MYCOPLANECINS, NOVEL ANTIMYCOBACTERIAL ANTIBIOTICS FROM *ACTINOPLANES AWAJINENSIS* SUBSP. *MYCOPLANECINUS* SUBSP. NOV.

II. ISOLATION, PHYSICO-CHEMICAL CHARACTERIZATION AND BIOLOGICAL ACTIVITIES OF MYCOPLANECIN A

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New antibiotics, mycoplanecins, were found in the culture broth of an actinomycete identified as *Actinoplanes awajinensis* subsp. *mycoplanecinus* subsp. nov.

Mycoplanecin complex was extracted with organic solvents both from the culture filtrate and mycelium and purified by column chromatography on silica gel and Florisil. Mycoplanecin A, a major component, was separated by high performance liquid chromatography on Prep PAK-500/C_{18} column. The physico-chemical characterization revealed that mycoplanecin A was a new cyclic peptide antibiotic.

Mycoplanecins exhibited strong activities primarily against mycobacteria and related microorganisms.

As described in the preceding paper[1], mycoplanecins, new antimycobacterial antibiotics, are produced by a new subspecies of Actinoplanes designated as *Actinoplanes awajinensis* subsp. *mycoplanecinus* subsp. nov. Physico-chemical properties coupled with biological properties of mycoplanecin A revealed it to be a new antibiotic.

This paper describes the isolation and physico-chemical and biological properties of mycoplanecin A.

Isolation

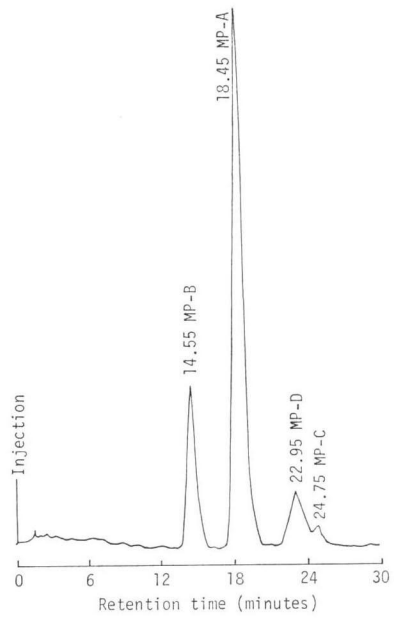
Six hundred liters of the culture broth from two 600-liter fermentors were filtered, yielding 420 liters of filtrate and 140 kg of mycelial cake mixed with 30 kg of diatomaseous earth (Celite 545, Johns-Manville Products Corp., U.S.A.). The filtrate was extracted with an equal volume of ethyl acetate. The mycelial cake was extracted twice with 400 liters of 80% (v/v) aqueous acetone, yielding 750 liters of extract, which was evaporated to remove acetone under reduced pressure, then the residue was extracted with 210 liters of ethyl acetate. The ethyl acetate extracts from the filtrate and the mycelium were combined and concentrated to 2 liters under reduced pressure. The concentrate was washed once with one liter of 0.05 N hydrochloric acid and then twice with 2 liters of 1% (w/v) aqueous sodium bicarbonate and saturated aqueous sodium chloride. After drying over anhydrous sodium sulfate, the washed concentrate was evaporated under reduced pressure to give 400 g of oily product.

The oily material was dissolved in 300 ml of ethyl acetate and chromatographed on a column of 1 kg of silica gel (Mallinkrodt Co., U.S.A.) packed and developed with ethyl acetate. The oily product obtained by evaporation of 5 liters of the active eluate was dissolved in 350 ml of a mixture of benzene and ethyl acetate (1: 1) and applied onto a column of 1 kg of silica gel packed and developed with the

same solvent system. The active eluate (8.5 liters) was concentrated to give 45 g of slightly yellowish powder. For further purification of the mycoplanecin complex, the powder dissolved in 100 ml of ethyl acetate was adsorbed on a 300 g of Florisil column (Iwai Chemical Co., Ltd., Japan) and developed with the same solvent. Mycoplanecins in the eluates were monitored by chromatography on silica gel TLC (Merck Co., Ltd., Silica gel 60 F-254, Art 5715, solvent; $CHCl_3$ - MeOH, 10: 1) and by visualization with iodine. The eluates showing a single spot of mycoplanecin complex on TLC were pooled and concentrated to dryness under reduced pressure to give 41.7 g of mycoplanecins as colorless amorphous powder. Overall recovery of mycoplanecin complex from the culture broth was 55 per cent.

High performance liquid chromatography of mycoplanecin complex thus obtained revealed it to have at least one major and four minor components; the major component was designated as mycoplanecin A, and the minor components as B, C and D as shown in Fig. 1. The separation of mycoplanecin A from the mycoplanecin complex was achieved by preparative high performance liquid chromatography (System 500, Waters Ltd., U.S.A.). Two grams of the mycoplanecin complex were dissolved in 20 ml of 70% aqueous acetonitrile and injected into Prep PAK-500/C_{18} cartridge equilibrated and eluted with 60% aqueous acetonitrile at a flow rate of 200 ml per minute, and the eluates were monitored by refractive index meter. Mycoplanecin A was eluted from 22 to 28 minutes after the injection of the sample and B from 16 to 19 minutes. Each active eluate was concentrated to dryness to yield 1 g of A and 300 mg of B, respectively. Other minor components such as C and D were eluted in the same fraction from 28 to 36 minutes together with large amounts of A. Further purification studies on these components as well as their characterization and structural elucidation will be reported elsewhere.

Fig. 1. High performance liquid chromatogram of mycoplanecins.
Column; μBondapak C_{18} (4 mm × 30 cm)
Solvent; 55% CH_3CN - H_2O.
Flow rate; 1.5 ml/minute.
Detector; UV at 222 nm.



Physico-chemical Properties

Mycoplanecin A was obtained as a neutral, lipophilic, colorless powder, soluble in alcohol, acetone, ethyl acetate and chloroform, but only slightly soluble in water. The antibiotic showed positive reactions to iodine, potassium permanganate - sulfuric acid and sulfuric acid on silica gel TLC plates. The molecular formula of mycoplanecin A was estimated to be $C_{61}H_{102}N_{10}O_{18}$(mol. wt. 1,182) from elementary analysis and the number of peaks of ^{13}C NMR spectrum together with measurement of the molecular weight by FD/MS spectrometry. These results as well as other physico-chemical properties are summarized in Table 1.

The IR, ^1H and ^{13}C NMR spectra of mycoplanecin A are shown in Figs. 2, 3 and 4, respectively.

Mycoplanecin A showed no characteristic absorption in the ultraviolet region and its IR spectrum

indicated the presence of carbonyl groups at 1760 and 1720 cm^{-1} and amide bond at 1670~1640 cm^{-1}. In the ^1H NMR spectrum, four singlets at 2.87, 2.99, 3.15 and 3.32 ppm due to *N*-methyl groups and some broad signals in the lower field which belong to amide protons were observed. Amino acid analysis and GC/MS analysis of the acid hydrolysate of mycoplanecin A (conc. HCl - CH_3COOH, 1: 1, at 105°C, 20 hours) indicated the presence of glycine, leucine, proline, *N*-methylvaline, *N*-methylleucine, *N*-methylthreonine and three unidentified amino acids. These physico-chemical properties suggested that mycoplanecin A is a peptide composed of nine amino acids including three *N*-methyl amino acids.

Fig. 2. IR spectrum of mycoplanecin A in KBr.

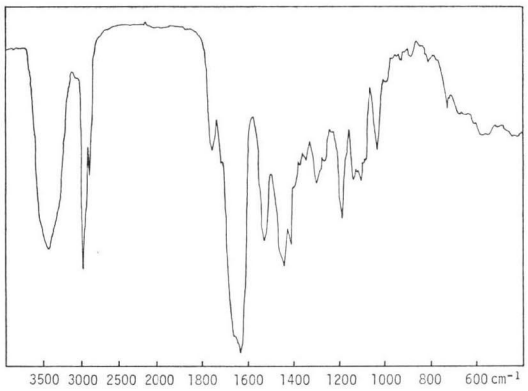


Fig. 3. ^1H NMR spectrum of mycoplanecin A in $CDCl_3$ (100 MHz).

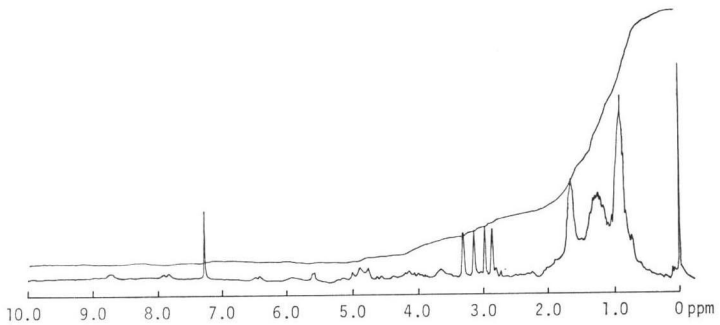


Table 1. Physical and chemical properties of mycoplanecin A.

Nature	Neutral, colorless powder
mp	161~167°C
$[\alpha]_D^{25}$	−66° (*c* 0.4, $CHCl_3$)
Elementary analysis (%)	Found: C 61.78, H 8.48, N 11.75.
	Calcd. for $C_{61}H_{102}N_{10}O_{13}$: C 61.93, H 8.63, N 11.84.
MW	1,182
Solubility	Soluble in MeOH, EtOAc, $CHCl_3$, benzene.
	Slightly soluble in *n*-hexane.
	Insoluble in H_2O.
Color reaction	(+) $KMnO_4$
	(−) Ninhydrin
Rf (Merck No. 5715)	0.15, EtOAc
	0.64, $CHCl_3$ - MeOH (10: 1)
Acid hydrolysis	Gly, Leu, Pro, *N*-MeLeu, *N*-MeThr, *N*-MeVal, 3unidentified amino acids
UV λ_{max}^{MeOH}	End absorption.
IR ν_{max}^{KBr}	1760, 1720 (sh), 1670~1640 cm^{-1}
NMR $\delta_{ppm}^{CDCl_3}$	2.87, 2.99, 3.15, 3.32 (*N*-Me)

Biological Properties

The minimal inhibitory concentration (MIC) of mycoplanecin A against bacteria, yeasts and molds were determined by a serial two-fold agar dilution method. The medium used was heart infusion agar supplemented with 1% glycerol for bacteria, WAKIMOTO[2] agar for Xanthomonas and Sabouraud-dextrose agar for yeasts and molds. The MICs were determined after incubation for 24 or 48 hours at 37°C for bacteria and 26°C for Xanthomonas, and for 2 or 14 days at 26°C for yeasts and molds.

As shown in Table 2, mycoplanecin A was very active against Mycobacteria and *Micrococcus luteus*, moderately active against *Xanthomonas oryzae*, and inactive against other bacteria, and against yeasts and molds tested, even at a concentration of 400 μg/ml. Mycoplanecin A was also tested against several

Fig. 4. ^{13}C NMR spectrum of mycoplanecin A in $CDCl_3$.

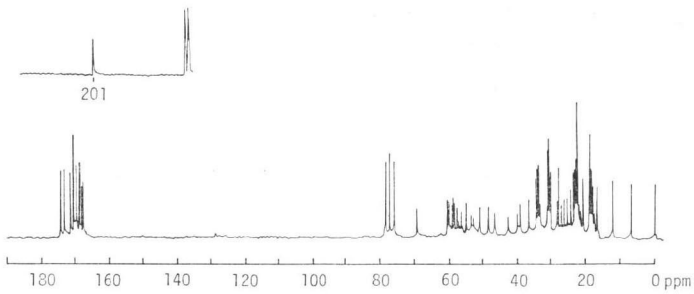


Table 2. Antimicrobial spectrum of mycoplanecin A.

Test organism	MIC (μg/ml)	Medium
Staphylococcus aureus FDA 209P JC-1	>400	a
S. aureus 56	>400	〃
S. aureus 337	>400	〃
Bacillus subtilis PCI 219	>400	〃
Micrococcus luteus PCI 1001	0.0125	〃
Mycobacterium smegmatis ATCC 607	0.025	〃
M. phlei IFM 2052	0.025	〃
Escherichia coli NIHJ JC-2	>400	〃
Klebsiella pneumoniae PCI 602	>400	〃
Pseudomonas aeruginosa SANK 73860	>400	〃
Proteus vulgaris OX19	>400	〃
Candida albicans YU 1200	>400	b
Aspergillus oryzae SANK 11262	>400	〃
Penicillium chrysogenum SANK 12768	>400	〃
Trichophyton mentagrophytes SANK 11868	>400	〃
Pellicularia filamentosa SANK 16376	>400	〃
Pyricularia oryzae SANK 10975	>400	〃
Xanthomonas oryzae SANK 70274	12.5	c
X. oryzae SANK 71772	12.5	〃
X. citri SANK 70876	>100	〃
X. campestris SANK 70561	>100	〃
X. pruni	>100	〃
X. phaseoli	>100	〃

a; Heart infusion agar +1% Glycerol.
b; Sabouraud-dextrose agar.
c; WAKIMOTO agar.

Table 3. Antimycobacterial activity of mycoplanecin A.

Test organism	MIC (μg/ml)
Mycobacterium kansasii IFM 2069	0.1
M. fortuitum IFM 2079	1.56
M. intracellulare IFM 2073	0.39
M. intracellulare IFM 2083	0.39
M. tuberculosis Matsudo IFM 2026	0.195
M. tuberculosis Maru IFM 2027	0.39
M. tuberculosis H37 IFM 2028	0.78
M. tuberculosis H37Rv IFM 2029	0.39
M. tuberculosis H2 IFM 2030	0.78
M. bovis B.C.G. IFM 2031	0.78
M. bovis Ushi-10 IFM 2032	0.1
M. tuberculosis SM-R IFM 2033	0.1

Medium; Dubos with 10% calf serum.

Table 4. Antimicrobial activity of mycoplanecin A against organisms related to mycobacterium.

Test organism	MIC (μg/ml)
Nocardia asteroides SANK 61865	>400
N. brasiliensis SANK 62065	>400
N. madurae SANK 64765	3.12
N. otitidis-caviarum SANK 63565	50
Actinoplanes philippinensis SANK 63577	200~400
A. awajinensis subsp. *mycoplanecinus* No. 41042, mycoplanecins producer	400
Streptosporangium roseum SANK 60768	25~50
Ampullariella regularis SANK 62175	>400
A. digitata SANK 61175	0.2
Micromonospora chalcea SANK 60268	0.1
Streptomyces fradiae SANK 69270	0.2
S. griseus SANK 90570	3.12~6.25
Corynebacterium equi SANK 73460	3.12
Mycobacterium smegmatis ATCC 607	0.8

Medium; Yeast extract - malt extract agar +1% glycerol.

strains of *M. tuberculosis* including clinical isolates and a streptomycin-resistant strain. Typical clinical isolates from recent mycobacterial lesions were also tested their sensitivity to mycoplanecin A. Determination of MICs for these mycobacteria was carried out by a serial two-fold dilution method using Dubos liquid medium and Kirchner semi-liquid medium both supplemented with 10% of calf serum; incubation was at 37°C for 3 to 6 weeks. The results are presented in Table 3. The MIC values against *M. tuberculosis* H37Rv, including clinical isolates and a streptomycin-resistant strain, were 0.1~0.78 μg/ml, indicating strong activity.

The antibiotic was also strongly active against atypical mycobacteria, especially *M. intracellulare*, which is resistant to most antituberculous drugs now in use and is isolated more and more frequently from consumptives.

The MIC against Mycobacterium-related microorganisms was determined on yeast extract - malt extract agar medium supplemented with 1% of glycerol after incubation at 28°C for two weeks. The antibiotic was also active against *Nocardia madurae, Ampullariella digitata, Micromonospora chalcea, Streptomyces fradiae* and *Corynebacterium equi* as shown in Table 4.

Mycoplanecin A has extremely low toxicity; all mice tolerated intravenous, subcutaneous and oral administrations of the antibiotic at dose of 400 and 3,000 mg/kg, respectively.

From these physico-chemical as well as biological activities, mycoplanecin A was easily distinguished from known antibiotics and identified as a new member of the peptide antibiotic group. The structural elucidation of mycoplanecin A and the identification of three other components of the antibiotic complex are described in the next paper.

Acknowledgments

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