHERTY, J. X. HE, P. M. DEPUE, L. A. WAITE, J. G. TOPLISS, S. HALEEN, D. LADONCEUR, M. A. FLYNN, K. E. HILL & E. E. REYNOLDS: The rational design of a highly potent combined ET_A and ET_B receptor antagonist. Med. Chem. Res. 3: 154~162, 1993

23) 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. Comm. 178: 132~137, 1991

24) 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

25) LAM, Y. K. T.; D. L. WILLIAMS Jr, J. M. SIGMUND, M. SANCHEZ, O. GENILLOND, L. KONG, S. STEVENS-MILES, L. HUANG & G. M. GARRITY: Cochinmicins, novel and potent cyclodepsipeptide endothelin antagonists from a *Microbispora* sp. 1. Production, isolation and characterisation. J. Antibiotics 45: 1709~1716, 1992

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

27) JACKSON, M.; N. S. BURRES, J. P. KARWOWSKI, L. A. ALDER, P. E. HUMPHREY, W. L. KOHL & J. B. MCALPINE: Aselacins, novel compounds that inhibit binding of endothelin to its receptor 1. Producing organism, fermentation and biological activity. J. Antibiotics 47: 523~527, 1994

28) MIHARA, S.-I. & M. FUJIMOTO: The endothelin ET_A receptor-specific effect of 50-235, a non-peptide endothelin antagonist. Eur. J. Pharm. (Mol. Pharm. Sec.) 246: 33~38, 1993

29) FUJIMOTO, M.; S.-I. MIHARA, S. NAKAJIMA, M. VEDA, M. NAKAMURA & K.-S. SAKWAI: A novel non-peptide endothelin antagonist isolated from bayberry, *Myrica cerifera*. FEBS Lett. 305: 41~44, 1992

30) SAMBROOK, J.; E. F. FRITSCH & T. MANIATIS: Molecular cloning: a laboratory manual. Second edition. Cold Springs Harbour Laboratory Press, Plainview, NY, 1989

31) ZHU, G.; L. H. WU, C. MAUZY, A. M. EGLOFF, T. MIRZADEGAN & F.-Z. CHUNG: Replacement of lysine-181 by aspartic acid in the third transmembrane region of the endothelin type B receptor reduces its affinity to endothelin peptides and sarafotoxin 6c without affecting

G protein coupling. J. Cell. Biochem. 50: 159~164, 1992

32) SAKAMOTO, A.; M. YANAGISAWA, G. TSUJIMOTO, K. NAKAO, T. TOYO-OKA & T. MASAKI: Pseudo-noncompetitive antagonism by BQ123 of intracellular calcium transients mediated by Human ET_A receptor, Biochem. Biophys. Res. Comm. 200: 679~686, 1994

33) REYNOLDS, E. E.; L. L. S. MOK & S. KUROKAWA: Phorbol ester dissociates endothelin-stimulated phosphoinositide hydrolysis and arachidonic acid release in vascular smooth muscle cells. Biochem. Biophys. Res. Comm. 160: 868~873, 1989

34) PITT, J. I.: A laboratory guide to common *Penicillium* species, 2nd Edn. CSIRO, North Ryde, Australia, 1988

35) NATSUME, M.; Y. TAKAHASHI & S. MARUMO: Chlamydospore-like cell-inducing substances of fungi: close correlation between chemical reactivity with methylamine and biological activity. Agric. Biol. Chem. 52: 307~312, 1988

36) EADE, R. A.; H. PAGE, A. ROBERTSON, K. TURNER & W. B. WHALLEY: The chemistry of fungi. Part XXVIII. Sclerotiorin and its hydrogenation products. J. Chem. Soc.: 4913~4924, 1957

37) CHONG, R.; R. R. KING & W. B. WHALLEY: The chemistry of fungi. Part LXI. The synthesis of (±)-sclerotiorin, of (±)-4,6-dimethylocta-*trans*-2-*trans*-4-dienoic acid and of an analogue of rotiorin: J. Chem. Soc. (C): 3566~3571, 1971

38) FUJIMOTO, H.; T. MATSUDO, A. YAMAGUCHI & M. YAMAZAKI: Two new fungal azaphilones from *Talaromyces luteus*, with monoamine oxidase inhibitory effect. Heterocycles 30: 607~616, 1990

39) SATOH, Y. & M. YAMAZAKI: Studies on the monoamine oxidase (MAO) inhibitory potency of TL-1, isolated from a fungus, *Talaromyces luteus*. Chem. Pharm. Bull. 37: 206~207, 1989

40) SETO, H. & M. TANABE: Utilization of ^{13}C-^{13}C coupling in structural and biosynthetic studies. III. Ochrephilone —a new fungal metabolite. Tet. Letts. 651~654, 1974

41) ITABASHI, T.; K. NOZAWA, M. MIYAJI, S. UDAGAWA, S. NAKAJIMA & K. KAWAI: Falconensins A, B, C and D, new compounds related to azaphilone, from *Emericella falconensis*. Chem. Pharm. Bull. 40: 3142~3144, 1992

42) NAKAMURA, K.; T. KINO, K. NIKO, S. KYOTOO & M. OKUHARA: Phospholipase A2 inhibitors containing sclerotiorin for treatment of inflammatory pancreatitis, and allergy JP 02255615, Mar 27, 1989

43) ŌMURA, S.; H. TANAKA, K. MATSUZAKI, H. IKEDA & R. MASUMA: Isochromophilones I and II, Novel inhibitors against gp120-CD4 binding from *Penicillium* sp. J. Antibiotics 46: 1908~1912, 1993