ISOLATION AND CHARACTERIZATION OF NEW ANTIBIOTICS RESORCINOMYCINS A AND B

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New antibiotics, resorcinomycins A and B, were isolated from the culture broth of a streptomycete strain identified as *Streptoverticillium roseoverticillatum*. The antibiotics are water-soluble amphoteric substances, positive to SAKAGUCHI's reagent. The molecular formulas $C_{14}H_{20}N_4O_5$ and $C_{13}H_{18}N_4O_5$ for A and B were indicated by elemental analysis and secondary ion MS. The structures of these antibiotics were determined by ^1H and ^{13}C NMR spectrometry and some chemical evidences to be *N*-[(*S*)-α-guanidino-3,5-dihydroxy-4-isopropylphenylacetyl]glycine and *N*-[(*S*)-α-guanidino-3,5-dihydroxy-4-ethylphenylacetyl]-glycine, respectively.

In the course of our screening program for new antibiotics, a streptomycete strain numbered DO-248 and identified as *Streptoverticillium roseoverticillatum* was found to produce new antibiotics named resorcinomycins A and B††.

These antibiotics are not active against common bacteria, but show weak activity against *Mycoplasma* and strong activity against a variety of acid-fast bacteria. Total synthesis of resorcinomycin A and its stereoisomers have been accomplished[1]. The antibacterial properties of resorcinomycin A was examined in comparison with the stereoisomers. The result will be presented in an accompanying paper[2].

In this paper, the taxonomy of the producing strain, isolation, physico-chemical properties and structure elucidation of resorcinomycins A and B are presented.

Taxonomy

The strain numbered DO-248 was isolated from a soil sample collected at Okinawa Prefecture, Japan. The strain showed the following taxonomic characters.

Morphological Characteristics

The morphology of the culture was microscopically observed on BENNETT's agar at 28°C for 14 days. The aerial mycelium of strain DO-248 branches and exhibits typical whorl formation. The surfaces of spores are smooth under the electron microscope.

Cultural and Physiological Characteristics

The cultural and physiological characteristics of strain DO-248 on various media are summarized

†† Resorcinomycins A and B have been formerly reported as DO-248-A and B in Jpn. Kokai 218396 ('85), Nov. 1, 1985; U.S. 4,647,693, Mar. 3, 1987 and Eur. Pat. Appl. 0,159,004, Oct. 23, 1985.

Table 1. Cultural characteristics of strain DO-248.

Media	Growth	Aerial mycelium		Reverse color	Soluble pigment
		Formation	Color		
Sucrose - nitrate agar	Good	Good	Pink	None to pink	None
Glucose - asparagine agar	Good	Good	Pink to pale pink	Light red	None
Glycerol - asparagine agar	Good	Good	Pink	Pale red	None
Inorganic salts - starch agar	Good	Good	Pinkish white to pale pink	Dull red	None
Tyrosine agar	Good	Good	Pale pink to pink	Dull red	Brownish black (trace)
Nutrient agar	Good	None	—	Grayish brown	Grayish brown
Yeast extract - malt extract agar	Good	Good	Pale pink to pale brown	Reddish orange	Yellowish brown
Oatmeal agar	Good	Good	Pale pink to pale brown	Light red	None
BENNETT's agar	Good	Good	Pale pink to pale orange	Dark reddish orange	Yellowish brown (trace)

in Tables 1 and 2, respectively. The color names described in the table were designated on the basis of the color table in "Guide to Color Standard", published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

Table 2. Physiological properties of strain DO-248.

Production of melanoid pigment	Positive
Tyrosinase reaction	Positive (weak)
Coagulation of milk	Negative
Peptonization of milk	Positive (weak)
Starch hydrolysis	Positive
Gelatin liquefaction	Negative

The utilization of carbon sources by the strain was studied on PRIDHAM and GOTTLIEB's basal agar. D-Glucose, D-fructose, inositol and D-mannitol were utilized; L-arabinose, D-xylose, sucrose, L-rhamnose and raffinose were not utilized. Cell walls contain LL-diaminopimelic acid.

From above characteristics, the strain DO-248 was assigned to the genus *Streptoverticillium*, and closely resembles *S. roseoverticillatum*[3,4] among known species of the genus *Streptoverticillium*. The characteristics of DO-248 were compared with those of the standard strain of *S. roseoverticillatum*, and good agreements were obtained except coagulation of milk. Therefore, strain DO-248 was identified as a strain of *Streptoverticillium roseoverticillatum*. It has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession number FERM BP-745.

Fermentation

The spores of the strain DO-248 were inoculated into a 2-liter Erlenmeyer flask containing 800 ml of a medium consisting of starch 0.5%, glucose 0.5%, Polypeptone 0.5%, meat extract 0.5%, yeast extract 0.25% and NaCl 0.25% (pH 7.0), and cultured at 28°C for 1 day on a rotary shaker. The culture was then transferred into 30-liter jar fermentor containing 20 liters of a medium consisting of starch 2.0%, glucose 2.0%, soy bean meal 2.0%, Bacto-soyton 1.0% and NaCl 0.25% (pH 7.0). Fermentation was carried out at 28°C for 5 days under aeration of 20 liters per minute and agitation of 300 rpm.

Isolation

The culture broth (100 liters) was centrifuged at pH 3.0, and the supernatant was passed through

a Dowex 50W-X4 (NH_4^+) column (10 liters). After water-washing, the column was eluted with 0.3 N NH_4OH. The active eluate was evaporated to remove excess ammonia and adsorbed on a Dowex 1X-2 (Cl^-) column (5 liters) at pH 9.0. The column was eluted with 50% methanol containing 5% NaCl. The active eluate was concentrated to a nearly aqueous solution and then adsorbed on a Diaion HP-20 column (2.5 liters) at pH 7.0. The antibiotics adsorbed were eluted with 50% MeOH. Concentration and freeze-dry of the eluate gave a crude powder (2.2 g).

The crude powder was subjected to chromatography on a QAE-Sephadex A-25 column (200 ml) by linear gradient elution with 20 mM phosphate buffer, pH 7.0 and the same buffer containing 1 M NaCl. The active eluate from the column was then applied to a Diaion CHP-20P column (100 ml). After washing the column with water, the activity was eluted by linear gradient manner with water and 80% MeOH. The active eluate was concentrated and freeze-dried to give a partially purified powder (100 mg).

The powder was then subjected to preparative HPLC on a Nucleosil 30 C_{18} column (20×250 mm) with 50 mM phosphate buffer - methanol (9 : 1). The peak fractions of resorcinomycins A and B were collected respectively. Desaltation with a Diaion CHP-20P column, concentration and freeze-dry afforded colorless powders of resorcinomycins A (60 mg) and B (6 mg).

Physico-chemical Properties and Structure Elucidation

Resorcinomycins A (**1**) and B (**2**) are hardly distinguishable by TLC, but easily separable by reversed phase HPLC showing retention volumes of 8.25 ml for resorcinomycin A and 4.15 ml for resorcinomycin B on a Nucleosil 7 C_{18} column (4×240 mm) with MeOH - 20 mM phosphate buffer, pH 6.5 (3 : 7).

These antibiotics are amphoteric in nature, and the free forms are obtained as colorless powders, soluble in water, methanol, ethanol and dimethylformamide, but sparingly soluble or insoluble in acetone, ethyl acetate, chloroform and ethyl ether. They shows positive reactions with SAKAGUCHI's reagent and ferric chloride. Their IR spectra shows a broad and strong absorption at approximately

Fig. 1. IR spectra of resorcinomycins A (**1**) and B (**2**) (KBr).

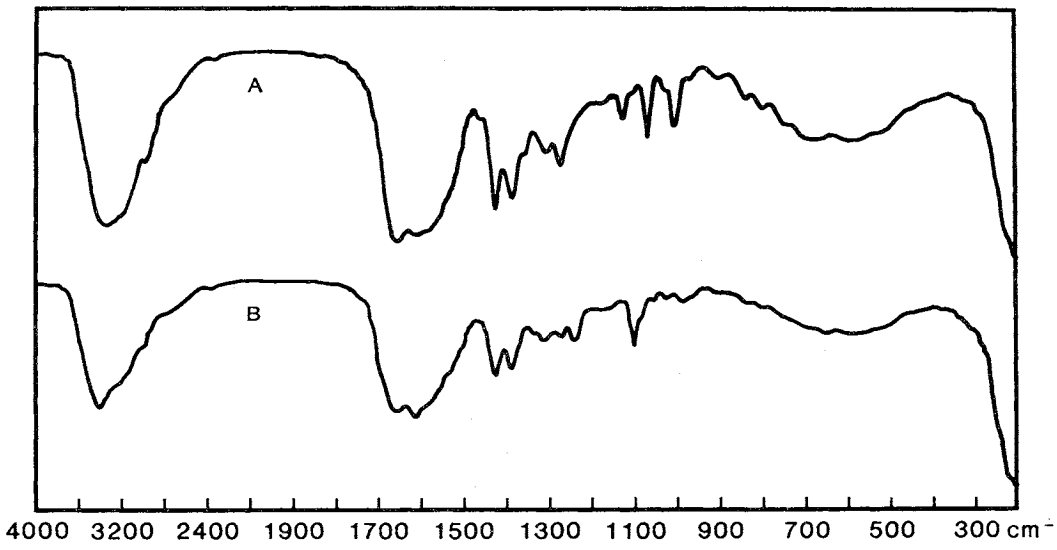


Table 3. Physico-chemical properties of resorcinomycins A and B.

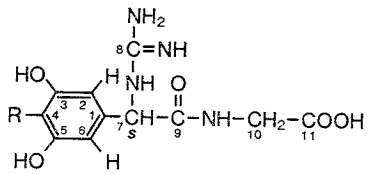
	A	B
MP (°C)	194~200	189~192
Molecular formula	$C_{14}H_{20}N_4O_5 \cdot 2H_2O$	$C_{13}H_{18}N_4O_5 \cdot 2H_2O$
Anal Calcd:	C 46.66, H 6.71, N 15.58.	C 45.08, H 6.40, N 16.18.
Found:	C 47.01, H 6.15, N 15.44.	C 45.26, H 6.13, N 16.13.
SI-MS (M+1) (*m/z*)	325	311
UV λ_{max}^{MeOH} nm (ε)	End absorption, 276 (1,166), 283 (1,166)	End absorption, 276 (1,166), 281.5 (1,116)
$\lambda_{max}^{dil\ HCl\text{-}MeOH}$ nm (ε)	End absorption, 276 (1,166), 283 (1,166)	End absorption, 276 (1,166), 281.5 (1,116)
$\lambda_{max}^{dil\ NaOH\text{-}MeOH}$ nm (ε)	End absorption, 297 (2,398)	End absorption, 297 (2,232)
CD (0.5 N HCl)	$[\theta]_{199}$ 0, $[\theta]_{211}$ +54,500, $[\theta]_{295}$ 0	$[\theta]_{200}$ 0, $[\theta]_{211}$ +51,600, $[\theta]_{295}$ 0

Table 4. ^1H and ^{13}C NMR data for resorcinomycins A and B[a].

Assignment	A		B	
	^1H[b]	^{13}C[c]	^1H[b]	^{13}C[c]
1		134.8 (s)		134.8 (s)
2, 6	6.48 (s)×2	108.1 (d)×2	6.50 (s)×2	107.4 (d)×2
3, 5		156.4 (s)×2		156.0 (s)×2
4		123.8 (s)		120.1 (s)
7	5.10 (s)	59.3 (d)	5.10 (s)	59.4 (d)
8		157.3 (s)		157.3 (s)
9		171.0 (s)		171.0 (s)
10	3.61 (d, 17)	44.4 (t)	3.61 (d, 17)	44.5 (t)
	3.83 (d, 17)		3.83 (d, 17)	
11		177.0 (s)		177.0 (s)
$(CH_3)_2CH$–	3.35 (q),	25.0 (d),		
	1.23 (d, 7.1)×2	20.5 (q)×2		
CH_3CH_2–			2.52 (q, 7.2),	16.9 (t),
			1.00 (t, 7.3)	13.6 (q)

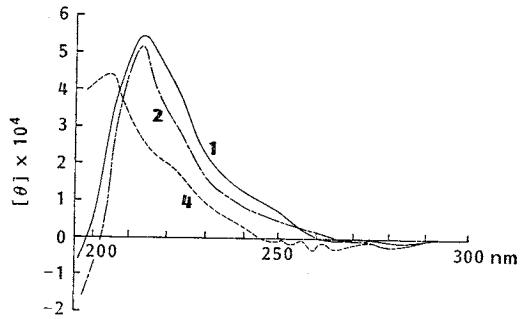
[a] The data measured in D_2O at room temperature were listed.
[b] 200 MHz; δ in ppm relative to external TMS, J in Hz.
[c] 25 MHz; δ in ppm relative to internal dioxane (δ 67.4).

Fig. 2. Structures of resorcinomycins A (**1**) and B (**2**).



1 R=$(CH_3)_2CH$–
2 R=CH_3CH_2–

Fig. 3. CD spectra of resorcinomycins A (**1**) and B (**2**) and *N*-[(*S*)-α-guanidinophenylacetyl]glycine (**4**) (in 0.5 N HCl).



1670~1550 cm^{-1} (Fig. 1), which is probably due to amide, carboxyl and guanidino groups. Other physico-chemical properties are listed in Table 3.

When **1** was hydrolyzed with 6 N HCl in the usual manner and the hydrolysate was analyzed by an amino acid analyzer, only glycine was detected. The ^1H and ^{13}C NMR of **1** suggested the presence

of the following structural units other than glycine; a tetra-substituted phenyl ring, a methine, a guanidino group, a carbonyl group and an isopropyl group. Long range selective decoupling experiments clarified the relations of these structural units, *i.e.* long range coupling relations were observed between 7-methine proton and carbons of 1, 2, 6 and 9 positions in Fig. 2. The ^{1}H and ^{13}C NMR spectra of **2** were closely similar to those of **1**, except for the occurrence of signals due to an ethyl group in place of an isopropyl group in **1**. Thus, the structures of **1** and **2** were deduced as shown in Fig. 2, and all the signals were assigned as in Table 4.

When **1** was hydrolyzed with 6 N HCl in the presence of hydroquinone, α-guanidino-α-(3,5-dihydroxy-4-isopropylphenyl)acetic acid (**3**) was produced as in the case of deoxypheganomycin[5].

In order to determine the configuration, a model compound, *N*-[(*S*)-α-guanidinophenylacetyl]-glycine (**4**), was synthesized from L-phenylglycine. From the similarity of the CD spectra of **1** and **2** to that of **4** (Fig. 3), the configuration of the α-methine of the guanidino acid residue in **1** and **2** was deduced to be *S*. Thus, the structures of **1** and **2** were concluded to be *N*-[(*S*)-α-guanidino-3,5-dihydroxy-4-isopropylphenylacetyl]glycine and *N*-[(*S*)-α-guanidino-3,5-dihydroxy-4-ethylphenylacetyl]-glycine, respectively.

Resorcinomycins A and B are essentially similar to pheganomycins[5,6] in possessing α-guanidino-3,5-dihydroxy-4-alkyl phenylacetyl moiety which is considered to be essential to the antimicrobial activity. However, molecular sizes of resorcinomycins are considerably small in comparison with those of pheganomycins.

Experimental

The UV absorption spectra were measured with a Hitachi 323 spectrophotometer, IR absorption spectra with a Jasco DS-403G spectrometer, CD spectra with a Jasco J-40C automatic recording spectropolarimeter and secondary ion (SI)-MS with a Hitachi M-68 mass spectrometer (Shionogi version of M-80). ^{1}H and ^{13}C NMR spectra were recorded with a Varian XL-200 spectrometer.

Acid Hydrolysis of 1 in the Presence of Hydroquinone

Some 25 mg of **1** was dissolved in 6 N HCl (2 ml) in a test tube. After 20 mg of hydroquinone was added and the air in the tube was substituted by N_2, the tube was sealed. Hydrolysis was carried out at 110°C for 15 hours. The hydrolysate was passed through a Diaion CHP-20P column. In the effluent, glycine was confirmed by amino acid analysis and TLC experiments. After washing the column with water, another compound was eluted with MeOH. The eluate was evaporated to dryness and purified by TLC (Merck Silica gel GF Plate, $CHCl_3$ - MeOH - H_2O (30 : 10 : 1), Rf *ca.* 0.20) to give **3** as a colorless powder (14 mg). Positive to SAKAGUCHI's reaction, ^{1}H NMR (CD_3OD) δ 1.24 (3H, d), 3.46 (1H, q), 4.70 (1H, s), 6.20 (2H, s).

Synthesis of *N*-[(*S*)-α-Guanidinophenylacetyl]glycine (4)

Benzyloxycarbonyl-L-phenylglycine (1.43 g, 5.0 mmol), prepared from L-phenylglycine and benzyloxycarbonyl chloride, was treated with dicyclohexylcarbonyldiimide (1.23 g, 6.0 mmol) and *N*-hydroxysuccinimide (0.58 g, 5.0 mmol) in dichloromethane (20 ml) at 0°C to give the corresponding *N*-hydroxysuccinimide ester, followed by treatment with glycine benzyl ester obtained by treatment of dichloromethane solution of the corresponding *p*-toluenesulfonate (2.0 g, 6.0 mmol) with 10% potassium carbonate solution, and purification by silica gel column chromatography to afford benzyloxycarbonyl-L-phenylglycylglycine benzyl ester as colorless powder (1.72 g, yield 80%).

Hydrogenation of the protected dipeptide (862 mg, 2.0 mmol) in acetic acid gave L-phenylglycyl-glycine acetate (413 mg, yield 77%), which was treated with 1-nitroguanyl-3,5-dimethylpyrazole (280 mg, 1.53 mmol) and sodium bicarbonate (256 mg, 3.06 mmol) in 50% aqueous MeOH at 50°C for 16 hours. Extraction with ethyl acetate at acidic condition and purification by TLC (Merck Silica

gel GF plate, $CHCl_3$ - MeOH - CH_3COOH (50 : 50 : 1), Rf 0.27) gave *N*-[(*S*)-α-nitroguanidinophenylacetyl]glycine (207 mg, yield 46%) as a colorless syrupy residue.

Hydrogenation of the nitroguanidino derivative (50 mg) in acetic acid and purification by TLC (Merck Silica gel GF plate, $CHCl_3$ - C_2H_5OH - 10% aqueous CH_3COOH (4 : 7 : 2), Rf 0.30) gave *N*-[(*S*)-α-guanidinophenylacetyl]glycine as a colorless powder (20 mg, yield 47%).

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