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Isolation and Structure of Two Metabolites of a Macrolide Antibiotic, SF-837 Substance

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M_1 Substance, one of the main metabolites of SF-837, was isolated from the urine of rats, or digested solution of SF-837 with a liver homogenate, and identified as 4″-depropionyl SF-837 by direct comparison. Another major metabolite, M_2 substance was isolated from the bile of rats, and its structure was proposed to be 14-hydroxyl M_1 on the basis of the mass and nuclear magnetic resonance analyses.

SF-837 Substance is a new macrolide antibiotic isolated in this laboratories,[2] and its structure has been established to be a glycoside of sixteen membered lactone (**1**) belonging to leucomycin-carbomycin family.[3] The metabolism of this new antibiotic was studied in relation to the mechanism of action, and it was shown that the main metabolic pathway involved the conversion of SF-837 into two metabolites, M_1 and M_2 substances.[4] As schematically shown in Fig. 1, M_1 substance was found mainly in blood and urine, and M_2 substance in bile.[4]

The present paper describes the isolation and structure determination of these two principal metabolites.

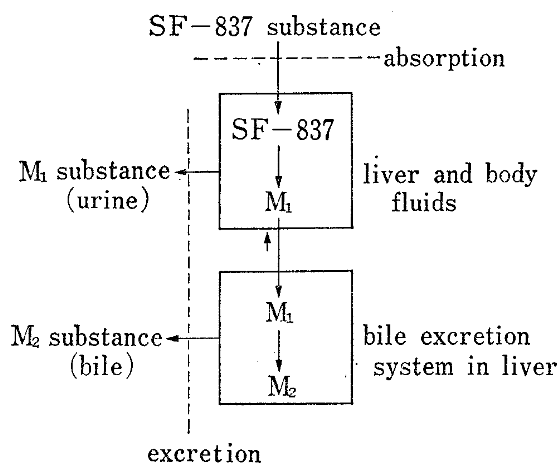
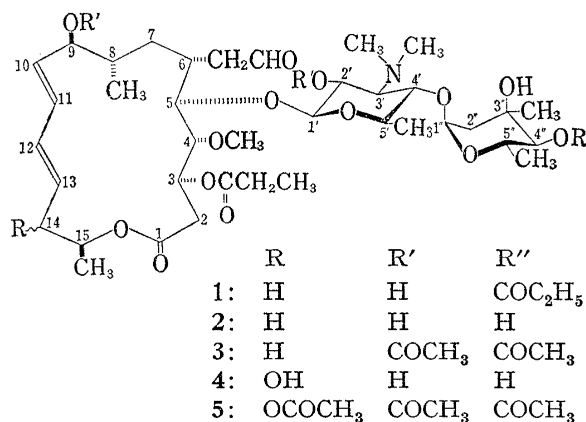


Fig. 1. Metabolic Pathway of SF-837 *in Vivo*



	R	R′	R″
1:	H	H	COC_2H_5
2:	H	H	H
3:	H	$COCH_3$	$COCH_3$
4:	OH	H	H
5:	$OCOCH_3$	$COCH_3$	$COCH_3$

Chart 1

Isolation and Structure of M_1 Substance (2)

M_1 Substance was conveniently isolated from the urine of rats administered SF-837 subcutaneously. Solvent extraction of the urine at pH 9—10, followed by transfer of the extracts into water at pH 2—3 and reextraction with ethyl acetate at pH 9 gave a mixture

1) Location: *Morooka, Kohoku-ku, Yokohama.*

2) T. Niida, T. Tsuruoka, N. Ezaki, T. Shomura, E. Akita, and S. Inouye, *J. Antibiotics*, **24**, 319 (1971).
3) S. Inouye, T. Tsuruoka, T. Shomura, S. Omoto, and T. Niida, *J. Antibiotics*, **24**, 460 (1971).
4) T. Shomura, I. Komiya, and K. Umemura, Presented at the 92nd Annual Meeting of Japan Pharmaceutical Association, Abstracts of Papers, IV-75p (1972): *Yakugaku Zasshi*, in preparation.

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of metabolites. This was separated by the preparative thin-layer chromatography (TLC) using silica gel plates, and M_1 substance was isolated as an amorphous powder from a main band absorbing ultraviolet (UV) light. M_1 Substance was prepared also with high yield by digesting aqueous solution of SF-837 with a liver homogenate of rats *in vitro* or with low yield from the bile of rats as described in the experimental part of M_2 substance.

M_1 Substance shows UV_{max} at 232 mμ, and has the antibacterial activity, about one-fourth of the parent antibiotic (**1**) *in vitro*.[5] The UV, infrared (IR) and nuclear magnetic resonance (NMR) spectra of M_1 were very close to those of SF-837, indicating only slight modification of the structure.

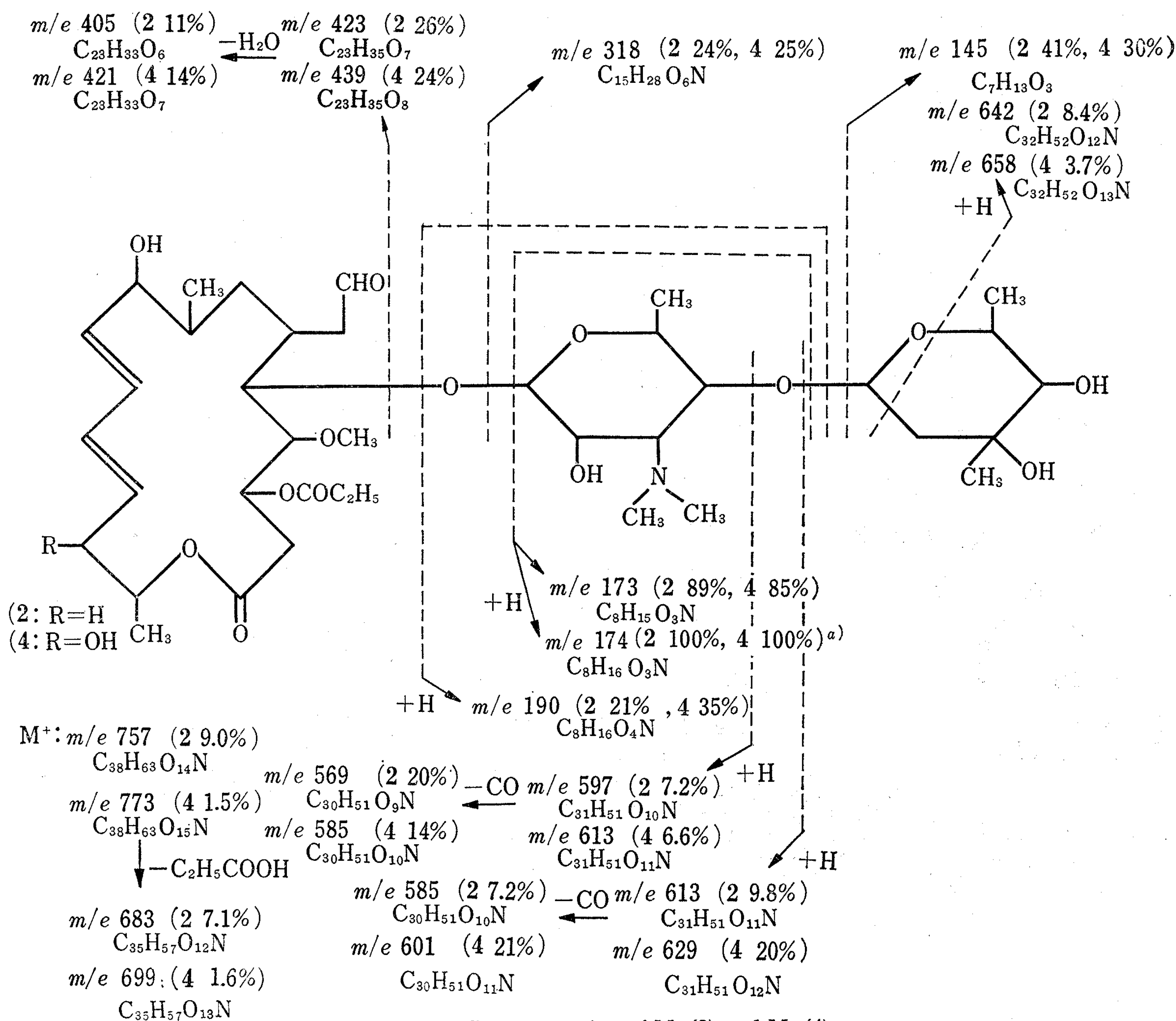

Chart 2. Fragmentation of M_1 (2) and M_2 (4)

The spectra were taken at 190°. The base peak was arbitrarily selected from the peaks above *m/e* 145.
a) Intensity increased markedly upon raising temperature above 200°

The mass spectrum of M_1 showed the M^+ at *m/e* 757, O-propionyl lactone fragments at *m/e* 423 and 405, a mycarosyl-mycaminose ion at *m/e* 318, mycaminose ions at *m/e* 174 and 173, and a mycarose ion at *m/e* 145 (Chart 2). Analysis of these fragment ions suggested that M_1 substance was 4″-depropionyl SF-837. Therefore, the physical properties of M_1

5) K. Kawarajo, T. Yoshida, T. Watanabe K. Miyauchi, B. Nomiya, S. Tada, and S. Kuwahara, *Nihon Kagakuryoho Gakukaishi*, **20**, No. 5 (1972).

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were compared with those of 4″-depropionyl SF-837 derived from microbiological transformation of SF-837 with *Mucor spinescense*.[6] IR, mass and NMR spectra, optical rotations and *Rf* values of both compounds were identical, thereby establishing the structure of M_1 (**2**). The identity was further confirmed by comparing the crystalline tri-O-acetyl M_1 (**3**) with the authentic sample of 2′,4″,9-tri-O-acetyl-4″-depropionyl SF-837.[6]

Isolation and Structure of M_2 Substance (4)

M_2 Substance was formed only *in vivo* so far examined, and no *in vitro* formation was demonstrated by treating SF-837 or M_1 either with a liver homogenate or with bile alone.[4] Accordingly, M_2 substance was isolated from the bile of rats administered SF-837 subcutaneously. An aqueous solution of the bile collected from the rats cannulated the common

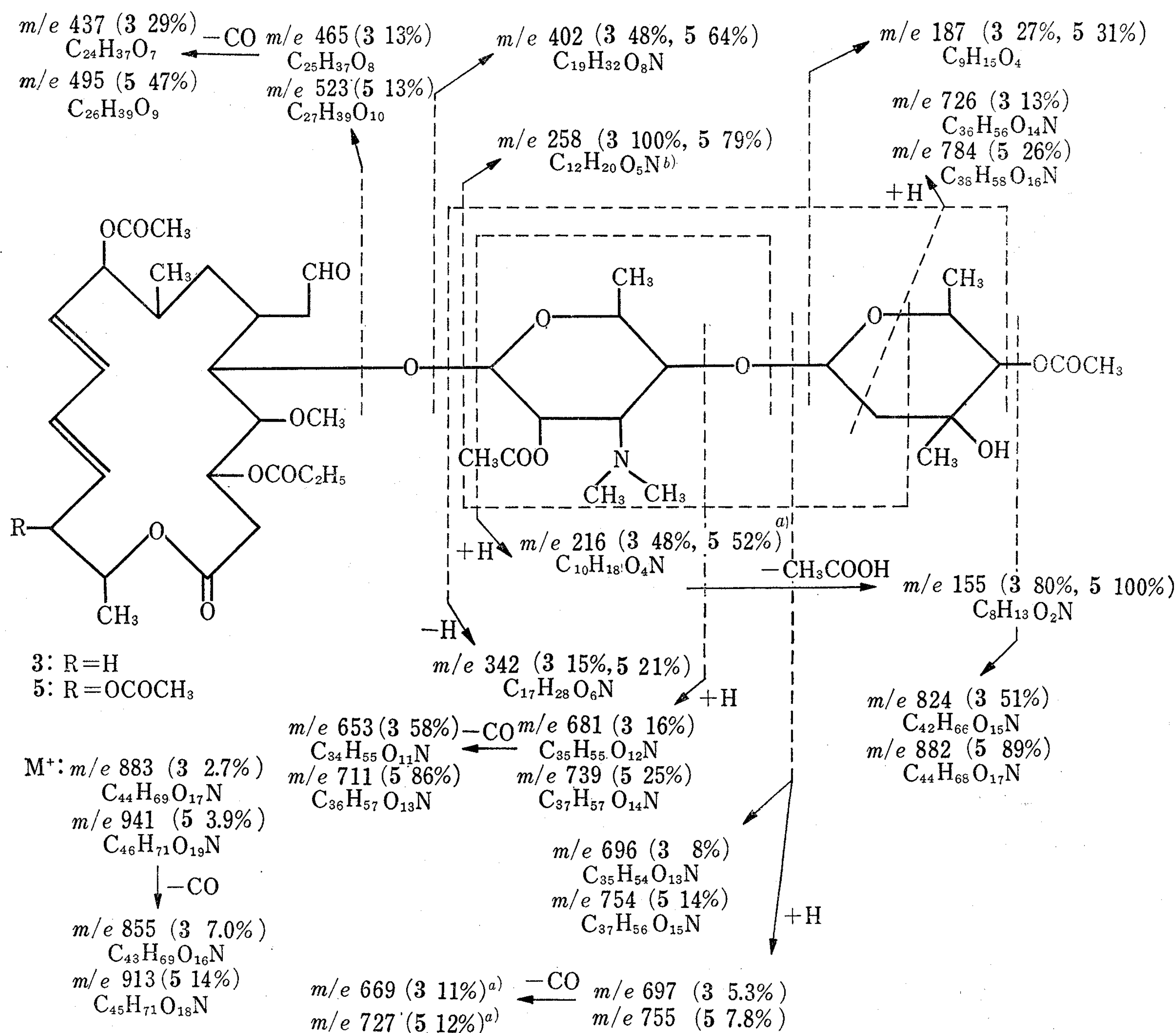

Chart 3. Fragmentation of Tri-O-acetyl M_1 (3) and Tetra-O-acetyl M_2 (5)

The spectra were taken at 150° (**3**) and 180° (**5**). The base peak was arbitrarily selected from the peaks above *m/e* 155.

a) Intensity increased markedly upon raising temperature above 190°

b) a doublet with the $C_{13}H_{24}O_4N$ ion

6) T. Tsuruoka, S. Inouye, T. Shomura, N. Ezaki, and T. Niida, *J. Antibiotics*, **24**, 526 (1971).

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bile ducts was extracted with ethyl acetate under alkaline condition. The extracts were purified by the procedure similar to that employed for the preparation of M_1.

M_2 Substance was obtained as an amorphous powder, after column chromatography over silicic acid. It shows UV_{max} at 232 mμ, and the antibacterial spectrum similar to that of SF-837, with the *in vitro* activity one-eighth of the parent antibiotic (**1**).[5] Treatment of M_2 with acetic anhydride in pyridine gave the crystalline tetra-O-acetyl M_2 (**5**).

Mass fragmentation data of M_2 and tetra-O-acetyl M_2 were summarized in Chart 2 and 3, together with those of M_1 and tri-O-acetyl M_1. The sugar fragment ions of M_2 were identical with those of M_1, showing a mycarose ion at *m/e* 145, mycaminose ions at *m/e* 173, 174 and 190, and a mycarosyl-mycaminose ion at *m/e* 318. However, the fragment ions involving the lactone moiety of M_2 increased 16 mass units more than the corresponding ions of M_1 (Chart 2). The same argument was valid for the mass fragmentation of tetra-O-acetyl M_2 and tri-O-acetyl M_1. No increment of the *m/e* value was observed for the sugar fragment ions, whereas the lactone ions of acetyl M_2 showed the increment of 58 mass units (CH_3COO minus H) compared to those of M_1 (Chart 3). Thus, the mass spectrometric analysis revealed that M_2 was a hydroxyl derivative of M_1, and that a newly introduced OH group was located on the lactone ring, but not on the sugar moieties. More detailed mass analysis suggested that a propionyl group on the lactone remained intact, since a depropionic acid ion formed by thermal decomposition was observed at *m/e* 699.

The substituted position of the hydroxyl group on the lactone ring was examined by NMR spectroscopy. Figures 2 and 3 showed NMR spectra of M_2 and M_1. Utilizing the

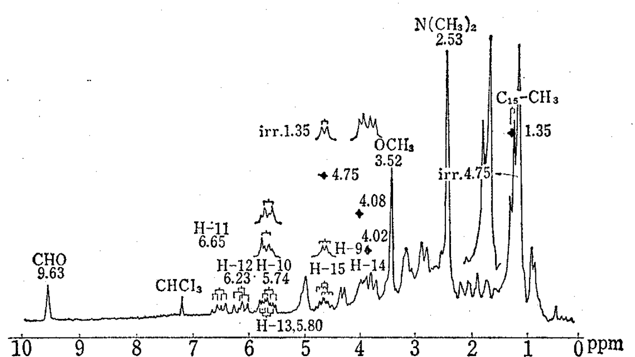

Fig. 2. NMR Spectra of M_2 Substance in Deuteriochloroform at 100 MHz

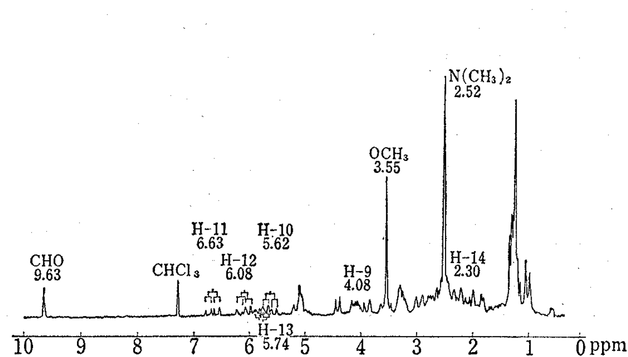

Fig. 3. NMR Spectrum of M_1 Substance in Deuteriochloroform at 100 MHz

decoupling technique, the chemical shifts of H-9, H-10, H-11, H-12, H-13, H-14 and H-15 signals of the lactone ring were estimated. Thus, the NMR spectrum of M_2 showed a quartet at δ 6.65 assignable to H-11, a quartet of H-12 at 6.23, and two quartets of H-10 and H-13 in 5.74 and 5.80, respectively.

An octet corresponding to one proton appeared at 4.75, well separated from other signals in the spectrum of M_2. Irradiation of this signal caused the alteration of signals around 4.0 and the collapse of a doublet at 1.35 into a singlet. Reversely, irradiation of the 1.35 signal caused the collapse of an octet into a doublet.[7] These results indicated the coupling between methine and methyl protons of -CH-CH_3 group. There present four -CH-CH_3 groups in the molecules of M_2 and M_1, of which two are on the lactone ring at C-8 and C-15, and the other two on the sugar moieties at C-5′ and C-5″. The 4.75 signal was not present in the same field in the spectrum of M_1, and apparently caused to shift by the effect of the newly introduced

7) Irradiation at 1.35 caused simultaneous alteration of the complex signals around 4.0. This was explained by the decoupling of the overlapped signals of 5′- or 5″-CH-CH_3 of the sugar moieties. Measurement of the signal intensity indicated the presence of at least four proton signals from 3.8 to 4.1.

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hydroxyl group. Therefore, it seemed reasonable to assign it to the methyl-methine group of the lactone ring, since the spectral pattern of the sugar moieties of M_2 should follow the pattern of M_1.[8] Considering from the chemical shift of this signal, a possible assignment to the C-8 methine was easily excluded, and the 4.75 signal was finally ascribed to the C-15 methine proton of the lactone ring.

Irradiation at 4.02 caused the collapse of an octet of the H-15 signal into a quartet, together with spectral change of the H-13 signal as shown in Fig. 2. On the other hand, irradiation at 4.08, slightly lower field than the 4.02 signal position, caused spectral change of the H-10 signal at 5.74, but no change of the H-15 signal at 4.75. Accordingly, the 4.02 and 4.08 signals were assigned to the H-14 and H-9 signals, respectively.

Chemical shift of the H-14 signals at 2.30 in the spectrum of M_1 was determined by irradiating the H-13 signal at 5.74. Reverse irradiation at 2.30 caused the change of an octet of the H-13 into a doublet, thereby confirming the assignment. Chemical shift of the H-9 signal of M_1 at 4.08 was determined similarly by the decoupling experiment with the H-10 signal at 5.62.

Comparison of the chemical shifts of the lactone methine signals of M_2 and M_1 indicated that the H-14 signal of M_2 was down-field shifted about 1.7 ppm compared to that of M_1, while the H-9 signal remained constant in M_2 and M_1. The large down-field shifting of the H-14 signal could be explained only by the substitution of a polar group at C-14, which must be a hydroxyl group as assessed by the mass spectrometric analysis. The coupling constants determined on the basis of the decoupling experiments were $J_{14,15}$ 10 Hz and J_{15,CH_3} 6 Hz. The latter value was comparable to those observed for the CH-CH_3 groups in erythromycin,[10] and the large $J_{14,15}$ value suggested *trans* diaxial or *trans* diequatorial relationship between H-14 and H-15 in solution.[10]

Therefore, the structure of M_2 (**3**) was shown to be 14-hydroxyl M_1 by the mass and NMR analyses. Chemical proof of this structure is in progress.

Concerning the metabolism of macrolide antibiotics, deacylation[11] and de-N-alkylation[12] have been reported. The formation of M_1 was undoubtedly due to depropionylation by an esterase distributed in a liver and body fluids. However, to the best of our knowledge, the hydroxylation of antibiotics *in vivo* has not been reported, and this paper presents the first example of the metabolic pathway of the macrolide antibiotics *via* hydroxylation.

Experimental

NMR Spectra were measured at 100 MHz with a Varian HA-100 spectrometer, with TMS as internal standard. Mass spectra were recorded on a JMS-01SG high resolution mass spectrometer at an ionizing potential of 75 eV, and with electric recording for the low-resolution spectra and with photographic recording for the high-resolution data.

Isolation of M_1 Substance from the Urine of Rats——An aqueous solution of SF-837 tartarate (1 g/8 ml) was administered subcutaneously to 8 male albino rats (Donryu, 250—350 g) cannulated urinary bladder. The urines excreted for 48 hr after injection were combined, and pH was raised to 9—10. After saturation with $(NH_4)_2SO_4$, metabolites were extracted three times with AcOEt (each two volumes). The extracts were combined, and concentrated to one-tenth volume. The metabolites in the organic layer were transfered to water acidified with HCl (pH 3), and then reextracted with fresh AcOEt at pH 9. The final extract was concentrated, and placed on two sheets of preparative TL silica gel plates (20×20×1 cm). The plates were developed three times with $CHCl_3$–MeOH (3:1). A band containing M_1 was taken up, and extracted

8) Detailed NMR analysis of SF-837 and M_1 substance will be published separately.[9]

9) S. Omoto, S. Inouye, T. Tsuruoka, T. Niida, and S. Satoh, to be published.
10) T.J. Perun, R.S. Egan, P.H. Jones, J.R. Martin, L.A. Mitsher, and B.J. Slater, Antimicrobial Agents & Chemoth, 1969, 116 (1970).
11) H. Takahira, H. Kato, N. Sugiyama, S. Ishii, T. Haneda, K. Uzu, K. Kumabe, and R. Kojima, *Japanese J. Antibiotics, Ser. B*, **19**, 95 (1966).
12) J.S. Welles, R.C. Anderson, and K.K. Chen, *Antibiotic Annual*, 1954—1955, 291 (1956).

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with MeOH. The MeOH extract was evaporated to dryness, and the residue dissolved in AcOEt. Evaporation of the solvent yielded a white powder of M_1. 150 mg. mp 123—125°, $[\alpha]_D^{22}$ −56.4° (c=1, EtOH). UV λ_{max}^{EtOH} mμ ($E_{1cm}^{1\%}$): 232 (386). IR ν_{max}^{KBr} cm^{-1}: 1735 ($\nu_{C=O}$). *Anal.* Calcd. for $C_{38}H_{63}O_{14}N$ (757.93): C, 60.22; H, 8.38; N, 1.85. Found: C, 60.63; H, 8.30; N, 1.77.

Crystalline tri-O-acetyl M_1 was prepared according to the procedure reported for tri-O-acetyl-depropionyl SF-837.[6] mp 123—125°.

Preparation of M_1 Substance by incubating SF-837 with a Rat Liver Homogenate——A fresh liver obtained from male albino rat (200—220 g) was suspended in saline (1 g/4 ml), cutted into small pieces, and homogenated by the use of a Potter-Elvehjem type Teflon homogenizer. Insoluble materials were removed by centrifugation (3000 rpm) for 15 min, and the supernatant was used as a liver homogenate.

To a solution of SF-837 tartarate (590 mg) in water (20 ml) was added a liver homogenate (110 ml), and a mixture was adjusted to pH 7 by adding 7.5% $NaHCO_3$, and incubated at 37° for 3 hr. Water (300 ml) was added, and the pH was raised to 9—10. Extraction with AcOEt three times followed by transfer to acidic water and reextraction with fresh AcOEt under alkaline condition yielded a white powder of M_1, 420 mg (90%).

Isolation of M_2 Substance from the Bile of Rats——An aqueous solution of SF-837 tartarate (7.1 g/45 ml) was administered subcutaneously to 45 male albino rats (Donryu, 250—350 g), who were received cannulation of common bile ducts. Biles were collected for 48 hr, and 1.15 liter were obtained in all. H_2O (1.15 liter), 20% $(NH_4)_2SO_4$ (600 ml) and 5N NaOH (170 ml) were added, and the alkaline solution (pH 9—10) was extracted twice with AcOEt (each, 3 liter). The extracts were combined and concentrated to 1.2 liter, and washed with alkaline H_2O.

The organic solvent layer was extracted twice with dilute HCl (each 200 ml). The aqueous extracts were combined (400 ml, pH 2—3), adjusted to pH 10 by adding 5N NaOH, and saturated with $(NH_4)_2SO_4$. Twice extraction with AcOEt (each 900 ml) followed by evaporation of the solvent gave a yellowish white powder (1.39 g). Further powder (215 mg) was recovered from the alkaline washing described above.

This powder (0.8 g) was dissolved in $CHCl_3$ (10 ml), and passed through a column of silicic acid (350 ml). After washing with 1.2 liter of $CHCl_3$–MeOH (50:1), the column was developed with $CHCl_3$–MeOH (10:1), and the effluents were collected in 15 ml-fractions. Fractions No. 145—262 were combined, and evaporated to dryness to yield a white powder of M_2 (250 mg). mp 137—141°, $[\alpha]_D^{25}$ −72.1° (c=1, EtOH). UV λ_{max}^{EtOH} mμ ($E_{1cm}^{1\%}$): 232 (371), IR ν_{max}^{KBr} cm^{-1}: 1730 ($\nu_{C=O}$). *Anal.* Calcd. for $C_{38}H_{63}O_{15}N$ (773.93): C, 58.97; H, 8.21; N, 1.81. Found: C, 58.75; H, 8.37; N, 1.78.

From fraction No. 115—135 were recovered 60 mg of M_1 substance.

Preparation of Tetra-O-acetyl M_2 Substance (5)——A solution of M_2 (100 mg) and acetic anhydride (0.5 ml) in pyridine (1 ml) was kept at 28° for 30 hr, and then poured into ice-water. A crude product precipitated was collected by filtration, and dried *in vacuo*. Crystallization from CCl_4 gave colorless needles of tetra-O-acetyl M_2 (68 mg). mp 183—185°, $[\alpha]_D^{23}$ −68.3° (c=1, EtOH). NMR ($CDCl_3$) δ: 2.02 (9- and 2'-$COCH_3$), 2.05 (14-$COCH_3$), 2.17 (4''-$COCH_3$), 2.46 ($N(CH_3)_2$), 3.49 (OCH_3), 5.70 (H-10), 5.80 (H-13), 6.33 (H-12), 6.75 (H-11), and 9.62 (CHO). *Anal.* Calcd. for $C_{46}H_{71}O_{19}N$ (942.08): C, 58.65; H, 7.60; N, 1.49. Found: C, 58.45; H, 7.60; N, 1.39.

Acknowledgement We are indebted to Dr. Shiroh Satoh of Nippon Electric Varian Ltd. for carrying out the decoupling experiments in NMR spectroscopy.

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