ISOLATION AND CHARACTERIZATION OF MILDIOMYCIN, A NEW NUCLEOSIDE ANTIBIOTIC

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A new antibiotic mildiomycin, strongly active against powdery mildew, was isolated from the culture filtrate of *Streptoverticillium rimofaciens* B-98891. It is a water-soluble basic antibiotic and was purified by ion-exchange and adsorption chromatography. The molecular formula of the purified compound was determined to be $C_{19}H_{30}N_8O_9(H_2O)$ from physical and chemical data. The UV and NMR spectra suggested that this antibiotic is a nucleoside. On acidic hydrolysis it gave 5-hydroxymethyl cytosine which has not previously been found in nucleoside antibiotics.

In our screening program for useful agricultural antibiotics *Streptoverticillium rimofaciens* B-98891 was isolated from a soil sample collected in Papua New Guinea.[1] It produced an antibiotic which showed remarkable activity against powdery mildews. Isolation and purification were carried out based on the results of *in vivo* tests using *Erysiphe graminis* on barley.[1] The product thus obtained was determined to be a new nucleoside antibiotic from its physico-chemical properties and from its release of 5-hydroxymethyl cytosine on acidic hydrolysis. This new antibiotic was named mildiomycin from its biological activity.

This report deals with the isolation and chemical characterization of mildiomycin.

Isolation Procedure

In preliminary experiments, mildiomycin (MIL) was found to be adsorbed on weak cation-exchange resins and activated charcoal and to be eluted with appropriate solvents. MIL was purified by the following procedure: Activity in the culture filtrate was adsorbed on Amberlite IRC-50 and eluted with 2% ammonia. From this eluate it was adsorbed on a column of activated charcoal and eluted with acetone-water. This eluate was concentrated and a crude precipitate obtained by addition of acetone. The crude powder was adsorbed on a column of Amberlite CG-50 and fractionated by eluting with 0.5~1.0% ammonia. Active fractions were concentrated and the product was adsorbed on a column of activated charcoal pretreated with 0.1 M ammonium formate. The active substance was recovered with acetone - 0.1 N formic acid (2: 8). After concentration of the active fractions, mildiomycin formate was precipitated with methanol as a white powder. Mildiomycin formate was passed through Amberlite IRA-410 to give free base. MIL was esterified with benzoyl chloride in dilute sodium bicarbonate solution to yield mildiomycin N-monobenzoate as colorless plates.

Initially, active fractions were detected by *in vivo* inhibition of *Erysiphe graminis* on barley and by thin-layer chromatography (TLC) using UV light at 254 nm and GREIG-LEABACK reagent to visualize the antibiotic. In later stages of the work samples were estimated with a diffusion assay using *Rhodotorula rubra* as the test organism[1] and with high-speed liquid chromatography (HLC).

High-Speed Liquid Chromatography

Application of high-speed liquid chromatography was carried out to assess purity of MIL and to analyse it quantitatively. It is known that cation-exchange resins are suitable for analysis of purine and pyrimidine bases and anion-exchange resins are also used for analysis of nucleosides. However, the best column packing for aminoacyl nucleosides has not been determined. Trials showed that a fine reversed-phase adsorbent is remarkably suited for this purpose.

MIL and related antibiotics were analyzed using μ Bondapak C_{18} (Waters) with the solvent systems as shown in Table 1. For MIL the mobile phase of 2.5% methanol - 0.005 M citrate buffer (pH 5.8) gave the best correlation between injection amount and the peak height by UV detection as shown in Fig. 1. From the standard curve the content of MIL in crude or purified samples, in culture filtrates or in solutions that had decomposed could be readily determined. The results could be used for injection weights as low as 50 ng.

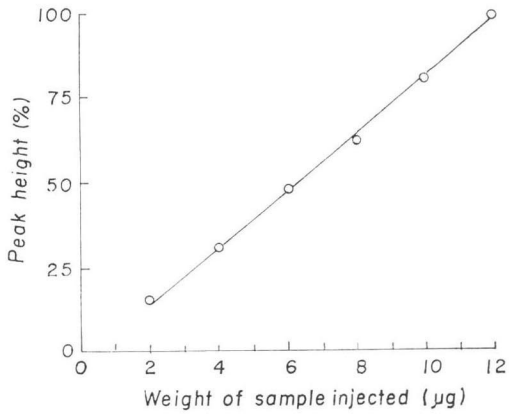
Table 1. HLC of mildiomycin and related antibiotics

Column: μ Bondapak C_{18}
Flow rate: 1.0 ml/min.
Column pressure: 1,500~2,000 psi

Solvent system	Retention time (min.)			
	(1)	(2)	(3)	(4)
MIL Free	6.0	4.0	6.2	8.6
Formate	6.0	4.1	6.4	8.6
N-Benzoate	14.7	12.9	N.E.*1	
Dihydro MIL	6.6	4.3	5.1	13.5
Blasticidin S	8.8	B.P.*2	B.P.	B.P.
Gougerotin	5.2	4.1	5.1	5.2
Amipurimycin	3.4	3.2	4.9	9.0
Polyoxin L	2.8	2.6	3.0	3.4
Aristeromycin	6.8	6.4	N.E.	
5-Hydroxy-methyl cytosine	3.2	3.2	3.6	5.0

Solvent system:
(1) MeOH - 0.01 M phosphate buffer (pH 5.7) (2: 8)
(2) MeOH - 0.005 M citrate buffer (pH 6.0) (2: 8)
(3) MeOH - 0.005 M citrate buffer (pH 5.8) (5: 95)
(4) 0.005 M citrate buffer (pH 5.8)
*1 Not eluted
*2 Broad peak

Fig. 1. The standard curve of mildiomycin on HLC (μ Bondapack C_{18}, 2.5% MeOH - 0.005 M citrate buffer, 1.0 ml/min.)
The sample was measured by the UV range, 0.2, at a sample concentration of 2 mg/ml.



Chemical Characterization

MIL was precipitated from methanol as a hygroscopic basic substance. Its mobilities by TLC on silica gel, paper partition chromatography (PPC) and paper electrophoresis (PE) are shown in Table 2. MIL gave a single spot in all solvent systems used, and a single peak was observed in HLC under various conditions. MIL is easily soluble in water and sparingly soluble in pyridine, dimethyl sulfoxide, dimethyl acetamide, dioxane or tetrahydrofuran. It shows positive color reactions with SAKAGUCHI, GREIG-LEABACK,[2] potassium permanganate and ninhydrin reagents, pseudo-positive with triphenyl tetrazolium chloride, aniline-phthalate and *p*-dimethylaminobenzaldehyde,[3] and negative with DRAGENDORFF, EHRLICH, BARTON, PAULY reagents, periodic acid-benzidine and ferric chloride-sulfosalicylic acid. In aqueous solution the antibiotic is stable when neutral, slightly unstable when basic (pH 9) and relatively

Fig. 2. ^{13}C-NMR spectrum of mildiomycin (XL-100, in D_2O)

The signals 4 and 5 overlapped in mildiomycin, however, these signals were clearly separated in mildiomycin formic acid salt and N-benzoate.

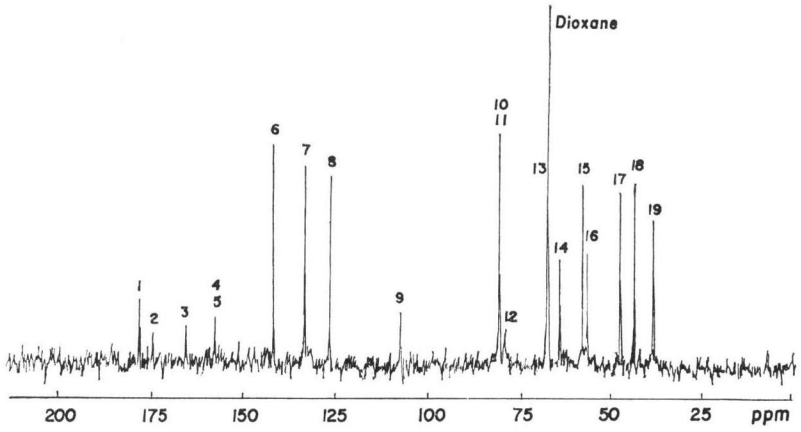
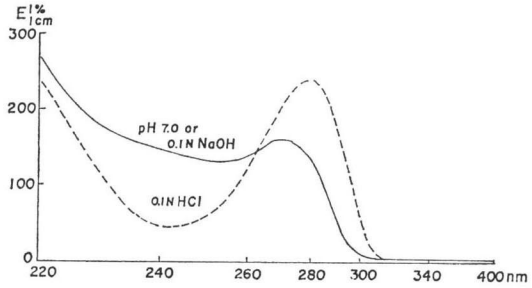


Table 2. Mobilities of mildiomycin on TLC, PPC and paper electrophoresis (PE)

	Solvent system	Rf value
TLC*1	$CHCl_3$ - MeOH - 17% NH_4OH (2: 1: 1, upper layer)	0.34
	PrOH - Pyridine - AcOH - H_2O (15: 10: 3: 10)	0.15
	AcOEt - Acetone - AcOH - H_2O (45: 5: 5: 45, lower layer)	0.37
PPC*2	BuOH - AcOH - H_2O (2: 2: 1)	0.13
	PrOH - H_2O (7: 3)	0.27
	iso-PrOH - 5% NH_4OH (6: 4)	0.26
		cm
PE*3	0.1 M Citrate buffer (pH 3.62)	−3.3
	0.15 M Phosphate buffer (pH 7.05)	−1.1
	0.1 M Glycine/NaCl - NaOH (pH 9.32)	−0.7

*1 Kieselgel F_{254} (Merck AG.)
*2 Whatmann No. 1 (W and R. Balston Ltd.)
*3 Whatmann No. 1, 500 v, 2 hours

Fig. 3. UV spectrum of mildiomycin



unstable when acidic (pH 2).

The specific rotation, $[\alpha]_D^{20}$, was +100° (*c* 0.5, H_2O) and +78.5° (*c* 0.5, 0.1 N HCl). Its pKa′ values were estimated by titration as 2.8, 4.3, 7.2 and >12. The molecular weight measured by titration at pKa′ value 7.2 was 529. The water of adhesion of this sample was 4.00% by thermogravimetric analysis. The elemental analysis was C, 42.73; H, 6.01; N, 20.48; O, 30.63%. In the ^{13}C-NMR spectrum of MIL the signals for 19 carbons were observed as shown in Fig. 2. Mildiomycin formic acid salt containing 0.38% water of adhesion showed C, 43.08; H, 6.41; N, 20.22; O, 31.18% in elemental analysis. And the crystalline mildiomycin N-monobenzoate containing 5.66% water of crystallization showed C, 47.67; H, 5.36; N, 16.88; O, 29.25%. In the ^{13}C-NMR of these derivatives 20 and 26 carbon atoms were observed. From these data the molecular formula of MIL was deduced to be $C_{19}H_{30}N_8O_9 \cdot H_2O$ (M. W. 532.53, Calcd. C, 42.85; H, 6.02; N, 21.04; O, 30.05; H_2O, 3.38%).

The UV spectrum of MIL indicated the maximum at 271 nm ($E_{1cm}^{1\%}$ 164) in neutral and basic (0.1 N

Fig. 4. IR spectrum of mildiomycin (KBr)

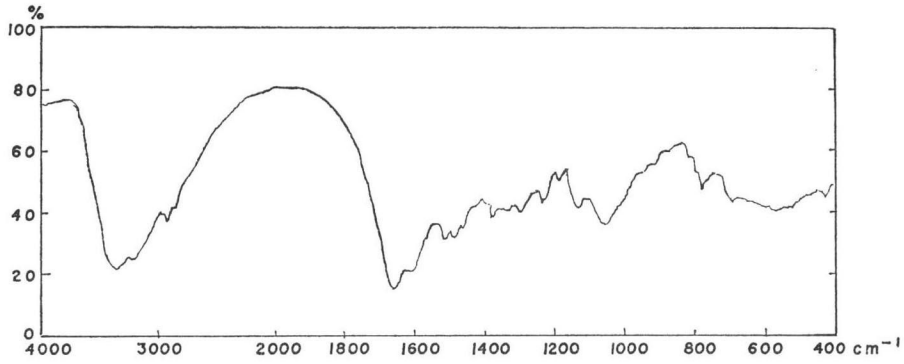
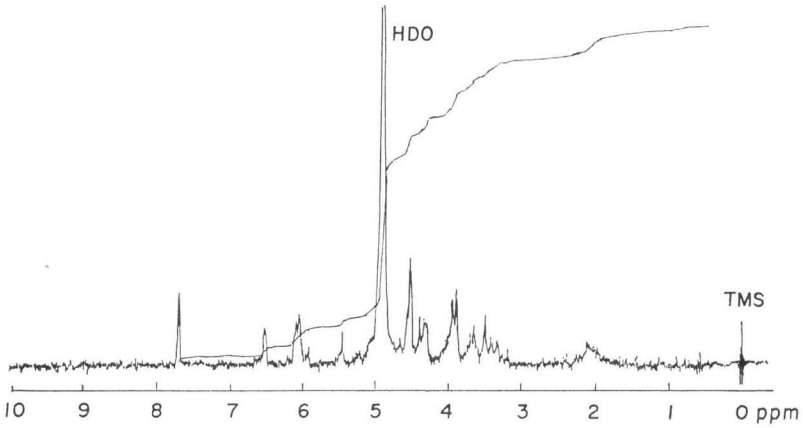


Fig. 5. ^{1}H-NMR spectrum of mildiomycin (100 MHz, D_2O)



NaOH) aqueous solutions and 280 nm ($E^{1\%}_{1cm}$ 247) in acidic (0.1 N HCl) aqueous solution as shown in Fig. 3. The IR (in KBr) and ^{1}H-NMR (in D_2O) spectra of MIL are shown in Figs. 4 and 5.

Discussion

MIL showed weak activity against Gram-positive and negative bacteria, phytopathogenic fungi and some yeasts.[1] It inhibited strongly the growth of various powdery mildews.

Preliminary acute toxicity of MIL (LD_{50}) in rats and mice was estimated to be 500~1,000 mg/kg by intravenous and subcutaneous injections, and 2.5~5.0 g/kg by oral administration*[1]. At a concentration of 1,000 ppm there was no irritation to cornea and skin in rabbits during 10 days observation*[2]. In a toxicity test using killifish no significant effect was observed at a concentration of 20 ppm for 7 days*[3]. Thus, MIL is considered to have low toxicity.

Blasticidin S,[4] gougerotin[5] (aspiculamycin[6]), ezomycins[7] and anthelmycin[8] (hikizimycin[9]) are similar to MIL in the UV spectra. These antibiotics have the maxima at 268 nm in basic aqueous solution and at 276 nm in acidic aqueous solution, due to a constituent cytosine moiety. MIL is clearly different from these compounds in that it affords 5-hydroxymethyl cytosine on acidic hydrolysis. Although this base was firstly found as the minor base of DNA in bacteriophage T_2,[10] it has never been reported in nucleoside antibiotics.

From these findings mildiomycin is considered to be a new nucleoside antibiotic.

* We thank Drs. 1) S. CHIBA, 2) T. HORI, 3) M. SAKAI for their biological examinations.

Plate 1. *Rectus-Flexibilis* or *Retinaculum-Apertum* sporophores of strain No. 13912 on oatmeal agar, 10 days.

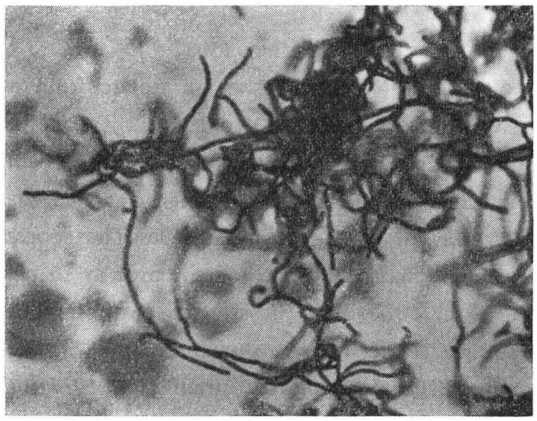


Plate 2. Smooth spores of strain No. 13912, scanning electron micrograph from 10-day culture on soil extract agar. A mark equals 1 μ.

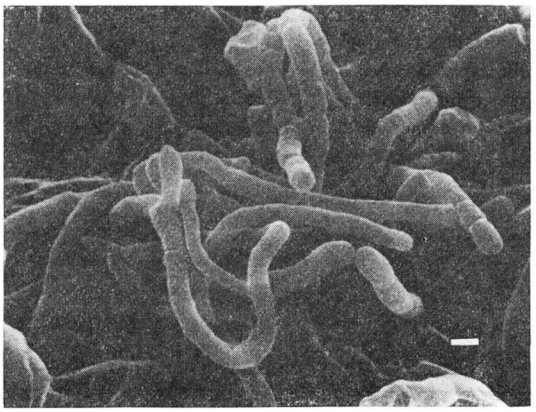


Table 1. Cultural characteristics of strain No. 13912 and *Streptomyces halstedii* ATCC 13499

	Strain No. 13912	*S. halstedii*
Yeast extract-malt extract agar (ISP 2)	G: Abundant AM: Brownish white R: Dark yellowish brown SP: None	G: Abundant AM: Gray R: Yellowish brown SP: None
Oatmeal agar (ISP 3)	G: Abundant AM: Brownish white R: Olive gray SP: None	G: Abundant AM: Gray R: Yellowish gray SP: None
Inorganic salts-starch agar (ISP 4)	G: Abundant AM: Light brownish white R: Pale yellowish brown to brownish gray SP: None	G: Abundant AM: Gray R: Brownish gray SP: None
Glycerol-asparagine agar (ISP 5)	G: Good AM: Light brownish white R: Yellowish brown SP: None	G: Good AM: Grayish white R: Yellowish brown SP: None

G: Growth. AM: Aerial mycelium. R: Reverse. SP: Soluble pigment.
Color names were assigned according to "Guide to Color Standard", a manual published by Nippon Shikisai Kenkyusho, Tokyo, Japan.

of the media, the color of substrate mycelia was yellowish brown and the mass color of aerial mycelia was white to brownish white. Physiological properties and the result of carbon utilization test are shown in Tables 2 and 3, respectively.

From these characteristics strain No. 13912 was classified as a member of genus *Streptomyces* and *S. halstedii*[2] was selected as the most closely related one. The results of simultaneous cultivation of *S. halstedii* ATCC 13499 with strain No. 13912 are shown in Tables 1~3. Morphological as well as physiological properties of these two strains were in good agreement, except for some grayish color of aerial hyphae of *S. halstedii* and non-utilization of D-cellobiose by strain No. 13912. The

ment throughout this work. We thank also the members of large scale fermentation and physical analysis.

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