SF2446, NEW BENZO[*a*]NAPHTHACENE QUINONE ANTIBIOTICS

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

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New antibiotics SF2446A1, A2, A3, B1 and B2 have been isolated from the culture of *Streptomyces* sp. SF2446 and antibiotic SF2446B3 has been obtained by methanolysis of SF2446B1 or B2. SF2446A1, A2 and B1 showed strong inhibitory activities against mycoplasmas and Gram-positive bacteria. Empirical molecular formulae of antibiotics SF2446-A1, A2, A3, B1, B2 and B3 were determined to be $C_{34}H_{35}NO_{15}$, $C_{34}H_{35}NO_{15}$, $C_{26}H_{21}NO_{11}$, $C_{34}H_{35}NO_{14}$, $C_{34}H_{35}NO_{14}$ and $C_{26}H_{21}NO_{10}$, respectively.

In the course of our screening for antibiotics active against mycoplasmas, we found new antibiotics SF2446A1, A2, A3, B1 and B2 produced by *Streptomyces* sp. SF2446. The antibiotics possess high activities against mycoplasmas including macrolide-resistant strain and Gram-positive bacteria. Antibiotic SF2446B3 having weak activity was obtained by methanolysis of SF2446B1 or B2.

In this paper, we report the taxonomy and fermentation of the producing organism, isolation, physico-chemical properties, and biological activities of antibiotics SF2446. The structural elucidation of antibiotics SF2446 will be reported in an accompanying paper[1].

Materials and Methods

Taxonomic Studies of Antibiotics Producing Strain

The producing organism, strain SF2446, was isolated from a soil sample collected at Hyogo Prefecture, Japan. For the taxonomic characterization of the strain, the methods and media recommended by the International Streptomyces Project (ISP)[2] and those recommended by WAKSMAN[3] were used.

The procedure of BECKER *et al.*[4] was used for the preparation of cells and chromatographic detection of the isomers of diaminopimelic acid.

Fermentation of *Streptomyces* sp. SF2446

A slant culture of strain SF2446 was inoculated into 20 ml of a seed medium consisting of 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) in a 100-ml Erlenmeyer flask and cultured on a rotary shaker (220 rpm) at 28°C for 72 hours. The first seed culture (4 ml) was inoculated into 80 ml of the same medium in a 500-ml Erlenmeyer flask and shaken at 28°C for 48 hours to give the second seed culture. The second seed culture (50 ml) was added to a 5-liter Erlenmeyer flask containing 1 liter of the same medium. After shaking at 28°C for 24 hours, an obtained third seed culture (1 liter) was added to a 50-liter jar fermentor containing 25 liters of the same medium and cultured at 28°C for 24 hours to afford the fourth seed culture. The fourth seed culture (25 liters) was transferred to a

300-liter fermentor containing 200 liters of the production medium consisting of glucose 3.0%, wheat germ 1.5%, soybean meal 0.5%, corn steep liquor 1.0%, $CaCO_3$ 0.1% and $CoCl_2 \cdot 6H_2O$ 0.001% in tap water (pH 7 before sterilization).

Fermentation was executed at 28°C for 120 hours with an air-flow rate of 100 liters/minute, and an agitation rate of 100 rpm for the first 48 hours and then 130 rpm for the remaining course. The concentration of the antibiotics was determined by a paper-disc agar diffusion assay against *Micrococcus luteus* PCI 1001 using SF2446A1 as the assay standard. Most of the antibiotics SF2446 were accumulated in the mycelia and the antibiotic production in the broth filtrate reached a maximum (*ca.* 10 μg/ml) at 120 hours.

Isolation of Antibiotics SF2446

Packed cells obtained from the fermentation broth (two fermentors: *ca.* 400 liters) were extracted with 70% aq Me_2CO and the extract (158 liters, 200 μg/ml) was concentrated to 54 liters. The concentrated extract was adjusted to pH 2.0 and immediately extracted with EtOAc (54 liters×2). The extracts were combined and concentrated to 2 liters. The concentrated solution was kept for a week at 5°C and then filtered. The precipitate, thus obtained, was washed with chilled MeOH and *n*-hexane and dried under reduced pressure to give SF2446A1 (**A1**, 8.59 g, 960 μg/mg) as a dark red crystalline powder. The filtrate (2.3 liters, 3,084 μg/ml) was concentrated to dryness and the residual oil was chromatographically purified three times on silica gel columns (Wakogel C-300, 1.2 kg) with $CHCl_3$ and $CHCl_3$ - MeOH (100 : 1 ~ 10 : 1) to give five active fractions. Fraction 2 was concentrated to give a dark red powder (10.51 g) which, when crystallized three times from $CHCl_3$ - MeOH (1 : 1), afforded **A1** (2.39 g, 970 ~ 1,050 μg/mg). The filtrate from these crystallizations was further purified by Sephadex LH-20 chromatography developed with $CHCl_3$ - MeOH (1 : 1) followed by chromatography on a silica gel column (30 g) eluted with $CHCl_3$ - MeOH (30 : 1) to give SF2446A3 (**A3**, 23.7 mg, 6 μg/mg) as a dark red powder. Fraction 4 was concentrated to dryness to give SF2446A2 (**A2**, 5.07 g, 70.6 μg/mg) as a dark red powder. Fraction 5 (5.72 g) was chromatographed twice on a silica gel column (300 g) developed with $CHCl_3$ - MeOH (50 : 1) and *n*-hexane - Me_2CO (2 : 1) followed by Sephadex LH-20 chromatography with $CHCl_3$ - MeOH (1 : 1) as eluant yielding pure **A2** (1.13 g, 74.3 μg/mg). Fraction 1 (650 mg), containing SF2446B1 (**B1**), was further purified by column chromatography on silica gel (60 g) with *n*-hexane - Me_2CO (2 : 1) followed by preparative TLC developed with *n*-hexane - Me_2CO (1 : 1) to afford **B1** (80.5 mg, 355 μg/ml) as a dark red powder. Purification of fraction 3 (722 mg), containing SF2446B2 (**B2**), was achieved by silica gel (80 g) column chromatography with $CHCl_3$ - MeOH (30 : 1). The crude powder, thus obtained, was purified twice by preparative TLC developed with toluene - EtOAc (1 : 2) and $CHCl_3$ - MeOH (15 : 1) to give **B2** (78.8 mg, 22 μg/mg) as a dark red powder.

Methanolyses of **B1** and **B2**

A solution of **B1** (15.7 mg) in 1 N HCl - MeOH (2.4 ml) was refluxed for 4 hours and then concentrated *in vacuo*. The residue was purified by preparative TLC ($CHCl_3$ - MeOH, 15 : 1) to yield the aglycone SF2446B3 (**B3**, 5.4 mg, 46%) and a methyl 2,4-di-*O*-methyl-α-L-rhamnopyranoside (3.6 mg, 76%, $[\alpha]_D^{25}$ −50.0° (*c* 0.36, $CHCl_3$)).

Methanolysis of **B2** (12.3 mg) under the same condition gave **B3** (4.5 mg, 49%) and the methylglycoside (2.2 mg, 59%).

Results and Discussion

Taxonomy of Strain SF2446

Morphological observations were made on the cultures grown at 28°C for 21 days on glucose - asparagine agar and oatmeal agar (ISP medium 3). Vegetative mycelium was well developed and branched. The hyphae did not fragment into coccoid or bacillary elements. The strain produced aerial mycelium with curved, hooked or spiral spore chains. Spores were ellipsoidal to cylindrical, 0.5 ~ 1.1 × 0.6 ~ 1.3 μm, with spiny surfaces (Fig. 1). Sporangia, flagellated spores and sclerotic

granules were not observed. Cultural characteristics of strain SF2446 on various media are summarized in Table 1. Mature aerial mass color was in the gray color series. No characteristic reverse color was observed. A distinct soluble pigment was not produced except for a faint pink color in tyrosine agar (ISP medium 7). Hydrolysis of starch, liquefaction of gelatin, peptonization and coagulation of skim milk, reduction of nitrate, and formation of melanoid pigment were all negative. Poor growth was observed on agar medium containing 4% NaCl, and no growth occurred on media containing more than 5% NaCl. The strain was aerobic and grew between 20°C and 37°C with an optimum growth at around 28°C. Utilization of carbon sources was performed on ISP medium 9. Strain SF2446 utilized D-glucose and glycerol, but did not utilize D-xylose, L-arabinose, D-mannitol, *myo*-inositol, raffinose and sucrose. Utilizations of D-fructose and L-rhamnose were doubtful. LL-Diaminopimelic acid was detected in the whole-cell hydrolysates.

Based on the taxonomic properties described above, strain SF2446 is considered to belong to the genus *Streptomyces*. Strain SF2446 has been deposited in Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. SF2446 and the accession number FERM P-8980.

Fig. 1. Scanning electron micrograph of spore chains of strain SF2446 on glucose - asparagine agar incubated at 28°C for 21 days (×10,000).

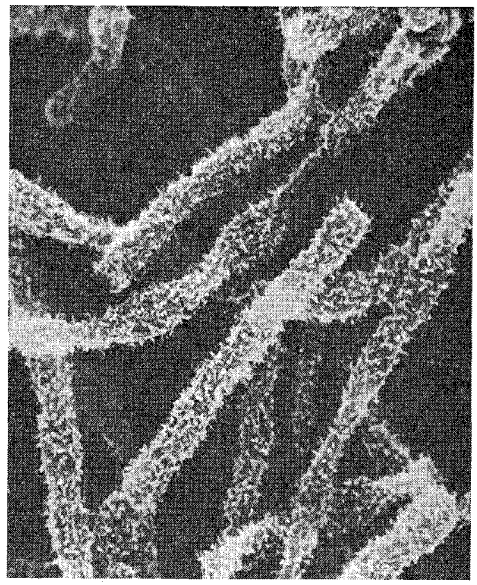


Table 1. Cultural characteristics of strain SF2446.

Medium	Growth	Aerial mycelium	Reverse color	Soluble pigment
Sucrose - nitrate agar	Moderate	Scant, gray (2dc~d)	Colorless~ivory (2db)	None
Glucose - asparagine agar	Moderate	Scant, gray (d)	Ivory (2db)	None
Glycerol - asparagine agar (ISP medium 5)	Moderate	Scant, light gray (2dc)	Ivory (2db)	None
Calcium - malate agar	Poor~moderate	None	Ivory (2db)	None
Inorganic salts - starch agar (ISP medium 4)	Poor	Scant, gray (e)	Colorless	None
Oatmeal agar (ISP medium 3)	Moderate	Scant, gray (e)	Colorless~ivory (2db)	None
Yeast extract - malt extract agar (ISP medium 2)	Good	Scant, gray (e)	Light amber (3ic)	None
Tyrosine agar (ISP medium 7)	Moderate	Scant, gray (e)	Light yellow (2ec)	Faint pink
Nutrient agar	Moderate	Very scant, white	Light yellow (2ec)	None
Bennett agar	Good	Gray (2dc~g)	Light brown (4ng)	None

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

Table 2. Physico-chemical properties of SF2446.

	A1	A2	A3	B1	B2	B3
Appearance	Dark red prismatic crystal	Dark red powder	Dark red powder	Dark red powder	Dark red powder	Dark red powder
Molecular formula	$C_{34}H_{35}NO_{15}$	$C_{34}H_{35}NO_{15}$	$C_{26}H_{21}NO_{11}$	$C_{34}H_{35}NO_{14}$	$C_{34}H_{35}NO_{14}$	$C_{26}H_{21}NO_{10}$
FD-MS (*m/z*)	698, 175	697, 175	523	682, 174	681, 175	507
Anal Calcd C	58.54,	58.54,	59.66,	59.91,	59.91,	
H	5.06,	5.06,	4.04,	5.18,	5.18,	
N	2.01	2.01	2.68	2.05	2.05	
Found C	58.28,	58.10,	59.04,	59.72,	59.16,	
H	5.19,	5.26,	4.12,	5.35,	5.18,	
N	1.97	1.90	2.53	2.21	2.05	
MP (°C)	203~205*	180~184*	>220	188~192*	177~181*	187~192
UV λ_{max}^{MeOH} nm (ε)	217 (43,400), 227 (sh, 33,500), 251 (30,000), 272 (sh, 23,200), 300 (sh, 12,900), 418 (5,200), 472 (5,500)	216 (45,200), 228 (sh, 34,400), 251 (31,200), 270 (sh, 25,800), 300 (sh, 14,700), 417 (5,900), 472 (6,100)	214 (30,700), 228 (sh, 24,100), 258 (sh, 16,800), 272 (sh, 14,100), 300 (sh, 9,700), 412 (2,500), 516 (2,500)	217 (42,500), 230 (sh, 31,700), 252 (31,400), 275 (sh, 23,200), 305 (sh, 11,600), 419 (5,300), 471 (5,600)	217 (42,000), 228 (sh, 32,000), 250 (31,000), 270 (sh, 24,200), 300 (sh, 13,000), 418 (5,700), 471 (6,000)	217 (34,500), 227 (sh, 26,200), 251 (23,500), 275 (sh, 17,500), 300 (sh, 11,700), 409 (4,400), 489 (3,700)
$\lambda_{max}^{MeOH-HCl}$ nm (ε)	217 (40,400), 227 (sh, 32,100), 251 (30,100), 270 (sh, 25,100), 300 (sh, 14,000), 418 (6,000), 472 (6,100)	217 (42,500), 227 (sh, 33,100), 250 (31,400), 270 (sh, 26,200), 300 (sh, 14,700), 415 (6,400), 470 (6,500)	216 (26,800), 230 (sh, 20,900), 255 (sh, 16,800), 275 (sh, 13,600), 302 (sh, 9,900), 410 (3,300), 494 (2,600)	218 (40,000), 227 (31,400, sh), 252 (32,200), 270 (sh, 22,500), 300 (sh, 13,000), 419 (5,900), 469 (6,000)	218 (39,900), 227 (sh, 31,400), 251 (31,600), 270 (sh, 25,200), 305 (sh, 12,300), 416 (5,800), 470 (6,100)	218 (32,700), 227 (sh, 25,400), 251 (24,000), 272 (sh, 18,300), 300 (sh, 12,200), 410 (4,600), 493 (3,800)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	213 (85,300), 233 (sh, 38,400), 240 (sh, 35,600), 275 (sh, 19,500), 300 (sh, 14,800), 570 (7,600)	213 (84,400), 240 (sh, 37,300), 300 (sh, 14,800), 574 (8,600)	214 (60,500), 232 (24,300), 300 (sh, 10,100), 460 (sh, 1,600), 581 (4,300)	215 (83,500), 246 (35,800), 300 (13,300), 589 (8,800)	214 (80,100), 246 (34,100), 300 (13,300), 585 (8,700)	215 (61,700), 240 (sh, 25,900), 285 (sh, 12,400), 460 (2,400), 587 (6,500)
IR (KBr) cm^{-1}	1725, 1680, 1655, 1620, 1120, 1090	1720, 1680, 1650, 1615, 1110, 1095	1720, 1680, 1650, 1625	1720, 1675, 1650, 1620, 1110, 1085	1720, 1680, 1650, 1620, 1110	1720, 1685, 1650, 1625

FD-MS: Field desorption mass spectrometry. * Partial isomerization.

Physico-chemical Properties of Antibiotics SF2446

A1 was crystallized from chloroform - methanol (1 : 2) as dark red prisms which were gradually broken under the atmosphere. **A1** is slightly soluble in methanol, chloroform, ethyl acetate and dioxane and soluble in dimethyl sulfoxide, *N*,*N*-dimethylformamide and pyridine but insoluble in water. Other SF2446 antibiotics are soluble in organic solvents mentioned above but insoluble in *n*-hexane and water. They show reddish orange or reddish purple spots on TLC and positive color reactions with $Mg(OAc)_2$, H_2SO_4, $KMnO_4$ and Na_2MoO_4 reagents and negative with ninhydrin reaction. Optical rotations of these antibiotics could not be measured even in the concentration of 0.5 mg/ml due to their own colors. Other physico-chemical properties of **A1, A2, A3, B1, B2** and **B3** are listed in Table 2. MP's were determined with a Yanaco MP-S3 micro mp apparatus and are uncorrected. UV and IR spectra were recorded on a Shimadzu UV-260 spectrophotometer and a Hitachi 260-10 IR spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were recorded on a

Fig. 2. UV spectra of SF2446A1 in MeOH (49.5 μg/mg).

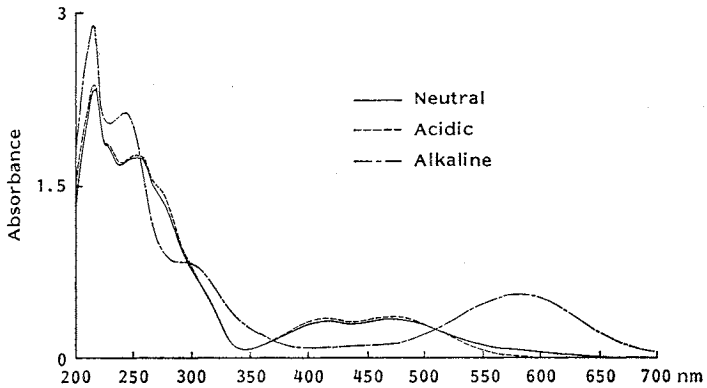


Fig. 3. IR spectrum of SF2446A1 (KBr).

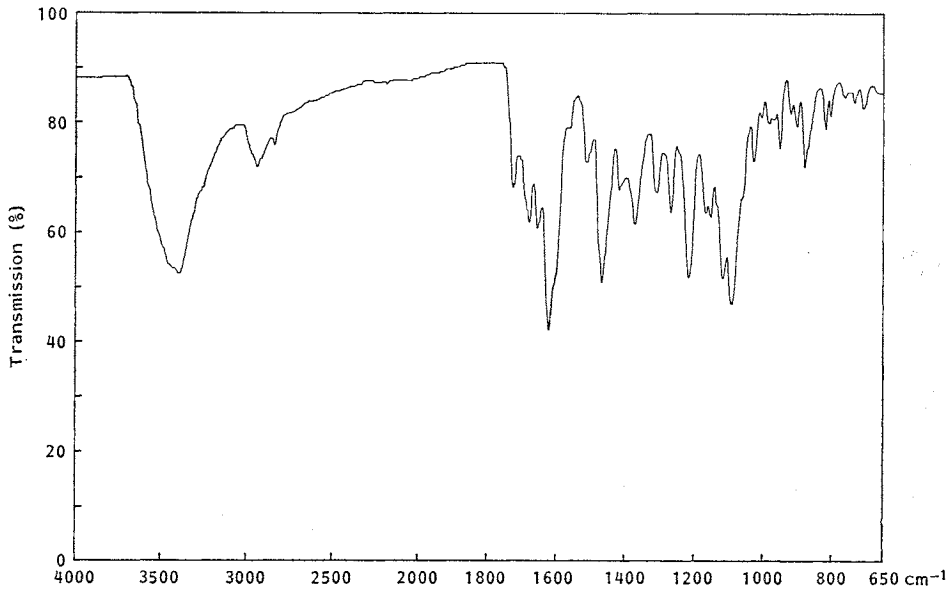


Fig. 4. ^{1}H NMR spectrum of SF2446A1 in $CDCl_3$ (400 MHz),

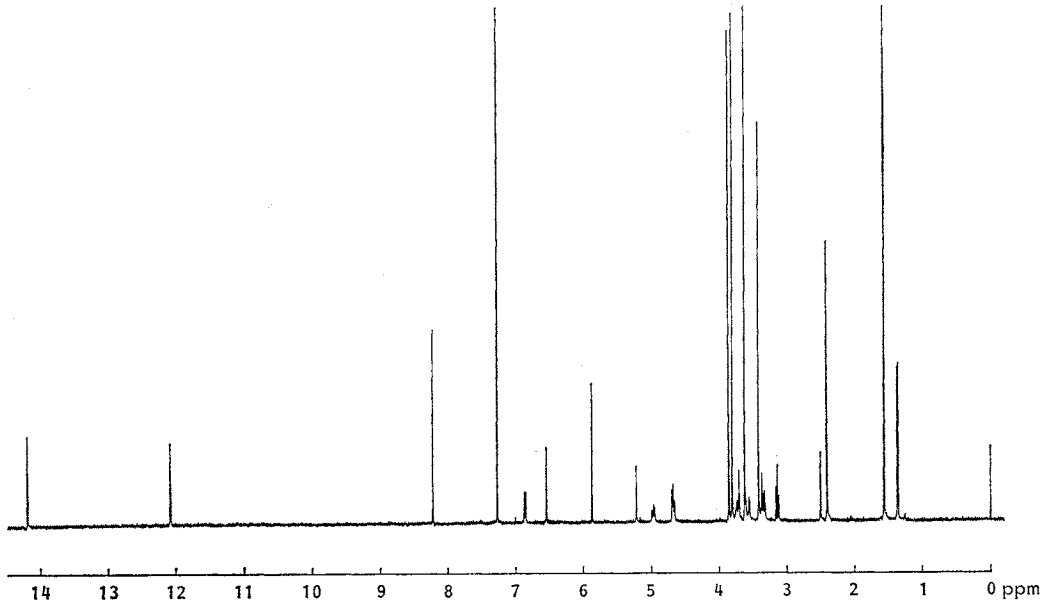
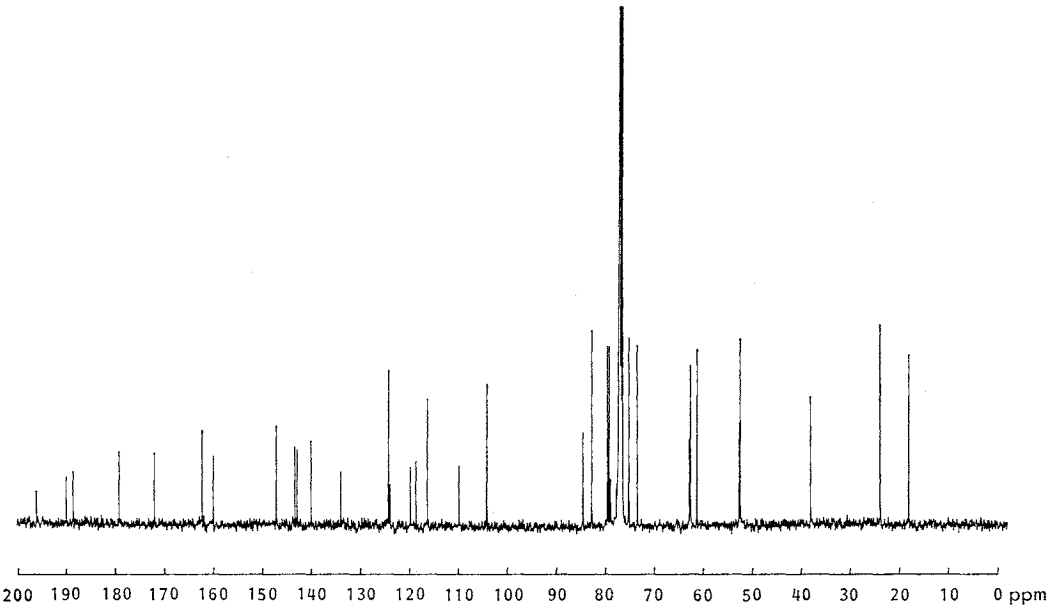


Fig. 5. ^{13}C NMR spectrum of SF2446A1 in $CDCl_3$ (100 MHz).



Jeol JNM-GX400 spectrometer with tetramethylsilane as an internal standard in $CDCl_3$. Mass spectra were recorded with a Hitachi M-80B mass spectrometer.

UV, IR, ^{1}H and ^{13}C NMR spectra of **A1** are shown in Figs. 2, 3, 4 and 5, respectively. Antibiotics SF2446 have a unique benzo[*a*]naphthacene quinone structure and an additional *N*-glycosyl sugar except **A3** and **B3**. Structural elucidation of antibiotics SF2446 will be described in the next paper[1].

Table 3. Antimicrobial activities of SF2446.

Test organisms	MIC (μg/ml)					
	A1	**A2**	**A3**	**B1**	**B2**	**B3**
Staphylococcus aureus 209-P JC-1	<0.025	<0.025	0.39	<0.025	0.10	3.13
S. aureus Smith S-424	<0.025	<0.025	0.20	<0.025	0.10	1.56
S. aureus No. 26	<0.025	0.05	0.39	0.10	0.20	3.13
S. epidermidis ATCC 14990	<0.025	<0.025	0.39	<0.025	0.39	3.13
S. epidermidis 109	<0.025	<0.025	0.20	0.05	0.20	3.13
Enterococcus faecalis ATCC 8043	50	100	>100	50	>100	100
Bacillus anthracis No. 119	<0.025	<0.025	<0.025	<0.025	<0.025	0.10
Escherichia coli JC-2	100	>100	>100	>100	>100	>100
E. coli No. 29	50	>100	>100	>100	>100	>100
E. coli W3630 RGN823	25	>100	>100	>100	>100	>100
E. coli JR66/W677	25	>100	>100	>100	>100	>100
Citrobacter freundii GN 346	50	>100	>100	>100	>100	>100
Salmonella typhi O 901-W	12.5	100	>100	25	>100	>100
S. enteritidis No. 11	12.5	50	>100	12.5	>100	>100
S. typhimurium LT-2	50	>100	>100	100	>100	>100
Salmonella sp. D-0001	50	>100	>100	>100	>100	>100
Shigella sonnei EW 33 Type 1	50	>100	>100	>100	>100	>100
Klebsiella pneumoniae PCI 602	50	>100	>100	>100	>100	>100
K. pneumoniae 22 No. 3038	50	>100	>100	>100	>100	>100
Proteus vulgaris OX 19	50	>100	>100	100	>100	>100
P. mirabilis GN 310	50	>100	>100	>100	>100	>100
Providencia rettgeri J-0026	25	>100	>100	50	>100	>100
Morganella morganii Kono	50	>100	>100	>100	>100	>100
Serratia marcescens MB-3848	100	>100	>100	>100	>100	>100
Pseudomonas aeruginosa MB-3829	50	>100	>100	100	>100	>100
P. cepacia M-0527	25	>100	>100	>100	>100	>100
Xanthomonas maltophilia M-0627	100	>100	>100	>100	>100	>100

Table 4. Anti-mycoplasmal activities of SF2446.

Test organisms	MIC (μg/ml)					
	A1	**A2**	**A3**	**B1**	**B2**	**B3**
Mycoplasma gallisepticum S-6	0.01	0.03	>1.56	0.10	0.20	>1.56
M. gallisepticum KP 13	0.01	0.05	>1.56	0.01	0.10	1.56
M. gallisepticum CH-3T	0.01	0.05	>1.56	0.05	0.20	>1.56
M. gallisepticum 4AS	0.01	0.03	>1.56	0.20	0.20	1.56

Biological Activities of Antibiotics SF2446

Antibiotics SF2446, especially **A1**, **A2** and **B1** have an excellent activity against Gram-positive bacteria and mycoplasmas including macrolide-resistant strains such as *Mycoplasma gallisepticum* 4AS, but show little activity against Gram-negative bacteria and fungi. The antimicrobial and anti-mycoplasmal activities of antibiotics SF2446 are shown in Tables 3 and 4, respectively. Oral administrations of 300 mg/kg of **A1** and **A2** produced no toxicity in mice. When tested in mice by the ip route, the acute LD_{50} of **A1** and **A2** were less than 50 mg/kg and about 100 mg/kg, respectively.

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