RAVIDOMYCIN (AY-25,545), A NEW ANTITUMOR ANTIBIOTIC*

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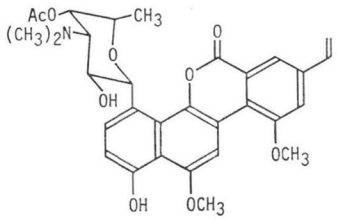
A streptomycete was isolated from a Guatemala soil sample and found to inhibit Gram-positive bacteria including mycobacteria. The antibiotic-producing microorganism was characterized, identified as a new species and named *Streptomyces ravidus*. The antibiotic principle was extracted with organic solvent from the mycelium, isolated in crystalline form and named ravidomycin. Ravidomycin is mainly active against Gram-positive bacteria including mycobacteria. It shows only weak activity against Gram-negative organisms and no activity against fungi. Ravidomycin exhibits potent antitumor activity against P388 lymphocytic leukemia, Colon 38 tumor and CD8F1 mammary tumor. Acute toxicity in mice is low.

Culture of a streptomycete isolated from a soil sample from Guatemala was found to inhibit Gram-positive bacteria. The active principle was isolated from the mycelium of the streptomycete and crystallized after purification. Based on classification of the producing organism, the antibiotic was named ravidomycin. FINDLAY *et al.*[1] have recently reported the structure of ravidomycin (Fig. 1). It is a polycyclic aromatic hydrocarbon with a *C*-glycosyl group. The amino sugar moiety is 3,6-dideoxy-3-*N*,*N*-dimethylamino pseudo altropyranose (ravidosamine). The aglycone moiety is identical with that of toromycin[2] and gilvocarcin[3].

Fig. 1. Ravidomycin (AY-25,545).



This paper deals with the characterization of the producing streptomycete, the isolation and purification of the antibiotic and its biological properties.

Identification of the Ravidomycin-producing Streptomycete

Streptomycete strain AY B-1198 was isolated from a soil sample collected in Guatemala: the soil was diluted with distilled water and the resulting suspensions were plated on yeast-starch agar according to the double-layer technique of PORTER *et al.*[4]. After one week of incubation at 28°C the streptomycete colonies were purified by repeated streaking and the pure strains grown separately on yeast-starch agar plates to yield confluent growth. After 7 days of incubation, 7-mm discs were cut and transferred onto the surface of plates of Bacto-Nutrient agar (Difco Laboratories, Detroit, Mich.) inoculated with test bacteria, and Sabouraud dextrose agar inoculated with *Candida albicans*. The Nutrient agar plates were inoculated with a standardized bacterial inoculum grown at 37°C for 5 hours in Bacto-Nutrient broth; the Sabouraud dextrose agar plates were inoculated with a standardized ino-

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culum of *C. albicans*. All plates were incubated at 37°C. Activity was read as the diameter of inhibition zone after incubation of bacteria and *C. albicans* for 18 and 48 hours respectively. When tested under these conditions, strain AY B-1198 showed the following antimicrobial activity (diameter of inhibition zone in mm): *Micrococcus luteus,* 30; *Staphylococcus aureus* (penicillin resistant), 20; *Bacillus* sp. ATCC 12480, 20; *Pseudomonas aeruginosa, Escherichia coli* and *C. albicans*, no zone of inhibition. Strain AY B-1198 was then maintained on tomato paste - oatmeal agar[5] and preserved by lyophilization[6].

The methods used for characterization were those of the International Streptomyces Project, published by SHIRLING and GOTTLIEB[7].

Morphological Characteristics

Streptomycete strain AY B-1198 grows and sporulates well on most media studied. The morphology as revealed by phase contrast microscopy and electron microscopy is illustrated in Figs. 2 and 3. Aerial mycelium is monopodially branched; sporophores are terminated by long spore chains which form open loops, hooks and sometimes extended spirals of wide diameter. When spore chains are very long, they form very loose spirals without typical arrangements and without a typical number of turns; at 14 days of incubation, spore chains always consist of more than 10 conidia. Therefore, *Streptomyces* AY B-1198 characteristically belongs to PRIDHAM's[8] group *Retinaculum Apertum*. The organism differs considerably, in its physiological characteristics, from all other gray streptomycetes and is considered a new species. It was named *Streptomyces ravidus* (*ravidus*: gray) and was deposited as NRRL 11300. The substrate mycelium is light brown to yellowish brown to rusty depending on the medium, and the presence of melanin has not been observed. Under the electron microscope, spores are cylindrical to oval in the same chain; they are smooth, 0.9 to 1.5 μm in length.

Cultural and Physiological Characteristics

The cultural characteristics of strain AY B-1198 are reported in Table 1. The numbers accompanying colors refer to the Color Harmony Manual Chips used in the guide available from Color Standards Department, Container Corporation of America. Tomato paste - oatmeal agar (PRIDHAM *et al.*[5]) yields rapid growth and abundant sporulation. ISP media also give satisfactory growth and sporulation, but growth is slower on ISP medium 3 than on other media.

The physiological characteristics of *S. ravidus* AY B-1198 are summarized in Table 2. Good

Fig. 2. Photomicrograph of *Streptomyces ravidus* AY B-1198 (ISP medium 2, 14 days at 25°C, ×400).

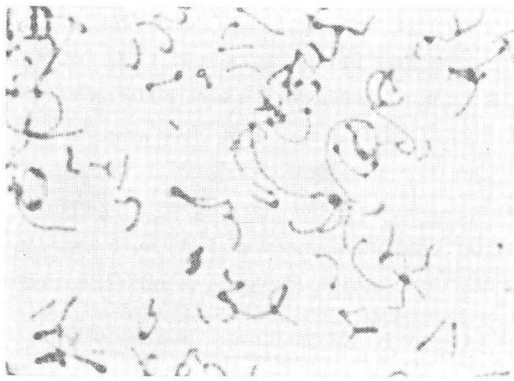

Fig. 3. Electron micrograph of *Streptomyces ravidus* AY B-1198 (ISP medium 2, 14 days at 25°C, ×10,000).

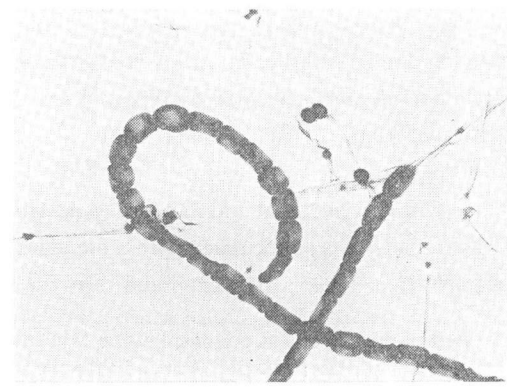

Table 1. Cultural characteristics of *Streptomyces ravidus* AY B-1198.

Culture media[a]	Vegetative mycelium (growth and color)[b]	Aerial mycelium		Pigment[b]
		Development and color[b]	Spores/spiral; shape of sporophores	
Tomato paste - oatmeal agar[5]	Rapid and abundant; cream to gray to grayish brown	Abundant, grayish	>10 spores/spiral	Yellowish brown (3 ie)
Tryptone - yeast extract agar (ISP medium 1, solidified)	Rapid and abundant; yellowish brown	Abundant, grayish	>10 spore/extended spiral	Yellowish brown (3 ie)
Yeast extract - malt extract agar (ISP medium 2)	Rapid and abundant; yellowish brown	Abundant, cream 3 ba	>10 spores/extended spiral	Yellowish brown (3 ie)
Oatmeal agar (ISP medium 3)	Slow but eventually abundant; yellowish brown	Slow but eventually abundant; gray 3 fe	>10 spores/extended spiral	Light brown (3 ie)
Inorganic salts - starch agar (ISP medium 4)	Rapid and very abundant; light brown	Rapid and very abundant; gray 2 ih		None
Glycerol - aspa-ragine agar (ISP medium 5)	Relatively slow but eventually very abundant; rust 4 ng	Very abundant; gray 2 fe	>10 spores/extended spiral	Brown (4 1g)

[a] For composition of ISP media refer to SHIRLING and GOTTLIEB[7]; in this study Bacto dehydrate media (Difco Laboratories, Detroit, Mich.) were used.

[b] Numbers and letters following color refer to Color Harmony Manual Chips, Color Standards Department, Container Corporation of America, 38 South Dearborn Street, Chicago, Ill. 60603, U.S.A.

Table 2. Physiological characteristics of *Streptomyces ravidus* AY B-1198.

Parameters	Reactions and other observations
Hydrolysis of starch (ISP medium 4)	Weak
Decomposition of cellulose	Negative
Production of hydrogen sulfide (ISP medium 6)	Negative
Production of tyrosinase (ISP medium 7)	Negative (melanin-negative)
Nitrate reduction (ISP medium 8)	Positive at 14 days, negative at 7 days
Carbohydrate utilization (ISP medium 9)[a]	Good growth on: D-glucose, D-xylose, *i*-inositol Slight growth (14 days) on: D-fructose No growth on: L-arabinose, rhamnose, raffinose, D-galactose, sucrose, D-mannitol, cellulose, salicin
Streptomycin (10 μg/ml)	Resistant
Reaction to pH	Growth at pH 5~8
Reaction to temperature	Growth at 20~35°C; no growth at 37°C

[a] Bacto - carbon utilization agar was used as the basal medium; slight growth without carbohydrate added.

growth and sporulation are observed in the temperature range of 20 to 32°C, but no growth takes place at 37°C (even after 14 days of incubation). Good growth and sporulation are also observed in the pH range of 5 to 8. No melanoid pigment is produced and no H_2S can be detected. Nitrate is reduced after 14 days, but not after 7 days of incubation. The organism is resistant to 10 μg streptomycin per ml.

S. ravidus AY B-1198 does not utilize a wide variety of carbohydrates. It differs in its carbohydrate utilization and other characteristics from all species of streptomycetes of the gray series. Cellulose is not utilized, and starch is only weakly hydro-lyzed, if at all. Some growth is observed on carbohydrate utilization medium (ISP medium 9) with no added carbohydrate even when the spore inoculum has been washed twice in distilled water, indicating that this streptomycete possibly fixes atmospheric CO_2. Results in Table 3 take this observation into account. D-Glucose, D-xylose and *i*-inositol support rapid and abundant growth, whereas D-fructose supports slight growth at 14 days. All other sugars tested and salicin are not utilized.

Table 3. Production of ravidomycin in aerated-agitated fermenters.

Fermentation time (hours)	pH	Packed cell volume (%)	Yield (μg/ml)
0	7.0	2	—
48	6.0	8	—
72	6.0	16	280
96	6.8	26*	560

* Cell volume measured after pH was adjusted to 4.0.

Production of Ravidomycin

S. ravidus AY B-1198 was grown and maintained on tomato paste - oatmeal agar. Good growth and sporulation were obtained in 7 days incubation at 28°C. Spores from one Roux bottle were washed off and suspended into 50 ml of sterile distilled water to constitute the spore inoculum.

Unbaffled, 500-ml Erlenmeyer flasks were filled with 100 ml of Emerson broth consisting of (g/liter): peptone 4.0, sodium chloride 4.0, yeast extract 1.0, glucose 10.0, and tap water to 1 liter (pH 7.0). The flasks were sterilized at 121°C for 30 minutes, cooled at 28°C and inoculated with 5 ml of the spore inoculum. The inoculated flasks were incubated for 30 hours at 28°C on a gyrotory shaker at 240 rev/minute, 5 cm-stroke to constitute the first stage inoculum.

Unbaffled, 24-liter round bottom flasks were filled with 3.4 liters of the same medium and autoclaved at 121°C for 30 minutes, cooled to 28°C and inoculated with 78 ml (2%) of the first stage inoculum. The inoculated flasks were incubated for 18 hours at 25°C on a reciprocating shaker at 65 strokes per minute and 10 cm-stroke. These flasks were used to inoculate the production stage.

Fermenters (Model F-250, New Brunswick Scientific Co.), 250-liter capacity, equipped with automatic antifoam addition systems and pH recorders-controllers, were filled with 160 liters of the production medium consisting of (g/liter): glycerol 10, tomato paste 10, corn meal 5, "Cerelose" 10, $MgSO_4$ 0.25, "blackstrap" molasses 20, N-Z Case 5, Mazer DF143PX (antifoam) 1 ml, and tap water to 1 liter. The fermenters were sterilized at 121°C for 45 minutes under an agitation of 150 rev/minute and cooled to 28°C (pH after sterilization: 7.0 to 7.2). The fermenters were inoculated with 3.2 liters (2%) of the second stage inoculum. The fermentation was run at 28°C under an agitation of 250 rev/minute and an aeration of 0.5 v/v/minute. Sterile Mazer DF143PX antifoam was added on demand. After 30~35 hours of incubation, the pH started to drop, but was controlled at 6.0 by the addition of a 10 N NaOH solution on demand. The antibiotic titers were determined at 72 and 96 hours of incubation. The maximum titers were usually obtained at 96 hours. The results of a typical fermentation are shown in Table 3.

A spectrophoto-fluorometric method was devised and used to assay ravidomycin in fermentation broths. A methanolic solution of ravidomycin, when excited at 390 nm, emits fluorescence at 490 nm (both values uncorrected). A linear relationship was observed between arbitrary fluorescence units and concentration of ravidomycin for concentrations of 0 to 8 μg/ml. For the assay of fermenta-

tion broths, 1 ml of unknown sample was diluted to 10 ml with reagent grade methanol and the mixture shaken thoroughly. The methanolic extract was centrifuged to remove the suspended particles and suitably diluted with reagent grade methanol so that the final concentration of ravidomycin fell between 2 and 6 μg/ml. The inoculated broth extract did not give any fluorescence and did not quench fluorescence of a standard solution. The titers of antibiotic in fermentation broths were read from the standard curve and corrected for dilution.

Isolation of Ravidomycin

The fermentation broth was adjusted to pH 4.0 with a 30% sulfuric acid solution and filtered on a vacuum rotary filter coated with Celite. The mycelium containing the antibiotic was extracted twice by stirring for 1 hour with 2 volumes of trichloroethane. The trichloroethane extracts were pooled and evaporated to a small volume under reduced pressure, dehydrated with anhydrous sodium sulfate and further concentrated to an oily residue. Typically, a 160-liter fermentation run yielded about 180 g of oily residue. The product was recovered from the concentrate by silica gel G (Merck) column chromatography with a solvent system consisting of varying proportions of acetone (from 20 to 40%) in hexane. The product was eluted with 40% acetone in hexane. The fractions containing ravidomycin were combined and evaporated to dryness. The final crystalline ravidomycin was obtained by crystallization with diethyl ether from the residue. Typically, a 160-liter fermentation run yielded about 30 g of pure product.

Physico-chemical Properties of Ravidomycin

Ravidomycin (AY-25,545) is a bright yellow crystalline compound with the molecular formula $C_{31}H_{33}NO_9$ (M^+ 563.2148), mp 248~250°C. Its optical rotation is $[\alpha]_D -100°$ (CH_3CN). Ravidomycin exhibits the following characteristic ultraviolet absorption maxima (methanol) (log ε): 244 (4.68), 263 (4.54, sh), 277 (4.60), 285 (4.65), 308 (4.33), 320 (4.30), 335 (4.20), 350 (4.08) and 392 nm (4.24). Its structure is given[1] in Fig. 1.

Biological Activity of Ravidomycin

The minimum inhibitory concentration of ravidomycin for selected Gram-positive and Gram-negative bacteria is given in Table 4. Ravidomycin exhibits marked activity against Gram-positive bacteria. It shows only weak activity against Gram-negative bacteria and no activity against fungi.

Table 4. Minimum inhibitory concentration (MIC) of ravidomycin.

Bacteria	MIC (μg/ml)	Bacteria	MIC (μg/ml)
Staphylococcus pyogenes (penicillin^S)	3.2	*Klebsiella pneumoniae*	25
Staphylococcus pyogenes (penicillin^R)	3.2	*Serratia marcescens*	50
Streptococcus faecalis	<0.2	*Mycobacterium tuberculosis* var. *hominis*	1~5
Escherichia coli	100	Photochromogenic mycobacteria (Group I)[a]	25
Enterobacter aerogenes	50		
Salmonella pullorum	50	Scotochromogenic mycobacteria (Group II)[a]	5
Pseudomonas aeruginosa	50		
Proteus mirabilis	25	*Mycobacterium fortuitum* (Group IV)[a]	0.5
Proteus vulgaris	25		

[a] According to the classification of Runyon cited by V. BEER and V. BONIFAS 1975. Les mycobactéries. Schweiz. Med. Wochenschr. 105 (31): 984~987, 1975

Table 5. Effect of ravidomycin on the survival time of mice implanted with lymphocytic leukemia P388[a].

Dose/injection (mg/kg)	Average weight difference of animals (T−C, g)	Survivors on day 5	MST (days)		T/C (%) (MST)
			T	C	
400	−3.4	6/6	7.9	12.7	—
200	−4.6	5/6	11.0	12.7	86
100	−1.0	6/6	19.0	12.7	149
100	−1.2	6/6	25.0	10.5	238
50	−1.4	6/6	14.3	10.5	136
25	−0.3	5/6	12.0	10.5	114

[a] Evaluation: T/C (%)=median survival time (MST) in days of treated animals (T)/control animals (C) ×100. A T/C (%) of 125 or greater is considered significant prolongation of host survival. Evaluation done on day 30.

Table 6. Effect of ravidomycin on Colon 38 tumor in mice and CD8F1 mammary tumors in rats[a].

Tumor	Dose/ injection (mg/kg)	Average weight difference of animals (T−C, g)	Survivors on day 5	MTW (mg)		T/C (%) (MTW)
				T	C	
Colon 38	400	3.4	10/10	0	1,273	0
	200	0.4	10/10	175	1,273	13
	100	3.2	10/10	384	1,273	30
	50	3.8	10/10	1,080	1,273	84
	25	7.2	10/10	661	1,273	51
	12.5	6.2	10/10	668	1,273	52
CD8F1	400	−4.8	0/10	0	1,116	—
	200	−7.6	5/10	0	1,116	—
	100	−7.4	10/10	1	1,116	0
	50	−3.8	10/10	1	1,116	0
	25	−2.4	9/10	576	1,116	51
	12.5	−0.5	9/10	864	1,116	77

[a] Evaluation: T/C (%)=median tumor weight (MTW) estimated from tumor diameter of treated animals (T)/control animals (C)×100. A T/C (%) of 42 or less is considered significant inhibition of tumor growth. Evaluation done on day 30.

Ravidomycin was submitted to the National Cancer Institute for antitumor screening. The activity of ravidomycin in mice implanted with P388 lymphocytic leukemia is reported in Table 5. The mice were treated with single intraperitoneal injections of ravidomycin (in hydroxypropylcellulose) on days 1, 5 and 9. The effect of ravidomycin on Colon 38 tumor in mice and on CD8F1 mammary tumor in rats is given in Table 6. Mice were treated with a single intraperitoneal injection on days 2, 9 and 16 (in hydroxypropylcellulose), and rats were treated on days 1, 8, 15, 22 and 29 in the same vehicle.

The acute intraperitoneal LD_{50} in mice is 400 mg/kg of body weight.

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