NEW ANTIBIOTICS SF2315A AND B PRODUCED BY AN *EXCELLOSPORA* SP.

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

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Two new antibiotics SF2315A and B have been isolated from culture filtrate of an actinomycete strain, *Excellospora viridilutea* SF2315. They are weakly active against Gram-positive bacteria and inhibited reverse transcriptase of avian myeloblastosis virus. Empirical molecular formula of antibiotics SF2315A and B were determined to be $C_{19}H_{16}O_5$ and $C_{19}H_{20}O_6$, respectively.

In the course of screening for new antibiotics from soil microorganisms, it was found that an actinomycete strain SF2315 produced two new antibiotics SF2315A and B which are active against Gram-positive bacteria. In this paper, the taxonomy and fermentation of the producing organism, isolation, and properties of the antibiotics are reported. The structural elucidation of antibiotics SF2315A and B are described in an accompanying paper[1].

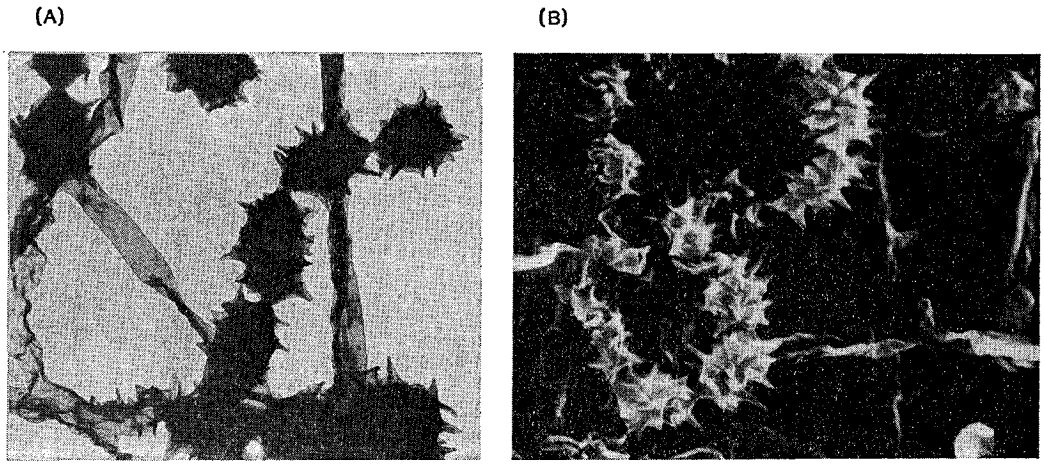
Taxonomy of the Producing Organism

The producing organism, strain SF2315, was isolated from a soil sample collected at Kasagake, Gunma Prefecture, Japan. For the taxonomic characterization of the strain SF2315, the methods and media recommended by the International Streptomyces Project (ISP)[2] and by WAKSMAN[3] were used. Diaminopimeric acid and sugars in whole-cell hydrolysates were determined by the method of BECKER *et al.*[4] and LECHEVALIER[5], respectively. Phospholipids and menaquinones were analyzed by the procedure of LECHEVALIER *et al.*[6] and COLLINS *et al.*[7], respectively.

Morphological observation was made on the cultures grown at 37°C for 10 to 15 days on inorganic salts - starch agar (ISP medium 4), glycerol - asparagine agar (ISP medium 5) and calcium - malate agar. Vegetative mycelium was well developed and branched. Spore formation on vegetative mycelium was sometimes observed, but no fragmentation of hyphae occurred. Aerial mycelium was simply branched and formed short, loops or spiral spore chains. Spore chains had 2 to 20 spores per chain. Spores were spherical to elliptical ($0.5 \sim 0.8 \times 0.7 \sim 1.2$ μm) with spiny surfaces as shown in Fig. 1. Sclerotic granules, sporangia and flagellated spores were not observed.

Cultural characteristics of strain SF2315 are shown in Table 1. The results were recorded after 14 days of incubation at 37°C. Aerial mass color was in the blue color series of TRESNER and BACKUS[8]. The reverse side of colony was colorless to yellow and no soluble pigment was formed. Physiological properties of strain SF2315 are summarized in Table 2. In whole-cell hydrolysates *meso*-diaminopimeric acid and madurose were detected. The strain had phospholipids of type PI and contained MK-9 (H_6) and MK-9 (H_8) as its major menaquinones.

Fig. 1. *Excellospora viridilutea* SF2315.



Spiny spores on calcium - malate agar; 14 days incubation.
(A) Transmission electron microscope, ×10,000.
(B) Scanning electron microscope, ×10,000.

Table 1. Cultural characteristics of strain SF2315.

Medium	Growth	Aerial mycelium*	Reverse*	Soluble pigment
Sucrose - nitrate agar	Moderate	Very scant, white	Colorless to pearl (2ba)	None
Glucose - asparagine agar	Poor	None	Colorless	None
Glycerol - asparagine agar (ISP medium 5)	Moderate	Abundant, aqua gray (19fe)	Ivory (2db)	None
Inorganic salts - starch agar (ISP medium 4)	Moderate to good	Abundant, aqua gray (19fe)	Ivory (2db)	None
Calcium - malate agar	Moderate	Abundant, aqua gray (19fe)	Colorless to cream (1ca)	None
Oatmeal agar (ISP medium 3)	Moderate to good	Scant, white	Parchment (1db)	None
Yeast extract - malt extract agar (ISP medium 2)	Good	Scant, white	Light gold (2ic)	None
Tyrosine agar (ISP medium 7)	Moderate	Scant, white	Light ivory (2ca)	None
Nutrient agar	Moderate	Scant, white	Bamboo (2gc)	None
BENNETT's agar	Good	Scant, white	Tan (3ie)	None

* The color scheme used was Color Harmony Manual, 4th Ed., 1958 (Container Corporation of America, Chicago, U.S.A.).

The above morphological characteristics and chemotaxonomic properties of strain SF2315 indicate that this isolate belongs to the genus *Excellospora* AGRE and GUZEVA[9]. Among the known species of *Excellospora*, *E. viridilutea*[9] is similar to strain SF2315, except for some physiological properties, as shown in Table 2. These differences are not sufficient to designate strain SF2315 as a new species. Therefore, the strain is considered to be a new strain of *E. viridilutea*. Strain SF2315 has been deposited

Table 2. Comparison of taxonomic characteristics of strain SF2315 and *Excellospora viridilutea*.

	Strain SF2315	*E. viridilutea* IFO 14480
Spore chain	Short to spiral	Short to spiral
Spores/chain	2~20	1~20
Spore surface	Spiny	Spiny
Spores on vegetative mycelium	Present	Present
Cell-wall type	IIIB	IIIB
Major menaquinone*	MK-9 (H_6), MK-9 (H_8)	MK-9 (H_6)
Phospholipid type*	PI	PI
Aerial mass color	Blue	Blue
Reverse color	Yellow	Yellow
Soluble pigment	None	None
Temperature for growth (°C)		
Minimum	25	37
Optimum	37~45	45~55
Maximum	60	65
Liquefaction of gelatin	Negative	Positive
Hydrolysis of starch	Positive	Positive
Coagulation of milk	Positive	Positive
Reduction of nitrate	Positive	Negative
Formation of melanin	Negative	Negative
Carbon utilization on CZAPEK's agar medium*		
D-Glucose	+	+
L-Arabinose	+	−
D-Xylose	+	+
D-Fructose	±	+
D-Mannitol	−	+
i-Inositol	−	±
L-Rhamnose	+	+
Raffinose	−	−
Sucrose	±	+

* The data were obtained from comparative experiments with the type strain.
Symbols: +, Positive; ±, doubtful; −, negative.

in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, with an accession number of FERM P-8735.

Fermentation

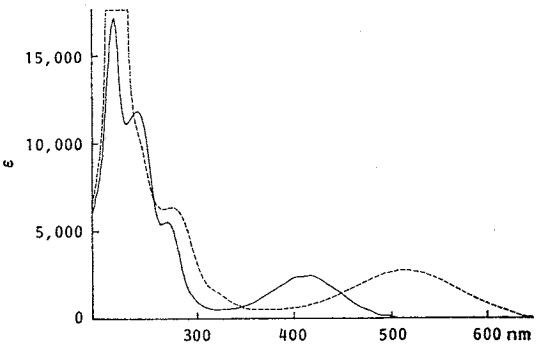
Two Erlenmeyer flasks (100-ml) each containing 20 ml of the seed medium consisting of glucose 1.0%, starch 1.0%, Polypepton (Daigo Eiyo Co., Ltd., Osaka) 0.5%, yeast extract 0.2%, meat extract 0.2%, soybean meal 0.2%, and $CaCO_3$ 0.1% were inoculated with the culture of strain SF2315 grown on an agar slant. The inoculated flasks were shaken at 37°C for 2 days on a rotary shaker (220 rpm). Thirty ml of the seed culture were transferred to 1 liter of the same medium in a 5-liter Erlenmeyer flask. After shaking at 28°C for 24 hours, 700 ml of the second seed was transferred to a 50-liter fermentor containing 35 liters of the production medium (starch 3.0%, cotton seed meal 0.5%, wheat germ 1.0%, meat extract 1.0%, $MgSO_4 \cdot 7H_2O$ 0.1%, $CaCO_3$ 0.3% and $CoCl_2 \cdot 6H_2O$ 0.001%). Fermentation was carried out at 28°C for 96 hours with an air-flow rate of 35 liters per minute and an agitation rate of 300 rpm for the first 41 hours and 500 rpm for the remaining course of fermentation. The pH

Table 3. Physico-chemical properties of antibiotics SF2315A and B.

	SF2315A	SF2315B
Appearance	Orange prisms	Pale yellow prisms
MP (°C)	175~177	185~186
Molecular formula	$C_{19}H_{16}O_5$	$C_{19}H_{20}O_6$
HR-MS (*m/z*)		
Calcd:	324.0997	344.1258
Found:	324.1062	344.1195
Anal Calcd:	C 70.36, H 4.97.	C 66.27, H 5.85.
Found:	C 70.29, H 4.93.	C 66.24, H 5.86.
$[\alpha]_D^{25}$ (*c* 0.1, MeOH)	+229°	+103°
UV λ_{max}^{MeOH} nm (ε)	237 (13,100), 269 (6,100), 419 (2,500)	267 (4,200), 339 (2,200)
$\lambda_{max}^{MeOH-NaOH}$ nm (ε)	272 (7,300), 515 (2,800)	232 (6,100), 268 (2,300), 376 (2,600)
IR ν_{max} (KBr) (cm^{-1})	3400, 1690 (sh), 1660, 1640	3400, 3300, 1640

was continuously kept below pH 6.5 with 9 N H_2SO_4. Antibacterial activity was assayed by an agar diffusion method using *Micrococcus luteus* PCI 1001 as a test organism.

Fig. 2. UV spectrum of antibiotic SF2315A.
— MeOH, --- MeOH - NaOH.



Isolation Procedures

The fermented broth of four 50-liter jar fermentors was combined and filtered with the aid of Hyflo Super-Cel (Johns-Manville). The filtrate (80 liters) was passed through a column of Diaion HP-20 (8 liters) and the column was washed with water (30 liters) and 50% aqueous methanol (30 liters). The antibiotics were eluted from the resin with 70% aqueous acetone. The active eluate was concentrated under reduced pressure and the concentrate was extracted twice with ethyl acetate (10 liters). The combined extract was washed with water (10 liters) and concentrated. The resulting syrup was applied to a column of silica gel (Wakogel C-200, 230 g) packed with chloroform. After washing with chloroform the column was developed with a mixture of chloroform - metanol (50 : 1). Two active fractions were obtained. After the first active fraction was evaporated to dryness, the residue was crystallized from hot ethanol to yield antibiotic SF2315A (53.4 mg) as orange prisms. After concentration of the second active fraction to dryness, the residue was crystallized from hot ethanol to give antibiotic SF2315B (1.075 g) as pale yellow prisms.

Physico-chemical Properties

Physico-chemical properties of antibiotics SF2315A and B are listed in Table 3. Antibiotics SF2315 are easily soluble in dimethyl sulfoxide, soluble in methanol, chloroform, acetone and aqueous alkali, but insoluble in aqueous acid.

The molecular formula of antibiotic SF2315A was determined to be $C_{19}H_{16}O_5$ from the elemental analysis and high resolution mass spectrometry (HR-MS) (Table 3). The UV spectrum of SF2315A in methanol exhibited maxima at 237, 269 and 419 nm shifting to 272 and 515 nm in basic methanol as shown in Fig. 2. The IR spectrum in KBr and ^1H NMR at 400 MHz spectrum in DMSO-d_6 are

Fig. 3. IR spectrum of antibiotic SF2315A (KBr disk).

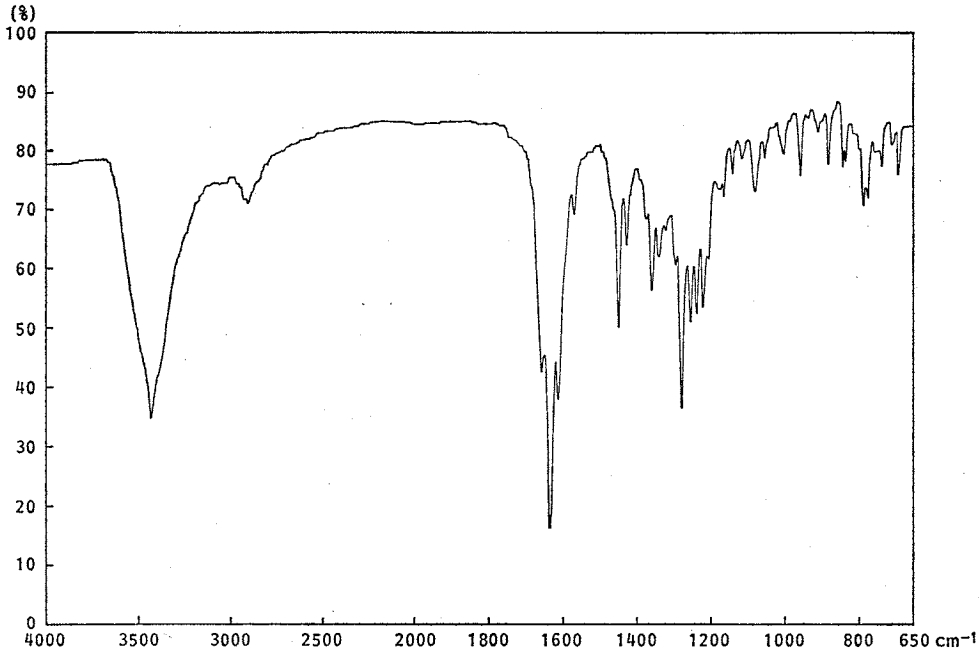
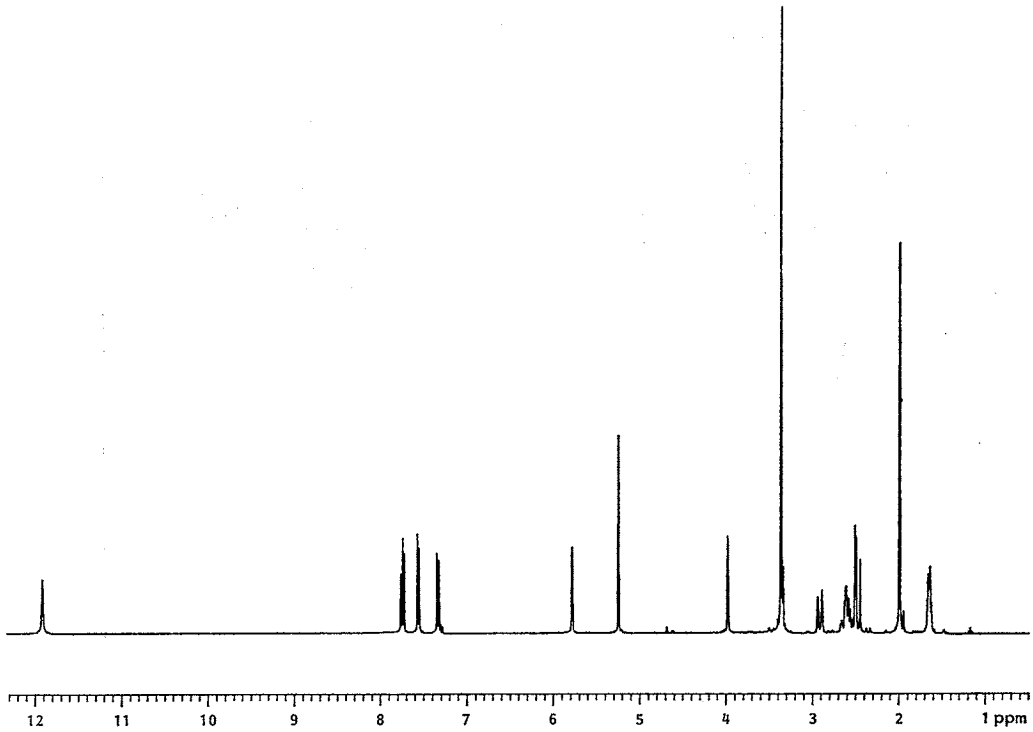


Fig. 4. ^{1}H NMR spectrum of antibiotic SF2315A in DMSO-d_6 (400 MHz).



shown in Figs. 3 and 4.

The molecular formula of antibiotic SF2315B was determined to be $C_{19}H_{20}O_6$ from the elementary analysis and HR-MS (Table 3). The UV spectrum of SF2315B in methanol exhibited maxima at 267 and 339 nm shifting to 232, 268 and 376 nm in basic methanol as shown in Fig. 5. The IR spectrum and ^1H NMR at 400 MHz spectrum in DMSO-d_6 are shown in Figs. 6 and 7.

From the physico-chemical properties mentioned above, it was concluded that SF2315A and B were new antibiotics having the carbon skeleton of benz[a]anthracene. Details of the structural elucidation will be reported in a separate paper[1].

Biological Properties

Antibiotics SF2315A and B are weakly active against some Gram-positive bacteria but are not active against Gram-negative bacteria at the concentration of lower than 100 μg/ml as shown in Table 4.

Inhibitory activities of antibiotics SF2315A and B against reverse transcriptase of avian myeloblastosis virus (Life Sciences Inc.) were determined by the method reported by HOUTS *et al.*[10]. The IC_{50} values of antibiotics SF2315A and B were 100 and 40 μg/ml, respectively. DNA directed DNA polymerase of *Escherichia coli* (Klenow) was not inhibited by the antibiotics

Fig. 5. UV spectrum of antibiotic SF2315B.
— MeOH, --- MeOH - NaOH.

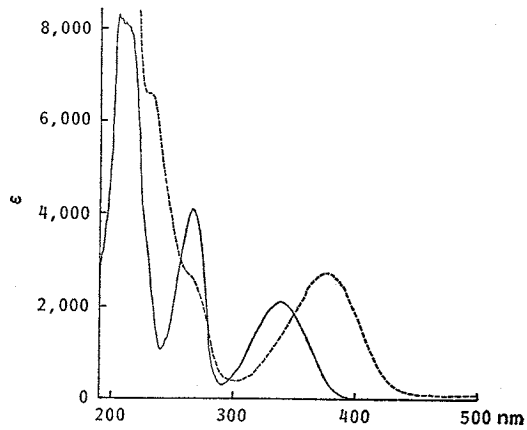


Fig. 6. IR spectrum of antibiotic SF2315B (KBr disk).

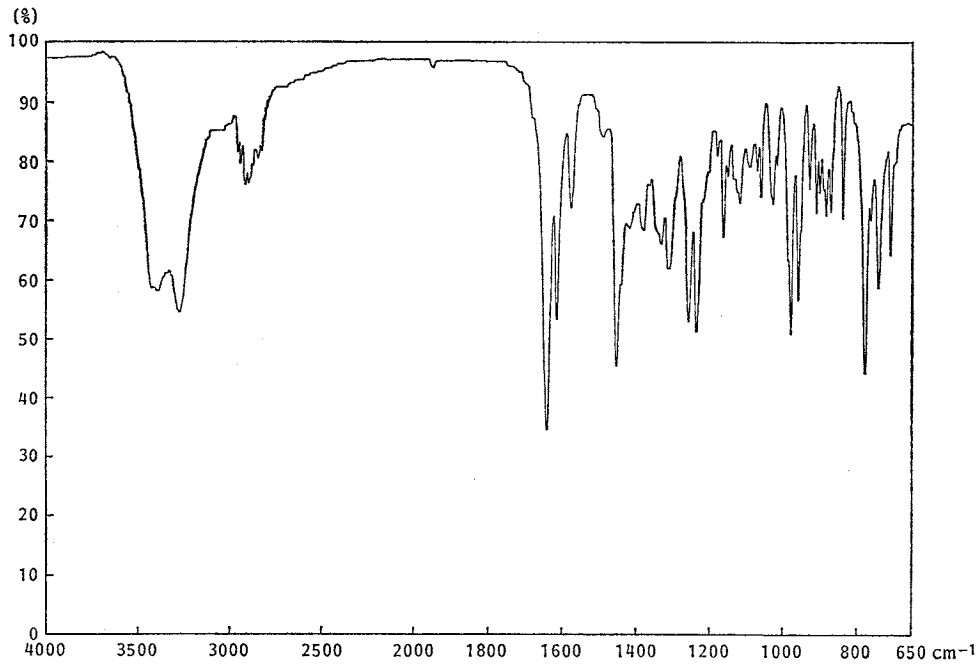


Fig. 7. ^{1}H NMR spectrum of antibiotic SF2315B in DMSO-d_6 (400 MHz).

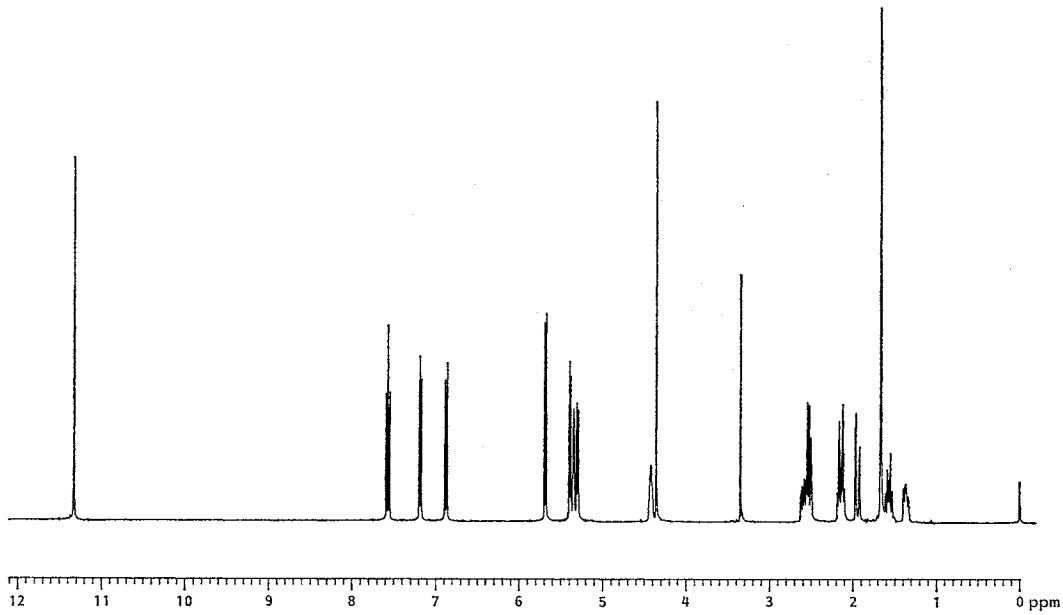


Table 4. Antimicrobial activities of antibiotics SF2315A and B.

Test organisms	MIC (μg/ml)	
	SF2315A	SF2315B
Staphylococcus aureus 209P JC-1	50	25
S. aureus Smith S-424	6.25	25
S. aureus No. 26	50	50
S. epidermidis ATCC 14990	12.5	50
S. epidermidis 109	12.5	50
Bacillus anthracis No. 119	3.13	12.5
Enterococcus faecalis ATCC 8043	>100	>100
Escherichia coli NIHJ JC-2	>100	>100
Citrobacter freundii GN346	>100	>100
Salmonella typhi O-901-W	>100	100
Klebsiella pneumoniae PCI 602	>100	>100
Proteus vulgaris OX 19	>100	>100
Providencia rettgeri J-0026	>100	>100
Serratia marcescens MB-3848	>100	>100
Pseudomonas aeruginosa MB-3829	>100	>100
P. cepacia M-0527	100	>100

at the concentration of lower than 100 μg/ml.

No antitumor activities of SF2315A and B were observed against P388 leukemia in mouse by intraperitoneal administration of 400 mg/kg for SF2315A and 50 mg/kg for SF2315B.

The acute toxicity (LD_{50}) of SF2315A was more than 400 mg/kg and that of SF2315B was 100 to 200 mg/kg when administered intraperitoneally in mice.

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References

1) SASAKI, T.; S. GOMI, M. SEZAKI, Y. TAKEUCHI, Y. KODAMA & K. KAWAMURA: New antibiotics SF2315A and B produced by an *Excellospora* sp. II. The structural elucidation. J. Antibiotics 41: 843~848, 1988
2) SHIRLING, E. B. & D. GOTTLIEB: Methods for characterization of *Streptomyces* species. Int. J. Syst. Bacteriol. 16: 313~340, 1966
3) WAKSMAN, S. A. (*Ed.*): The Actinomycetes. Classification, Identification and Descriptions of Genera and Species. Vol. 2. Williams & Wilkins Co., Baltimore, 1961
4) BECKER, B.; M. P. LECHEVALIER, R. E. GORDON & H. A. LECHEVALIER: Rapid differentiation between *Nocardia* and *Streptomyces* by paper chromatography of whole-cell hydrolysates. Appl. Microbiol. 12: 421~423, 1964
5) LECHEVALIER, M. P.: Identification of aerobic actinomycetes of clinical importance. J. Lab. Clin. Med. 71: 934~944, 1968
6) LECHEVALIER, M. P.; C. DEBIEVRE & H. A. LECHEVALIER: Chemotaxonomy of aerobic actinomycetes: Phospholipid composition. Biochem. Syst. Ecol. 5: 249~260, 1977
7) COLLINS, M. D.; T. PIROUZ & M. GOODFELLOW: Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100: 221~230, 1977
8) TRESNER, H. D. & E. J. BACKUS: System of color wheels for streptomycete taxonomy. Appl. Microbiol. 11: 335~338, 1963
9) AGRE, N. S. & L. N. GUZEVA: New actinomycetes genus: *Excellospora* gen. nov. Mikrobiologiya 44: 518~523, 1975
10) HOUTS, G. E.; M. MIYAGI, C. ELLIS, D. BEARD & J. W. BEARD: Reverse transcriptase from avian myeloblastosis virus. J. Virol. 29: 517~522, 1979