STUDIES ON A NEW ANTIBIOTIC FR-900109

1. TAXONOMY, ISOLATION AND CHARACTERIZATION

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FR-900109 is a new antibiotic obtained from fermentation broth of a streptomyces which was identified as *Streptomyces prunicolor*. Its elementary analysis and mass spectroscopic measurement suggest that the molecular formula is $C_{27}H_{32}O_9$. It has an ultraviolet absorption maximum at 254 nm. The antibiotic is active against Gram-positive bacteria. Acute toxicity in mice is very low.

In the course of a screening program directed towards the isolation of new antibiotics, a strain of actinomycetes, No. 9261, was found to produce an antibiotic active against Gram-positive bacteria.

From its chemical and biological properties, the antibiotic, called by the code number of FR-900109, has been judged new. In this paper, we report the characteristics of the producing strain, No. 9261, fermentation and isolation procedures, and chemical and biological properties of FR-900109.[1]

Taxonomy of Strain No. 9261

Strain No. 9261 was isolated from a soil sample collected at Tsu city, Mie Prefecture, Japan. The microorganism was identified as a strain of *Streptomyces prunicolor*[2,3]. It has the fundamental characteristics of the organism, namely, reverse side color is light brown to reddish brown on some media. Mature aerial mass color is in the red color series on most media[4] commonly used in taxonomic studies (Table 1). The spore chain morphology is classified in *Rectiflexibiles* section (Fig. 1). Straight to flexous spore chains are generally long with 10 to often more than 50 spores per chain. The spores are cylindrical, $0.4 \sim 0.9 \times 1.0 \sim 2.0$ μm. The spore surfaces are smooth as determined from the electron micrograph (Fig. 2). Whole cell hydrolysates of strain No. 9261 showed that it contained LL-diaminopimelic acid. Melanoid pigment are not formed in peptone - yeast extract - iron agar, tyrosine

Table 1. Cultural characteristics of strain No. 9261.

Medium	Aerial mycelium	Reverse side color	Soluble pigment
Sucrose - nitrate agar	White to pale pink, powdery	Pale yellow	None
Glucose - asparagine agar	Pale pink, powdery	Pale yellow	None
Glycerin - asparagine agar	Pale pink to grayish pink	Pale yellowish brown	None
Inorganic salts - starch agar	Pale pink, short cottony	Pale brown	None
Nutrient agar	None	Colorless	None
Tyrosine agar	Pale pink, powdery	Brown	None
Yeast - malt extract agar	Pinkish gray, thin powdery	Pale yellow	None
Oatmeal agar	Pale brown, powdery	Pale yellow	None
Peptone - yeast extract - iron agar	None	Colorless	None

Fig. 1. Aerial mycelium of strain No. 9261 .(×400).

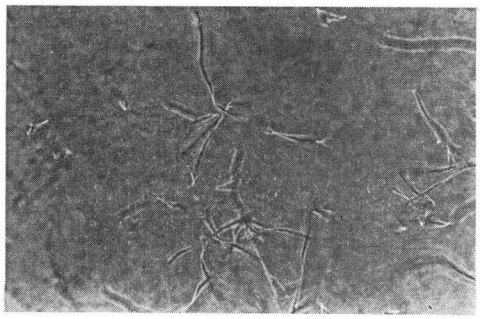


Fig. 2. Electron micrograph of the spores of strain No. 9261.

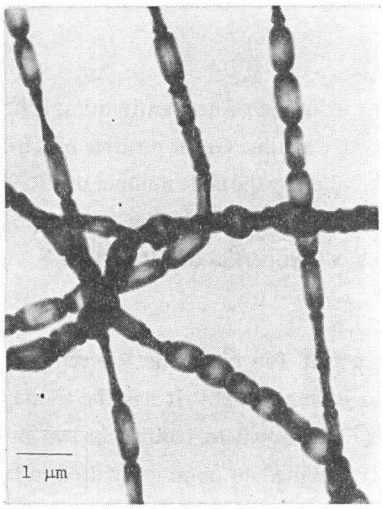


Table 2. Physiological properties of strain No. 9261.

Temperature range for growth	9~40°C
Optimum temperature	26°C
Starch hydrolysis	Positive
Gelatin liquefaction	Negative
Milk peptonization	Weakly positive
Milk coagulation	Negative
Melanin production	Negative

Table 3. Carbon sources utilization of strain No. 9261.

L-Arabinose	+
D-Xylose	+
D-Glucose	++
D-Fructose	+
Sucrose	+
Inositol	+
L-Rhamnose	+
Raffinose	+
D-Mannitol	+
D-Mannose	+
Salicin	+

Symbols: ++=good utilization, +=utilization.

Table 4. Media used for production of FR900109.

Seed medium		Fermentation medium	
Soluble starch	1%	Sucrose	6%
Glycerine	1%	Cotton seed meal	1.5%
Cotton seed meal	1%	Dried yeast	0.25%
Dried yeast	1%	pH	6.8
pH	6.3		

agar and tryptone - yeast extract broth (Table 2). No soluble pigment is produced. D-Glucose, L-arabinose, D-xylose, inositol, D-mannitol, D-fructose, L-rhamnose and raffinose are utilized for growth (Table 3).

S. prunicolor No. 9261 has been deposited at the American Type Culture Collection, as ATCC 31315.

Production of FR-900109

For production of the antibiotic, 30-liter fermentors with 20 liters of medium shown in Table 4 were inoculated with 2% of mature seed broth. Seed flasks (500 ml) containing 100 ml of the seed medium were inoculated with spores from slant cultures and incubated at 30°C on a rotary shaker with 7.6-cm throw at 180 rev/minute for 48 hours to obtain good growth.

Fermentations were run for 72 hours under the following condition; temperature, 30°C; agitation, 250 rpm; aeration, 20 liters/minute; tank back pressure, 0.5 kg/cm^2. Progress of the fermentation was monitored by diffusion plate assays performed on supernatant fluid and acetone extract of mycelium obtained from centrifuged broth (3,000 rpm, 15 minutes). *Staphylococcus aureus* 209P was used as a test organism for the bioassay.

Isolation Procedure

A procedure for isolation of FR-900109 is outlined in Fig. 3. Most of the antibiotic activity was found in the mycelium extract. After fermentation was completed, the culture broth (140 liters, 900 μg/ml) was filtered with the aid of Radiolite (filter aid). The collected cake (25 kg) was extracted twice with 40 liters of acetone. The acetone extract (80 liters) was concentrated under reduced pressure and remaining aqueous solution (10 liters) was adjusted to pH 2.0 with 6 N hydrochloric acid and extrated twice with 10 liters of ethyl acetate. The broth filtrate was concentrated under reduced pressure to 5 liters and 20 liters of methanol was added. The supernatant solution was again concentrated under reduced pressure to 2 liters, adjusted to pH 2.0 with 6 N hydrochloric acid and extracted with 2 liters of ethyl acetate.

The ethyl acetate extracts from filtered cake and filtrate were combined and concentrated under

Fig. 3. Isolation procedure for FR-900109.

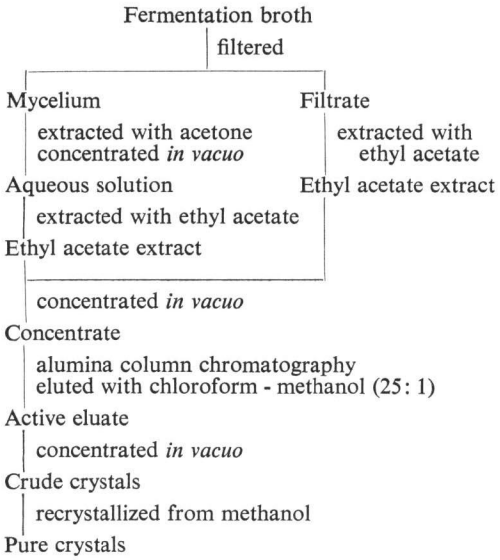


Fig. 4. UV absorption spectrum of FR-900109.

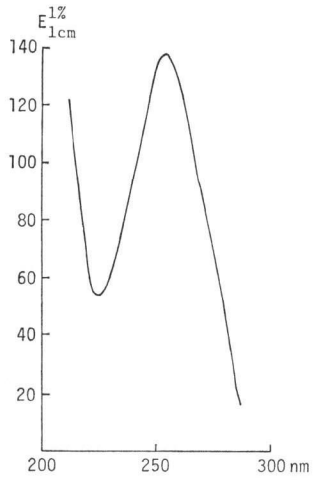
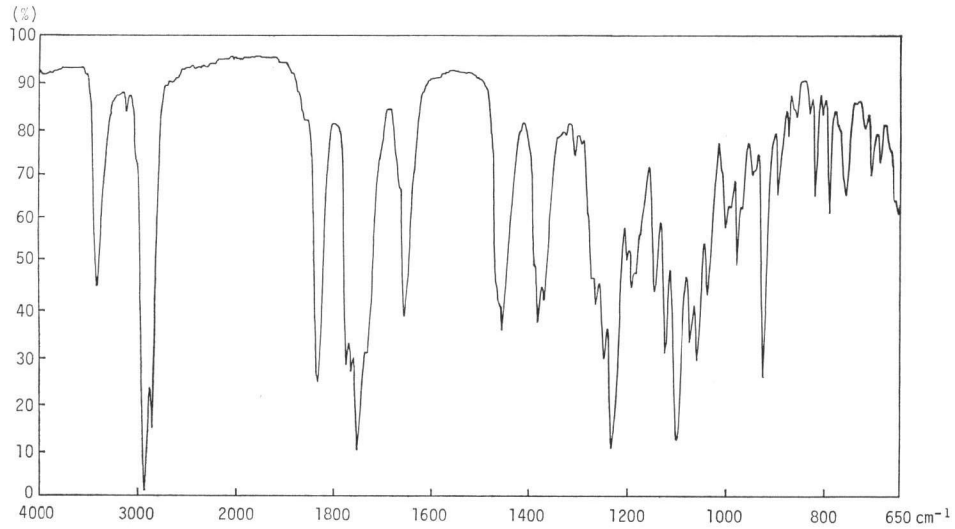


Fig. 5. IR spectrum of FR-900109.



reduced pressure to 1 liter. The concentrate was washed with 1 M phosphate buffer pH 6.98 and concentrated under reduced pressure to dryness. The active syrup was purified by chromatography on a column of alumina (Woelm, acidic, activity grade V, 1,000 ml). The column was washed with benzene and chloroform successively and the active substance was eluted with chloroform - methanol solution (25: 1, 10 liters). The eluate was concentrated under reduced pressure to yield crude crystals (9.6 g). Recrystallization from methanol gave 7.7 g of pure crystals.

Chemical Properties of FR-900109

FR-900109 is acidic, colorless crystalline material, soluble in acetone, chloroform, DMSO, slightly soluble in methanol, ethyl acetate and insoluble in benzene, ether and hexane. It decomposes at 263 ~ 264°C. The optical rotation is $[\alpha]_D^{26}$ −80° (*c* 1, chloroform). The antibiotic shows ultraviolet absorption maximum at 254 nm ($E_{1cm}^{1\%}$ 104, methanol) (Fig. 4). Color reaction is as follows; positive to $KMnO_4$, I_2 tests, negative to ninhydrin, $FeCl_3$, Ehrlich, $AgNO_3$-NH_4OH, dinitrophenylhydrazine tests.

Elementary analysis gave the following data;

Anal. Calcd. for $C_{27}H_{32}O_9$: C 64.79, H 6.44
Found: C 64.76, H 6.45

The molecular formula of FR-900109 has been confirmed to be $C_{27}H_{32}O_9$ by the parent peak in the mass spectrum at *m/z* 500. The infrared absorption spectrum in a Nujol (Fig. 5) shows absorption bands at 3370, 3100, 2930, 2850, 1830, 1770, 1750, 1740, 1720, 1650, 1580, 1510, 1455, 1380, 1370, 1310, 1260, 1245, 1225, 1220, 1195, 1185, 1140, 1120, 1095, 1080, 1070, 1065, 1055, 1035, 1000, 980, 965, 940, 920, 900, 875, 860, 820, 790, 760, 700, 680, 650, 620 and 610 cm^{-1}.

The ^1H NMR and ^{13}C NMR of the antibiotic in $CDCl_3$ are shown in Fig. 6 and Fig. 7, respectively. The Rf values in thin-layer chromatography are shown in Table 5.

Fig. 6. ^1H NMR spectrum of FR-900109.

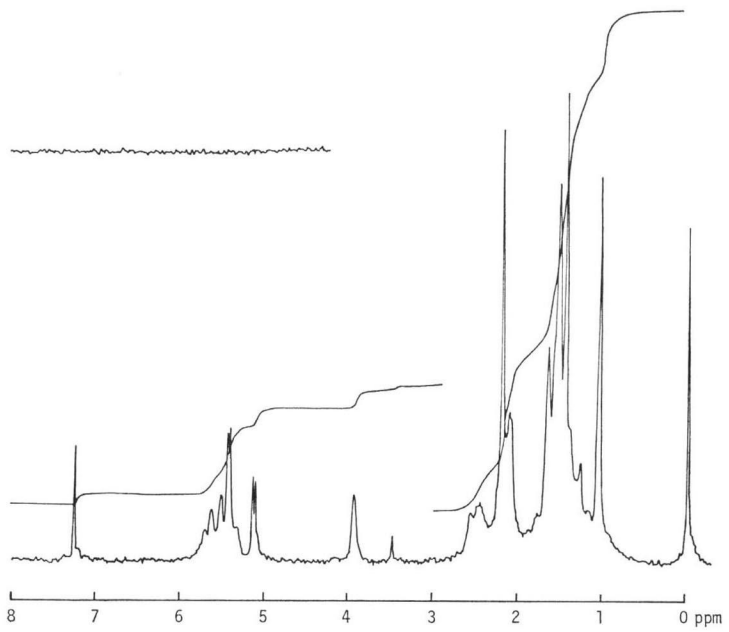


Fig. 7. ^{13}C NMR spectrum of FR-900109.

s: singlet, d: doublet, t: triplet, q: quartet.

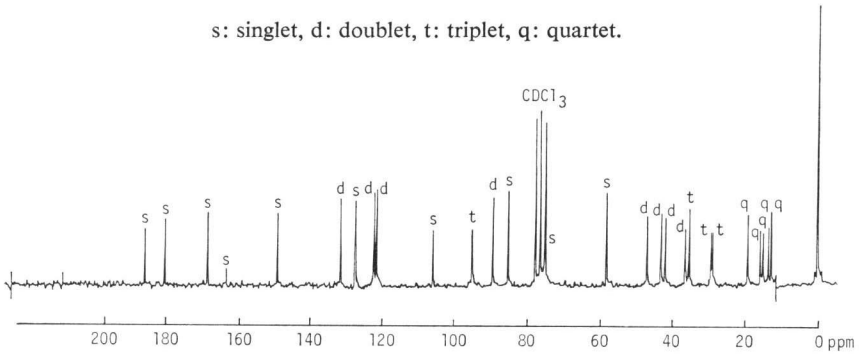


Table 5. Rf values of FR-900109 on silica gel sheet (Eastman silica gel sheet No. 13179).

Solvent	Rf
Chloroform - methanol (20: 1)	0.3
Benzene - ethyl acetate (1: 1)	0.6

Table 6. Antibacterial spectrum of FR-900109.

Test organism	MIC (μg/ml)
Staphylococcus aureus 209P JC 1	0.2
Bacillus subtilis ATCC 6633	0.4
Micrococcus luteus PCI 1001	0.2
Corynebacterium diphteriae A7 NIHJ	8.0
Staphylococcus aureus 1601-6	0.2
〃 1601-13	0.1
〃 1601-26	0.1
〃 1601-30	0.1
〃 1601-32	0.2
Escherichia coli NIHJ JC 2	>100
Proteus vulgaris IAM 1025	>100
Pseudomonas aeruginosa NCTC 10490	>100
Candida albicans	>100
Penicillium chrysogenum	>100
Mycobacterium phlei	>100

Biological Characteristics

Antibacterial spectrum of FR-900109 is shown in Table 6. This test was conducted by the serial agar dilution method. One loopful of an overnight culture of each test strain in Trypticase - soy broth (about 10^8 viable cells/ml) was streaked on heart-infusion agar containing graded concentration of the drug and the minimal inhibitory concentration (MIC) was expressed in terms of μg/ml after incubation at 37°C for 20 hours except for Mycobacterium and fungi in which MIC was determined after 3 days of incubation.

FR-900109 shows antibacterial activity against Gram-positive bacteria such as Staphylococcus and Bacillus, but it is ineffective against Gram-negative bacteria. Addition of serum or blood to the medium greatly reduced the activity of FR-900109 on test organisms. This may explain its ineffectiveness on Streptococci which require addition of rabbit serum for growth.

FR-900109 underwent no cross-resistance with benzylpenicillin, cephalosporin C, streptomycin, kanamycin, chloramphenicol, tetracycline, erythromycin and lincomycin.

Intraperitoneal administration of 1,000 mg/kg of FR-900109 into mice did not result in any toxic symptom for 2 weeks after injection.

Discussion

FR-900109 is an antibiotic with activity against Gram-positive bacteria and low toxicity in experimental animals. It shows UV absorption maximum at 254 nm and decomposed at 263~264°C. Its molecular formula has been determined to be $C_{27}H_{32}O_9$. From these characteristics, FR-900109 can be differentiated from any of the antibiotics so far reported and, therefore, considered to be a new antibiotic.

References

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