MICROBIAL CONVERSION OF ANTIBIOTICS. I

DEACYLATION OF MARIDOMYCIN BY BACTERIA

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Maridomycin, a macrolide antibiotic, was hydrolyzed to 4''-deacylmaridomycin by esterases obtained from many bacteria. Among these bacteria, *Bacillus megaterium* 91277 showed the highest activity. The properties of the esterase were investigated by using cell-free extract obtained from *B. megaterium* 91277. Optimum pH was 8.5. 9-Propionylmaridomycin was also hydrolyzed to 4''-deacyl-9-propionylmaridomycin by the esterase. The hydrolytic rate of 9-propionylmaridomycin was more rapid than that of maridomycin. Substrates bearing 4''-acyl group of C_3 or C_4 were most rapidly hydrolyzed. 4''-Depropionylmaridomycin III and 4''-depropionyl-9-propionylmaridomycin III were prepared from maridomycin III and 9-propionylmaridomycin III, respectively, in large scale by using culture broth of *B. megaterium* 91277. 4''-Deisovaleryljosamycin was also prepared from josamycin by using the same strain.

Recently, MUROI *et al.*[1] reported that 9-propionylmaridomycin (PMDM) and maridomycin (MDM) were hydrolyzed by rat liver homogenate to 4''-deacyl-9-propionylmaridomycin (PMDM-M) and 4''-deacylmaridomycin (MDM-M), respectively. Deacylation of macrolide antibiotics, leucomycin[2,3] and SF-837,[4] by fungi has also been reported.

After oral administration of PMDM to human, PMDM-M, MDM and MDM-M were isolated from urine as metabolites of PMDM.[1] Therefore, the deacylation products (PMDM-M and MDM-M) are indispensable for the studies on the metabolism of PMDM *in vivo*. The deacylation products would also serve as substrates for the synthesis of new 4''-acylated derivatives of macrolide antibiotics.

In the course of studies on microbial conversion of macrolide antibiotics, it has been found that maridomycin III (MDM III) is hydrolyzed to 4''-depropionylmaridomycin III (MDM III-M) by esterases of many bacteria.

This paper deals with the distribution of the esterases among bacteria and the some properties of the esterase obtained from *Bacillus megaterium* 91277 which showed the highest esterase activity among bacteria tested. Large scale preparation of the deacylation products by using the same strain is also reported.

Materials and Methods

Antibiotics

Maridomycin I (MDM I),[5] maridomycin III (MDM III),[5] maridomycin V (MDM V),[5] 9-propionylmaridomycin I (PMDM I),[6] 9-propionylmaridomycin III (PMDM III),[6] 9-propionylmaridomycin V (PMDM V),[6] 4''-butyryl-4''-depropionyl-9-propionylmaridomycin III (PMDM VII) and 4''-isobutyryl-4''-depropionyl-9-propionylmaridomycin III were prepared in our research

laboratories. Josamycin (JM)[7,8] was extracted from commercial preparations.

Microorganisms and culture conditions

Bacteria were obtained from the Institute for Fermentation, Osaka, or isolated in our research laboratories. Bacteria were grown in DB medium for 48 hours at 28°C on a rotary shaker. DB medium (pH 7.2) contained 2% dextrin, 0.5% peptone, 0.5% yeast extract, 0.5% beef extract and 0.5% $CaCO_3$ in deionized water.

Assay of esterase

Esterase activity was assayed by measuring the amount of MDM III-M formed from MDM III according to the equation in Fig. 1.

For the assay of esterase activity of various bacteria, a reaction mixture containing 5 ml of the culture broth and 1 mg of MDM III was incubated for 18 hours at 28°C with shaking. The mixture was then adjusted to pH 8.5 with dil. NaOH, and extracted with ethyl acetate. MDM III-M in the extract was determined by bioautography.

For the assay of esterase obtained from *B. megaterium* 91277, a reaction mixture containing 2 mg of MDM III, 400 μmoles of glycine-NaOH buffer (pH 9.0) and enzyme solution in a total volume of 4 ml was incubated for 60 minutes at 37°C with gentle shaking, and then extracted with 4 ml of ethyl acetate. MDM III-M in the extract was determined by bioautography.

Fig. 1. Deacylation of MDM III to MDM III-M by esterases

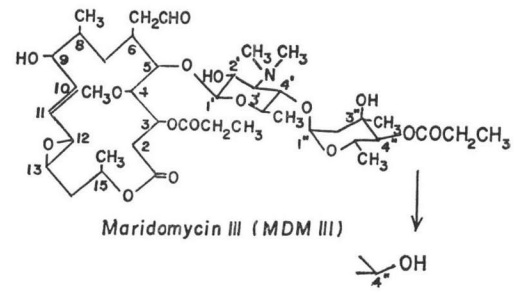
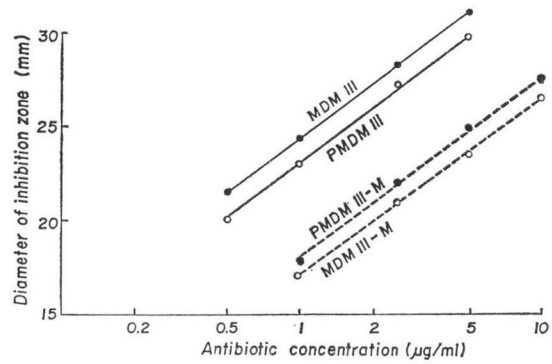


Fig. 2. Standard curves of MDM III, MDM III-M, PMDM III and PMDM III-M by bioautography



Bioautography

Nutrient agar plate for bioautography was prepared as follows: One ml of *Sarcina lutea* PCI 1001 cell suspension giving an optical density at 660 nm of 0.8 measured by a Hitachi spectrophotometer Model EPO-B was inoculated into 200 ml of a nutrient agar (pH 8.0) containing 0.1% glucose, 0.6% peptone, 0.3% yeast extract, 0.15% beef extract and 1.2% agar in deionized water, and the mixture was poured into a bioautograph plate (24×32 cm).

Samples were chromatographed on silica gel TLC plates (Tokyo Kasei) using benzene-acetone (1:1, v/v) as solvent. After the development, the TLC plate was placed for 10 minutes on the nutrient agar plate, which was then incubated for 16 hours at 37°C.

The amounts of antibiotics were determined by measuring the diameters of inhibition zones. Standard curves of MDM III, MDM III-M, PMDM III and PMDM III-M are shown in Fig. 2.

Thin-layer chromatography (TLC)

Samples were chromatographed on silica gel TLC plates (Merck) using benzene-acetone (1:2, v/v) as solvent. The spots of antibiotics were detected by heating the plates after spraying 10% H_2SO_4.

Results

Distribution of Esterase among Bacteria

Esterase activity of various bacteria was assayed by using the culture broth as enzyme

Table 1. Distribution of esterases among bacteria

Genus	Total	Conversion rate (%)*				
		0~10	11~30	31~50	51~70	71~100
Pseudomonas	87	87	0	0	0	0
Azotobacter	4	4	0	0	0	0
Alcaligenes	3	3	0	0	0	0
Achromobacter	4	3	0	0	1	0
Flavobacterium	15	12	0	1	0	2
Escherichia	48	48	0	0	0	0
Aerobacter	15	15	0	0	0	0
Erwinia	2	2	0	0	0	0
Serratia	7	7	0	0	0	0
Micrococcus	20	10	4	1	0	5
Staphylococcus	12	12	0	0	0	0
Sarcina	4	4	0	0	0	0
Sporosarcina	2	1	0	0	0	1
Brevibacterium	51	44	3	1	1	2
Corynebacterium	45	28	13	1	2	1
Arthrobacter	18	17	0	0	0	1
Bacillus	108	105	1	0	0	2
Mycobacterium	13	6	4	2	0	1
Total	458	408	25	6	4	15

* Conversion rate was expressed as percentage of MDM III-M (μmoles) formed per MDM III (μmoles) added to the reaction mixture.

source. The activity was found in many Gram-positive bacteria and in a few Gram-negative bacteria. Of 458 strains tested, 50 strains showed measurable activity with the conversion rates of more than 10%. The high activity with the conversion rates of more than 50% was found in bacteria belonging to one species of *Achromobacter*, 2 species of *Flavobacterium*, 5 species of *Micrococcus*, one species of *Sporosarcina*, 3 species of *Brevibacterium*, 3 species of *Corynebacterium*, one species of *Arthrobacter*, one species of *Bacillus* (2 strains) and one species of *Mycobacterium* (Table 1). Among them *B. megaterium* 91277 showed the highest activity, and was chosen for further studies.

Preparation and Properties of Esterase from *B. megaterium* 91277

Esterase was obtained from *B. megaterium* 91277 as follows: The cells were harvested by centrifugation from 1 liter of the culture broth, washed twice with 0.02 M phosphate buffer (pH 7.2), and suspended in 100 ml of the same buffer. The cell suspension was treated for 15 minutes at 2°C with a sonic oscillator (Kubota Model 200 M), and centrifuged for 30 minutes at 30,000 *g* to remove cell debris. The supernatant fluid (cell-free extract) was served as the enzyme solution for the assay of esterase. The protein concentration of the cell-free extract was 20.0 mg/ml, determined by the method of LOWRY *et al.*[9]

The amount of MDM III-M formed was proportional to the incubation time (Fig. 3) and the enzyme concentration (Fig. 4), respectively.

Optimum pH for the reaction was 8.5 in phosphate-glycine-NaOH buffer of various pH

Fig. 3. Effect of incubation time on MDM III-M formation

A reaction mixture containing 2 mg of MDM III, 400 μmoles of glycine-NaOH buffer (pH 9.0) and 0.1 ml of the enzyme solution in a total volume of 4 ml was incubated for the indicated time at 37°C.

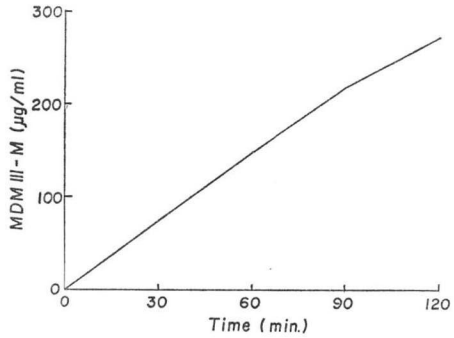


Fig. 4. Effect of enzyme concentration on MDM III-M formation

A reaction mixture containing 2 mg of MDM III, 400 μmoles of glycine-NaOH buffer (pH 9.0) and the indicated volume of the enzyme solution in a total volume of 4 ml was incubated for 60 minutes at 37°C.

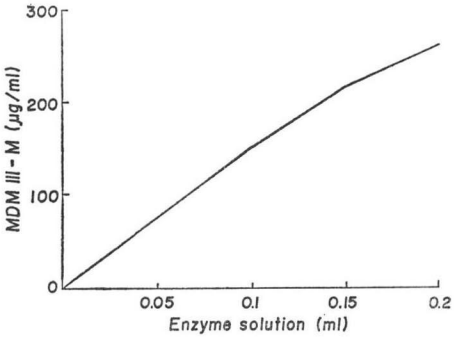


Fig. 5. Effect of pH

A reaction mixture contained 2 mg of MDM III, 400 μmoles of phosphate-glycine-NaOH buffer and 0.1 ml of the enzyme solution in a total volume of 4 ml.

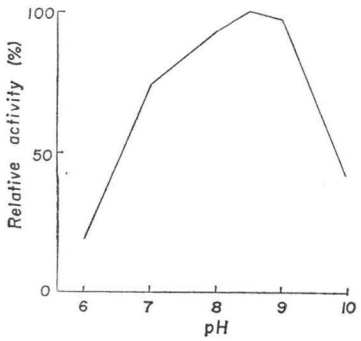


Fig. 6. Effect of pH on stability

The enzyme solution was added to an equal volume of 0.2 M buffer solutions, and the mixture was incubated for 17 hours at 4°C. After the incubation, 0.2 ml of the mixture was served as enzyme solution for assay of esterase.

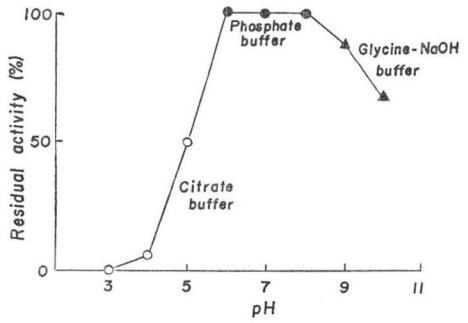


Fig. 7. Effect of temperature on stability

The enzyme solution was added to an equal volume of 0.2M phosphate buffer (pH 7.2), and the mixture was incubated for 15 min. at the indicated temperature. After the incubation, 0.2 ml of the mixture was served as enzyme solution for assay of esterase.

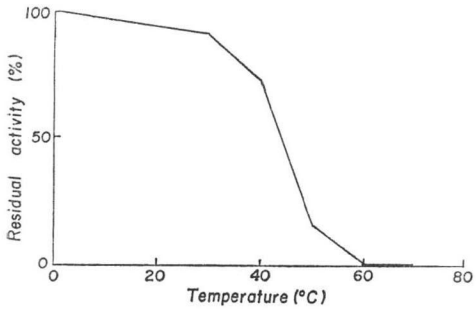


Fig. 8. Hydrolytic rates of MDM and PMDM

A reaction mixture containing 2 mg of the indicated substrates, 400 μmoles of glycine-NaOH buffer (pH 9.0) and 0.2 ml of the enzyme solution in a total volume of 4 ml was incubated for the indicated time at 37°C.

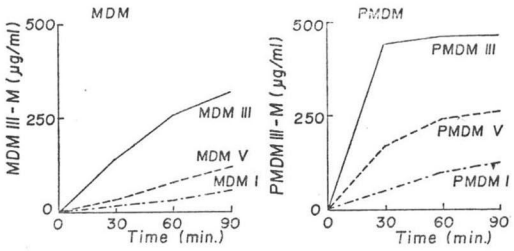
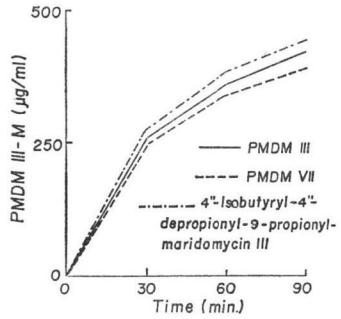


Fig. 9. Hydrolytic rates of PMDM III, PMDM VII and 4″-isobutyryl-4″-depropionyl-9-propionylmaridomycin III

A reaction mixture containing 2 mg of the following substrates, 400 μmoles of glycine-NaOH buffer (pH 9.0) and 0.05 ml of the enzyme solution in a total volume of 4 ml was incubated for the indicated time at 37°C.



values (Fig. 5).

The enzyme was stable in the range of pH 6 to 8 at 4°C for 17 hours (Fig. 6). The activity was completely lost by heating at 60°C for 15 minutes in 0.1M phosphate buffer (pH 7.2) (Fig. 7).

The esterase hydrolyzed MDM I (4″-isovaleryl), MDM III (4″-propionyl) and MDM V (4″-acetyl) to MDM III-M. These substrates were hydrolyzed at a rate in the order MDM III>MDM V>MDM I. It also hydrolyzed PMDM I (4″-isovaleryl), PMDM III (4″-propionyl) and PMDM V (4″-acetyl) to PMDM III-M. The order of the hydrolytic rate of these substrates was PMDM III>PMDM V>PMDM I. Compared MDM and PMDM possessing the same acyl group at $C_{4''}$, PMDM was hydrolyzed more rapid than MDM (Fig. 8). PMDM VII (4″-butyryl) and 4″-isobutyryl-4″-depropionyl-9-propionylmaridomycin III, like PMDM III in Fig. 8, were completely hydrolyzed to PMDM III-M after 30-minute incubation. Since the hydrolytic rates of these substrates were too rapid to determine the order, the concentration of enzyme was reduced to 1/4. Under this condition, the hydrolytic rates of PMDM III, PMDM VII and 4″-isobutyryl-4″-depropionyl-9-propionylmaridomycin III were determined to be almost equal (Fig. 9).

Preparation of MDM III-M

B. megaterium 91277 was grown in 500 ml of DB medium in a 2-liter SAKAGUCHI flask for 2 days at 28°C on a reciprocating shaker. The contents of six flasks were transferred to 100 liters of DB medium in a 200-liter fermentor, and the cultivation was carried out for 42 hours at 28°C with stirring (230 rpm) and aeration (100 liters/min.).

To 100 liters of the culture broth, 100 g of MDM III dissolved in 2 liters of methanol was added, and the mixture was immediately adjusted to pH 8.5 with dil. NaOH. The reaction mixture was incubated for 5 hours at 28°C with stirring (230 rpm). Thin-layer chromatogram of the reaction

Fig. 10. Thin-layer chromatogram of reaction mixture

After the incubation, the reaction mixture containing MDM III (1) or PMDM III (2) was extracted with ethyl acetate at pH 8.5, and the extract was chromatographed on a TLC plate.

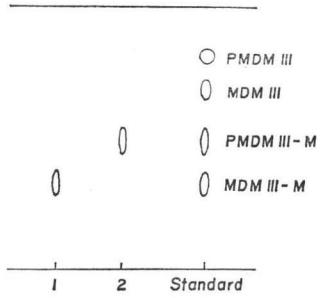
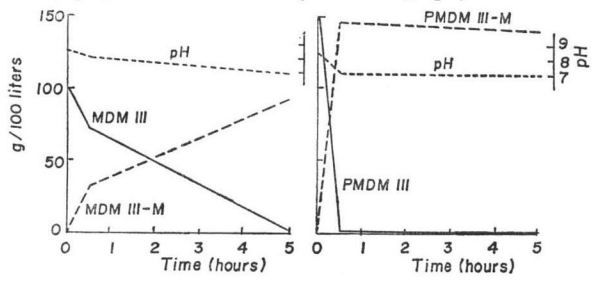


Fig. 11. Time courses of deacylation

Deacylation of MDM III (left) and PMDM III (right) was monitored by bioautography.



mixture indicated that MDM III added was converted to MDM III-M (Fig. 10). The disappearance of MDM III and the formation of MDM III-M were monitored by bioautography. After 5-hour incubation, 100 g of MDM III was converted to 92 g of MDM III-M (Fig. 11).

The reaction mixture (100 liters) was adjusted to pH 8.5 with dil. NaOH, and extracted twice with 50 liters each of ethyl acetate. MDM III-M in the extract was transferred into 1/3 M KH_2PO_4 solution adjusted to pH 3.0 with aqueous phosphoric acid, and was transferred again into ethyl acetate at pH 8.5. The extract was concentrated *in vacuo* to give MDM III-M as a white powder (61.5 g), which was more than 90 % in purity. The white powder (15 g) was chromatographed on a silica gel column using benzene-acetone (2:1, v/v) as the solvent system to obtain MDM III-M fraction. The concentrate of the fraction was crystallized from acetone-*n*-hexane to give 2 g of MDM III-M as colorless prisms. m.p. 157~158°C (dec.), $[\alpha]_D^{21}$ −75.9° (*c* 0.98, EtOH).

Anal. Calcd. for $C_{38}H_{63}NO_{15}\cdot H_2O$: C, 57.63; H, 8.28; N, 1.77.
Found: C. 57.93; H, 8.21; N, 1.78.

The compound was identical with the authentic sample of MDM III-M[1] in the IR, NMR and mass spectra.

Preparation of PMDM III-M

Deacylation of PMDM III was carried out in the same manner as that of MDM III, except that 150 g of PMDM III dissolved in 3 liters of methanol was added to 100 liters of the culture broth. Thin-layer chromatogram of the reaction mixture indicated that PMDM III added was converted to PMDM III-M (Fig. 10). After 30-minute incubation, PMDM III was quantitatively converted to PMDM III-M. The amount of PMDM III-M formed in the reaction mixture remained unchanged for 30 minutes ~ 5 hours. The yield of PMDM III-M was 140 g at the end of the incubation (Fig. 11).

Purification of PMDM III-M was carried out in the same manner as that of MDM III-M, and 99 g of PMDM III-M was obtained as a white powder, which was more than 90 % in purity. The white powder (5 g) was chromatographed on a silica gel column using benzene-acetone (3:1, v/v) as the solvent system to obtain PMDM III-M fraction. The concentrate of the fraction was crystallized from ethyl acetate-*n*-hexane to give 1 g of PMDM III-M as colorless prisms. m.p. 142~143°C (dec.), $[\alpha]_D^{22}$ −63.6°C (*c* 1.01, EtOH).

Anal. Calcd. for $C_{41}H_{67}NO_{16}$: C, 59.33; H, 8.14; N, 1.69.
Found: C, 59.14; H, 8.18; N, 1.73.

The compound was identical with the authentic sample of PMDM III-M[1] in the IR, NMR and mass spectra.

Preparation of JM-M

To 4 liters of the culture broth of *B. megaterium* 91277, 2 g of JM dissolved in 40 ml of methanol was added. The reaction mixture was distributed into twenty 1-liter Erlenmeyer flasks, and the flasks were shaken for 24 hours at 28°C on a rotary shaker. JM was converted to JM-M during the incubation.

After the reaction mixture was centrifuged for 15 minutes at 20,000 *g*, the supernatant fluid was extracted three times at pH 8.5 with 700 ml each of ethyl acetate. JM-M in the

extract was transferred into 0.05 M citrate buffer (pH 3.0), and was reextracted into ethyl acetate at pH 8.5. The concentrate of the extract was chromatographed on a silica gel culumn using benzene-acetone (2:1, v/v) as the solvent system, and the eluate containing JM-M was concentrated *in vacuo* to give 780 mg of white powder. $[\alpha]_D^{24}$ −68.7° (*c* 1.12, EtOH), λ_{max}^{MeOH} 231 ~232 nm ($\varepsilon=3.09\times10^4$).

Anal. Calcd. for $C_{37}H_{61}NO_{14}$: C, 59.74; H, 8.27; N, 1.88.
Found: C, 59.37; H, 8.51; N, 1.64.

The compound was identical with the authentic sample of JM-M in the UV, IR, NMR and mass spectra. The authentic sample was prepared from JM by using rat liver homogenate as follows:

Rats (Sprague-Dawley, female, 150~200 g) were sacrificed by cervical dislocation. Livers were excised, added to ten volumes of 1/15 M phosphate buffer (pH 7.2) and homogenized to give liver homogenate. To 300 ml of the liver homogenate, 600 mg of JM dissolved in 20 ml of glycofurol was added, and the mixture was incubated for 4 hours at 37°C with shaking. At the end of the incubation, the mixture was added to 600 ml of acetone, and centrifuged. The supernatant fluid was concentrated *in vacuo*, adjusted to pH 8~9 and extracted with ethyl acetate. JM-M in the extract was transferred into aqueous layer at pH 3, and the aqueous solution was reextracted with ethyl acetate at pH 9. The extract was concentrated *in vacuo*, and the concentrate was chromatographed on a silica gel column using benzene-acetone (2:1, v/v) as the solvent system. The eluate containing JM-M was concentrated *in vacuo* to give 320 mg of JM-M as a white powder. $[\alpha]_D^{22}$ −67.5° (*c* 0.5, EtOH), λ_{max}^{MeOH} 231 nm ($\varepsilon=2.87\times10^4$). Anal. Found: C, 59.55; H, 8.05; N, 1.89. The IR spectrum of JM-M showed intense bands at 1720~1740 cm^{-1} (C=O), 1050~1200 cm^{-1} (C-O-C) and a band at 2740 cm^{-1} (-CHO) similarly to that of JM. The NMR spectrum showed the signals assigned to $-N(CH_3)_2$ at δ 2.51(6 H, s), $-OCH_3$ at δ 3.54 (3 H, s), $-OCOCH_3$ at δ 2.28 (3 H, s), $C_{1''}$ proton at δ 4.42 (1 H, d) and aldehyde at δ 9.64 (1H, s), which were almost identical with those of JM. Both compounds differed in that JM-M showed the signals assigned to $C_{4''}$ proton (δ *ca.* 3.2) and $C_{5''}$ proton (δ *ca.* 4.0) at higher field than those of JM ($C_{4''}$, δ 4.61; $C_{5''}$, δ 4.41). Furthermore, protons due to isovaleryl group disappeared in JM-M. The mass spectrum of JM-M showed molecular ion peak at *m/e* 743. The fragment ion peaks reasonable for the structure were observed at *m/e* 409 (macrolactone), *m/e* 318 (mycarosylmycaminose), *m/e* 173 (mycaminose) and *m/e* 145 (mycarose). An isovalerylmycarose ion peak and an isovalerylmycarosyl mycaminose ion peak appearing in JM were not observed in JM-M. These data clearly indicate that JM-M is 4''-deisovaleryl derivative of JM.

Discussion

Macrolide antibiotics, SF-837[10] and PMDM,[1] have been shown to be hydrolyzed by rat liver homogenate to 4''-depropionyl SF-837 (M_1 substance) and PMDM-M, respectively. When PMDM was administered to human, PMDM-M was found in urine as one of metabolites of the antibiotic.[1] Thus, it seems that esterases which catalyze the deacylation of the antibiotics exist in various animals. Several fungi hydrolyzed leucomycin[2,3] and SF-837.[4] In this report, various bacteria have been shown to exhibit the esterase activity, which hydrolyzes MDM to MDM-M. Thus, it is interesting that the esterases which hydrolyze macrolide antibiotics are

widely distributed among animals and microorganisms.

Hydrolytic rate of PMDM by the esterase obtained from *B. megaterium* 91277 was more rapid than that of MDM (Fig. 8). It is interesting that the hydrolytic rate is affected by the presence of propionyl group at C_9 of the lactone moiety which is remote from acyl group at $C_{4''}$ of the mycarose moiety.

It has been found that rat liver homogenate hydrolyzes PMDM to PMDM-M at a rate in the order PMDM III (4''-propionyl)>PMDM V (4''-acetyl)>PMDM I (4''-isovaleryl) (FUGONO, T. & Y. KITA: personal communication). The same order was shown with esterase obtained from *B. megaterium* 91277 (Fig. 8). In this respect, the esterase of *B. megaterium* 91277 resembles that of rat liver homogenate.

The hydrolytic rates of PMDM III, PMDM VII (4''-butyryl) and 4''-isobutyryl-4''-depropionyl-9-propionylmaridomycin III by the esterase obtained from *B. megaterium* 91277 were almost equal, and were more rapid than those of PMDM I and PMDM V (Figs. 8, 9). Thus, the esterase seems to hydrolyze PMDM bearing 4''-acyl group of C_3 or C_4 most rapidly.

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