MICROBIAL TRANSFORMATION OF MARIDOMYCIN III BY *SERRATIA MARCESCENS*†

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Two transformation products of maridomycin (MDM) III, MDM-S_1 and MDM-S_2 named after *Serratia marcescens*, were isolated by silica gel chromatography. NMR and IR analysis revealed that MDM-S_1 and -S_2 had no aldehyde group at C-18 on the macrolactone ring, and that the hydroxyl group at C-9 seemed to disappear. Although MDM-S_1 and -S_2 are less active against Gram-positive bacteria than starting MDM III, they are interesting materials in view of the introduction of nitrogen into each molecule, and that the transformation products are produced by Gram-negative bacteria which are thought to be insensitive to macrolide antibiotics.

Studies on microbial transformations of antibiotics[1~3] have been carried out in order to improve the antimicrobial activity of the existing compounds, or to make them less-toxic or more effective against resistant strains. In our recent paper[4], it was discussed that recently isolated, maridomycin (MDM) III-insensitive strains transformed MDM III into three derivatives[1]: 18-dihydro MDM III (by reduction), 4″-depropionyl MDM III (by deacylation) and 18-dihydro-4″-depropionyl MDM III (by reduction and deacylation). Among these reactions, the reduction of the macrolide antibiotics is thought to be a detoxification mechanism by these MDM III-insensitive streptomycetes.

Moreover, the investigation of the transformation of MDM III by Gram-negative bacteria made it clear that *Serratia marcescens* transformed MDM III into new transformation products. The new transformation product complex was found to consist of two components by thin-layer chromatography. This paper describes the isolation and characterization of the transformation products.

Materials and Methods

Antibiotics

Maridomycin (MDM) III was kindly supplied by Takeda Chemical Industries, Ltd. The structure of MDM III is shown in Fig. 1[5,6].

Cultural Conditions

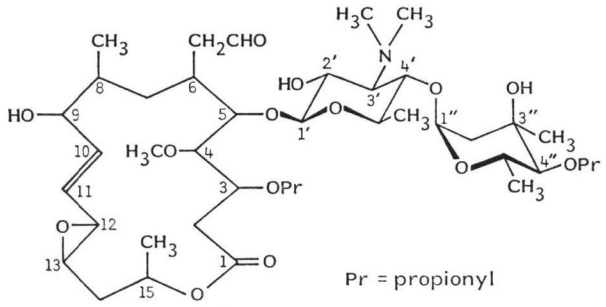
Fermentation was carried out with 30 ml of the medium in a 200-ml Erlenmeyer flask. The seed medium containing 5% glycerol, 1% peptone, 0.5% meat extract, 0.3% NaCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCO_3$ (pH 7.0) was inoculated from slant culture. *S. marcescens* IFO 3046 was grown at 37°C for 24 hours on a rotary shaker. The resultant culture (1.5 ml) was transferred to 30 ml of the transformation medium (the same composition as the seed culture). After incubation for 24 hours, 30 mg of MDM III was added to the culture (final concentration of MDM III; 1 mg/ml medium). The cultivation was continued for an addtional 48 hours after the addition of MDM III.

Thin-Layer Chromatography (TLC)

Filtered broth was extracted with ethyl acetate at pH 8.2 and the extract was chromatographed on

† Microbial transformation of antibiotics. V.

Fig. 1. Structure of maridomycin III.



silica gel G (Merck) plate using $CHCl_3$ - MeOH - NH_4OH (40: 3: 20, lower layer) or C_6H_6 - Me_2CO (1: 1) as solvent. The position of the antibiotics was detected by heating the plates after spraying with 10% H_2SO_4.

Minimum Inhibitory Concentration (MIC)

MIC's were determined by agar dilution method using glucose bouillon agar (pH 7.0). Test organisms were grown at 37°C for 18 hours.

Results

Isolation of MDM-S_1 and MDM-S_2

The isolation of MDM III and transformation products, MDM-S_1 and -S_2, was carried out using a general procedure as outlined in Fig. 2. The antibiotic complex was extracted from a filtered broth of *S. marcescens* with ethyl acetate at pH 8.2. The ethyl acetate extract was concentrated *in vacuo* and precipitated to give a crude material by addition of *n*-hexane. The precipitate was named crude S which, in addition to the transformation products, also contained MDM III.

The MDM-S fraction was isolated from the above mixture by column chromatography on silica gel developed with $CHCl_3$ - MeOH - NH_4OH (100: 0.5: 25, lower layer). This fraction contained

Fig. 2. Purification procedure of MDM-S_1 and MDM-S_2.

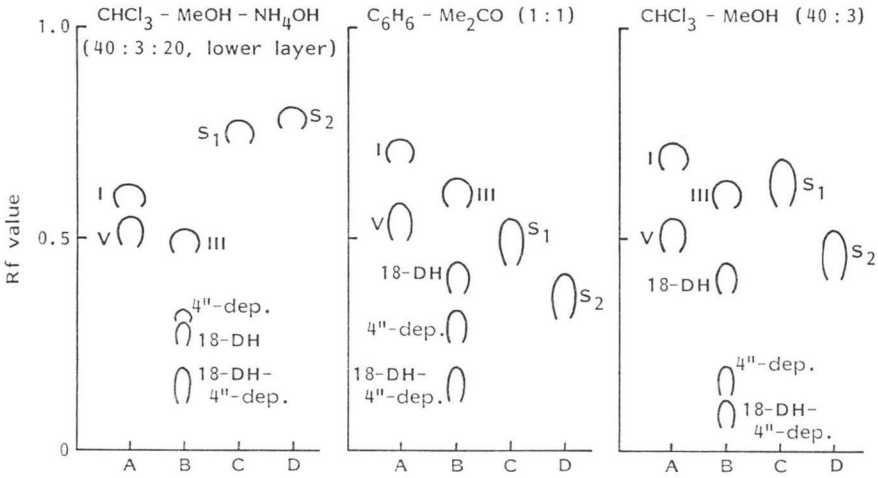
Culture filtrate
- extracted with EtOAc at pH 8.2
- dried *in vacuo*
- dissolved in chloroform
- decolorized with activated charcoal
- dried *in vacuo*
- dissolved in benzene
- precipitated with *n*-hexane

Crude S
- silica gel chromatography
  $CHCl_3$ - MeOH - NH_4OH (100: 0.5: 25, lower layer)

MDM-S fraction
- silica gel chromatography
  C_6H_6 - Me_2CO (7: 3→3: 2→1: 1, stepwise elution)

MDM-S_1 fraction	MDM-S_2 fraction
silica gel chromatography $CHCl_3$ - MeOH - NH_4OH (100: 0.1: 25, lower layer) or C_6H_6 - Me_2CO (7: 3→3: 2) stepwise elution	silica gel chromatography C_6H_6 - Me_2CO (3: 2→1: 1) stepwise elution
Purified MDM-S_1	Purified MDM-S_2

Fig. 3. TLC of MDM-S_1, MDM-S_2 and MDM III derivatives.
Sample A; MDM I and MDM V
Sample B; MDM III, 4″-depropionyl MDM III (4″-dep.), 18-dihydro MDM III (18-DH) and 18-dihydro-4″-depropionyl MDM III (18-DH-4″-dep.)
Sample C; MDM-S_1, Sample D; MDM-S_2



MDM-S_1, MDM-S_2 and a small amount of MDM III. MDM-S_1 and MDM-S_2 were isolated by further column chromatography on silica gel eluting with 1) C_6H_6 - Me_2CO (7: 3), 2) C_6H_6 - Me_2CO (3: 2) and finally with C_6H_6 - Me_2CO (1: 1). However, separation of MDM-S_1 and -S_2 was difficult on account of their similar mobilities but was accomplished by repeated column chromatography to give pure S_1 and S_2 in low yields.

Fig. 3 shows typical thin-layer chromatograms of transformation products by *S. marcescens* IFO No. 3046. Rf values of MDM-S_1 and -S_2 on TLC developed with C_6H_6 - Me_2CO (1: 1) were 0.52 and 0.38, respectively. However, these values were reversed on TLC developed with $CHCl_3$ - MeOH - NH_4OH (40: 3: 20); each value of MDM-S_1 and -S_2 was 0.76 and 0.80, respectively. Table 1 gives an example of yields of individual components obtained from starting MDM III.

Table 1. Yields of MDM-S_1 and MDM-S_2 after purification by column chromatography on silica gel.

Derivative	Amount (mg)	Yield (%)
MDM III	3,700	100.0
Crude S	3,004	81.2
MDM-S	304	8.2
MDM-S_1	117	3.2
MDM-S_2	53	1.4

Physicochemical Properties and Structures of Transformation Products

The physicochemical properties of transformation products MDM-S_1, MDM-S_2 and MDM III are summarized in Table 2. MDM-S_1 and -S_2 were crystallized as basic colorless needles from *n*-hexane. Their melting points are 130~133°C and 129~134°C, respectively. The UV spectra showed only end absorption. The elementary analysis of MDM-S_1 and -S_2 suggested that each compound contained two nitrogen atoms, in contrast to one nitrogen atom in starting MDM III. Table 3 shows the mass fragmentation patterns of MDM III, -S_1 and -S_2. The mass spectra of both MDM-S_1 and -S_2 exhibited a molecular ion peak at 898, which was 69 mass units greater than that of MDM III. The elementary analysis and the molecular weights indicate that the probable molecular form is either $C_{45}H_{74}N_2O_{16}$ or

Table 2. Some properties of MDM-S_1 and MDM-S_2.

	MDM-S_1	MDM-S_2	MDM III
Appearance	Colorless needles	Colorless needles	Colorless needles
Nature	Basic	Basic	Basic
mp (°C)	130~133	129~134	135~138
UV λ_{max} (EtOH)	End	End	End
Analysis (%)			
Found C	58.71	58.49	58.07
H	8.44	8.11	8.20
N	3.02	3.04	1.65
Molecular wt. (Mass)	898	898	829

Table 3. Mass fragmentation patterns of MDM III, MDM-S_1 and MDM-S_2.

	MDM III	MDM-S_1	MDM-S_2
M^+	829	898	898
(1)	756	825	825
(2)	658	727	727
(3)	628	698	698
(4)	613	682	682
(5)	682	751	751
(6)	584	653	653
(7)	554	623	623
(8)	539	608	608
(9)	738	807	807
(10)	439	508	508
(11)	174	174	174
(1)	201	201	201
(13)	300	300	300
(14)	374	374	374
(15)	57	57	57

$C_{44}H_{70}N_2O_{17}$. From the mass fragmentation patterns, the sugar moieties of MDM-S_1 and -S_2 were identical with those of MDM III, and the macrolactone moiety was different from that of MDM III. The macrolactone peaks of both MDM-S_1 and -S_2 were m/z 508; 69 mass units more than that of MDM III. These data suggest that the moieties of mycaminose and mycarose are unchanged, and that the transformation occurred on the macrolactone ring. The IR spectra of MDM III, -S_1 and -S_2 are shown in Fig. 4. The IR spectra of two transformation products are similar to MDM III and exhibit strong absorption at 1730 cm^{-1} (C=O), and 1050~1200 cm^{-1} (C-O-C). However, the absorption at 2700 cm^{-1} (CHO) has disappeared. Also, decreased absorption at 3500 cm^{-1} indicates that hydroxyl group at C-9 has been transformed.

The NMR spectra at 100 MHz are shown in Fig. 5. Drawing of MDM-S_1 and -S_2 was partly omitted in the figure, since both compounds showed a similar pattern to MDM III in the region between δ 2

Fig. 4. IR spectra of MDM III, MDM-S_1 and MDM-S_2.

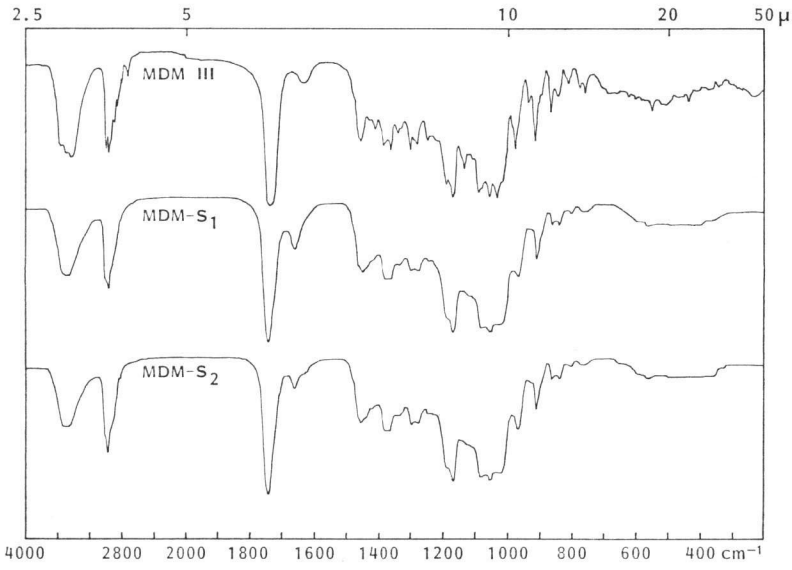
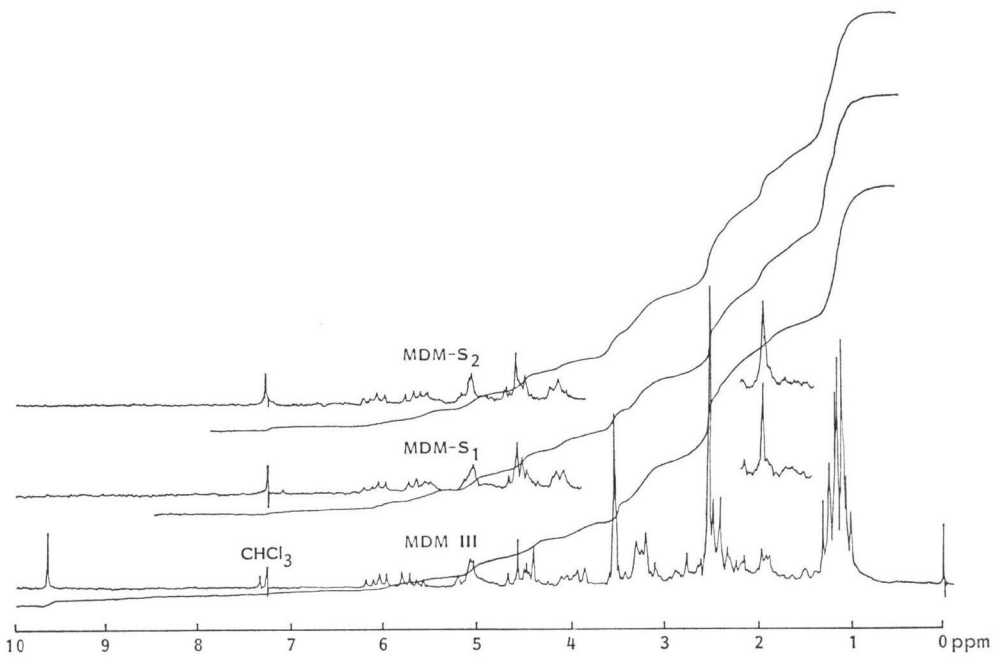


Fig. 5. NMR spectra of MDM III, MDM-S_1 and MDM-S_2.



and 4 ppm, and around 1 ppm. The NMR spectra of both MDM-S_1 and -S_2 showed signals assigned to $-N(CH_3)_2$ at δ 2.52 ppm (6H, s), $-OCH_3$ at δ 3.53 ppm (3H, s), which were observed in the spectrum of MDM III.

The NMR spectra of these products differ from that of MDM III mainly in two regions; a signal at δ 9.62 ppm which is the aldehyde proton in starting MDM III has disappeared, and a new signal has appeared at δ 1.95 ppm in the spectra of MDM-S_1 and -S_2.

Qualitative Analyses of MDM-S_1 and -S_2

Several qualitative analyses were carried out to examine the form of nitrogen introduction. They showed the same color reactions on MDM III, -S_1 and -S_2 except the EHRLICH reaction; Positive DRAGENDORFF reaction, and negative nitrous acid reaction, Na-nitroprusside test, β-naphthol reaction, ninhydrin test and ferric chloride reaction. MDM III is negative against EHRLICH reaction, whereas spots of MDM-S_1 and -S_2 on TLC appeared to be pink after the reaction. It suggesested the possibility of a secondary amine or its derivative.

Isolation of the Aglycone

In order to isolate each aglycone, MDM III, -S_1 and -S_2 were hydrolyzed under the following reaction conditions; each antibiotic was dissolved in 0.36 N H_2SO_4 solution and heated at 90°C for 24 hours. Further details for the isolation of each aglycone are shown in Fig. 6. After purification of each aglycone by column chromato-

Fig. 6. Isolation of aglycone from MDM III, MDM-S_1 and MDM-S_2.

MDM III (or MDM-S_1 or -S_2)
↓ dissolved in 0.36 N H_2SO_4 solution
heated at 90°C for 24 hours
Reaction mixture
↓ extracted with EtOAc at pH 6.0
concentrated *in vacuo*
Crude aglycone
↓ column chromatography
1) on silica gel
solvent system; C_6H_6 - Me_2CO (4: 1)
2) on Sephadex LH-20
solvent system; $CHCl_3$
Aglycone

Table 4. Antimicrobial activity of MDM-S_1, -S_2 and related derivatives.

Test organism	MIC (μg/ml)					
	MDM-S_1	MDM-S_2	MDM III	4″-Dep**	18-DH**	18-DH-4″-dep**
Staphylococcus aureus	50	20	0.5	5	>100	>100
S. aureus-4R*	>100	>100	10	100	>100	>100
Bacillus subtilis	50	20	0.5	5	>100	>100
B. megaterium	50	50	0.5	—	—	—
Micrococcus luteus	5	10	0.2	0.5	20	20
Escherichia coli	>100	>100	>100	>100	>100	>100
Proteus vulgaris	>100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa	>100	>100	>100	>100	>100	>100
S. marcescens	>100	>100	>100	>100	—	—

* Streptomycin, erythromycin, chloramphenicol, chlortetracycline-resistant strain.
** 4″-Dep; 4″-depropionyl MDM III.
18-DH; 18-dihydro MDM III.
18-DH-4″-dep; 18-dihydro-4″-depropionyl MDM III.

graphy on silica gel, then on Sephadex LH-20, they were developed and detected on TLC. Each aglycone had almost the same Rf value. These three aglycones were negative against anisaldehyde and DRAGENDORFF reagents and were positive towards $KMnO_4$ reagent. By the detection of above $KMnO_4$ reagent on TLC, recovery of aldehyde group in the aglycones from hydrolysates of MDM-S_1 and -S_2 was recognized.

From these results, the aglycones of S_1 and S_2, obtained from hydrolysis, are thought to be identical with that of MDM III.

Antimicrobial Activity of MDM-S_1 and -S_2

MDM III is active against Gram-positive bacteria including acid-fast bacteria. As shown in Table 4, antimicrobial activities of both MDM-S_1 and -S_2 were much less than that of MDM III; they retained about 1/50 of the activity. However, these derivatives were more active than 18-dihydro MDM III,[7] although they had no aldehyde group in their molecules.

Discussion

Molecular weights of both MDM-S_1 and -S_2 were 898, which is 69 daltons greater than that of MDM III. IR and NMR spectra of these transformation products were quite similar to each other. Therefore, MDM-S_1 and -S_2 were considered to be isomers. It is apparent that the aldehyde group at C-18 on the macrolactone ring is transformed from the disappearance of both the absorption band at 2700 cm^{-1} in the IR spectrum, and the signal at δ 9.62 ppm in the NMR spectrum. Reemergence of the aldehyde group at C-18 by acid hydrolysis suggests the existence of an acetal. Decrease in the absorption at 3500 cm^{-1} (OH) in the IR spectra of MDM-S_1 and -S_2 suggests a hydroxyl group may be transformed. In MDM III there are three hydroxyl groups present. Since the mass spectra suggests that the moieties of mycaminose and mycarose are not transformed, the hydroxyl group at C-9 on the macrolactone is probably transformed. The new signal at δ 1.95 ppm in NMR spectra of -S_1 and -S_2 are similar to those of an allyl methyl group ($\rangle C{=}CH{-}CH_3$). In addition, MDM-S_1 and -S_2 are thought to contain structures similar to a pyrrole ring as indicated by the EHRLICH reaction.

Further examinations are still in progress. Complete elucidation of these structures will be reported in the future. It should be noted that there is a kind of detoxification mechanism of macrolide antibiotics by *S. marcescens*, a Gram-negative bacterium. With regard to this kind of experiment, the late Dr. PERLMAN reported erythromycin degradation by *Pseudomonas* sp. 56[8].

Acknowledgment

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