NOVEL ANTIFUNGAL ANTIBIOTICS MANIWAMYCINS A AND B

I. TAXONOMY OF THE PRODUCING ORGANISM, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL PROPERTIES

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Two antifungal antibiotics maniwamycins A and B were isolated from the culture broth of a strain of actinomycetes, which were classified as *Streptomyces prasinopilosus*. These antibiotics were isolated by resin absorption and extraction with EtOAc and purified by column chromatography. Both antibiotics were found to be new azoxy substances from their physico-chemical properties. They showed broad antifungal spectra.

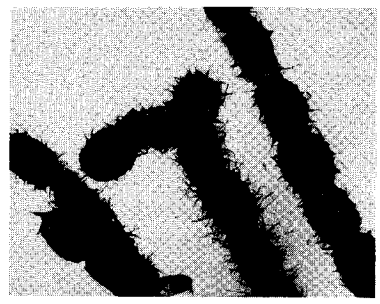
In the course of screening for new antifungal agents from culture broths, we have discovered the novel antifungal antibiotics, maniwamycins A and B†, produced by a *Streptomyces* sp. This paper details the taxonomy of the producing organism, isolation, and physico-chemical and biological properties of these antibiotics. The structural elucidation of antibiotics maniwamycins A and B are described in an accompanying paper.[1]

Taxonomy of the Producing Organism

Strain KC-7367 was isolated from a soil sample collected in Maniwa-gun, Okayama Prefecture, Japan. For the taxonomic characterization of the strain KC-7367, the methods and media recommended by the International Streptomyces Project (ISP)[2] and WAKSMAN[3] were used. Color names and hue numbers indicated in Table 1 are those of the Color Harmony Manual (4th Ed.) published by Container Corporation of America. Diaminopimelic acid in whole cell hydrolysate was determined by the method of BECKER *et al.*[4] The cultural characteristics of strain KC-7367 on various media are shown in Table 1. Aerial mycelium was often poorly developed and mass color was almost reseda green on some media tested. Vegetative mycelium developed on most of the media and was usually light yellow to dark brown. The spore chains have more than ten spores per chain. The spores are cylindrical in shape, $1.1 \sim 1.3 \times 1.3 \sim 1.5\,\mu m$ in size and have hairy surfaces as shown in Fig. 1.

Fig. 1. Scanning electron micrograph of spore chain of strain KC-7367.

Bar represents 1 μm.



† Maniwamycins A and B were reported as KA-7367 A and B at the 109th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, Japan, Apr. 4~6, 1989.

Table 1. Cultural characteristics of strain KC-7367.

Yeast extract-malt extract agar	G:	Good
(ISP medium 2)	R:	Beige brown (3ig) to light melon yellow (3ea)
	AM:	Pearl gray (13cb) to reseda green (24ig)
	SP:	Squash yellow (2ia)
Oatmeal agar	G:	Moderate
(ISP medium 3)	R:	Dark brown (3nl)
	AM:	Smoke (13dc)
	SP:	Nude tan (4gc)
Inorganic salts-starch agar	G:	Moderate
(ISP medium 4)	R:	Dusty yellow (1 1/2gc) to dark olive (1 1/2nl)
	AM:	Light olive gray (1 1/2ge) to reseda green (24ig)
	SP:	None
Glycerol-asparagine agar	G:	Moderate
(ISP medium 5)	R:	Covert tan (2ge)
	AM:	Orchid mist (10cb)
	SP:	None
Peptone-yeast extract-iron agar	G:	Poor
(ISP medium 6)	R:	Light tan (3gc)
	AM:	White (a)
	SP:	None
Tyrosine agar	G:	Good
(ISP medium 7)	R:	Beige (3ge) to dark brown (3nl)
	AM:	White (a) to reseda green (24ig)
	SP:	None
Sucrose-nitrate agar	G:	Poor
	R:	Light ivory (2ca)
	AM:	White (a)
	SP:	None
Glucose-asparagine agar	G:	Poor
	R:	White (a)
	AM:	White (a)
	SP:	None
Nutrient agar	G:	Moderate
	R:	Light yellow (1 1/2ea)
	AM:	White (a)
	SP:	None

Abbreviations: G, Growth of vegetative mycelium; R, reverse; AM, aerial mycelium; SP, soluble pigment.

Table 2. Physiological properties of KC-7367.

Melanin formation	−
Tyrosinase reaction	−
H_2S production	−
Liquefaction of gelatin	−
Peptonization of milk	+
Coagulation of milk	−
Starch hydrolysis	+
Temperature range for growth	18~40°C

Table 3. Utilization of carbon sources by strain KC-7367.

L-Arabinose	±
D-Xylose	+
D-Glucose	+
D-Fructose	+
D-Mannitol	+
Sucrose	−
Inositol	+
Raffinose	−
L-Rhamnose	+

+: Utilized, ±: weakly utilized, −: not utilized.

The physiological properties and the utilization of carbon sources of strain KC-7367 are shown in Tables 2 and 3, respectively. The whole cell hydrolysate contained L,L-diaminopimelic acid.

Based on the taxonomic properties described above, strain KC-7367 belongs to the genus *Streptomyces*. The characteristics of this strain were compared with the published descriptions[5~8] of various *Streptomyces*

species. It was considered strain KC-7367 to be closely related to *Streptomyces prasinopilosus*. Therefore, strain KC-7367 was classified as a strain of *S. prasinopilosus*, named *S. prasinopilosus* KC-7367. This strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession No. of FERM BP-1277.

Fermentation

Spores of strain KC-7367 were inoculated into 100 ml of a medium (pH 7.0) composed of starch 1.0%, glucose 1.0%, soybean meal 1.0%, corn steep liquor 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.3% and $CoCl_2 \cdot 6H_2O$ 0.0005% in a 500-ml Erlenmeyer flask, and cultured at 28°C on a rotary shaker for 2 days. The seed culture was transferred into a 400-liter fermenter containing 250 liters of a medium (pH 7.0) consisting of mannitol 1.0%, glucose 1.0%, soybean meal 0.6%, soybean oil 0.8%, $MgSO_4 \cdot 7H_2O$ 0.04%, $CaCl_2 \cdot 2H_2O$ 0.8% and silicon oil 0.02%. The fermentation was conducted at 28°C for 2 days under aeration of 250 liters/minute, agitation of 180 rpm and inner pressure of 0.5 kg/cm^2.

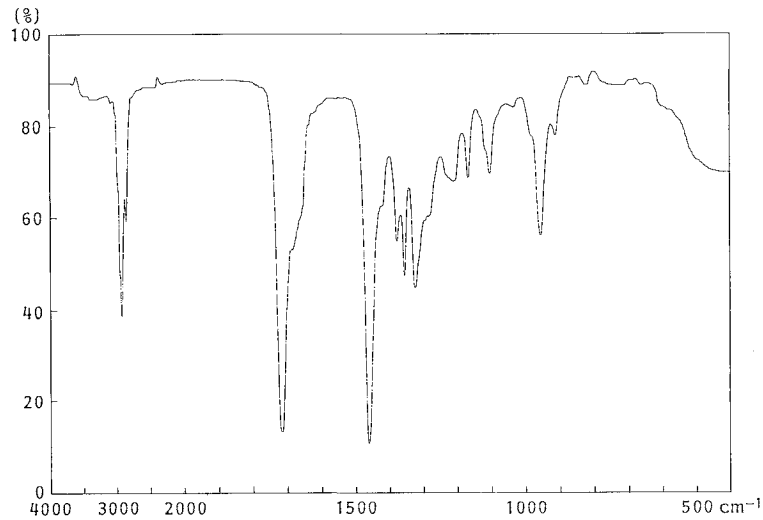
Isolation

The whole broth (*ca.* 250 liters) was adjusted to pH 6.0 with conc HCl and then filtered. The filtrate was passed through a column of Diaion HP-20. The adsorbed antibiotics were eluted with MeOH. The eluate was concentrated *in vacuo*. The concentrate was extracted with EtOAc. The extract was washed with 5% (w/v) $NaHCO_3$ and 0.05 N HCl, and then evaporated *in vacuo* to give a crude oily material. The residue was chromatographed on a silica gel column with benzene-EtOAc (25:1~10:1). Further purification was accomplished by gel filtration on Sephadex LH-20 (eluting with MeOH) to give two active compounds, maniwamycins A (2.5 g) and B (200 mg).

Physico-chemical Properties

Maniwamycins A and B are colorless oils. The antibiotics are soluble in MeOH, acetone, EtOAc and benzene but insoluble in water. The IR spectra and ^{1}H NMR spectra of these compounds are shown in Figs. 2, 3, 4 and 5 respectively. Maniwamycin A is stable in acidic conditions but undergoes decomposition at pH above 7.0. Maniwamycin B is stable in the range pH 2.0~10.0.

Fig. 2. IR spectrum of maniwamycin A ($CHCl_3$).



Maniwamycin A possessed the following physical constants: $[\alpha]_D^{22}$ $-144°$ (*c* 1.0, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 235 (12,400); fast atom bombardment (FAB)-MS *m/z* 199 (M+H); high resolution (HR)-MS *m/z* 198.1363 (M, calcd for $C_{10}H_{18}N_2O_2$ 198.1369).

Maniwamycin B possessed the following physical constants: $[\alpha]_D^{22}$ $+108°$ (*c* 1.0, $CHCl_3$); UV λ_{max}^{EtOH} nm (ε) 230 (15,200); FAB-MS *m/z* 201 (M+H); HR-MS *m/z* 201.1607 (M+H, calcd for $C_{10}H_{21}N_2O_2$ 201.1604).

The characteristic band at 1465 cm^{-1} in the IR spectrum and the UV absorption maximum suggest the presence of an azoxy group.

Fig. 3. IR spectrum of maniwamycin B ($CHCl_3$).

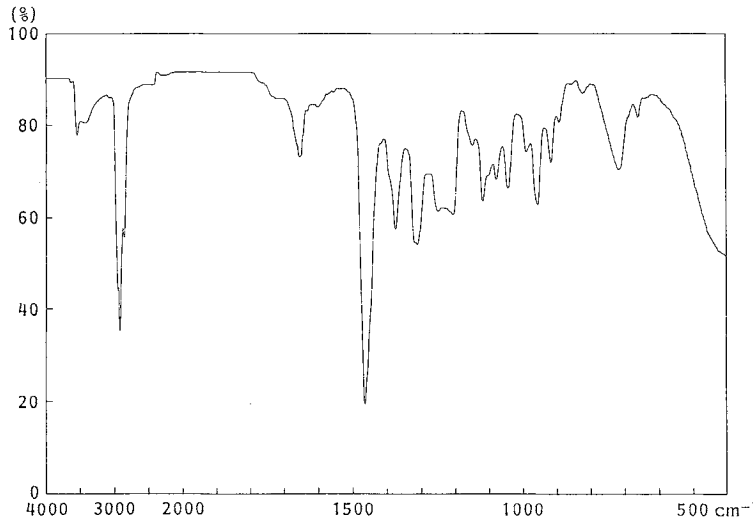


Fig. 4. ^1H NMR spectrum of maniwamycin A (270 MHz, $CDCl_3$).

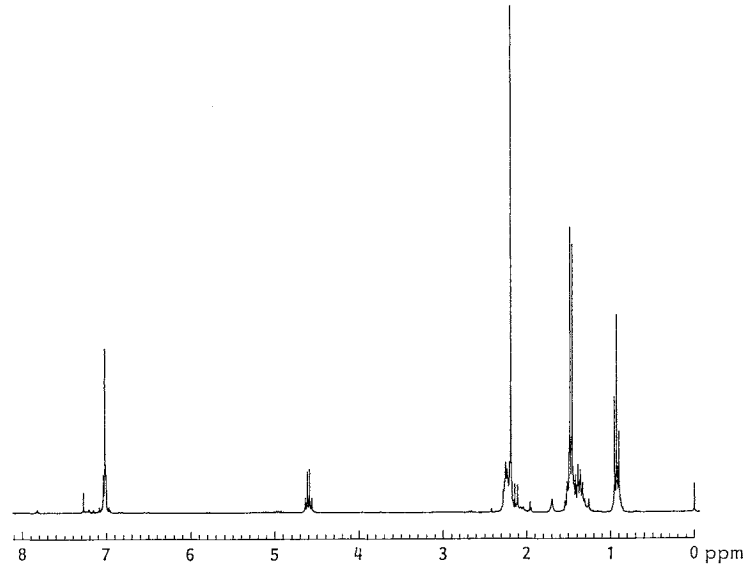


Fig. 5. ^{1}H NMR spectrum of maniwamycin B (270 MHz, $CDCl_3$).

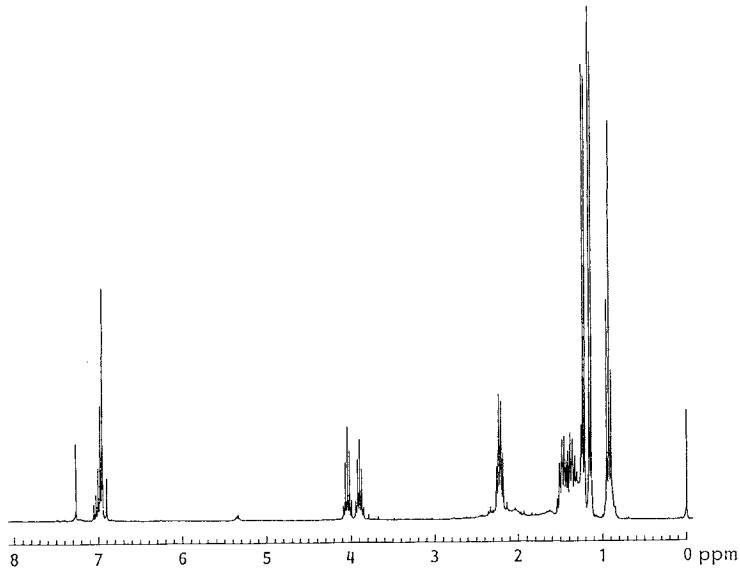


Table 4. Antimicrobial spectra of maniwamycins A and B.

Test organisms	MIC (μg/ml)		Test organisms	MIC (μg/ml)	
	A	B		A	B
Candida albicans IFM 40001	3.1	50	*Nannizzia otae* JCM 1909	1.6	100
C. albicans N 508	1.6	50	*Trichophyton mentagrophytes* IFM 40769	3.1	100
C. albicans TIMM 0228	1.6	50			
C. albicans TIMM 0237	1.6	50	*T. mentagrophytes* IFM 40771	3.1	100
C. glabrata chuken 86-22384	12.5	100	*T. rubrum* IFM 40768	3.1	100
C. glabrata chuken 88-9502	12.5	100	*Staphylococcus aureus* FDA 209P	>100	>100
Cryptococcus neoformans IFM 40038	3.1	100	*Escherichia coli* NIHJ JC-2	>100	>100

Fungi: Sabouraud-dextrose agar, 27°C, 72 hours.
Bacteria: Mueller-Hinton agar, 37°C, 20 hours.

Biological Properties

The antimicrobial activities of maniwamycins A and B against bacteria and fungi by the agar dilution method are presented in Table 4. Although both antibiotics exhibited no activity against bacteria, they showed strong activity against fungi. The toxicity (LD_{50}, iv) of maniwamycins A and B in rats are *ca.* 10 mg/kg and *ca.* 100 mg/kg, respectively.

Discussion

There are only a few azoxy antibiotics reported so far. Among them are elaiomycin,[9] LL-BH872α,[10] valanimycin[11] and jietacins A and B.[12] Elaiomycin is effective against *Mycobacterium tuberculosis*, LL-BH872α has antifungal activity, valanimycin is active against Gram-positive and Gram-negative bacteria and tumors, and jietacins A and B exhibit nematocidal activity. There are no compounds which have the same physico-chemical properties as maniwamycins A and B. Therefore, these antibiotics are new members of this class.

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