WS009 A AND B, NEW ENDOTHELIN RECEPTOR ANTAGONISTS ISOLATED FROM *Streptomyces* sp. No. 89009

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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WS009 A and B[†] novel endothelin receptor antagonists, have been isolated from the fermentation broth of *Streptomyces* sp. No. 89009. These antagonists were purified from the culture filtrate followed by Diaion SP-207, DEAE Toyopearl column chromatography and HPLC. WS009 A and B showed selective activity in an endothelin receptor binding assay with IC_{50} of 5.8×10^{-6} M and 6.7×10^{-7} M, respectively. On the basis of spectroscopic and chemical evidence, the structures of WS009 A and B have been established as **1** and **3**, and are highly hydroxylated benz[*a*]anthraquinone chromophores.

Endothelin (ET) is a 21-amino-acid residue polypeptide that has been originally isolated from the supernatant of cultured vascular endothelial cells and shown to be one of the most potent vasoconstrictors[1]. Subsequent studies of gene cloning led to the discovery of three ET gene products designated ET-1, ET-2 and ET-3[2]. It is well known now that the ET family of isopeptides exert diverse biological effects through specific receptors in a wide variety of tissues and cell types[3,4].

Many factors, such as ET and endothelium-derived relaxing factor (EDRF)[5], affect the circulatory status of animals by modulating vascular-resistance. ET-1 is the most potent vasoconstrictor identified to date. It also induces mitogenesis and increases the expression of proto-oncogenes in vascular smooth muscle cells, fibroblast, and mesangial cells. Thus, ET-1 may be involved in the regulation of vascular tone as well as in the pathogenesis of vasospasm, hypertension and atherosclerosis[4].

In the course of a screening program for ET-1 receptor antagonists from culture broths of microorganisms, we found new compounds from the fermentation broth of *Streptomyces* spp.

In this paper, we describe the taxonomic studies, fermentation of producing strain, isolation, physico-chemical properties and biological activities of the ET antagonists, WS009 A and B.

Materials and Methods

Taxonomic Studies

Strain No. 89009 was isolated from a soil sample obtained from Narita City, Chiba Prefecture, Japan. The methods described by Shirling and Gottlieb[6] were employed for the taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 14 days on

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yeast extract - malt extract agar, inorganic salts - starch agar and oatmeal agar. Cultural characteristics were observed on the media described by SHIRLING and GOTTLIEB[6], and WAKSMAN[7]. Incubation was carried out at 30°C for 21 days. The color names used in this study were taken from the Methuen Handbook of Colour[8]. Wall analysis was performed by the methods of BECKER et al.[9], and YAMAGUCHI[10]. The temperature range for growth was determined on yeast extract - malt extract agar using a temperature gradient incubator (Advantec Toyo Co., Ltd.). Utilization of carbon sources was examined by the method of PRIDHAM and GOTTLIEB[11].

Fermentation

A loopful of Streptomyces sp. No. 89009 on mature slant culture was transferred into twenty five 500-ml Erlenmeyer flaks each containing 160 ml of sterile seed medium composed of soluble starch 1%, sucrose 1%, glucose 1%, Pharmamedia (Traders Protein Co., Ltd.) 1%, peptone 0.5%, soybean meal 0.5% and $CaCO_3$ 0.2%. The medium was adjusted to pH 6.5 prior to addition of $CaCO_3$. These flasks were shaken on a rotary shaker (220 rpm, 5.1 cm-throw) for 3 days at 30°C. The resultant first stage seed culture was transferred to a sterilized second stage of 200 liters of the seed medium supplemented with Adekanol LG-109 (defoaming agent, Asahi Denka Co., Ltd.) 0.07% and Silicone KM-70 (defoaming agent, Shin-Etsu Chemical Co., Ltd.) 0.05% in a 500-liter jar fermenter. The second stage seed was cultured for 2 days at 30°C with aeration at 200 liters per minute, back pressure of $1.0\,kg/cm^2$ and agitation speed of 200 rpm. The 60 liters of seed, preparaed as above, was inoculated into a 4,000-liter fermenter containing 3,000 liters of a production medium of sucrose 4%, peanut powder 1%, dried yeast 0.2%, wheat germ 0.5%, potato protein 0.5% and Adekanol LG-109 0.07% and Silicone KM-70 0.05%. The fermentation ran for 7 days at 30°C with aeration at 3,000 liters per minute, back pressure of $1.0\,kg/cm^2$ and agitation speed of 100 rpm. The progress of fermentation was monitored by high performance liquid chromatography (HPLC) using an Hitachi Model 655 pump. A steel column (4.00 mm inside diameter, 250 mm length) packed with LiChrospher RP-18 (5 μm, E. Merck Co., Ltd.) was used with flow rate of 1.0 ml/minute. The mobile phase used was an aqueous solution of 25% acetonitrile with 0.1% trifluoroacetic acid (TFA). The detector wavelength was set at 230 nm. The sample for the HPLC assay was prepared as follows: The fermentation broth was centrifuged at 1,500 × g for 20 minutes and then 5 μl of supernatant was injected into the injector of a Hitachi Model 655 HPLC. WS009 A and B were eluted at about 4 minutes and 8 minutes, respectively.

General Procedures

MP's were determined on a Yanagimoto micro melting point apparatus and are reported uncorrected. Optical rotations were measured on a Jasco DIP-140 polarimeter in a 10-cm microcell. IR spectra were recorded on a Jasco A-102 infrared spectrometer. Low-resolution FAB-MS and HRFAB-MS spectra were measured on a VG ZAB-SE mass spectrometer. NMR spectra were acquired on a Bruker AM400wb spectrometer. The standard Bruker software library was employed for a series of 2D NMR experiments. The chemical shift are reported in ppm relative to internal tetramethylsilane and coupling constants in Hz. A conventional amino acid analysis was achieved with a Hitachi 835 automatic amino acid analyzer.

ET Receptor Assay

(a) Crude Receptor Membrane Preparation: Porcine aorta was purchased from Pel-Freeze Biologicals (U.S.A.) and stored at -80°C until use. Porcine aorta (50 g) was thawed and dissected free from fatty tissue, minced with scissors and then homogenized with a polytron (Brinkmann PT-20, maximal speed for 3 × 10 seconds) in 100 ml buffer (0.25 M sucrose, 10 mM Tris-HCl, 0.1 mM EDTA). The homogenate was centrifuged at 10,000 × g for 20 minutes at 4°C. The supernatant, containing the plasma membranes fraction, was centrifuged at 100,000 × g for 60 minutes at 4°C, and the resultant pellets were referred to as crude membrane fractions. The pellets were resuspended in 25 ml of binding assay buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, 1.5 μg/ml phenylmethylsulfonyl fluoride (PMSF), 120 μg/ml bacitracin, 12 μg/ml leupeptin, 6 μg/ml chymostatin, 0.1% bovine serum albumin (BSA), pH 7.5). The aorta membrane fractions were stored at -80°C until use.

(b) $[^{125}I]$-ET-1, $[^{125}I]$-ET-2 and $[^{125}I]$-ET-3 Binding Assay: 1.67×10^{-11} M of $[^{125}I]$-ET-1, $[^{125}I]$-ET-2 or $[^{125}I]$-ET-3 (Amersham Japan, specific activity: 2,000 Ci/mmol) was incubated with 50 μl

of aorta membranes preparation in binding assay buffer at room temperature (20~22°C) for 60 minutes in a final volume of 250 μl. After incubation, the mixtures were filtered through a glass-fiber GF/C filter (pretreatment with 0.1% polyethyleneimine for 3 hours prior to use) using a cell harvester (Brandel M-24S). The filters were then washed ten times with a total of 5 ml of the washing buffer (50 mM Tris-HCl, pH 7.5) at 4°C. The filters were counted in a gamma counter (Packard Auto Gamma Model 5650). Specific .binding was defined as total binding minus nonspecific binding in the presence of 10^{-5} M unlabeled ET-1, ET-2 or ET-3.

Antimicrobial Activity

The antimicrobial activity of WS009 A and B were determined by a serial broth dilution method in bouillon medium for bacteria and in SABOURAUD's medium for fungi and yeasts. The antimicrobial activity was observed after overnight incubation at 37°C for bacteria and 48~72 hours incubation at 28°C for a filamentous fungus and a yeast.

Results

Taxonomy of the Producing Strain

The vegetative mycelium developed well without fragmentation. The aerial mycelium branched monopodially and formed loose spiral chains and *Rectiflexibiles* chains of spores with more than 30 spores per chain. The spores had a smooth surface and were cylindrical in shape with a size of 0.5~0.8 × 0.7~1.0 μm. No sclerotic granules, sporangia or zoospores were observed (Fig. 1).

The results of cultural characteristics are shown in Table 1. The aerial mycelium was brownish gray on yeast extract - malt extract agar and glycerol - asparagine agar. Part of the colony became black and moist, and exhibited hygroscopic characteristics on most agar media. On the reverse side, the growth was violet brown on yeast extract - malt extract agar and glycerol - asparagine agar, and reddish brown on oatmeal agar. This mycelial pigment was pH sensitive, changing from red to dark violet with addition of 0.05 N NaOH. Melanoid pigments were produced in Tryptone - yeast extract broth, peptone - yeast extract - iron agar and tyrosine agar. A trace of pale pink pigment was observed in oatmeal agar and glycerol - asparagine agar. This soluble pigment was pH sensitive, changing from pink to violet with addition of 0.05 N NaOH.

Analysis of whole cell hydrolysates of strain No. 89009 showed the presence of LL-diamino-pimelic acid.

Physiological properties and utilization of carbon sources of strain No. 89009 are shown in Tables 2 and 3, respectively.

Based on the taxonomic properties described above, strain No. 89009 is considered to belong to the genus *Streptomyces* and to be a strain of the gray color series of the PRIDHAM and TRESNER grouping[12]. A culture of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM

Fig. 1. Scanning electron micrograph of aerial mycelia of strain No. 89009 grown on yeast extract - malt extract agar 30°C for 14 days.
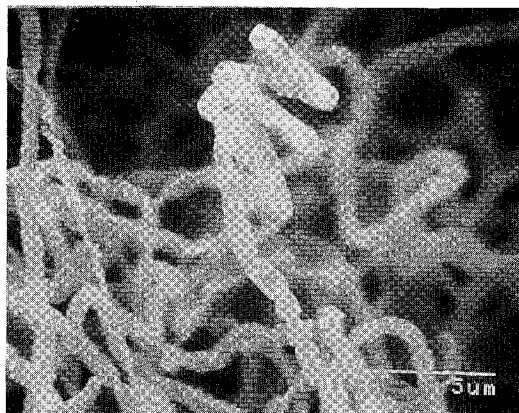
Bar represents 5 μm.

Table 1.   Cultural characteristics of strain No. 89009.

Medium		Cultural characteristics
Yeast extract - malt extract agar	G:	Good
	A:	Abundant, brownish gray (8E2) to black
	R:	Violet brown (11F6)
	S:	None
Oatmeal agar	G:	Good
	A:	Abundant, brownish gray (9E2, 9F2)
	R:	Reddish brown (9E7)
	S:	Pale pink
Inorganic salts - starch agar	G:	Good
	A:	Abundant, brownish gray (7E2) to black
	R:	Grayish orange (6B5)
	S:	None
Glycerol - asparagine agar	G:	Good
	A:	Abundant, brownish gray (6E2)
	R:	Violet brown (11F8)
	S:	Trace of pale pink
Peptone - yeast extract - iron agar	G:	Moderate
	A:	None
	R:	Brown (6E4)
	S:	Pale brown
Tyrosine agar	G:	Good
	A:	Abundant, brownish gray (9E2) to black
	R:	Dark violet (15F8)
	S:	Brown
Sucrose - nitrate agar	G:	Moderate
	A:	None
	R:	Yellowish white (4A2)
	S:	None

Abbreviation: G, growth; A, aerial mycelium; R, reverse side color; S, soluble pigment.

Table 2.   Physiological properties of strain No. 89009.

Conditions	Characteristics
Temperature range for growth	13 ~ 42°C
Optimum temperature range for growth	30 ~ 35°C
Gelatin liquefaction	Negative
Milk coagulation	Negative
Milk peptonization	Positive
Starch hydrolysis	Positive
Production of melanoid pigments	Positive
Decomposition of cellulose	Negative
Production of H_2S	Negative

Table 3.   Carbon utilization of strain No. 89009.

Compounds	Growth
D-Glucose	+
Sucrose	+
D-Xylose	+
D-Fructose	+
L-Rhamnose	+
Raffinose	±
L-Arabinose	+
Inositol	+
D-Mannitol	+

+: Utilization, ±: doubtful utilization.

BP-2474 (deposited date: 14th, June, 1989).

Production of WS009 A and B

Fig. 2 presents the data from a typical 4,000-liter fermentation and gives information regarding WS009 A and B production, pH and packed cell volume. WS009 A and B production reached a maximum after 6 days of cultivation and the yields were 35 μg/ml and 0.7 μg/ml, respectively.

Isolation and Purification

The isolation scheme is shown in Fig. 3. The cultured broth was filtered with the aid of diatomaceous earth (50 kg). The pH of the filtrate (2,550 liters) was adjusted to 2.0, and then the filtrate was passed through a Diaion SP-207 (170 liters, Mitsubishi Chemical Ind. Co., Ltd.) column. The column was washed with water (1,600 liters) and eluted with 50% aqueous methanol (800 liters; WS009 B fraction) and methanol (440 liters; WS009 A fraction).

i)   WS009 A Fraction: The methanol eluate fraction was diluted with 30 liters of water and then concentrated to about 20 liters *in vacuo*. An aqueous concentrated eluate was adjusted to pH 2.0 with 6 N HCl and then extracted with 60 liters of ethyl acetate. The ethyl acetate extract was concentrated *in vacuo*. The resultant material was dissolved with 50% aqueous methanol (2 liters) and then applied to DEAE-Toyopearl 650C (30 liters; Cl⁻ type, Tosoh Co., Ltd.). The column was washed with water (300 liters) and then eluted with 300 liters of 0.03 M NaCl. The rich cut from DEAE-Toyopearl 650C separation was absorbed to Diaion SP-207 (10 liters) column. The column was washed with water (100 liters), 50%
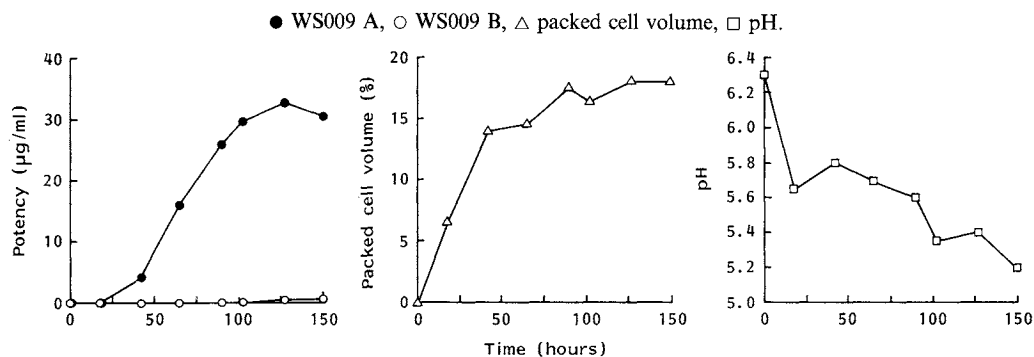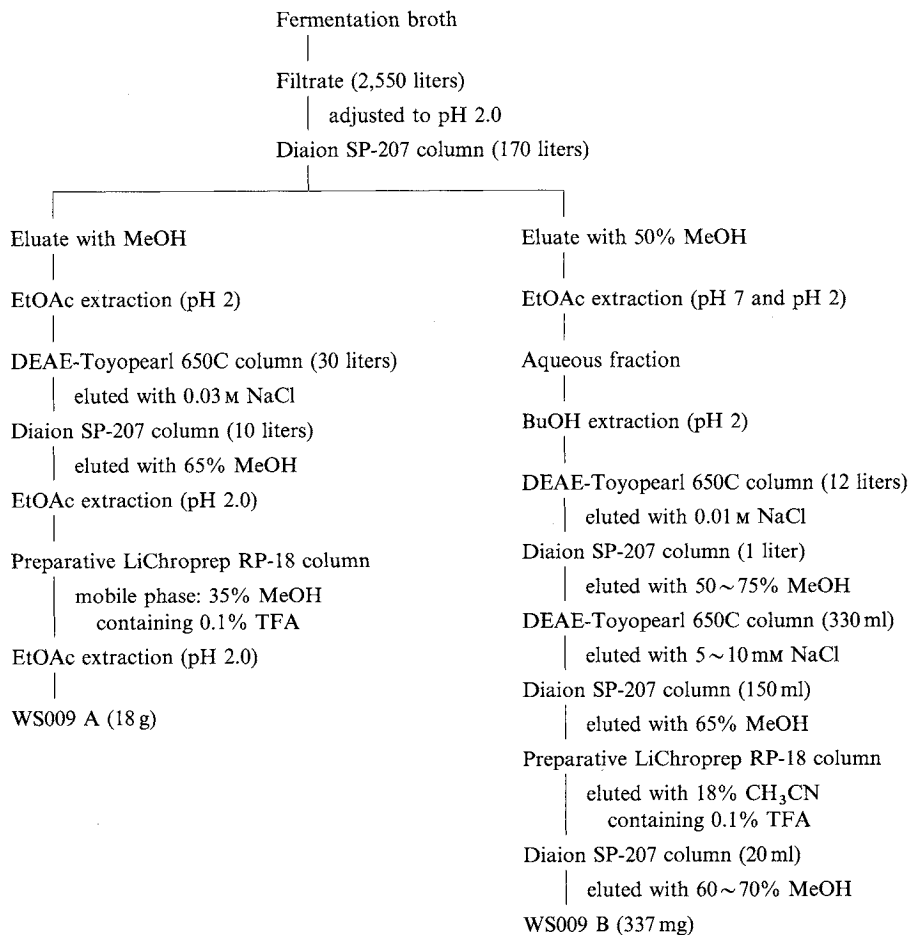
Fig. 2.   Time course of fermentation.

● WS009 A, ○ WS009 B, △ packed cell volume, □ pH.

Time (hours)

Fig. 3.   Isolation procedure of WS009 A and B.

Fermentation broth
|
Filtrate (2,550 liters)
| adjusted to pH 2.0
Diaion SP-207 column (170 liters)
|

Eluate with MeOH
|
EtOAc extraction (pH 2)
|
DEAE-Toyopearl 650C column (30 liters)
| eluted with 0.03 M NaCl
Diaion SP-207 column (10 liters)
| eluted with 65% MeOH
EtOAc extraction (pH 2.0)
|
Preparative LiChroprep RP-18 column
| mobile phase: 35% MeOH
| containing 0.1% TFA
EtOAc extraction (pH 2.0)
|
WS009 A (18 g)

Eluate with 50% MeOH
|
EtOAc extraction (pH 7 and pH 2)
|
Aqueous fraction
|
BuOH extraction (pH 2)
|
DEAE-Toyopearl 650C column (12 liters)
| eluted with 0.01 M NaCl
Diaion SP-207 column (1 liter)
| eluted with 50~75% MeOH
DEAE-Toyopearl 650C column (330 ml)
| eluted with 5~10 mM NaCl
Diaion SP-207 column (150 ml)
| eluted with 65% MeOH
Preparative LiChroprep RP-18 column
| eluted with 18% CH₃CN
| containing 0.1% TFA
Diaion SP-207 column (20 ml)
| eluted with 60~70% MeOH
WS009 B (337 mg)

aqueous methanol (40 liters) and then eluted with 65% aqueous methanol (130 liters). The eluate was concentrated to a volume of 3 liters, adjusted to pH 2.0 with 1 N HCl and then extracted with ethyl acetate (6 liters). After concentration of the ethyl acetate extract, the concentrate was dissolved in methanol (1.2 liters). This solution was diluted with water (1.2 liters), and then applied to a pre-packed column

of LiChroprep RP-18 (43 ~ 62 μm, size C, E. Merck Co., Ltd.) equilibrated with 35% aqueous methanol in 0.1% TFA solution. The column was washed with 7 liters of the same solution and eluted with 35% aqueous methanol in 0.1% TFA. The elution was monitored by UV at 230 nm. The required fractions were combined, concentrated to 1,000 ml and then loaded onto a Diaion SP-207 column (200 ml). After washing with water, it was eluted with methanol. The active eluate was concentrated *in vacuo* and weighed to give 18 g of pure WS009 A substance.

ii) WS009 B Fraction: The 50% aqueous methanol fraction was neutralized to pH 7.00 with 6 N NaOH and then concentrated to about 20 liters *in vacuo*. This concentrate was extracted with ethyl acetate (40 liters) and the solvent was discarded. The aqueous layer was adjusted to pH 2.0 with 6 N HCl and then extracted with 40 liters of ethyl acetate and the solvent extract was discarded. The remaining aqueous layer was extracted with *n*-butanol (20 liters). This extract containing WS009 B was concentrated to a small volume and then diluted to 60 liters with water. This solution was applied to DEAE-Toyopearl 650C (12 liters; Cl⁻ type, Tosoh). The column was washed with water (70 liters) and then eluted with 116 liters of 0.01 M NaCl. The rich cut from DEAE-Toypearl 650C separation was absorbed to Diaion SP-207 (1 liter) column. The column was washed with water (10 liters), 25% aqueous methanol (4 liters) and 30% aqueous methanol (2 liters) and then eluted with 50% aqueous methanol (4 liters) and 75% aqueous methanol (2 liters). The eluate was concentrated to a volume of 1.6 liters (pH 3). This solution was rechromatographed on DEAE-Toyopearl 650C (330 ml). The column washed with water (900 ml) and 0.002 M NaCl (900 ml), was eluted with 0.005 M NaCl (900 ml) and 0.01 M NaCl (2,400 ml). Active fractions were combined, adjusted to pH 2.1 with 1 N HCl and then desalted by using a Diaion SP-207 column (150 ml). The eluate from Diaion SP-207 was concentrated to 10 ml, and then applied to a pre-packed column of LiChroprep RP-18 (43 ~ 62 μm, size C, E. Merck) equilibrated with 18% acetonitrile in 0.1% TFA solution. The column was washed with 1,000 ml of the same solution and eluted with 18% acetonitrile in 0.1% TFA. The elution was monitored by UV at 230 nm. The required fractions were combined, concentrated to 200 ml and then loaded onto a Diaion SP-207 column (20 ml). After washing with water and 40% aqueous methanol, it was eluted with 60% aqueous methanol and 70% aqueous methanol. The active eluate was concentrated *in vacuo* and weighed to give 337 mg of pure WS009 B substance.

Physico-chemical Properties

The physico-chemical properties of WS009 A and B are summarized in Table 4. WS009 A and B are soluble in methanol and slightly soluble in water and ethyl acetate and insoluble in hexane. The color reactions of WS009 A and B are as follows: Positive to iodine vapor and cerium sulfate, though negative to ninhydrin and Molisch reagent.

The ^{1}H NMR spectra of WS009 A and B are shown in Figs. 4 and 5. ^{13}C NMR spectra of WS009 A and B are shown in Figs. 6 and 7.

Structure Elucidation of WS009 A (1)

HRFAB-MS measurement of WS009 A (1) established its molecular formula to be $C_{24}H_{25}NO_{10}S$ (Table 4). The ^{13}C and DEPT NMR experiments showed the presence of 2 CH$_3$, 4 CH$_2$, 1 sp^3 CH, 4 sp^2 CH, 4 sp^3 quaternary, 4 sp^2 quaternary and 5 carbonyl carbons (Table 5). The remaining four unsaturations not accounted for by the 13 sp^2 carbons had to be satisfied by four rings.

Treatment of **1** with diazomethane gave its methyl ester (**2**) (FAB-MS m/z 534 (M+H)$^+$; IR 1740 cm^{-1}) in high yield (89%). A combined analysis of ^{1}H-^{1}H COSY and ^{13}C-^{1}H COSY of **2** suggested the

Table 4. Physico-chemical properties of WS009 A and WS009 B.

	WS009 A	WS009 B
Appearance	Colorless powder	Colorless powder
Melting point	$170 \sim 172°C$	$156 \sim 159°C$ (decomposition)
$[\alpha]_D^{23}$	$+92°$ (c 1.0, MeOH)	$+148°$ (c 0.5, MeOH)
UV λ_{max}^{MeOH}	233, 245 (sh), 354	232, 248 (sh), 354
$\lambda_{max}^{MeOH+HCl}$	233, 245 (sh), 354	234, 248 (sh), 354
$\lambda_{max}^{MeOH+NaOH}$	233, 410	234, 412
Molecular formula	$C_{24}H_{25}NO_{10}S$	$C_{24}H_{25}NO_{11}S$
HRFAB-MS Calcd $(M+H)^+$	520.1277	536.1227
Found $(M+H)^+$	520.1279	536.1223
Elemental analysis	(Calcd for $C_{24}H_{25}NO_{10}S \cdot H_2O$)	(Calcd for $C_{24}H_{25}NO_{11}S \cdot H_2O$)
Calcd:	C 53.63, H 5.06, N 2.61, S 5.97	C 52.08, H 4.92, N 2.53, S 5.79
Found:	C 53.93, H 5.28, N 2.55, S 4.50	C 52.16, H 5.24, N 2.76, S 5.08
Solubility		
Soluble:	MeOH, acetone	MeOH, EtOH
Slightly soluble:	H_2O, EtOAc	H_2O, EtOAc, acetone
Insoluble:	n-Hexane	n-Hexane
Color reaction		
Positive:	Cerium sulfate, iodine vapor	Cerium sulfate, iodine vapor
Negative:	Ninhydrin, Molisch, Dragendorff	Ninhydrin, Molisch, Ehrlich
Rf value (TLC, RP-18)	0.50*	0.52**

*   Solvent system: $CH_3CN - H_2O - TFA$ (40 : 60 : 0.1).
** Solvent system: $CH_3CN - H_2O - TFA$ (20 : 80 : 0.1).

Fig. 4. ^1H NMR spectrum of WS009 A (400 MHz in DMSO-d_6).
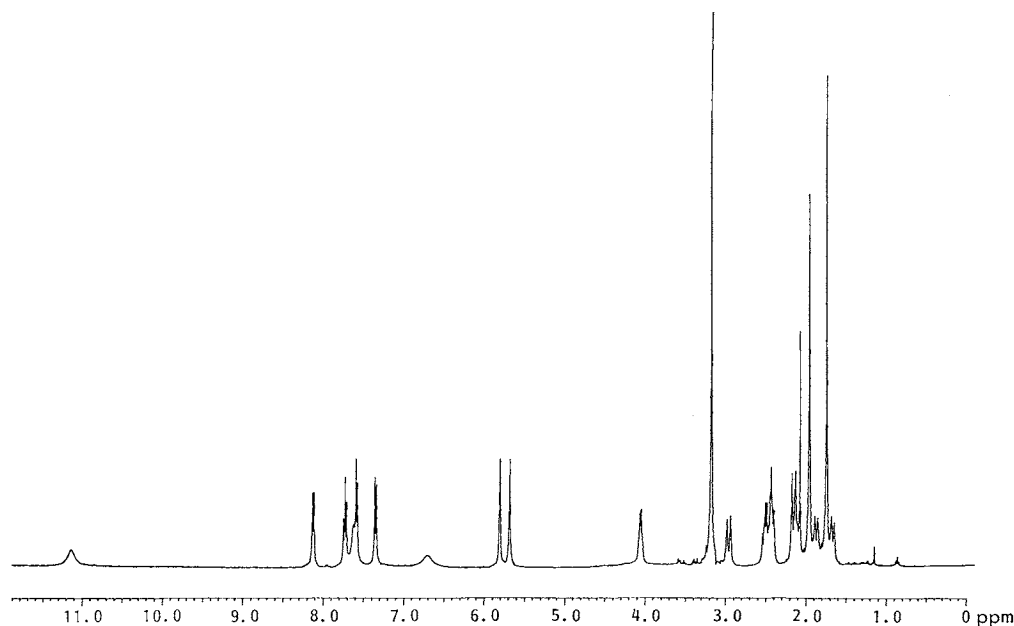
following fragments: Three contiguous aromatic protons: $-CH_2-CH_2-$; $-CO-CH=C(CH_3)CH_2-$; $-CH_2-CH(NH-)-$. The fragments were extended to partial structures **a ~ c** by the extensive analysis of long-range $^{13}C\text{-}^1H$ coupling patterns of **2**, derived from COLOC NMR experiments[13].

The presence of N-acetyl crysteine moiety, inferred from long-range $^{13}C\text{-}^1H$ coupling relationship as

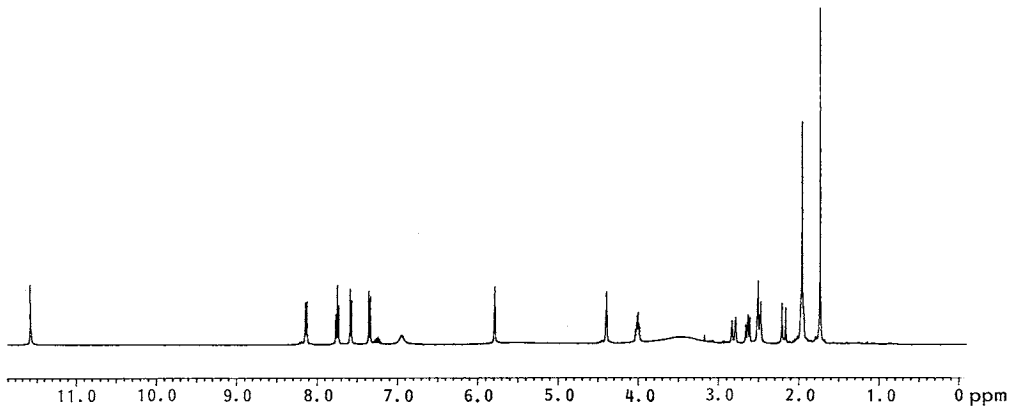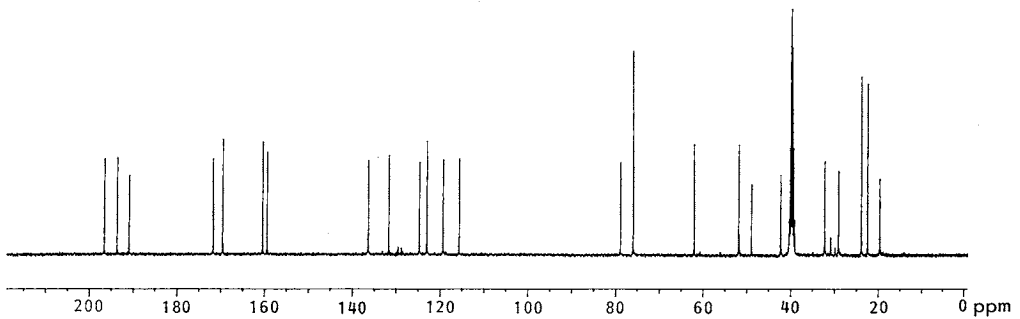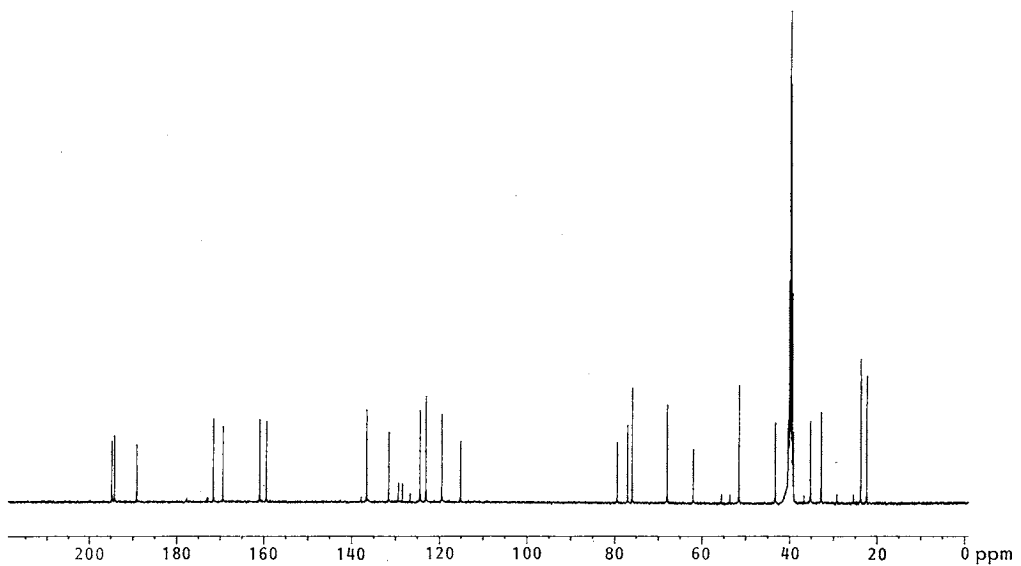Fig. 5. ^{1}H NMR spectrum of WS009 B (400 MHz in DMSO-d_6).

Fig. 6. ^{13}C NMR spectrum of WS009 A (100 MHz in DMSO-d_6).

Fig. 7. ^{13}C NMR spectrum of WS009 B (100 MHz in DMSO-d_6).

Table 5. ^{1}H and ^{13}C NMR data for WS009 A (**1**) and WS009 B (**3**).

Position	1			3		
	^{1}H		^{13}C	^{1}H		^{13}C
	δ^a	Mult. (J, Hz)	δ^b	δ^a	Mult. (J, Hz)	δ^b
1			193.6			194.1
2	5.80	br s	122.9	5.79	m	123.0
3			159.3			159.3
3-CH$_3$	1.96	br s	23.8	1.95	br s	23.7
4	2.96,	d, (18),	42.2	2.80,	d, (18),	43.2
	2.15	d, (18)		2.17	d, (18)	
4a			75.9			75.9
5	2.12,	m,	29.0	2.48,	m,	35.2
	1.67	m		1.90	m	
6	2.40,	m,	19.6	4.39	m	67.9
	1.87	m				
6a			61.9			62.0
7			196.5			194.7
7a			115.6			115.0
8			160.3			160.9
8-OH	11.12	s		11.60	s	
9	7.34	br d, (8)	124.6	7.34	br d, (8)	124.3
10	7.72	dd, (8, 8)	136.3	7.73	dd, (8, 8)	136.5
11	7.58	br d, (8)	119.2	7.58	br d, (8)	119.3
11a			131.6			131.5
12			190.9			189.0
12a			78.8			79.3
12b			75.9			76.9
1'			171.6			171.4
2'	4.06	m	51.8	4.00	m	51.5
3'	2.55,	m,	32.2	2.63,	dd, (13, 8),	32.7
	2.40	m		2.48	m	
NH	8.12	d, (8)		8.12	d, (8)	
COCH$_3$	1.74	s	22.4	1.74	s	22.3
COCH$_3$			169.5			169.3

^{a} 400 MHz in DMSO-d_6.
^{b} 100 MHz in DMSO-d_6.
Mult.: Multiplicity.

Fig. 8. Partial structures **a**, **b** and **c** for **2**.

The arrows indicate the long-range ^{13}C-^{1}H correlations.

a   b   c
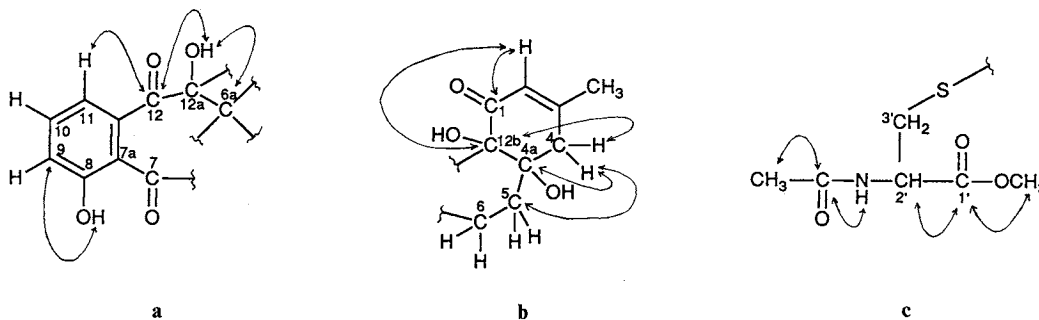
shown in partial structure **c** in Fig. 8, was finally confimed by the detection of cystine in the acid hydrolysate of **1** on an automatic amino acid analyzer. The low field OH signal at 11.26 ppm (OH on C-8) was indicative of strongly hydrogen-bonded phenol hydroxy proton and hence a carbonyl group was presumed to be attached at position 7a. In conjunction with this, the correlations between 8-OH (11.26 ppm) and C-9 (124.8 ppm), 11-H (7.67 ppm) and C-12 (191.9 ppm) and from 12a-OH (6.79 ppm) to C-12 (191.9 ppm) and to C-6a (60.9 ppm) indicated the presence of partial structure **a**. The presence of the cyclohexenone moiety (**b**) was suggested by the long-range coupling patterns shown in Fig. 8.

The connectivity of C-3′ and C-6a through the sulfur atom of **c** was ascertained by the long-range coupling between one of 3′-H$_2$ (2.74 ppm) and C-6a (60.9 ppm). The long-range couplings of one of 6-H$_2$ (1.88 ppm) to C-6a (60.9 ppm) and another of 6-H$_2$ (2.53 ppm) to C-7 (196.0 ppm) enabled us to link C-6 to C-6a and C-6a to C-7. The assumption of a bond between C-12a and C-12b would give the complete carbon skeleton of **2** and was therefore the structure of **1** elucidated to be 2-acetamido-3-[[1,4,4a,5,6,6a,-7,12,12a,12b-decahydro-4a,8,12a,12b-tetrahydro-xy-3-methyl-1,7,12-trioxobenz[*a*]anthracen-6a-yl]-thio]propionic acid (Fig. 9). The complete ^{1}H and ^{13}C chemical shift assignments of **2** are summarized in Table 6.

Structure Elucidation of WS009 B (3)

The molecular formula of the minor congener WS009 B (**3**) was determined to be C$_{24}$H$_{25}$NO$_{11}$S by HRFAB-MS (Table 4). The ^{13}C NMR data of **3** are shown in Table 5 along with those of **1**. The

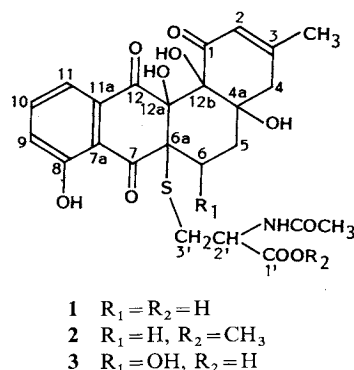Fig. 9. Structures of WS009 A (**1**), WS009 A methyl ester (**2**) and WS009 B (**3**).

1 R$_1$ = R$_2$ = H
2 R$_1$ = H, R$_2$ = CH$_3$
3 R$_1$ = OH, R$_2$ = H

Table 6. ^{1}H and ^{13}C chemical shift assignments of WS009 A methyl ester (**2**).

Position	^{1}H δ^a	Mult. (J, Hz)	^{13}C δ^b	Position	^{1}H δ^a	Mult. (J, Hz)	^{13}C δ^b
1			193.5	9	7.24	br d, (8)	124.8
2	5.87	br s	123.5	10	7.58	dd, (8, 8)	135.9
3			158.6	11	7.67	br d, (8)	119.9
3-CH$_3$	1.96	br s	24.1	11a			131.0
4	2.92,	d, (18),	42.2	12			191.9
	2.16	d, (18)		12a			77.9
4a			76.2	12a-OH	6.79	s	
4a-OH	4.50	br s		12b			76.0
5	2.20,	m,	28.5	12b-OH	5.77	br s	
	1.75	m		1′			170.3
6	2.53,	m,	19.4	2′	4.63	m	51.1
	1.88	m		3′	2.74,	dd, (13, 5),	33.0
6a			60.9		2.49	dd, (13, 6)	
7			196.0	NH	6.13	d, (8)	
7a			114.9	COCH$_3$	1.82	s	22.7
8			161.2	COCH$_3$			170.0
8-OH	11.26	s		OCH$_3$	3.57	s	52.5

^{a} 400 MHz in CDCl$_3$.
^{b} 100 MHz in CDCl$_3$.

^{13}C NMR spectrum of **3** was similar to that of **1**, but displayed a new CH carbon (67.9 ppm) at the expense of CH$_2$ carbon (19.4 ppm, C-6) in **1**. The difference of molecular formulae between **1** and **3** is ascribable to the replacement of CH$_2$ in **1** with CHOH in **3** and thus the structure of **3** was elucidated to be 2-acetamido-3-[[1,4,4a,5,6,6a,7,-12,12a,12b-decahydro-4a,6,8,12a,12b-pentahydro-xy-3-methyl-1,7,12-trioxobenz[*a*]anthracen-6a-yl]thio]propionic acid (Fig. 9). The position of an additional OH group in **3** was consistent with that inferred from polyketide biosynthetic considerations.

Table 7. Comparison of IC$_{50}$ values of WS009 A and B for porcine aorta receptors of ETs.[a]

Compounds	IC$_{50}$ (M)	
	ET-1	ET-2
WS009 A	5.8×10^{-6}	6.9×10^{-6}
WS009 B	6.7×10^{-7}	8.0×10^{-7}

[a] No existence of specific binding for [^{125}I]-ET-3.

Experimental

Preparation of 2

To a solution of **1** (100 mg) in MeOH (10 ml) was added trimethylsilyldiazomethane and the mixture was allowed to stand at room temperature for 30 minutes. Removal of the solvent gave an oil which was purified by preparative TLC (CHCl$_3$ - MeOH, 95:5) to give 92 mg of **2** as a colorless oil: IR ν_{max} (CHCl$_3$) cm^{-1} 3410, 2990, 1740, 1660, 1500; FAB-MS *m/z* 534 (M + H)$^+$; ^{1}H NMR (400 MHz, CDCl$_3$) δ consult Table 6; ^{13}C NMR (100 MHz, CDCl$_3$) δ consult Table 6.

The Detection of Cystine in the Acid Hydrolysate of 1

The suspension of **1** (1 mg) in 6 N HCl (1 ml) was heated at 110°C for 18 hours and evaporated to dryness. The residue was dissolved in 0.1 N HCl (1 ml) and air was bubbled into the mixture for 15 minutes to change cysteine to cystine and then analyzed on a Hitachi 835 automatic amino acid analyzer.

Biological Properties

The biological activities of WS009 A and B in an ET receptor binding assay using porcine aorta membranes are shown in Table 7. The IC$_{50}$ values of WS009 A and B were evaluated for ET-1 and ET-2 binding to porcine aorta membrane receptors.

We could not detected specific binding for ET-3 in the porcine aorta membranes. Our results suggested that ET-3 receptors were not present in the porcine aorta membranes.

Antimicrobial activities of WS009 A and B were evaluated by the serial broth dilution method. No antibiotic activities have been found against *Escheichia coli* NIHJ JC-2, *Staphylococcus aureus* 209P JC-1, *Bacillus subtilis* ATCC 6633, *Candida albicans*, *Aspergillus fumigatus* IFO 5840 at a concentration of 1,000 μg/ml.

Acute toxicity studies with WS009 A were conducted in *dd*Y mice and its LD$_{50}$ value given intraperitoneally was greater than 300 mg/kg and did not show either toxicity or abnormal movements until day 14.

Discussion

There has been much attention on ET antagonists because of the pathological action of ETs. Recently, we[14,15] as well as IHARA *et al.*[16] have reported on the ET antagonists composed of cyclic pentapeptides. In our extensive screening, we have further succeeded in discovering the non-peptidic ET antagonists, WS009 A and B, as described in this paper. Whereas WS009 B and WS-7338 B[15] are almost equally active in antagonizing the effect of ET-1.

As can be shown in Fig. 9, there seem to be no structural similarities between ET and WS009 substances and it is not possible to deduce a mechanism to account for their ET antagonistic activities. WS009 B containing one more-OH residues than WS009 A was approximately 10-fold more potent than WS009 A, which may provide information to investigate the structure-activity relationships of these compounds in the following study of the chemical modifications.

The carbon skeletons of **1** and **3** exhibit well-known benz[a]anthraquinone chromophores, for which HAYAKAWA et al.[17] have proposed the generic name "isotetracenone". The stereochemistry of **1** and **3** remains to be established and its determination will be the subject of future work.

There are many reports on the antibiotics containing benz[a]anthraquinone chromopheres[17~19], most of which are reported to be cytotoxic against various cells. WS009 A and B were shown to have almost no toxicity both *in vitro* and *in vivo*, suggesting that these ET antagonists may be applicable to treating cardiovascular diseases.

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