SPOREAMICIN A, A NEW MACROLIDE ANTIBIOTIC

I. TAXONOMY, FERMENTATION, ISOLATION AND CHARACTERIZATION

SATOSHI YAGINUMA, ATSUKI MORISHITA, KENYA ISHIZAWA, SAKAE MUROFUSHI, MITSUO HAYASHI and NAOKI MUTOH

Research Labolatories, Toyo Jozo Co., Ltd.,
Ohito, Shizuoka 410-23, Japan

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Sporeamicin A, a novel antibiotic, was isolated from the culture filtrate of an actinomycete. The producing organism, strain L53-18, was taxonomically assigned as a species of the genus *Saccaropolyspora*. The antibiotic was extracted with chloroform and was then purified by crystallization. It was obtained as colorless prisms from ethanolic solutions. Sporeamicin A exhibited a strong UV absorption peak at 276 nm. The molecular formula of sporeamicin A was determined to be $C_{37}H_{63}NO_{12}$.

An antimicrobial agent having a new structure is always desired as a lead in the development of novel chemotherapeutic agents. In the course of our screening for new antibiotics from actinomycetes, a novel antibiotic, sporeamicin A, was found. The antibiotic was produced by a species of *Saccharopolyspora*. It exhibited antimicrobial activity against Gram-positive bacteria. In this paper, we describe the taxonomy of the producing strain, strain L53-18, and its fermentation, as well as the isolation and characterization of sporeamicin A.

Results

Taxonomy

Strain L53-18 was isolated from a soil sample collected at Setouchi-cho, Kagoshima Prefecture, Japan. Stock slant cultures of this strain were maintained on yeast extract - malt extract agar. The methods described by SHIRLING and GOTTLIEB[1] were employed for the taxonomic study. Morphological observations were made by light and electron microscopy using cultures grown at 30°C for 10~20 days on inorganic salts - starch agar and yeast extract - malt extract agar.

Strain L53-18 was a Gram-positive and non-acid fast actinomycete which produced both substrate and aerial hyphae on most of the media used in this study. The substrate hyphae (0.4~0.6 μm in diameter) spread out to form long branches which tangled complicatedly with each other. In the latter term of the cultivation, typical nocardioform fragmentation was observed in the colony. The aerial hyphae (0.5~0.7 μm in diameter), which grew from substrate hyphae, formed curves or straights and simple-branches. The aerial hyphae was segmented into bead-like chains of spores (usually 10 or more spores per chain) which often were connected by 'empty' hyphae. The spore chains formed loops, hooks or spirals with two or three turns, but frequently straight to flexuous chains were formed. The spores were oval (0.5~0.7 × 0.7~1.3 μm) to short cylindrical, and the surfaces were covered with a sheath growing long spines (Fig. 1). Sporangia and zoospores were not observed.

Cultural characteristics were observed on several media described by SHIRLING and GOTTLIEB[1], and

WAKSMAN[2]. Incubation was carried out at 30°C for 20 days. The color names used in this study were taken from the Color Harmony Manual[3]. Results are shown in Table 1. The substrate mycelium was oak brown to cinnamon. Aerial mycelium was formed on most media except on glucose-asparagine agar, and was powdery in appearance and light fawn to bisque on most media. Copper brown to oak brown soluble pigments were produced on most media.

Cell wall analysis was performed by the method of STANECK *et al.*[4]. Analysis of the whole cell hydrolysate showed the presence of *meso*-diaminopimeric acid, arabinose and galactose, but did not reveal xylose. Phospholipid analysis was

Fig. 1. Transmission electron micrograph of spores of strain L53-18 on yeast extract-malt extract agar incubated at 30°C for 14 days.

Bar represents 1 μm.

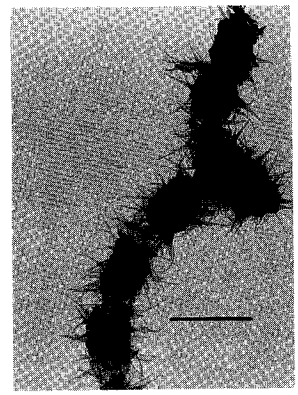


Table 1. Cultural characteristics of strain L53-18.

Medium	Growth	Substrate mycelium	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	Good	Copper brown (5pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Copper brown (5pi)
Glucose-asparagine agar	Poor	Colorless to light ivory (2ca)	None	None
Glycerol-asparagine agar	Good to moderate	Cinnamon (3le)	Good to moderate: Powdery, light fawn (4ge)	Dark luggage tan (4pg)
Inorganic salts-starch agar	Good to moderate	Cinnamon (3le)	Good to moderate: Powdery, light fawn (4ge) to bisque (4ec)	Dark luggage tan (4pg), slightly
Tyrosine agar	Good	Oak brown (4pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Copper brown (5pi)
Oatmeal agar	Good to moderate	Light tan (4gc)	Good to moderate: Powdery, light fawn (4ge) to bisque (4ec)	Nude tan (4gc)
Yeast extract-malt extract agar	Good	Oak brown (4pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Copper brown (5pi)
Nutrient agar	Moderate	Light wheat (2ea)	Poor: White (a) to pearl (3ba)	None
Glycerol-nitrate agar	Good	Oak brown (4pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Copper brown (5pi)
BENNET's agar	Good	Oak brown (4pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Oak brown (4pi)
EMMERSON's agar	Good	Cinnamon (3le)	Moderate to good: Powdery, light fawn (4ge) to bisque (4ec)	Oak brown (4pi)
HICKEY & TRESNER's agar	Good	Oak brown (4pi)	Good: Powdery, light fawn (4ge) to bisque (4ec)	Copper brown (5pi)

performed by the procedure of MINNIKIN *et al.*[5], and showed the presence of phosphatidyl choline. Accordingly, the cell wall and phospholipid patterns of this strain are classified as type IV[6] and type PIII[7], respectively.

Lipid LCN-A and nocardiomycolic acid were not found to be present in this strain when tested by the method of MORDARSKA *et al.*[8], and MINNIKIN *et al.*[9].

Menaquinones were analyzed by the method of COLLINS *et al.*[10], and the major components were found to be MK-9(H_4) and MK-10(H_4).

Physiological properties of strain L53-18 are shown in Table 2. The temperature range for growth and the oxygen requirement test were determined on yeast-malt extract agar[11]. Gelatin liquefaction was examined for 5~20 days on a medium consisting of gelatin 20%, glucose 2% and peptone 0.5% (pH 7.0). The medium was placed at 5°C after cultivation to detect liquefaction. H_2S production and resistance to lysozyme were determined by the methods of TRESNER *et al.*[12] and GORDON *et al.*[13], respectively. Salt tolerance was determined on yeast-malt extract agar containing NaCl prepared at concentrations of 0, 3, 5, 7, 9, 10, 12 and 14%, respectively.

Antibiotics resistance testing was performed by the method of IWASAKI *et al.*[14]. The results are shown in Table 3. All aminocyclitol antibiotics except spectinomycin showed MIC values in a range between 16 and 63 μg/ml when tested against strain L53-18.

Degradation was examined by the methods of GORDEN[15], GOODFELLOW[16] and LACEY *et al.*[17]. The results are shown in Table 4.

Utilization of sugars and organic acids were examined according to the methods of PRIDHAM *et al.*[18] and GORDON *et al.*[19], respectively. The results were determined after 20 days incubation at 30°C, and are shown in Tables 5 and 6.

From the results of these studies, the main characteristics of strain L53-18 are summarized as

Table 2. Physiological characteristics of strain L53-18.

Temperature range	24~45°C
Optimum temperature	28~35°C
Gelatin liquefaction	Positive
Starch hydrolysis	Positive
Milk peptonization	Positive
Milk coagulation	Doubtful
Melanoid pigment production	Negative
O_2 requirement	Aerobic
H_2S production	Positive
Lysozyme resistance	Sensitive
NaCl tolerance	≦10%

Table 3. Antibiotic sensitivities of strain L53-18.

Antibiotic	MIC (μg/ml)
Kanamycin sulfate	63
Gentamicin sulfate	16
Paromomycin sulfate	31
Streptomycin sulfate	31
Neomycin sulfate	63
Spectinomycin sulfate	>1,000
Rifampicin	<16
Leucomycin A_5	>1,000

Table 4. Substrate degradation by strain L53-18.

Response	Substrates
Positive	Tyrosine, casein, xanthine, hypoxanthine, adenine, aesculin, elastin, urea
Negative	Cellulose, keratin

Table 5. Sugar utilization by strain L53-18.

Response	Sugars
Positive	L-Arabinose, D-fructose, D-galactose, D-glucose, glycerol, inositol, D-mannitol, D-mannose, melezitose, melibiose, raffinose, L-rhamnose, D-ribose, trehalose, sucrose, D-sorbitol, D-xylose, cellobiose, starch, adonitol, erythritol
Negative	β-Lactose, L-sorbose, dulcitol, saricin, α-methyl-D-glycoside, cellulose

Table 6. Organic acid utilization by strain L53-18.

Response	Organic acids and salts
Positive	Sodium acetate, sodium benzoate, sodium butylate, sodium citrate, sodium fumarate, sodium malate, sodium propionate, sodium succinate, adipic acid, sodium pyruvate, sebacic acid
Negative	Sodium tartrate

follows: According to morphology, the aerial hyphae which form many spore chains were produced from the substrate hyphae with nocardioform fragmentation, and zoospores and sporangia were not formed. According to chemotaxonomy, this strain belongs to cell wall type IV and phospholipid type PIII. Neither lipid LCN-A nor nocardiomycolic acid were detected. MK9(H_4) and MK10(H_4) were detected as the major menaquinones.

For the reasons mentioned above, strain L53-18 belongs to the genus *Saccharopolyspora* LACEY and GOODFELLOW[17]. Concequently, strain L53-18 will be named *Saccharopolyspora* sp. L53-18, and has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under accession No. FERM BP-2231.

Fermentation

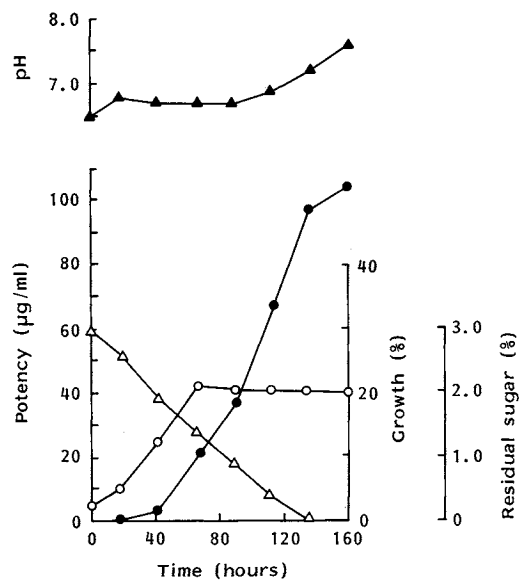
A seed medium (200 ml) consisting of glucose 1%, dextrin 1%, yeast extract 0.5%, casein hydrolysate 0.5% and calcium carbonate 0.1% (pH 6.5) in tap water was transferred into two 500-ml Erlenmeyer flasks and was sterilized at 120°C for 15 minutes. A loopful of *Saccharopolyspora* sp. L53-18 was inoculated into the seed medium and was incubated under shaking conditions at 28°C for 3 days. The primary seed culture (200 ml) was then inoculated into a 30-liter jar fermenter containing sterile seed medium (20 liters, pH 6.5 before sterilization at 120°C for 20 minutes) supplemented with 0.02% of FS-antifoam 028 (Dow Corning K.K., Japan). This secondary seed fermentation was incubated at 28°C for 2 days with agitation at 200 rpm, and was aerated at 20 liters per minute.

A production medium (200 liters) consisting of glucose 3%, corn steep liquor 1%, dry yeast 0.6%, cobalt chloride 0.001% and FS-antifoam 028 0.04% (pH 7.0) in tap water was transferred into a 250 liter-fermenter and was sterilized at 120°C for 30 minutes. The secondary seed culture (5 liters) was inoculated into the sterile production medium and was incubated at 28°C for 161 hours, with agitation at 200 rpm, and was aerated at 180 liters per minute. The amount of antibiotic produced was monitored by HPLC analysis using a Hitachi gel #3056 column (4.6 mm × 15 cm) with UV detection at 276 nm. The mobile phase was acetonitrile - methanol - 1/15 M ammonium acetate (50 : 25 : 35) with a flow rate of 0.8 ml per minute.

Typical fermentation profiles for sporeamicin A production are shown in Fig. 2. Mycelial growth was expressed as packed cell volume (PCV, ml) obtained after centrifugation of 5 ml of the culture broth at 3,000 rpm for 5 minutes. The maximum production titer of sporeamicin A reached about 104 μg/ml after 161 hours of fermentation.

Fig. 2. Time course of large scale fermentation for sporeamicin A production.

Potency (●). Growth (○). Packed cell volume (%). Residual sugar (△), determined by the glucose oxidase method. pH (▲).



Isolation

The culture broth (200 liters) was filtered using diatomaceous earth. The filtrate (180 liters) was extracted with one half volume of ethyl acetate at

pH 9.0 and was transferred to a 0.1 M potassium phosphate buffer (pH 4.0) solution (40 liters). The acidic buffer layer was extracted with chloroform at pH 9.0 (20 liters), and was then concentrated under reduced pressure to give 60 g of sporeamicin A as a crude powder. The crude material was dissolved in 20 ml of chloroform and was then mixed with hexane (180 ml). After standing overnight at room temperature, the mixture was centrifuged at 3,000 rpm for 10 minutes to remove insoluble material and the resulting supernatant was recovered. This supernatant solution was evaporated *in vacuo* to dryness. The resulting crude powder was dissolved in ethyl acetate (200 ml), and was evaporated under reduced pressure to about 30 ml. The concentrate when held at room temperature gave crystals of sporeamicin A (11.1 g). Recrystallization from hot ethanol gave pure sporeamicin A (8.3 g) as colorless prisms (44.3% rocovery).

Physico-chemical Properties

Sporeamicin A was found to be basic in nature and soluble in methanol, ethyl acetate, acetone, chloroform, benzene and acidic water, but barely soluble or insoluble in hexane and water. It gave positive color reactions to potassium permanganate, iodine, Dragendorff and Molisch, but was negative to ninhydrin and Sakaguchi reagents. The antibiotic was stable in citrate buffer solution of pH 4.0 at 60°C, but it was labile at pH 3.0 (Fig. 3). The molecular formula was found to be $C_{37}H_{63}NO_{12}$ by the high resolution FAB-MS measurement and elemental analysis. The other physico-chemical properties of sporeamicin A are summarized in Table 7. The IR spectrum of sporeamicin A in KBr disc (Fig. 4) showed the presence of a hydroxyl group (3460 cm^{-1}), as well as ester carbonyl (1740 cm^{-1}) and enone (1690, 1620 cm^{-1}) functions. The UV absorption maximum at 276 nm (Fig. 5) suggested the presence of an enone function.

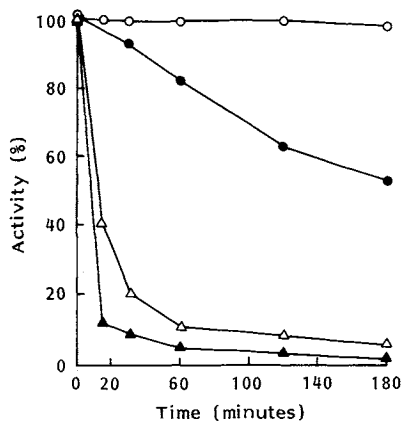
Antibacterial Activity

The antimicrobial activity of the sporeamicin A in comparison with erythromycin and leucomycin A_3 is shown in Table 8.

The antibacterial activity of sporeamicin A against macrolide-sensitive strain of *Staphylococcus aureus*

Fig. 3. Stability of sporeamicin A in aqueous solutions.

○ pH 4.0, sporeamicin A; ● pH 3.0, sporeamicin A; △ pH 4.0, erythromycin; ▲ pH 3.0, erythromycin.



Concentration of sample; 100 μg/ml in 0.1 M citrate buffer. Temperature: 60°C. Detection: HPLC method.

Table 7. Physico-chemical properties of sporeamicin A.

Appearance	Colorless prisms
FAB-MS (*m/z*)	714.4419 $(M+H)^+$
Formula	$C_{37}H_{63}NO_{12}$
Analysis	
Calcd for $C_{37}H_{63}NO_{12}$:	C 62.25, H 8.89, N 1.96
Found:	C 62.51, H 9.38, N 1.89
$[\alpha]_D^{22}$	−37° (*c* 0.8, $CHCl_3$)
MP (°C)	149~152
UV λ_{max}^{MeOH} nm (ε)	276 (10,550)
TLC (Rf)[a]	0.36
HPLC (Rt)[b]	6.70

[a] Adsorbent; Silica gel f spot-film (Tokyo Kasei Co.). Solvent system; $CHCl_3$ - MeOH - NH_4OH (10 : 0.5 : 0.05). Detection; UV lamp at 254 nm and bioautography using *Micrococcus luteus* ATCC 9341.

[b] Column; ODS, Hitachi gel #3056 (Hitachi, Ltd.). Equipment; Model 655 HPLC (Hitachi, Ltd.). Mobile pase; CH_3CN - MeOH - 1/15M $AcONH_4$ (50 : 25 : 35). Detection; UV absorbance at 275 nm. Flow rate; 0.8 ml/minute, Rt value is expressed in minutes.

Fig. 4. IR spectrum of sporeamicin A in KBr disc.

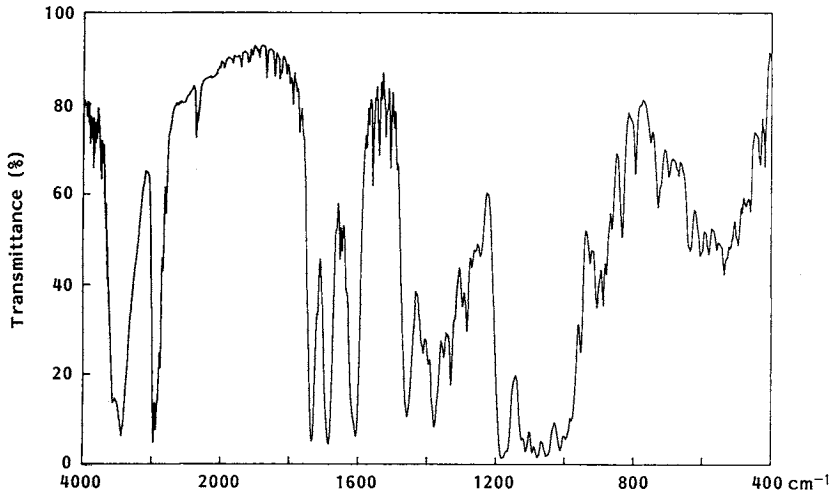


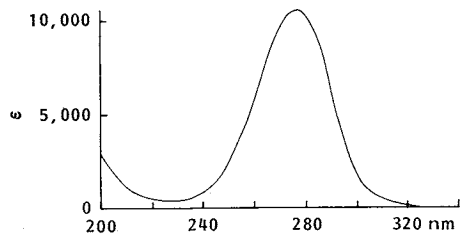
Table 8. Antibacterial spectra of sporeamicin A (SRM-A), erythromycin (EM) and leucomycin A_3 (LM-A_3).

Test organism	MIC (μg/ml)		
	SRM-A	EM	LM-A_3
Staphylococcus aureus MS 353	0.39	0.2	3.2
S. aureus MS 353 AO (Mac^r A)	>100	>100	>100
S. aureus 0126 (Mac^r B)	>100	>100	3.2
S. aureus MS 353 (Mac^r C)	>100	>100	3.2

Determined by the agar dilution method on heart infusion agar, pH 7.0. Inoculum size: 1×10^8 cells/ml.

MS 353, was superior to that of leucomycin A_3. The erythromycin-resistant strains, *S. aureus* MS 353 AO, *S. aureus* 0126 and *S. aureus* MS 353 C36 were cross-resistant to sporeamicin A.

Fig. 5. UV spectrum of sporeamicin A in methanol.



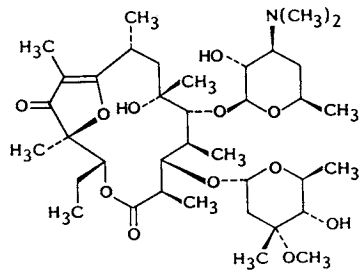
Discussion

From the taxonomic studies performed, it is evident that strain L53-18 should be classified in the genus *Saccharopolyspora*. Reports of antibiotic production by this genus have been few. Sporaricins[14], saccharocin[20], and nodusmicin[21] are known to be produced by members of this genus. Sporaricins and saccharocin are members of the aminocyclitol group antibiotics, and nodusmicin belongs to the lactone group. Recently, an erythromycin producing strain, *Streptomyces erythraeus*, was transferred to the genus *Saccharopolyspora* as *S. erythraea*[22]. Accordingly, erythromycin is the first basic macrolide antibiotic to be isolated from this genus. Now, we have also obtained a novel basic macrolide antibiotic termed sporeamicin A from a species of *Saccharopolyspora*. The characteristics of *Saccharopolyspora* sp. L53-18 were compared to those of the erythromycin producing strain, *Saccharopolyspora erythraea*. As a result, good agreement was obtained, except for utilization of L-arabinose, D-xylose, L-rhamnose and inositol[23].

The physico-chemical properties described above, and data published elsewhere[24,25], indicated that sporeamicin A may be a basic 14-membered macrolide antibiotic such as erythromycin. Sporeamicin A

was more stable than erythromycin in acidic solution (Fig. 3). Accordingly, sporeamicin A is easily distinguishable from erythromycin. The molecular formula of sporeamicin A was similar to that of a basic macrolide antibiotic such as dedesosaminyl-5-*O*-mycaminosyl-10,11-dihydromycinamicin IV[26]. This macrolide antibiotic showed UV absorption peaks at 211 nm and 275 nm. Sporeamicin A showed a UV absorption peak only at 276 nm. Accordingly, sporeamicin A is also distinguishable from dedesosaminyl-5-*O*-mycaminosyl-10,11-dihydromycinamicin IV. Of the known basic macrolide antibiotics, none showed a UV spectrum similar to that of sporeamicin A. Sporeamicin A is also distinguishable from other macrolide antibiotics on the basis of IR spectrum, molecular weight, molecular formula and specific rotation as shown in Table 7.

Fig. 6. The proposed structure of sporeamicin A.



Detailed chemical studies of sporeamicin A to be reported elsewhere[24] have confirmed the possibility, that it is a basic 14-membered macrolide antibiotic. The structure proposed for sporeamicin A is shown as Fig. 6.

Clinically isolated Staphylococci were classified into three groups (A, B and C) on the basis of their resistance to macrolide antibiotics. Group A strains were resistant to all of the macrolide antibiotics at high levels. Group B and C strains were resistant to both erythromycin and oleandomycin, or only erythromycin, respectively. As reported here (Table 8), sporeamicin A showed activity vs. sensitive *S. aureus* MS 353, but sporeamicin A showed cross-resistance against macrolide-resistant strains belonging to groups A, B and C. Details of biological properties will be reported in a separate paper[27].

Addendum in Proof

Compound L53-18 A has been identified as sporeamicin A[25].

Acknowledgment

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