Notes

ADDITIONAL COCHINMICINS FROM A *Microbispora* sp.

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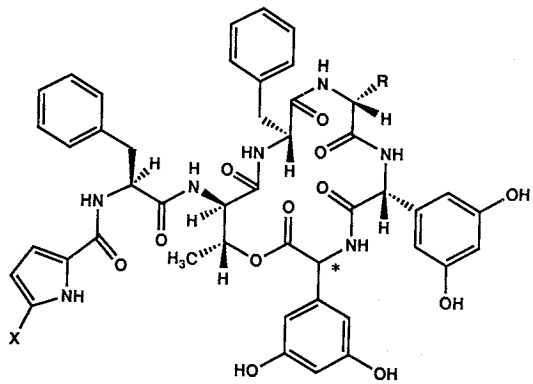
Recently, we have described the discovery of cochinmicins I (**1**), II (**2**) and III (**3**) (Fig. 1) as endothelin antagonists from the fermentation of a *Microbispora* sp., ATCC 55140[1,2]. Subsequently, in working with a directly scaled up fermentation, we found two additional components, IV (**4**) and V (**5**), in sufficient amounts for characterization. In this paper, we will present the isolation as well as physico-chemical and biochemical characterization of these two new metabolites.

In the present fermentation, the seed nutrient and production media employed were the same as those reported previously[1]. A two stage seed method of 4 and then 0.8% inoculum was used with incubation at 28°C and 220 rpm for 96 and 72 hours, respectively. Production of cochinmicins was carried out in 75-liter stainless steel fermenters containing 50 liters of the production medium. An inoculum of 3.0% was used. The fermentation was carried out at 25°C and pH 6.4~7.3, under an aeration of 15 liters/minute and a stirrer speed of 400~500 rpm. Production of **2** was followed by HPLC analysis. A time course of this fermentation in a 75-liter fermenter is shown in Fig. 2. Production of **2** starts during log phase and reaches a maximum at 9 to 10 days of fermentation followed by a sharp decline to 40% of the peak level in 24 hours.

The whole broth from two fermenters (100 liters) was immediately extracted with methylethyl ketone (MEK, 2×100 liters). Flash evaporation of the MEK layer to dryness under reduced pressure at <40°C gave 245 g of crude extract. This crude extract was mixed with CH_2Cl_2 (850 ml) and adsorbed on to an open column of 2.5 kg Silica gel 60 (E. Merck, 0.2~0.5 mm particle size) in the same solvent. Stepwise gradient elution with 7, 25 and 24 liters of 0, 5 and 10% MeOH - CH_2Cl_2, respectively, afforded 32.6 g of cochinmicin enriched fraction in the 10% MeOH - CH_2Cl_2 effluent. In this exercise, the enriched fraction was purified as two

Fig. 1. The structures of cochinmicins I~V (**1**~**5**).



	Cochinmicins	X	R	*
1	I	H	CH_3	*R*
2	II	Cl	CH_3	*S*
3	III	Cl	CH_3	*R*
4	IV	Cl	CH_2OH	*S*
5	V	H	CH_3	*S*

Fig. 2. Time course of production of cochinmicin II (**2**) in a 75-liter fermenter.

○ pH, ● **2**, ▲ dissolved oxygen, △ packed cell volume.

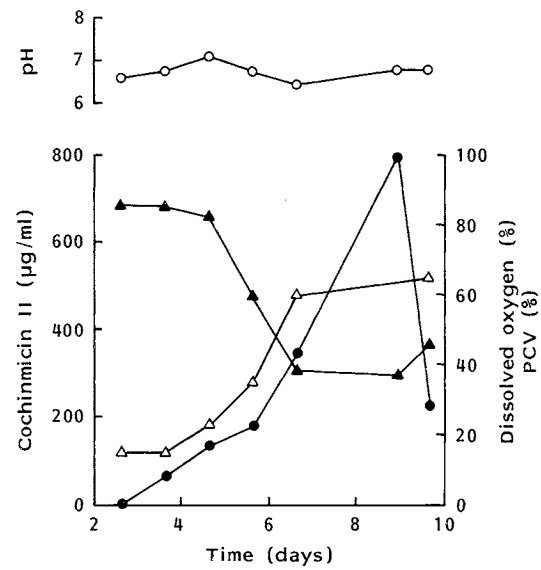


Table 1. Physico-chemical and biochemical characteristics of **4** and **5**.

	4	**5**
Molecular formula	$C_{46}H_{46}N_7O_{13}Cl$	$C_{46}H_{47}N_7O_{12}$
HRFAB-MS ($(M+H)^+$ m/z)		
Found:	940.2921	890.3360
Calcd:	940.2920	890.3361
$[\alpha]_D^{23}$	+30.0° (*c* 0.1 in MeOH)	+20.0° (*c* 0.1 in MeOH)
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	214 (485), 229 (sh, 240), 274 (234)	214 (489), 230 (sh, 237), 270 (224)
FT-IR (ZnSe) ν_{max} cm^{-1}	3309, 1738, 1661, 1607, 1525	3302, 1739, 1661, 1555, 1516
HPLC[a] Rt (minutes)	4.4	3.3
[^{125}I]Endothelin binding:		
IC_{50} (μM)		
Cow aorta	3	90
Rat hippocampus	2	25

sh: Shoulder.

[a] Whatman Partisil 5 ODS-3, 4.6 × 100 mm; MeOH - H_2O (45 : 55); flow rate, 1 ml/minute; 40°C; UV monitor at 215 nm.

injections on a Separations Technology C-18 column (7.6 × 91 cm, 0.02 mm particle size) using 55% MeOH (aq) as the mobile phase for elution at 200 ml/minute and room temperature, monitoring the effluent at 270 nm. This step afforded, in order of elution, 0.66 g of enriched **5**, 0.19 g of homogeneous **4**, 16 g of **2** and 1.47 g of **3** at elution volumes of 9.2 ~ 11.5, 17.4 ~ 19.2, 22.5 ~ 30.7 and 39.0 ~ 47.7 liters, respectively. Thus, using the present procedure, **2**, **3** and **4** can be isolated in two chromatographic steps from whole broth.

The above fraction enriched in **5** was purified as one sample on a Whatman Partisil 10 ODS-3 column (2.21 × 50 cm). Elution was performed with 45% MeOH (aq) at 15 ml/minute and room temperature. The effluent was monitored at 215 nm. Homogeneous **5** (0.49 g) was recovered from elution volumes of 480 ~ 675 ml.

Physico-chemical analyses were performed using methods previously described. The physico-chemical characteristics of **4** and **5** are summarized in Table 1.

Comparison of ^{1}H and ^{13}C NMR data (DMSO-d_6 and CD_3OD) with those reported for **1**, **2** and **3** suggested that **4** and **5** are new members of the cochinmicin family. Of importance, stereochemically both **4** and **5** are **2**-like rather than **3**-like. In addition, both **4** and **5**, like **2**, are dextrorotatory. Structures of the new members were determined by methods described previously.

Using the α-methylbenzyl isothiocyanate method described earlier[2], the total hydrolysate of **4** showed the presence of D-Ser, D-*allo*-Thr, L-dihydroxyphenylglycine, D-dihydroxyphenylglycine, L-Phe and D-Phe. D-Ala was absent. A D-Ser replacement of D-Ala accounted for an increase of 16 mass units of **4** from **2**. **4** possesses ^{1}H NMR (DMSO-d_6, 300 MHz) chemical shifts at δ 1.11 (d, J=6.5 Hz, 3H, D-*allo*-Thr H_γ), 3.71 (br s, 2H, D-Ser H_β), 4.02 (dd, J=5.5, 4.5 Hz, 1H, D-Ser H_α), and pyrrole protons at 6.03 (d, J=4 Hz, 1H), 6.90 (d, J=4 Hz, 1H) and 12.21 (br s, NH) ppm. The ^{13}C NMR spectra of **4** (DMSO-d_6, 75 MHz, coupled, decoupled and APT) showed a quartet at δ 16.6, three triplets at δ 37.1, 38.2 and 60.4, twenty four doublets at δ 53.7, 54.9, 55.7, 56.4, 56.7, 58.4, 70.7, 102.0, 102.2, 106.5 (2×), 107.1 (2×), 111.7, 126.26, 126.35, 128.0 (2×), 128.2 (2×), 129.1 (2×) and 129.2 (2×), and seventeen singlets at δ 117.2, 125.8, 136.9, 137.8, 138.4, 139.4, 158.1 (2×), 158.2 (2×), 159.1, 168.3, 168.5, 169.0, 170.0, 171.4, 171.5 ppm. These data for **4** are consistent with assigning it as [D-Ser^5]**2**.

5 is identical (HPLC retention time, ^{1}H and ^{13}C NMR, UV, IR and MS) to semisynthetic deschloro-**2**, obtained from catalytic hydrogenation of **2** using an analogous method reported for the conversion of **3** to **1**[1]. The ^{1}H NMR spectrum (DMSO-d_6, 300 MHz) of **5** revealed chemical shifts for pyrrole protons at δ 6.06 (dd, 1H, J=2.4, 6 Hz), 6.82 (m, 1H), 6.89 (m, 1H), and δ 11.40 (br d, J=1.8 Hz, NH) ppm. The ^{13}C NMR spectra of **5** (DMSO-d_6, 75 MHz, coupled, decoupled and APT) showed quartets at δ 16.6 and 16.9, two triplets at δ 36.7 and 38.0, twenty six doublets at δ 51.9, 53.9, 55.0, 55.8, 56.1, 56.2, 70.7, 101.9, 102.1, 106.6 (2×), 106.9 (2×), 108.6, 110.7, 121.5, 126.2, 126.3, 128.0 (2×), 128.2 (2×), 129.1 (2×) and 129.2 (2×), and sixteen singlets at δ 125.8, 137.0, 138.0, 138.3, 139.7, 158.0 (2×), 158.2 (2×), 160.2, 168.4, 168.7, 168.9, 171.2, 171.7 and 172.3 ppm.

The [^{125}I]endothelin binding data for **4** and **5**,

obtained by methods described previously, is also summarized in Table 1. **4** showed comparable **2**-like affinities, which are significantly weaker than **1** and **3** for both ET-A and ET-B sites. **5** is the least active member. These observations further corroborate with the notion that the *S* stereochemistry at the carbon marked * (*i.e.* L-dihydroxyphenylglycine) and chloro substitution at the 5-position in the pyrrole is deleterious to inhibiting endothelin binding.

At a 20 μg/disc level, **4** and **5** do not exhibit any antimicrobial activity against *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 6538P), *Micrococcus luteus* (ATCC 9341), *Escherichia coli* (ATCC 9637), *Proteus vulgaris* (ATCC 21100), *Pseudomonas aeruginosa* (ATCC 25619), *Cochliobolus miyabeanus* (ATCC 11608) and *Acholeplasma laidlawii* (ATCC 23206).

Acknowledgments

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