Polyketomycin, a New Antibiotic from *Streptomyces* sp. MK277-AF1

I. Taxonomy, Production, Isolation, Physico-chemical Properties and Biological Activities

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A new antibiotic designated polyketomycin was isolated from the culture broth of *Streptomyces* sp. MK277-AF1. It was purified by ethyl acetate extraction, Sephadex LH-20 column chromatography and centrifugal partition chromatography (CPC). It inhibited growth of Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA). Its MICs were less than 0.2 μg/ml. Polyketomycin exhibited cytotoxic activity against nine tumor cell lines at concentrations of 0.9~5.2 μg/ml.

During our screening for novel antibiotics, we have isolated a new antibiotic, polyketomycin which was produced by *Streptomyces* sp. MK277-AF1. In this paper, the taxonomy of the producing strain, production, isolation, physico-chemical properties and biological activities of polyketomycin are reported. The structural studies of polyketomycin will be described in the following paper[1].

Materials and Methods

Microorganism

The polyketomycin producing strain, MK277-AF1 was isolated from a soil sample collected at Miura city, Kanagawa prefecture, Japan and has been deposited in the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan under the accession number FERM P-15442.

Taxonomic Studies

Cultural and physiological characteristics were determined by the methods of SHIRLING and GOTTLIEB[2], and WAKSMAN[3]. Carbohydrate utilization was investigated by using the procedure of PRIDHAM and GOTTLIEB[4]. The substrate and aerial mycelium color including soluble pigments were assigned by the Color Harmony Manual, 1958 (Container Corporation of America, Chicago). Morphological characteristics of the spores and mycelia grown on SCM (spore chain morphology) agar medium[5] were observed with a scanning electron microscope (Hitachi S-570). 2,6-Diaminopimelic acid in the cell wall was analyzed by the method of BECKER *et al.*[6]. Phospholipids were analyzed by the procedure of MINNIKIN *et al.*[7]. Menaquinones were analyzed by HPLC and mass spectrometry as described by TAMAOKA *et al.*[8].

Production of Polyketomycin

A slant culture of strain MK277-AF1 was inoculated into a 500 ml Erlenmeyer flask containing 110 ml of culture medium consisting of potato starch (Yoshida Seiyaku) 2.0%, glucose 2.0%, yeast extract (Wako) 0.5%, NaCl 0.25%, Toasted soya (Nissin) 2%, $CuSO_4 \cdot 5H_2O$ 0.0005%, $MnCl_2 \cdot 4H_2O$ 0.0005%, $ZnSO_4 \cdot 7H_2O$ 0.0005% and $CaCO_3$ 0.32% (pH 7.4 before sterilization). The flask was shaken on a rotary shaker (180 rpm) at 27°C for 72 hours. This seed culture (3 ml) was transferred into 500-ml Erlenmeyer flasks each containing 110 ml of the same medium. Production of polyketomycin was carried out at 27°C for 96 hours on a rotary shaker.

HPLC Analysis

The amount of polyketomycin was measured by reverse phase HPLC as follows: column, Capcell Pak

UG (Shiseido, 4.6 × 150 mm); mobile phase, CH_3CN-H_2O (70:30) containing 0.1% TFA; flow rate, 1 ml/minute; detection, UV at 280 nm. Under these conditions, polyketomycin was eluted at 7.40 minutes.

Biological Activities

Antimicrobial activity of polyketomycin was assayed by the cup or paper-disk diffusion methods against *Bacillus stearothermophilus*. The MICs of polyketomycin against bacteria and yeast were determined by the agar dilution method in Mueller-Hinton agar (Difco).

Cytotoxic activity of polyketomycin against cultured tumor cell lines was determined by MTT assay method[9].

Results and Discussion

Taxonomy of the Polyketomycin Producing Strain

Strain MK277-AF1 formed well-branched substrate mycelia and aerial hyphae which bore spirals (3~8 turns). The mature spore chain consisted of 10 to 50 or more cylindrical spores. The spore was 0.5~0.7 × 0.8~1.1 μm in size with a smooth surface as shown in Fig. 1. No synnemata, sclerotia or sporangia were observed. The cultural characteristics of strain MK277-AF1 on various agar media are shown in Table 1. The color of growth was light brown to grayish red brown on some media. The color of aerial mycelia was white to brownish white on ISP media Nos. 3, 4, 5 and 7. Pale orange, pink or brown soluble pigments were produced. The physiological properties of strain MK277-AF1 are shown in Table 2.

Analysis of the whole-cell hydrolysate of the strain

Fig. 1. Scanning electron micrograph of spore chains of strain MK227-AF1.

Bar represents 1.5 μm.

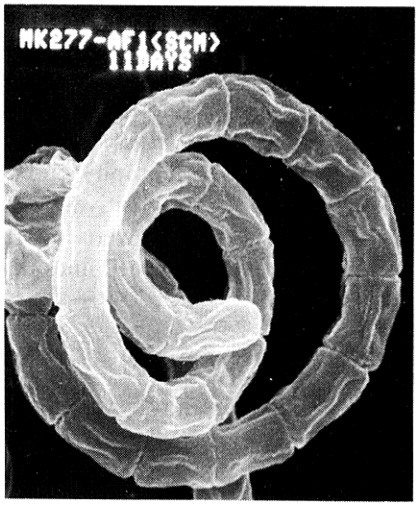


Table 1. Cultural characteristics of strain MK277-AF1.

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose nitrate agar	Pale brown [3gc, Lt Tan~4gc, Nude Tan]	None	None
Glucose-asparagine agar	Pale reddish orange[5le. Rust Tan] ~Light brown[5pi, Copper Brown]	None	Pink~Ligth red
Starch agar	Yellowish brown [3le, Cinnamon] ~ Pale brown [4ie, Cork Tan]	None	None
Yeast extract-malt extract agar (ISP No. 2)	Light brown [4pe, Orange Rust ~ 5pg, Lt Copper Brown]	None	Faint, Brown
Oatmeal agar (ISP No. 3)	Yellow orange[3pe, Amber ~ 3nc, Amber]	White	Pale reddish brown ~ Brown
Inorganic salts-starch agar (ISP No. 4)	Pale orange[3ca, Pearl Pink] ~Dark yellow[3pe, Amber] ~Light brown[4ng. Lt Brown]	White ~Brownish white	Faint, Brown
Glycerol asparagine agar (ISP No. 5)	Light brown[5le, Copper brown]~Grayish red brown[5pl, Deep Brown]	Scant, white	Pale reddish brown~Brown
Tyrosine agar (ISP No. 7)	Pale reddish brown [4lg, Lt Spice Brown] ~Grayish red brown[5ni, Cocoa Brown] ~Brown purple [8pl, Burgundy]	Partially, white	Grayish red brown ~ Brown purple

Observation after incubation at 27℃ for 21 days.

Table 2. Physiological characteristics of strain MK277-AF1.

		Utilization of	
Temperature range for growth (℃)	20 ~ 37	L-Arabinose	+
Optimum temperature (℃)	30	D-Fructose	+
Formation of melanoid pigment	+	D-Glucose	+
Liquefaction of gelatin (20℃)	+	Inositol	+
Liquefaction of glucose peptone gelatin	-	D-Mannitol	+
Coagulation of milk (37℃)	+	Raffinose	+
Peptonization of milk (37℃)	+	Rhamnose	+
Hydrolysis of starch	+	Sucrose	+
Reduction of nitrate	-	D-Xylose	+

+ : positive; - : negative

showed the presence of LL-diaminopimelic acid. The phospholipid type was PII, which contained phosphatidylethanolamine, but neither phosphatidylcholine nor unknown glucosamine-containing phospholipids. The major menaquinone was MK-9 (H_8) and the minor one was MK-9 (H_6).

On the basis of these characteristics, strain MK277-AF1 was found to belong to the genus *Streptomyces*.

Production

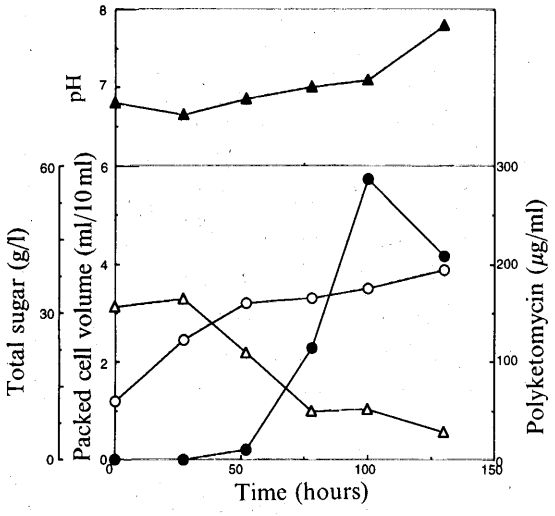
The production of polyketomycin was carried out in shake-flask culture. A typical time course of the polyketomycin production is shown in Fig. 2. Total sugar gradually decreased with mycelial growth. The production of polyketomycin began after about 50 hours and reached maximum after 100 hours cultivation. Thereafter, the accumulation in the culture broth decreased with rising pH.

Isolation

The culture broth (10 liters) was centrifuged to separate the supernatant and the mycelium cake. The supernatant (8 liters) was extracted with ethyl acetate (8 liters). The mycelium cake was extracted with methanol (2 liters). The methanol extract was concentrated to 300 ml under reduced pressure, diluted with water (700 ml) and extracted with ethyl acetate (1 liter). Both ethyl acetate extracts were combined and evaporated to dryness to give a brown oil (*ca.* 2 g). This oily substance was applied on a Sephadex LH-20 column and eluted with methanol. The active fractions were concentrated *in vacuo* to dryness. The crude compound was subjected to centrifugal partition chromatography (CPC). The chromatography was performed using CPC-L. L. N. model NMF (Sanki Engineering Limited) under the following condition: solvent system, chloroform - methanol - water (5 : 6 : 4, v/v); ascending mode; centrifugation, 700 rpm. The active substance was eluted with the lower phase of the solvent system to give pure polyketomycin (612 mg).

Fig. 2. The time course of polyketomycin production.

▲ pH, ● polyketomycin, ○ packed cell volume, △ total sugar.



Mycelial growth was expressed as packed cell volume obtained after centrifugation of 10 ml of the culture broth at 2500 rpm for 10 minutes. Total sugar was measured by the phenol-sulfuric acid method.

Table 3. Physico-chemical properties of polyketomycin.

Appearance	Orange powder
mp	194 ~ 196℃ (dec.)
$[\alpha]_D^{24}$	-58.9° (*c* 1.00, MeOH)
Molecular formula	$C_{44}H_{48}O_{18}$
FAB-MS (*m*/*z*)	865 $(M+H)^+$
	864 (M^-)
HRFAB-MS (*m*/*z*)	
Calcd:	864.2841 (as $C_{44}H_{48}O_{18}$)
Found:	864.2831 (M^-)
UV λ_{max} nm, (log ε) in	
MeOH	208 (4.56), 243 (4.56), 282 (4.34), 445(3.76)
MeOH-NaOH	250 (4.50), 279 (sh, 4.36), 569 (3.70)
MeOH-HCl	208 (4.56), 243 (4.56), 284 (4.31), 444 (3.76)
IR ν_{max} (KBr) cm^{-1}	3430, 2975, 2935, 1680, 1635, 1410, 1205, 1145, 1055, 860, 800
TLC (Rf value)	0.53

Silica gel TLC (Merck Art. 5715) : $CHCl_3$-MeOH-CH_3COOH (10 : 1 : 0.03)

Table 4. The antimicrobial activities of polyketomycin.

Test organism	MIC(μg/ml)	Test organism	MIC(μg/ml)
Staphylococcus aureus FDA209P	0.1	*E.coli* K-12	>100
S. aureus Smith	0.1	*Shigella dysenteriae* JS11910	>100
S. aureus MS9610	0.1	*Salmonella typhi* T-63	>100
S. aureus MS16526 (MRSA)	0.025	*Proteus vulgaris* OX19	>100
S. aureus TY-04282 (MRSA)	0.2	*Providencia rettgeri* GN311	>100
Micrococcus luteus IFO3333	0.1	*Serratia marcescens*	>100
Bacillus subtilis PCI219	<0.006	*Pseudomonas aeruginosa* A3	>100
Corynebacterium bovis 1810	0.1	*Klebsiella pneumoniae* PCI602	>100
		Mycobacterium smegmatis ATCC607 [a]	>100
		Candida albicans 3147	>100

Mueller Hinton agar (Difco) 37℃ 18 hours.

[a] 37℃ 42 hours.

Physico-chemical Properties

Physico-chemical properties of polyketomycin are shown in Table 3. The compound was soluble in most organic solvents such as acetone, ethyl acetate, methanol, chloroform and pyridine, but insoluble in hexane and water. The molecular formula for polyketomycin was established as $C_{44}H_{48}O_{18}$ by HRFAB-MS and NMR spectra. The UV spectrum of polyketomycin showed absorption maxima at 208, 243, 282 and 445 nm in MeOH, and was similar to that of dutomycin[10]. Polyketomycin gave a positive color reaction with molybdophosphoric acid-sulfuric acid, $FeCl_3$, 2,4-dinitrophenylhydrazine and anisaldehyde-sulfuric acid reagents, but a negative one with ninhydrin and Rydon-Smith reagents.

Biological Activities

The antimicrobial activities of polyketomycin are shown in Table 4. Polyketomycin inhibited the growth

Table 5. The cytotoxic activities of polyketomycin to tumor cell lines.

Cells	IC_{50}(μg/ml)
L1210 leukemia	3.3
EL-4 leukemia	2.1
P388 leukemia	5.2
Ehrlich carcinoma	1.0
IMC carcinoma	0.9
colon 26 adenocarcinoma	1.8
Meth A fibrosarcoma	2.4
FS-3 fibrosarcoma	1.5
B16-BL10 melanoma	1.6

of Gram-positive bacteria including multi-drug resistant strains such as *Staphylococcus aureus* MS9610 and methicillin-resistant *S. aureus* (MRSA). Its MICs were less than 0.2 μg/ml. However polyketomycin did not inhibit growth of Gram-negative bacteria and yeast at 100 μg/ml.

The cytotoxic activities of polyketomycin are shown in Table 5. Polyketomycin exhibited growth inhibition against several tumor cell lines and its IC_{50} was estimated to range from 0.9 ~ 5.2 μg/ml.

The acute toxicity (LD_{50}, ip) of polyketomycin in mice was estimated to be 6.25 ~ 12.5 mg/kg.

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