COCHINMICINS, NOVEL AND POTENT CYCLODEPSIPEPTIDE ENDOTHELIN ANTAGONISTS FROM A *Microbispora* sp.†

I. PRODUCTION, ISOLATION, AND CHARACTERIZATION

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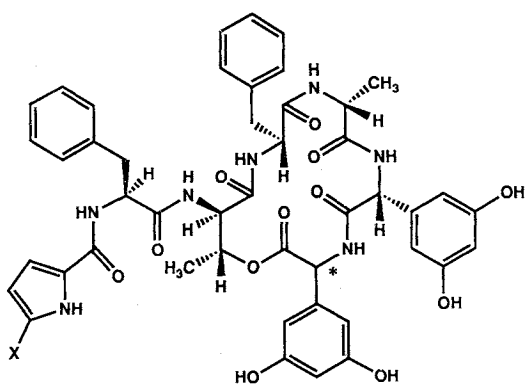
Cochinmicins I, II, III are novel peptolides produced in submerged-fermentation cultures of *Microbispora* sp. ATCC 55140. These closely related compounds are separated by HPLC and are novel competitive endothelin antagonists. Cochinmicins II and III are stereoisomeric to each other. Cochinmicin I is the deschloro analog of cochinmicin III.

Endothelin-1 (ET-1), and two closely related peptide hormones, endothelin-2 (ET-2) and endothelin-3 (ET-3), are widely distributed in mammalian tissues. They can induce numerous biological responses in vascular and non-vascular tissues by binding to two distinct endothelin receptor subtypes. ET-1 is a highly potent constrictor of vascular smooth muscle *in vitro* and pressor agent *in vivo*. Circulating levels of ET-1 have been found to be elevated in cyclosporin nephrotoxicity, myocardial infarction, uremia, diabetes mellitus, systemic hypertension, endotoxic shock, cardiac ischemia, post-ischemic renal failure and compromised renal flow. Coronary and cerebral artery vasospasm are also associated with elevated levels of ET-1. Antagonism at the ET receptor(s) can therefore be potential treatment of disease states caused by elevated levels of ET.

In our microbial screening for endothelin antagonists using a bovine aortic preparation, we discovered a new class of cyclodepsipeptides in a culture of a *Microbispora* sp. ATCC 55140. We gave them the trivial names cochinmicins I (**1**), II (**2**) and III (**3**) (Fig. 1). The most unusual structural features

Fig. 1. Structure of cochinmicins I~III.



	Cochinmicin	X	*
1	I	H	*R*
2	II	Cl	*S*
3	III	Cl	*R*

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in these metabolites are their high content of D-amino acid (especially in the macrocyclic lactone portion) and the presence of the rare amino acid residues; 3,5-dihydroxyphenylglycine (D and/or L) and pyrrole-2-carboxylic acid. This paper describes the cultural characteristics of the producer, fermentation, isolation, and physico-chemical and biochemical characteristics of these microbial products. Details of the structure determination, including chemical shift assignments, primary sequence and absolute stereochemistry, are described in ZINK *et al.*, succeeding paper[1].

Materials and Methods

Morphological and Cultural Characteristics of the Strain

The producing organism was isolated from a soil sample collected in Cochin, India.

Observations of growth and general cultural characteristics, as well as determination of carbon utilization pattern, were made in accordance with the methods of SHIRLING and GOTTLIEB[2]. Coloration of the culture was determined by comparison with color standards contained in the Inter-Society Color Council-National Bureau of Standards Centroid Color Charts[3]. Chemical composition of the cells was determined using the methods of LECHEVALIER and LECHEVALIER[4]. Identification is based upon a comparison of laboratory data with descriptive characteristics used for differentiation of *Microbispora* sp. in the literature[5]. The strain was deposited in the American Type Culture Collection, Rockville, Maryland, U.S.A., with the accession No. ATCC 55140.

Fermentation

A seed culture was produced by inoculating 50 ml of aqueous nutrient medium in a 250-ml triple baffled Erlenmeyer flask with 2 ml of refrigerated or thawed frozen vegetative mycelia. The nutrient medium consisted of glucose 10.0 g, soluble starch 20.0 g, yeast extract 5.0 g, N-Z amine E 5.0 g, $CaCO_3$ 1.0 g, beef extract 3.0 g and Bacto-peptone 5.0 g in 1 liter water, adjusted to pH 7.0 with NaOH prior to $CaCO_3$ addition. The culture vessel was incubated at 28°C and shaken at 220 rpm for 96 hours. 2 ml of seed culture was aseptically transferred to 44 ml of production medium in a 250-ml non-baffled Erlenmeyer flask. The production medium contained dextrin 50 g, soybean flour 30.0 g, Difco peptone 1.0 g and $CaCO_3$ 5.0 g in 1 liter water, adjusted to pH 7.0 with NaOH prior to $CaCO_3$ addition. The production medium was incubated at 28°C and 220 rpm for 10 days prior to harvest.

Isolation

Fractionation was guided by inhibition of [^{125}I]ET-1 binding to bovine aortic membranes. The fermentation broth (2.7 liters) was adjusted to pH 7.2 with dil hydrochloric acid and extracted for 1 hour with methyl ethyl ketone (3.8 liters). The inactive aqueous layer was discarded. The active organic layer (1.34 g dry weight) was purified on a column of Silica gel 60 (E. Merck, 50 g, 40~63 μm) in dichloromethane with stepwise elution of dichloromethane (180 ml), MeOH - dichloromethane, 5:95 (v/v, 5 × 100 ml), MeOH - dichloromethane, 1:9 (v/v, 2 × 100 and 200 ml) and 200 ml each of MeOH - dichloromethane, 1:4, 3:7, 1:1 and 1:0 (v/v). [^{125}I]ET-1/bovine aorta binding data showed that the second and third 10% MeOH - dichloromethane eluates were inhibitory. These fractions were pooled (dry weight = 264 mg) and purified on a Whatman Partisil 10 ODS-3 column (2.21 × 25 cm) at 40°C and 15 ml/minute using a 40~55~100% MeOH (aq) gradient elution (0~5 minutes, at 40%; 5~65 minutes, from 40 to 55%, linear; 65~120 minutes, from 55 to 100%, linear). One minute fractions were collected. Analytical HPLC suggested pooling fractions 41~46, 51~55, and 64~72. Thus flash evaporation of solvents gave, in order of elution, 8.8 mg of **1**, 88 mg of **2** and 33 mg of **3**.

Physico-chemical and HPLC Analyses

UV spectra were recorded with a Beckman model DU-70 spectrophotometer. IR spectra were recorded as a neat deposit on a Zn-Se crystal with a Perkin-Elmer model 1750 FT-IR spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were recorded in DMSO-d_6 on a Varian SC-300 or XL-400 NMR spectrometer. Chemical shifts are given in ppm relative to TMS at zero ppm using the solvent peaks at 2.49 ppm (for

^1H spectra) and 39.5 ppm (for ^{13}C spectra) as internal standard. MS data were recorded on a Finnigan-MAT90 (FAB) and TSQ70B (FAB, EI) mass spectrometers. Exact mass measurements were performed on the MAT90 at high resolution using CsI as the internal standard. Optical rotation was measured on a Perkin-Elmer model 241 polarimeter.

HPLC analyses were performed on a Whatman Partisil 5 ODS-3 column (4.6 × 100 mm). The column was eluted at 1 ml/minute flow rate with 45% MeOH (aq) at 40°C. The effluent was monitored at 215 or 270 nm.

Chemical Conversion of **3** to **1**

3 (0.80 g, 0.87 mmol) was dissolved in methanol (6 ml) and kept under H_2 (2.81 kg/cm^2) in the presence of 5% Pd/C (0.20 g) and MgO (0.20 g) for 15 hours. HPLC analysis revealed ~98% completion of conversion. The reaction mixture was filtered over Celite and Whatman No. 3 filter paper. Flash evaporation, under reduced pressure below 40°C, to dryness afforded 0.79 g of material. Purification on a Whatman Partisil 10 ODS-3 column (2.21 × 50 cm) was performed using 50% MeOH (aq) as the isocratic mobile phase for elution at 10 ml/minute at room temperature. The effluent was monitored at 215 nm. 1.5-minute fractions were collected. HPLC analyses suggested pooling fractions 34~40 (vol of elution = 495~600 ml), which was flash evaporated to dryness, under reduced pressure at below 40°C, to afford 0.40 g of semi-synthetic **1**, identical in HPLC retention time, ^1H NMR, UV, IR and MS to the natural product. 16 mg of starting **3** was recovered from fractions 61~80.

Biochemical Evaluation/Assays

The [^{125}I]ET-1 binding assays were performed essentially as described by AMBAR *et al.*[6] and KLOOG *et al.*[7] with minor modifications[8]. ET-1 was purchased from Peptides International (Louisville, KY). [^{125}I]ET-1 was purchased from Amersham (Arlington Heights, IL). Membranes were prepared from rat hippocampus (ET-B sites), and rat or cow aorta (ET-A site). Dissected tissue was homogenized twice for 30 seconds with a Brinkman Polytron (setting 10, Generator PTA 20 TS (Westbury, NY)) in ice cold 250 mM sucrose, 50 mM Tris-HCl pH 7.4 with 7 μg/ml pepstatin A and 0.5 μg/ml leupeptin. The crude particulate matter was removed by centrifugation at 750 × *g* for 10 minutes. The membranes were sedimented from the supernatant fraction by centrifugation at 48,000 × *g* for 30 minutes. Membrane pellets were resuspended in the above buffer with protease inhibitors. Aliquots of these suspensions were stored at −70°C.

Binding studies with [^{125}I]ET-1 were conducted in 50 mM potassium phosphate pH 7.5 with 0.1% bovine serum albumin (BSA) using 12-well Skatron (Lier, Norway) cell harvester tube strips. [^{125}I]ET-1 concentrations were 25 pM for hippocampus and 150 pM for aorta. Samples were dissolved in DMSO. Upon addition of the sample, the final DMSO concentration was 3%. Membranes were added last to start the binding reaction. The reaction mixture was incubated at 37°C for 30 or 60 minutes. Binding reactions were terminated using a Skatron cell harvester by filtration through glass fiber filter pads presoaked with 2% bovine serum albumin. The samples on the pads were immediately washed with 150 mM NaCl 0.1% bovine serum albumin. The pads were punched out and radioactivity was evaluated in a Beckman Gamma 5500 gamma counter (Fullerton, CA). Nonspecific binding was determined in the presence of 100 nM ET-1 and was found to be 10 and 35% of the maximum binding, for ET-B and ET-A respectively.

Other radioligand binding assays, including [^{3}H]oxytocin, [^{3}H]Arg-vasopressin, [^{125}I]angiotensin, [^{3}H]neurotensin and [^{3}H]bradykinin, were performed according to established methods[9~12].

The effect of cochinmicins on ET-1 induced phosphoinositide hydrolysis in rat atrial mince was determined as described by WILLIAMS *et al.*[8].

Results and Discussion

Morphological and Cultural Characteristics of Strain ATCC 55140

Strain ATCC 55140 is Gram stain positive and filamentous. The substrate mycelium is long, non-fragmenting, irregularly branched and approximately 0.76 μm in diameter. Aerial mycelia arises from the substrate mycelium and is monopodially branched. Spores are borne in pairs on short sporophores that attach to the aerial mycelium.

Table 1. Cultural characteristics of ATCC 55140.

Medium	Amount of growth	Aerial mycelia and/or sporophores	Soluble pigments	Reverse color
Yeast-malt extract	Moderate	Rudimentary aerial growth, no spores observed	None	Deep yellow brown (78d. yBr). Iodinin crystals present
Glucose asparagine	Moderate	Sparely developed, few bisporate chains	None	Pale yellow pink (31p. yPink). Iodinin crystals present
Inorganic salts-starch	Good, slight hydrolysis of starch	Sparely developed, few bisporate chains	None	Light yellow brown (76l. yBr). Iodinin crystals present
Oatmeal	Good	Well developed, numerous bisporate chains throughout	None	Light yellow brown (76l. yBr)
Σ-water-NZamine	Moderate	Well developed, bisporate chains throughout	None	Yellow white (92y White)
CZAPEK	Fair	Limited development, no spores observed	None	Light yellow brown (76l. yBr). Iodinin crystals present
Peptone iron	Good		Melanin negative	

Observation after incubation at 27°C for 25 days.

Color names and numbers (in parentheses) from Inter-Society Color Council-National Bureau of Standards Centroid Color Charts, US Dept. of Commerce National Bureau of Standards supplement to NBS Circular 553, 1985.

The cultural characteristics of ATCC 55140 grown at 27°C for 25 days on various media are described in Table 1. Moderate to good growth was observed on yeast-malt extract (YME), glucose asparagine (GAs), inorganic salts-starch (ISS), oatmeal and Σ-water-NZamine agars. Fair growth was observed on CZAPEK's agar. Culture also grows in YED broth. Growth is observed at 27 and 37°C with greater growth at the elevated temperature. On oatmeal agar for 25 days at 27°C the substrate mycelium is light yellow brown. The aerial mycelium is white and forms at the colony edge. Colonies are opaque and raised with an entire margin and rough surface. The colonies are rubbery in texture. On GAs aerial mycelia have a pale yellow pink coloration. Biotin is not required for growth. Iodinin crystals are formed on GAs, ISS, YME and CZAPEK's agars. Hydrogen sulfide is produced on peptone iron agar (lead acetate strip method). Starch is weakly hydrolyzed. Neither melanoid nor soluble pigments are produced under the stated conditions of growth.

Table 2. Carbohydrate utilization pattern of ATCC 55140.

Carbon source	Utilization by ATCC 55140
D-Arabinose	1
L-Arabinose	3
Cellobiose	3
D-Fructose	3
Inositol	3
D-Lactose	3
D-Maltose	3
D-Mannitol	3
D-Mannose	3
D-Raffinose	1
D-Rhamnose	1
Sucrose	3
D-Xylose	3
L-Xylose	1
D-Glucose (control)	3

Observation after incubation at 27°C for 21 days.

3, Good utilization; 2, moderate utilization; 1, poor utilization; 0, no utilization.

The carbon utilization pattern of ATCC 55140 is summarized in Table 2. Good utilization of L-arabinose, cellobiose, D-fructose, D-glucose, inositol, D-lactose, D-maltose, D-mannitol, D-mannose, sucrose and D-xylose, and poor utilization of D-arabinose, D-raffinose, L-rhamnose and L-xylose were observed.

Analysis of the peptidoglycans showed that the strain contained *meso*-diaminopimelic acid. Whole cell carbohydrate analysis revealed the presence of glucose, madurose and traces of galactose; supporting the assignment of cell wall type III.

The results of these chemotaxonomic, morphological and physiological studies indicate that ATCC 55140 is a strain belonging to the genus *Microbispora*. All members of this genus have a type III B cell wall (*meso*-diaminopimelic acid and madurose) and produce spores in logitudinal pairs on short sporophores on the aerial mycelium. A comparison of laboratory data with descriptive characteristics used for differentiation of *Microbispora* sp. was made. At present, there are eleven validly named species in this genus. Of the seven mesophillic species of *Microbispora* only two exhibit significant phenotypic similarity with ATCC 55140; *M. amethystogenes* and *M. parva*. Both of these species are reported to produce iodinin crystals on starch containing media and both have yellow brown substrate mycelia. These two species differ from ATCC 55140 with regard to the hydrolysis of starch (*M. amethystogenes* is negative), inositol utilization (*M. parva* is negative) and rhamnose utilization (*M. parva* is positive).

ATCC 55140 can be readily differentiated from *Streptomyces* sp. S-16328 which produces the immunosuppressant substances 55185 RP and 59451 RP[13]. *Streptomyces* sp. S-16328 posses a type I cell wall (LL-DAP and no diagnostic sugars), and a different mode of sporulation (long chams of spores on flexible sporophores). Based upon this information, it is clear that ATCC 55140 is the first microbispora reported to produce cochinmicins.

Isolation and Chemical Characterization

The isolation procedure described is a refined and practical version based on experience gained on a pilot fermentation.

UV, FT-IR and ^{1}H NMR spectra recorded on the HPLC homogeneous isolates suggested the three components to be closely structurally related. Their physico-chemical characteristics are shown in Table 3. Since **1** can be derived from **3** by hydrogenolytic deschlorination, **1** is deschloro-**3**. **2** and **3** are isomeric. In addition, **1** and **3** are levorotatory while **2** is dextrorotatory in optical rotatory power.

Of these three components, only the weakest endothelin binding inhibitor, but most abundant, **2**, might have been discovered previously from the *Streptomyces* sp. S-16328 as 55185 RP[13]. The stereochemistry of 55185 RP and its deschloro analog, 59451 RP, was not reported. Although a direct

Table 3. Physico-chemical properties of **1**, **2** and **3**.

	1	**2**	**3**
Molecular formula	$C_{46}H_{47}N_7O_{12}$	$C_{46}H_{46}N_7O_{12}Cl$	$C_{46}H_{46}N_7O_{12}Cl$
HRFAB-MS ($(M+H)^+$ m/z)			
Found:	890.3380	924.3005	924.2949
Calcd:	890.3361	924.2971	924.2971
$[\alpha]_D^{23}$	−10.0° (*c* 0.1, MeOH)	+20.0° (*c* 0.1, MeOH)	−10.0° (*c* 0.1, MeOH)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	212 (446), 230 (sh, 172), 269 (175)	213 (483), 230 (sh, 208), 275 (219)	212 (471), 230 (sh, 185), 274 (207)
FT-IR (ZnSe) ν_{max} cm^{-1}	3299, 1733, 1661, 1623, 1603, 1555, 1516	3304, 1734, 1657, 1607, 1521	3304, 1733, 1662, 1558, 1519
HPLC[a] Rt (minutes)	4.8	6.1	9.4

sh: Shoulder.

[a] Whatman Partisil 5 ODS-3, 4.6 × 100 mm; MeOH - H_2O (45 : 55); flow rate, 1 ml/minute; 40°C.

comparison of **2** with 55185 RP have not been made at this time, the ^{13}C NMR signals of these two compounds were almost identical. (The 9 chemical shift differences of $>0.2\sim<0.9$ ppm in their ^{13}C NMR spectra may be caused by difference in recording temperature.)

The more potent and biologically interesting (see below) but less abundant **1** and **3** are new and are isomeric with but stereochemically distinct from 59451 RP and 55185 RP[1].

Biochemical Characterization

The dose-response competition of [^{125}I]ET-1 and cochinmicins I, II and III in binding to rat aorta and rat hippocampus are depicted in Figs. 2 and 3, respectively.

Scatchard analysis of [^{125}I]ET-1 binding to rat aortic membranes, in the absence of cochinmicins

Fig. 2. Competition of [^{125}I]ET-1 and **1**, **2** and **3** in binding to rat aorta.
● **1**, □ **2**, ▲ **3**.

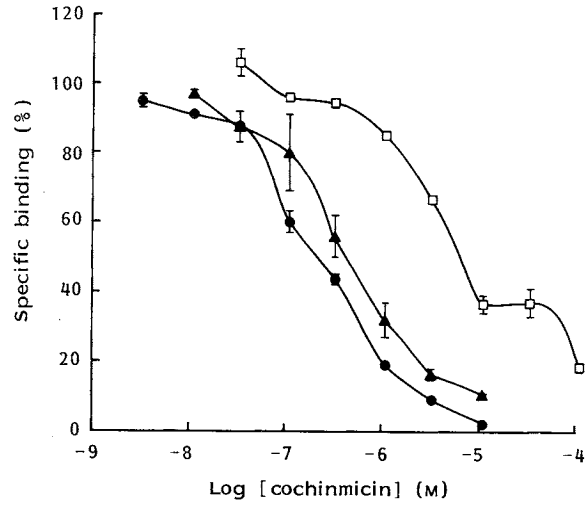


Fig. 3. Competition of [^{125}I]ET-1 and **1**, **2** and **3** in binding to rat hippocampus.
● **1**, □ **2**, ▲ **3**.

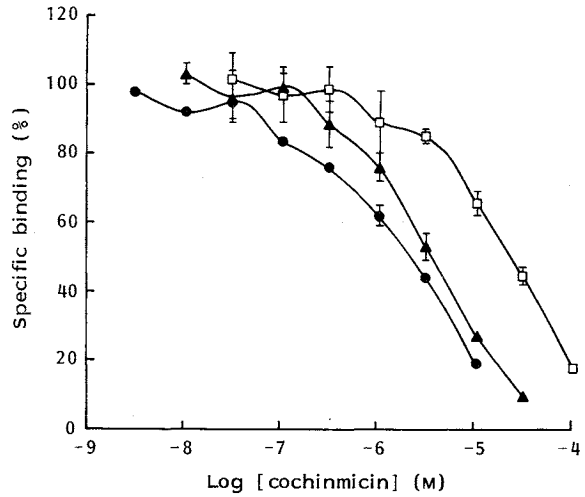


Table 4. Summary of radioligand binding affinities for **1**, **2** and **3**.

	Tissue	Ligand	**1**	**2**	**3**
IC_{50} (μM)	Rat aorta	[^{125}I]ET-1	0.24±0.02	6.2±0.03	0.42±0.11
~*Ki* (μM)			0.12	3.1	0.21
IC_{50} (μM)	Rat	[^{125}I]ET-1	1.5±0.1	22±2	3.7±0.6
~*Ki* (μM)	hippocampus		0.2	3	0.5
IC_{50} (μM)	Rat uterus	[^{3}H]Oxytocin	5.1	1.8	1.8
% Inhibition (μM)	Rat liver	[^{3}H]Arg-vasopressin	2 (2.2 μM)	91 (22 μM)	67 (5.4 μM)
% Inhibition (μM)	Rat kidney	[^{3}H]Arg-vasopressin	15 (2.2 μM)	91 (22 μM)	60 (5.4 μM)
% Inhibition (μM)	Rabbit aorta	[^{125}I]Angiotensin	8 (2.2 μM)	21 (22 μM)	19 (5.4 μM)
% Inhibition (μM)	Rat brain	[^{3}H]Neurotensin	23 (2.2 μM)	14 (22 μM)	21 (5.4 μM)
% Inhibition (μM)	Rat uterus	[^{3}H]Bradykinin	7 (11 μM)	7 (433 μM)	31 (32 μM)

Fig. 4. Scatchard analysis of [^{125}I]ET-1 binding to rat aortic membranes—effects of **2** and **3**.

○ Control, *Kd*=110 pM, □ 5 μM (**2**), *Kd*=320 pM, *Ki*=2~4 μM, ▲ 2 μM (**3**), *Kd*=950 pM, *Ki*=0.28 μM. Bmax ~8 pM (20 fmoles/mg).

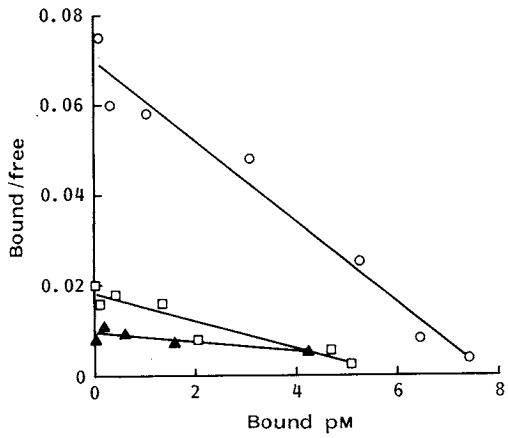
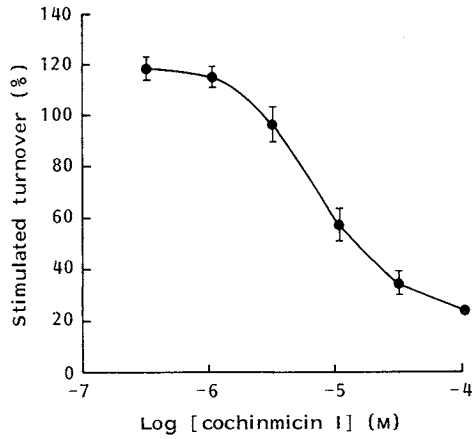


Fig. 5. Effect of **1** on rat atrial ET-1 stimulated phosphatidyl inositol turnover.

[ET-1]=10 nM.



and the presence of 5 μM **2** or 2 μM **3** (Fig. 4) suggests reversible inhibition by these compounds.

Radioligand binding affinities for **1**, **2** and **3** are summarized in Table 4. **1** is the most potent and selective for ET-1 binding.

Most importantly, **1** blocked ET-1 stimulated phosphatidyl inositol turnover in a dose-dependent fashion in a rat atria mince with an EC_{50} of 10.8 μM (Fig. 5). **1** is the first ET-A and ET-B sites nonselective antagonist reported.

55185 RP and 59451 RP were claimed to be suppressants of cellular immunity at 100 nM~10 μM concentrations *in vitro*. **2** did not show any T-cell activation response at 1.1 nM~11 μM levels in a primary murine T-cell culture and possessed little affinity for [^{125}I]charybdotoxin binding to Jurkat membranes[14] (data not shown).

Antibiotic Activity

1, **2** and **3** were tested for antimicrobial activity against a broad variety of bacteria and fungi on agar plates. Only **3** showed very weak/insignificant activity against some Gram-positive bacteria and fungi, but totally inactive against Gram-negative bacteria (data not shown). The other components were devoid of

antimicrobial activity.

All-D-magainin-2 was recently synthesized and reported to be highly resistant to proteolysis and exhibited antibacterial potency similar to that of its all-L-enantiomer[15]. We noticed that the more interesting components described in this paper, **1** and **3**, share all-D-amino acid residues in the macrocycle lactone core. It may be worthwhile to explore the therapeutic potential of the present compounds or their derivatives.

Acknowledgments

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