SWALPAMYCIN, A NEW MACROLIDE ANTIBIOTIC

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION AND BIOLOGICAL ACTIVITY

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A new macrolide antibiotic, swalpamycin, has been isolated from the culture broth of *Streptomyces* sp. Y-84,30967. Taxonomically the producing organism most closely resembles *Streptomyces anandii* and has therefore been named *S. anandii* subsp. *swalpus*. Swalpamycin is a neutral 16-membered macrolide active against Gram-positive bacteria including erythromycin-resistant strains.

In the course of screening for new macrolide antibiotics from actinomycetes, we detected and isolated a novel 16-membered compound which we named swalpamycin (swalpa meaning minor in Sanskrit). It is coproduced with chalcomycin. Swalpamycin[1,2] (Fig. 1) contains a novel macrolide aglycone which we have called swalpanolide and the two neutral sugars mycinose and aldgarose. In this paper we present the taxonomy of the producing organism *Streptomyces* sp. Y-84,30967 together with the fermentative production, isolation procedure and biological activity of swalpamycin.

Taxonomy of the Producing Strain

The swalpamycin producing strain *Streptomyces* sp. Y-84,30967 was isolated from a soil sample collected near Pune, Maharashtra State, India.

The strain has been deposited at the Deutsches Sammlung von Mikroorganismen, Göttingen, FRG, where it has been assigned the accession number DSM 3740.

Fig. 1. Structure of swalpamycin.

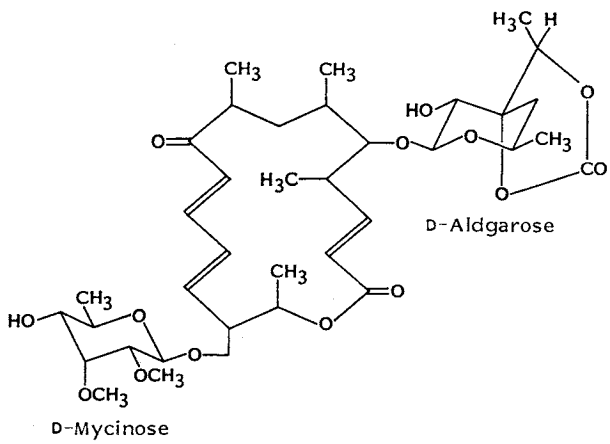
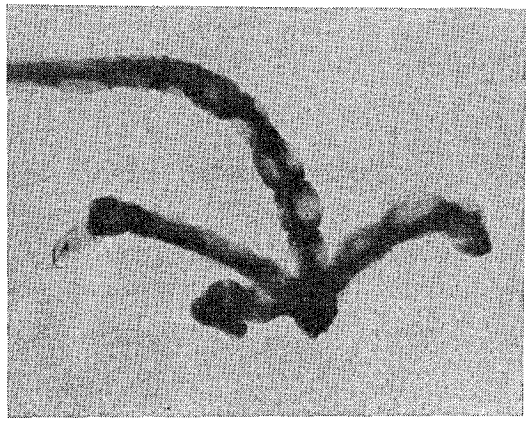


Fig. 2. Photomicrograph of the swalpamycin-producing strain on inorganic salts - starch agar.

Fig. 3. Electron micrograph of spore chains of *Streptomyces* sp. Y-84,30967.



The strain was characterized by the methods of the International Streptomyces Project (ISP) recommended by SHIRLING and GOTTLIEB[3] and WAKSMAN[4].

Morphological Properties

The vegetative mycelium of *Streptomyces* sp. Y-84,30967 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 spore chains in open spirals with 10 or more spores per chain (Fig. 2). Many imperfect spirals, hooks and loops are also present. Whirls are not observed. The spores are cylindrical ($0.3 \sim 0.4 \times 0.5 \sim 0.6$ μm) with a smooth surface (Fig. 3).

Chemical Composition

The chemical analysis of cell wall diaminopimelic acid isomers carried out by the method of LECHEVALIER and LECHEVALIER[5] showed the presence of LL-diaminopimelic acid.

Cultural and Physiological Characteristics

Cultural characteristics of *Streptomyces* sp. Y-84,30967 grown on various media at 27°C for 14 days are shown in Table 1. The reverse mycelial pigment had no pH indicator properties. Soluble melanoid pigment was produced in peptone - yeast extract - iron agar, tyrosine agar and Tryptone - yeast extract agar.

Physiological characteristics of the strain are summarized in Table 2. The utilization of carbon sources, which was tested by growth on PRIDHAM and GOTTLIEB's medium containing 1% of each carbon source at 27°C and observed for 16 days, is shown in Table 3.

Comparison with Other Related Species

On the basis of its characteristics, *Streptomyces* sp. Y-84,30967 belongs to the gray or red series. Among the species of *Streptomyces* described in the 8th Ed of BERGEY's manual[6], SHIRLING's

Table 1. Cultural properties of *Streptomyces* sp. Y-84,30967.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Yeast extract - malt extract agar	Abundant	Abundant, white to pinkish gray	Pale brown	None
Oatmeal agar	Abundant	Abundant, white to gray to pale brown	Pale yellow	None
Inorganic salts - starch agar	Abundant	Abundant, gray to brownish gray	Pale brown	None
Glycerol - asparagine agar	Good	Weak, white	Pale yellow	None
Peptone - yeast extract agar	Moderate	None	Dark brown to black	Brownish black
Tyrosine agar	Good	Good, white to pinkish gray	Brownish black	Pale brown
Sucrose - nitrate agar	Weak	Scant, white	Pale brown	None
Glucose - asparagine agar	Moderate	Weak, white to grayish brown	Pale yellow	None
Nutrient agar	Moderate	None	Pale brown	None

Table 2. Physiological properties of *Streptomyces* sp. Y-84,30967.

Temperature	
Range for growth	15~45°C
Optimum	27°C
Production of melanoid pigments	
Tryptone - yeast extract agar	Positive
Peptone - yeast extract - iron agar	Positive
Tyrosine agar	Positive
Hydrolysis of starch	Weakly positive
Liquefaction of gelatin	Positive
Peptonization of milk	Negative
Coagulation of milk	Positive
H_2S production	Positive
Nitrate reduction	Nagative
Cellulolytic activity	Negative
NaCl tolerance	>4%, <7%
pH tolerance	5.0~9.0
Streptomycin inhibition	Inhibition at ≥0.5 μg/ml
Growth on CZAPEK's solution agar	Poor

ISP reports[7~10], and NONOMURA's key[11], the one most closely resembling this producing organism is *Streptomyces anandii* Batra and Bajaj 1965.

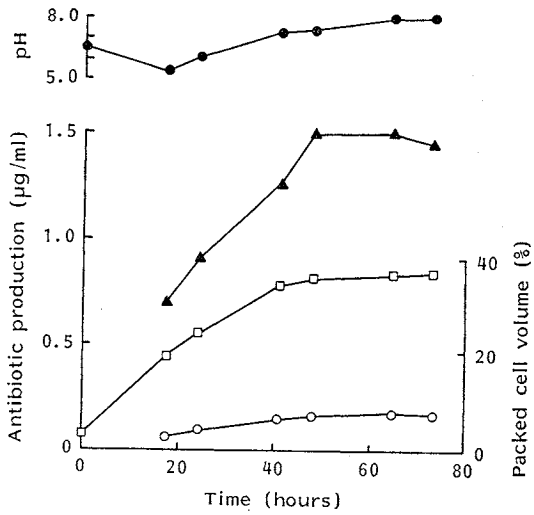
However, *Streptomyces* sp. Y-84,30967 differs from *S. anandii* in its growth on yeast extract - malt extract agar where *Streptomyces* sp. Y-84,30967 forms a whitish pinkish gray aerial mycelium while *S. anandii* produces a yellowish

Table 3. Carbohydrate utilization of *Streptomyces* sp. Y-84,30967.

D-Glucose	+	Cellulose	−
L-Arabinose	+	Galactose	+
Sucrose	±	Salicin	−
D-Xylose	+	Maltose	+
m-Inositol	+	Cellobiose	+
D-Fructose	+	D-Mannose	+
Rhamnose	−	Dulcitol	−
Raffinose	+	Lactose	+
D-Mannitol	+	Sodium glutamate	+

Fig. 4. Time course of the fermentation of *Streptomyces* sp. Y-84,30967.

○ Swalpamycin, ▲ chalcomycin, □ packed cell volume, ● pH.



gray aerial mass color. In addition, on CZAPEK's agar *Streptomyces* sp. Y-84,30967 grows poorly whereas *S. anandii* shows excellent growth. *S. anandii* is not known to produce chalcomycin but is reported[12] to produce a pentaenic antifungal antibiotic, which is absent in *Streptomyces* sp. Y-84,30967. It is appropriate, therefore, to classify *Streptomyces* sp. Y-84,30967 as a varient of *S. anandii* and designated *Streptomyces anandii* subsp. *swalpus*.

The microorganisms known to produce chalcomycin are *Streptomyces bikiniensis* and *Streptomyces goshikiensis*. *Streptomyces* sp. Y-84,30967 differs from *S. bikiniensis* in the spore chain morphology and melanin pigment production and from *S. goshikiensis* in the utilization of carbon sources.

Fermentation

Streptomyces sp. Y-84,30967 was cultured and maintained on yeast extract - malt extract agar slants.

A loopful of mature slant culture of *Streptomyces* sp. Y-84,30967 was inoculated into Erlenmeyer

Fig. 5. Isolation and purification.

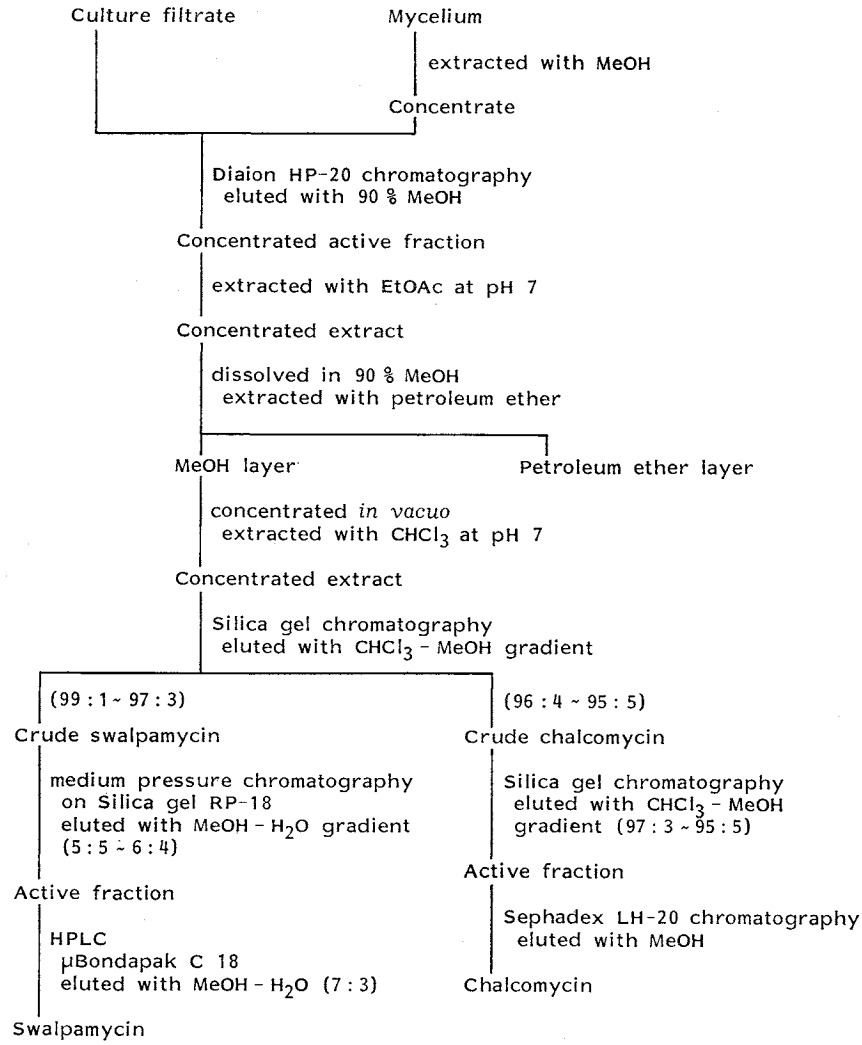


Fig. 6. TLC of swalpamycin and chalcomycin.
TLC SiO_2: Merck 5554, $CHCl_3$ - MeOH (93 : 7).
Detection: 254 nm.

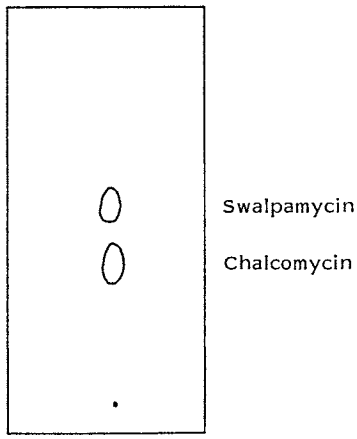
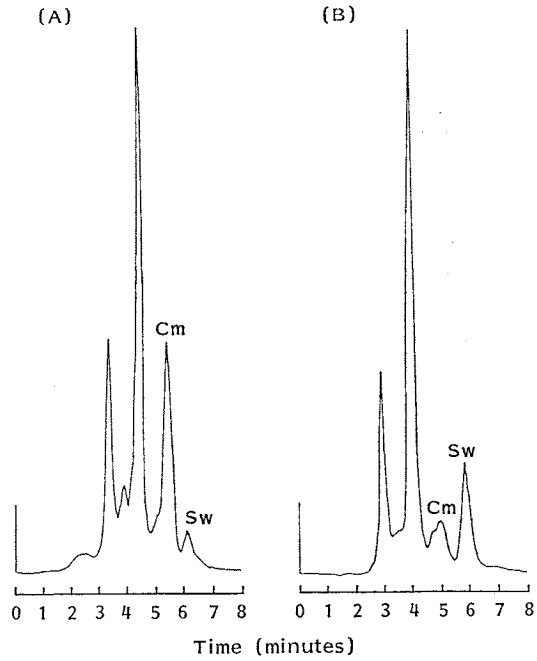


Fig. 7. Reverse phase chromatogram of extract containing swalpamycin (Sw) and chalcomycin (Cm).
Column: μBondapak C 18 (7.8×300 mm).
Mobile phase: MeOH - H_2O (7 : 3).
Detection at A: 216 nm, B: 280 nm.



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) and incubated at 27°C on a rotary shaker with a 4 cm-throw at 240 rpm for 72 hours. The resultant vegetative growth was used to inoculate at a rate of 8%, two 15-liter glass fermentors containing 10 liters each of the seed medium for the preparation of second stage seed culture. The fermentation was carried out at 27°C for 24 hours under aeration of 7 liters/minute and agitation of 180 rpm. This second stage seed culture was inoculated into a 390-liter fermentor containing 280 liters of production medium comprised of glucose 1.5%, soluble starch 2%, $(NH_4)_2SO_4$ 0.5%, Soyatone 0.3%, peptone 0.3%, $CaCO_3$ 0.2%, NaCl 0.2% and corn steep liquor 0.2% (pH 6.5). The fermentor was operated at 27°C for 48 hours under aeration of 170 liters/minute and agitation of 120 rpm.

The antibiotic level in the fermentation broth was assayed both by activity against *Staphylococcus aureus* 209 P and by high pressure liquid chromatography.

A typical time course of the fermentation in a 390-liter fermentor is presented in Fig. 4. The production of antibiotic compounds reached a maximum between 48~60 hours after inoculation. The antibiotic activity decreased with prolongation of fermentation.

Isolation and Purification

The flow diagram for the isolation of swalpamycin is presented in Fig. 5. The culture filtrate (230 liters) was adjusted to pH 6.0 and applied to a column of Diaion HP-20 (12 liters). The column was washed repeatedly with brine and water followed by two bed volumes of 30%, and then 50% aqueous methanol. The adsorbed antibiotics were eluted with 90% aqueous methanol. The active eluates were concentrated *in vacuo* and then extracted with ethyl acetate. The methanol extract of the mycelial cake was concentrated *in vacuo* to afford an aqueous solution which was diluted with water and chromatographed on Diaion HP-20 in a similar manner to that for the broth filtrate. The active

Table 4. Antimicrobial activity of erythromycin (Em), chalcomycin (Cm) and swalpamycin (Sw).

Test organism	MIC (μg/ml)		
	Em	Cm	Sw
Staphylococcus aureus 209 P	0.2	3.2	1.6
S. aureus 20424 Mac^R	>100.0	>100.0	6.3
S. aureus 3066 $Meth^R$	0.8	1.6	3.2
S. aureus R 85 Tet^R	0.4	1.6	6.3
S. aureus R 85/M Em^R	>100.0	>100.0	12.6
S. aureus 712 $Meth^R$	>100.0	1.6	6.3
S. aureus 789 $Meth^R$	>100.0	>100.0	6.3
S. aureus MLS 11 Em^R	>100.0	6.3	>50.0
S. aureus MLS 14 Em^R	>100.0	3.2	6.3
S. aureus MLS 16 Em^R	>100.0	1.6	3.2
S. aureus 011UC5 Mac^R	>100.0	>50.0	25.0
S. aureus 011GR5 Em^R	>100.0	3.2	6.3
Streptococcus faecalis UD8b Mac^R	>100.0	>100.0	>50.0
S. faecalis Eder Mac^R	>100.0	>100.0	>50.0
Micrococcus luteus ATCC 9341	0.1	0.1	0.8
Bacillus subtilis ATCC 6633	0.1	0.1	0.1
Escherichia coli 9632	>100.0	>100.0	>100.0
E. coli 250 GR2	>100.0	>100.0	>100.0
Alcaligenes faecalis HIL 38	>100.0	>100.0	>100.0
Pseudomonas aeruginosa M 35	>100.0	>100.0	>100.0
Proteus vulgaris HIL 22	>100.0	>100.0	>100.0
Enterobacter cloacae P 99	>100.0	>100.0	>100.0
Serratia marcescens 20460	>100.0	>100.0	>100.0
Citrobacter freundii HIL 39	>100.0	>100.0	>100.0
Candida albicans HIL 111	>100.0	>100.0	>100.0
Aspergillus niger HIL 113	>100.0	>100.0	>100.0

Mac^R, Macrolide-resistance; $Meth^R$, methicillin-resistance; Tet^R, tetracycline-resistance; Em^R, erythromycin-resistance.

eluates were concentrated, diluted with water and then extracted with ethyl acetate. The combined ethyl acetate extracts were concentrated and the residue obtained was dissolved in 90% aqueous methanol. After repeated extraction with petroleum ether, the aqueous methanol layer was concentrated *in vacuo* to remove methanol, diluted with water and extracted with chloroform. The chloroform extract was concentrated *in vacuo* to give 35 g crude material which was subjected to chromatography on silica gel (100~200 mesh, 3 kg) with a chloroform - methanol gradient. The eluates were analyzed by TLC (Fig. 6) and by HPLC (Fig. 7). Fractions eluted with chloroform - methanol (99:1~97:3) contained swalpamycin as a major component and those eluted with chloroform - methanol (96:4~95:5) contained chalcomycin predominantly.

Pure chalcomycin was obtained from the latter fractions by repeated chromatography on silica gel using a chloroform - methanol gradient followed by chromatography on Sephadex LH-20 using methanol as eluting solvent. Chalcomycin[13] was characterized by the identity of its physical and spectroscopic properties with those of an authentic sample.

Two g of swalpamycin-enriched powder was applied to Silica gel RP-18 in a Labomatic column (2.9×40 cm) and eluted with a water - methanol gradient using a Büchi 681 chromatographic pump, at a flow rate of 32 ml/minute, with detection *via* a UV detector at 216 nm. Fractions eluted with 50~60% aqueous methanol were concentrated to an aqueous solution and extracted with chloroform

to yield 600 mg semi-pure powder of swalpamycin. Further purification was achieved by preparative HPLC using a μBondapak C 18 column (7.8×300 mm), elution with methanol - water (7:3) at a flow rate of 2 ml/minute, with UV detection at 216 nm, to afford 180 mg pure swalpamycin.

Biological Activity

The minimum inhibitory concentrations of swalpamycin in comparison to erythromycin and chalcomycin, assayed by the agar dilution method, are given in Table 4. Swalpamycin is active against Gram-positive bacteria including strains of *Staphylococcus aureus* resistant to erythromycin and methicillin.

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