A NEW TETRACYCLINE ANTIBIOTIC WITH ANTITUMOR ACTIVITY

I. TAXONOMY AND FERMENTATION OF THE PRODUCING STRAIN, ISOLATION AND CHARACTERIZATION OF SF2575

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(Received for publication August 23, 1991)

A new antitumor antibiotic SF2575 has been isolated from a culture filtrate of *Streptomyces* sp. SF2575. The molecular formula was determined to be $C_{40}H_{43}NO_{15}$ by elemental analysis, mass and ^{13}C NMR spectral analyses. The spectral data revealed SF2575 to be a new tetracycline antibiotic. It was active against Gram-positive bacteria and exhibited antitumor activity against P388 leukemia in mice.

In the course of a screening program for new antitumor antibiotics, we found a new antibiotic SF2575 produced by *Streptomyces* sp. SF2575. The antibiotic was active against Gram-positive bacteria and effective against P388 leukemia in mice. In this paper, the taxonomy and fermentation of the producing organism, isolation, physico-chemical and biological properties of the antibiotic are described.

Taxonomy

Strain SF2575 was isolated from a soil sample collected at Nasu, Tochigi Prefecture, Japan. For the taxonomic characterization of the strain, the methods and media recommended by the International Streptomyces Project (ISP)[1] and those recommended by WAKSMAN[2] were used. The procedure of BECKER *et al.*[3] was used for the preparation of cells and chromatographic detection of isomers of diaminopimelic acid. Morphological observations were made on the cultures grown at 28°C for 14 to 21 days on calcium-malate agar and oatmeal agar (ISP medium 3). Carbon utilization test was performed in PRIDHAM and GOTTLIEB's basal medium omitting $CuSO_4$, because the strain showed no growth on the ISP medium 9.

Vegetative mycelium was well developed and branched. The hyphae did not fragment into coccoid or bacillary elements. The strain produced aerial mycelium with straight, curved, or rarely loose spiral spore chains. Spores were spherical, ellipsoidal to cylindrical, $0.7 \sim 0.9 \times 0.7 \sim 1.1\ \mu M$, with hairy surfaces (Fig. 1). Sporangia, flagellated spores and

Fig. 1. Scanning electron micrograph of spore chains of strain SF2575 on oatmeal agar incubated at 28°C for 21 days (× 10,000).

Table 1. Cultural characteristics of strain SF2575.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose - nitrate agar	Poor	Abundant, silver gray (3fe)	Colorless	None
Glucose - asparagine agar	Moderate to good	Scant, ivory (2db)	Yellow (3la) to amber (3nc)	None, or faint yellow
Glycerol - asparagine agar (ISP medium 5)	Poor	Gray (2ih)	Light gray (2dc)	None
Calcium - malate agar	Moderate	Abundant, beige brown (3ig)	Colorless	None
Inorganic salts - starch agar (ISP medium 4)	Moderate	Silver gray (3fe)	Old gold (2ne)	Faint yellow
Oatmeal agar (ISP medium 3)	Moderate to good	Covert tan (2ge)	Mustard (2le)	None
Yeast extract - malt extract agar (ISP medium 2)	Moderate	Scant, grayish white (2dc)	Amber (3pc)	Faint yellow
Tyrosine agar (ISP medium 7)	Moderate	Silver gray (3fe)	Light gray (2dc)	None
Nutrient agar	Moderate	None	Buff (2fb)	None
BENNETT agar	Moderate	Scant, grayish white (2dc)	Old gold (2ne)	None

( ): Color number designations taken from Color Harmony Manual, 4th Ed., Container Corporation of America, Chicago, Illinois, U.S.A., 1958.

sclerotia were not observed. Cultural characteristics of strain SF2575 on various media are summarized in Table 1. Mature aerial mass color was in the gray color series. Reverse color was yellow to grayish yellow. A distinct soluble pigment was not produced except for a faint yellow color in inorganic salts - starch agar (ISP medium 4) and yeast extract - malt extract agar (ISP medium 2). Hydrolysis of starch, liquefaction of gelatin and peptonization of skim milk were positive. Reduction of nitrate, coagulation of skim milk and formation of melanoid pigment were negative. Moderate growth was observed on agar medium containing 1.5% NaCl, and no growth occurred on media containing more than 3% NaCl. The strain was aerobic and grew between 15°C and 32°C. No growth occurred at 37°C. Strain SF2575 utilized D-glucose, glycerol, D-xylose, L-arabinose, L-rhamnose, but not D-mannitol, *myo*-inositol, D-fructose and sucrose. Utilizations of raffinose was doubtful. LL-Diaminopimelic acid was detected in the whole-cell hydrolysates. Based on the taxonomic properties described above, strain SF2575 is considered to belong to the genus *Streptomyces*. Further studies for species identification will be reported elsewhere. Strain SF2575 has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. SF2575 and the accession number FERM P-9830.

Fermentation

A well-grown culture of strain SF2575 on an agar slant was inoculated into 20 ml of a culture medium consisting of starch 1.0%, glucose 1.0%, wheat germ 1.0%, Pharmamedia (Traders Protein) 1.0%, soybean meal 1.0%, NaCl 0.2%, K_2HPO_4 0.2% and $MgSO_4 \cdot 7H_2O$ in a 100-ml Erlenmeyer flask. The flask was incubated at 28°C on a rotary shaker at 220 rpm, for 72 hours. The culture (4 ml) was inoculated into 80 ml of the same medium in a 500-ml Erlenmeyer flask and the flask was shaken at 28°C for 72 hours. The culture (50 ml) was transferred to a 5-liter Erlenmeyer flask containing 1 liter of the same medium and the flask was shaken at 28°C for 24 hours. The culture (1 liter) was retransferred to a 50-liter fermenter containing 35 liters of the production medium consisting of maltose syrup 3.0%, soybean meal 0.3%,

soluble vegetable protein 0.5%, wheat germ 1.0%, gluten meal 1.2%, $CaCO_3$ 0.2%, $FeSO_4 \cdot 7H_2O$ 0.0005% and $CoCl_2 \cdot 6H_2O$ 0.0005%. The medium was adjusted to pH 7.0 before sterilization. The fermentation was carried out at 28°C for 120 hours with an air-flow rate of 20 liters/minute and an agitation rate of 250 rpm.

Isolation and Purification

The fermentation broth was filtered with the aid of diatomaceous earth. The antibiotic in the filtrate (80 liters) was extracted with EtOAc (60 liters) at pH 2.0 adjusted with 6 N HCl. The extract was concentrated to a small volume, which was washed with water, dried over anhydrous sodium sulfate and evaporated to dryness under reduced pressure. To the oily residue, hexane was added to obtain a brown precipirate (16.30 g). The precipitate was dissolved in $CHCl_3$ and subjected to column chromatography on silica gel (500 g, Wakogel C-200). The column was successively developed with $CHCl_3$ and a mixture of $CHCl_3$-MeOH (100:1). The fractions containing SF2575 were combined and concentrated to dryness to give a crude powder. Crystallization from MeOH gave yellowish crystals (1.31 g). By recrystallization from MeOH, pure SF2575 was obtained as pale yellow needles.

Physico-chemical Properties

The physico-chemical properties of SF2575 are summarized in Table 2. The molecular formula of SF2575 was determined to be $C_{40}H_{43}NO_{15}$ by the

Table 2. Physico-chemical properties of SF2575.

Appearance	Pale yellow needles
MP	191~193°C
$[\alpha]_D^{24}$	+251.7° (*c* 1.0, $CHCl_3$), +163.6° (*c* 0.5, MeOH)
Molecular formula	$C_{40}H_{43}NO_{15}$
FD-MS (*m/z*)	777 $(M)^+$
Anal Calcd:	C 61.18, H 5.53, N 1.80
Found:	C 61.41, H 5.68, N 1.64
UV λ_{max}^{MeOH} nm (ε)	224 (31,100), 243 (sh, 25,900), 321 (13,200), 358 (17,500)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	241 (27,500), 387 (20,300)
IR (KBr) cm^{-1}	3560, 1720 (sh), 1700 (sh), 1670 (sh)
Solubility	Soluble in MeOH, $CHCl_3$, DMSO, EtOAc, Me_2CO, MeCN Insoluble in H_2O, hexane
Rf[a]	0.34 ($CHCl_3$-MeOH, 10:1), 0.05 (benzene-Me_2CO, 2:1), 0.78 ($CHCl_3$-MeOH-H_2O, 65:25:4)

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

Fig. 2. IR spectrum of SF2575 (KBr).

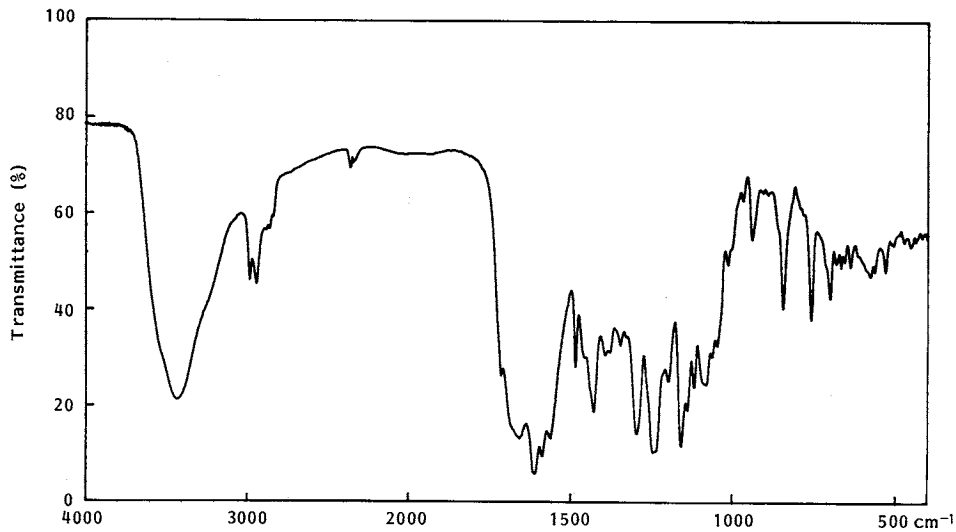


Fig. 3. ^{1}H NMR spectrum of SF2575 (400 MHz, DMSO-d_6).

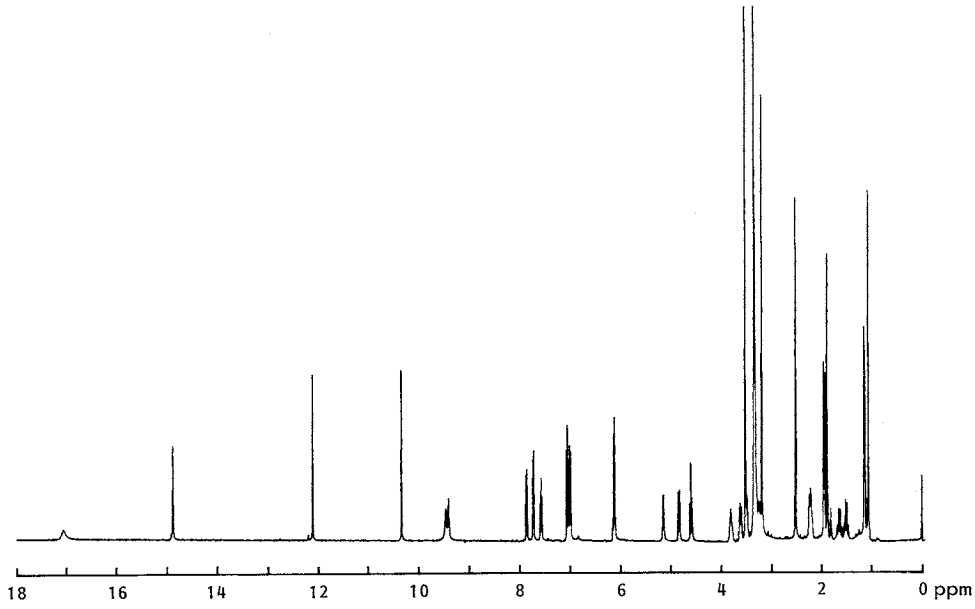
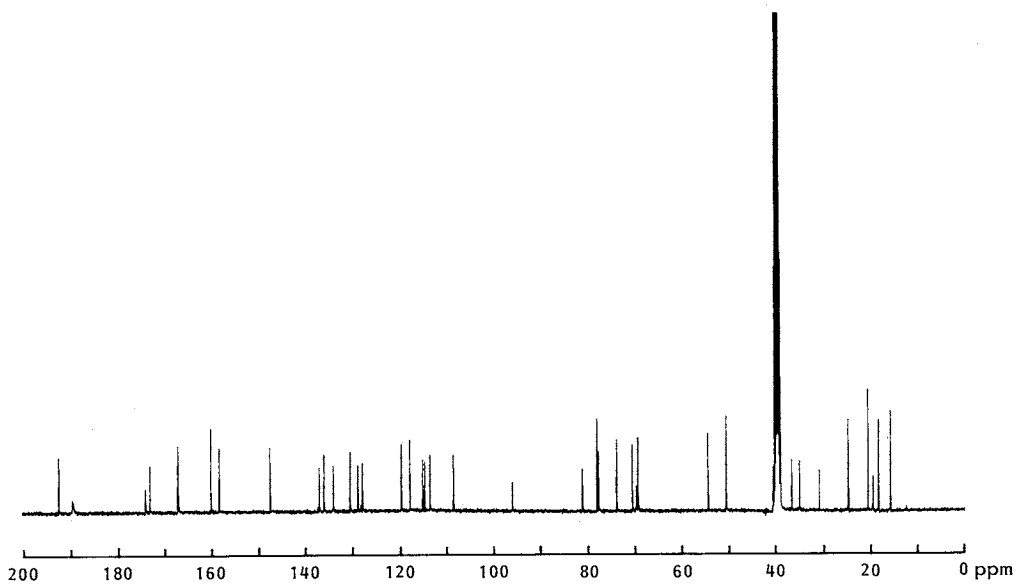


Fig. 4. ^{13}C NMR spectrum of SF2575 (100 MHz, DMSO-d_6).



elemental analysis, FD-MS and ^{13}C NMR spectrum. It shows a bright yellow fluorescence on a silica gel TLC plate and positive color reactions with sulfuric acid, sodium moribdate and iodine reagents. The IR spectrum in KBr showed the absorption bands at 3560 (NH and OH), 1720 (sh, ester), 1700 (sh), 1670 (sh), 1650 (amide) and 1610 cm^{-1}. The IR spectrum in KBr, ^{1}H and ^{13}C NMR spectra in DMSO-d_6 are shown in Figs. 2, 3 and 4, respectively. The spectral data were related to those of the tetracycline antibiotics, but sufficient to differentiate this antibiotic from known antibiotics. Therefore, SF2575 was

Table 3. Antimicrobial activity of SF2575.

Test organism	MIC (μg/ml)	Test organism	MIC (μg/ml)
Staphylococcus aureus 209P JC-1	25	*Salmonella typhi* O-901-W	>100
S. aureus Smith S-424	25	*Shigella sonnei* EW33 Type 1	>100
S. aureus No. 26	25	*Klebsiella pneumoniae* PCI 602	>100
S. epidermidis ATCC 14990	100	*K. pneumoniae* 22 No. 3038	>100
S. epidermidis 109	25	*Proteus vulgaris* OX19	>100
Enterococcus faecalis ATCC 8043	25	*P. mirabilis* GN310	>100
Bacillus anthracis No. 119	3.13	*Providencia rettgeri* J-0026	>100
Escherichia coli JC-2	>100	*Morganella morganii* Kono	>100
E. coli No. 29	>100	*Serratia marcescens* MB-3848	>100
E. coli W3630 RGN823	>100	*Pseudomonas aeruginosa* MB-3829	>100
E. coli JR66/W677	>100	*P. cepacia* M-0527	25
Citrobacter freundii GN346	>100	*Xanthomonas maltophilia* M-0627	>100

Determined on a Sensitivity Disk Agar medium (Nissui Seiyaku).

recognized to be a new antibiotic. Structural elucidation of SF2575 will be reported in an accompanying paper[4].

Biological Properties

Antibiotic SF2575 is weakly active against Gram-positive bacteria, but not active against Gram-negative bacteria as shown in Table 3. The antibiotic exhibited potent cytotoxicity against P388 leukemia cells *in vitro* with IC_{50} value of 7.5 ng/ml. Marked increases in life span (ILS) was observed in experiment with a single ip treatment of SF2575 against mice ip-implanted P388 leukemia cells, as shown in Table 4. The acute LD_{50} of SF2575 in mice by intraperitoneal injection was 11.8 mg/kg, but mice tolerated orally administration of 300 mg/kg.

Table 4. Antitumor activity of SF2575 against P388 leukemia in mice ($n=3$).

Dose (mg/kg, ip)	MSD	ILS (%)
20.0	14.0±0.0 (Toxicity)	67
10.0	16.0±0.0	91
5.0	13.0±1.4	55
2.5	12.0±1.4	43
0.0	8.4±0.5	—

MSD: Mean survival days (mean±SD).

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