NEW ANTIBIOTICS, METHYLENOMYCINS A AND B

I. PRODUCING ORGANISM, FERMENTATION AND ISOLATION, BIOLOGICAL ACTIVITIES AND PHYSICAL AND CHEMICAL PROPERTIES

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Methylenomycins A and B, two new antibiotics were found in the culture filtrate of a streptomycete strain No. 2416, which was identified as a strain of *Streptomyces violaceoruber*. Their elementary analyses and mass spectroscopic measurements suggested that the molecular formula of methylenomycin A was $C_9H_{10}O_4$ and that of methylenomycin B was $C_8H_{10}O_2$. Methylenomycins A and B exhibited activities against gram-positive and gram-negative bacteria, and especially against *Proteus*.

Methylenomycins A and B were obtained in the culture filtrate of a streptomycete No. 2416, which was isolated from a soli sample collected at Sagamihara, Kanagawa Prefecture, Japan. The biological and chemical data relevant to a description of methylenomycins A and B such as the taxonomy of the producing micro-organism, their preparation by fermentation, and the isolation and characterization of the antibiotics, are presented in this paper.

Producing Organism

Taxonomic studies of strain No. 2416 revealed that it was a *Streptomyces* which formed sporophores with open spirals as side branches of the mycelium. Spores were spherical to oval ($0.5\sim0.8\ \mu\times0.6\sim1.1\ \mu$) with smooth surface. The mass color of the aerial mycelia was in the red-color series on almost all of the media tested. The cultural characteristics of the strain No. 2416 are shown in Table 1. Observation of the culture was performed after incubation at 28°C for two weeks, except where otherwise mentioned.

On most of the tested media, vegetative mycelia developed abundantly, but the aerial mass color varied from bluish gray to purplish white with the changes of the medium. It formed a blue soluble pigment on sucrose-nitrate agar or inorganic salts-starch agar, and a purple soluble pigment on glycerol-asparagine or oatmeal agar. Physiological properties and utilization of carbon sources by the strain No. 2416 are shown in Tables 2 and 3. These characteristics were compared with those of the known species of *Streptomyces* described by WAKSMAN or with those presented in BERGEY's Mannual of Determinative Bacteriology (7th edition). Among them, *Streptomyces violaceoruber* was very similar to strain No. 2416. A comparison of the characteristics of both strains is shown in Table 4.

Differences in the color of aerial mycelium, soluble pigments and ability to produce methylenomycins were observed between these two strains, but no difference in the number of

Table 1. Cultural characteristics of *Streptomyces violaceoruber* No. 2416.

Medium	Growth	Aerial mycelium	Reverse	Soluble pigment
Sucrose-nitrate agar	Abundant	Purplish white	Dark blue	Dark blue
Glucose-asparagine agar	Good	None	Dark orange	None
Glycerol-asparagine agar	Abundant	Bluish gray	Purplish black	Brown purple
Inorganic salts-starch agar	Abundant	Light brownish gray	Light brown	Grayish blue
Nutrient agar	Abundant	Grayish white	Pale brown to dark brown	Olive gray
Tyrosine agar	Abundant	Brownish gray	Pale yellowish brown to brownish purple	None
Yeast extract-malt extract agar	Abundant	Brownish gray	Light brown to dark brown	None
Oat meal agar	Abundant	Light greenish gray	Dark red	Pale reddish brown
Peptone-yeast extract iron agar	Abundant	None	Yellowish brown	Light olive gray
Skim milk (25°C, 7 days)	Moderate	None	Deep reddish purple	None
Skim milk (37°C, 7 days)	Good	None	Deep purplish red	None
Glucose-peptone gelatin stab (18°C)	Good	Grayish white	Dark brown	Dark reddish brown

Table 2. Physiological properties of *Streptomyces violaceoruber* No. 2416.

Tyrosinase reaction	−
Nitrate reduction	+
Hydrolysis of starch	++
Liquefaction of gelatin	++
Coagulation of milk	−(25°C), +(37°C)
Peptonization of milk	−(25°C), +(37°C)
Temperature range for growth	10°−45°C

Table 3. Utilization of carbon source by *Streptomyces violaceoruber* No. 2416.

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

Table 4. Comparative studies of the strain No. 2416 and *S. violaceoruber*.

	Strain No. 2416	*S. violaceoruber*
Spore surface	Smooth	Smooth
Open spiral	+	+
Aerial mycelium	Grayish white to bluish gray	White to light gray
Soluble pigment	Olive gray to dark blue	Pale pink to bluish black
Growth	Pale yellowish brown to purplish black	Colorless to purplish black
Liquefaction of gelatin	++	++
Coagulation of milk	+	+
Peptonization of milk	+	+

open spirals, utilization of carbon sources, milk coagulation and peptonization was found between them. The differences observed herein were not sufficient to differentiate strain No. 2416 from *S. violaceoruber* as a new species. Therefore strain No. 2416 was named *Streptomyces violaceoruber* No. 2416.

Fermentation and Isolation

The antimicrobial activity of the cultured broth was determined by a cylinder-plate method using *Escherichia coli* NIHJ as a test organism. For the highest production of the antibiotics, a spore suspension of strain No. 2416 was used as a seed culture. The spore suspension was prepared by the following method. A two-liter Erlenmyer flask containing 100 g of rice, which had been immersed in water for 30 minutes and filtered to remove the excess of water, was autoclaved for 45 minutes at 120°C. An agar slant of the organism was suspended in water, inoculated onto this medium and incubated at 28°C under appropriately moistened atmosphere. After two weeks, it was suspended in 500 ml of sterilized water and transferred into 600 liters submerged tank containing 300 liters of a medium composed of glycerol 2 %, soy-bean meal 1 %, corn-steep liquor 1 %, KH_2PO_4 1 % and $CaCO_3$ 0.3 % (pH 7.2 before sterilization). Fermentation was carried out at 28°C for 48 hours under aeration of 300 liters per minute and agitation of 170 r.p.m.

Three hundred liters of the culture filtrate were adjusted to pH 2.0 with hydrochloric acid and extracted with an equal volume of ethylacetate. The concentrated extracts were dissolved in 500 ml of chloroform and passed through 1 kg of silica gel packed into a column with chloroform, and eluted with 5.5 liters of the same solvent. The active fraction was concentrated *in vacuo* to 100 ml and 175 g of methylenomycin A crystallized upon addition of 500 ml of carbon tetrachloride. The crude crystals were filtered and recrystallized as colorless needles (140 g) from a mixture of chloroform and carbon tetrachloride. The mother liquor obtained after filtration of the crude crystals of methylenomycin A was adsorbed on a column of silica gel (500 g) packed with benzene and eluted with the same solvent. Seven grams of crude methylenomycin B were thus obtained as slightly yellowish oil. The crude methylenomycin B was dissolved in a mixture of benzene and *n*-hexane (1:4) and was applied on a column of silica gel (350 g) packed with *n*-hexane and eluted with a mixture of benzene and *n*-hexane (1:1). Finally 5.3 g of purified methylenomycin B were obtained as neutral colorless oil.

Biological Activities of Methylenomycins

The antimicrobial spectra of methylenomycins A and B in the minimal inhibitory concentrations are shown in Table 5. Both A and B are active against gram-positive and gram-negative bacteria. Especially, methylenomycin A is active against *Proteus vulgaris* and *Proteus morganii* at lower concentrations in broth dilution than in agar dilution studies.

The acute toxicities (LD_{50} in mice) of methylenomycins A and B were 75 and 245 mg/kg intraperitoneally and 1,500 and 260 mg/kg orally, respectively.

The stability of methylenomycins A and B was examined at 1,000 mcg/ml in aqueous solution containing 10 % methanol adjusted to pH 2.0 to 10.0 at 60°C for 30~240 minutes. As shown in Table 6, both methylenomycins A and B were moderately labile in alkaline and

acidic solution and B was more labile than A. Removal of the organic solvent under reduced pressure from the preparation of methylenomycin B resulted in polymerization of the antibiotic with a loss of antimicrobial activity.

Table 5. Antimicrobial spectra of methylenomycins A and B.

Test organism	Broth dilution MIC mcg/ml		Agar dilution MIC mcg/ml	
	A	B	A	B
Staphylococcus aureus 209P JC-2	50	50	200	100
Staphylococcus aureus 56	>100		400	
Staphylococcus aureus 1557	100		400	
Sarcina lutea PCI 1001	12.5		25	50
Bacillus subtilis PCI 219			25	
Alcaligenes faecalis	0.8		100	100
Escherichia coli NIHJ JC-2	100	>100	200	200
Escherichia coli K-12	100		400	
Escherichia coli CM, TCf	1.56		25	25
Proteus vulgaris OX-19	0.39	1.5	25	50
Proteus mirabilis	12.5		200	100
Proteus morganii	0.39	50		
Proteus rettgeri	12.5		50	
Aerobacter aerogenes	400			
Shigella flexneri 2a	25			50
Salmonella typhosa	400			
Klebsiella pneumoniae PCI 602	25	>100	200	400
Pseudomonas aeruginosa	50		50	
Mycobacterium smegmatis ATCC 607	>400			
Aspergillus oryzae			>400	>400
Penicillium chrysogenum			>400	>400
Piricularia oryzae			>400	>400
Trichoyhyton mentagrophytes			>400	>400
Trichophyton interdigitale			>400	>400
Candida albicans			>400	>400

Table 6. Stability of methylenomycins A and B (at 60°C).

pH	Residual activities (%)							
	30 min.		60 min.		120 min.		240 min.	
	A	B	A	B	A	B	A	B
2.0	84	65	67	32	43	<10	35	<10
4.0	84	68	70	35	62	<10	50	<10
5.0	85	71	80	38	75	<10	54	<10
6.0	86	70	70	40	70	<10	47	<10
7.0	87	68	69	33	55	<10	38	<10
8.0	75	60	57	30	53	<10	30	<10
9.0	70	60	60	25	35	<10	23	<10
10.0	70	50	55	<10	28	<10	17	>10

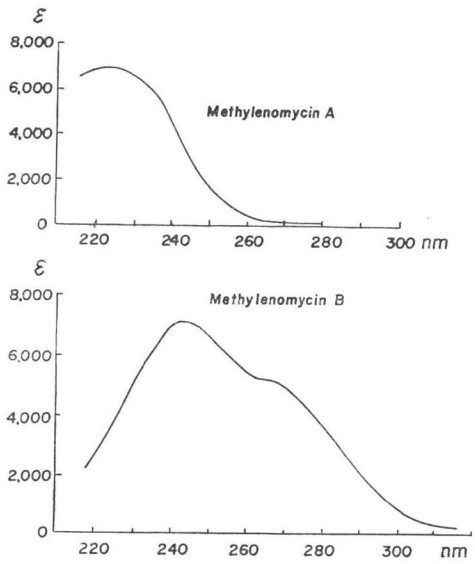
Activities were determined by the cylinder-plate method using *Escherichia coli* CM, TC fast as a test organism.

Physical and Chemical Properties of Methylenomycins

Methylenomycin A

Methylenomycin A is an acidic (pKa′ 3.65), lipophilic, colorless crystalline substance; m.p. 115°C (decomp.), $[\alpha]_D^{20}$+42.3° (*c* 1, in $CHCl_3$). It is slightly soluble in *n*-hexane, carbon tetrachloride and fairly soluble in benzene, chloroform, ethylacetate, acetone, methanol and water. The molecular weight was confirmed to be 182 by the parent peak in its mass spectrum. The elemental analysis and the molecular weight indicate that the molecular formula of methylenomycin A is $C_9H_{10}O_4$, Found: C 59.32, H 5.55%. Calcd. for $C_9H_{10}O_4$: C 59.33, H 5.53%. The UV spectrum in methanol exhibited a maximum at 224 nm (ε 6300) as shown in Fig. 1. The IR spectrum indicated two characteristic absorption bands at 1740 and 1720 cm^{-1} in the carbonyl region, and a band at 1650 cm^{-1} due to an unsaturated bond (Fig. 2). The NMR spectrum, taken at 100 MHz in $CDCl_3$ using TMS as internal standard, demonstrated all 10 protons of the molecule as shown in Fig. 3. Each proton was assigned as follows: $\delta_{ppm}^{CDCl_3}$—COO<u>H</u> 9.95 (1H, singlet, exchangeable with D_2O), <u>H</u>>C=C<6.27 (1H, doublet, J= 1.90 Hz), <u>H</u>>C=C<5.65 (1H, doublet, J= 1.68 Hz), <u>H</u>—C— 3.82 (1H, multiplet, J=1.90 and 1.68 Hz), <u>CH_3</u>—C— 1.58 (3H, singlet) and <u>CH_3</u>—C— 1.48 (3H, singlet).

Fig. 1. Ultraviolet absorption spectra of methylenomycins A and B.



Methylenomycin B

Methylenomycin B was obtained as a neutral colorless oily substance, $[\alpha]_D^{20}$ 0° (*c* 1, in $CHCl_3$). It is slightly soluble in *n*-hexane, petroleum ether and fairly soluble in ether, benzene, chloroform, ethylacetate, acetone and alcohols. The molecular weight of methylenomycin B was shown to be 138 from the parent peak in its mass spectrum. The elementary analysis and molecular weight indicate that the molecular formula is $C_8H_{10}O_2$, Found: C 69.56, H 7.32%. Calcd. for $C_8H_{10}O_2$: C 69.54, H 7.30%. The UV spectrum of methylenomycin B in methanol exhibited a maximum at 240 (ε 7650) and a shoulder at 270 nm as shown in Fig. 1. The IR spectrum contained a characteristic absorption band at 1720 cm^{-1} in the carbonyl region and another at 1650 cm^{-1} due to an unsaturated bond (Fig. 2).

The NMR spectrum showed all 10 protons of the molecule, and these were assigned as

Fig. 2. Infrared absorption spectra of methylenomycins A and B.

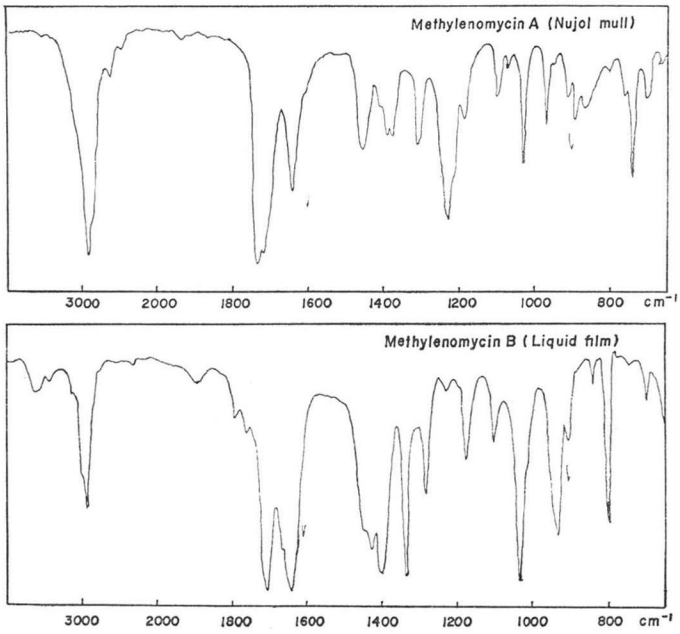
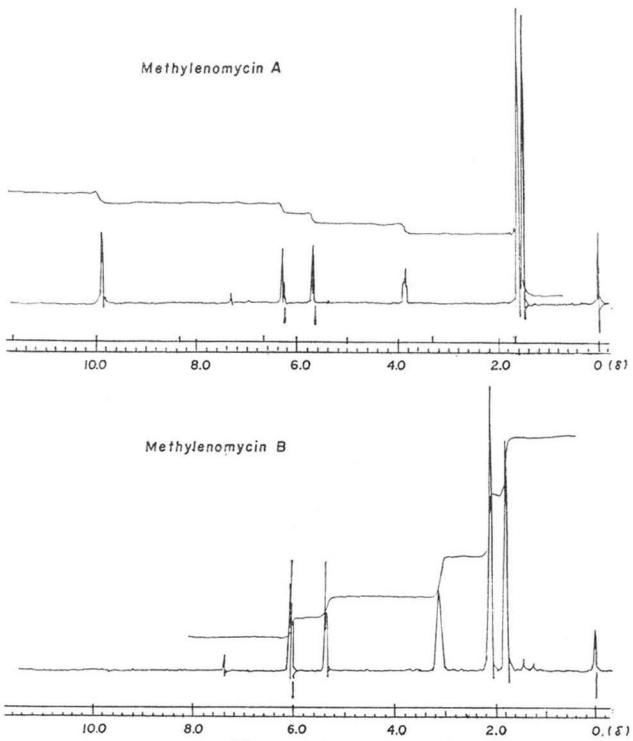


Fig. 3. NMR spectra of methylenomycins A and B in $CDCl_3$ (100 MHz).



follows (Fig. 3): $\delta^{CDCl_3}_{ppm}$ $\underline{H}$>C=C<6.03 (1H, doublet, J=1.45 Hz), $\underline{H}$>C=C<5.32 (1H, doublet, J=1.68 cps), —$\underline{C}H_2$ 3.08 (2H, multiplet, J=1.45 and 1.68 Hz), $\underline{CH_3}$—$\underline{C}$— 2.08 (3H, double doublet, J=0.92 and 1.1 Hz), $\underline{CH_3}$—$\underline{C}$— 1.79 (3H, double doublet, J=0.92 and 1.1 Hz).

In comparing the NMR spectra of methylenomycins A and B, the existence of a terminal methylene and two olefinic methyl group was observed in both antibiotics, but the signals of the carboxylic acid and methyne proton in A were not found in B. Instead of these two protons, a methylene group at δ=3.08 ppm was observed in B. This fact reasonably explains the difference in physical and chemical properties between them. The existence of a carboxylic acid function in methylenomycin A was confirmed by a pKa′ value of 3.65, the absorption band at 1720 cm^{-1} in the IR spectrum, the signal at 9.95 ppm in the NMR spectrum and the major fragment ion peak at *m/e* 137 (M^+—45) in the mass spectrum, while all these indications for carboxylic acid were lacking in the neutral substance, methylenomycin B. From the similarities of A with B observed in NMR spectra as well as in their other physical and chemical properties, they were assumed to be very closely related to each other.

Based on the biological, physical and chemical properties described above, methylenomycins A and B were differentiated from any of the known antibiotics. This conclusion was further supported by the structural elucidation of the antibiotics to be presented in the subsequent paper.