A NEW ANTITUMOR ANTIBIOTIC, AWAMYCIN

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A new antibiotic, awamycin, was isolated from the culture broth of *Streptomyces* sp. No. 80-217. It appeared to belong to the quinone indicator group from results of physicochemical studies and has the empirical formula $C_{38}H_{40}NO_{12}S$.

This antibiotic possessed antibacterial and antitumor activities against Gram-positive bacteria and experimental murine tumors. The antibiotic also showed direct cytotoxic activity against HeLa cells *in vitro*.

In the course of our screening for novel antibiotics showing antitumor activity, awamycin was isolated from the fermentation broth of *Streptomyces* sp. No. 80-217 which had been isolated from a soil sample collected in Chiba Prefecture. It was not only active against Gram-positive bacteria *in vitro*, but also showed activity against experimental murine tumors *in vivo*. This paper describes the taxonomy of the producing organism, fermentation, isolation, and physicochemical and biological properties of this antitumor antibiotic.

Materials and Methods

Taxonomic Studies

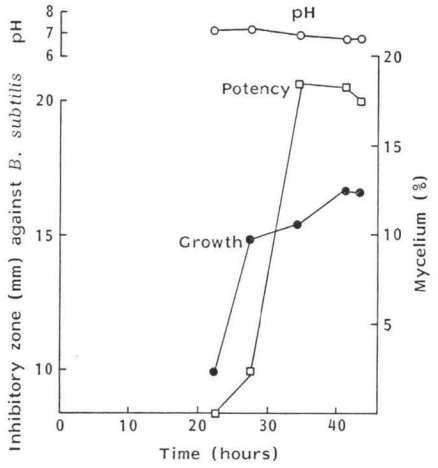
For taxonomic studies, most cultures were grown in accordance with methods adopted by the International Streptomyces Project.[1] For experiments on cultural properties, all cultures were incubated at 27°C and were observed for 15~20 days. The color recorded for mature cultures is described according to the "Color Harmony Manual".[2] Physiological properties including utilization of carbon sources were examined by the method of PRIDHAM and GOTTLIEB.[3] Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[4]

Fermentation and Isolation of Awamycin

Stock culture of the producing organism were inoculated into a 500-ml Sakaguchi flask containing 100 ml of the seed medium consisting of 0.5% Bacto-Tryptone and 0.33% Bacto Yeast extract (pH 7.0~7.2). The flasks were incubated at 27°C for 72 hours on a reciprocal shaker. Then 1 liter of the resulting culture was transferred to a 200-liter fermentor containing 130 liters of the production medium consisting of 1.5% starch, 0.2% glucose, 0.25% peptone, 0.15% yeast, 0.3% meat extract and 0.25% $CaCO_3$ (pH 7.0~7.2) and fermentation was carried out at 25°C for 35 hours, using an agitation rate of 160 rpm and an aeration rate of 130 liters/minute (Fig. 1).

The fermentation broth (117 liters) of *Streptomyces* sp. No. 80-217 was mixed with 2% of Hyflo Super-Cel (Johns-Manville Sales Co., U.S.A.), and then filtered with a filter press. The clear filtrate (113 liters) was adsorbed on a chromatographic column (2.5 liters) of Diaion HP-20 (Mitsubishi Chemical Industries Ltd., Japan) and the column was washed with 70% aqueous MeOH (7.5 liters), followed by elution with 80% aqueous acetone (7.5 liters). Antimicrobially active fractions were collected, combined and concentrated *in vacuo* to 500 ml. The residue was extracted with butyl acetate (500 ml) at pH 9.0 followed by concentration. Then crude awamycin (410 mg) was obtained by addition of *n*-hexane as a precipitant. The crude material was subjected to preparative HPLC (Waters Associates,

Fig. 1. Time course of awamycin production.



System 500A, silica gel) with ethyl acetate as an eluant, and a purified orange powder (45 mg) was obtained (Fig. 2).

As shown in Fig. 2, the second antibiotic was obtained by back extraction of the pH 9.0 BuOAc extract with water at pH 2.0.

Antimicrobial Activity

The antimicrobial spectrum of awamycin was determined by the ordinary agar dilution method using Mueller-Hinton agar medium for bacteria and potato agar for fungi. The minimum inhibitory concentrations (MIC) were observed after 24 hours incubation (for bacteria) and/or longer term incubation (for fungi).

Antitumor Activity

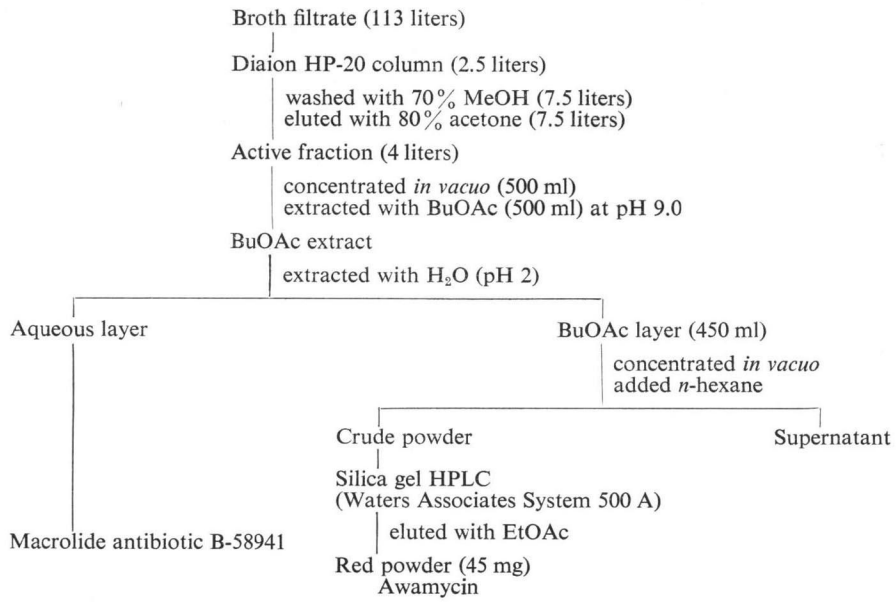
For determination of antitumor activity of awamycin, male ICR mice and CDF_1 mice weighing 20~24 g were purchased from the Shizuoka Agricultural Cooperation. Sarcoma 180 ascites and IMC carcinoma were obtained from the Sasaki Institute and Institute of Microbial Chemistry respectively.

Antitumor activity was evaluated by the increase in life span (ILS): $(T/C-1)\times 100\%$, where "T" was the median survival days (MSD) of the treated group and "C" the MSD of the control group. Survivors were scored 60 days after tumor inoculation and mice remaining alive after this period of observation were considered cured.

Effect of Awamycin on HeLa Cells

HeLa cells have been maintained in monolayers in Eagle's minimum essential medium (MEM) supplemented with 10% bovine serum and antibiotics (100 units/ml of benzylpenicillin and 100 μg/ml of streptomycin) at 37°C. To determine the cytotoxicity of awamycin on mammalian cells, HeLa cells

Fig. 2. Purification method of awamycin from culture broth.



(5×10^4) in 2 ml of medium were placed in a 30-mm Petri dish and incubated for 48 hours at 37°C in a 5% CO_2 - 95% air atmosphere. Each culture dish was filled with a fresh medium containing a different concentration of awamycin. Then HeLa cells were trypsinized to form a single cell suspension, and cells were counted in a hemocytometer.

Results

Taxonomic Studies

The chain of the mature spores consisted of 5 to 20 spores and formed hooks, loops and occasionally closed spirals (Plate 1). The spores were short ovoid, and $0.53\sim0.46\times0.8\sim1.0$ μm. Spore surfaces were warty.

Aerial mycelium was in the gray color series on yeast extract - malt extract agar, inorganic salts - starch agar, and nutrient agar. Beige colored soluble pigment (3ge~4ge) was produced on inorganic salts - starch agar, oatmeal agar, and tyrosine agar. Culture characteristics are shown in Table 1. The physiological properties and utilization of carbon sources of strain No. 80-217 was shown in Tables 2 and 3 respectively. Cell wall analysis showed the presence of LL-diaminopimelic acid and was classified at type I. Microscopic studies and cell wall type indicated that strain No. 80-217 belongs to the genus Streptomyces and it was designated as *Streptomyces* sp. No. 80-217. Further studies are in progress.

Physicochemical Properties of Awamycin

The physicochemical properties of awamycin are summarized in Table 4. UV and IR absorption

Plate 1. Electron micrograph of *Streptomyces* sp. No. 80-217.

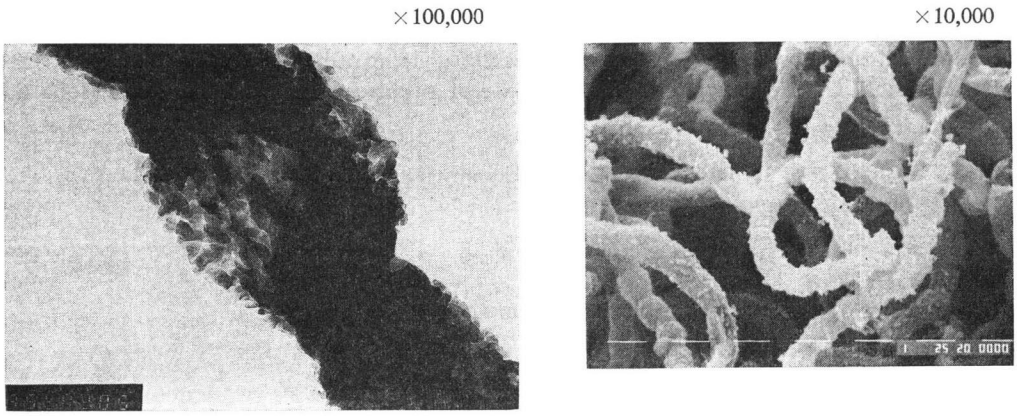


Table 1. Cultural properties of strain No. 80-217.

Medium	Growth	Aerial mycelium	Riverse	Soluble pigment
Glycerol - asparagine agar	Good	Colonial white (1fe)	Beaver (4li)	Rose beige (4ge)
Inorganic salts - starch agar	Good	Covert gray (2fe)	Light olive drab (1li)	Beige (3ge)
Tyrosine agar	Moderate	Dark laurel (22po)	Rose beige (4ge)	Rose beige (4ge)
Yeast extract - malt extract agar	Moderate	Olive gray (1ih)	Covert brown (2li)	
Oatmeal agar	Moderate	Olive (1ih)	Mistletoe gray (24½ih)	
Peptone - yeast extract - iron agar	Moderate	Bamboo (2gc)	Gold (2lc)	
Nutrient agar	Moderate	Silver gray (3fe)	Olive gray (1½ig)	

Table 2. Physiological characteristics.

Nitrate reduction	+
Liquefaction of gelatin	+
Coagulation of milk	+
Cellulolytic activity	+
Melanin formation	−
Production of H_2S	−

Table 3. Utilization of carbon sources.

Responses	Carbon source
Positive	L-Arabinose, D-xylose, inositol, L-rhamnose, cellulose
Negative	Sucrose, D-mannitol, raffinose
Doubtful	D-Fructose

Table 4. Physicochemical properties of awamycin.

Appearance	Red crystals
Molecular formula	$C_{38}H_{49}NO_{12}S \cdot H_2O$
Analysis(%) Found:	C 60.32, H 6.72, N 1.83, S 4.36
Calcd:	C 59.91, H 6.75, N 1.84, S 4.21
MW(FD-Mass)	M^+ 743
Mp	177~180°C
$[\alpha]_D^{20}$ (*c* 0.1, $CHCl_3$)	+1,010°
Solubility: Soluble	$CHCl_3$, C_6H_6, EtOAc, Me_2CO, MeOH
Insoluble	H_2O, *n*-hexane
TLC (SiO_2)	
C_6H_6 - MeOH, 6: 1	Rf 0.29
$CHCl_3$ - MeOH, 96: 4	Rf 0.36

spectra are shown in Figs. 3 and 4 respectively. This antibiotic was readily soluble in MeOH, acetone, benzene, ethyl acetate, tetrahydrofuran, dimethylformamide and dimethylsulfoxide, but practically insoluble in water, petroleum ether, and *n*-hexane. Awamycin gave positive reactions in magnesium acetate and alkali solutions but showed negative color reactions in the ninhydrin, MOLISCH and FEHLING reactions.

Fig. 3. UV spectrum of awamycin.

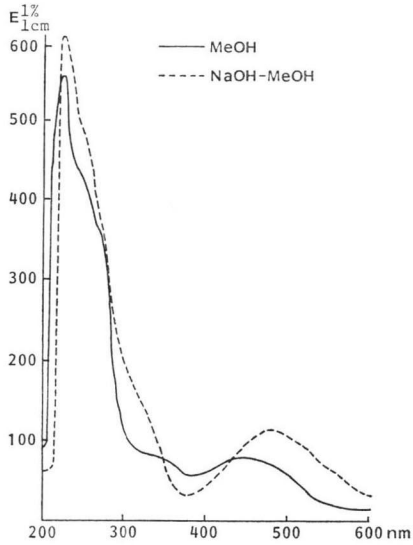


Fig. 4. IR spectrum of awamycin (KBr).

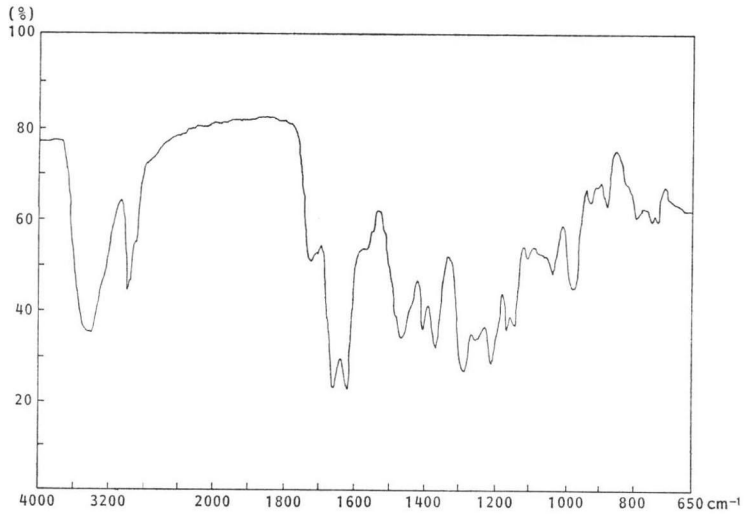


Table 5. Antimicrobial spectrum of awamycin.

Test organism	Minimum inhibitory concentration (μg/ml)
Staphylococcus aureus FDA 209P	0.8
Bacillus subtilis PCI 219	0.1
B. cereus IFO 3001	0.8
Micrococcus luteus PCI 1001	0.05
Escherichia coli NIHJ	>100
Shigella sonnei E-33	>100
Salmonella typhimurium	>100
Klebsiella pneumoniae PCI 602	>100
Pseudomonas aeruginosa P-3	>100
Saccharomyces sake	>100
Candida albicans	>100
Aspergillus niger	>100
Trichophyton rubrum	>100
Piricularia oryzae	>100

Table 6. Antitumor activity of awamycin on sarcoma 180 tumor.

Dose (mg/kg/day)	MSD (range)	ILS (%)
12.5	51 (32~61)	264
6.3	40 (19~>61)	186
3.1	26 (19~50)	86
1.5	20 (20~60)	43
—	14 (12~16)	0

Sarcoma 180 cells (1×10^6) were inoculated into i.p. of ICR mouse.

Mice were given i.p. with awamycin on days 1~9.

Table 7. Antitumor activity of awamycin on IMC carcinoma.

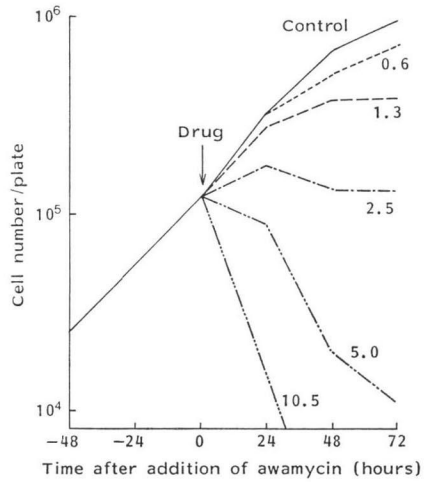
Dose (mg/kg/day)	MSD (range)	ILS (%)
25	53 (19~>60)	180
12.5	35 (26~47)	84
6.3	21 (16~26)	11
3.1	19 (19~26)	0
—	19 (13~26)	0

IMC carcinoma cells (1×10^6) were inoculated into i.p. of CDF_1 mouse.

Mice were given i.p. with awamycin on days 1~9.

Fig. 5. Cytocidal activity of awamycin on HeLa cells.

Numbers in figure indicate concentration (μg/ml) of the antibiotic.



Biological Properties of Awamycin

Awamycin was active against Gram-positive bacteria but inactive or only weakly active Gram-negative bacteria, yeast and fungi. Minimum inhibitory concentrations of awamycin are listed in Table 5.

Antitumor Activity

Antitumor activity of awamycin on sarcoma 180 and IMC carcinoma is shown in Tables 6 and 7 respectively. The antibiotic produced a prolongation of median survival time for both tumors.

The effect of adding awamycin to asynchronous exponentially growing cultures of HeLa cells was determined. Fig. 5 shows that a concentration of 2.5 μg/ml was effective in completely preventing cell growth.

Discussion

A new antibiotic, awamycin, was isolated from the culture filtrate of *Streptomyces* sp. No. 80-217. The physicochemical characteristics of awamycin suggest that this antibiotic belongs to the group of naphthalenoid ansamycins having sulfur atom in the molecule. Several antibiotics including 3-thiome-

thylrifamycin S,[5,6] 3-thiomethylrifamycin SV[5,6] and CP-50833[7] are known to possess a sulfur atom in their structures, but awamycin differs from those antibiotics in the other properties.

From the above data it is suggested that awamycin is a new antitumor antibiotic and the structural studies are in progress.

Besides awamycin, *Streptomyces* sp. No. 80-217 produced macrolide antibiotic identical to B-58941.[8,9]

As awamycin inhibited the growth of HeLa S3 cells *in vitro*, it seems that the antitumor effect *in vivo* is due to direct cytotoxic activity. We are studying the antitumor spectrum of awamycin on a variety experimental tumors, and the results will be reported elsewhere.

Acknowledgments

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