Agric. Biol. Chem., **48** (4), 881～885, 1984

Structure of Mildiomycin D

Shigeki TASHIRO, Norio SUGITA, Takashi IWASA* and Hidekazu SAWADA*

*Research Laboratories, Agricultural Chemicals Division and *Applied Microbiology Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Yodogawa-ku, Osaka 532, Japan*

Received July 29, 1983

A new nucleoside antibiotic, mildiomycin D, was isolated from the culture broth of *Streptoverticillium rimofaciens* B-98891 as a minor component. The molecular formula of the antibiotic purified by silica gel and ion exchange resin column chromatographies was determined to be $C_{19}H_{30}N_8O_8 \cdot (2H_2O)$ from its physicochemical data. The ultraviolet and infrared spectra were very similar to those of mildiomycin, a major component. On the basis of ^1H and ^{13}C-NMR spectra and acidic hydrolysates of the compound, the chemical structure of the antibiotic was determined as a deoxy compound at the $C_{8'}$ position in mildiomycin. Mildiomycin D showed weak activities against Gram-positive and negative bacteria, phytopathogenic fungi and some yeasts, and its activity against *Rhodotorula rubura* was about 40% that of mildiomycin.

Mildiomycin (MIL) is a unique aminoacyl nucleoside antibiotic[1～3] produced by *Streptoverticillium rimofaciens* as a major component.[5] MIL being developed for practical use shows potent activity against powdery mildews on various kinds of plants.[4,6,7] Subsequently, a minor component showing an antimicrobial activity against *Rhodotorula rubura* in the culture broth was isolated and named mildiomycin D (MIL-D). This paper is concerned with the isolation, physicochemical and biological characteristics, and the structural determination of MIL-D (Fig. 1).

The culture broth filtrate of *S. rimofaciens* was adsorbed on a column of activated charcoal. The fractions showing activity against *R. rubura* by a disk-plate method were eluted with acetone-water and followed by Amberlite CG-50(H^+) column chromatography, eluting with 0.5～1.0% ammonia. The crude powder of MIL containing MIL-D was obtained by freeze-drying the eluate, and dissolving in acetone–5% ammonia to subject to silica gel column chromatography. By eluting with the same solvent system, the fractionated eluate of MIL-D was concentrated and the residue was applied to an Amberlite CG-50(H^+) column, eluting with 0.5% ammonia. The fractions showing a single spot on the thin-layer chromatograph (TLC) of a silica gel and a single peak on the high-performance liquid-chromatograph (HPLC) were concentrated and freeze-dried to give pure MIL-D.

MIL-D has a melting point at 210° with decomposition. The specific rotation of MIL-D was $[\alpha]_D^{20} = +119.4°$ ($c=1.0$, H_2O), $+82.2°$ ($c=1.0$, N/10 HCl). It is freely soluble in water but practically insoluble in such organic solvents as methanol, ethanol and acetone. It is positive in Sakaguchi, Greig-Leaback, potassium permanganate and ninhydrin reagents, but negative in Dragendorff, Ehlrich, Barton and Pauly reagents. It is stable within a pH range from 5.5 to 7.0. The molecular weight of MIL-D was calculated as 517 ± 50 by titration (p*Ka'* 4.2 and 6.9). The elemental and thermogravimetric analyses of MIL-D were C, 42.70; H, 6.37; N, 20.97; and 6.7% in water adhesion, respectively. The ^{13}C-NMR spectrum showed the signals for 19 carbons (Table

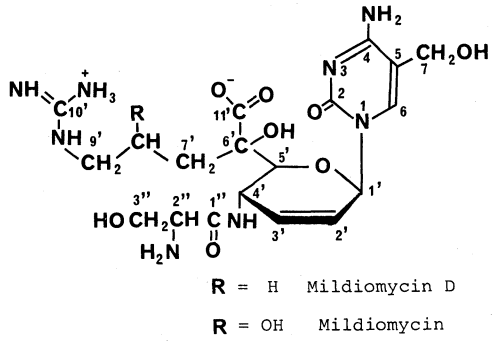

FIG. 1. Chemical Structures of Mildiomycin D and Mildiomycin.

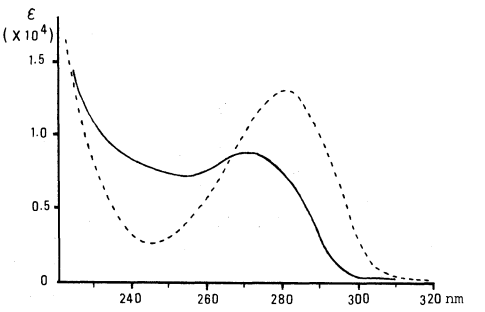

FIG. 2. UV Spectra of Mildiomycin D.
——, H_2O or N/10 NaOH; ----, N/10 HCl.

TABLE I. *Rf* VALUES AND RETENTION TIMES OF MILDIOMYCIN D AND ITS DERIVATIVES ON TLC, GLC AND HPLC SYSTEMS

Compound	TLC		GLC[c] (min)	HPLC[d] (min)
	a	*b*		
Mildiomycin D	0.08	0.39	—	7.0
Mildiomycin	0.06	0.37	—	7.3
5-Hydroxymethylcytosine	0.37	0.81	8.9	3.3
Serine	0.25	0.71	1.1	2.3
4-Guanidinobutyric acid	0.18	0.70	—	2.8[e]
4-Guanidino-2-hydroxy-butyric acid	0.16	0.67	—	2.6[e]

[a] Solvent system, *n*-PrOH–H_2O–NH_4OH (10:5:1).
[b] Solvent system, $CHCl_3$–MeOH–17% NH_4OH (2:1:1).
[c] 5% OV-17 ($\phi 3 \times 2000$ mm), column temp. 180°C, trimethylsilylated product (*i.e.*, trimethylsilyl derivative).
[d] Nucleosil $10C_{18}$ ($\phi 4 \times 250$ mm), 2% CH_3CN–0.003 M P.B. (pH 5.5).
[e] Detector, R.I.; —, not determined.

II). The molecular formula of MIL-D was therefore deduced to be $C_{19}H_{30}N_8O_8 \cdot 2H_2O$ (M.W. 534.55) and differed from MIL only by one oxygen atom; Calcd. C, 42.57; H, 6.70; N, 20.62; O, 24.78; H_2O, 7.40%. The UV spectra indicated the maxima at 271 nm (ε=9100, H_2O and N/10 NaOH) and at 280 nm (ε= 13,600, N/10 HCl) (Fig. 2). Therefore, MIL-D was found to be a very similar compound to MIL.

The structure of MIL-D was elucidated by comparing its spectral data and chemical degradation with those of MIL. The singlet signals at 7.61 (H_6) and 4.43 ppm (H_7) in the ^{1}H-NMR spectrum, and the signals at 157.8 (s, C_2), 165.6 (s, C_4), 107.8 (s, C_5), 142.4 (d, C_6) and 58.3 ppm (t, C_7) in the ^{13}C-NMR spectrum were reasonably assigned to a 5-hydroxymethylcytosine group in MIL-D. The absorptions in the UV spectrum also supported the presence of the chromophore. The signals at 3.53 (1H, t, $H_{2''}$) and 3.76 ppm (2H, d, $H_{3''}$) in the ^{1}H-NMR spectrum, and at 175.2 (s, $C_{1''}$), 57.1 (d, $C_{2''}$) and 64.5 ppm (t, $C_{3''}$) in the ^{13}C-NMR spectrum were attributed to a serine group in the antibiotic. These partial structures were confirmed by the acidic hydrolysis of MIL-D. The degradation products, 5-hydroxymethylcytosine and serine, were identified against authentic samples by means of TLC, GLC and HPLC systems (Table I). Tables II and III show the assignments of the signals in the NMR spectra for MIL-D and MIL. In the ^{13}C-NMR of MIL-D, the signals at 32.2 ppm (t, $C_{7'}$) and 42.0 (t, $C_{9'}$) showed up-field shifts compared with those at 39.2 and 48.1 ppm in that of MIL. Additionally in the spectrum of MIL, an oxymethine signal at 67.9 ppm (d) disappeared and a methylene signal at 24.1 ppm (t) appeared in that of MIL-D. In the ^{1}H-NMR spectrum of MIL-D two combined methylene signals at 1.3 ~ 1.8 ppm (2H × 2, m) were also observed. These spectral data suggest that MIL-D has $H_2N(HN)C$–NH–CH_2–CH_2–CH_2–, instead of $H_2N(HN)C$–NH–CH_2–CH(OH)–CH_2– in MIL, as the partial structure around the side chain. This assignment

Table II. ^{13}C-NMR Spectra of Mildiomycin D and Mildiomycin in D_2O (ppm)

Compound	Carbon number																			
	C-2	C-4	C-5	C-6	C-7	C-1′	C-2′	C-3′	C-4′	C-5′	C-6′	C-7′	C-8′	C-9′	C-10′	C-11′	C-1″	C-2″	C-3″
Mildiomycin D	157.8 (s)	165.6 (s)	107.8 (s)	142.2 (d)	58.3 (t)	80.6 (d)	126.8 (d)	133.8 (d)	43.9 (d)	80.9 (d)	81.3 (s)	32.2 (t)	24.1 (t)	42.0 (t)	157.3 (s)	179.3 (s)	175.2 (s)	57.1 (d)	64.5 (t)
Mildiomycin[1]	157.9 (s)	165.9 (s)	107.9 (s)	142.2 (d)	58.4 (t)	81.0 (d)	126.8 (d)	133.8 (d)	44.1 (d)	80.8 (d)	79.5 (s)	39.2 (t)	67.9 (d)	48.1 (t)	158.1 (s)	178.7 (s)	175.1 (s)	57.1 (d)	64.5 (t)

was supported by the proton spin-decoupling experiments of MIL-D in which the methylene signal at 3.16 ppm collapsed to a singlet when the methylene signals at 1.7 ppm were irradiated. From these findings, the partial structure of MIL-D was reasonably established as $H_2N(HN)C–NH–CH_2–CH_2–CH_2–$. On oxidation by periodic acid and hydrochloric acid,[1] MIL-D gave 4-guanidinobutyric acid, which was identified against a synthetic sample[8] by their ^{1}H-NMR spectrum and HPLC analysis (Fig. 3). As for the absolute configuration of the 6-membered ring, the stereochemistry of $H_{4'}$ and $H_{5'}$ should be diaxial from the coupling constant of $J_{4',5'}=10$ Hz, and $H_{1'}$ was also assigned to an axial form from $J_{1',2'}=2$ Hz. MIL-D showed a negative Cotton effect ($[\theta]_{273}-8600$) quite similar to that of MIL in the CD spectrum, indicating that the configuration of the 6-membered ring should be β-D. The chemical structure of MIL-D was thus confirmed by the spectral and decomposition studies shown in Figs. 1 and 3.

In Table IV, MIL-D shows weak activities against Gram-positive and negative bacteria, phytopathogenic fungi and some yeasts. When assayed by the disk-plate method against *R. rubura*, the activity of MIL-D was about 40% that of MIL. The activity of MIL-D against *Erysiphe graminis* on barley seedlings was 20~40% that of MIL (Table V).

In preliminary studies, the acute toxicity (LD_{50}) of MIL-D in mice was estimated to be >2500 and >1000 mg/kg by an oral administration and an intraperitoneal injection, respectively.

EXPERIMENTAL

The melting point was determined with a micro hot stage apparatus (Yanagimoto Co., Ltd.). TLC was carried out using a silica gel 60 F_{254} (E. Merck). The IR spectrum was measured with an EPI-G3 spectrometer (Hitachi) in a KBr pellet. ^{1}H and ^{13}C-NMR were determined with an XL-100 spectrometer (Varian), using deuterium oxide with tetramethylsilane as an external standard. GLC was conducted with a GC-7AG (Shimadzu) and HPLC with an ALC/GPC 244 Waters Associate).

TABLE III. ^{1}H-NMR SPECTRA OF MILDIOMYCIN D AND MILDIOMYCIN IN D_2O (ppm)

Compound	Proton number											
	H_6	H_7	$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$	$H_{7'}$	$H_{8'}$	$H_{9'}$	$H_{2''}$	$H_{3''}$
Mildiomycin D	7.61	4.43	6.40	6.02	5.88	4.89	4.19	1.3~1.8		3.16	3.53	3.76
Mildiomycin[2)]	7.61	4.42	6.43	6.05	5.88	4.88	4.24	1.95	3.93	3.36	3.58	3.82

TABLE IV. ANTIMICROBIAL SPECTRUM OF MILDIOMYCIN D

Test organism	Condition of assay			MIC[b] (μg/ml)	Inhibition zone[c] (mm)
	Media[a]	Temp. (°C)	Time (hr)		
Aspergillus niger IFO 4066	A	28	40	>500	−
Penicillium chrysogenum IFO 4626	A	28	40	>500	+
Pyricularia oryzae IFO 5279	A	28	40	>500	+
Cochliobolus miyabeanus IFO 5277	A	28	40	>500	14
Sclerotinia sclerotiorum IFO 9395	A	28	40	>500	+
Botrytis cinerea TKF 12	A	20	88	>500	14
Guignardia laricina IFO 7888	A	28	88	>500	−
Trichophyton mentagrophytes IFO 5809	D	28	88	>500	+
Candida albicans IFO 0583	A	28	40	>500	−
Saccharomyces cerevisiae IFO 0209	A	28	40	>500	10
Rhodotorula rubra IFO 0870	A	28	40	100	50
Bacillus subtilis IFO 3513	B	37	20	500	15
Staphylococcus aureus IFO 3061	B	37	20	>500	10
Escherichia coli IFO 3044	B	37	20	250	16
Proteus vulgaris IFO 3045	B	37	20	250	13
Pseudomonas aeruginosa IFO 3080	B	37	20	>500	−
Mycobacterium phlei IFO 3158	C	37	40	50	29
Mycobacterium smegmatis ATCC 607	C	37	40	250	26

[a] A, modified Pfeffer's agar; B, nutrient agar; C, glycerol-nutrient agar; D, 1.0% glucose, 0.4% $(NH_4)_2HPO_4$, 0.07% $MgSO_4 \cdot 7H_2O$, 0.1% KH_2PO_4, 0.1% NaCl, 0.005% $FeSO_4$ and 1.5% agar.
[b] MIC, minimal inhibitory concentration.
[c] A paper disk dipped in mildiomycin D solution (5 mg/ml) was subjected to the medium and the diameter of the inhibition zone was determined.

Isolation of MIL-D. A crude powder of MIL (38 g), obtained from the culture broth of *S. rimofaciens* B-98891 according to the procedure of Harada *et al.*,[5)] was dissolved in water and loaded on a silica gel (3 kg) column ($\phi 10 \times 77$ cm) after washing with acetone–5% aqueous ammonia. The eluate by the same solvent system was fractionated and the fractions containing MIL-D were detected by a TLC analysis of each fraction. The fractions were concentrated to be purified with Amberlite CG-50 (H^+) (100 ml) column chromatography. After washing with water and 0.5% ammonia (500 ml each), MIL-D was eluted with 1% ammonia (350 ml). The eluate was freeze-dried to give a white powder of MIL-D (3.4 g).

Acidic hydrolysis of MIL-D. A solution of MIL-D in 2 N HCl was refluxed for 2 hr. After neutralization with 2 N NaOH, the reaction mixture was subjected to Amberlite CG-50 (H^+) column chromatography. The fraction washed with water was concentrated and the residue was subjected to a silica gel column, and then allowed to elute with *n*-PrOH–H_2O (9:1). The ninhydrin and UV absorption positive fractions were individually purified with TLC. The compounds obtained were identified as serine and 5-hydroxymethylcytosine, respectively, by comparison with authentic samples on TLC, GLC and HPLC systems. For GLC determination, both compounds were trimethylsilylated to the trimethylsilyl derivatives with TMS-BA (Tokyo Kasei).

Periodic acid oxidation of MIL-D. A solution of MIL-D (300 mg) in 2 N HCl (14 ml) and 6% periodic acid (17 ml) was refluxed for 2 hr. After neutralization with Amberlite

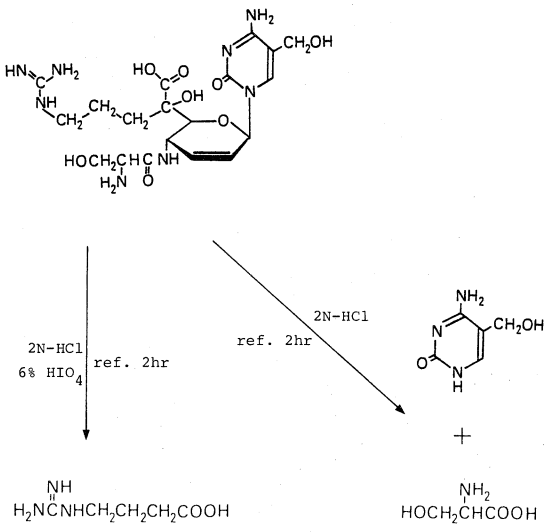

FIG. 3. Acidic Hydrolysates of Mildiomycin D.

IR-45 (OH^-) (50 ml), the mixture was loaded on an Amberlite CG-50 (H^+) (70 ml) column. The eluate of 0.2~0.3% ammonia was concentrated after washing with water (350 ml). The residue was subjected to silica gel column chromatography by eluting with *n*-BuOH–AcOH–H_2O (4:1:2). The positive fractions in a Sakaguchi reagent were concentrated and freeze-dried to give a white powder of 4-guanidinobutyric acid (32 mg, mp>240° dec.), and the compound obtained was identified against the authentic sample (Fig. 6 and Table I). *Anal.* Found: C, 41.04; H, 7.39; N, 28.67. Calcd. for $C_5H_{11}N_3O_2$ (M.W.145.16): C, 41.37; H, 7.64; N, 28.95%.

Synthesis of 4-guanidinobutyric acid. *S*-Ethylurea hydrobromide (9.25 g) in a flask was immersed in an ice bath, and 2 N NaOH (25.5 ml) was added. A hot (80°C) solution of 4-aminobutyric acid (5.63 g, 10 ml of H_2O) was added rapidly and stirred at room temperature for 3 hr. The reaction mixture was extracted 3 times with diethylether (50 ml). The aqueous layer was then chilled for 2 hr in an ice bath and allowed to crystallize. 4-Guanidinobutyric acid in white needle crystals (4.0 g) was obtained (mp>266° dec.). *Anal.* Found: C, 41.11; H, 7.67; N, 29.03. Calcd. for $C_5H_{11}N_3O_2$ (M.W. 145.16): C, 41.37; H, 7.64; N, 28.95%.

Acknowledgments. The authors wish to thank Drs. T. Kishi, M. Asai and S. Harada for their valuable suggestions throughout this work, and Mr. E. Mizuta for determination of the NMR spectra. We are also grateful to our collaborators for their support in this work.

TABLE V. EFFECT OF MILDIOMYCIN D AND OTHER CHEMICALS ON THE POWDERY MILDEW OF BARLEY SEEDLINGS[a]

Chemical	Concn. (ppm)	% of lesion area
Mildiomycin D	100	15.1
	200	0.8
	400	0.4
Mildiomycin	20	2.9
	40	1.2
	80	0.9
Morestan®[b]	62.5	0.2
	125	0.3
Control	—	61.3

[a] Barley seedlings (*Hordeum vulgare* L.C.V., Shiga Hakkoku No. 5) at 10 days after sowing were treated with a specific concentration of the chemical solution on the first leaf, then inoculated with conidia of *Erysiphe graminis* on the day. The seedlings were grown at 20~25°C in a greenhouse for 13 days to evaluate the percentage area of the lesion according to the official criteria.[9]

[b] Chinomethionat, 6-methyl-1,3-dithiolo[4,5-*b*]-quinoxalin-2-one.

REFERENCES

1) S. Harada, E. Mizuta and T. Kishi, *J. Am. Chem. Soc.*, **100**, 4895 (1978).
2) S. Harada, E. Mizuta and T. Kishi, *Tetrahedron*, **37**, 1317 (1981).
3) K. Kamiya, Y. Wada and M. Takamoto, *Tetrahedron Lett.*, 4277 (1978).
4) T. Iwasa, K. Suetomi and T. Kusaka, *J. Antibiot.*, **31**, 511 (1978).
5) S. Harada and T. Kishi, *J. Antibiot.*, **31**, 519 (1978).
6) T. Kusaka, K. Suetomi and T. Iwasa, *J. Pesticide Sci.*, **4**, 345 (1979).
7) K. Suetomi and T. Kusaka, *J. Pesticide Sci.*, **4**, 349 (1979).
8) Org. Syntheses, Coll. Vol. 3, 1955, p. 440.
9) The criteria for the assessment of injuries by diseases and insect pests. The Ministry of Agriculture, Forestry and Fisheries, Japan Plant Protection Association, Tokyo, 1974.