WS-7338, NEW ENDOTHELIN RECEPTOR ANTAGONISTS ISOLATED FROM *Streptomyces* sp. No. 7338

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

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WS-7338 A, B, C and D, novel endothelin receptor antagonists, have been isolated from fermentation broth of *Streptomyces* sp. No. 7338. These antagonists were purified from the culture mycelium by extraction with acetone, followed by carbon column chromatography and HPLC. Among them, WS-7338 B showed good activity in an endothelin receptor binding assay with an IC_{50} of 2.7×10^{-7} M.

Vascular endothelium has received great attention as a modulator of vascular tone, since endothelium-derived relaxing factor (EDRF) was described by FURCHGOTT in 1980[1]. In recent years it has been observed that vascular endothelium can also mediate vasoconstriction. At present, the known endothelium-derived contracting factors (EDCF) are thromboxane A_2, angiotensin II and endothelin (ET).

ET is a 21-amino-acid polypeptide that has been isolated from supernatants of cultured porcine endothelial cells[2]. A family of mammalian ET isopeptides (ET-1, ET-2 and ET-3) has been identified[3]. Endothelial cells produce and release ET-1, which has potent and long-lasting vasoconstrictor activity *in vivo* and *in vitro*.

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

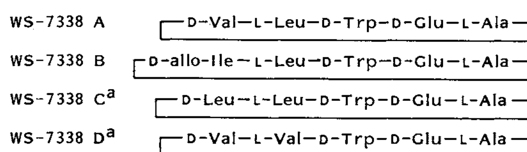
In this paper, we describe the taxonomic studies and fermentation of producing strain, isolation, and physico-chemical properties and biological activities of the ET antagonists, WS-7338 A, B, C and D. As shown in Fig. 1, WS-7338 A, B, C and D are the cyclic pentapeptides. A full description of the structural elucidation of these compounds and total synthesis of WS-7338 B will be discussed in the succeeding paper[4].

Materials and Methods

Taxonomic Studies

Strain No. 7338 was isolated from a soil sample obtained from Yubari, Hokkaido, Japan. The methods described by SHIRLING and GOTTLIEB[5] were empolyed for the taxonomic study. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 14 days on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine

Fig. 1. Structures of WS-7338 A, B, C and D.



[a] Structures of WS-7338 C and D are the tentative structures.

agar. Cultural characteristics were observed on the media described by SHIRLING and GOTTLIEB[5], and WAKSMAN[6]. 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[7]. Wall analysis was performed by the methods of BECKER *et al.*[8], and YAMAGUCHI[9]. 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[10].

Fermentation

A loopful of *Streptomyces* sp. No. 7338 on mature slant culture was transferred into twenty five 500-ml Erlenmeyer flasks each containing 160 ml of sterile seed medium composed of soluble starch 1%, glucose 1%, Pharmamedia (Traders Protein) 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 seed culture was used to inoculate 160 liters of production medium containing starch 2%, gluten meal 0.5%, meat meal 0.5%, Pharmamedia (Traders Protein) 0.3% and dried yeast 0.1% in a 200-liter jar fermenter and this was cultured at 30°C for 3 days under aeration of 160 liters/minute with agitation of 200 rpm. The amount of WS-7338 substances in the fermentaion broth was quantified by HPLC using an Hitachi Model 655 pump. A steel column (4.00 mm inside diameter, 250 mm length) packed with LiChrosorb RP-18 (E. Merck) was used with flow rate of 1.0 ml/minute.

ET Receptor Assay

a) Crude Receptor Membranes 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 homogenates were centrifuged at $10,000 \times g$ for 20 minutes at 4°C. The supernatant, containing the plasma membranes fraction, was centrifuged at $100,000 \times g$ for 60 minutes at 4°C, and then 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.

Porcine brain membranes, kidney membranes, lung membranes and human aorta membranes were prepared using the same procedures mentioned above.

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/m mol) was incubated with 50 μl of aorta membranes preparation in binding assay buffer at room temperature ($20 \sim 22°C$) for 60 minutes in a final volume of 250 μl. After incubation, the mixture were filtered through a glassfiber GF/C filters (pretreatment with 0.1% polyethylene imine 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).

Antimicrobial Activity

The antimicrobial activity of WS-7338 A, B, C and D were determined by a serial broth dilution method in bouillon medium for bacteria and Sabouraud medium for fungi and yeasts. The antimicrobial activity was observed after overnight incubation at 37°C for bacteria and $48 \sim 72$ hours incubation at 28°C for a filamentous fungus and an 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 *Rectus-Flexibilis* chains of spores with more than 20

Fig. 2. Scanning electron micrograph of aerial mycelia of strain No. 7338 grown on yeast extract-malt extract agar at 30°C for 14 days.

(A) Bar represents 20 μm. (B) Bar represents 2 μm.

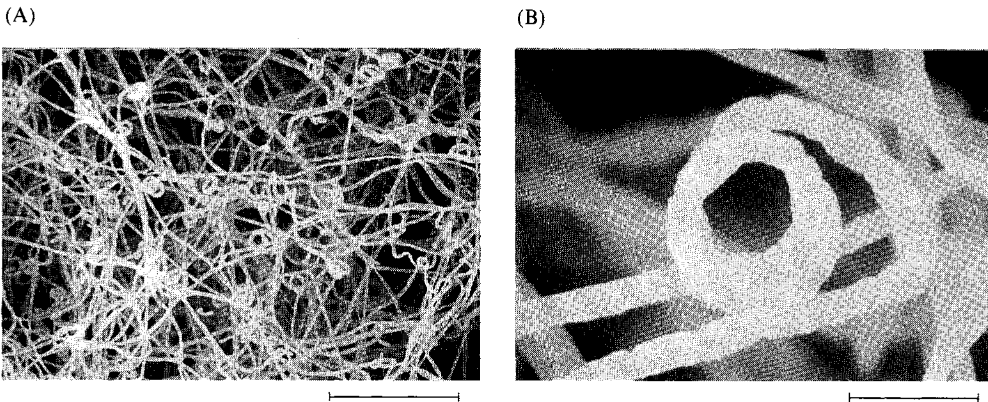


Table 1. Cultural characteristics of strain No. 7338.

Medium		Cultural characteristics	Medium		Cultural characteristics
Yeast extract-malt extract agar	G:	Good	Peptone-yeast extrat agar	G:	Moderate
	A:	Moderate, reddish gray (7B2)		A:	None
	R:	Dark brown (7F6)		R:	Grayish brown (6B4)
	S:	None		S:	None
Oatmeal agar	G:	Moderate	Tyrosine agar	G:	Moderate
	A:	Poor, white		A:	Poor, pale gray (B2)
	R:	Pale yellow (3A3) to reddish brown (9E4)		S:	Trace of reddish brown
	S:	None	Nutrient agar	G:	Moderate
Inorganic salts-starch agar	G:	Good		A:	None
	A:	Abundant, white		R:	Pale yellow (3A3)
	R:	Violet brown (11E4) to dark brown (9F6)		S:	None
	S:	None			
Glycerol-asparagine agar	G:	Good			
	A:	Moderate, reddish gray (12C2)			
	R:	Violet brown (11F8)			
	S:	Pale brown			

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

spores per chain. The spores had a smooth surface and were cylindrical in shape with a size of $0.5 \sim 0.7 \times 1.0 \sim 1.3\ \mu m$. No sclerotic granules, sporangia or zoospores were observed (Fig. 2).

The results of cultural characteristics are shown in Table 1. The aerial mycelium was reddish gray on yeast extract-malt extract agar and glycerol-asparagine agar. On the reverse side the growth was dark brown on yeast extract-malt extract agar and glycerol-asparagine agar, and reddish brown on oatmeal agar. This mycelial pigment was not pH sensitive. Melanoid pigments were not produced in Tryptone-yeast extract broth, peptone-yeast extract-iron agar and tyrosine agar. A trace of pale reddish brown pigment was observed in glycerol-asparagine agar and tyrosine agar. This soluble pigment was pH sensitive, changing slightly from brown to yellow with addition of 0.05 N HCl, and from brown to pink with addition of 0.05 N NaOH.

Table 2. Physiological properties of strain No. 7338.

Conditions	Characteristics
Temperature range for growth	11°C~35°C
Optimum temperature for growth	30°C
Gelatin liquefaction	Negative
Milk coagulation	Negative
Milk peptonization	Negative
Starch hydrolysis	Positive
Production of melanoid pigments	Negative
Decomposition of cellulose	Negative
Production of H_2S	Negative

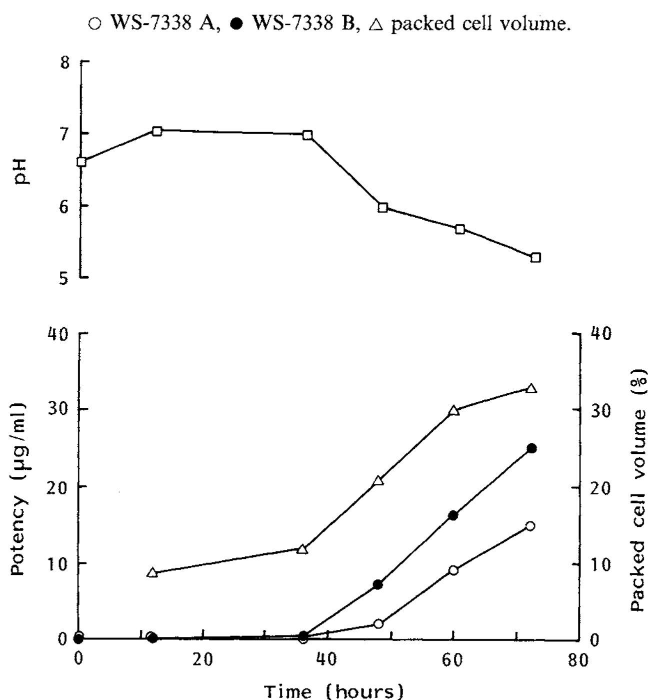
Table 3. Carbon utilization of the strain No. 7338.

Compound	Growth
D-Glucose	+
Sucrose	+
D-Xylose	±
D-Fructose	+
L-Rhamnose	−
Inositol	+
Mannitol	+

+: Utilization, ±: doubtful utilization; −: no utilization.

Fig. 3. Time course of fermentation.

○ WS-7338 A, ● WS-7338 B, △ packed cell volume.



Analysis of whole cell hydrolysates of strain No. 7338 showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

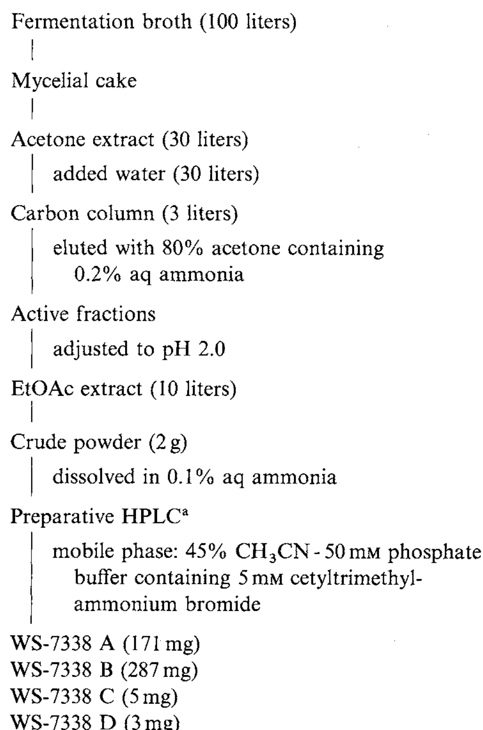
Physiological properties and utilization of carbon sources are shown in Tables 2 and 3, respectively.

Based on the taxonomic properties described above, strain No. 7338 is considered to belong to the genus *Streptomyces* and to be a strain of gray or red color series of the PRIDHAM and TRESNER grouping[11]. A culture of this strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM BP-2550 (deposited date: August 11, 1989).

Production of WS-7338 A, B, C and D

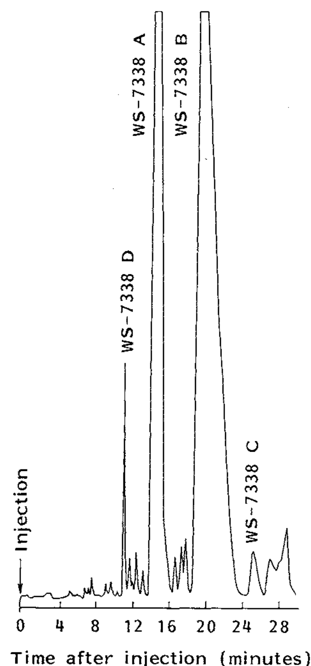
Fig. 3 presents the data from a typical 200-liter fermentation and gives information regarding WS-7338 A and B production, pH and packed cell volume. WS-7338 A and B production reached a maximum after 3 days of cultivation and the yields were 25 μg/ml and 15 μg/ml, respectively. The yields of WS-7338 C

Fig. 4. Isolation procedure of WS-7338 A, B, C and D.



[a] HPLC profile is shown in Fig. 5.

Fig. 5. HPLC profile of WS-7388 A, B, C and D.



Column: ODS-5 (Yamamura Chem., 250 × 20 i.d.), mobile phase: 45% acetonitrile - 50 mM phosphate buffer containing 5 mM cetyltrimethylammonium bromide, flow rate: 15 ml/minute, detection: UV 230 nm, apparatus: Jasco 880-PU.

and D were less than 1 μg/ml.

Isolation and Purification

The isolation scheme is shown in Fig. 4. The cultured broth (100 liters) was filtered with the aid of diatomaceous earth (1 kg). The mycelial cake was treated with 30 liters of acetone and stirred for 60 minutes. The mycelial extract (30 liters) was diluted with 30 liters of water and the solution was absorbed on an activated carbon column (3 liters). After washing with 15 liters of water the active fractions were eluted with 10 liters of 80% aqueous acetone containing 0.2% ammonia. The eluate was adjusted to pH 2.0 with 6 N HCl and extracted with 10 liters of ethyl acetate. The extract was concentrated *in vacuo* to give a crude powder (2 g). This powder was dissolved in 100 ml of 0.1% ammonia water and the solution was applied to a preparative HPLC (column; YMC pack ODS-5, 20 i.d. × 250 mm, flow rate: 15 ml/minute) and the column was eluted with 45% acetonitrile - 50 mM phosphate buffer (pH 7.0) containing 5 mM cetyltrimethylammonium bromide. The HPLC profile of WS-7338 A, B, C and D is shown in Fig. 5. The active fractions of WS-7338 A, B, C and D were collected. Each of the active fractions were passed through a Dowex 50W (H^+ type, 400 ml) column and then applied to SP-207 (Mitsubishi Chemical Ind. Co., Ltd.) column (50 ml). The active fractions were eluted with 80% methanol (200 ml) and concentrated under reduced pressure to give white powder. The yields of pure WS-7338 A, B, C and D from 100 liters of whole broth were 171 mg, 287 mg, 5 mg and 3 mg, respectively.

Table 4. Physico-chemical properties of WS-7338 A, B, C and D.

	WS-7338 A	WS-7338 B	WS-7338 C[a]	WS-7338 D[a]
Appearance	Colorless powder	Colorless powder	Colorless powder	Colorless powder
Optical rotation $[\alpha]_D^{23}$	$-10°$ (*c* 1.0, DMSO)	$-7°$ (*c* 1.0, DMSO)		
UV λ_{max}^{MeOH} nm	280, 289	280, 289	280, 289	280, 289
IR cm^{-1} (KBr)	3270, 2950, 1700, 1650, 1635, 1540, 1440, 1380, 1340, 1220, 1170, 1150, 1120, 1090, 1000	3270, 2950, 1700, 1650, 1635, 1540, 1440, 1380, 1340, 1220, 1170, 1150, 1120, 1090, 1000		
Molecular formula	$C_{30}H_{42}N_6O_7$	$C_{31}H_{44}N_6O_7$	$C_{31}H_{44}N_6O_7$	$C_{29}H_{40}N_6O_7$
HRFAB-MS				
Calcd $(M+Na)^+$	621.3027	635.3169	635.3169	607.2856
Found $(M+Na)^+$	621.3013	635.3167	635.3166	607.2845
Elemental analysis	Calcd for $C_{30}H_{42}N_6O_7 \cdot H_2O$	Calcd for $C_{31}H_{44}N_6O_7 \cdot \frac{1}{2}H_2O$		
Calcd:	C 58.43, H 7.19, N 13.63	C 59.89, H 7.30, N 13.52		
Found:	C 58.83, H 7.13, N 13.33	C 60.13, H 7.14, N 12.47		
Composed amino acids	D-Glu, L-Ala, D-Val, L-Leu, D-Trp	D-Glu, L-Ala, D-aIle, L-Leu, D-Trp	D-Glu, L-Ala, D-Leu, L-Leu, D-Trp	D-Glu, L-Ala, D-Val, L-Val, D-Trp
TLC (RP-18) Rf value[b]	0.34	0.30	0.35	0.28

[a] Because of small quantities of WS-7338 C amd WS-7338 D, select data are listed.

[b] Acetonitrile - 50 mM phosphate buffer in 5 mM cetyltrimethylammonium bromide (pH 7.0) (45 : 55).

Physico-chemical Properties

The physico-chemical properties of WS-7338 A, B, C and D are summarized in Table 4. WS-7338 A, B, C, and D are soluble in DMSO and aqueous ammonia and insoluble in chloroform and hexane. Color reactions of WS-7338 A, B, C and D are as follows; positive to iodine vapor, cerium sulfate and Ehrlich reagent, though negative to ninhydrin, Molisch reagent, Dragendorff reagent and ferric chloride.

WS-7338 C and D are the minor components and their fermentation productivity is very poor, so only selected data of WS-7338 C and D are listed in Table 4.

The ^{1}H NMR spectra of WS-7338 A and B are shown in Figs. 6 and 7. ^{13}C NMR spectra of WS-7338 A and B are shown in Figs. 8 and 9.

Fig. 6. ^{1}H NMR spectrum of WS-7338 A (400 MHz in DMSO-d_6).

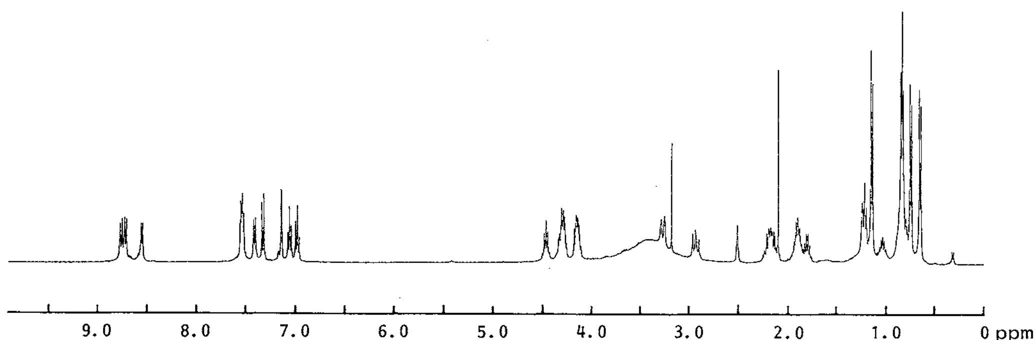


Fig. 7. ^{1}H NMR spectrum of WS-7338 B (400 MHz in DMSO-d_6).

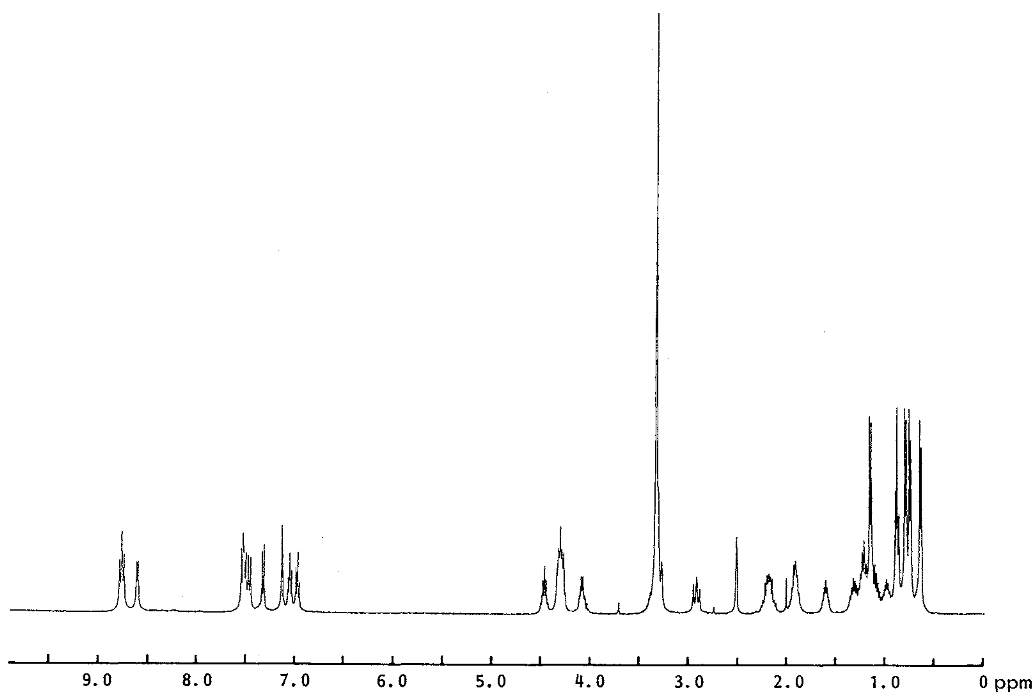


Fig. 8. ^{13}C NMR spectrum of WS-7338 A (100 MHz in DMSO-d_6).

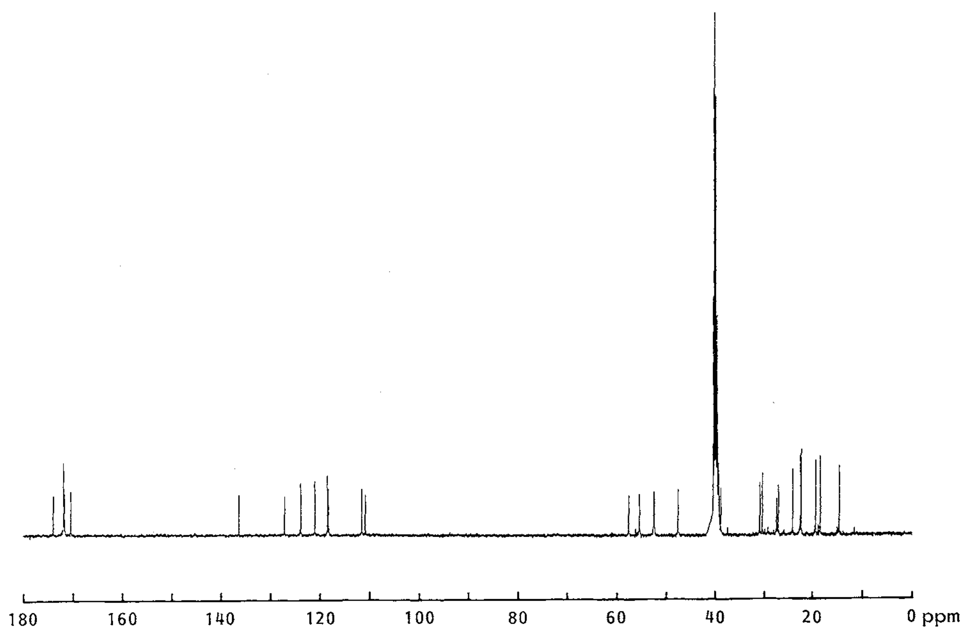
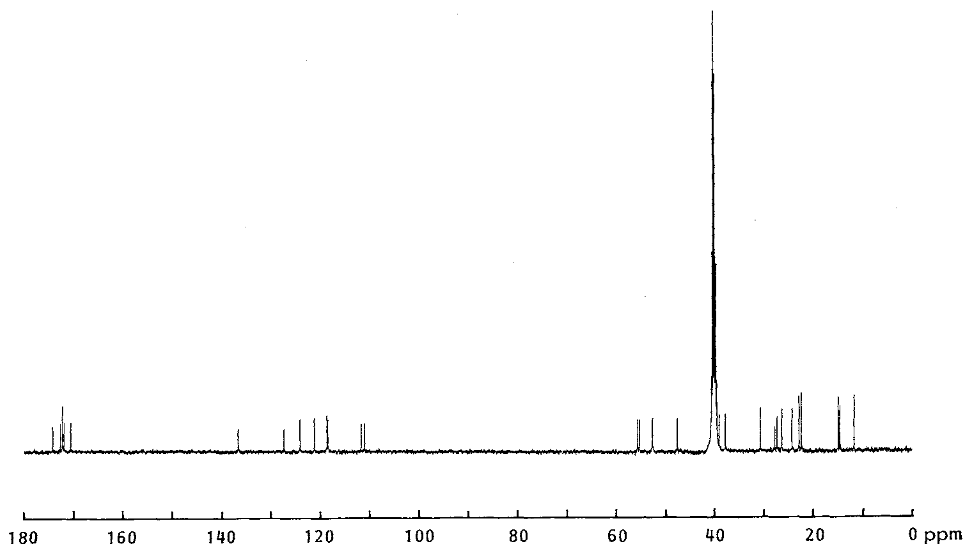


Fig. 9. ^{13}C NMR spectrum of WS-7338 B (100 MHz in DMSO-d_6).



Of these WS-7338 compounds, WS-7338 B† was the most active ET-1 binding antagonist, and a total synthesis of this congener was carried out to confirm the chemical structure of WS-7338 B. The amino acid analyses showed each of these compounds to be composed of 5 amino acids. They compose a closely related family of cyclic pentapeptides. Structural elucidation and total synthesis of WS-7338 B will be

† Added by Editorial Office: After preparation of this paper it was noticed that Banyu group reported a compounds BE-18257B in Biochem. Biophys. Res. Commun. 178: 132~137, July 15th, 1991 which is identical with WS-7338 B.

published elsewhere[4].

Biological Properties

The antagonistic activities of WS-7338 A, B, C and D in the ET receptor binding assay were shown in Table 5. The IC_{50} values of WS-7338 A, B, C and D were evaluated for ET-1 and ET-2 binding to porcine aorta membrane receptors. Of these WS-7338 compounds, WS-7338 B was the most potent ET-1 binding antagonist.

Table 5. Comparison of IC_{50} values of WS-7338 A, B, C and D for porcine aorta receptors of ETs[a].

Compound	IC_{50} (M)	
	ET-1	ET-2
WS-7338 A	9.3×10^{-7}	2.3×10^{-6}
WS-7338 B	2.7×10^{-7}	4.8×10^{-7}
WS-7338 C	2.0×10^{-6}	4.4×10^{-6}
WS-7338 D	4.3×10^{-5}	8.1×10^{-5}

[a] ET-3 receptors were not present in the porcine aorta membrane.

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 WS-7338 A and B were evaluated by serial broth dilution method. No antibiotic activities have been found against *Escherichia coli* NIHJ JC-2, *Staphylococcus aureus* 209P JC-1, *Bacillus subtilis* ATCC 6633, *Candida albicans* and *Aspergillus fumigatus* IFO 5840 at 1,000 μg/ml.

Discussion

On inspection of the amino acid sequence of WS-7338 A, B, C and D (Fig. 1), the common amino acid sequence (D-Trp-D-Glu-L-Ala) is conserved, so this sequence may be involved in the essential role for receptor antagonist activity. It may be worthwhile investigating the chemical modification of WS-7338 A, B, C and D in order to increase the ET-1 antagonist activity or to study the structure-activity relationship of derivatives of these unique lead compounds.

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