SF2487, A NEW POLYETHER ANTIBIOTIC PRODUCED BY *ACTINOMADURA*

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A new antibiotic SF2487 has been isolated from the culture broth of *Actinomadura* sp. SF2487. The structure of antibiotic SF2487 was determined by spectroscopic analyses of the sodium salt and X-ray diffraction analysis of the silver salt. The antibiotic represents a new member of polyether group antibiotics known as the acyltetronic acid type 4. The antibiotic is weakly active against Gram-positive bacteria and exhibits antiviral activity against influenza virus *in vitro*.

In the course of our screening program for new antibiotics active against *Bacillus subtilis* phage ϕ29[1] produced by soil actinomycetes, a new polyether antibiotic SF2487 has been isolated from the fermentation broth of *Actinomadura* sp. SF2487. The antibiotic represents a new member of polyether group antibiotics known as the acyltetronic acid type 4[2], such as ICI139603[3~5] (M139603) and tetronomycin[6]. It has moderate activity against Gram-positive bacteria and exhibits antiviral activity against influenza virus *in vitro*. This paper describes the taxonomy and fermentation of the producing organism and the isolation, characterization and structure determination of SF2487.

Taxonomy

Strain SF2487 was isolated from a soil sample collected at Mobara city, Chiba Prefecture, Japan. The taxonomic characteristics[7] of strain SF2487 are given below. The vegetative mycelium on agar medium was well developed and irregularly branched. Fragmentation of hyphae did not occur either on agar or under submerged growth conditions. The monopodially-branched aerial mycelia were formed moderately on yeast extract - malt extract agar, tyrosine agar and inorganic salts - starch agar. On inorganic salts - starch agar, the strain produced short spore chains which contained 5 to 10 spores and were flexuous, hooked or looped. The spores were elliptical to cylindrical in shape and measured 0.5 to 0.8 by 0.8 to 1.5 μm with warty surfaces (Fig. 1). Spores were not motile, and sclerotia or sporangia (including pseudosporangia) were not observed. Aerial mass color was white, and reverse was colorless to pale brown. Distinctive soluble pigments were not formed on the media tested. The observed physiological characteristics of the strain are as follows: Hydrolysis of starch and reduction of nitrate were positive. Liquefaction of gelatin, peptonization and coagulation of milk, and formation of melanoid pigment were negative. The strain grew in media containing up to 4% NaCl, but 5% NaCl was inhibitory. The temperature range for growth was 15 to 42°C, with best growth occurring between 25 and 30°C. On International Streptomyces Project (ISP)

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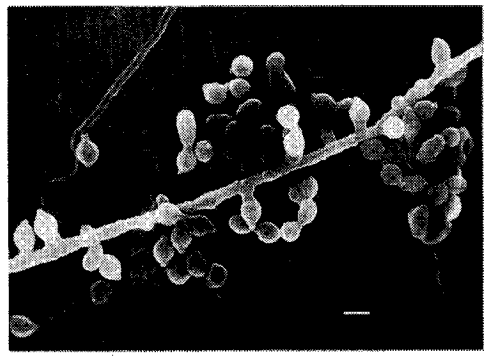
medium 9, good growth was obtained with D-glucose, D-fructose, D-xylose, L-arabinose and D-mannitol. No growth or only a trace of growth was observed with glycerol, *myo*-inositol, L-rhamnose, sucrose and raffinose. Whole-cell hydrolysates contained *meso*-diaminopimelic acid and a small amount of madurose, but no arabinose or xylose. Phospholipid pattern was a type PI. Mycolic acids were not present. An MK-9(H_6) menaquinone was detected as the major component (more than 90%). The whole-cell fatty acids consisted of hexadecanoic acid (16:0) 27%, octadecenoic acid (18:1) 19%, 14-methylpentadecanoic acid (iso-16:0) 12%, 10-methyloctadecanoic acid (10Me-19:0) 11% and heptadecanoic acid (17:0) 9% with other minor components. From the morphological and chemotaxonomic properties of strain SF2487, it is considered to belong to the genus *Actinomadura*. Strain SF2487 was deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Actinomadura* sp. SF2487 and the accession No. of FERM P-9063.

Fig. 1. Scanning electron micrograph of spore chain of strain SF2487 on oatmeal agar incubated at 28°C for 21 days.

Bar = 1 μm.



Fermentation

A well-grown slant culture of strain SF2487 was inoculated into 20 ml of a seed culture medium consisting of soluble starch 2.0%, glucose 1.0%, wheat germ 0.6%, Polypepton (Daigo Eiyo Kagaku) 0.5%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.1% (pH 7.0) in a 100-ml Erlenmeyer flask. The flask was shaken at 28°C for 4 days on a rotary shaker (220 rpm) and then transferred into 80 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 28°C for 2 days, 50 ml of the culture was re-transferred into 1 liter of the same medium in a 5-liter Erlenmeyer flask. After shaking for 2 days at 28°C, the seed culture was transferred into a 50-liter jar fermenter containing 35 liters of a production medium consisting of maltose syrup 3.0%, soluble vegetable protein 1.5%, cotton seed meal 0.5%, soybean oil 0.2%, $CaCO_3$ 0.1%, $FeSO_4 \cdot 7H_2O$ 0.0005% and $CaCl_2 \cdot 6H_2O$ 0.0005%. The medium was adjusted to pH 7.0 before sterilization. The fermentation was carried out at 28°C for 6 days with an air flow rate of 20 liters per minute and an agitation rate of 250 rpm.

Isolation

The fermentation broth (25 liters, pH 7.6) was filtered with the aid of diatomaceous earth. The antibiotic in mycelial cake was extracted with 50% aqueous acetone (20 liters) and the extract was concentrated to remove acetone. The broth filtrate (20 liters) and the concentrate of mycelial extract was combined and the antibiotic was adsorbed on a column of Diaion HP-20 (4 liters). After washing the column with water and 50% aqueous methanol, the antibiotic was eluted with 50% aqueous acetone. The eluate (20 liters) was concentrated to remove acetone and the concentrate (8 liters) was extracted twice with 10 liters of ethyl acetate. After drying over anhydrous sodium sulfate, the ethyl acetate was evaporated to dryness yielding 3.5 g of an oily residue. The residue was dissolved in chloroform and chromatographed on a silica gel column (Wakogel C-200, 200 g) packed with chloroform. After washing the column with chloroform, the antibiotic was eluted with a mixture of chloroform - methanol (100 : 1). The active fractions

were combined and concentrated to dryness. Further purification by chromatography on a Sephadex LH-20 (350 ml) column developed with methanol gave a white powder (208 mg). The powder was dissolved in chloroform (100 ml) and washed twice with an equal volume of 0.1 N HCl, followed by washing with an equal volume of 1 N NaOH. The chloroform solution was dried over anhydrous sodium sulfate and evaporated to dryness. Crystallization from hot methanol gave pure SF2487 sodium salt (102 mg) as colorless prisms.

Physico-chemical Properties

The physico-chemical properties of SF2487 sodium salt are summarized in Table 1. Sodium salt of SF2487 is soluble in chloroform, ethyl acetate and dimethyl sulfoxide, slightly in methanol and ethanol, but almost insoluble in water. It gave positive color reactions with sulfuric acid,, iodine and sodium molybdate reagents and negative ninhydrin. The IR spectrum in KBr is shown in Fig. 2. ^{1}H NMR data at 400 MHz and ^{13}C NMR data at 100 MHz in $CDCl_3$ are shown in Tables 2 and 3, respectively.

Structure

The molecular formula of SF2487 sodium salt was determined to be $C_{42}H_{63}O_{12}Na$ (MW 782) from the elemental analysis, FD-MS and ^{13}C NMR spectrum. In the ^{1}H NMR spectrum, characteristic signals due to seven methyl groups (one CH_2CH_3, three $\overset{|}{C}HCH_3$, one $\overset{|}{C}CH_3$, one $=\overset{|}{C}CH_3$ and one OCH_3), two

Table 1. Physico-chemical properties of SF2487 sodium salt.

Appearance	Colorless crystals	UV λ_{max}^{MeOH} nm (ε)	252 (17,100), 299 (9,300)
MP	250~252°C (dec)	$\lambda_{max}^{MeOH-HCl}$ nm (ε)	248 (11,800)
$[\alpha]_D^{20}$	−57.3° (*c* 0.1, MeOH)	$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	240 (10,900), 305 (6,800)
Molecular formula	$C_{42}H_{63}O_{12}Na$	IR ν_{max}(KBr) cm^{-1}	3400~3300, 1740, 1680,
Anal Calcd:	C 64.43, H 8.11		1610, 1450
Found:	C 64.16, H 8.16	Rf[a]	0.73 (Diethyl ether)
FD-MS	783 $(M+H)^+$		0.48 ($CHCl_3$-MeOH, 50:1)
SI-MS	805 $(M+Na)^+$		0.27 (Hexane-acetone, 8:2)

[a] Silica gel TLC (E. Merck, Art. No. 5715).

Fig. 2. IR spectrum of SF2487 sodium salt in KBr.

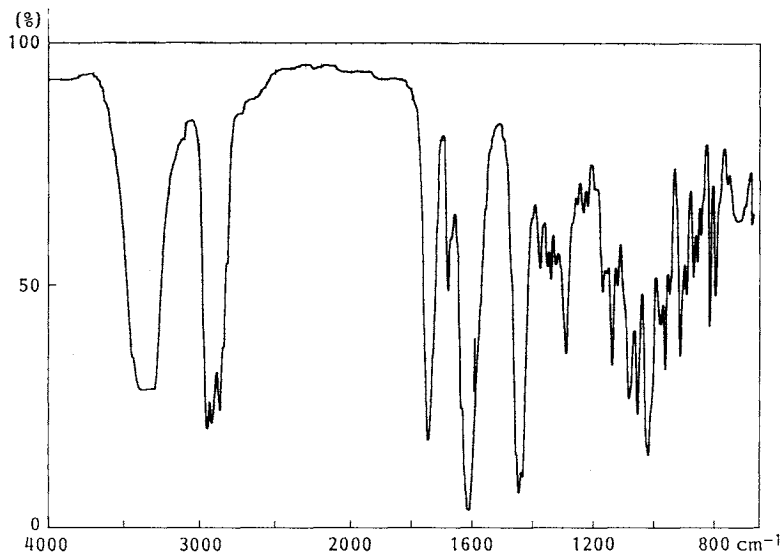


Table 2. ^{1}H NMR data for SF2487 sodium salt in $CDCl_3$.

Proton	ppm (*J*, Hz)	Proton	ppm (*J*, Hz)
4-H	4.67 br s	23-H_2	1.30, 1.96 dd (9.4, 12.4)
5-H	2.45 t (9.8)	25-H	3.62~3.72
6-H	1.64	26-H	1.85~1.95
7-H	4.02 br s	27-H_2	1.66, 1.80
8-H	1.52	28-H	3.62~3.72
9-H_2	0.98, 1.36	29-H	3.97 dt (2.6, 7.2)
10-H	2.72 dq (3.6, 9.8)	30-H_2	1.23, 1.35
11-H	5.24 d (10.4)	31-H_3	0.93 t (7.2)
13-H	3.62~3.72	32-H_3	1.06 s
14-H_2	1.18, 1.80	33-H_3	1.00 d (6.4)
15-H_2	1.20, 1.34	34-H_3	1.72 s
16-H	1.58	35-H_3	0.66 d (7.6)
17-H	3.42 d (10.8)	36-H_2	3.94, 4.25 d (11.2)
19-H	5.84 br dd (6.0, 8.0)	37-H_3	0.91 d (7.2)
20-H_2	2.26 m	38-H_2	3.88, 4.36 dd (2.6, 13.0)
21-H	3.62~3.72	41-H_2	4.78, 5.18 d (2.8)
22-H	1.80	42-H_3	3.34

Table 3. ^{13}C NMR data for SF2487 sodium salt in $CDCl_3$.

Carbon	δ (ppm)	m	Carbon	δ (ppm)	m
C-1	171.2	s	C-22	40.2	d
C-2	96.2	s	C-23	44.5	t
C-3	196.0	s	C-24	82.4	s
C-4	83.3	d	C-25	82.9[b]	d
C-5	37.2	d	C-26	25.2[c]	t
C-6	43.5	d	C-27	22.8[c]	t
C-7	76.3	d	C-28	81.3	d
C-8	35.9	d	C-29	70.5	d
C-9	34.9	t	C-30	28.2	t
C-10	36.1	d	C-31	10.5	q
C-11	137.6	d	C-32	25.6	q
C-12	135.9	s	C-33	16.2	q
C-13	85.0	d	C-34	10.4	q
C-14	32.3[a]	t	C-35	17.3	q
C-15	31.3[a]	t	C-36	55.9	t
C-16	31.2	d	C-37	17.8	q
C-17	92.1	d	C-38	65.6	t
C-18	134.5	s	C-39	180.2	s
C-19	131.1	d	C-40	153.5	s
C-20	32.2	t	C-41	90.1	t
C-21	83.3[b]	d	C-42	58.9	q

[a~c] Signals could be interchanged.
m: Multiplicity.

hydroxymethyl groups and a terminal methylene group were observed (Table 2). In the ^{13}C NMR spectrum, the signals of 42 carbons were resolved and assigned to seven methyl carbons including one methoxy, eleven methylene carbons including two hydroxymethyls and one terminal methylene, sixteen methine carbons including eight $\underset{|}{C}HO$ and two $CH^{=}$, and eight quarternary carbons including three carbonyl carbons (Table 3). The UV spectrum (Table 1) resembled those of M139603[3] and tetronomycin[6] possessing the tetronic acid moiety. The presence of the tetronic acid moiety in SF2487 was confirmed by the observation of the signals of a terminal methylene at δ_H 4.78 and 5.18 (41-H_2), δ_C 90.1 (C-41), two

Fig. 3. HMBC experiments of SF2487 sodium salt.

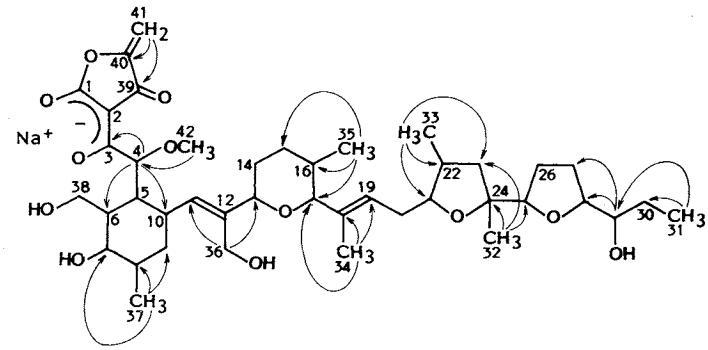
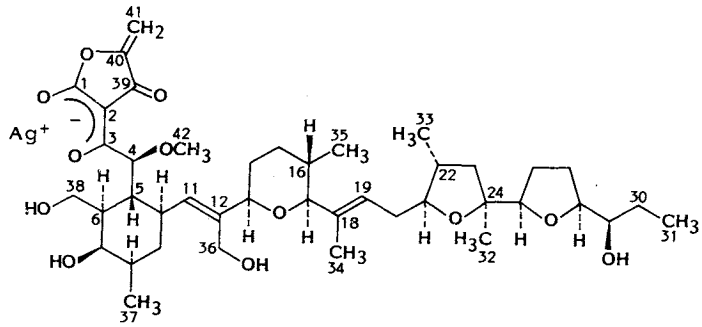


Fig. 4. The structure of SF2487.



quarternary sp^2 carbons at δ_C 96.2 (C-2) and 153.5 (C-40) and three carbonyl carbons at δ_C 171.2 (C-1), 196.0 (C-3) and 180.2 (C-39) in the ^1H and ^{13}C NMR spectra (Tables 2 and 3). On comparison of ^1H and ^{13}C NMR spectra of SF2487 with those of tetronomycin[6] and M139603[4,5], the signal due to one of olefin group of tetronomycin, CHCH=CCH$_2$OH was observed in SF2487, but the other *trans* substituted olefin group, CHCH=CHCH$_2$ of tetronomycin was replaced by CHC(CH$_3$)=CHCH$_2$ in SF2487. Assignment of the ^1H and ^{13}C NMR spectra were obtained from the 2D (^1H-^1H and ^1H-^{13}C COSY) spectrum including long range ^1H-^{13}C COSY spectrum and hetronuclear multiple-bond correlation (HMBC) experiments (Fig. 3). However the four methine protons at C-13, C-21, C-25 and C-28 carbons, and methylene protons at C-14, C-15, C-25 and C-27 carbons were tentatively assigned because of the overlapping of these signals.

Treatment of the antibiotic with $AgCO_3$ afforded colorless prisms of the silver salt. The structure and absolute configuration of SF2487 were determined by X-ray crystallographic analysis of the silver salt as shown in Fig. 4. An ORTEP drawing of SF2487 is shown in Fig. 5. Absolute stereochemistry of the chiral centers of SF2487 were more similar to those of the corresponding chiral centers of ICI139603[3] than those of tetronomycin[6].

After the completion of our work[8] (priority date to Japan, Feb. 6, 1987), an antibiotic, A80577 has appeared in a patent application[9] (priority data to U.S., Aug. 13, 1987) and the structure is identical with that of SF2487.

Biological Properties

Antibiotic SF2487 sodium salt showed moderate activity against Gram-positive bacteria, but is not

Table 4. Antimicrobial activity of SF2487.

Organisms	MIC (μg/ml)	Organisms	MIC (μg/ml)
Staphylococcus aureus 209P JC-1	0.10	*Citrobacter freundii* GN 346	>100
S. aureus Smith S-424	0.10	*Salmonella typhi* O-901-W	>100
S. aureus No. 26	0.10	*Salmonella* sp. D-0001	>100
S. epidermidis ATCC 14990	0.39	*Klebsiella pneumoniae* PCI 602	>100
Enterococcus faecalis ATCC 8043	0.39	*Proteus vulgaris* OX-19	>100
Bacillus anthracis No. 119	0.10	*Serratia marcescens* MB-3848	>100
Escherichia coli NIHJ JC-2	>100	*Pseudomonas aeruginosa* MB-3829	>100
E. coli No. 29	>100		

Medium: Sensitivity Disk Agar-N (Nissui).
Inoculum size: 10^6 cfu/ml.

active against Gram-negative bacteria as shown in Table 4. SF2487 exhibited antiviral activity against influenza virus *in vitro* measured by the plaque reduction method (Table 5). The LD_{50} value of SF2487 was 25 mg/kg by ip injection in mice.

Table 5. Antiviral activity of SF2487 against influenza virus.

Dose of SF2487 (μg/ml)	Mean No. of plaques n=3	Rate of inhibition (%)
1.0	0	100
0.5	2	95
0.25	15	62
0.13	22	44
0.06	31	21
0	39	0

Cell: Canine kidney cells (MDCK).
Virus: Influenza virus A/PR/8/34.

Experimental

General

IR spectrum in KBr disc was measured on a Hitachi 260-10 IR spectrophotometer. UV spectra were recorded on a Shimadzu UV-260 spectrophotometer. MS spectra were measured on a Hitachi M-80B spectrometer. Optical rotation was recorded with a Perkin Elmer 141 polarimeter. ^1H and ^{13}C NMR spectra were measured on a Jeol JNM-GX400 spectrometer at 400 and 100 MHz, respectively. Antimicrobial activity (MIC values) was determined by agar dilution method according to the method of Japan Society of Chemotherapy[10]. Antiviral activity was assayed by plaque reduction method reported by TOBITA[11].

SF2487 Silver Salt

SF2487 sodium salt (100 mg) in 20 ml of chloroform was shaken with 10 ml of 0.1 N HCl. The organic layer was washed six times with 10 ml of water and then treated twice with 10 ml of saturated Ag_2CO_3 solution. The organic phase was concentrated to dryness. The residue (98 mg) was crystallized from methanol to give SF2487 silver salt as colorless prisms: MP 244~246°C (dec), $[\alpha]_D^{22}$ −67.4° (*c* 0.4, MeOH), FD-MS *m/z* 686.

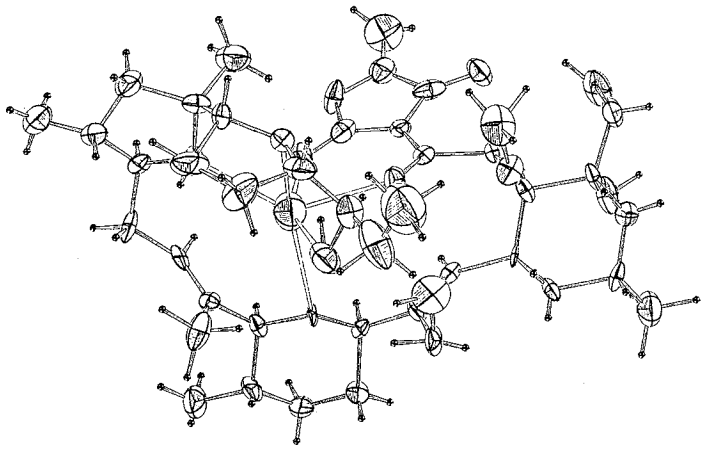
X-Ray Diffraction Analysis

SF2487 silver salt was recrystallized from methanol as transparent prismatic crystals. A crystal of approximate dimensions 0.15×0.2×0.2 mm was mounted on a Philips PW-1100 X-ray diffractometer. All X-ray measurements were made using graphite-monochromated CuKα radiation. The lattice constants were derived from setting angles of 16 higher angle (θ=18.4°~29.5°) reflections.

Crystal data: $C_{42}H_{63}O_{12}Ag$, MW 867.82, orthorhombic, space group $P2_12_12_1$, a=14.721(7), b=23.226 (12), c=12.593(6) Å, U=4305.7 Å^3, Z=4, D_{calc}=1.339 g·cm^{-3}, μ for CuKα radiation=42.9 cm^{-1}.

Intensities were measured by a $2\theta-\omega$ scan method with the scan speed 0.1°/sec in ω. Backgrounds were measured at each end of the scan for half of the total scan time. For the weak reflections whose intensities were less than 3,000 counts during the single scan, the scans were repeated once. A total of 3,468 reflections in the 2θ range 6°~130° were measured.

Fig. 5. An ORTEP drawing of SF2487.



The structure was solved by means of a combination of MULTAN[12] and heavy-atom techniques. In the final refinement, the non-hydrogen atoms were refined anisotropically by block-diagonal least-squares. The hydrogen atoms were eventually included in calculated positions (most were detectable in a difference map) but were not refined. The final R value was 0.082 for 3,468 observed reflections. The absolute configuration was determined by the anomalous dispersion method. The dispersion corrections for CuKα radiation were applied to silver, oxygen and carbon atoms. The calculations were done on an IBM 4381 computer at the Meiji Information System Center, Ltd., using the UNICS III program[13]. A computer-generated perspective drawing of the final X-ray model of SF2487 silver salt is shown in Fig. 5.

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