STUDIES ON ANTIBIOTIC SF-837, A NEW ANTIBIOTIC. I
THE PRODUCING MICROORGANISM AND ISOLATION AND CHARACTERIZATION OF THE ANTIBIOTIC*

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A new antibiotic, SF-837, having potential utility as a chemotherapeutic agent was discovered in the culture broth of *Streptomyces mycarofaciens* SHOMURA and NIIDA nov. sp. Antibiotic SF-837 is a basic macrolide showing a UV maximum at 232 mμ, and is different from the leucomycins, josamycin, the spiramycins and the tertiomycins. It has a molecular formula $C_{41}H_{67}NO_{15}$ (MW, 813), and liberated 2 moles of propionic acid on hydrolysis.

In the continuing search for new antibiotics in this laboratory, a new antibiotic, SF-837, was isolated from the fermentation broth of a *Streptomyces* strain, which, based on taxonomic study, is considered to be a new species, and is named *Streptomyces mycarofaciens* nov. sp.

Antibiotic SF-837 is active mainly against Gram-positive bacteria, and belongs to the macrolide group. This report deals with the producing microorganism and the isolation and characterization of antibiotic SF-837.

Producing Microorganism

Streptomyces strain SF-837 which produces antibiotic SF-837 was isolated from soil collected at Onomichi City of Hiroshima Prefecture in Japan. For the taxonomic characterization of strain SF-837, the procedures described by SHIRLING and GOTTLIEB[2] (supplemented with additional media recommended by WAKSMAN[3]) were used.

Strain SF-837 shows the following properties:

1. Morphological properties:

When grown on media such as glycerine-nitrate agar, glycerine-calcium malate agar, inorganic salts-starch agar (ISP)** and oatmeal agar (ISP), it forms abundant aerial mycelia which develop into many long coils as shown in Fig. 1. The spores are oval or ellipsoidal, and measure 0.5~0.7×0.8~1.0 μ. The surfaces of spores are spiny as shown in Fig. 2.

2. Cultural properties:

Cultural properties on various media are listed in Table 1.

* A part of this work has been reported in preliminary form.[1]
** ISP=International *Streptomyces* Project.

Fig. 1. Aerial mycelium of *Streptomyces mycarofaciens*, strain SF-837

Fig. 2. Electron micrograph of spores of *Streptomyces mycarofaciens*, strain SF-837

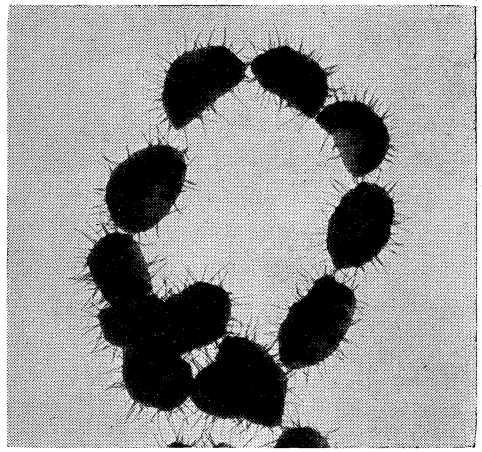


Table 1. Cultural properties of *Streptomyces mycarofaciens*, strain SF-837

Medium	Growth	Aerial mycelium	Soluble pigment
Sucrose-nitrate agar (28°C)	Poor, colorless~cream	Scant, cottony, whitish gray	None
Glycerine-nitrate agar (28°C)	Dark brown	White, later becoming gray, cottony	None, or faint light brown
Glucose-asparagine agar (28°C)	Light brown~reddish brown	Rose~white	None, or faint brownish yellow
Glycerine-asparagine agar (ISP) (28°C)	Light brown~light reddish brown	None, or very scant, white	None
Ca-malate agar (28°C)	Poor, cream	None	None
Glycerine - Ca-malate agar (28°C)	Light brown	Rose, later becoming gray, cottony	None
Inorganic salts-starch agar (ISP) (28°C)	Purplish dark brown	Reddish rose, later becoming gray, cottony	None
Nutrient agar (28°C)	Yellowish brown	Very scant, white	None
Tyrosine agar (ISP) (28°C)	Reddish brown	White	None
Yeast extract-malt extract agar (ISP) (28°C)	Brown with reddish tinge	Whitish gray	None
Oatmeal agar (ISP) (28°C)	Good, light brown~brown	Abundant, rose~white, later becoming gray	None
BENNETT's agar (28°C)	Brown~dark brown	Rose	Light brown
Potato plug (28°C)	Good, thick, brown to almost black	Abundant, grayish brown	Dark brown

3. Physiological properties:

Physiological properties, including utilization of carbon sources, are summarized in Tables 2 and 3.

From the observations described above, strain SF-837 may be characterized as follows: It forms coils consisting of chains of spiny-surfaced spores. The color of vegetative growth on synthetic media is cream to brown to reddish brown. On some media, the aerial mass color is rose, later becoming gray and cottony. No distinct

diffusible pigment forms on either synthetic or organic media, suggesting that the strain is non-chromogenic.

4. Comparison of strain SF-837 with related *Streptomyces*:

With respect to the characteristics mentioned above, strain SF-837 has some similarity to *Streptomyces griseoflavus* (KRAINSKY, 1914) WAKSMAN and HENRICI, 1948[3,5,6], *Streptomyces rubiginosus* (PREOBRAZHENSKAYA *et al.*) PRIDHAM *et al.*[7,23], *Streptomyces fasiculatus* PITTENGER and NELMS in SHIRLING and GOTTLIEB 1968[8] and *Streptomyces fungicidicus* (Group G) OKAMI *et al.*, 1954[9]. These four species and strain SF-837 are similar in the morphology of their sporophores and the spores. It was found, however, that these four species were different from strain SF-837 in the following points.

Table 2. Physiological properties of *Streptomyces mycarofaciens*, strain SF-837

Production of hydrogen sulfide	negative
Tyrosinase reaction	negative
Reduction of nitrate	positive
Coagulation of skimmed milk	positive
Peptonization of skimmed milk	positive
Hydrolysis of starch	positive
Liquefaction of gelatin	positive (slow)
Liquefaction of LOEFFLER's coagulated serum	negative
Cellulolytic activity	negative
Temperature range (oatmeal agar)	15~38°C

Table 3. Carbon source utilization of *Streptomyces mycarofaciens*, strain SF-837 in PRIDHAM and GOTTLIEB's synthetic agar[4]

Positive utilization	glucose, galactose, fructose, mannose, maltose, lactose, dextrin, starch, glycerol, inositol, salicin
Doubtful utilization	arabinose, rhamnose
Negative utilization	xylose, sucrose, raffinose, mannitol, sorbitol, inulin, dulcitol, cellulose

1) The aerial mass color of immature cultures of *Streptomyces griseoflavus* is yellow, whereas that of strain SF-837 is rose in color. Furthermore, *Streptomyces griseoflavus* shows yellow to orange yellow growth when grown on yeast extract-malt extract agar and inorganic salts-starch agar. The growth of strain SF-837 is reddish or purplish brown on these media.

2) The growths of *Streptomyces rubiginosus* and *Streptomyces fasiculatus* show no distinctive color (yellowish brown, grayed orange brown or yellowish gray) on yeast extract-malt extract and inorganic salts-starch agars, whereas strain SF-837 is reddish or purplish brown color on those media. Furthermore, strain SF-837 and other two organisms differ in the utilization of D-mannitol and sucrose.

3) *Streptomyces fungicidicus* (Group G) shows colorless growth on glycerine-nitrate agar, glucose-asparagine agar and inorganic salts-starch agar. Strain SF-837 shows brown to reddish or purplish brown growth on these agars. In addition, the two organisms differ in their utilization of D-mannitol, xylose and sucrose.

When strain SF-837 is compared with the organisms producing basic macrolides similar to antibiotic SF-837, there is a clear difference in spore morphology among them. That is, the spores of the leucomycins-producing organism, *Streptomyces kitasatoensis* HATA 1953[10]; josamycin-producing organism, *Streptomyces narbonensis* var. *josamyceticus* OSONO *et al.* 1967[11]; spiramycins-producing organism, *Streptomyces ambofaciens* PINNERT-SINDICO 1954[10]; miamycin-producing organism, *Streptomyces ambofaciens*[10]; and the tertiomycins-producing organisms, *Streptomyces eurocidicus* OKAMI *et al.*[10] and *Streptomyces albireticuli* NAKAZAWA 1955[10] all have smooth

surfaces. On the other hand, the spores of strain SF-837 have spiny surface. Also *Streptomyces eurocidicus*, *S. albireticuli* and *S. kitasatoensis* are streptoverticillia and quite different from strain SF-837 in morphology.[24]

As a result of the above comparisons, strain SF-837 is considered a new species, and the name *Streptomyces mycarofaciens* nov. sp. SHOMURA and NIIDA is proposed. The specific epithet "*mycarofaciens*" means mycarose-making. A culture of the new taxon has been deposited in the American Type Culture Collection, where it has been given accession number ATCC 21454.

Production and Isolation

Strain SF-837 was grown in submerged culture in a 50-liter jar fermentor at 28°C in a medium containing 3.0 % glucose, 1.0 % peptone, 0.5 % meat extract, 0.4 % soluble vegetable protein, 0.3 % soybean oil, 0.2 % NaCl and 0.3 % $CaCO_3$ (pH 7). The deep-aerated culture under these conditions yielded 400~500 mcg/ml of antibiotic SF-837 after 65 hours.

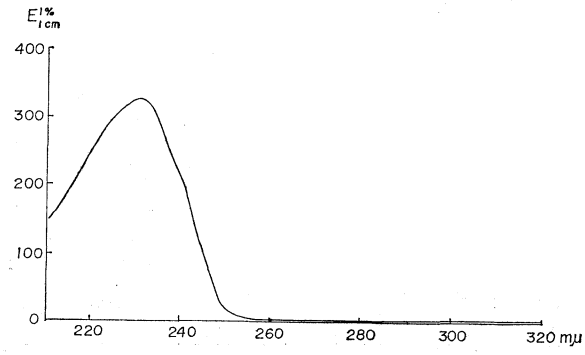
The yield of the antibiotic was determined by the conventional paper disc-agar diffusion assay method, using *Bacillus subtilis* ATCC 6633 as a test organism. The assay agar (pH 7.8) contained 0.5 % peptone and 0.3 % meat extract.

The fermented broth was filtered at pH 3.5. The filtrate was extracted with one-fourth volume of ethyl acetate at pH 8, the ethyl acetate layer was washed with water, and then reextracted with dilute hydrochloric acid (pH 2). The antibiotic in the aqueous layer was transferred into fresh ethyl acetate at pH 8. The final organic solvent layer was passed through a column of activated carbon, and effluents were collected and concentrated to dryness to yield a crude preparation. The material thus obtained was dissolved in a small volume of benzene, the insoluble portion was removed by filtration, and the filtrate was applied to a silica gel column and chromatographed using benzene-acetone (4:1) as the developing solvent. The bioactive fractions were combined and concentrated to dryness to give a preparation of antibiotic SF-837.

Physical and Chemical Properties

Antibiotic SF-837 obtained by the above process is a white powder, with melting point of 122~124°C. Crystallization from benzene-cyclohexane gives colorless needles, which melted at 155~156°C after drying at 80°C for 8 hours *in vacuo*. All melting points were determined with glass capillary tubes in an oil bath, and are uncorrected.

Fig. 3. UV spectrum of antibiotic SF-837



Antibiotic SF-837 is soluble in methanol, ethanol, acetone, chloroform, ethyl acetate, butyl acetate, acidic water, benzene and ethyl ether; and almost insoluble in *n*-hexane, petroleum ether and water.

Fig. 4. IR spectrum of antibiotic SF-837 (KBr pellet)

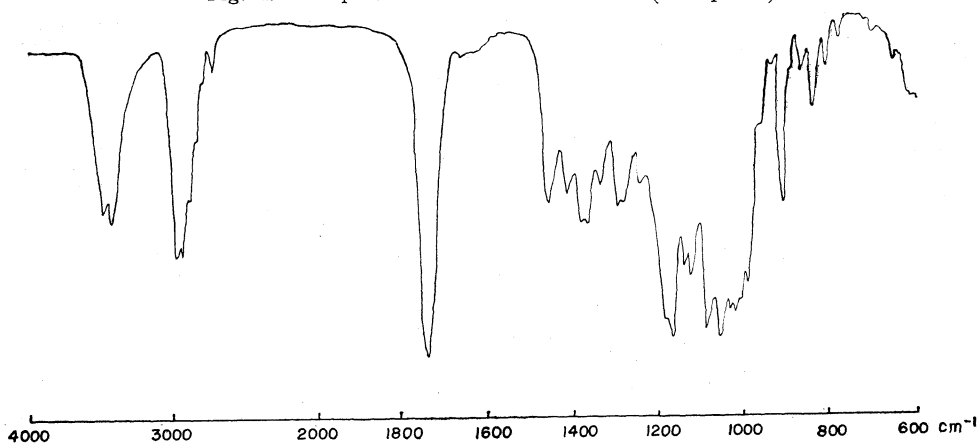
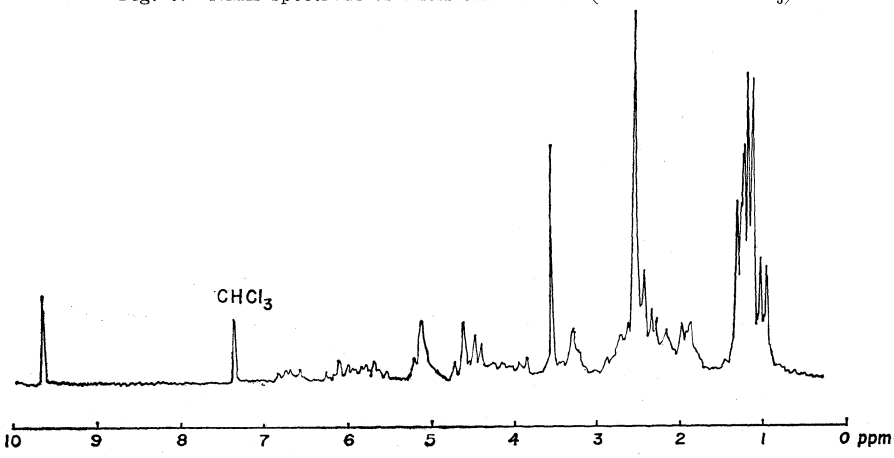


Fig. 5. NMR spectrum of antibiotic SF-837 (100 MHz in $CDCl_3$)



It is optically active, $[\alpha]_D^{22}$ −67° (*c* 1, ethanol). Potentiometric titration in 50 % aqueous ethanol gave a neutral equivalent of 830 as a monoacidic base, and mass spectrometry showed the parent peak at *m/e* 813. Elemental analysis gave the following composition: C 60.78, H 8.35, N 1.65, O 29.62 %. A molecular formula of $C_{41}H_{67}NO_{15}$ (MW, 813) requires: C 60.52, H 8.24, N 1.72, O 29.39 %.

Antibiotic SF-837 is a weak base, with pKa′ of 6.9 in 50 % aqueous ethanol. The ultraviolet absorption spectrum in ethanol has a maximum at 232 mμ ($E_{1cm}^{1\%}$ 325), as shown in Fig. 3. The IR spectrum is illustrated in Fig. 4, which shows characteristic bands at 3500, 2970, 2930, 1735, 1460, 1408, 1376, 1360, 1330, 1295, 1275, 1190, 1165, 1120, 1082, 1050, 1015, 910, 860, 840, 805, 780, 735, and 680 cm^{-1}. The NMR spectrum is shown in Fig. 5.

Antibiotic SF-837 gives positive erythromycin and carbomycin tests[12], but negative FEHLING, ferric chloride and ninhydrin tests. On silica gel thin-layer plates (Camag, Switzerland) developed with various solvent systems, the antibiotic showed the following Rf values: 0.45 with benzene - acetone (2 : 1), 0.67 with 1-butanol - acetic acid - water (3 : 1 : 1), 0.82 with methanol, 0.92 with acetone - water (49 : 1). The antibiotic on thin-layer plates was visualized as a dark purple spot by spraying with 10 %

sulfuric acid followed by heating.

Treatment of antibiotic SF-837 (200 mg) with acetic anhydride (0.25 ml) and pyridine (1 ml) at 5°C for 20 hours, gave the di-O-acetyl derivative, which was crystallized from carbon tetrachloride as white needles, 220 mg. m.p. 123~124°C, $[\alpha]_D^{22}$ −75° (*c* 1, chloroform).

Anal. Calcd. for $C_{45}H_{71}NO_{17}$ (MW, 897) : C 60.20, H 7.90, N 1.56 %.
Found : C 60.38, H 7.26, N 1.54 %.

Refluxing antibiotic SF-837 (100 mg) in ethanol (2 ml) with thiosemicarbazide (10 mg) for 3 hours yielded the crystalline thiosemicarbazone, which was recrystallized from ethanol, 65 mg. m.p. 147~148°C, $[\alpha]_D^{22}$ −132° (*c* 1, methanol).

Anal. Calcd. for $C_{42}H_{71}N_5O_{14}S$: C 55.91, H 7.92, N 7.76 %.
Found : C 56.39, H 7.68, N 7.35 %.

Alkaline hydrolysis of antibiotic SF-837 with 0.1 N ethanolic sodium hydroxide at 75°C for 40 minutes liberated an acid, which, after addition of phosphoric acid, was analyzed by GLC using a column of Chromosorb 101. Two moles of propionic acid per mole of antibiotic SF-837 were obtained. It was found later that direct insertion of a sample of antibiotic SF-837, acidified with phosphoric acid, into the flash heater of the GLC apparatus gave similar results.

Biological Properties

The antimicrobial spectrum of antibiotic SF-837 was obtained by conventional two-fold agar dilution assays. The results are summarized in Table 4. The antibiotic is primarily active against Gram-positive bacteria, with little activity against Gram-negative bacteria, yeasts and molds. Antibiotic SF-837 does not show cross-resistance with penicillin, tetracycline, novobiocin, streptomycin or kanamycin, but does show cross-resistance with erythromycin as indicated by the MIC values against *Staphylococcus aureus* resistant to each of the antibiotics mentioned above.

Table 4. Antimicrobial spectrum of antibiotic SF-837 by agar dilution method

Test organisms	M.I.C. (mcg/ml)	Medium
Staphylococcus aureus 209 P	0.39	1
〃 〃 〃 penicillin-R	0.78	1
〃 〃 〃 streptomycin and A-249 substance-R	0.39	1
〃 〃 〃 kanamycin-R	3.125	1
〃 〃 〃 novobiocin-R	3.125	1
〃 〃 Smith	0.39	1
〃 〃 Terajima	0.78	1
〃 〃 193	1.56	1
〃 〃 streptomycin, tetracycline and penicillin-R	1.56	1
〃 〃 erythromycin-R	>25	1
Bacillus subtilis ATCC 6633	0.39	1
Sarcina lutea	<0.05	1
Mycobacterium phlei	12.5	2
Escherichia coli	>25	1
Shigella dysenteriae	>25	1
Pseudomonas aeruginosa	>25	1
Candida albicans	>25	3
Aspergillus niger	>25	3

Medium : 1. Heart infusion agar (Difco); 2. Glycerine bouillon agar; 3. SABOURAUD's agar.

The antibiotic exhibits low toxicity. Oral administration of 6,000 mg/kg to mice caused no deaths. When given orally or subcutaneously, antibiotic SF-837 protected mice against death caused by experimental infections with *S. aureus*. Details of *in vivo*

experiments will be reported elsewhere.

Discussion

The physico-chemical and biological properties described above point out the macrolide nature of antibiotic SF-837. As is well known, most of the known macrolide antibiotics can be conveniently classified into five groups based on their UV spectra:

a) macrolides with a weak maximum at 280~290 mμ,
b) macrolides with a relatively strong maximum at about 225 mμ,
c) macrolides having a strong maximum at about 232 mμ,
d) macrolides having a strong maximum at about 240 mμ,
e) macrolides having a strong maximum at 280~290 mμ.

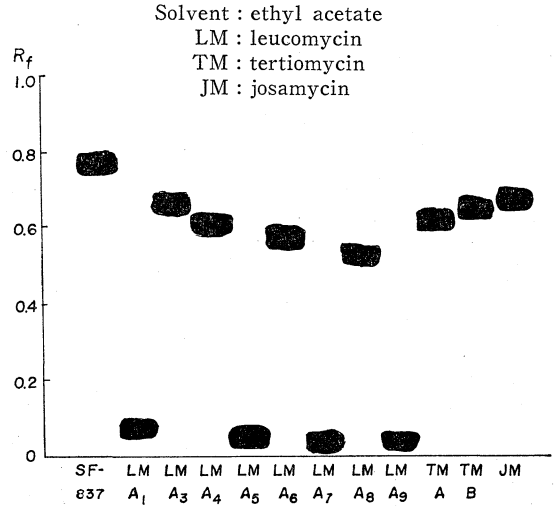
Antibiotic SF-837 exhibits a strong UV maximum at 232 mμ and, therefore, belongs to group c which includes the leucomycins[13,14,15,16], josamycin[11], the spiramycins[17], miamycin[18] and the tertiomycins[19,20].

Leucomycins B_1, B_2, B_3 and B_4 are distinguished from antibiotic SF-837 by their high melting points and different molecular formulae. Three members of the spiramycins (I, II and III) are relatively strong bases with pKa' 7.6~7.7, and have higher nitrogen contents (3.05~3.1 %), whereas antibiotic SF-837 is a weak base with lower nitrogen content.

Miamycin is reported to be optically inactive in chloroform, and accordingly differs from the optically active antibiotic SF-837. The leucomycin A group, josamycin*, and tertiomycins A and B are more closely related antibiotics, but, differ from antibiotic SF-837 with respect to their melting points, molecular weights and molecular formulae except for leucomycin A_4. The molecular weight and molecular formula of leucomycin A_4 are identical with those of antibiotic SF-837. However, the NMR spectrum of the latter (shown in Fig. 5) lacks an acetyl signal that is present in the spectrum of leucomycin A_4 at 2.22 ppm[16]. In this context, it was shown by GLC that antibiotic SF-837 liberates two moles of propionic acid on hydrolysis, while leucomycin A_4 should liberate one mole of acetic acid and one mole of *n*-butyric acid[16]. Definite differentiation of antibiotic SF-837 from the leucomycins, josamycin and the tertiomycins was accomplished by direct comparisons on alumina thin-layer chromatograms. Because Rf values of macrolides are affected markedly by the quantity of sample, relative mobilities of the compared antibiotics were determined under exactly similar conditions, *i. e.*, an equal amount of each sample was developed in streak, overlapping partially with an equal amount of antibiotic SF-837 on the same plate. The results are shown in Fig. 6. Of the macrolides tested, antibiotic SF-837 gave the highest Rf value (0.78), whereas the Rf values of the leucomycins, josamycin and the tertiomycins are all less than 0.66.

Thus, antibiotic SF-837 was differentiated from these known materials, and, therefore, is considered to be a new antibiotic. This conclusion was supported by structural studies to be described in a subsequent paper[21].

Fig. 6. Comparison of antibiotic SF-837 with the leucomycins, the tertiomycins, and josamycin on alumina thin-layer chromatogram.



* Josamycin was recently reported to be identical with leucomycin A_3[22].

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