ALISAMYCIN, A NEW ANTIBIOTIC OF THE MANUMYCIN GROUP

I. TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL ACTIVITY

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Alisamycin is a new member of the manumycin group of antibiotics produced by *Streptomyces* sp. HIL Y-88,31582, which taxonomically appears to be *Streptomyces actuosus*. Alisamycin is active against Gram-positive bacteria and fungi, and has a weak antitumour activity.

In the course of screening for new bioactive metabolites from actinomycetes, we detected a new member of the manumycin group of antibiotics which we named alisamycin.[1] Other members of this group include manumycin,[2] asukamycin,[3] U-56,407,[4] U-62162[5] and colabomycin.[6] Alisamycin (**1**) was detected by bioautography using TLC plates. In this paper, we present the taxonomy of the producing organism *Streptomyces* sp. Y-88,31582 together with the fermentative production, isolation and biological activity of alisamycin. The structure elucidation will be presented elsewhere.[7]

Taxonomy of the Producing Strain

Streptomyces sp. Y-88,31582 was isolated from a soil sample collected in the coastal region near Alibag, Maharashtra State, India. The strain has been deposited at the German Collection of Microorganisms and Cell Cultures, Braunschweig, where it has been assigned the accession number DSM 5559. The methods of the International Streptomyces Project (ISP) recommended by SHIRLING and GOTTLIEB[8] and WAKSMAN[9] were employed in the characterization of the strain.

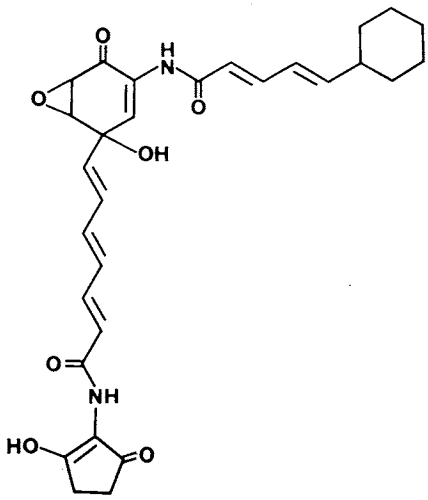

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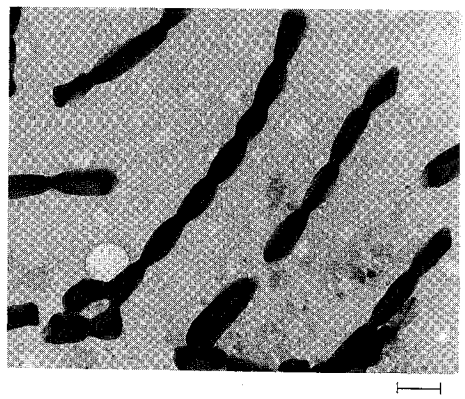
Fig. 1. Electronmicrograph of *Streptomyces* sp. Y-88,31582 (magnification × 12,000).

Bar represents 1 μm.



Morphological Properties

The vegetative mycelium of *Streptomyces* sp. Y-88,31582 grows abundantly on both synthetic and complex agar media and does not show fragmentation into bacillary or coccoid forms. After cultivation on yeast extract-malt extract agar and inorganic salts-starch agar at 27°C for 14 days the following morphological properties were observed: The aerial mycelium branched monopodially with sporophores forming straight spore chains of section *Rectiflexibiles* with 10~50 spores per chain. The spores are cylindrical and regular shaped (0.6~0.7×1.2~1.5 μm) with a smooth surface (Fig. 1).

Chemical Composition

The chemical analysis of cell wall diaminopimelic acid isomers carried out by the method of LECHEVALIER and LECHEVALIER[10] showed the presence of LL-diaminopimelic acid.

Cultural and Physiological Characteristics

Cultural characteristics of *Streptomyces* sp. Y-88,31582 grown on various media at 27°C for 14 days are shown in Table 1. The reverse mycelium had no pH indicator properties. Melanoid pigment was produced in peptone-yeast extract-iron agar and tyrosine agar. The physiological characteristics of the strain are shown in Table 2. The utilization of carbon sources, which was tested by growth on PRIDHAM

Table 1. Cultural properties of *Streptomyces* sp. Y-88,31582.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract-malt extract agar (ISP 2)	Good, elevated, dry	Abundant, grayish white, powdery	Pale yellowish brown	None
Oatmeal agar (ISP 3)	Good, flat, dry	Good, powdery, gray	Pale yellowish brown	None
Inorganic salts-starch agar (ISP 4)	Good, raised, dry	Good, powdery gray	Pale white	None
Glycerol-asparagine agar (ISP 5)	Moderate, wrinkled, dry	Scant to none	Pale brown	None
Peptone-yeast extract-iron agar (ISP 6)	Moderate, elevated	None	Pale brown	Light purple
Tyrosine agar (ISP 7)	Good, wrinkled, dry	Abundant, cottony, grayish white	Black	Light purple
Sucrose-nitrate agar	Moderate, raised	Scant, white	Pale white	None
Peptone-beef extract agar	Good, flat, dry	None	Pale white	Pale brown

Table 2. Physiological properties of *Streptomyces* sp. Y-88,31582.

Optimum growth temperature	25~37°C	Tyrosinase reaction	Positive
Nitrate reduction	Negative	Production of H_2S	Negative
Liquefaction of gelatine	Positive	Cellulolytic activity	Negative
Starch hydrolysis	Positive	Growth on CZAPEK's solution agar	Moderate
Coagulation of milk (37°C)	Negative	Streptomycin inhibition	>0.8 μg/ml
Peptonization of milk (37°C)	Positive	NaCl tolerance	>6, <8%
Melanin formation	Positive	pH tolerance	5.0~9.0

and GOTTLIEB's medium containing 1% of each carbon source at 27°C and observed for 16 days, is shown in Table 3.

Table 3. Utilization of carbon sources by *Streptomyces* sp. Y-88,31582.

Response	Carbon source
Positive	D-Glucose, D-xylose, D-fructose, D-mannose, L-arabinose, D-mannitol, *m*-inositol, galactose, lactose, sucrose, maltose, cellobiose, sodium glutamate, L-rhamnose, raffinose, salicin
Negative	Cellulose, dulcitol, melezitose

The basal medium used was ISP-9.

On the basis of the observed characteristics, *Streptomyces* sp. Y-88,31582 belongs to the gray color series. Among the species described in the 8th Edition of BERGEY's Manual,[11] SHIRLING's ISP reports[12~15] and NONOMURA's key,[16] the one most closely resembling this producing organism is *Streptomyces actuosus* Pinnert, Ninet and Preud'homme 1964. *Streptomyces* sp. Y-88,31582 differs from *S. actuosus*[14] only in that the former produces a scanty aerial mycelium on ISP medium 5 while the latter is reported to have a good aerial mycelium on this medium. Therefore, *Streptomyces* sp. Y-88,31582 is most probably *S. actuosus*.

Fermentation

Streptomyces sp. Y-88,31582 was cultured and maintained on yeast extract-malt extract agar slant. Inoculum was prepared in Erlenmeyer flasks (500 ml) containing 100 ml of a seed medium consisting of glucose 1.5%, soyabean meal 1.5%, corn steep liquor 0.5%, NaCl 0.5% and $CaCO_3$ 0.2% (pH 6.5), which was incubated at 27°C on a rotary shaker with a 4-cm throw at 240 rpm for 72 hours after inoculation with a loopful of mature slant culture.

Fermentation was carried out both in 500-ml Erlenmeyer flasks containing 100 ml production medium and in 15-liter fermenters containing 10 liters production medium consisted of sucrose 2.0%, $CaCO_3$ 0.25%, KNO_3 0.1%, K_2HPO_4 0.05%, $MgSO_4 \cdot 7H_2O$ 0.05% and NaCl 0.05% (pH 7.2). In fermenters 2 ml Desmophen was added as antifoaming agent.

Shake flasks, inoculated with 4% inoculum, were incubated on the shakers described above for 44 hours when optimum production was obtained. In fermenters the fermentation was carried out at 27°C under aeration of 6~8 liters/minute and stirrer speed of 120 rpm. An inoculum of 9.0% was used. Detection of alisamycin production in the fermentation broth was monitored by activity against *Staphylococcus aureus* 209 P, and quantified by TLC of the ethyl acetate extract of the filtered broth followed by densitometric analysis using a Shimadzu CS-930 TLC scanner set at 280 nm adsorbent wavelength. Monitoring of antibiotic production during the fermentation was critical as maximum antibiotic production levels are maintained for a short time period, especially in the fermenters. A typical time-course of this fermentation in the 15-liter fermenters is shown in Fig. 2. Production of alisamycin starts during log phase and reaches a maximum between 23~27 hours after which a loss in activity sets in. The fermentative production profile of alisamycin resembles that of manumycin from *Streptomyces parvulus* in that there is a rapid increase in production followed by a sharp decline.[2]

Isolation

The culture filtrate from two fermenters (17 liters) was extracted twice with 10 liters each of ethyl acetate at pH 7.0. The ethyl acetate extract was concentrated to dryness to give 4.5 g crude compound which was dissolved in a minimum amount of ethyl acetate and precipitated with *n*-hexane. This procedure was repeated thrice to obtain a dry powder (1.2 g) which was charged onto a 8×15 cm glass column packed with 400 g silica gel (100~200 mesh). Alisamycin eluted out with a chloroform-ethyl acetate (70:30 to

Fig. 2. Time course of the fermentative production of alisamycin in 15-liter fermenters.

○ pH, ▲ activity, △ packed cell volume.

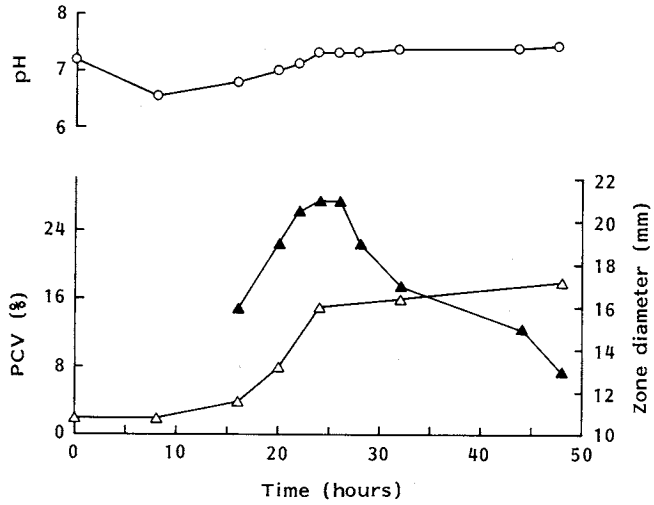


Table 4. MICs of alisamycin.

Test organism	Zone diameter (mm)		MIC (μg/ml)
	500 μg/ml	32 μg/ml	
Staphylococcus aureus 209 P	26	18.5	1.6
S. aureus 20424	25	19	3.2
S. aureus E88	27	14	6.4
Streptococcus faecalis UD8b	16	13	>50
S. faecalis Eder	23	16	6.4
S. faecalis 23241	22	13	6.4
Escherichia coli 9632	—	—	>50
Candida albicans	10	—	50
Piricularia oryzae	15	11	>50
Botrytis cinerea 47	20	—	>50

65:35) gradient. The active eluates containing alisamycin were concentrated to dryness (0.42 g) and then dissolved in a minimum amount of ethyl acetate to which *ca.* 50 ml methanol was added. 0.5 ml double distilled water was added to this methanolic solution and the solution kept overnight at −20°C. The resultant precipitate was dissolved in a minimum amount of ethyl acetate followed by 50 ml acetonitrile and kept at −20°C for at least 48 hours. 110 mg pure yellow crystalline alisamycin was obtained as first crop.

Biological Activity

Alisamycin is active primarily against Gram-positive bacteria but also shows an antifungal activity when tested by the agar-well method in Antibiotic Assay medium. The activities both by this method and by the agar-dilution method in Mueller-Hinton agar (MIC) are shown in Table 4. In addition to this antimicrobial activity alisamycin also displayed a weak cytotoxic effect in the proliferation assay (IC_{50} 0.78 μg/ml) and in the stem cell assay (IC_{50} 2.0 μg/ml) against murine L1210 leukemia cells.

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