MICROBIAL CONVERSION OF ANTIBIOTICS. III

HYDROXYLATION OF MARIDOMYCIN I AND JOSAMYCIN

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Maridomycin I (MDM I), a macrolide antibiotic, was hydroxylated at β-position of 4″-isovaleryl group to 3‴-hydroxymaridomycin I (HMDM I) by culture broth of *Streptomyces olivaceus* 219. The structure of HMDM I was elucidated from the results of its NMR and mass spectra, methanolysis and alkaline hydrolysis. Josamycin (JM) was also hydroxylated to 3‴-hydroxyjosamycin (HJM) by the same strain. Antimicrobial activities of these hydroxylated products (HMDM I and HJM) were 1/2~1/5 of their substrates (MDM I and JM). Protective effect of HJM against *Staphylococcus aureus* by oral administration, however, was similar to that of JM. HJM and HMDM I were resistant to rat liver homogenate and bacterial esterase, which hydrolyzed JM and MDM I to 4″-deisovaleryljosamycin and 4″-deisovalerylmaridomycin I, respectively.

In our previous papers, *Bacillus megaterium* 91277[1] and *Streptomyces olivaceus* 219[2] have been shown to hydrolyze maridomycin III (MDM III) to 4″-depropionylmaridomycin III (MDM III-M). It has also been reported that *B. megaterium* 91277 hydrolyzes maridomycin I (MDM I) and josamycin (JM) to 4″-deisovalerylmaridomycin I (MDM I-M) and 4″-deisovaleryljosamycin (JM-M), respectively.[1] On the other hand, we have recently found that *S. olivaceus* 219 does not hydrolyze MDM I and JM but converts them to new active compounds.

This paper presents the preparation of the new compounds from MDM I and JM by using *S. olivaceus* 219 and the evidences for identification as 3‴-hydroxymaridomycin I (HMDM I) and 3‴-hydroxyjosamycin (HJM), respectively, and deals with the biological properies of these new compounds.

Materials and Methods

Antibiotics

MDM I[3], MDM III[3], maridomycin V (MDM V)[3], 9-propionylmaridomycin I (PMDM I)[4], and 9-propionylmaridomycin III (PMDM III)[4] and 9-propionylmaridomycin V (PMDM V)[4] were prepared in our research laboratories. JM[5,6] was extracted from the commercial preparations. As MDM I-M is identical with MDM III-M, it was prepared from MDM III by using *B. megaterium* 91277[1]. 4″-Depropionyl-9-propionylmaridomycin III (PMDM III-M) and JM-M were also prepared from PMDM III and JM, respectively, by the same procedure[1].

Microorganism and culture conditions

S. olivaceus 219 was isolated from the soil of Kyoto City. The strain was grown in 200 ml of a medium in a 1-liter Erlenmeyer flask at 28°C for 48 hours on a rotary shaker. The 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.

Thin-layer chromatography (TLC)

Samples were chromatographed on silica gel TLC plates (Merck) using benzene-acetone

(1 : 1, v/v) as the solvent system. The spots of antibiotics were detected by heating the plates after spraying 10% H_2SO_4.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatographic analysis was performed with a JGC-20 K instrument (Nihon Denshi) using a 2.3 mm×10 cm column packed with Chromosorb 101. The carrier gas was helium. The column temperature was 240°C, and the inlet temperature was 260°C.

Mass spectra were recorded on a JMS-01SC instrument (Nihon Denshi). The electron ionization energy was 40 eV. The ionization source temperature was maintained at 250°C.

Minimum inhibitory concentration (MIC)

MIC was determined by agar dilution method using glucose bouillon agar (pH 7.0). Test organisms were grown at 37°C for 18 hours, except that *Mycobacterium* sp. ATCC 607 was grown at 37°C for 2 days.

Protective effect

Protective effect against *Staphylococcus aureus* infection in mice was determined as described by KONDO *et al.*[7]

Preparation of rat liver homogenate

Liver was excised from a rat (SD/JCL, male, 400 g). The liver (5 g) was added to 10 ml of 0.1 M phosphate buffer (pH 7.5), homogenized for 1 minute on an ice bath, and filtered through antiseptic gauze. The residue was washed with the same buffer. The filtrate and the washing were combined to give 25 ml and used as rat liver homogenate.

Preparation of cell-free extract of *B. megaterium* 91277

Cell-free extract of *B. megaterium* 91277 was prepared as previously described[1]. The protein concentration was 20.3 mg/ml, determined by the method of LOWRY *et al.*[5]

TLC-Bioautography

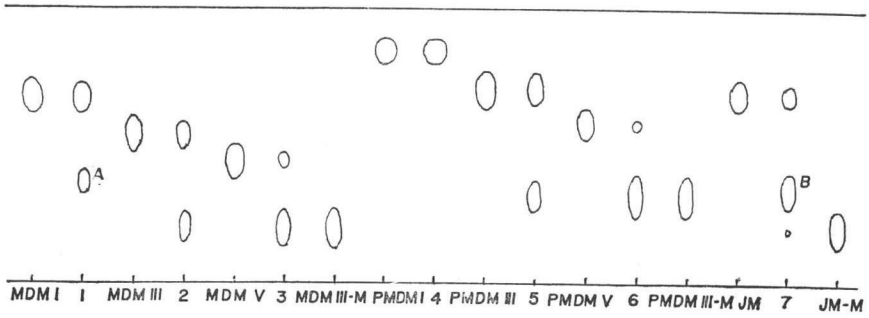
TLC-Bioautography was carried out as previously described[1].

Results

Conversion of Maridomycins, 9-Propionylmaridomycins and JM by *S. olivaceus* 219

Antibiotic (2 mg) in methanol (0.1 ml) was added to the culture broth of *S. olivaceus* 219 (4 ml). The reaction mixture was incubated at 28°C for 48 hours with shaking, and then extracted with ethyl acetate. The extract was chromatographed on TLC plates. MDM III (4″-propionyl) and MDM V (4″-acetyl) were hydrolyzed to MDM III-M. However, MDM I (4″-isovaleryl) was not hydrolyzed to MDM III-M, and was converted to an unknown compound (compound A). PMDM III (4″-propionyl) and PMDM V (4″-acetyl) were hydrolyzed to PMDM III-M. PMDM I (4″-isovaleryl) was neither hydrolyzed to PMDM III-M nor converted to other

Fig. 1. Conversion of maridomycins, 9-propionylmaridomycins and JM by *S. olivaceus* 219.
The reaction mixture contained 4 ml of the culture broth and 2 mg of the following antibiotics.
1, MDM I; 2, MDM III; 3, MDM V; 4, PMDM I; 5, PMDM III; 6, PMDM V; 7, JM



compounds. JM was converted to an unknown compound (compound B) and slightly to JM-M (Fig. 1).

Preparation of Compound A

MDM I (4 g) dissolved in methanol (80 ml) was added to the culture broth of *S. olivaceus* 219 (8 liters), and the reaction mixture was incubated at 28°C for 48 hours with shaking. The reaction mixture was adjusted to pH 8.5 with dil. NaOH and extracted with ethyl acetate. The extract was washed with water and extracted with 0.05 M citrate buffer (pH 3.0). The buffer layer was reextracted with ethyl acetate at pH 8.5. The extract was washed with water and concentrated *in vacuo*. The concentrate was chromatographed on a silica gel column using a solvent system consisting of benzene-acetone (2 : 1, v/v), and the eluate containing compound A was concentrated *in vacuo* to give compound A (391 mg) as a white powder. $[\alpha]_D^{25}$ −75.7° (*c* 1.02, EtOH). Anal. Calcd. for $C_{43}H_{71}NO_{17}$: C, 59.08; H, 8.19; N, 1.60. Found: C, 58.77, H, 8.28; N, 1.69.

Structure of Compound A

The IR spectrum of compound A (Fig. 2) showed intense bands at 1730 cm^{-1} (C=O), 1050

Fig. 2. IR spectrum of compound A in KBr disk

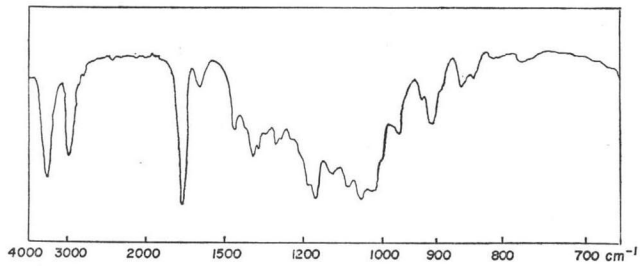
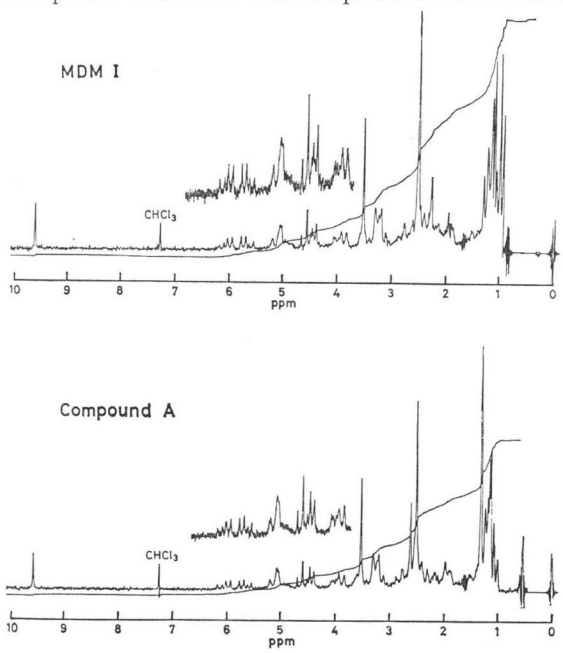


Fig. 3. NMR spectra of MDM I and compound A at 100 MHz in $CDCl_3$

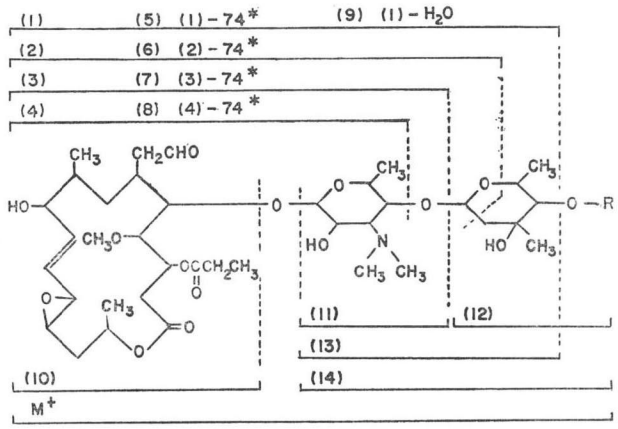


$\sim$1200 cm^{-1} (C-O-C) and a band at 2730 cm^{-1} (-CHO) similarly to that of MDM I.

The NMR spectrum of compound A showed the presence of $-N(CH_3)_2$ at δ 2.50 (6H, s), $-OCH_3$ at δ 3.51 (3H, s), $C_{1'}$ proton at δ 4.41 (1H, d), $C_{4''}$ proton at δ 4.63 (1H, d) and -CHO at δ 9.60 (1H, s) similarly to that of MDM I. The NMR spectrum of compound A differed from that of MDM I mainly in two regions. A doublet at δ 0.97 assigned to geminal methyl protons of isovaleryl group in MDM I shifted downfield to a singlet at δ 1.30 in the spectrum of compound A. A signal at δ 2.26 assigned to methylene protons at α position of isovaleryl group shifted downfield to a singlet at δ 2.60 in the spectrum of compound A (Fig. 3).

The mass spectrum of compound A showed the molecular ion peak at *m/e* 873, which was 16 mass units greater than that of MDM I. The fragment ion peaks reasonable for the structure were observed at *m/e* 439 (macrolactone), *m/e* 174 (mycaminose) and *m/e* 245 (acylmycarose). The fragment ions involving 4''-acyl group shifted to higher region by 16 mass units compared with the corresponding ions of MDM I (Fig. 4).

Fig. 4. Mass fragmentation patterns of MDM I and HMDM I (compound A)



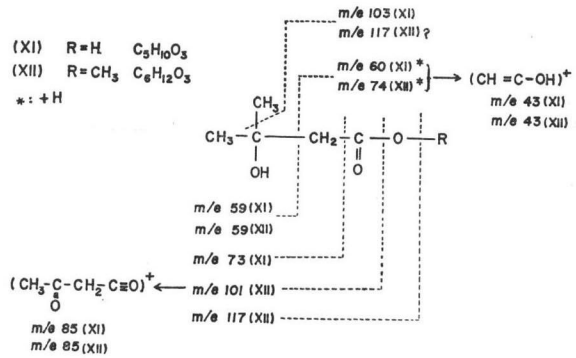
	M†	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
MDM I	857	756	658	628	613	682	584	554	539	738	439	174	229	300	402
HMDM I	873	756	658	628	613	682	584	554	539	738	439	174	245	300	418

These data suggested that 4''-acyl group of compound A was β-hydroxyisovaleryl group. Furthermore, in order to confirm the presumption, alkaline hydrolysis[9,10] and methanolysis[9,10,11] were carried out.

Compound A was hydrolyzed with 0.5 N KOH, and the hydrolyzate was analyzed with GC-MS. The gas-liquid chromatogram of the hydrolyzate showed the presence of propionic acid and another fatty acid (**XI**). The retention time of **XI** (13.7 minutes) was identical with that of authentic β-hydroxyisovaleric acid prepared by the method of COFFMAN *et al.*[12] The

mass spectrum of **XI** was identical with that of authentic β-hydroxyisovaleric acid, and showed that the fragment ion peaks were reasonable for the structure (Fig. 5). **XI** was further methylated to **XII**. The fragment ion peaks of **XII** also were reasonable for the structure (Fig. 5).

Fig. 5. Mass fragmentation patterns of **XI** and **XII**



Methanolysis of compound A (955 mg) with methanol-hydrochloric acid yielded neutral sugars (215 mg), which were further separated by silica gel column chromatography into **XIIIa** (42 mg) and **XIIIb** (79 mg) as liquids. **XIIIa**: $[\alpha]_D^{29}$ −130.5° (*c* 0.77, $CHCl_3$). Anal. Calcd. for $C_{13}H_{24}O_6$: C, 56.50; H, 8.75; O, 34.74. Found: C, 56.12; H, 8.78; O, 34.34. **XIIIb**: $[\alpha]_D^{29}$ +12.3° (*c* 0.84, $CHCl_3$). Anal. Calcd. for $C_{13}H_{24}O_6$: C, 56.50; H, 8.75; O, 34.74. Found: C, 56.20; H, 8.97; O, 34.95. The NMR spectrum of **XIIIa** (Fig. 6) showed the presence of C_3 $-CH_3$ at δ 1.05 (3H, s), C_5 $-CH_3$ at δ 1.13 (3H, d, J=6 Hz), geminal methyl protons at δ 1.26 (6H, s), C_2 methylene protons at *ca.* δ 1.9 (2H), $C_{2'}$ methylene protons at δ 2.50 (2H), $-OCH_3$ at δ 3.35 (3H, s), -OH at δ 3.56 (1H) which disappeared by addition of D_2O, C_5 proton at δ *ca.* 3.9 (1H, m), C_4 proton at δ 4.51 (1H, d, J=10 Hz) and C_1 proton at δ *ca.* 4.7 (1H). The spectrum differed from that of α-methyl-4-O-isovalerylmycaroside[11] in two regions. A doublet at δ 0.95 assigned to geminal methyl protons of isovaleryl group in α-methyl-4-O-isovalerylmycaroside shifted downfield to a singlet at δ 1.26 in the spectrum of **XIIIa**. A signal at δ *ca.* 2.2 assigned to methylene protons of isovaleryl group in α-methyl-4-O-isovalerylmycaroside shifted downfield to δ 2.50 in the spectrum of **XIIIa**. The NMR spectrum of **XIIIb** (Fig. 6) showed the presence of C_3 $-CH_3$ at δ 1.12 (3H, s) C_5 $-CH_3$ at δ 1.12 (3H, d, J=6 Hz), geminal methyl protons at δ 1.29 (6 H, s), C_2 methylene protons at δ 1.52 (1 H, dd, J=9, 14 Hz; ax.) and δ 1.90 (1H, dd, J=2.5, 14 Hz; eq.), $C_{2'}$ methylene protons at δ 2 48 (2H, d, J=2 Hz), -OH at δ 2.71 (1H) which disappeared by addition of D_2O, $-OCH_3$ at δ 3.37 (3H, s), C_5 proton at δ *ca.* 3.8 (1H, m), C_4 proton at δ 4.50 (1H, d, J=9 Hz) and C_1 proton at δ 4.58 (1H, dd, J=2.5, 9 Hz). The spectrum differed from that of β-methyl-4-O-isovalerylmycaroside[11] in two regions. A doublet at δ 0.95 assigned to geminal methyl protons of isovaleryl group in β-methyl-4-O-isovalerylmycaroside shifted downfield to a singlet at δ 1.29 in the spectrum of **XIIIb**. A signal at δ *ca.* 2.2 assigned to methylene protons of isovaleryl group in β-methyl-4-O-isovalerylmycaroside shifted downfield to δ 2.48 in the spectrum of **XIIIb**. Thus, it was apparent that **XIIIa** and **XIIIb** were α-methyl-4-O-3′-hydroxyisovalerylmycaroside and β-methyl-4-O-3′-hydroxyisovalerylmycaroside, respectively.

From these results, compound A was identified as 3‴-hydroxymaridomycin I (HMDM), a hydroxylated derivative of MDM I at β position of the isovaleryl group.

Fig. 6. NMR spectra of **XIIIa** and **XIIIb** at 100 MHz in CCl_4

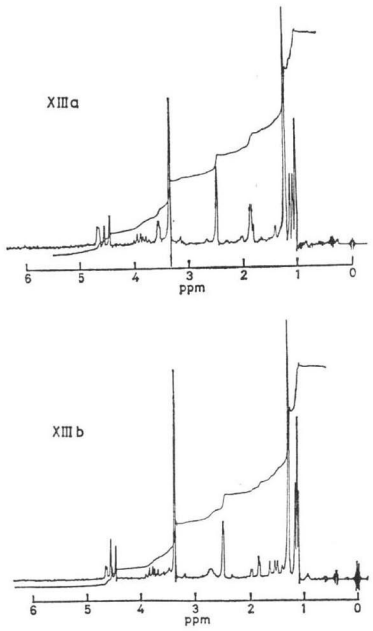


Fig. 7. IR spectrum of compound B in KBr disk

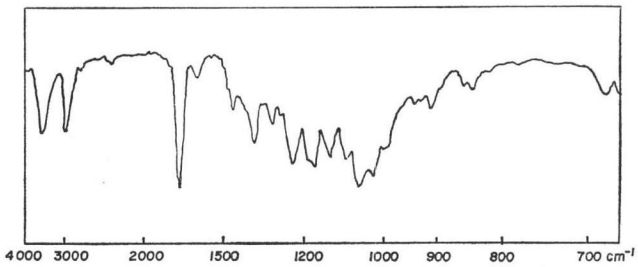
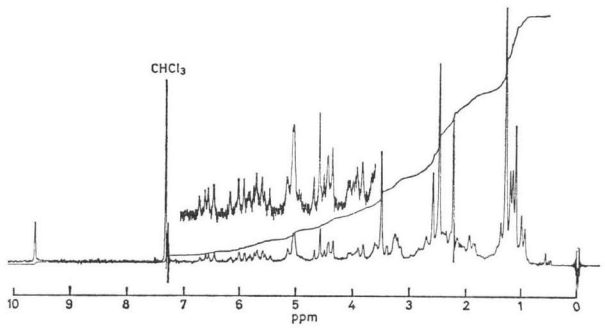


Fig. 8. NMR spectrum of compound B at 100 MHz in $CDCl_3$



Preparation of Compound B

JM (2.5 g) dissolved in methanol (50 ml) was added to the culture broth of *S. olivaceus* 219 (5 liters), and the reaction mixture was incubated at 28°C for 48 hours with shaking. The purification of compound B formed in the reaction mixture was carried out in the same manner as that of compound A to give compound B (396 mg) as a white powder. $[\alpha]_D^{27}$ −63.3° (*c* 1.12, EtOH), λ_{max}^{MeOH} 231 nm ($\varepsilon = 2.92 \times 10^4$). Anal. Calcd. for $C_{42}H_{69}NO_{16}$: C, 59.77; H, 8.24; N, 1.66. Found: C, 59.81; H, 8.28; N, 1.37.

Structure of Compound B

The IR spectrum of compound B (Fig. 7) was similar to that of JM. The NMR spectrum of compound B (Fig. 8) differed from that of JM mainly in two regions. A doublet at δ 0.97 assigned to geminal methyl protons of isovaleryl group in JM shifted downfield to a singlet at δ 1.30 in the spectrum of compound B. A singlet at δ 2.59 assigned to methylene protons of β-hydroxyisovaleryl group appeared in the spectrum of compound B, though it was not observed in the spectrum of JM.

The mass spectrum of compound B showed the molecular ion peak at *m/e* 843, which was 16 mass units greater than that of JM. The fragment ion peaks reasonable for the structure were observed at *m/e* 409 (macrolactone), *m/e* 174 (mycaminose) and *m/e* 245 (acylmycarose). The fragment ions involving 4″-acyl group shifted to higher region by 16 mass units compared with the corresponding ions of JM.

Hydrolysis of compound B with 0.5 N KOH gave acetic acid and β-hydroxyisovaleric acid.

Methanolysis of compound B with methanol-hydrochloric acid yielded neutral sugars, which were further separated into α-methyl-4-O-3′-hydroxyisovalerylmycaroside and β-methyl-4-O-3′-hydroxyisovalerylmycaroside.

From these results, compound B was identified as 3'''-hydroxyjosamycin (HJM), a hydroxylated derivative of JM at β position of the isovaleryl group.

Antimicrobial Activity

MDM I, HMDM I and MDM I-M showed similar antimicrobial spectra. The antimicrobial activity of HMDM I against Gram-positive bacteria was 1/2~1/5 of MDM I. MDM I-M showed the lowest activity among them. Similar results were obtained in JM, HJM and JM-M (Table 1).

Protective Effect

Fifty percent effective doses (ED_{50}, oral administration) of HJM and JM against *S. aureus* 308 A-1 were 224 and 200 mg/kg, respectively. The result indicated that the protective effect of HJM was similar to that of JM.

Table 1. Antimicrobial activity

Test organism	MIC (μg/ml)					
	MDM I	HMDM I	MDM I-M	JM	HJM	JM-M
Staphylococcus aureus FDA 209 P	1	5	20	1	2	10
S. aureus Terajima	1	5	20	0.5	2	20
S. aureus No. 87*	>100	>100	>100	>100	>100	>100
S. aureus OE-R*	100	>100	>100	50	>100	>100
Micrococcus flavus IFO 3242	0.2	0.5	0.5	0.1	0.2	2
Sarcina lutea PCI 1001	0.1	0.1	1	0.05	0.1	1
Bacillus subtilis PCI 219	0.2	0.5	5	0.5	1	5
Pseudomonas aeruginosa IFO 3080	>100	>100	>100	100	>100	>100
Escherichia coli NIHJ	>100	>100	>100	>100	>100	>100
Proteus vulgaris IFO 3045	>100	>100	>100	>100	>100	>100
Mycobacterium sp. ATCC 607	20	100	>100	50	100	>100

* Macrolide resistant strains

Deacylation by Rat Liver Homogenate

A reaction mixture containing 1 mg of JM or HJM, 5 ml of 0.9% NaCl and 5 ml of rat liver homogenate in a total volume of 10 ml was incubated at 37°C for 4 hours. At intervals, the aliquots were withdrawn and then assayed for antimicrobial activity by paper disk method using *Bacillus subtilis* PCI 219 as the test organism. The antimicrobial activity of the reaction mixture containing JM decreased gradually and became much lower than that containing HJM after 4 hours. The amounts of antibiotics in the aliquots were determined by TLC-bioautography. JM was gradually hydrolyzed to JM-M, whereas HJM was not (Fig. 9).

Deacylation by Esterase Obtained from *B. megaterium* 91277

Esterase from *B. megaterium* 91277 have been found to hydrolyze MDM I and JM to MDM I-M and JM-M, respectively[1]. Then, the hydrolysis of HMDM I and HJM by the esterase was examined. A reaction mixture containing 2 mg of antibiotic, 400 μmoles of glycine-NaOH buffer (pH 9.0) and 8.0 ml of cell-free extract of *B. megaterium* 91277 in a total volume of 4 ml was incubated at 37°C for 4 hours. At intervals, the aliquots were withdrawn, and then the amounts of antibiotics were determined by TLC-bioautography. Contrary to active hydrolysis of MDM I and JM, no hydrolysis of HMDM I and HJM was observed (Fig. 10).

Fig. 9. Deacylation of JM and HJM by rat liver homogenate

The reaction mixture containing JM (left) or HJM (right) was incubated at 37°C for 4 hours. At intervals, the aliquots were withdrawn and extracted with ethyl acetate at pH 8.0. The amounts of antibiotics in the extracts were determined by TLC-bioautography.

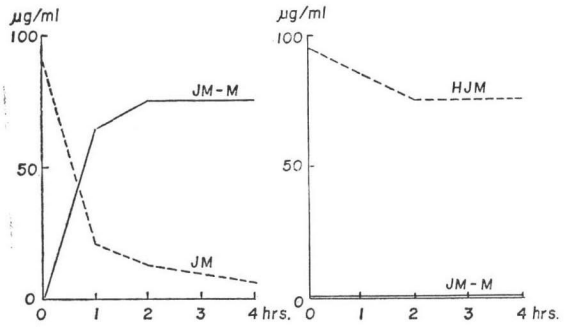
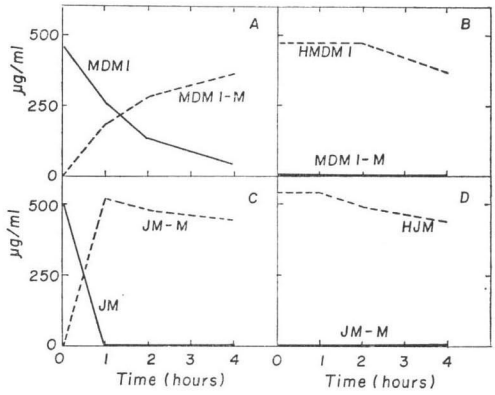


Fig. 10. Deacylation of MDM I, HMDM I, JM and HJM by esterase from *B. megaterium* 91277

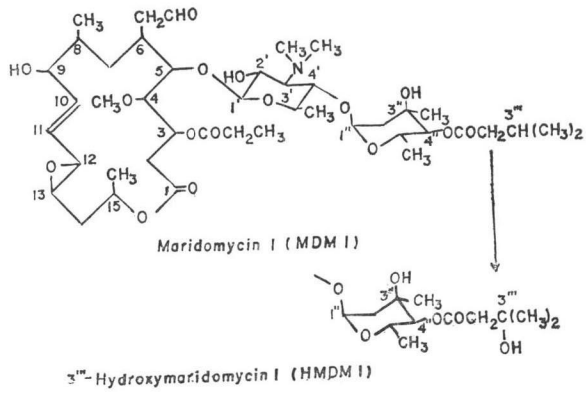
The reaction mixture contained 2 mg of the following antibiotic, 400 μmoles of glycine-NaOH buffer (pH 9.0) and 0.8 ml of cell-free extract in a total volume of 4 ml. A, MDM I; B, HMDM I; C, JM; D, HJM



Discussion

Although MDM III (4″-propionyl) and MDM V (4″-acetyl) were hydrolyzed to MDM III-M by *S. olivaceus* 219, MDM I (4″-isovaleryl) was not hydrolyzed (Fig. 1). As shown in Fig. 11, MDM I was hydroxylated to HMDM I by the same strain. Thus, it is evident that propionyl and acetyl groups at $C_{4''}$ is hydrolyzed by *S. olivaceus* 219 but isovaleryl group is hydroxylated without hydrolysis.

Fig. 11. Hydroxylation of MDM I to HMDM I by *S. olivaceus* 219



Of 125 strains of actinomycetes tested, 11 strains were found to show the hydroxylation activity against MDM I and JM. Among them, *S. olivaceus* 219, *S. platensis* IFO 12901, *S. rimosus* IFO 12907 and *Actinomyces rubiginosus* IFO 12913 showed relatively high activity. Recently, TACHIBANA *et al.*[18] reported the isolation of a metabolite, metabolite O, in human urine after oral administration of JM. The structure of the metabolite appears to be identical with that of HJM. Thus, the enzymes catalyze the hydroxylation of MDM I and JM seem to be widely distributed among microorganisms and animals.

The protective effect of HJM by oral administration was similar to that of JM, whereas the antimicrobial activity of HJM *in vitro* was 1/2~1/4 of JM. It may be due to the stability of HJM *in vivo* in contrast with the instability of JM. In practice, HJM was not hydrolyzed to JM-M by rat liver homogenate, though JM was readily hydrolyzed to JM-M, whose antimicrobial activity was 1/5~1/10 of JM (Fig. 9 and Table 1).

Similarly to rat liver homogenate, esterase from *B. megaterium* 91277 was able to hydrolyze

JM and MDM I but not HJM and HMDM I (Fig. 10). Thus, it is thought that isovaleryl group at $C_{4''}$ becomes resistant to these esterases owing to hydroxylation at β position.

After oral administration of HJM to rat, HJM and unknown metabolites showing antimicrobial activity were observed by TLC-bioautography in other experiments. The Rf values of the unknown metabolites were lower than that of JM-M by using a solvent system consisting of chloroform - methanol - pyridine - water (15 : 2 : 1 : 1, v/v). The unknown metabolites were not detected in the reaction mixture after incubation of HJM with rat liver homogenate, namely, *in vitro*. Thus, it seems that the metabolism of HJM differs between *in vivo* and *in vitro*.

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