SAKYOMICINS A, B, C AND D: NEW QUINONE-TYPE ANTIBIOTICS PRODUCED BY A STRAIN OF *NOCARDIA*

TAXONOMY, PRODUCTION, ISOLATION AND BIOLOGICAL PROPERTIES

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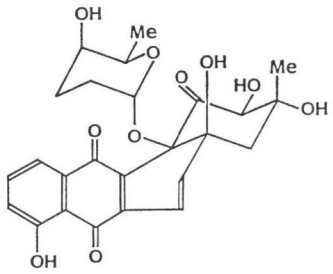
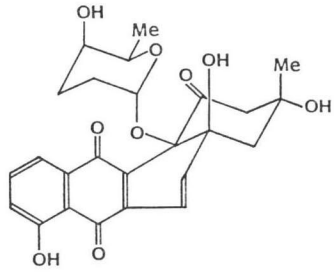
Actinomycete strain M-53, a new soil isolate, was found to produce four quinone-type antibiotics. Antibiotic sakyomicin components A, B, C and D were isolated from the fermentation broth of strain M-53 by XAD-2 column chromatography, silica gel column chromatography and Sephadex LH-20 column chromatography. The components are active against Gram-positive bacteria. Strain M-53 was identified as a strain of genus *Nocardia*.

A soil isolate, strain M-53, was found to produce four quinone antibiotics, and they have been designated sakyomicins A, B, C and D. In the preceding paper[1], the structure of a new antibiotic substance, sakyomicin A, was elucidated by X-ray crystallographic analysis and the structures for its congeners, sakyomicins B, C and D were proposed from their spectroscopic properties (Fig. 1).

Fig. 1. Chemical structure of sakyomicins A, B, C and D.



Sakyomicin A



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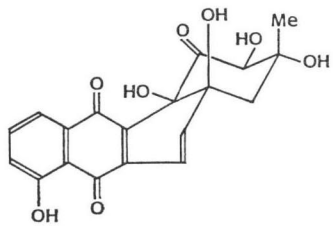

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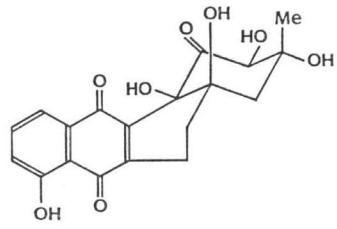


Sakyomicin D

We described here the taxonomy of strain M-53, and the production, isolation and biological properties of sakyomicin.

Results and Discussion

Taxonomic Studies on Strain M-53

Antibiotic sakyomicin-producing strain M-53 is an actinomycete isolated from a soil sample collected in Sakyo-ku, Kyoto City, Kyoto Prefecture, Japan.

Morphological, cultural and physiological properties of the strain M-53 were examined primarily according to the methods described by SHIRLING and GOTTLIEB[2], along with several supplementary tests. The characteristics of the strain were compared with those of the known species of actinomycetes described in "The Actinomycetes, Vol. 2" by WAKSMAN[3], "BERGEY's Manual of Determinative Bacteriology, 8th Edition"[4], the "ISP Reports" by SHIRLING and GOTTLIEB[5~8] and other recent publications concerning taxonomy of *Nocardiae* and *Streptomycetes*. The morphological characteristics of the hyphae on Czapek, Bennett and inorganic salts - starch media at 28°C for 7 to 21 days were examined under a light microscope.

Strain M-53 was non-motile and Gram-positive. Vegetative hyphae of strain M-53 were fully developed with branching (Fig. 2). In older culture, they fragmented into bacillary elements, averaging 0.5~1.5 μm in size. Spores were negative. The aerial hyphae developed poorly, and were short.

Observations of cultures on various agar media were made after cultivation at 28°C for 7 to 21 days. The mass colors of mycelium are described in common terminology. Strain M-53 showed a brownish gray aerial mycelium was only rudimentarily present or absent on almost all media employed. The characteristic properties on the 14th day of cultivation at 28°C in a variety of media are shown in Table 1.

The colony was round, creamy and raised. The margin of colony was irregular (Fig. 2). The size of the colony was 1 mm diameter on yeast extract - malt extract medium (ISP medium 2) after the incubation for 6 days at 25°C.

The young mycelia grown under submerged culture were used to determine acid fastness as described in the Manual of Clinical Microbiology[9]. Decomposition of casein, tyrosine and urea, acid production from carbohydrate and the resistance to lysozyme were studied by the procedures described by GORDON *et al.*[10]. The effect of temperature on growth was investigated by streaking the inoculum over the surface of ISP medium 2 and incubating it for 21 days in a incubator. The test for sodium chloride

Fig. 2.

(a) Colony of strain M-53 (on glycerol - asparagine agar, 28°C, 14 days).

(b) Light micrograph of strain M-53 (on glycerol - asparagine agar, 14 days) ×750.

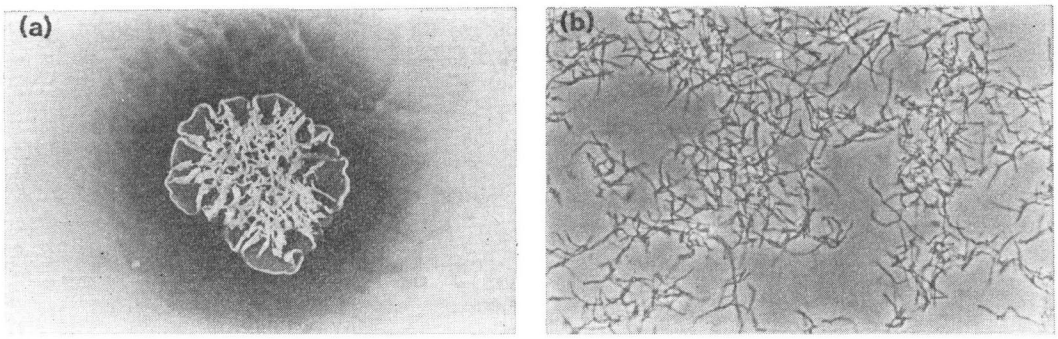


Table 1. Cultural characteristics of strain M-53.

Medium	Characteristics
Czapek agar	Growth fair, white; no aerial mycelium; no soluble pigment
Glucose - asparagine agar	Growth fair, white; aerial mycelium poor; no soluble pigment
Glycerol - asparagine agar	Growth fair, pale brown; aerial mycelium poor; no soluble pigment
Inorganic salts agar	Growth abundant, yellow to pale cream; no aerial mycelium; soluble pigment (reddish brown)
Tyrosine agar	Growth fair, white; no aerial mycelium; no soluble pigment
Yeast extract - malt extract agar	Growth abundant, yellowish brown; aerial mycelium poor; soluble pigment (reddish brown)
Oat meal agar	Growth fair, white; no aerial mycelium; soluble pigment (pale yellow)
Peptone - yeast extract - iron agar	Growth abundant, yellowish brown; no aerial mycelium; soluble pigment (reddish brown)

Table 2. Physiological properties of strain M-53.

Properties observed	Characteristics	Properties observed	Characteristics
Gram staining	+	7% NaCl	+
Acid fastness	+	10% NaCl	−
Decomposition of:		43°C	−
Casein	+	40°C	+
Tyrosine	+	37°C	+
Urea	+	30°C	+
Hydrolysis of starch	−	25°C	+
Liquefaction of gelatin	+	20°C	−
Tolerance to lysozyme	+	Action on milk:	
Production of nitrate	± (slight)	Peptonization	+
Growth at/in/on:		Coagulation	−
anaerobic	−	Melanoid pigment formation on:	
1% NaCl	+	ISP medium 1	−
2% NaCl	+	ISP medium 6	−
3% NaCl	+	ISP medium 7	−
5% NaCl	+	Cell wall pattern	IV[a]

[a] *meso*-diaminopimelic acid.

tolerance was examined by streaking the inoculum on the same medium as used for the temperature study, but containing sodium chloride at 1.0, 2.0, 3.0, 5.0, 7.0 and 10.0%, and incubating at 28°C for 21 days. The media used for tests were as follows: ISP media 1, 6 and 7 for melanoid pigment formation, nitrate broth (Difco) for nitrate reductase, ISP medium 4 for starch hydrolysis, gelatin stab for gelatin liquefaction, and dehydrated skim milk for coagulation and peptonization. The cultures on all of the media tested were incubated at 28°C for 14 days except for those on milk (37°C, 10 days) and gelatin (25°C, 21 days) media. These physiological characteristics of the strains are summarized in Table 2. Carbohydrate utilization was studied by the procedures by PRIDHAM and GOTTLIEB[11]. D-Glucose, D-xylose, D-fructose, L-rhamnose, D-mannitol, inositol were readily utilized and L-arabinose and sucrose were slightly utilized for growth of the organism. Raffinose was not utilized. The procedure of BECKER *et al.*[12] was used for preparation of cells and chromatographic detection of the isomers of diaminopimelic acid. Whole cell hydrolysates contain *meso*-diaminopimelic acid.

Microscopic and cultural studies as well as cell wall components of strain M-53 indicate that this

strain belongs to the genus *Nocardia*. Based on the various taxonomic criteria examined, it has not been possible to assign *Nocardia* M-53 to any of the previously described species of *Nocardia*. However, at this time we defer assigning the species until further studies are performed.

Production of Sakyomicin

The stock culture of strain M-53 on inorganic salts - starch agar was added to 100 ml of a medium consisting of glycerol 1.0%, Casamino Acids 0.2%, yeast extract 0.04%, KH_2PO_4 0.05%, L-asparagine 0.02% and L-arginine 0.02%, pH 7.0, in a 500-ml Sakaguchi-flask. After incubation at 28°C for 48 hours on a reciprocal shaker, the subculture was transferred to 500 ml of the same medium in a 2-liter Sakaguchi-flask and the fermentation was carried out at 28°C for 70 hours with aeration. Thus, a high amount of seed culture was necessary for the formation of sakyomicin in the main culture.

Fermentation was monitored by measuring the absorbance of the supernatant obtained from centrifuged broth samples (at 12,000 rpm for 10 minutes) at 415 nm (Fig. 3), because sakyomicin congeners show the marked yellow color. The increase in the formation of yellow pigments corresponded to the increase in the antibacterial activity when *Bacillus subtilis* IFO 3022 was used as a test organism for the bioassay.

Isolation Procedures

The isolation method used for sakyomicins A, B, C and D is outlined in Fig. 4. Most of the antibiotic activity was found in the broth filtrate. After the fermentation was completed, the culture broth was centrifuged at 12,000×*g* for 10 minutes. The active principle was adsorbed to Amberlite XAD-2 column (3×40 cm) and the column was fully washed with water - methanol (7: 3). Sakyomicin was extracted from Amberlite XAD-2 with methanol. The active eluate was concentrated under reduced pressure and applied to the silica gel column (Wakogel C-300, 2×40 cm) that was equilibrated with benzene - ethyl acetate (1: 2), and then developed with the same solvent. Two active fractions (I and II) were obtained.

Fraction I was evaporated to dryness and dissolved in a small amount of acetone, and then crystallization was induced by adding *n*-hexane. Red prisms were obtained (sakyomicin A).

Fig. 3. Time course of production of sakyomicin congeners by *Nocardia* M-53.
The fermentation was carried out in a 2-liter Sakaguchi-flask containing 600 ml of the medium.

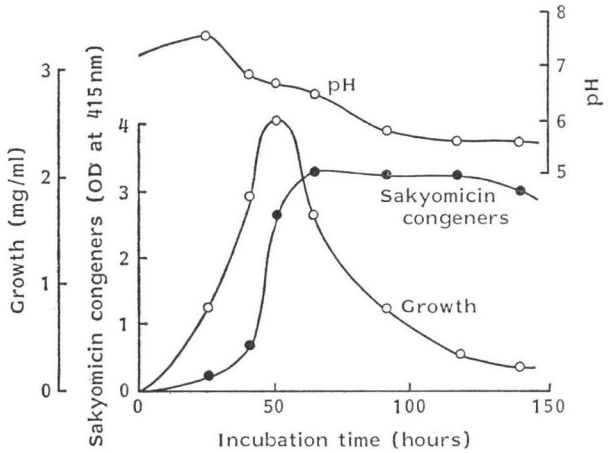
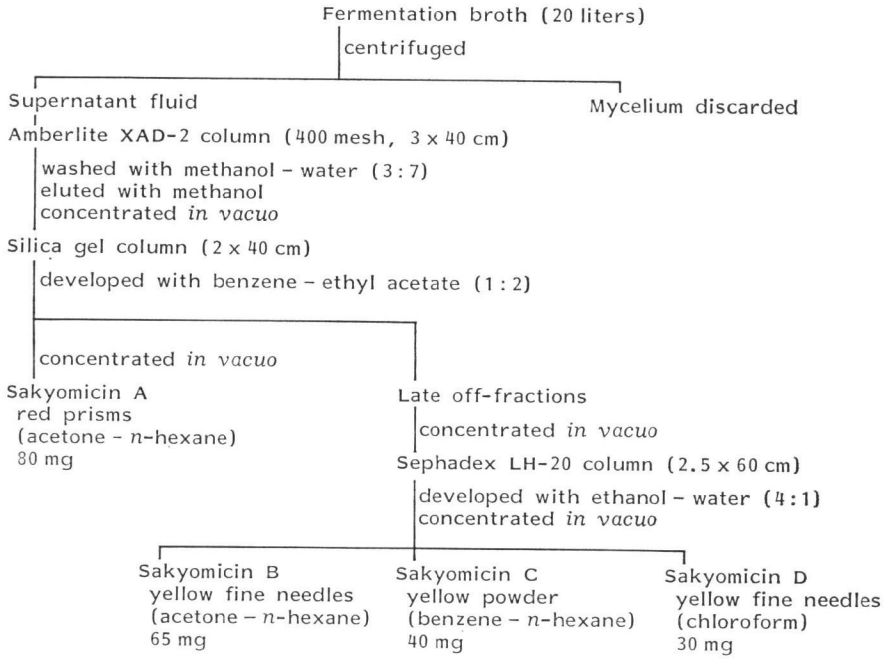


Fig. 4. Isolation procedure for sakyomicins A, B, C and D.



Fraction II was evaporated to dryness and dissolved in a small amount of water - ethanol (1 : 4). The solution was applied to Sephadex LH-20 column (2.5×60 cm) that was equilibrated with water - ethanol (1 : 4), and then eluted with the same solvent. Three active fractions (IIa, IIb and IIc) were obtained. Evaporation of fraction IIa yielded yellow fine needles (sakyomicin B) from acetone - *n*-hexane. Fraction IIc was evaporated and yellow fine needles (sakyomicin D) were obtained from chloroform. Each of the three crude crystals were recrystallized from the corresponding solvent system. Sakyomicin C was obtained as a powder from benzene - *n*-hexane. Twenty liters of fermentation broth yielded 80 mg of sakyomicin A, 65 mg of sakyomicin B, 40 mg of sakyomicin C and 30 mg of sakyomicin D.

Structures and physico-chemical properties of sakyomicins A, B, C and D were described in detail in the preceding paper[1]. The aglycone portion of sakyomicin A is enantiomeric to that of P-1894B (vineomycin A_1)[13], and it is the first naturally occurring compound containing (+)-rhodinose. The structure of sakyomicin B was similar to that of yoronomycin[14,15], but not identical. Physico-chemical studies suggest that yoronomycin is in fact a diastereoisomer of sakyomicin B at C(2) and/or C(3).

Biological Characteristics

The antibacterial spectra of sakyomicins A, B and C are shown in Table 3. One loopful of an overnight culture of each test strains in peptone - meat extract broth (about 10^8 viable cells/ml) was streaked on antibiotic medium 2 (Difco) graded concentrations of the drugs and the minimal inhibitory concentration (MIC) was expressed in terms of μg/ml after incubation at 37°C for 20 hours except for *Mycobacterium* and fungi, in which MIC was determined after 3 days of incubation. For fungi malt extract medium was used and spore suspension (10^6/ml) was streaked.

Sakyomicins A, B and C show selective antibacterial activity, especially against Gram-positive bac-

Table 3. Antimicrobial spectra of sakyomicins A, B and C.

Test microorganisms	MIC (μg/ml)		
	A	B	C
Bacillus megaterium IFO 3970	3.13	3.13	4.69
Bacillus subtilis IFO 3022	12.5	4.69	9.38
Staphylococcus aureus IFO 12732	9.38	4.69	6.25
Micrococcus roseus IFO 3964	3.13	1.56	4.69
Corynebacterium fascians IFO 12077	1.56	0.78	1.56
Mycobacterium phlei IFO 3158	1.56	1.56	1.56
Escherichia coli K12 IFO 3301	>100	>100	>100
Klebsiella pneumoniae IFO 3319	>100	>100	>100
Proteus mirabilis IFO 3849	>100	>100	>100
Salmonella typhimurium AKU 90	>100	>100	>100
Pseudomonas aeruginosa IFO 3445	>100	>100	>100
Aspergillus niger IFO 6661	>200	>200	>200
Candida albicans IFO 1060	>200	>200	>200

teria including *Bacillus, Staphylococcus, Micrococcus, Corynebacterium* and *Mycobacterium.* It has no inhibitory effect on Gram-negative bacteria, fungi or yeast.

The antitumor activity of sakyomicins A and C were examined by using an *in vivo* assay system with sarcoma 180A cells and lymphocytic leukemia P338 cells in mouse. Intraperitoneal administration of 10 mg/kg of sakyomicin A and 6 mg/kg of sakyomicin C into mice did not exhibit any antitumoral effects.

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