

## A NEW ANTITUMOR ANTIBIOTIC, SPORAMYCIN

KANKI KOMIYAMA, KEN'ICHI SUGIMOTO, HIDEO TAKESHIMA  
and IWAO UMEZAWA

The Kitasato Institute  
Shirokane, Minatoku, Tokyo, 108, Japan

(Received for publication December 8, 1976)

A new antitumor antibiotic, sporamycin, was isolated from the culture broth of *Streptosporangium pseudovulgare* strain No. PO-357.\* Sporamycin was prepared as a white powder by ammonium sulfate precipitation and chromatographic techniques using DEAE-cellulose and Sephadex G-50. The producing organism was similar to *Streptosporangium pseudovulgare* in a number of properties but differed in the biological activity of the culture broth and the color of the aerial mycelium on agar. Sporamycin is active against most Gram-positive bacteria and Sarcoma-180 solid tumors. It is heat labile, but stable at low temperature.

In our search for new antitumor substances, we isolated from the culture filtrate of *Streptosporangium pseudovulgare* strain No. PO-357, a new antibiotic, sporamycin, which was briefly reported previously.<sup>1)</sup> This paper describes a taxonomic study of the producing organism in detail, and gives some physico-chemical and biological properties of sporamycin.

### Materials and Methods

#### Taxonomic Studies

Strain No. PO-357 was isolated from a soil sample collected in Setagaya-ku, Tokyo, Japan. For taxonomic studies, most cultures were grown in accordance with method adopted by the International Streptomyces Project.<sup>2)</sup> For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for mature cultures is described according to the "Color Harmony Manual".<sup>3)</sup> Physiological properties, including the utilization of carbon sources, were examined by the method of PRIDHAM and GOTTLIEB.<sup>4)</sup> Since the organism did not grow on PRIDHAM and GOTTLIEB agar supplemented with carbon sources, yeast extract was added to the medium at a final concentration of 0.05%. Diaminopimelic acid in the cell wall and the sugar pattern of whole cells were analysed by the method of BECKER *et al.*<sup>5)</sup> and of LECHEVALIER and LECHEVALIER.<sup>6,7)</sup>

#### Fermentation and Isolation of Sporamycin

Sporamycin was isolated by a slight modification of the method reported previously<sup>1)</sup>. Stock cultures of the producing organism were inoculated into a 500-ml SAKAGUCHI flask containing 100 ml of the medium consisting of 2% glucose, 0.5% peptone, 0.3% dried yeast, 0.5% meat extract, 0.5% NaCl and 0.3% CaCO<sub>3</sub>. The flask was incubated at 27°C for 3 days on a reciprocal shaker, and 10 ml of the resulting culture was transferred into 500-ml SAKAGUCHI flasks each containing 100 ml of the medium consisting of 0.2% glucose, 0.2% peptone, 1.5% starch, 0.15% yeast extract, 0.25% CaCO<sub>3</sub>, and 0.3% meat extract. The flasks were incubated at 27°C for 48 hours on a reciprocal shaker. To 20 liters of fermentation medium containing 2% glucose, 0.5% peptone, 0.3% dried yeast, 0.5% meat extract, 0.5% NaCl and 0.3% CaCO<sub>3</sub> was added 400 ml of the resulting inoculum and the culture was incubated at 27°C in a 30-liter fermentor for 45 hours, aerated at 16 liters/min and agitated at 300 rpm (Fig. 1). To the culture filtrate (36 liters) was added 25 kg of ammonium sulfate, and the mixture

\* The strain was deposited in the Fermentation Research Institute, Chiba, and assigned accession number FERM-P No. 3571.

was allowed to stand overnight at 5°C. The precipitate containing sporamycin was collected by centrifugation and dissolved in ice-cold distilled water. The resulting solution was transferred into a cellophane tube and dialysed against ice-cold distilled water for 3 days. The non-dialysable solution was subjected to DEAE-cellulose column chromatography. The column was previously equilibrated with 0.002 M phosphate buffer (pH 7.4). The antibiotic was eluted with a linear gradient of 0~0.6 M NaCl in 0.002 M phosphate buffer at pH 7.4. Active fractions were collected, and saturated with ammonium sulfate; and the resulting precipitate was dissolved in deionized water. The solution was placed in a cellophane tube, dialysed against ice-cold water for 3 days, and rechromatographed on a DEAE-cellulose column equilibrated with 0.002 M phosphate buffer (pH 5.4). The antibiotic was eluted with the linear gradient described above except that the pH of the solution was adjusted to 5.4. The active substance was precipitated with ammonium sulfate and dialysed for 2 days. After lyophilization, it was applied to a Sephadex G-50 column and eluted with water; the eluate was lyophilized to yield 620 mg of a white powder.

#### Ultracentrifugation and Electrophoresis

The homogeneity of the purified sporamycin was confirmed by analytical ultracentrifugation and by polyacrylamide gel electrophoresis. For analytical ultracentrifugation, 10 mg of sporamycin was applied to a synthetic boundary cell. Polyacrylamide gel (7.5%, pH 4.0) was prepared in a glass tube according to the method of REISFELD *et al.*,<sup>10)</sup> and 10 mg of sporamycin was applied to the tube. For paper electrophoresis, 0.2 M acetate buffer and 0.2 M phosphate buffer were used for pH 4.2 and 8.0, respectively, and electrophoresis was carried out at 250 V for 3.5 hours.

#### Determination of Biological Activity

The antimicrobial spectrum of sporamycin was determined by the agar dilution method using nutrient agar for bacteria and potato agar for fungi. The minimum inhibitory concentration was observed after 48 hour-incubation. The antitumor activity of sporamycin was examined using Sarcoma-180 solid tumor.

## Results

### Taxonomic Studies

Microscopic examination revealed that the aerial mycelium was straight with most sporangia on the top of the mycelium (Plate 1). Viewed under the electron microscope, the sporangia were spherical with a diameter of 5~10  $\mu$ , averaging 7.5  $\mu$  (Plate 2). Spores were round or ovoid with a smooth surface and a diameter of 0.9~1.4  $\mu$ . Flagella were not observed (Plate 3). Table 1 shows the culture properties of strain No. PO-357. Growth on chemically defined media was poor compared with that on natural nutrient media. Clear diffusible pigment was not observed in the media used. The physio-

Fig. 1. Fermentation pattern of sporamycin. Antibacterial activity was measured by the paper disk diffusion method.

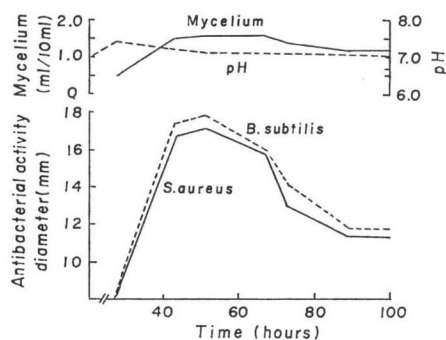


Plate 1. Photomicrograph of aerial mycelia of strain No. PO-357.

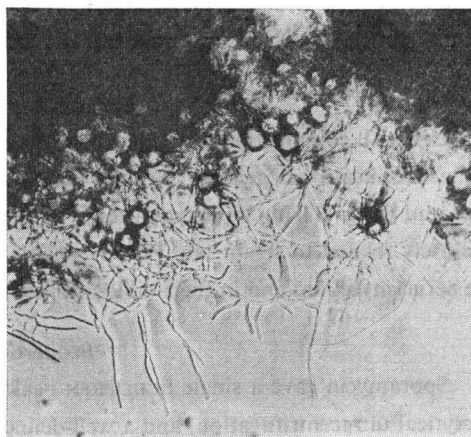


Plate 2. Electronmicrograph of sporangia of strain No. PO-357

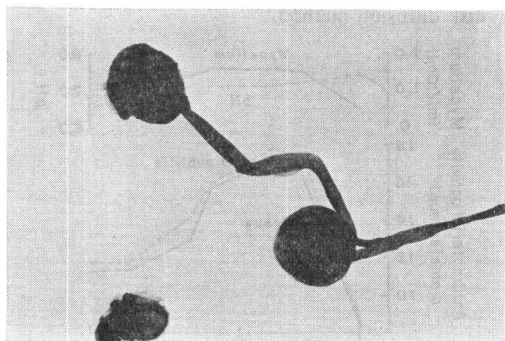


Plate 3. Electronmicrograph of spores of strain No. PO-357

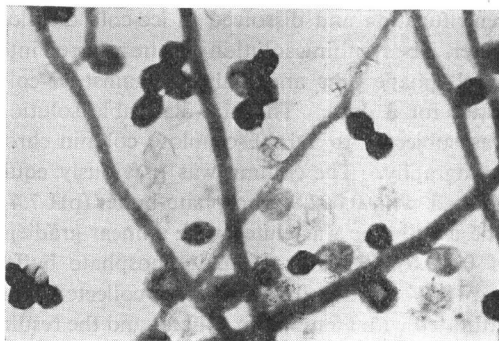


Table 1. Culture properties of strain No. PO-357

Medium	Growth	Aerial mycelium
Sucrose-nitrate agar	Poor	Brownish white~white
Glucose-asparagine agar	Poor	Brownish white~white
Glycerol-asparagine agar	Poor	White~pale yellowish brown
Inorganic salt-starch agar	Poor	Brownish white~white
Tyrosine agar	Moderate	White
Nutrient agar	Good	Orange
Yeast extract-malt extract agar	Good	Light yellowish red
Oatmeal agar	Good	Orange
Oatmeal-yeast agar	Good	Light yellowish red

logical properties of strain No. PO-357 are shown in Table 2. The temperature range for good growth was 26~38°C, but moderate growth was observed at 42°C after cultivation for 7 days on a medium consisting of 2% glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl, and 1.2% agar. Analysis of the cell walls showed *meso*-diaminopimelic acid and madurose but not arabinose and galactose.

#### Comparison with Known Species

Cell wall composition and sugar patterns of the whole cell have become widely accepted as an aid in the identification of genera. Strain No. PO-357 was classified as Type III from its cell wall composition, and group B from its sugar pattern.<sup>7)</sup> Among known species, *Streptosporangium pseudovulgare*<sup>8,9)</sup> is closely related to strain No. PO-357 (Table 4), but there are differences, especially in the color of the aerial mycelium and in their biological activities.

#### Properties of Sporamycin

Sporamycin gave a single SCHLIEREN peak during sedimentation at 52,000 rpm for 72 minutes by analytical ultracentrifugation, and a well-defined single band staining with Amido Black was observed

Table 2. Physiological properties of strain No. PO-357

Temperature for good growth	26~38°C
Liquefaction of gelatine	Positive
Hydrolysis of starch	Positive
Peptonization of milk	Positive
Melanin production	Negative
H <sub>2</sub> S formation	Negative
Nitrate reduction	Positive

Table 3. Carbon source utilization of strain No. PO-357

Carbon source	Growth
D-Glucose, L-arabinose, D-xylose, D-mannose.	+
D-Galactose, raffinose, L-rhamnose, D-fructose.	±
Inositol, saccharose.	-

+: utilization, ±: probable utilization, -: no utilization.

Table 4. Comparison of strain No. PO-357 with *Streptosporangium pseudovulgare*

	PO-357	<i>S. pseudovulgare</i>
Sporangium	5~10 $\mu$	7~10 $\mu$
Sporangiospore	0.9~1.4 $\mu$	1.2~1.5 $\mu$
Yeast extract-malt extract agar	#Light yellowish red	Orange
Oatmeal-yeast agar	Light yellowish red	Pale orange
Oatmeal agar	Orange-coral red	Pale orange
Liquefaction of gelatin	+	+
Hydrolysis of starch	+	+
Nitrate reduction	+	+
Melanin production	-	-
Peptonization of milk	+	+
Growth at 42°C	+	+
Antimicrobial activity*	Strong	Weak
Antitumor activity*	+	-

\* The fermentation medium containing 2% glucose, 0.5% peptone, 0.3% dried yeast, 0.5% meat extract, 0.5% NaCl, and 0.3% CaCO<sub>3</sub> was used for the culture for 72 hours. The activities were determined on *Staph. aureus* (disk method) and mouse leukemia P-388 *in vivo*.

# Color of aerial mycelium.

Table 6. Antimicrobial spectrum of sporamycin

Test organism	Minimum inhibitory concentration ( $\mu$ g/ml)
<i>Staphylococcus aureus</i> FDA 209P	0.1
<i>Staphylococcus aureus</i> JC-1	0.05
<i>Bacillus subtilis</i> PCI 219	2.1
<i>Bacillus cereus</i> IFO 3001	10.3
<i>Sarcina lutea</i> PCI 1001	50.0
<i>Mycobacterium smegmatis</i> ATCC 607	> 100
<i>Escherichia coli</i> NIHJ	> 100
<i>Shigella sonnei</i> E33	> 100
<i>Salmonella typhimurium</i>	> 100
<i>Klebsiella pneumoniae</i> PCI 602	> 100
<i>Aerobacter aerogenes</i> ATCC 9621	> 100
<i>Pseudomonas aeruginosa</i> P-3	> 100
<i>Candida albicans</i>	> 500
<i>Saccharomyces sake</i>	> 500
<i>Aspergillus niger</i>	> 500
<i>Trichophyton interdigitale</i>	> 500
<i>Penicillium notatum</i>	> 500

Table 5. The amino acid composition of sporamycin

	mg/g		mg/g
Lysine	59.74	Alanine	139.20
Aspartic acid	86.39	Valine	146.44
Threonine	108.81	Isoleucine	12.61
Serine	85.89	Leucine	72.52
Glutamic acid	88.42	Tyrosine	13.07
Glycine	83.01	Phenylalanine	59.56

Sporamycin was hydrolysed with 6 N HCl at 110°C for 15 hours.

Fig. 2. UV spectra of sporamycin.

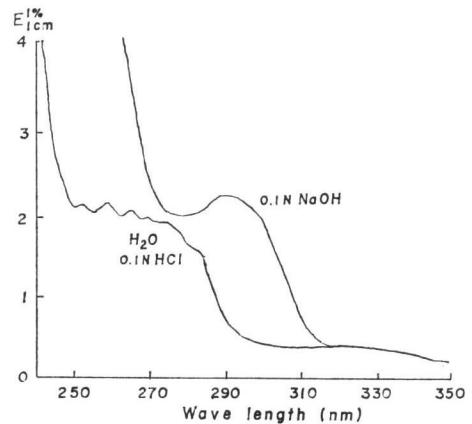
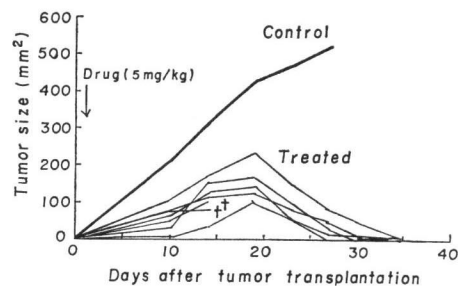


Fig. 3. Effect of sporamycin on Sarcoma-180 solid tumor.

A small piece of Sarcoma-180 solid tumor was transplanted into the axillar region of *ddN* mice weighing 20~22 g. Forty eight hours after the transplantation, mice were given with 5 mg/kg of sporamycin in the caudal vein. Control: (mean of 7 mice).



by polyacrylamide gel electrophoresis. The UV spectrum of sporamycin was shown in Fig. 2. Sporamycin was identified as a basic substance

by paper electrophoresis. Table 5 shows the amino acid composition of sporamycin.

#### Biological Activity of Sporamycin

As shown in Table 6, the antibiotic was effective against most Gram-positive bacteria, but was inactive against *Mycobacterium*, Gram-negative bacteria, fungi and yeasts. No growth of *Sarcina lutea* was observed at a sporamycin concentration of 2.1  $\mu\text{g/ml}$  within 24 hours, but very slight growth was observed on the agar containing 25  $\mu\text{g/ml}$  after 48 hours incubation. As shown in Fig. 3, the tumor was strongly inhibited by a single injection with sporamycin. It is interesting that the tumor continued to grow for approximately 18 days after the treatment, and then regressed.

Table 7. Effect of pH on the stability of sporamycin at 5°C in the dark

Incubation (hours)	Residual activity (%) at pH				
	4.0	5.0	6.0	7.0	8.0
0	100	100	100	100	100
24	100	100	100	100	100
72	95	99	95	100	100
96	60	65	65	60	68
120	60	57	57	53	53

#### Stability of Sporamycin

In order to determine the stability of sporamycin under various conditions, the antibiotic was dissolved in M/30 phosphate buffer (pH 6.0, 7.0 and 8.0) and M/30 acetate buffer (pH 4.0 and 5.0). The solution was placed in a dark room at 5°C, and the residual activity was determined by the paper disk diffusion method against *Staphylococcus aureus* FDA 209P. The activity was unchanged for 72 hours under these conditions (Table 7). As shown in Fig. 4, sporamycin was rapidly inactivated at relatively high temperature, but its activity was not reduced at -20°C for 90 days. Sporamycin is sensitive to UV light (Fig. 5). The UV spectrum of a sample treated with UV light differed from that of the intact antibiotic.

Fig. 4. Stability of sporamycin at different temperatures.

The antibiotic was dissolved in 1/30 M phosphate buffer of pH 7.0.

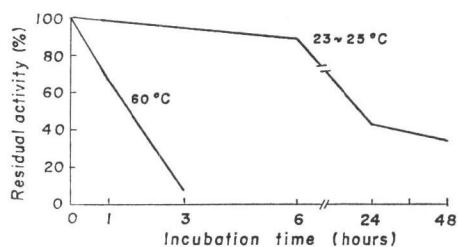
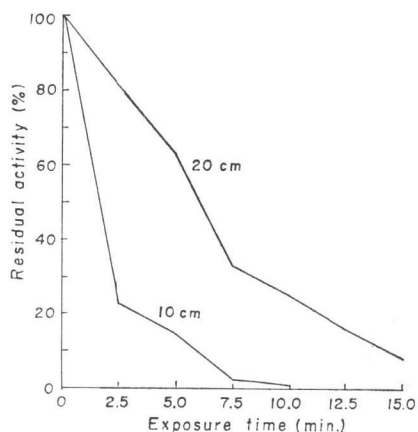


Fig. 5. Inactivation of sporamycin by UV light. Ten mg of sporamycin dissolved in 10 ml of water in a Petri dish (10 cm in diameter) was irradiated with light from a UV lamp (Tōshiba Co., Type GL-15) distance of 10 or 20 cm.



#### Discussion

Taxonomic studies of strain No. PO-357 indicate that this organism belongs to the genus *Streptosporangium* COUCH 1955.<sup>11)</sup> When compared with the known species of *Streptosporangium* from their descriptions in BERGEY's manual and in the report by NONOMURA,<sup>8)</sup> strain No. PO-357 shows a close relationship to *Streptosporangium pseudovulgare* as reported by NONOMURA and OHARA.<sup>8,9)</sup> There are some differences and these are listed in Table 4, but it seems likely that No. PO-357 is a strain of *S. pseudovulgare*.

Many macromolecular antitumor antibiotics have been reported. Neocarzinostatin,<sup>12)</sup> macromomycin,<sup>13)</sup> mitomalcin,<sup>14)</sup> carcinocidin,<sup>15)</sup> and A-280<sup>16)</sup> are acidic polypeptides, while sporamycin is a basic substance judging from its behavior during paper electrophoresis. Furthermore, the UV absorption spectrum of sporamycin differed from that of the substances described above. Actinocarcin,<sup>17)</sup> phenomycin<sup>18)</sup>, enomycin<sup>19)</sup> and A-216<sup>16)</sup> belong to the group of basic antitumor substances, but they have no antibacterial activity. Pluralline<sup>20)</sup> and actinogan<sup>21)</sup> are glycoproteins. Peptimycin<sup>22)</sup> did not show any growth inhibition against bacteria and HeLa cells *in vitro*. Actinoxanthin<sup>23)</sup> is active against *Staphylococcus aureus* at a concentration of 0.001  $\mu\text{g/ml}$  and its LD<sub>50</sub> for mice is 0.24 mg/kg by intraperitoneal injection. These values are completely different from those of sporamycin. To our knowledge, therefore, there is no known substance identical with sporamycin.

#### Acknowledgements

We wish to thank Prof. SATOSHI ŌMURA and Dr. JUICHI AWAYA for their kind advice, and Mr. ROKUROU MASUMA and Miss FUJIKO KIMURA for their technical assistance.

This work was partially supported by a Grant-in-aid for Cancer Research from the Ministry of Health and Welfare, the Ministry of Education, Culture and Science, and by funds from Japan Keirin Association.

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