STUDIES ON NEW ANTIBIOTICS SF2415

I. TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL PROPERTIES AND BIOLOGICAL ACTIVITIES

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(Received for publication January 9, 1987)

A new species of *Streptomyces* is described for which the name *Streptomyces aculeolatus* is proposed. The organism produces new antibiotics SF2415A1, A2, A3, B1, B2 and B3 active against Gram-positive bacteria. Empirical molecular formulae of the antibiotics SF2415A1, A2, A3, B1, B2 and B3 were determined to be $C_{26}H_{31}N_2O_5Cl$, $C_{26}H_{30}N_2O_5$, $C_{26}H_{30}N_2O_5Cl_2$, $C_{26}H_{33}O_5Cl$, $C_{26}H_{32}O_5$ and $C_{26}H_{32}O_5Cl_2$, respectively.

In the course of our screening program for new antibiotics, we have isolated a strain of *Streptomyces*, designated strain SF2415, which produces new antibiotics SF2415.

In this paper, we report the taxonomy of the producing organism, fermentation, isolation, physico-chemical properties and biological activities of the antibiotics SF2415. The structural elucidation of the antibiotics SF2415 is described in accompanying paper.[1]

Materials and Methods

Taxonomic Studies of Antibiotics Producing Strain

The producing organism, strain SF2415, was isolated from a soil sample collected in Tottori Prefecture, Japan.

Methods adopted by the International Streptomyces Project (ISP)[2] were used for taxonomic characterization and carbohydrate utilization studies. Cultural characteristics were determined on media recommended by the ISP and WAKSMAN.[3] Observations were made after incubation at 28°C for 14 days.

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

Fermentation of *Streptomyces aculeolatus*

A slant culture of strain SF2415 was inoculated into a 100-ml Erlenmeyer flask that contained 20 ml of a seed medium consisting of starch 2%, glucose 1%, wheat germ 0.6%, Polypeptone 0.5%, yeast extract 0.3%, soybean meal 0.2% and $CaCO_3$ 0.1% (pH 7). The inoculated flask was cultured on a rotary shaker (220 rpm) at 28°C for 3 days. The first seed culture (4 ml) was inoculated into 80 ml of the same medium in a 500-ml Erlenmeyer flask. After shaking at 28°C for 2 days, the second seed culture was obtained. The second seed (50 ml) was added to a 5-liter Erlenmeyer flask containing 1 liter of the same medium. The third seed culture was shaken at 28°C for 24 hours, and 1 liter of

the culture was added to a 50-liter jar fermentor containing 35 liters of the production medium (starch 2%, soybean oil 1%, cotton seed meal 1.5%, corn gluten meal 0.7%, $CaCO_3$ 0.3% and $FeSO_4 \cdot 7H_2O$ 0.001% in a tap water (pH 7 before sterilization)).

Fermentation was carried out at 28°C for 72 hours with an air-flow rate of 20 liters per minute, and an agitation rate of 250 rpm for first 41 hours and 400 rpm for the remaining course of the fermentation. Packed cell volumes were determined by centrifuging at 3,000 rpm for 15 minutes in 15 ml, conical tubes. Antibiotic activity was determined by a paper-disc agar diffusion assay, using *Micrococcus luteus* PCI 1001 as an assay organism. Antibiotic SF2415A1 was used as an assay standard.

Isolation of Antibiotics SF2415

The fermentation broth in a 50-liter jar fermentor was filtered by using Hyflo Super-Cel (Johns-Manville) as the filter aid and the filtrate (30 liters) and packed cells (10 liters) were obtained. The antibiotics in the packed cells were extracted with a mixture (1 : 1, 30 liters) of Me_2CO and water and the extract was concd to 10 liters. The antibiotics in the filtrate were adsorbed on a column of Diaion HP-20 (3 liters) and the antibiotics were eluted with a mixture (1 : 1, 30 liters) of 0.5 N NH_4OH and Me_2CO. The active eluates were collected and concd to 5 liters. Concentrated extracts and eluates were combined and the antibiotics in the mixture extracted with EtOAc (15 liters). The resulting extract was then concd under reduced pressure to give a dark red oil (38 g). The residual oil was adsorbed on 50 g of silica gel and charged on a silica gel column (200 g). The column was eluted stepwise with toluene - EtOAc (75 : 1, 300 ml), (50 : 1, 340 ml), (20 : 1, 1,000 ml) and (10 : 1, 1,100 ml). Approximately 170-ml fractions were collected. The fractions (Nos. 3~5) containing SF2415B2 (B2) were concd to give a brownish oil (1.26 g) and further purification was achieved by column chromatography on silica gel (150 g) eluting with toluene - EtOAc (20 : 1) to afford a yellow oil (180 mg) of B2. The fractions (Nos. 6~7) containing SF2415B1 (B1) and SF2415B3 (B3) were concd to give a brownish oil (1.09 g). The crude oil was further purified by Sephadex LH-20 (800 ml) chromatography developed with a mixture (9 : 1) of $CHCl_3$ and MeOH followed by column chromatography on silica gel (50 g) eluted with toluene - EtOAc (75 : 1) to give a pale yellow oil (280 mg) of B1 and hygroscopic yellow needles (250 mg) of B3. The fractions (Nos. 9~11) on the first silica gel column chromatography containing SF2415A2 (A2) and SF2415A3 (A3) were concd to give a dark red oil (1.16 g). The residual oil was purified by chromatography on Sephadex LH-20 (800 ml) developed with a mixture (9 : 1) of $CHCl_3$ and MeOH to give a crude oil (470 mg). The crude oil was further purified by column chromatography on silica gel (40 g) eluted with toluene - EtOAc (75 : 1) and (50 : 1) followed by preparative TLC developed with hexane - Me_2CO (3 : 1) to afford a red oil (54 mg) of A2 and a hygroscopic red powder (50 mg) of A3. The fractions (Nos. 13~15) on the first silica gel column chromatography containing SF2415A1 (A1) was concd to give a dark orange oil (1.14 g) and was further purified by Sephadex LH-20 (800 ml) chromatography developed with a mixture (9 : 1) of $CHCl_3$ and MeOH followed by chromatography on silica gel (30 g) eluted with toluene - EtOAc (10 : 1) to afford an orange powder (132 mg), which was recrystallized from hexane to give 70 mg of yellow needles of A1.

Results and Discussion

Taxonomy of Strain SF2415

Vegetative mycelium was well developed and branched. The hyphae did not fragment into coccoid or bacillary elements. Aerial mycelium was simply branched and terminated in open or closed coils. Mature spore chains have 10 or more spores per chain. This morphology was observed in sucrose - nitrate agar, glycerol - asparagine agar (ISP medium 5), and inorganic salts - starch agar (ISP medium 4). Spores were ellipsoidal in shape, 0.8~1.2 by 1.0~1.6 μm in size. Surface irregularities on spores were intermediate between warts and spines (Figs. 1 and 2). Sporangia, flagellated spores, and sclerotic granules were not observed.

Cultural characteristics of strain SF2415 are shown in Table 1. Aerial mass color was in the

Fig. 1. Transmission electron micrograph of a spore chain of strain SF2415 on inorganic salts - starch agar (ISP medium 4) incubated at 28°C for 14 days. × 20,000.

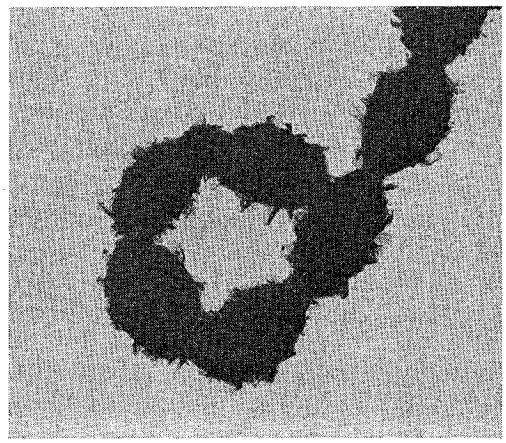


Fig. 2. Scanning electron micrograph of a spore chain of strain SF2415 on glycerol - asparagine agar (ISP medium 5) incubated at 28°C for 14 days. ×15,000.

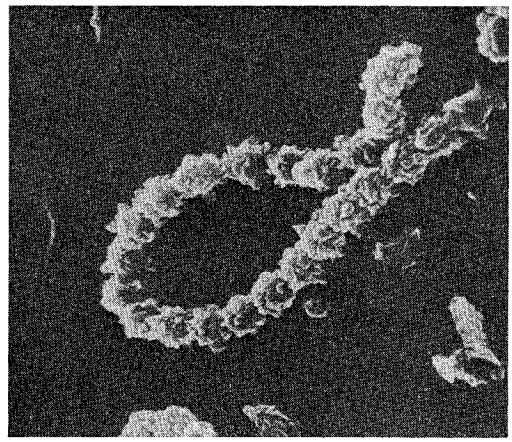


Table 1. Cultural characteristics of strain SF2415.

Medium	Growth	Aerial mycelium*	Reverse*	Soluble pigment
Sucrose - nitrate agar	Poor to moderate	White (a)	Colorless to light yellow (1ea)	None
Glucose - asparagine agar	Poor	None	Colorless	None
Glycerol - asparagine agar (ISP medium 5)	Moderate	White (a)	Colorless to light yellow (1ea)	None
Inorganic salts - starch agar (ISP medium 4)	Moderate to good	White (a) to shell (3ca)	Light melon yellow (3ea)	Light apricot
Calcium - malate agar	Poor to moderate	White (a) to ivory (2db)	Maple (4le)	None, or pale brown
Oatmeal agar (ISP medium 3)	Poor to moderate	Scant, white (a)	Orange (4la)	None
Yeast extract - malt extract agar (ISP medium 2)	Good	None, or scant, ivory (2db)	Maple (4le) to dusty orange (4lc)	Pale brownish orange
Tyrosine agar (ISP medium 7)	Moderate	White (a)	Bamboo (2gc)	None
Nutrient agar	Moderate	None	Cinnamon (3le)	None
Bennett agar	Good	None	Pastel orange (4ic) to light brown (3lg)	None

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

white, yellow or red color series of TRESNER and BACKUS.[5] The reverse side of colonies varied from pale yellow to orange depending on the medium. This orange color was somewhat pH sensitive, changing from orange to reddish with addition of 0.05 N NaOH and from orange to yellowish with addition of 0.05 N HCl. Light brownish orange, water-soluble pigment was occasionally produced.

Table 2. Comparison of strain SF2415 with related *Streptomyces* species.

	Strain SF2415	*Streptomyces arabicus*	*Streptomyces erythrogriseus*
Aerial mass color on			
ISP medium 4	R, W	Gy	Gy
ISP medium 5	W	R	Gy, R, W
Utilization of			
i-inositol	−	+	+
sucrose	−	±	−
raffinose	+	−	−

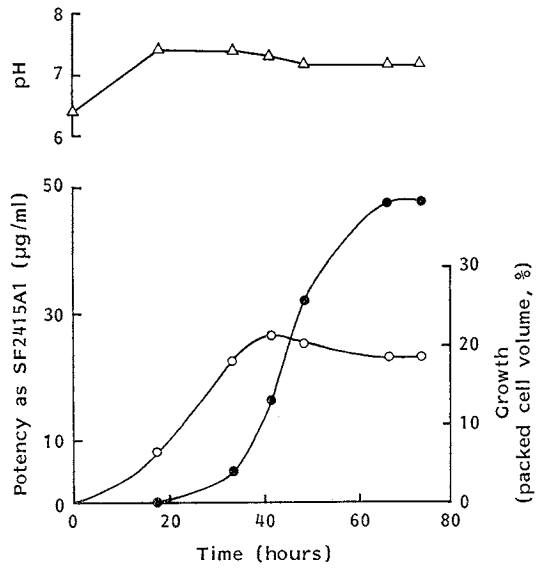
Abbreviations: R; Red, W; white, Gy; gray.
Symbols: +; Positive, ±; doubtful, −; negative.

Strain SF2415 grew within a temperature range of 15°C to 37°C, with an optimum range of 26°C to 30°C. Hydrolysis of starch and liquefaction of gelatin were positive. Reduction of nitrate, peptonization and coagulation of milk, and formation of melanoid pigment were all negative. Strain SF2415 tolerated 3% NaCl, but no growth occurred on more than 4% NaCl. On ISP medium 9 the strain utilized D-glucose, D-fructose, D-xylose, L-arabinose, D-mannitol, raffinose and L-rhamnose, but did not utilized *i*-inositol and sucrose.

LL-Diamino-pimeric acid was detected in whole-cell hydrolysates of the culture.

Based on the taxonomic properties described above, strain SF2415 is considered to belong to the genus *Streptomyces*. A comparison of the description of strain SF2415 with those of the *Streptomyces* species described previously showed that no known species are identical to strain SF2415 based on the following combination of criteria: White, yellow or red color series, spiral spore chain, warty to spiny spore surface, non-chromogenic reaction on ISP media 1, 6 and 7, and carbon utilization pattern. Organisms that resemble this strain are *Streptomyces arabicus*[6] and *Streptomyces erythrogriseus*.[7] As summarized in Table 2, however, strain SF2415 differs clearly from these two species in their aerial mass color and utilization of some carbon sources, such as *i*-inositol, sucrose and raffinose.

Fig. 3. Time course of fermentation in a 50-liter jar fermentor.
○; Growth, ●; potency, △; pH.



Therefore, we regard strain SF2415 as a new species, for which we propose the name *Streptomyces aculeolatus* (acu.le.o.la'tus. L. adj. *aculeolatus* somewhat spiny, referring to the spore surfaces). Strain SF2415, the type strain of *S. aculeolatus*, have been deposited in the Japan Collection of Microorganisms, with accession number of JCM 6055.

Fermentation of *S. aculeolatus*

The time course of antibiotics SF2415 fermentation in a 50-liter jar fermentor is shown in Fig. 3.

Table 3. Physico-chemical properties of SF2415.

	A1	A2	A3	B1	B2	B3
Appearance	Yellow needles	Red oil	Red powder	Pale yellow oil	Yellow oil	Yellow needles
Molecular formula	$C_{26}H_{31}N_2O_5Cl$	$C_{26}H_{30}N_2O_5$	$C_{26}H_{30}N_2O_5Cl_2$	$C_{26}H_{33}O_5Cl$	$C_{26}H_{32}O_5$	$C_{26}H_{32}O_5Cl_2$
FD-MS (*m/z*)	486, 487, 488	450	521, 523, 524	460, 461, 462	424	494, 495, 496
SI-MS (*m/z*)	487, 488, 489	451			425	
HR-MS Calcd				460.2015	424 (EI)	459.1936 $(M-Cl)^+$
Found				460.2055		459.1900
Anal Calcd	C 64.12, H 6.42, N 5.75, Cl 7.29	C 69.31, H 6.71, N 6.22	C 59.89, H 5.80, N 5.37, Cl 13.60	C 67.73, H 7.21, Cl 7.70	C 73.56, H 7.60	C 63.03, H 6.51, Cl 14.31
Found	C 64.39, H 6.53, N 5.54, Cl 7.44	C 69.09, H 6.73, N 5.64	C 59.20, H 5.62, N 5.23, Cl 13.36	C 67.15, H 7.24, Cl 7.06	C 72.63, H 7.58	C 63.56, H 6.80, Cl 14.48
$[\alpha]_D^{22}$ (*c* 0.5, MeOH)	+133°	+49°	+195°	−82°	+122°	+33°
UV λ_{max}^{MeOH} nm (ε)	204 (20,500), 254 (19,200), 299 (19,900), 364 (5,500), 440 (3,900)	203 (19,900), 232 (14,400, sh), 256 (16,500), 308 (11,900), 371 (4,200), 453 (2,800)	204 (19,500), 240 (16,900, sh), 254 (18,300), 302 (18,900), 376 (4,900), 450 (3,800)	204 (20,200), 260 (20,300), 313 (7,800), 347 (7,100)	205 (23,900), 263 (22,600), 360 (7,400)	205 (21,400), 266 (20,900), 327 (7,800), 360 (7,400)
$\lambda_{max}^{MeOH-HCl}$ nm (ε)	203 (15,300), 253 (19,200), 299 (20,800), 366 (5,500), 437 (4,100)	204 (13,200), 235 (13,100, sh), 255 (15,600), 308 (11,500), 371 (3,600), 452 (2,500)	204 (14,900), 240 (17,500, sh), 252 (18,400), 301 (19,200), 375 (5,200), 442 (3,800)	204 (16,000), 260 (21,000), 323 (7,700), 345 (7,200, sh)	205 (20,700), 264 (23,100), 361 (7,600)	205 (16,600), 267 (21,600), 332 (7,900), 356 (7,600)
IR (cm^{-1})	2155, 1690, 1645, 1600	2150, 1685, 1640, 1605	2160, 2140, 1690, 1650, 1615	1690, 1630, 1600	1680, 1630, 1600	1685, 1640, 1600

FD-MS; Field desorption mass spectra, SI-MS; secondary ion mass spectra, HR-MS; high resolution mass spectra, EI; electron impact.

The production of antibiotics was maximum at 65 hours after inoculation, reaching 48 μg/ml estimated as the antibiotic SF2415A1.

Physico-chemical Properties of Antibiotics SF2415

The physico-chemical properties of antibiotics SF2415A1, A2, A3, B1, B2 and B3 are listed in Table 3. UV spectra were measured on a Shimadzu UV-260 spectrophotometer. IR spectra were recorded on a Hitachi 260-10 infrared spectrophotometer. MS spectra were measured on a Hitachi M-80B mass spectrometer. Optical rotations were measured with a Perkin Elmer model 141 polari-

Fig. 4. UV spectrum of SF2415A1 in MeOH (20 μg/ml).

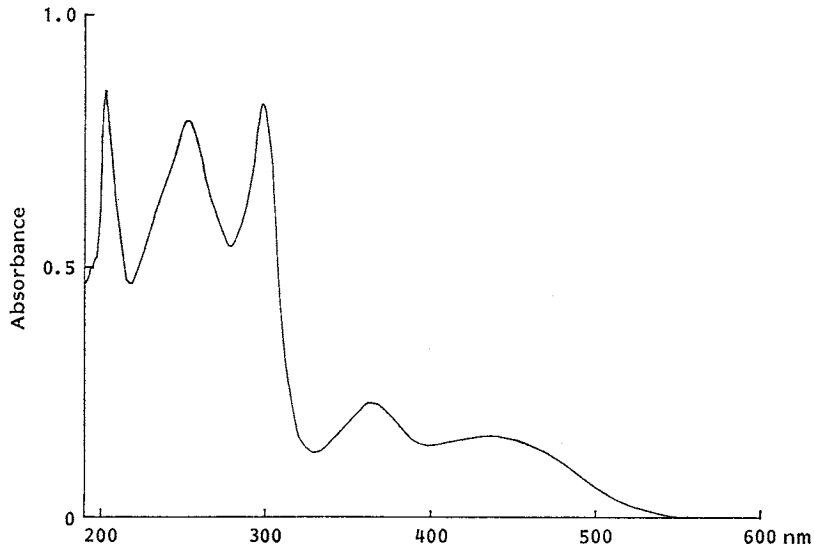


Fig. 5. IR spectrum of SF2415A1 (KBr).

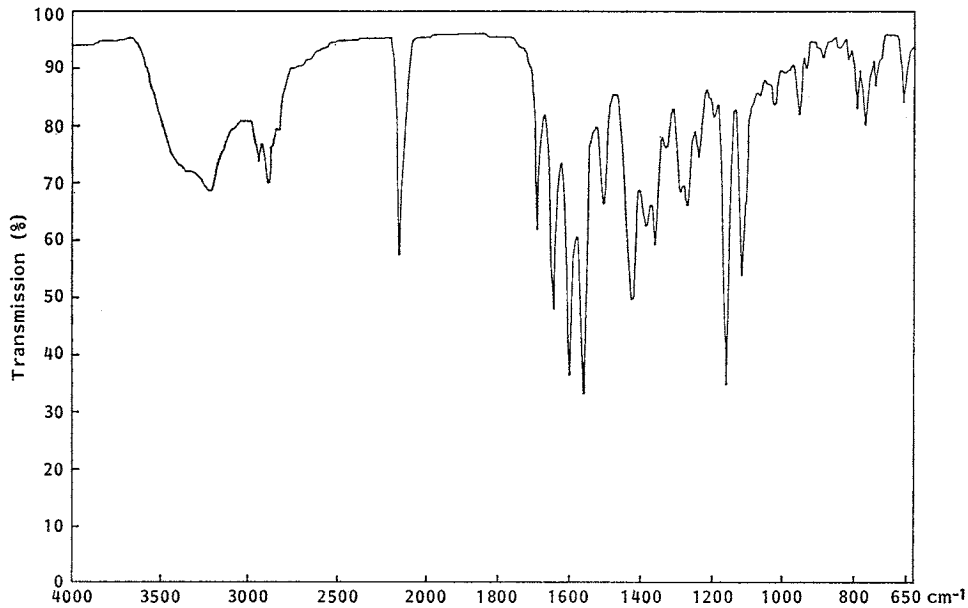


Fig. 6. ^{1}H NMR spectrum of SF2415A1 in $CDCl_3$ (400 MHz).

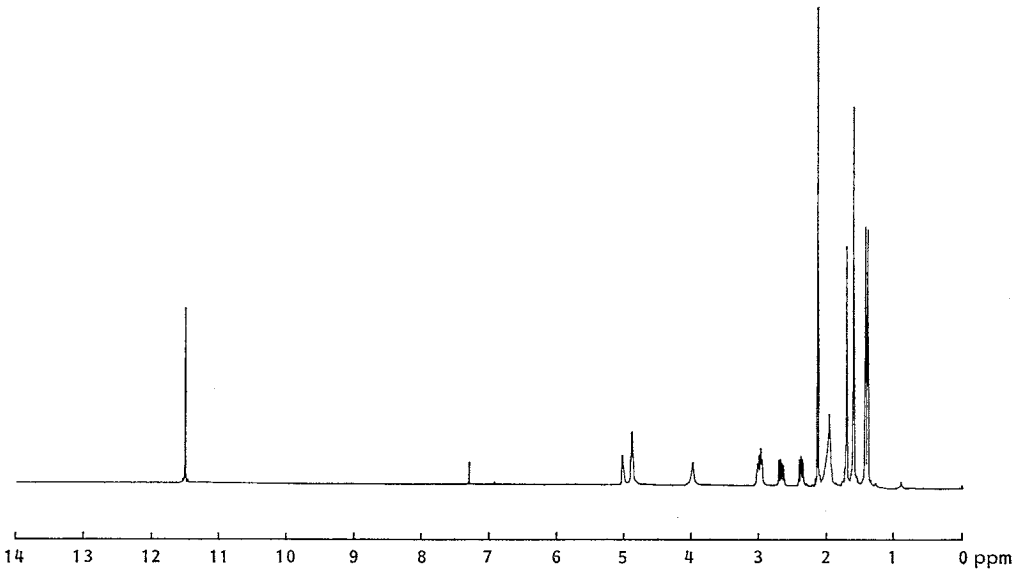
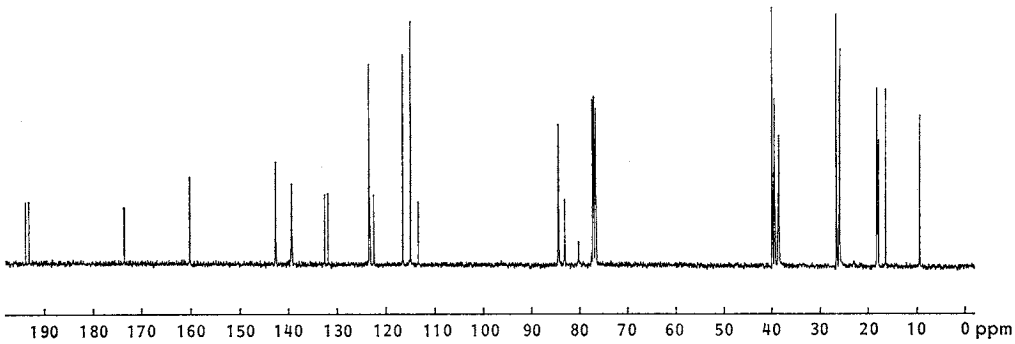


Fig. 7. ^{13}C NMR spectrum of SF2415A1 in $CDCl_3$ (100 MHz).



meter. Antibiotics SF2415 are soluble in organic solvents including methanol, chloroform, ethyl acetate and acetone, but almost insoluble in water. They show reddish purple colors with magnesium acetate in methanol and positive color reactions with H_2SO_4, $KMnO_4$ and $NaMoO_4$ reagents and negative with ninhydrin reaction. A1 showed melting point at 89~90°C. Melting points of A3 and B3 could not be measured because of their hygroscopicities. IR spectra of A1, A3 and B3 were measured using KBr pellets and those of other SF2415 antibiotics were measured on NaCl plates. ^{1}H and ^{13}C NMR spectra were recorded on a Jeol JNM-GX400 spectrometer. The chemical shifts in $CDCl_3$ refer to an internal standard of tetramethylsilane (0 ppm). UV, IR, ^{1}H and ^{13}C NMR spectra of A1 are shown in Figs. 4, 5, 6 and 7, respectively. Antibiotics SF2415 have the novel semi-naphthoquinone structure, especially A1, A2 and A3 have additional unique α-diazoketone structure for natural products. Structural elucidation of antibiotics SF2415 will be reported in the next paper.[1]

Biological Activities of Antibiotics SF2415

Antibiotics SF2415 have moderate activities against Gram-positive bacteria, but are not active against Gram-negative bacteria including *Escherichia coli* JC-2, *E. coli* No. 29, *E. coli* W3630 RGN823,

Table 4. Antimicrobial activities of SF2415.

Test organisms	MIC (μg/ml)					
	A1	A2	A3	B1	B2	B3
Staphylococcus aureus 209-P JC-1	3.13	3.13	0.78	1.56	6.25	1.56
S. aureus No. 26	3.13	3.13	0.78	3.13	6.25	1.56
S. epidermidis ATCC 14990	3.13	3.13	0.78	3.13	6.25	3.13
S. epidermidis 109	6.25	6.25	3.13	3.13	50	3.13
Enterococcus faecalis ATCC 8043	12.5	6.25	6.25	6.25	6.25	6.25
Bacillus anthracis No. 119	0.10	0.78	0.20	1.56	6.25	1.56

E. coli JR66/W677, *Citrobacter freundii* GN346, *Salmonella typhi* 0-901-W, *S. enteritidis* No. 11, *S. typhimurium* LT-2, *Salmonella* sp. D-0001, *Shigella sonnei* EW33 Type 1, *Klebsiella pneumoniae* PCI 602, *K. pneumoniae* 22#3038, *Proteus vulgaris* OX19, *P. mirabilis* GN310, *Providencia rettgeri* J-0026, *Morganella morganii* Kono, *Serratia marcescens* MB-3848, *Pseudomonas aeruginosa* MB-3829, *P. cepacia* M-0527 and *Xanthomonas maltophilia* M-0627 at concentrations of 100 μg/ml. The antibacterial activities of antibiotics SF2415 are shown in Table 4. When tested in mice by intraperitoneal administration, A1, A2 and A3 were found to have LD_{50} of less than 15 mg/kg and LD_{50} of B1, B2 and B3 were 141, >200 and >100 mg/kg, respectively.

Acknowledgment

The authors deeply thank to Dr. SHINICHI KONDO for his kind advice and his critical review of this manuscript.

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