MARIDOMYCIN, A NEW MACROLIDE ANTIBIOTIC. VII*

INCORPORATION OF LABELED PRECURSORS INTO MARIDOMYCIN AND PREPARATION OF ^{14}C-LABELED 9-PROPIONYLMARIDOMYCIN

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Incorporation of several ^{14}C-compounds into maridomycin was investigated by using *Streptomyces hygroscopicus*. Of some compounds, L-methionine-methyl-^{14}C was well incorporated into maridomycin molecule with 27 % of the added radioactivity. Degradation studies of the labeled maridomycin revealed that one methyl group in aglycone, two methyl groups in mycaminose and one methyl group in mycarose were derived from the methyl group of L-methionine.

Maridomycin (MDM) components have been isolated from culture broth of *Streptomyces hygroscopicus*[1,2] and their chemical structures elucidated as a new group of macrolide antibiotics.[3]

9-Propionylmaridomycin (PMDM) showed improved chemotherapeutic effect and low toxicity.[4,5,6] The preparation of radioactive MDM was undertaken for the study of metabolism in experimental animals,** mode of action in bacteria, and the biosynthesis.

This paper deals with the incorporation of labeled precursors into MDM and the preparation of ^{14}C-labeled PMDM.

Materials and Methods

Microorganism

A strain of *Streptomyces hygroscopicus* No. B-5050 which produced MDM was used in this experiment.

^{14}C-Compounds

The ^{14}C-compounds employed were: D-glucose-^{14}C(U), glycerol-^{14}C(U), starch-^{14}C(U), Na-acetate-^{14}C(U), Na-propionate-1-^{14}C, Na-propionate-2-^{14}C, Na-*n*-butyrate-1-^{14}C, glycine-^{14}C(U), L-alanine-^{14}C(U), L-leucine-^{14}C(U), L-isoleucine-^{14}C(U), L-methionine-methyl-^{14}C, L-lysine-^{14}C(U), L-threonine-^{14}C(U), methanol-^{14}C and ethanol-1-^{14}C (The Radiochemical Centre, Amersham, England), Na-propionate-3-^{14}C and L-proline-^{14}C(U) (New England Nuclear Corporation, U.S.A.), *n*-propanol-1-^{14}C (Tracer Lab. Calif., U.S.A.).

Measurement of radioactivity

Liquid scintilation counter (Aloka LSC 502, Nihon Musen Co., Tokyo) was used for the measurement of radioactivity. The composition of scintilator used were as follows: naphthalene (1,000 g), PPO (120 g), POPOP (3 g), dioxane (7.2 liters), toluene (1.35 liters) and MeOH (0.45 liter). TLC (thin-layer chromatography)-scanner (Aloka Model TRM-1B, Nihon Musen Co., Tokyo) was used for the detection of radioactivity.

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* The preceding papers IV, V and VI in this series correspond to the reference Nos. 4, 5 and 6 in the present paper, respectively.

** ^{14}C-Labeling position of MDM was presented to the 20th Annual Meeting of Japan Society of Chemotherapy, in Osaka, June 1972.

Calculation of incorporation ratio

The incorporation ratio was calculated from the ratio of the total radioactivity of crude powder of MDM-^{14}C to the total radioactivity of added ^{14}C-compounds.

Preparation of MDM-^{14}C

The seed culture medium (100 ml) was inoculated with the spore of *Streptomyces hygroscopicus* grown on glucose-asparagine agar and incubated at 28°C for 48 hours on a rotary shaker.[1] The production medium (400 ml) in SAKAGUCHI-flask was inoculated with the seed culture (100 ml) and incubated at 28°C for 48 hours with aeration of 0.1 liter/minute on a reciprocal shaker. Air was filtered through the sterilized cotton and was passed through 1 N NaOH solution before use. The waste air containing ^{14}C–CO_2 was washed twice with 0.2 M Hyamine X-10 (Packard Instrumental Co., Inc., U.S.A.) methanol solution and 1 N NaOH solution. The ^{14}C-compound used as precursor was dissolved in sterilized water or methanol and added to the fermentation broth at zero time.

The broth filtrate was extracted with ethyl acetate at pH 9 and the extract was washed with water, dried with Na_2SO_4 and concentrated *in vacuo*.[3] The concentrate was precipitated with the addition of *n*-hexane to give a crude powder. The crude powder was applied to silica gel TLC plate and developed in benzene-acetone (3 : 2). The band corresponding to MDM was collected and extracted with ethyl acetate.

After concentration of the extract MDM-^{14}C was crystallized from benzene.

Results

Incorporation of ^{14}C-Compounds

Each ^{14}C-compound including glucose, starch, glycerin and several amino acids was added into fermentation broth and its incorporation ratio into MDM was measured. Reults were shown in Table 1.

L-Methionine-methyl-^{14}C, Na-propionate-1-^{14}C, Na-propionate-2-^{14}C, Na-propionate-3-^{14}C and propanol-1-^{14}C were markedly incorporated into MDM. L-Leucine-^{14}C(U), L-isoleucine-^{14}C(U), Na-*n*-butyrate-1-^{14}C, Na-acetate-^{14}C(U) and glycerol-^{14}C(U) were also incorporated. Less incorporation were observed with the other compounds tested. As L-methionine-methyl-^{14}C showed the highest incorporation ratio among the markedly incorporated compounds, attempts were made to prepare ^{14}C-labeled MDM and ^{14}C-labeled PMDM from this compound.

Preparation of MDM-^{14}C and PMDM-^{14}C

L-Methionine-methyl-^{14}C (10 mCi) was added to the culture medium and incubated at 28°C for 18 hours on a reciprocal shaker. From the culture fluid, 103 mg of the crude

Table 1. Incorporation of various ^{14}C-compounds into maridomycin

^{14}C-Compound	Incorporation ratio (%)
Na-Acetate-^{14}C(U)	3.18
Na-Propionate-1-^{14}C	19.07
Na-Propionate-2-^{14}C	17.09
Na-Propionate-3-^{14}C	19.77
Na-*n*-Butyrate-1-^{14}C	3.37
L-Methionine-methyl-^{14}C	27.40
L-Leucine-^{14}C(U)	2.05
L-Isoleucine-^{14}C(U)	3.15
L-Lysine-^{14}C(U)	0.06
L-Alanine-^{14}C(U)	0.03
L-Proline-^{14}C(U)	0.02
L-Threonine-^{14}C(U)	0.61
Glycine-^{14}C(U)	0.25
Starch-^{14}C(U)	0.12
D-Glucose-^{14}C(U)	0.26
Glycerol-^{14}C(U)	2.70
Methanol-^{14}C	0.03
Ethanol-1-^{14}C	0.28
n-Propanol-1-^{14}C	12.41

Each radioactive compound was added to the culture medium at zero time and the culture medium was incubated at 28°C for 48 hours. The filtrate of the fermented broth was used for the assay of antibiotic activity and for the extraction of MDM-^{14}C. The incorporation ratio represents: (Total dpm of MDM-^{14}C)/(Total dpm of added ^{14}C-compound) × 100.

Chart 1. Degradation pathway of maridomycin III

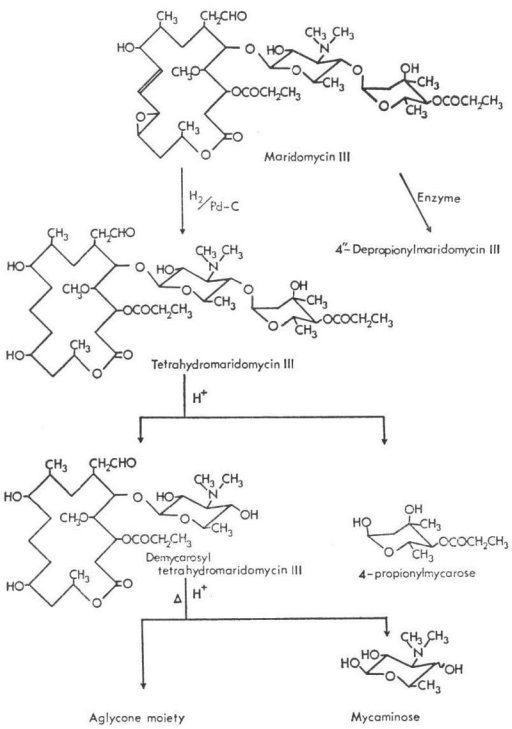


Fig. 1. Thin-layer chromatograms of maridomycin-^{14}C prepared from L-methionine-methyl-^{14}C.

Solvent system,
A; chloroform-dimethylamine-H_2O (10 : 3 : 10), lower layer
B; benzene-acetone (1 : 2)
C; benzene-acetone (3 : 2)

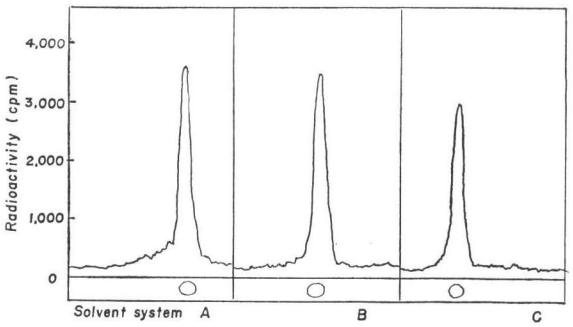
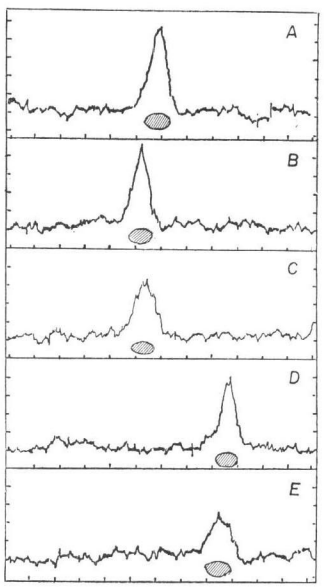


Fig. 2. Thin-layer chromatograms of the degradation products of maridomycin-^{14}C

A; 4''-Depropionylmaridomycin III; benzene-acetone (1 : 1)
B; Tetrahydromaridomycin III; benzene-acetone (1 : 1)
C; Propionylmycarose; benzene- acetone (3 : 2)
D; Demycarosyltetrahydromaridomycin III; chloroform-methanol (5 : 1)
E; Mycaminose; *n*-butanol-acetic acid-H_2O (3 : 1 : 1)



powder with the specific radioactivity of 26.4 μCi/mg was obtained (total radioactivity, 2.74 mCi). The crude powder was purified by TLC described above. The total radioactivity of MDM-^{14}C thus obtained was 1.14 mCi.

After the addition of non-labeled MDM, MDM-^{14}C was crystallized from benzene to yield 1.27 g of pure MDM-^{14}C (0.82 μCi/mg). As shown in Fig. 1, MDM-^{14}C showed the single peak on the radioautgrams by TLC using several solvent systems.

To a solution of MDM-^{14}C (3.9 mCi, 3.5 g) in pyridine (14 ml) propionyl chloride (1.2 ml) was added dropwise at 5~10°C and the mixture was stirred for 2 hours.[4] After addition of 2% $NaHCO_3$ in ice water, the reaction mixture was extracted with ethyl acetate (400 ml). The extract was washed with water, dried and concentrated *in vacuo*. Addition of petroleum ether gave PMDM-^{14}C (0.965 μCi/mg, 3.0 g). The purity of PMDM-^{14}C was calculated to be more than 97% by the TLC-radioautograhy.

To determine the labeled position in MDM-^{14}C prepared from L-methionine-methyl-^{14}C, degradation studies of MDM-^{14}C by the chemical and enzymatic reaction were carried out as shown in Chart 1. MDM III-^{14}C, a main component of MDM-^{14}C was purified according to the procedure previously reported[2] and was used for this experiment.

Preparation of 4''-Depropionyl MDM III-^{14}C

Bacillus megaterium[7] was grown for 3 days in nutrient broth on a rotary shaker. The cells were harvested by centrifugation, resuspended in the fresh medium (20 ml) and incubated with the addition of MDM III-^{14}C (0.68 μCi) for 18 hours at 37°C by shaking. After incubation, the supernatant was extracted with ethyl acetate at pH 8. The extract was washed with water and concentrated *in vacuo*. The residue was applied to TLC according to the same method described in the isolation of MDM-^{14}C. The recovery of 4''-depropionyl MDM III-^{14}C was 92.9 % from the TLC-radioautogram as shown in Fig. 2, A.

The specific radioactivity of 4''-depropionyl MDM III-^{14}C purified with TLC was 679 μCi/mM, equal to that of the starting material, MDM III-^{14}C (681 μCi/mM). From the result, it is apparent that L-methionine-methyl-^{14}C is not incorporated into 4''-propionyl group of MDM III-^{14}C.

Preparation of Tetrahydro MDM III-^{14}C

To prepare the tetrahydro (TH)-MDM III-^{14}C, the mixture of MDM III (600 mg) and MDM III-^{14}C (200 mg, 200 μCi) in ethanol (20 ml) was hydrogenated with 10 % palladium-charcoal (200 mg, Engelhard Co.). After the addition of non-labeled TH-MDM III (20 g), the reaction mixture was concentrated *in vacuo* and *n*-hexane was added to the concentrated solution to give TH-MDM III-^{14}C (20.4 g, 190 μCi). The specific radioactivity of TH-MDM III-^{14}C was 7.77 μCi/mM and its purity was 94.2 % from TLC-radioautogram (Fig. 2, B).

Table 2. Distribution of ^{14}C in the degradation products of maridomycin-^{14}C biosynthesized from L-methionine-methyl-^{14}C

Degradation product	Specific radioactivity	
	μCi/mM	%
a) Tetrahydro MDM III-^{14}C	7.77	100
b) Propionylmycarose-^{14}C	2.20	28
c) Demycarosyl tetrahydro MDM III-^{14}C	5.29	68
d) Mycaminose-^{14}C	3.47	45
b)+c)=Tetrahydro MDM III-^{14}C	7.49	97
c)−d)=Aglycone part	1.82	23

* Expressed as relative value, taking the specific radioactivity of tetrahydro MDM III-^{14}C as 100 (%).

Hydrolysis of Tetrahydro MDM III-^{14}C

For the hydrolysis of TH-MDM III-^{14}C into propionylmycarose (PM)-^{14}C and demycarosyl (DM)-TH-MDM III-^{14}C,[3] a solution of TH-MDM III-^{14}C (15 g) in 0.5 N HCl (300 ml) was kept at 25°C for 5 hours. The reaction mixture was adjusted to pH 2.0 and extracted with ethyl acetate (1,000 ml×2). The aqueous layer was then readjusted to pH 8.5 and extracted with chloroform (1,500 ml×2). Each extract was washed with water and concentrated separately. Crystallization of the residue of the ethyl acetate at pH 2.0 from *n*-hexane gave PM-^{14}C (1.6 g, 2.20 μCi/mM) and its purity was 100 % estimated by TLC-radioautogram (Fig. 2, C). From the chloroform extract, crude DM-TH-MDM III-^{14}C (6.5 g) was obtained. This crude material (1.5 g) was purified with column chromatography of silica gel (50 g, 0.05~0.2 mm, Merck A. G., Germany) and eluted with a solvent system of benzene-

ethyl acetate (1 : 1). The eluate was concentrated to obtain the pure DM-TH-MDM III-^{14}C (1.0 g, 5.29 μCi/mM). The purity of DM-TH-MDM III-^{14}C assayed by TLC-radioautogram was 93.6 % (Fig. 2, D). And also, mycaminose (MC) hydrochloride was obtained by acid hydrolysis of DM-TH-MDM III-^{14}C.[3] A solution of DM-TH-MDM III-^{14}C (5.0 g) in 2 N HCl (100 ml) was refluxed for 3 hours. After filtration, the reaction mixture was diluted with H_2O (500 ml) and washed with *n*-butanol (300 ml×3), and then, concentrated *in vacuo*. The residue was chromatographed on a column of Dowex 50 W×2 (30 ml, 100~200 mesh, Dow Chem. Co., U.S.A.) and eluted stepwise with H_2O, 0.5 N HCl and 1 N HCl. Radioactivity was detected in 0.5 N HCl eluate and 95 % of the radioactivity was recovered. The radioactive fraction was collected and concentrated *in vacuo*. The residue washed with acetone (100 ml×3) was extracted with methanol. The extract was concentrated to give MC·HCl-^{14}C (368 mg). It showed the specific radioactivity of 3.47 μCi/mM and the purity of 91.7 % from TLC-radioautogram (Fig. 2, E).

These results are summarized in Table 2.

Since no radioactivity was found in 4″-propionyl group of MDM III-^{14}C prepared from L-methionine-methyl-^{14}C as a precursor, the labeling ratio of mycarose: mycaminose: aglycone moiety is accounted to be 28 : 45 : 23.

Discussion

Biosynthetic studies of erythromycin, a 14-membered macrolide antibiotic by *Streptomyces erythreus*, have been revealed that the aglycone of this antibiotic, erythronolide, was derived from acetate units, N-methyl group of desosamine and C-methyl and O-methyl groups in L-cladinose from L-methionine-methyl and cladinose and desosamine from D-glucose.[8,9]

In 16-membered macrolide antibiotics, the biosynthesis of carbomycin (magnamycin) was investigated. It has been found that the aglycone moiety of carbomycin was derived from acetate and propionate, mycarose and mycaminose were from D-glucose, four methyl groups (3″-methyl, N-dimethyl and 4-O-methyl groups) from L-methionine-methyl and isovaleryl group at C-4 of mycarose from L-leucine.[10,11,12] ACHENBACH *et al.*[13] have reported that the radioactivity of carbomycin-^{14}C derived from L-methionine-methyl-^{14}C distributed in a O-methyl group in the aglycone (27 %), N-dimethyl in mycarose (46 %), 3″-methyl in mycarose (24 %) and isovaleryl in mycarose (0 %).

Prior to the detailed studies on the biosynthesis of MDM having 16-membered aglycone, the incorporation of various ^{14}C-compounds into MDM was investigated.

L-Methionine-methyl-^{14}C, Na-propionate-1-^{14}C, Na-propionate-2-^{14}C, Na-propionate-3-^{14}C, Na-acetate-^{14}C(U), Na-*n*-butyrate-1-^{14}C, *n*-propanol-1-^{14}C, glycerol-^{14}C(U), L-leucine-^{14}C(U) and L-isoleucine-^{14}C(U) were resulted to be incorporated into MDM to a significant extent.

MDM was efficiently labeled by the fermentation of *Streptomyces hygroscopicus* in the presence of L-menthionine-methyl-^{14}C. Thus, L-methionine-methyl-^{14}C was selected for the preparation of ^{14}C-labeled MDM. MDM-^{14}C prepared from L-methionine-methyl-^{14}C was divided into two parts, one part for the preparation of PMDM-^{14}C and the other for the degradation studies.

The degradation studies of MDM III-^{14}C prepared from L-methionine-methyl-^{14}C revealed that the labeling ratio of 4″-acyl group-mycarose-mycaminose-aglycone was accounted to be 0 : 28 : 45 : 23. The labeling ratio of MDM III-^{14}C thus obtained shows good agreement with that of carbomycin-^{14}C. It is assumed that radioactivity of L-methionine-methyl-^{14}C is incorporated into one methyl group of aglycone, two methyl groups of mycaminose and one methyl group of mycarose in MDM III.

It was found that Na-propionate-1-^{14}C, Na-propionate-2-^{14}C and Na-propionate-3-^{14}C were

incorporated into MDM in approximately the same incorporation ratio (17~19 %). The incorporation at the same ratio might be interpreted as the direct incorporation of one propionate unit into macrocyclic lactone of MDM without the decarboxylation of propionate, as reported in the biosynthesis of carbomycin.[11]

Na-Acetate-^{14}C(U) was incorporated into MDM. It seems to serve as the precursor of MDM lactone ring. The lower incorporation ratio of acetate-^{14}C than propionate-^{14}C might be depend on the cultural conditions or the physiological characteristics of this producing organism.

The incorporation ratio of *n*-propanol-1-^{14}C into MDM was 12.4 %. The incorporation of *n*-propanol-1-^{14}C into MDM in the same degree as that of labeled propionate might predict that both precursors were metabolized on the same pathway.

Of interest is the efficient incorporation of Na-*n*-butyrate-1-^{14}C in MDM even though no 4''-*n*-butyryl-4''-deacyl MDM was formed in the fermented broth under the cultural conditions used. It seems that *n*-butyrate does not serve as the C-4'' acylating unit but is incorporated into MDM aglycone moiety through a metabolic pathway similar to propionate. If the incorporation of *n*-butyrate-^{14}C into MDM was related to the origin of C-6, C-17 and C-18 carbon atoms, it may be conceivable that aldehyde group at C-18 was formed by the oxidation of methyl group in the biosynthesis of this antibiotic. This interpretation might be supported by the fact that FURUMAI *et al.* recently isolated the biogenetic intermediates of macrolide which have methyl group at C-18.[14]

Glycerol-^{14}C(U) was well incorporated into MDM under the condition used, but no significant incorporation was observed in D-glucose-^{14}C(U). The direct comparison of incorporation ratio between glycerol-^{14}C and D-glucose-^{14}C is not suitable, because D-glucose-^{14}C(U) was diluted with the fermentation medium cotaining non-labeled D-glucose.

Our attention is being directed to the fact that L-leucine-^{14}C(U) and L-isoleucine-^{14}C(U) were also significantly incorporated into MDM.

The role of L-leucine and L-isoleucine for the MDM biosynthesis was investigated and will be published in near future by M. UCHIDA *et al.* and by the authors.

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