RESPINOMYCINS A1, A2 B, C AND D, A NOVEL GROUP OF ANTHRACYCLINE ANTIBIOTICS

I. TAXONOMY, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITIES

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Respinomycins are a novel group of anthracycline antibiotics produced by *Streptomyces xanthocidicus*. Respinomycins A1, A2, B, C and D were isolated by EtOAc extraction, silica gel column chromatography, centrifugal partition chromatography and preparative silica gel thin layer chromatography. Respinomycins A1 and A2 induced the terminal differentiation of human leukemia K-562 cells.

In the course of our screening for new antibiotics, we found that a strain of *Streptomyces* sp. RK-483 (FERM P-11622) isolated from a soil sample collected in Suwa-shi, Nagano prefecture, Japan, produces novel anthracycline antibiotics designated respinomycins A1, A2, B, C and D. The antibiotics induced the terminal differentiation of human leukemia K-562 cells.

In this paper, we wish to describe the taxonomy of the producing organism, the production, isolation and biological activities of these compounds. Physico-chemical properties and structure determination of these compounds are reported in the following paper.[1] A preliminary communication on respinomycin A1 has appeared.[2]

Materials and Methods

Taxonomic Studies

Methods and media recommended by International Streptomyces Project (ISP)[3] were used to examine the taxonomic characterization of the strain RK-483. Morphology on ISP media was observed after incubation at 28°C for 14 days. The Color Harmony Manual (4th Ed., 1958, Container Corporation of America, Chicago, Illinois) was used to identify the color of mycelial and soluble pigments. A scanning electron microscope (SEM) was used to study the morphology of the spore chains. Whole-cell sugars were identified by the method of LECHEVALIER and LECHEVALIER[4] and diaminopimelic acid (DAP) isomers were analyzed by the method of BECKER *et al.*[5]

Bacterial Strain

The producing strain RK-483 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-11622.

Fermentation

The composition of the seed medium is as follows; glucose 2%, soluble starch 1%, meat extract 0.1%, dry yeast 0.4%, soybean flour 2.5%, NaCl 0.2% and K_2HPO_4 0.005%. The medium was adjusted to pH 7.0 prior to sterilization. Two seed cultures were incubated on a rotary shaker at 250 rpm for 48 hours in two 500-ml cylindrical flasks containing each 70 ml of the seed medium. Then 140 ml of the culture was transferred to 30-liter jar fermentors charged with 18 liters of the same medium containing 0.01% of DF 40P antifoam. The fermentors were agitated at 200 rpm, aerated at 10 liters/minute for 72 hours and the temperature was maintained at 27°C.

The fermentation was monitored by antimicrobial activity against sensitive bacteria *Pseudomonas aeruginosa* L and *Escherichia coli* BE1186.

Tank Fermentation

The composition of the seed medium for tank fermentation is as follows; glucose 2%, corn starch 2%, soybean meal 2.5%, beer yeast 0.4%, meat extract 0.1%, NaCl 0.2% and K_2HPO_4 0.005%. The medium was adjusted to pH 7.3 prior to sterilization. Three seed cultures were incubated on a rotary shaker at 250 rpm for 48 hours in three 500-ml cylindrical flasks containing each 70 ml of the seed medium. Then 180 ml of the culture was transferred to a 30-liter jar fermentor charged with 18 liters of the same medium containing 0.1% of CA-123 and 0.025% KM-68 antifoam. The fermentors were agitated at 200 rpm, aerated at 10 liters/minute for 48 hour and the temperature was maintained at 27°C. Sixteen liters of the culture were transferred to a 600-liter tank fermentor charged with 400 liters of the same medium containing 0.1% CA-123 and 0.025% of KM-68 antifoam. The fermentor was agitated at 200 rpm, aerated at 200 liters/minute for 66 hours and the temperature was maintained at 27°C.

The fermentation was monitored by antimicrobial activity against sensitive bacterium *Staphylococcus aureus* FDA 209P.

Isolation of Respinomycins A1, B, C and D

The culture broth (36 liters) was filtered on Celite and the mycelial cake was extracted with 80% aq acetone. After removal of acetone, the aqueous extract was combined with the filtrate and the mixed solution was adjusted to pH 2 with HCl and extracted with EtOAc, which was discarded. The aqueous layer was adjusted to pH 7.5 and extracted with $CHCl_3$. The organic layer was evaporated *in vacuo* giving 1.57 g of a yellow powder. The crude material (680 mg) was partitioned on a centrifugal partition chromatograph (Sanki Model CPC-LLN) employing the following conditions: BuOH - MeOH - 0.01 M NH_4OAc (upper phase stationary, 4 : 1 : 5) 4 ml/minute in the descending mode at 900 rpm, BuOH - $CHCl_3$ - MeOH - 1% diethylamine/formic acid (pH 3.9) buffer (lower phase stationary, 10 : 100 : 100 : 60) 4 ml/minute in the ascending mode at 700 rpm and EtOAc - MeOH - 0.01 M formic acetate (pH 7.5) (upper phase stationary, 7 : 2 : 6) 4 ml/minute in the ascending mode at 1,300 rpm. After further purification by preparative silica gel TLC (Rf 0.35, $CHCl_3$ - MeOH - H_2O - AcOH (80 : 20 : 6 : 14); Silica gel $60F_{254}$, Merck), 41.25 mg of respinomycin A1 and 55.95 mg of respinomycin D were isolated. The remaining crude power (872 mg) was chromatographed on a silica gel column (Silica gel 60, 70~230 mesh, Merck) with $CHCl_3$ - MeOH - H_2O - AcOH (80 : 20 : 6 : 14) to give 39 mg of another crop of respinomycin A1, 6.4 mg of respinomycin B, 12.8 mg of respinomycin C, and 32.8 mg of additional respinomycin D.

Isolation of Respinomycin A2 from a Tank Fermentation

The fermentation broth (390 liters) was filtered by 24 inch press (23 papers) with the aid of 39 kg of Celite to give a mycelial cake, which was extracted with 360 liters of 80% acetone. The extract was concentrated to 170 liters and combined with 350 liters of broth filtrate. The aqueous solution was adjusted to pH 2 and extracted with ethyl acetate. The aqueous layer was adjusted to pH 7.5 and extracted with chloroform. The organic layer was washed with water and concentrated to 1.1 liters. After removal of the aqueous layer, the organic layer was dried over Na_2SO_4, filtered and concentrated *in vacuo* to give 15 g of crude powder. The crude powder was subjected to silica gel column chromatography (Silica gel 60, 70~230 mesh, Merck) with $CHCl_3$ - MeOH - H_2O - AcOH (80 : 20 : 6 : 14). After rechromatography using the same procedure, 4.4 g of respinomycin A2 and 427 mg of respinomycin A1 were obtained.

Biological Activity

K-562 cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY., U.S.A.). Two hundred μl of the cells (5×10^4 cells/ml) were put into each well of a 96 well plate, and various concentrations of respinomycins in 10 μl of 50% methanol were added to the cells, which were incubated for 96 hours at 37°C in a 5% CO_2 incubator. The morphological change of the cells was observed under a microscope. The inducing activity on K-562 cell differentiation was estimated by benzidine staining.[6]

Antibacterial activity was determined by the conventional paper disk method using nutrient agar medium.

Antibacteriophage and antimicrobial activity was measured by the modified paper disk-agar plate method.[7]

Results and Discussion

Taxonomy

The producing strain RK-483 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with the accession No. FERM P-11622.

RK-483 strain produced melanoid pigments. The carbon utilization of the strain RK-483 is listed in Table 1. The cultural and physiological characteristics of RK-483 are shown in Table 2.

The strain had rectiflexible spore chains with over 50 spores per chain (Fig. 1). The spores were cylindrical or ellipsoidal with smooth surfaces. Whole-cell hydrolysate of the strain RK-483 contained LL-diaminopimelic acid which suggests that the strain belongs to cell wall type I.[8] The major menaquinone are MK-9 (H_8) and MK-9 (H_6).

The properties of strain RK-483 suggested that the strain belongs to the genus *Streptomyces*.[9] As a result, it was found that strain RK-483 closely

Table 1. Utilization of carbohydrates by strain RK-483.

D-Glucose	++	D-Fructose	++
L-Arabinose	++	Rhamnose	−
D-Xylose	+	Sucrose	−
i-Inositol	−	Raffinose	++
D-Mannitol	−		

Table 2. Cultural characteristics of strain RK-483 on various media.

Yeast extract - malt extract agar (ISP-2)	G:	Abundant	Glucose - asparagine agar	G:	Good
	AM:	Shadow gray (5ih)		AM:	None
	SM:	Golden brown (3pi)		SM:	Colorless
	P:	None		P:	None
Oatmeal agar (ISP-3)	G:	Abundant	Sucrose - nitrate agar (WAKSMAN's-1)	G:	Good
	AM:	Ashes (5fe)		AM:	Parchment (1cb)
	SM:	Golden brown (3pg)		SM:	DK luggage tan (4pg)
	P:	Brown		P:	Brown
Inorganic salts - starch agar (ISP-4)	G:	Abundant	V-8 juice agar	G:	Abundant
	AM:	Ashes (5fe)		AM:	Shadow gray (5ih)
	SM:	Golden brown (3pg)		SM:	Golden brown (3pg)
	P:	Brown		P:	None
Glycerol - asparagine agar (ISP-5)	G:	Abundant	Potato - carrot agar	G:	Good
	AM:	Ashes (5fe)		AM:	Parchment (5cb)
	SM:	Bamboo (2gc)		SM:	Maple (4le)
	P:	None		P:	Brown
Yeast extract - starch agar	G:	Abundant			
	AM:	Ashes (5fe)			
	SM:	Clove brown (3ni)			
	P:	Brown			

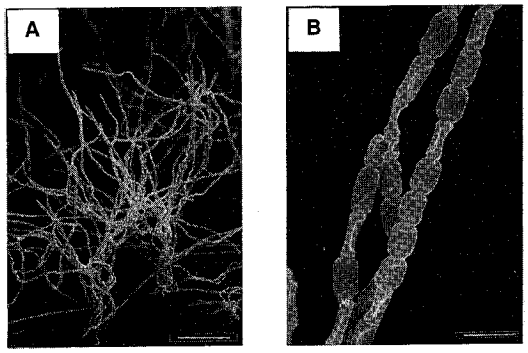
Observations after incubation at 28°C for 2 weeks.

Abbreviations: G, growth; AM, aerial mycelium; SM, substrate mycelium; P, diffusible pigment.

resembled *Streptomyces xanthocidicus* Asahi, Nagatsu and Suzuki 1966[10] with a little difference about producing of soluble pigment. The strain RK-483 produce melanoid pigment and brownish soluble pigment, but production of melanoid pigment by *S. xanthocidicus* is variable and it does not produce distinctive soluble pigments. Since these differences are too small to regard RK-483 as a different species, strain RK-483 was identified as a strain of *Streptomyces xanthocidicus*.

Fig. 1. Scanning electron micrograph of spores chains of strain RK-483 on V-8 juice agar incubated at 28°C for 14 days.

A: Bar represents 13 μm; B: bar represents 1.5 μm.



Purification of Respinomycins

The fermentation of respinomycins was carried out as described in Materials and Methods. Maximum antibiotic production was obtained after 72 hours of fermentation. From 36 liters of the culture broth, 80.3 mg of respinomycin A1, 6.4 mg of respinomycin B, 12.8 mg of respinomycin C, and 88.8 mg of respinomycin D were isolated as shown in Fig. 2. In order to obtain the *in vivo* data of biological activities of respinomycins, a tank

Fig. 2. Isolation and purification of respinomycins A1, B, C and D.

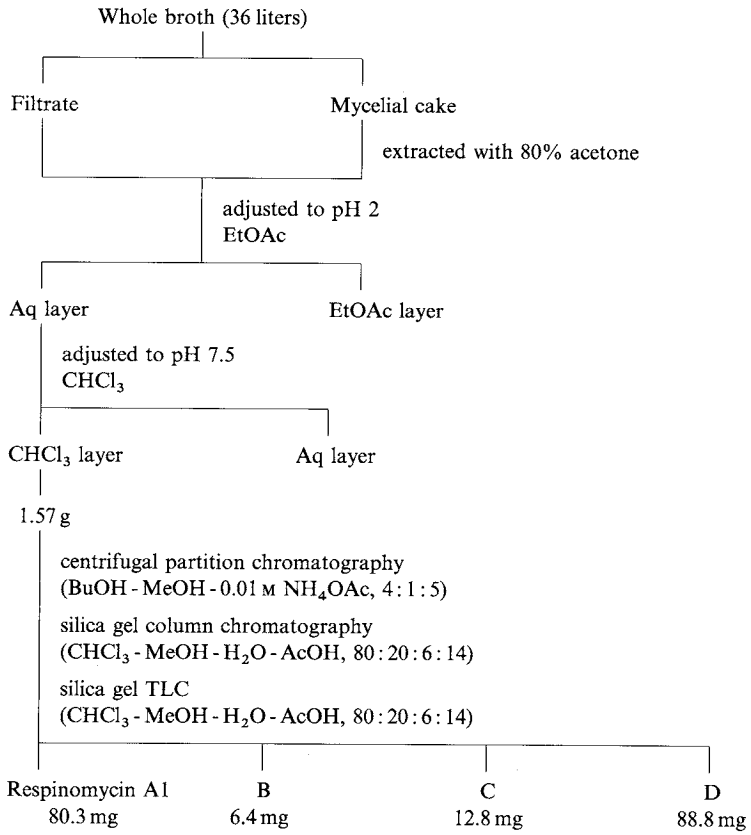


Fig. 3. Isolation and purification of respinomycin A2.

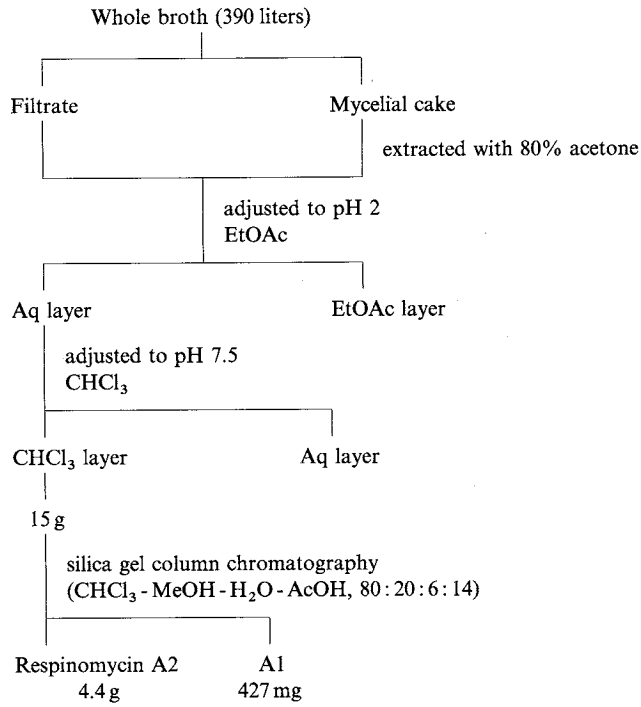


Table 3. Effects on human leukemia K-562 cells.

Respinomycin	Cell differentiation MEC (induction rate)	Cytotoxicity MIC
A1	1.58 μg/ml (48%)	50 μg/ml
A2	5.0 μg/ml (76.3%)	50 μg/ml
B	5.0 μg/ml (<10%)	15.8 μg/ml
C	5.0 μg/ml (<10%)	15.8 μg/ml
D	0.5 μg/ml (<10%)	1.58 μg/ml

Abbreviations: MEC, minimal effective concentration; MIC, minimal inhibitory concentration.

Table 4. Antiphage and antimicrobial activities against actinophage B and its host actinomycete *Streptomyces griseus*.

Respinomycin	Antiphage activity	Antimicrobial activity
A1	+	−
A2	+++	+
B	+	−
C	+	−
D	++	+++

Modified paper disk-agar plate method was used to evaluate antiphage and antimicrobial activity.[7] The paper disks contained 40 μg of each antibiotic.

Abbreviations: +++, strong activity; ++, moderate activity; +, weak activity; −, no activity.

fermentation was carried out. After the solvent extraction, it was apparent that the production ratio of respinomycins completely changed. Surprisingly, a new major component which posses little higher Rf value than that of respinomycin A1 was detected by silica gel thin layer chromatography ($CHCl_3$-MeOH-H_2O-AcOH = 80 : 60 : 6 : 14; Silica gel $60F_{254}$, Merck). After characterization, the new component was designated as respinomycin A2. Isolation of the respinomycin A2 from the tank fermentation is illustrated in Fig. 3. From the fermentation broth (390 liters), 4.4 g of respinomycin A2 and 427 mg of respinomycin A1 were obtained. Precise physico-chemical properties and structures of respinomycins A1, A2, B, C and D are described in the succeeding paper.

Biological Activities

The effect of respinomycins on human leukemia K-562 cells are shown in Table 3. Respinomycins

Table 5. Antimicrobial activity of respinomycins.

Test organism	Diameter of inhibition zone (mm)					
	A1	A2	B	C	D	DM
Pseudomonas aeruginosa L-mutant N-10	19	−	(11)	18	22	13
Escherichia coli BE1186	(12)	+	13	+	(10)	29
Staphylococcus aureus FDA 209p JC-1	13	−	+	(10)	15	16

The susceptibility of bacteria to respinomycins was determined by the conventional paper disk method using nutrient agar medium. The paper disks contained 40 μg of each antibiotic.

Abbreviations: DM, daunomycin; +, weak inhibition zone; −, no inhibition zone; ( ), turbid zone,

A1 and A2 induced the terminal differentiation of human leukemia K-562 cells. Especially, respinomycin A2 showed high activity on the differentiation induction. On the contrary, respinomycin D showed the strongest cytotoxicity among these antibiotics, however, it did not induce K-562 differentiation. Since anthracycline antibiotics inhibit nucleic acid synthesis, anti-phage activity of respinomycins were examined as shown in Table 4. Respinomycin A2 showed a strong anti-phage activity against actinophage B of *Streptomyces griseus*. The antimicrobial activity of respinomycins is summarized in Table 5. Respinomycins are inhibitory to hypersensitive mutants of Gram-negative and Gram-positive bacteria. Respinomycin A2 showed only weak antimicrobial activity against *E. coli* BE1186. All these antibiotics did not show antimicrobial activity against fungi and yeasts. Respinomycin A1 is toxic to mice: LD_{50} is approximately 37.5 mg/kg by intraperitoneal administration. However respinomycin A2 did not show toxicity to mice at the dose of 480 mg/kg by intraperitoneal administration.

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