A NEW ANTIBIOTIC, SETOMIMYCIN, PRODUCED BY A STRAIN OF *STREPTOMYCES*

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(Received for publication August 2, 1978)

A new antibiotic, setomimycin, was isolated from the culture broth of strain AM-2947, which was identified as *Streptomyces pseudovenezuelae*. The compound is a weakly acidic substance, and has UV-absorptions at 228, 268 and 422 nm and a molecular formula of $C_{34}H_{28}O_9$ (MW 580). It is active against Gram-positive bacteria including *Mycobacteria*, and has antitumor activity against Sarcoma-180 solid tumor in mice.

In the course of our screening program for new antibiotics from actinomycetes, a new antibiotic, setomimycin, effective against Gram-positive bacteria including *Mycobacteria* and Sarcoma-180 solid tumor in mice, was obtained from the culture broth of an actinomycete (strain AM-2947) which had been isolated from a soil sample collected at Uchinomi-chō, Shōdo Island (The Setonai Sea), Japan. In the present paper, taxonomy of the producing strain, production, isolation, characterization and biological properties of the antibiotic are described.

Characteristics of the Producing Strain

Morphological Characteristics

The morphology of the strain cultured on yeast extract-malt extract agar or inorganic salts-starch agar was observed microscopically (Plates 1 and 2). The aerial mycelium was observed to be moderate on inorganic salts-starch and yeast extract-malt extract agars, but absent or poor on glucose-nitrate, peptone-yeast extract-iron, nutrient and glucose-peptone agars. It forms no whorls, but extends straight aerial hyphae. The spore chain is *Rectus-flexibilis*. The spores are elliptical or cylindrical ($0.4 \sim 0.8 \times 0.8 \sim 1.3\ \mu$). The spore surface is smooth.

Cultural and Physiological Characteristics

Strain AM-2947 was cultivated on various media described by WAKSMAN[1] and International Streptomyces Project (ISP)[2] at 27°C, and the changes of growth, aerial mycelium and soluble pigment were observed after periods of 7, 14 and 21 days. Utilization of carbon sources was tested by growth on PRIDHAM and GOTTLIEB's medium[2] containing 1% of various carbon sources. Color names and hue numbers indicated in Tables 1 and 4 were those of the Color Harmony Manual (4th edition)[3].

The cultural and physiological characteristics of strain AM-2947 are listed in Tables 1 and 2, respectively. The utilization of carbon sources by the strain is shown in Table 3. The cultural characteristics can be summarized as follows: growth is moderate or good, and is pale yellow to pale yellowish brown; aerial mass color is brownish gray to brownish white; soluble pigment is yellow brownish white; melanoid pigment is produced. A cell wall preparation of the strain was found to contain LL-diaminopimelic acid and glycine but no *meso*-diaminopimelic acid.

Plate 1. Photomicrograph of the sporophores of strain AM–2947
Yeast extract-malt extract agar, 27°C, 1 week.

Plate 2. Electronmicrograph of the spores of strain AM–2947
Inorganic salts-starch agar, 27°C, 2 weeks.

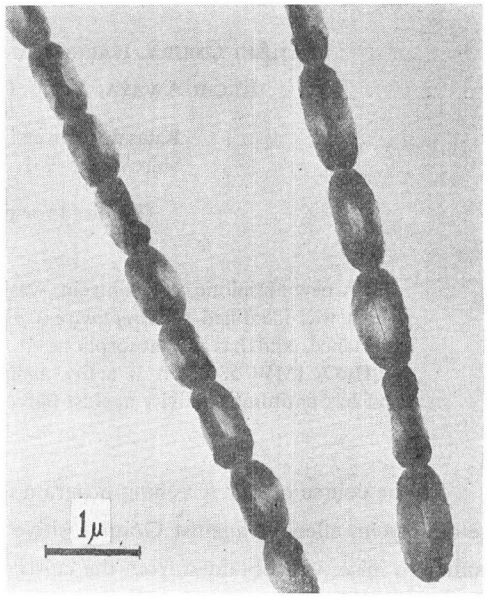


Table 1. Cultural characteristics of strain AM–2947

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar	moderate, gold (21c)	abundant, velvety cream (1½ca)	none
Glucose-nitrate agar	moderate, wrinkled light maize (2ea)	none	none
Glycerol-calcium malate agar	moderate dull gold (2ng)	moderate, cottony light antique gold (1½ic)	antique gold (1½le)
Glucose-asparagine agar (ISP)	moderate mustard gold (2ne)	moderate, velvety light ivory (2ca)	poor light antique gold (1½ic)
Glycerol-asparagine agar (ISP)	abundant, wrinkled yellow maple (3ng)	partially, velvety oyster white (b)	none
Inorganic salts-starch agar (ISP)	moderate, bamboo (2gc)	moderate, velvety covert gray (2fe)	none
Tyrosine agar (ISP)	moderate dark brown (3pn)	moderate, velvety natural (3dc)	dark brown (3nl)
Nutrient agar	moderate, camel (3ie)	poor, white (a)	yellow maple (3ng)
Glucose-peptone agar	moderate, wrinkled mustard brown (2ni)	none	poor, topaz (3ne)
Yeast extract-malt extract agar (ISP)	moderate, wrinkled natural (2dc)	moderate, velvety sand (3cb)	poor, cinnamon (31e)
Oatmeal agar (ISP)	moderate, honey gold (2ic)	abundant, cottony pussywillow gray (5dc)	honey gold (2ic)
Peptone-yeast extract agar (ISP)	abundant, wrinkled yellow maple (3ng)	partially, velvety oyster white (b)	none

Table 2. Physiological characteristics of strain AM-2947

Melanin formation	+
Tyrosinase reaction	+
H_2S production	+
Nitrate reduction	−
Liquefaction of gelatin	−
Peptonization of milk	+
Coagulation of milk	−
Hydrolysis of starch	+
Cellulolytic activity	−
Temperature range for growth	20~38°C

Table 3. The utilization of carbon sources by strain AM-2947

Carbon source	Response
D-Glucose	+
L-Arabinose	+
D-Xylose	+
D-Fructose	+
L-Rhamnose	+
i-Inositol	+
D-Mannitol	+
Sucrose	+
Raffinose	+

Table 4. Comparison of strain AM-2947 with *Streptomyces pseudovenezuelae* ISP 5215

	Strain AM-2947	*S. pseudovenezuelae* ISP 5215
Morphology	*Rectus-flexibilis*, smooth	*Rectus-flexibilis*, smooth
Cultural characteristics		
1) Inorganic salts-starch agar		
G*	bamboo (2gc)	bamboo (2gc)
AM*	cottony, covert gray (2fe)	cottony, light gray (c)
SP*	none	none
2) Glycerol-asparagine agar		
G	yellow maple (3ng)	biscuit (2ec)
AM	velvety, oyster white (b)	velvety, light gray (c)
SP	none	none
3) Glucose-asparagine agar		
G	mustard gold (2ne)	mustard gold (2ne)
AM	velvety, light ivory (2ca)	velvety, silver gray (3fe)
SP	poor	very poor
Production of the antibiotic	+	−

* G, growth; AM, aerial mycelium; SP, soluble pigment.

From the above results, the strain is chromogenic and belongs to the gray or red series of the genus *Streptomyces* classified by PRIDHAM and TRESNER[4]. Among known *Streptomyces* species described in "BERGEY's Manual of Determinative Bacteriology", 8th ed.,[4] "The Actinomycetes", Vol. II by WAKSMAN[1] and ISP reports by SHIRLING and GOTTLIEB[5~8], *Streptomyces pseudovenezuelae*[6] is closely related to strain AM-2947. In comparison of strain AM-2947 with *S. pseudovenezuelae* ISP 5212, all of the morphological and physiological characteristics and almost all of the cultural characteristics of the former were in agreement with those of the latter. As shown in Table 4, in some cultural characteristics only a little difference is recognized. Strain AM-2947 produces setomimycin, but strain ISP 5212 does not.

Therefore, strain AM-2947 should be identified as *S. pseudovenezuelae*, and was designated as *S. pseudovenezuelae* strain AM-2947. The strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Chiba, Japan, with the acession number FERM-P No. 3430, and at United States Department of Agriculture, Agricultural Research Service, Northern Regional Research Center (Peoria, Illinois 61604) with the acession number NRRL 11269.

Production of Setomimycin

The stock culture of strain AM-2947 (*S. pseudovenezuelae*) was maintained as an agar slant (KRAINSKY's agar medium). A 7-day culture of the agar slant was incubated into a seed medium (100 ml) in a 500-ml Sakaguchi flask and incubated for 2 days at 27°C. The seed culture (200 ml) was transferred into 20 liters of a production medium in a 30-liter jar fermentor and incubated for 2 days at 27°C (agitation, 300 rpm; aeration, 10 liters per minute). The composition of the seed and production media was 2.0% glycerol, 2.0% soybean meal and 0.3% sodium chloride (the pH was adjusted to 7.0 with 6 N sodium hydroxide before sterilization). In the incubation using a jar fermentor, Adekanol LG-109 (Asahi Electro-Chemical Co., Ltd.) was used as an antifoam agent. The antibiotic activity of the culture broth was assayed by the paper disc method using *Bacillus subtilis* PCI 219 as a test organism.

A typical time course of the fermentation is shown in Fig. 1. The antibiotic production started one day after the inoculation and reached the maximum at the 2nd day. The amount of the antibiotic accumulated was about 150 μg/ml.

Fig. 1. A typical time course of setomimycin production by *S. pseudovenezuelae* strain AM-2947
The medium and culture conditions are described in the text.

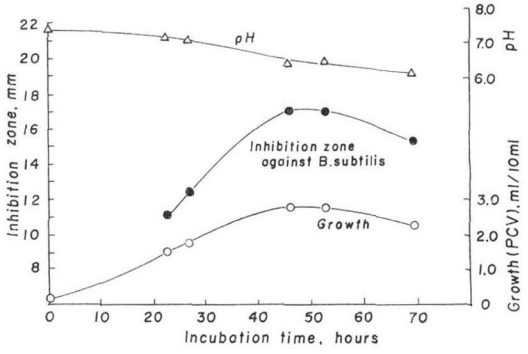
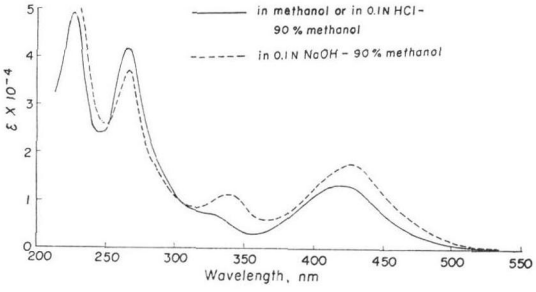


Fig. 2. UV-absorption spectra of setomimycin



Isolation of Setomimycin and Preparation of its Acetate

Culture broth (20 liters) of *S. pseudovenezuelae* strain AM-2947, obtained by incubation in a 30-liter jar fermentor, was used as a starting material for the isolation of the antibiotic. The

Table 5. Physicochemical properties of setomimycin and its diacetate

	Setomimycin	Setomimycin diacetate
Nature	reddish orange powder	yellow powder
mp (°C)	176~178°	185~187°
$[\alpha]_D^{24}$	+502° (*c* 1.0, $CHCl_3$)	+392° (*c* 1.0, $CHCl_3$)
Anal.	Found Calcd.	Found Calcd.
C%	69.88 70.34	68.80 68.67
H	5.09 4.86	5.07 4.85
N	0 0	0 0
Formula	$C_{34}H_{28}O_9$ (MW 580)	$C_{38}H_{32}O_{11}$ (MW 664)
EI-MS		664 (M^+), 622 (M^+-42), 580 (M^+-84)
FD-MS	581 ($M^+ +1$)	
UV λ_{max}^{MeOH} nm (ε)	228 (49,400), 268 (42,500) 422 (13,600)	225 (57,100), 266 (40,800) 385~395 (10,300)
IR ν_{max}^{KBr} (cm^{-1})	1690, 1580	1760, 1720, 1700, 1610, 1580

Fig. 3. IR spectrum of setomimycin (KBr method)

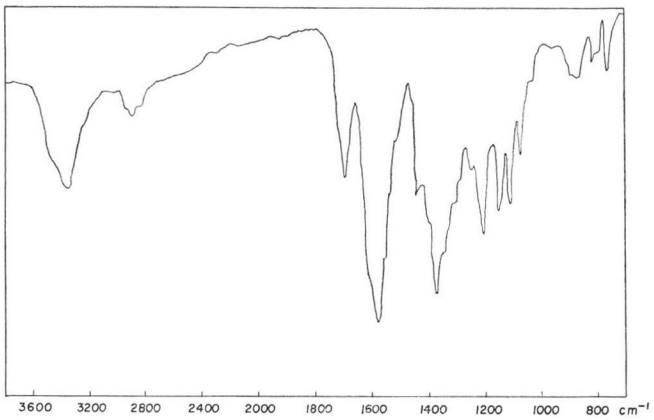


Fig. 4. PMR spectrum of setomimycin in $CDCl_3$

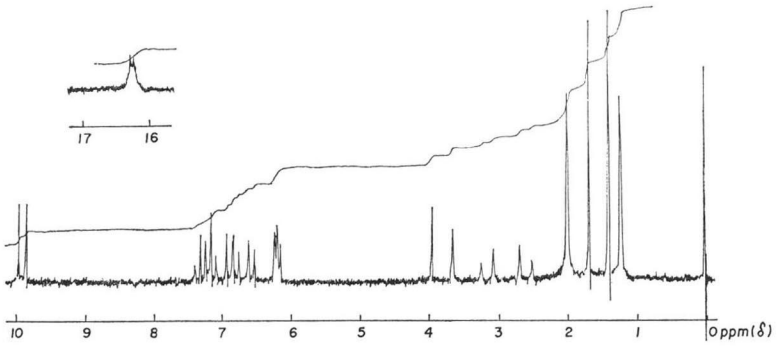
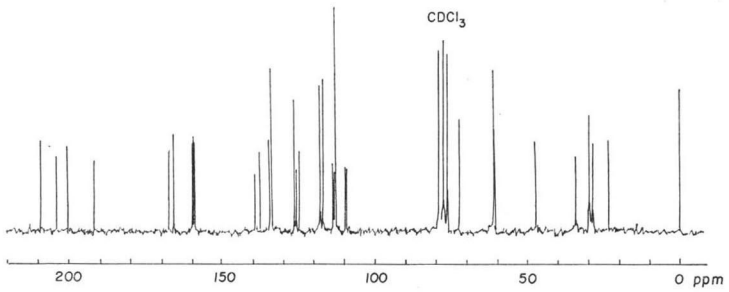


Fig. 5. CMR spectrum of setomimycin in $CDCl_3$



antibiotic was detected by the antimicrobial activity against *Bacillus subtilis* PCI 219 and silica gel thin-layer chromatography. After the pH value of the culture broth was adjusted to 2.0 with 6 N hydrochloric acid, ethyl acetate (10 liters) was added to the culture broth containing the mycelia, stirred vigorously, and centrifuged to give the ethyl acetate layer. After the ethyl acetate layer was concentrated under reduced pressure, it was applied on an acid-treated silica gel column (250 g) and then developed with chloroform and methanol (200: 1). The active eluate from the column was evaporated under reduced pressure to give a reddish orange powder (2.34 g). The powder was further purified

Table 6. The microbial spectra of setomimycin and its acetate

Test organisms	Medium*1	MIC (μg/ml)*2	
		Setomimycin	Setomimycin acetate
Staphylococcus aureus FDA 209P	N	3.13	
	H	25	25
S. aureus FS 1277 (PC-R*3)	N	3.13	
	H	12.5	12.5
S. aureus KB 64 (EM, TC-R*3)	N	3.13	
	H	12.5	12.5
Bacillus subtilis PCI 219	N	3.13	
	H	6.25	6.25
B. cereus T	N	3.13	
	H	6.25	6.25
Sarcina lutea PCI 1001	N	3.13	
	H	6.25	100
Mycobacterium smegmatis ATCC 607	N	1.56	
	H	6.25	1.56
Nocardia asteroides KB 49	N	1.56	
	H	3.13	12.5
Proteus vulgaris IFO 3167	N	>100	
	H	>100	>100
Escherichia coli NIHJ	N	>100	
	H	>100	>100
Salmonella typhimurium KB 20	N	100	
	H	>100	>100
Shigella sonnei E 33	N	>100	
	H	>100	>100
Pseudomonas aeruginosa P-3	N	>100	
	H	>100	>100
Candida albicans	P	>100	
Saccharomyces sake	P	>100	
Aspergillus niger	P	>100	
Piricularia oryzae	P	50	
Microsporum gypseum	P	>100	
Trichophyton interdigitale	P	>100	
T. rubrum	P	>100	

*1 N, nutrient agar (37°C, 24 hours); H, heart infusion agar (37°C, 24 hours); P, potato agar (27°C, 72 hours).

*2 Minimum inhibitory concentration determined by agar dilution method.

*3 PC, penicillin; EM, erythromycin; TC, tetracycline; R, resistant.

by preparative thin-layer chromatography on silica gel (Merck's plate, thickness: 2 mm) with chloroform and methanol (10: 1). The Rf value with the system is 0.52. The Rf values of the antibiotic on thin-layer chromatography with chloroform – methanol (5: 1), benzene – acetone (4: 1), benzene – ethyl acetate (1: 3) and benzene – methanol (4: 1) were 0.59, 0.18, 0.37 and 0.31, respectively.

The preparation of an acetate of the antibiotic was performed as follows. The pure powder of the antibiotic (200 mg) was dissolved in acetic anhydride (4 ml) and pyridine (5 ml) was added to the solution. After standing the mixture in room temperature for 40 minutes, 2 volumes of ethyl acetate were added to the solution together with some ice. The ethyl acetate layer was washed with water, concentrated under reduced pressure, and then applied on preparative thin-layer chromatography on silica gel with benzene and acetone (3: 1) to give a yellow powder (148 mg). The Rf value of the acetate on the chromatogram was 0.48.

Physicochemical Properties of Setomimycin and its Acetate

The antibiotic, setomimycin, obtained as described above is weakly acidic in nature and is soluble in lower alcohols, lower alkyl acetates, acetone, chloroform and benzene, but insoluble in water, petroleum ether and *n*-hexane. The compound is positive to ferric chloride and ferric hydroxamate, but negative to ninhydrin, RYDON-SMITH and EHRLICH reagents. It contains no nitrogen and no halogen. The other physicochemical properties of setomimycin and its acetate are listed in Table 5. The UV, IR, PMR and CMR spectra of setomimycin are given in Figs. 2~5.

Table 7. Antitumor activity of setomimycin

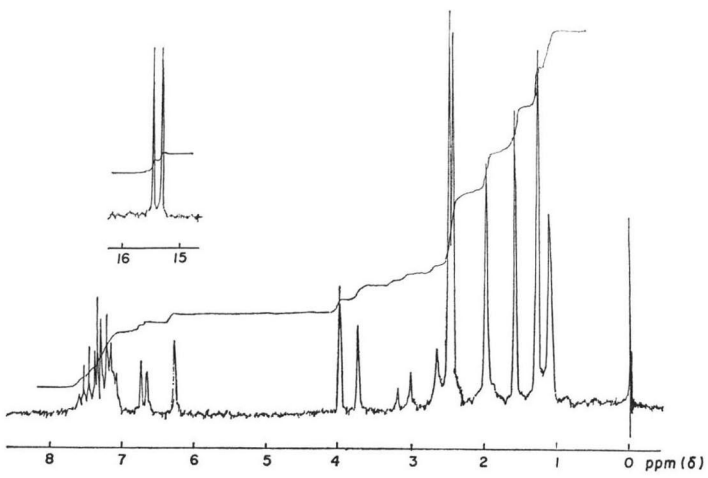
Tumor: Sarcoma-180 ascite tumor (5×10^6 cells) was transplanted subcutaneously to a ddY mouse.
Treatment: Setomimycin was dissolved in ethanol and then some Tween-80 was added to the solution. The solution was evaporated to remove ethanol and dissolved in 0.9% NaCl to give a colloid, which was administered intraperitoneally at 1 day after the transplantation.
Judgement: A tumor size ($ab^2/2$) at 7th day after the administration of the antibiotic was compared with that of control: *a*, length; *b*, width.

Antibiotic	Dose (mg/kg)	Tumor size (T/C*)	Body weight change (g)
None (control)	—	1.0 (1154.5 mm^3)	+5.0
Setomimycin	100	0.66	+2.0
	200	0.43	−1.0
Mitomycin	4.2	0.37	+2.0

* Treated/control

The UV absorptions of setomimycin (Fig. 2) show that it has a naphthocyclinone or anthracyclinone skeleton as a chromophore. The CMR and PMR spectra (Figs. 5 and 4) suggest that the compound contains 34 carbons and 28 protons: 12 protons from 4 methyl groups, 4 protons from two methine groups and a methylene group, 8 protons from olefinic and/or aromatic moieties, and 4 protons from 4 hydroxyl groups. It contains no sugar moiety. In the PMR spectrum of the acetate (Fig. 6) the signals of 2 hydroxyl groups disappeared and those of two additional acetyl groups (2.46 and 2.49 ppm) appeared. This shows that the acetate is a diacetyl derivative of setomimycin. The MS-fragmentation of the acetate [*m/e* 664 (M^+), 622 (M^+ −42), 580 (M^+ −84)] and the IR absorptions (1760 and 1720 cm^{-1}) also support this. From the data of PMR, CMR and MS-spectra of setomimycin and its acetate, their formulae are shown to be $C_{34}H_{28}O_9$ (MW 580) and $C_{38}H_{32}O_{11}$ (MW 664), respectively.

Fig. 6. PMR spectrum of diacetylsetomimycin in $CDCl_3$



Biological Properties of Setomimycin and its Diacetate

The antimicrobial activities of setomimycin and its diacetate were determined by the conventional agar dilution method using nutrient and heart infusion agars (37°C, 24 hours). As shown in Table 6, the antibiotic and its diacetate inhibited Gram-positive bacteria including *Mycobacteria*. But they are scarcely active against Gram-negative bacteria and fungi. The acute toxicity (LD_{50}, iv) of setomimycin is 60 mg/kg in mice.

Setomimycin exhibits antitumor activity against Sarcoma-180 solid tumor on ddY mice. As shown in Table 7, when 200 mg/kg of setomimycin was injected intraperitoneally once at 1 day after transplantation of tumor, the tumor size (treated/control) at the 7th day after administration was 0.43.

Discussion

From the above results, the antibiotic, setomimycin, isolated from the culture broth of *S. pseudovenezuelae* strain AM-2947, was found to be an antibiotic active against Gram-positive bacteria including *Mycobacteria* and Sarcoma-180 solid tumor. It was also found to have a naphthocyclinone or anthracyclinone chromophore but no sugar and no nitrogen. Among known antibiotics, nanaomycin A[9,10] and griseusins[11] show some resemblance to setomimycin in respect to UV absorptions in methanol. However, these antibiotics are different from setomimycin in molecular weight, melting point, UV spectrum in an alkaline solution, and so on.

Therefore, the antibiotic, setomimycin, was concluded to be a new antibiotic. Further investigations on its chemical structure are in progress.

Acknowledgements

We wish to thank Dr. T. HIGUCHI (JEOL) for FD-mass spectrometry and Mr. S. ŌKUBO (Kyowa Hakko Kogyo Co., Ltd.) for assays of antitumor activity and LD_{50}. Thanks are also due to Mr. H. MIYASHITA and Miss Y. UCHIYAMA for their helpful assistance.

This work was partially supported by a fund from Japan Keirin Association.

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