BIOSYNTHESIS AND THE METABOLIC FATE OF CARBON-14 LABELED SPIRAMYCIN I

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Spiramycins are 16-membered macrolide antibiotics produced by *Streptomyces ambofaciens*[1], the structures of which are shown in Fig. 1. For studying metabolic behaviors of the antibiotics in animals, radioactive spiramycins have been prepared by biosynthetic means.

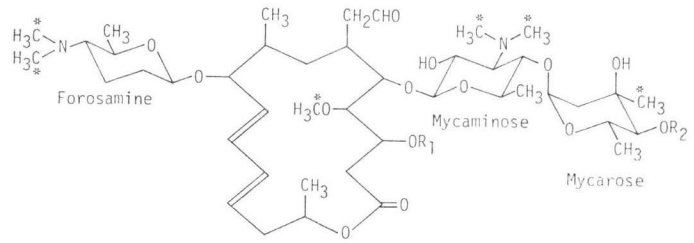
In the present paper we wish to report on the preparation of ^{13}C or ^{14}C labeled spiramycin I (SPM-I) from L-[methyl-^{13}C or ^{14}C]methionine, the biosynthetic origin of methyl groups in SPM-I and the formation of polar metabolites of SPM-I in rat. SPM-I, -II and -III were labeled by the fermentation of *S. ambofaciens* in the presence of L-[methyl-^{14}C]methionine (54.1 mCi/mmole, 2 mCi/1.5 liters medium). About 51% of the radioactivity of L-[methyl-^{14}C]methionine was incorporated into spiramycins. From the resulting mixture spiramycin I was isolated by silica gel chromatography. The specific activity and radiochemical purity of [^{14}C]SPM-I were 0.42 mCi/mmole and 96.5%, respectively.

Biosynthetic labeling of SPM-I was attempted using L-[methyl-^{13}C]methionine (50 mg/1.5 liters medium) in the same manner as [^{14}C]SPM-I. ^{13}C NMR spectra (JNM-FX-100, JEOL Co., Ltd.) of enriched SPM-I indicated that the methoxy carbon of the aglycone, the dimethylamino carbons of mycaminose and forosamine and the C-3″ methyl carbon of mycarose were enriched by ^{13}C, which appeared at 61.8, 42.0, 40.7 and 25.4 ppm, respectively. Whereas no significant incorporation was observed in the other methyl groups. Therefore, these six methyl carbons of SPM-I were derived from methionine.

Our results are consistent with the findings on magnamycin group[2,3,4]. Further it has been found that *N*-dimethyl group of forosamine was also derived from L-methionine-methyl.

The stability of SPM-I, SPM-II and 4″-*O*-acetylspiramycin II (ASPM) in liver homogenate (40 μg/g) and plasma (20 μg/ml) at 37°C are shown in Fig. 2. No significant inactivation of SPM-I, SPM-II or ASPM was observed in liver homogenate as estimated by microbial assay (Fig. 2-b). Although ASPM maintained its initial antimicrobial activity in liver homogenate, chromatographic analysis revealed that the reaction mixture contained solely SPM-II; ASPM was in fact deacetylated at C-4″ position. The apparent stability of ASPM was due to the antibacterial activity of SPM-II, which is more potent

Fig. 1. Chemical structures of spiramycins.



	R_1	R_2
Spiramycin I	H	H
Spiramycin II	$COCH_3$	H
Spiramycin III	COC_2H_5	H
4″-*O*-Acetylspiramycin II (ASPM)	$COCH_3$	$COCH_3$

* The methyl group derived from [methyl-^{14}C or ^{13}C]methionine.

Fig. 2. Stability of SPM-I, SPM-II and ASPM in rat plasma (a) and liver homogenate (b) at 37°C.

Liver homogenate was prepared by homogenizing rat liver in three volumes of 0.01% phosphate buffer (1.15% KCl, pH 7.4). One hundred μg of SPM-I, SPM-II or ASPM was added to 5 ml rat plasma (a: 20 μg/ml) or 10 ml liver homogenate (b: 40 μg/g wet tissue) and incubated at 37°C. The antimicrobial activity was estimated by an agar diffusion assay with *Micrococcus luteus* ATCC 9341.

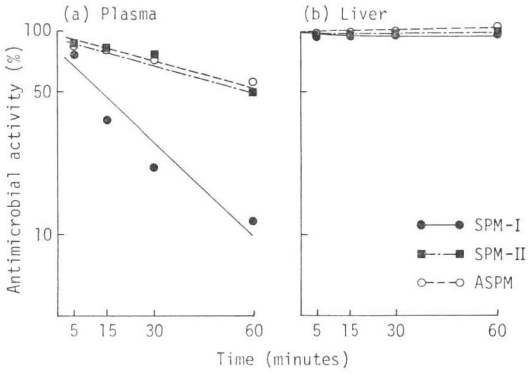
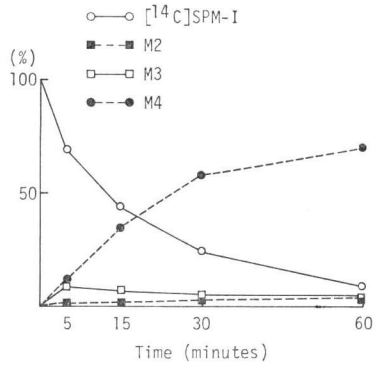


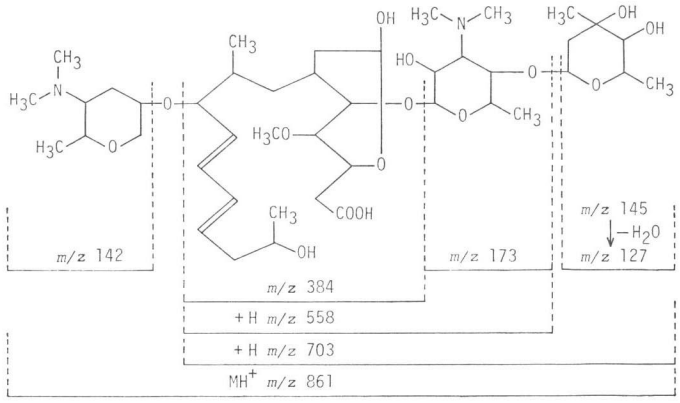
Fig. 3. Degradation of [^{14}C]SPM-I and liberation of its metabolites in rat plasma at 37°C.

One mg of [^{14}C]SPM-I was added to 1 ml rat plasma, and incubated at 37°C for 5, 15, 30 and 60 minutes. The separation of [^{14}C]SPM-I, non-polar metabolites (M2, M3) and a polar metabolite (M4) were carried out as described in the text. The percent radioactivity of a compound over the total radioactivity in the reaction mixture plotted against time.



in vitro than ASPM (K. KITAURA *et al.*, unpublished results). However, in plasma SPM-I was less stable than SPM-II or ASPM and was degraded mainly to a polar metabolite. The degradation product of SPM-I was isolated as follows. To the reaction mixture an equal volume of acetone was added and centrifuged at 3,000 rpm for 10 minutes. The supernatant fluid was concentrated *in vacuo* and the resulting aqueous layer was extracted with ethyl acetate. The ethyl acetate layer was concentrated *in vacuo* to dryness, dissolved in a small amount of methanol, developed on a silica gel thin layer (F_{254} plate, E. Merck) with methanol - carbon tetrachloride (9 : 1). The aqueous layer was applied to a column of Amberlite XAD-2 and eluted with methanol. The eluate was concentrated *in vacuo*, dissolved in a small amount of methanol and isolated by silica gel thin layer chromatography (F_{254} plate, E. Merck) with methylethylketone - acetic acid - water (3 : 1 : 1). The metabolites were detected by autoradiography with industrial X-ray films (Fuji). The radioactivity of the metabolites was determined by a liquid scintillation spectrometer (Aloka LSC-653). Fig. 3 shows the time-course of

Fig. 4. Diagnostic fragmentation of M4 (FAB mass spectrum).



[^{14}C]SPM-I degradation to non-polar metabolites (M2, M3) and a polar metabolite (M4). The degradation of SPM-I followed Michaelis-Menten kinetics. The apparent kinetic constants were determined by conventional Lineweaver-Burk plots to be a *Km* of 0.77 mM and *Vmax* of 6.78×10^{-5} mmole/minute.

Fast atom bombardment (FAB) mass spectrum of M4 was taken with a JEOL JMS-DX-300. The diagnostic fragmentation of M4 is shown in Fig. 4. The metabolite showed an intense protonated molecule ion peak (MH^+) at *m/z* 861, and a peak at *m/z* 883 resulted from association of a sodium ion to the M4 molecule (MNa^+). The fragment ions arising from forosamine, mycaminose and mycarose were observed at *m/z* 142, 173 and 145, respectively. These fragment patterns indicated the sugar moieties of M4 were the same as those of SPM-I.

Carbon chemical shifts due to the sugar moieties of M4 were consistent with those of SPM-I in the ^{13}C NMR spectra. Whereas, the chemical shifts due to C-1 which appeared at 174.1 ppm in SPM-I shifted to 180.5 ppm in M4, indicating that the lactone ring was hydrolyzed in the latter. This was also supported by the ^{1}H NMR spectrum, namely, H-15 of M4 was observed at around 3.9 ppm which is about 1.4 ppm higher than that of SPM-I. In addition, in the ^{13}C NMR spectrum of M4 no signal due to an aldehyde was observed while a new signal appeared at 93.9 ppm assignable to a hemiacetal carbon. In the field of SPM chemistry, it is known that a hemiacetal ring can be formed between 17-aldehyde and 3-OH with the macrolide ring retained intact (K. SHIRAHATA *et al.*, unpublished result). Thus the chemical structure of M4 was proposed as shown in Fig. 4.

The *in vitro* metabolism data indicated that SPM-II and ASPM were stable in rat plasma, but the 4″-*O*-acetyl group of ASPM was removed in liver to give more potent SPM-II. On the other hand, SPM-I was inactivated in rat plasma to give the polar metabolite, M4. Thus it was assumed that the 3-*O*-acetyl group played an important role in influencing the rate of hydrolysis of the lactone moiety of spiramycins.

From these results it is suggested that the high antimicrobial activity observed for ASPM in experimental mice infections[5] and the long biological half-life ($T_{1/2}$)[6] of ASPM might be caused by the resistance against the hydrolysis of the macrolide ring.

Acknowledgment

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References

1) PINNERT-SINDICO, S.; L. NINET, J. PREND'S HOMME & C. COSAR: A new antibiotic-spiramycin. Antibiot. Ann. 1954/1955: 724~729, 1955
2) ACHENBACH, H. & H. GRISEBACH: Zur Biogenese der Makrolide. XI. Weitere Untersuchungen zur Biosynthese der Magnamycins. Z. Naturforch. 19b: 561~568, 1964
3) ONO, H.; S. HARADA & T. KISHI: Maridomycin, a new macrolide antibiotic. VII. Incorporation of labeled precursors into maridomycin and preparation of ^{14}C-labeled 9-propionylmaridomycin. J. Antibiotics 27: 442~448, 1974
4) GERSCH, D.; H. BOCKER & H. THRUM: Biosynthetic studies on the macrolide antibiotic turimycin using ^{14}C-labeled precursors. J. Antibiotics 30: 488~493, 1977
5) TAKAHIRA, H.: The studies on spiramycin derivatives. II. Jap. J. Antibiotics 23: 429~433, 1970
6) INOUE, A. & T. DEGUCHI: The pharmacokinetic studies on spiramycin and acetylspiramycin. Jap. J. Antibiotics 35: 1998~2004, 1982