MICROBIAL TRANSFORMATION OF ANTIBIOTICS

I. ISOLATION AND CHARACTERIZATION OF THE TRANSFORMATION PRODUCTS OF MARIDOMYCIN III

MOTOO SHIBATA, MASARU UYEDA and SHINYA MORI

Sciences, Laboratory of Medicinal Microbiology, Faculty of Pharmaceutical
Kumamoto University, Kumamoto City, Japan

(Received for publication November 21, 1974)

Three transformation products of maridomycin (MDM) III, a macrolide antibiotic, by *Streptomyces lavendulae* were isolated by silica gel and alumina chromatography, and designated as spots 1 (starting MDM III), 2, 3 and 4, in the order of their decreasing Rf values on thin-layer chromatogram. By mass- and NMR-spectrometry and thin-layer chromatography, spot 2 was identified as 18-dihydro-MDM III, spot 3 as 4″-depropionyl-MDM III, and spot 4 as 18-dihydro-4″-depropionyl-MDM III. The relationship between starting MDM III and these transformation products were also discussed.

The studies on microbial transformation of antibiotics have been carried out in order to improve antimicrobial activity of the existing ones, or making them less-toxic or effective against resistant strains. It has been reported in the review of SEBEK and PERLMAN[1] that many antibiotics were susceptible to microbial attack or degradation. Only a limited effort has been invested in using enzymes to produce new and potentially useful antibiotic derivatives.

In the course of studies on microbial transformation of macrolide antibiotics, it has been reported that 9-propionylmaridomycin (PMDM) and maridomycin (MDM) were hydrolyzed by rat liver homogenate[2] or by many bacteria[3] to 4″-deacyl-PMDM and 4″-deacyl MDM, respectively. Deacylation, dehydrogenation and acylation of T-2636 antibiotics[4] were demonstrated in such enzyme systems. Deacylation on macrolide antibiotics, leucomycin[5] and SF-837[6,7] by fungi has also been reported.

In the course of the screening, MDM III[8,9] was found to be transformed into three derivatives by *Streptomyces lavendulae* strain No. K-122 as judged from its thin-layer chromatograms on silica gel. They were isolated by silica gel and alumina chromatography and designated as spots 1 (starting MDM III), 2, 3 and 4, in the order of their decreasing Rf values on thin-layer chromatogram. Of these three transformation products, spots 2 and 3 were identical with those previously reported by NAKAHAMA *et al.*[3,10] In addition, our culture produced a new compound namely 18-dihydro-4″-depropionyl-MDM III (spot 4). This paper deals mainly with the isolation and characterization of the transformation products and with the relationship between the substrate (MDM III) and its products.

Materials and Methods

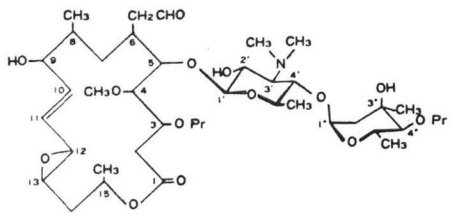
Antibiotics

Maridomycin III (Fig. 1), and 4″-depropionylmaridomycin III were kindly supplied by Takeda Chemical Industries, Ltd.

Microbiological Properties of *Streptomyces* sp. strain No. K-122

1) The strain was isolated from a soil sample collected in Kumamoto City, Japan and used throughout this work.

Fig. 1. Structure of maridomycin III.



2) Morphological observation: After incubation on a glucose-asparagine agar at 28°C for 7 days, the organism was examined by a light-microscope and an electron-microscope (JEM-50 B, Japan Electron Optics Laboratory, Co., Ltd.)

3) Cultural characteristics: Each of the media used in this study was prepared according to the description of WAKSMAN[11]. Spores of the strain collected from the 7-day culture grown on glucose-asparagine agar were suspended in sterile water and a loopful of the suspension was added to each medium. The strain was cultured at 28°C for 14 days and examined after 7 and 14 days of incubation. The color names based on RAYNER's description[12] were used.

4) Utilization of carbon sources: Examination of carbon utilization was made according to PRIDHAM's method.[13]

Cultural Conditions

Fermentation was carried out with 10 ml of a medium in 200-ml Erlenmeyer flasks. The seed culture medium containing 2% glucose, 3% soluble starch, 1% soybean flour, 1% corn steep liquor, 0.5% Polypepton, 0.3% NaCl and 0.5% $CaCO_3$(pH 7.0), was inoculated with spores from the slant culture. *Streptomyces* sp. strain No. K-122 was grown at 28°C for 48 hours on a rotary shaker (180 rpm, 5-cm radius). The resultant culture (0.5 ml) was transferred to 10 ml of the transformation medium containing 5% glucose, 1% Polypepton, 0.5% Ehrlich meat extract, 0.3% NaCl and 0.5% $CaCO_3$(pH 7.0).

After 48 hours of incubation, 20 mg of MDM III was added to the culture (final concentration of MDM III: 2 mg/ml medium). The cultivation was continued for more 48 hours.

Assay of Antibacterial Activity

The cup assay method was employed for the estimation of transformation process using *Bacillus subtilis* PCI-219 as test organism.

Thin-layer Chromatography (TLC)

Filtered broth was extracted with ethyl acetate at pH 8 and the solvent layer was chromatographed on silica gel G (Merck) plates using benzene - acetone (1:2, v/v) as solvent. The position of antibiotics were detected by heating the plates after spraying 10% H_2SO_4.

Results

Taxonomical Characteristics of *Streptomyces* sp. No. K-122

As summarized in Table 1, strain No. K-122 was identified as *Streptomyces lavendulae* (WAKSMAN and CURTIS) WAKSMAN and HENRICI, 1948. It was reported that strains belonging to *S. lavendulae* produce streptothricin[14,15], actithiazic acid[16,17] *etc.* Our strain, however, produced only very low concentration of antibacterial material which was not further investigated.

Transformation of MDM III in the Growing System

Typical thin-layer chromatogram was shown in Fig. 2a. Besides starting MDM III (Rf 0.81), three clearly defined spots were detected. We designated these products temporarily as spots 1 (starting MDM III), 2, 3 and 4, in the order of their decreasing Rf values on TLC.

Table 1. Taxonomical characteristics of *Streptomyces* sp. strain No. K-122.

Spore chain morphology	Spore chains straight (to form tuft), some coils of wide diameter and spirals. Oval shaped spores.
Spore surface	Smooth.
Color of colony	Characteristic color is pale-vinaceus lavender of RAYNER's color sheet 4, IV-85 on oatmeal agar, salt-starch agar and glycerol-asparagine agar.
Reverse side of colony	No distinctive pigment on yeast malt agar, oatmeal agar, salts-starch agar or glycerol-asparagine agar.
Color in the medium	Melanoid pigments formed in peptone-yeast-iron agar and usually in tyrosine agar (Shinobu) and in melanin formation agar of WAKSMAN. Pigments other than melanoids not formed in yeast-malt agar, oatmeal agar, salts-starch agar or glycerol-asparagine agar.
Carbon utilization	D-Glucose, D-fructose and rhamnose are utilized for growth. No growth on L-arabinose, sucrose, D-xylose, *meso*-inositol, D-mannitol and raffinose.

Fig. 2. Typical thin-layer chromatograms of transformation products by *S. lavendulae* No. K-122.

a: Sample transformed in the medium of 10 ml in 200-ml Erlenmeyer flask.

b: Sample transformed in the medium of 50 ml in 200-ml Erlenmeyer flask.

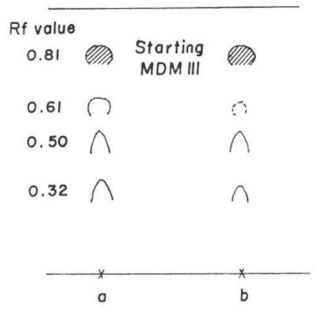
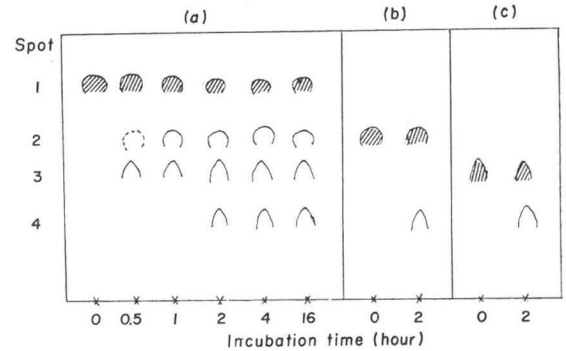


Fig. 3. Time course changes in the transformation by washed cell.

Starting material (spots with oblique lines); MDM III in Fig. (a). spot 2 in (b) or spot 3 in (c), respectively.

Incubation mixture for the transformation by washed cell: 10 mg of MDM III, 2 ml of phosphate buffer (0.5 M, pH 8) 5 ml of cell suspension (0.3 g/ml), 3 ml of sterile water (10 ml, in total). Incubated at 28°C on rotary shaker.



When this strain was grown in 50 ml of the medium in 200-ml Erlenmeyer flask, only partial transformation took place and particularly spots 2 and 4 were rarely detectable (Fig. 2b).

Transformation in the Washed Resting-Cell System

Enzymes which transform MDM III are thought to be intracellular since they were not detected in the culture filtrate. To clarify the sequence of the products formed, the transformation was carried out in the washed resting-cell system. Cells were harvested after 2 days of incubation, washed twice with sterile saline and suspended in 0.05 M phosphate buffer-saline (pH 8). For starvation, this suspension was shaken on rotary shaker at 28°C for 3 hours, cells were centrifuged and resuspended in sterile phosphate buffer (0.05 M, pH 8).

The progress of transformation of MDM III by the washed resting-cell are shown in Fig. 3a. Spot 3 was formed first (half an hour), followed by spot 2 (one hour) and 4 (two hours). Moreover, as shown in Figs. 3b and 3c, spot 4 was formed not only from purified spot 2 but

Table 2. Time course change of antibacterial activity during transformation.

	Incubation time (hr)				
	0	1	2	4	16
Antibacterial activity (mm)*	33.5	–	31.8	30.5	29.0

* Length of inhibition diameter (mm) by the cup assay using *B. subtilis* PCI-219 as test organism. Samples were diluted by 20 folds, therefore, assayed as the order of 100 μg/ml of starting MDM III.

also from purified spot 3.

As recorded in Table 2 transformation products may have lower antibacterial activities than the starting MDM III.

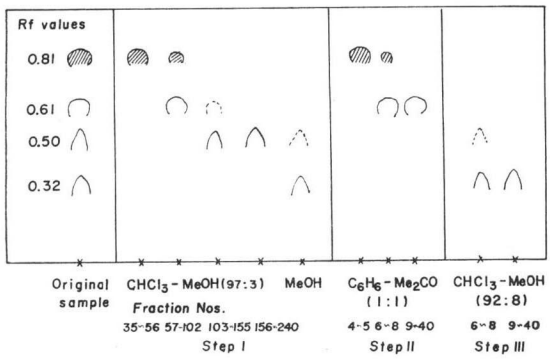
Isolation of Transformation Products

The isolation of starting MDM III and its transformation products was carried out by general procedures used for the isolation of lipophilic basic substances. A mixture of transformation products was extracted from a filtered broth with ethyl acetate at pH 8 and transferred to water at pH 3 (adjusted with citrate buffer), then back again into ethyl acetate at pH 8. A concentrate of this ethyl acetate extract gave a crude material upon the addition of *n*-hexane.

Table 3. Column chromatography for isolation of MDM III and its derivatives.

Step	I	II	III
Column size	2×60 (cm)	1×25 (cm)	1×40 (cm)
Adsorbent	Silica gel	Alumina (grade III)	Silica gel
Solvent	1. $CHCl_3$-MeOH (99:1) 2. 〃 (97:3) 3. MeOH	C_6H_6-Me_2CO (1:1)	$CHCl_3$-MeOH (92:8)
Flow rate (ml/min)	1	0.4	0.4
Fraction size (ml)	5	2	5
Sample applied	1, 2, 3 & 4 (mixture)	1<2	3<4
After chromatography	1, 1<2, 3, 3<4	1, 2	3, 4

Fig. 4. Typical thin-layer chromatograms of isolated products.



For the isolation of each component, three steps of column chromatography were used as summarized in Table 3.

Step I: A preliminary separation of the mixture of transformation products was accomplished by column chromatography on silica gel developed first with chloroform - methanol (99:1), then with the same solvent pair (97:3), and finally with methanol. Fractions of pure spot 1, spot 2 contaminated with spot 1, pure spot 3 and spot 4 contaminated with spot 3 were obtained.

Step II: For the isolation of spot 2 from the fraction of spot 2 contaminated with spot 1 on step I, column chromatography on alumina developed with benzene - acetone (1:1) was used.

Step III: Spot 4 was isolated from the fraction of spot 4 contaminated with spot 3 on step I by column chromatography on silica gel developed with chloroform - methanol (92:8).

Typical thin-layer chromatograms of the three steps are summarized in Fig. 4. The corresponding fractions of each component after column chromatography were combined, concentrated,

Table 4. Yields of individual components of crude transformation extracts.

	I		II	
	mg	Yield (%)	mg	Yield (%)
Mixture of 4 components	700	100	710	100
Spot 1 (MDM III)	266	38	341.5	48
Spot 2	110	15	91.4	12.8
Spot 3	79	11.3	96.5	13.5
Spot 4	51	7.3	59.5	8.4

redissolved in ethyl acetate and decolorized by activated charcoal. The concentrated sample was precipitated with the addition of *n*-hexane, thus affording a colorless white powder. Table 4 gives two examples of yields of individual components obtained from crude extracts.

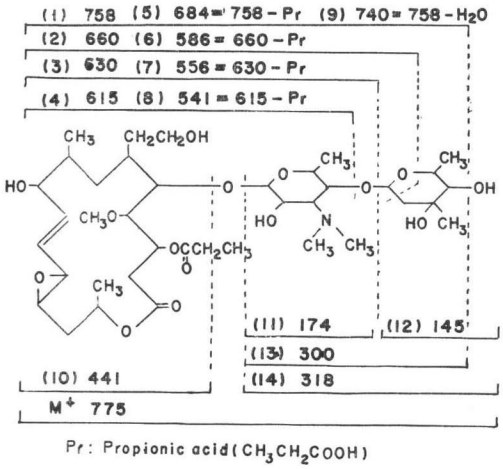
Physicochemical Properties and Structures of Transformation Products

The physicochemical properties of transformation products and of MDM III are summarized in Table 5. The IR spectra of these products are quite similar to MDM III and exhibit strong absorption band at about 3500 cm^{-1}(ν_{OH}), 1730 cm^{-1}($\nu_{C=O}$) and 1050~1200 cm^{-1} (ν_{C-O-C}).

Table 5. Physicochemical properties of maridomycin III (Spot 1) and its transformation products.

	Spot 1	Spot 2	Spot 3	Spot 4
m.p. (°C) (decomp.)	135~138	—	157~158	—
Molecular wt. Mass M^+(m/e)	829	831	773	775
Elementary analysis (%)				
Found C	57.93	58.82	57.63	58.50
H	8.18	8.33	8.28	8.50
N	1.69	1.70	1.77	1.74
Calcd. for	$C_{41}H_{67}NO_{13}H_2O$	$C_{41}H_{69}NO_{13}$	$C_{33}H_{53}NO_{13}H_2O$	$C_{38}H_{65}NO_{13}$
C	58.07	59.19	57.93	58.82
H	8.20	8.36	8.21	8.44
N	1.65	1.68	1.78	1.81

Fig. 5. Mass spectrum of 18-dihydro-4″-dipropionyl-MDM III.



The structures of MDM antibiotics including MDM III have been reported by MUROI *et al.*[8,9] The structures of transformation products and of MDM III as reported in this paper were obtained by mass- and NMR-spectrometry and TLC (Fig. 5 and Table 6).

Of these transformation products, spot 2 and spot 3 have the same physicochemical characteristics of the previously described substances[8,10]. The former is 18-dihydro-MDM III and the latter is 4″-depropionyl-MDM III.

The mass spectrum of spot 4 differs from that of MDM III mainly in two regions, in fragment ion peaks at macrolactone (*m/e* 439→441) and at acylmycarose (*m/e* 201→145). Difference in macrolactone was same as spot 2 by the mass- and NMR-spectrometry. On the other hand, the difference in acylmycarose is the same as spot 3. NMR spectrum of this

Table 6. Mass fragmentation patterns of MDM III and its derivatives.

	M+	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)
Spot 1	829	756	658	628	613	682	584	554	539	738	439	174	201	300	374	57
Spot 2	831	758	660	630	615	684	586	556	541	740	441	174	201	300	374	57
Spot 3	773	756	658	628	613	682	584	554	539	738	439	174	145	300	318	
Spot 4	775	758	660	630	615	684	586	556	541	740	441	174	145		318	

Spot 1: MDM III, Spot 3: 4″-Depropionyl-MDM III,
Spot 2: 18-Dihydro-MDM III, Spot 4: 18-Dihydro-4″-depropionyl-MDM III.

Fig. 6. NMR spectrum of 18-dihydro-4″-depropionyl-MDM III.

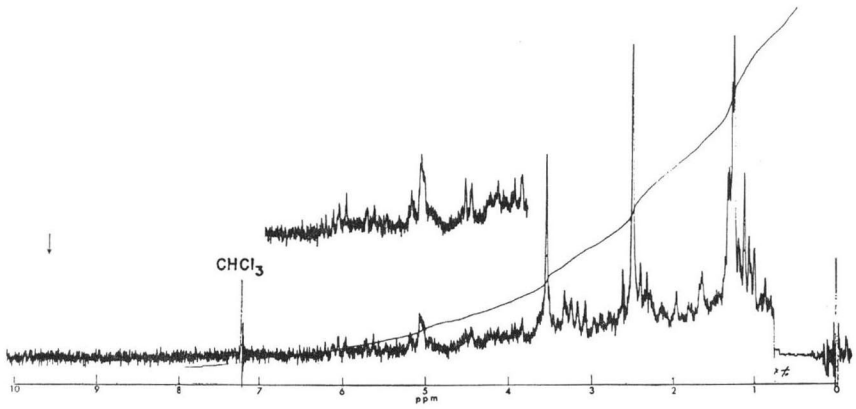
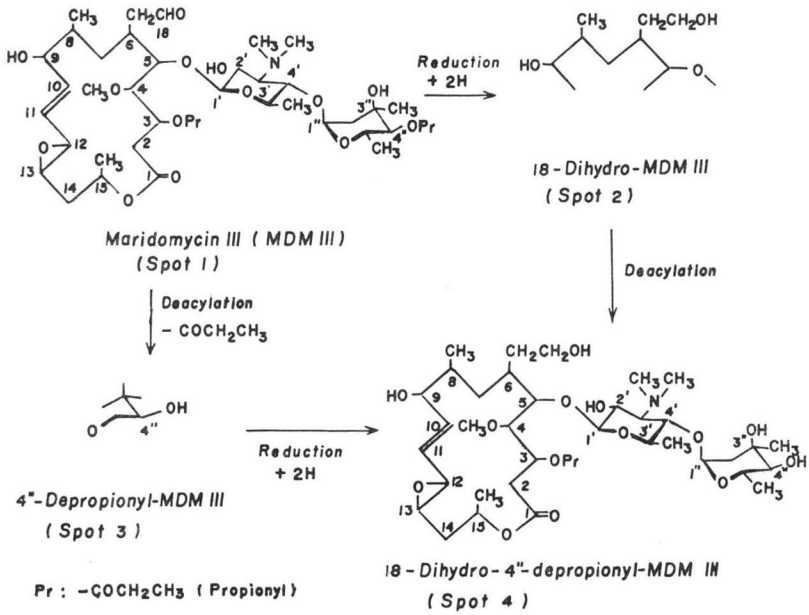


Fig. 7. Transformation of maridomycin III into its derivatives.



derivative is shown in Fig. 6. A proton at δ 9.62 which was recognized as an aldehyde proton in starting MDM III[8] disappeared in this derivative (marked by an arrow in Fig. 6).

From these results, spot 4 was identified as 18-dihydro-4″-depropionyl-MDM III and is a

Table 7. Antimicrobial spectra of maridomycin III and its derivatives.

Test organism	Minimum inhibitory conc. (μg/ml)			
	(1)	(2)	(3)	(4)
B. subtilis	0.5	>100	2	>100
S. aureus	1	>100	5	>100
S. aureus SM, EM, CP, CTC-R	20	>100	>100	>100
Sarcina lutea	0.2	20	0.5	20
E. coli	>100	>100	>100	>100
Proteus vulgaris	>100	>100	>100	>100
Ps. aureofaciens	>100	>100	>100	>100
Ps. aeruginosa	>100	>100	>100	>100
Mycobact. avium NM-R	50	>100	>100	>100
Mycobact. avium SM-R	50	>100	>100	>100
Candida albicans	>100	>100	>100	>100
Crypt. neoformans	>100	>100	>100	>100
Sacch. cerevisiae	>100	>100	>100	>100
Asp. niger	>100	>100	<100	>100

(1): Maridomycin III, (2): 18-Dihydro-MDM III, (3): 4''-Depropionyl-MDM III, (4): 18-Dihydro-4''-depropionyl-MDM III.

new transformation product of MDM III.

Relationship between MDM III and its Transformation Products

As we have already described, the sequence of the transformation products formed from MDM III by washed resting-cells was spot 3, spot 2 and spot 4. It was also found that spot 4 was formed from spot 2 and from spot 3.

From these results, the relationship between MDM III and its transformation products was considered as shown in Fig. 7: that is, 4''-depropionyl MDM III was formed by deacylation of MDM III and 18-dihydro-MDM III by reduction of MDM III. 18-Dihydro-4''-depropionyl-MDM III was formed either from 4''-depropionyl-MDM III by reduction or from 18-dihydro-MDM III by deacylation.

Antimicrobial Activity of MDM III and its Transformation Products

MDM III is active against Gram-positive bacteria including acid-fast bacteria. As shown in Table 7, spot 3, 4''-depropionyl-MDM III retained 20~30% of activity of MDM III. Spot 2 and spot 4, 18-dihydro derivatives, however, lost almost all (99%) activity.

These results suggest that the aldehyde group at C 18 of the macrolactone ring of maridomycins is essential for the antimicrobial activity of these antibiotics, (spiramycin[18], leucomycin A_3[19], maridomycins[10]).

Discussion

Of 360 strains of actinomycetes tested, about 70 strains showed some transformation of MDM III. In general, spot 3 (4''-deacyl derivative of MDM III), was formed quite frequently which indicates that this hydrolase is widely distributed among actinomycetes. On the other hand, it was found that several strains gave other transformation products which were detected by TLC. These results will be presented elsewhere. NAKAHAMA *et al.* have reported that *Bacillus megaterium*[8], *S. pristinaespiralis* IFO 13074 and *S. olivaceus* 219[20] transformed MDM III into 4''-depropionyl-MDM III and that *Nocardia mexicana*[10] transformed MDM III into 18-dihydro-MDM III. It is interesting that in our case both reactions (deacylation and reduction) were carried out by *S. lavendulae*.

The three transformation products especially the 18-dihydro derivatives have a greatly reduced antibacterial activity. On the other hand MDM I[21], which has isovaleryl group instead

of propionyl group at 4 position of mycarose, had higher bioactivity than MDM III. We have shown that the deacylation products can serve as substrates for the synthesis of new 4″-acylated derivatives of macrolide antibiotics. When various functional group including fatty acids have been introduced into the deacylation products, antibiotics with improved properties were obtained as will be reported in a separate communication.

Acknowledgement

We wish to thank Takeda Chemical Industries, Ltd. for the supply of maridomycin III. We also thank Miss S. NAITO, Miss M. MAENOSONO and Miss S. KISHINO for their technical assistance.

References

1) SEBEK, O.K. & D. PERLMAN: Microbiological transformation of antibiotics. Adv. Appl. Microbiol. 14: 123~150, 1971
2) MUROI, M.; M. IZAWA & T. KISHI: Maridomycin, a new macrolide antibiotic. VIII. Isolation and structures of metabolites of 9-propionylmaridomycin. J. Antibiotics 27: 449~459, 1974
3) NAKAHAMA, K.; M. IZAWA, M. MUROI, T. KISHI, M. UCHIDA & S. IGARASI: Microbial conversion of antibiotics. I. Deacylation of maridomycin by bacteria. J. Antibiotics 27: 425~432, 1974
4) FUGONO, T.; E. HIGASHIDE, T. SUZUKI, H. YAMAMOTO, S. HARADA & T. KISHI: Interconversion of T-2636 antibiotics produced by *Streptomyces rochei* var. *volubilis*. Experientia 26: 26, 1970
5) ŌMURA, S.; Macrolytic lactones produced by microorganisms, macrolide. Kagaku to Seibutsu 8: 139~150, 1970 (in Japanese)
6) INOUYE, S.; T. TSURUOKA, T. SHOMURA, S. OMOTO & T. NIIDA: Studies on antibiotic SF-837, a new antibiotic. II. Chemical structure of antibiotic SF-837. J. Antibiotics 24: 460~475, 1971
7) TSURUOKA, T.; S. INOUYE, T. SHOMURA, N. EZAKI & T. NIIDA: Studies on antibiotic SF-837, a new antibiotic. IV. Structures of antibiotics SF-837 A_2, A_3 and A_4. J. Antibiotics 24: 526~536, 1971
8) MUROI, M.; M. IZAWA & T. KISHI: Structures of maridomycin I, III, IV, V and VI, macrolide antibiotics. Experientia 28: 129~131, 1972
9) MUROI, M.; M. IZAWA, H. ONO, E. HIGASHIDE & T. KISHI: Isolation of maridomycins and structure of maridomycin II. Experientia 28: 878~880, 1972
10) NAKAHAMA, K. & S. IGARASI: Microbial conversion of antibiotics. IV. Reduction of maridomycin. J. Antibiotics 27: 605~609, 1974
11) WAKSMAN, S.A.: The actinomycetes. Vol. II. pp. 328~334. The Williams & Wilkins Co., Baltimore, M.D., 1961
12) RAYNER, R.W.: A mycological color chart. Commonwealth Mycological Inst., Kew, Surrey & British Mycological Soc., 1970
13) PRIDHAM, T. & D. GOTTLIEB: The utilization of carbon compounds by some Actinomycetales as an aid of species determination. J. Bact. 56: 107~114, 1948
14) WAKSMAN, S.A. & H.B. WOODRUFF: Streptothricin, a new selective bacteriostatic and bacteriocidal agent, particularly active against gram-negative bacteria. Proc. Soc. Exptl. Biol. Med. 49: 207~210, 1942
15) WAKSMAN, S.A.: Production and activity of streptothricin. J. Bact. 46: 299~310, 1943
16) MCLAMORE, W.M.; W.D. CELMER, V.V. BOYERT, F.C. PENNINGTON & I.A. SOLOMONS: The structure and synthesis of a new thiazolidone antibiotic. J. Am. Chem. Soc. 74: 2946, 1952
17) SOBIN, B.A.: A new streptomyces antibiotic. J. Am. Chem. Soc. 74: 2947~2948, 1952
18) ADAMSKI, R.J.; H. HEYMANN, S.G. GEFTIC & S.S. BARKULIS: Preparation and antibacterial activity of some spiramycin derivatives. J. Med. Chem. 9: 932~934, 1966
19) ŌMURA, S. & M. TISHLER: Relationship of structures and microbiological activities of the 16-membered macrolides. J. Med. Chem. 15: 1011~1015, 1972
20) NAKAHAMA, K.; T. KISHI & S. IGARASI: Microbial conversion of antibiotics. II. Deacylation of maridomycin by actinomycetes. J. Antibiotics 27: 487~488, 1974
21) ONO, H.; T. HASEGAWA, E. HIGASHIDE & M. SHIBATA: Maridomycin, a new macrolide antibiotic. I. Taxonomy and fermentation. J. Antibiotics 26: 191~198, 1973