STUDIES ON A NEW ANTIBIOTIC FR-900336

TAXONOMY, ISOLATION AND CHARACTERIZATION

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A new lipophilic antibiotic, FR-900336 was isolated from a culture of *Streptomyces sioyaensis* subsp. *tanegashimaensis*. FR-900336 is light yellow and has a molecular formula $C_{30}H_{30}NO_{13}Cl$. The characterization by IR, UV, ^{1}H NMR and ^{13}C NMR spectra makes a quinone structure very probable.

FR-900336 is active against Gram-positive bacteria and fungi.

In the course of the screening program directed towards the isolation of new antibiotics, a strain of actinomycetes, No. 9979, was found to produce an antibiotic active against Gram-positive bacteria and fungi.

Based on chemical and biological properties, the antibiotic, designated FR-900336, has been determined to be new. In this paper, we report the characteristics of the producing strain, No. 9979, fermentation and isolation procedures, and chemical and biological properties of FR-900336.

Taxonomy of Strain No. 9979

Strain No. 9979 was isolated from a soil sample collected on Tanegashima island, Kagoshima Prefecture, Japan.

The methods described by SHIRLING and GOTTLIEB[1] were employed for the taxonomic studies. Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 14 days on yeast - malt extract agar, oatmeal agar and inorganic salts - starch agar. Aerial mycelia of strain No. 9979 are characterized by forming monopodial branching and hygroscopic disintegration of spore chains. The mature sporophores formed Spirales (Fig. 1) with more than ten spores in each chain. The spores were determined by electron microscopy to be oval to short cylindrical and measured $0.4 \sim 0.6 \times 0.6 \sim 0.9$ μm in size. Spore surfaces were smooth (Fig. 2).

Fig. 1. Aerial mycelium of strain of No. 9979 (×400).

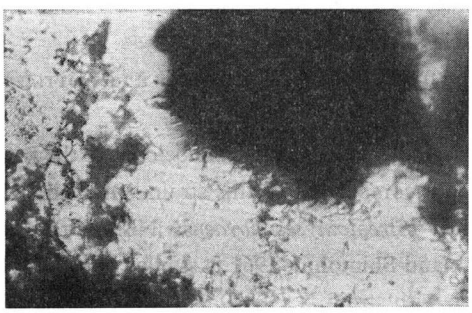


Fig. 2. Electron micrograph of the spores of strain No. 9979.

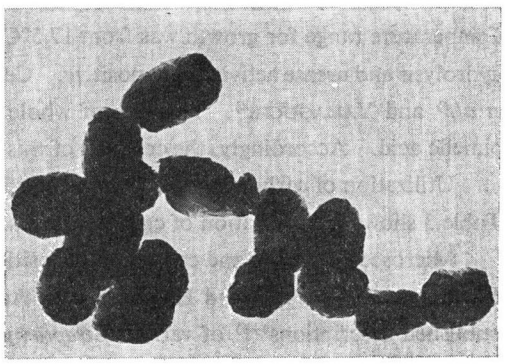


Table 1. Cultural characteristics of strain No. 9979.

Medium	Growth	Aerial mass color	Reverse side color	Soluble pigment
Sucrose - nitrate agar	Moderate	Light gray	Light reddish yellow	None
Glucose - asparagine agar	Good	Light gray, cottony	Pale yellow	None
Glycerol - asparagine agar	Poor	White, powdery	Colorless	None
Inorganic salts - starch agar	Good	Light gray, cottony	Pale yellow	None
Tyrosine agar	None	None	None	None
Nutrient agar	Moderate	White	Pale yellow	None
Yeast extract - malt extract agar	Good	White, cottony	Light reddish yellow	None
Oatmeal agar	Moderate	Dark yellowish brown, powdery	Pale yellow	None
Peptone - yeast extract - iron agar	Moderate	White	Pale yellow	None
Potato - dextrose agar	Poor	White	Light reddish yellow	None
Czapek agar	None	None	None	None

Table 2. Physiological properties of strain No. 9979.

Nitrate reduction	Negative
H_2S production	Negative
Urease activity	Positive
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Negative
Melanin production	Negative
Gelatin liquefaction	Negative
Temperature range for growth	17.5~35.4°C
Optimum temperature for growth	29°C
pH range for growth	5~9
Optimum pH for growth	7.7

Table 3. Carbon utilization of strain No. 9979.

Glycerol	++	D-Galactose	±
D-Xylose	+	L-Arabinose	−
Na-Citrate	+	D-Glucose	+
Lactose	−	Mannitol	+
D-Fructose	++	D-Mannose	+
Rhamnose	−	Sucrose	+
Maltose	+	Cellulose	−
Na-Succinate	−	D-Trehalose	+
Inulin	−	Salicin	±
Inositol	+	Chitin	−
Raffinose	+	Na-Acetate	−

Symbols: ++ Good utilization, + utilization, ± doubtful utilization, − no utilization.

Cultural characteristics were observed on eleven media described by SHIRLING and GOTTLIEB[1] and WAKSMAN[2]. Incubation was at 30°C for 10 days. The color names used in this study were taken from the Color Standard (Nihon Shikisai Co., Ltd.). Aerial mass color was in the gray color series, and appeared cottony. The reverse side was colorless to pale yellow. No soluble pigment was produced as shown in Table 1.

Physiological properties of strain No. 9979 are shown in Table 2. Temperature range and optimum temperature for growth were determined on yeast - malt extract agar after 10 days incubation. Temperature range for growth was from 17.5°C to 35.4°C with optimum temperature at 29°C. Starch hydrolysis and urease activity were positive. Cell wall analysis was performed by the methods of BECKER *et al.*[3] and YAMAGUCHI[4]. Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly, the cell wall of this strain is classified as type I.

Utilization of carbon sources was examined according to the methods of PRIDHAM and GOTTLIEB[5]. Table 3 shows the utilization of carbon sources.

Microscopic studies and cell wall composition analysis, indicate that this strain belongs to the genus *Streptomyces* Waksman and Henrici 1943. Accordingly, a comparison of this strain was made with published descriptions[6~9] of various *Streptomyces* species. *Streptomyces sioyaensis* Nishimura, Okamoto, Mayama, Ohtsuka, Nakajima, Tawara, Shimaoka and Shimohira 1961 is similar to strain No. 9979. Therefore, further detailed comparison was made with strain No. 9979 and *S. sioyaensis* IFO

12820. *S. sioyaensis* IFO 12820 was different from strain No. 9979 in the following characteristics: *S. sioyaensis* can utilize lactose, rhamnose and inulin but not D-fructose, and can reduce nitrate.

Based on the above comparison, it was concluded that strain No. 9979 is a strain of *S. sioyaensis*, and can be considered a new subspecies designated as *Streptomyces sioyaensis* subsp. *tanegashimaensis* subsp. nov., referring to Tanegashima island from which the organism was isolated.

Production of FR-900336

Production of the antibiotic is as follows: 30-liter fermentors with 20 liters of medium (Table 4) were inoculated using 0.1% 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 revolutions/minute for 72 hours.

Fermentations were run for 72 hours under the following conditions: temperature 30°C; agitation 250 rpm; aeration 20 liters/minute; tank back pressure 1 kg/cm^2. Progress of the fermentation was monitored by diffusion plate assays performed on supernatant fluid obtained from centrifuged broth (2,000 rpm, 10 minutes). *Candida albicans* was the test organism for the bioassay.

Table 4. Media used for production of FR-900336.

Seed medium		Fermentation medium	
Soluble starch	3%	Corn starch	2%
Glucose	0.2%	Corn steep liquor	0.25%
Cotton seed meal	1%	Cotton seed meal	0.5%
Gluten meal	1%	Dried yeast	0.25%
Dried yeast	1%	Wheat germ	0.5%
$CaCO_3$	0.2%	pH	7.0
pH	7.0		

Isolation Procedure

A procedure for isolation of FR-900336 is outlined in Fig. 3. After fermentation was completed, the cultured broth (18 liters) was filtered with aid of the Radiolite (filter aid). The filtrate was adjusted to pH 7.0 with 6 N NaOH and adsorbed on a column of Diaion HP-20 (1.5 liters). The column was washed with 50% methanol - water (5 liters), and then eluted with 50% tetrahydrofuran - water. This

Fig. 3. Isolation procedure for FR-900336.

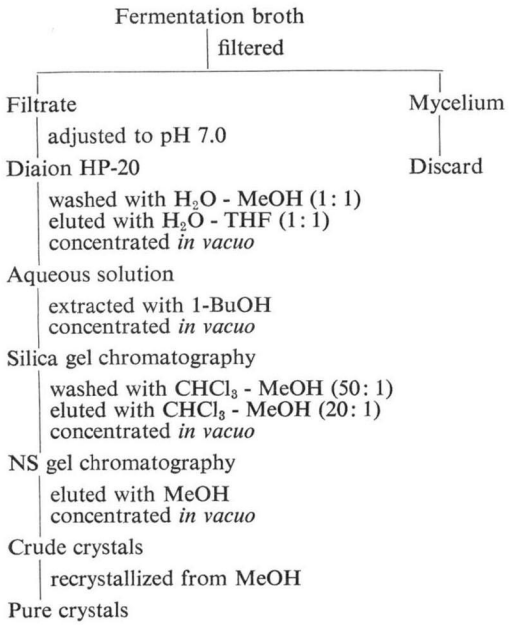


Fig. 4. UV spectra of FR-900336.

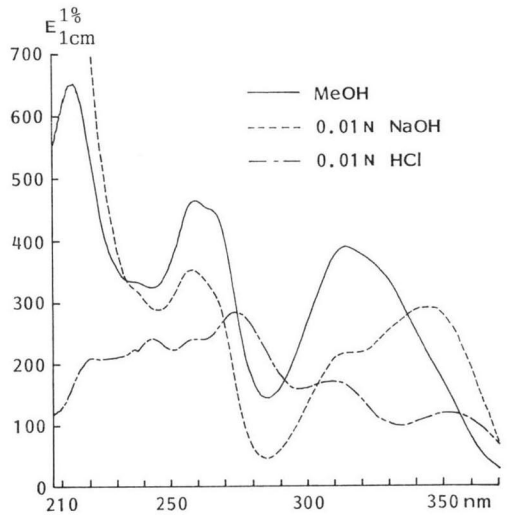
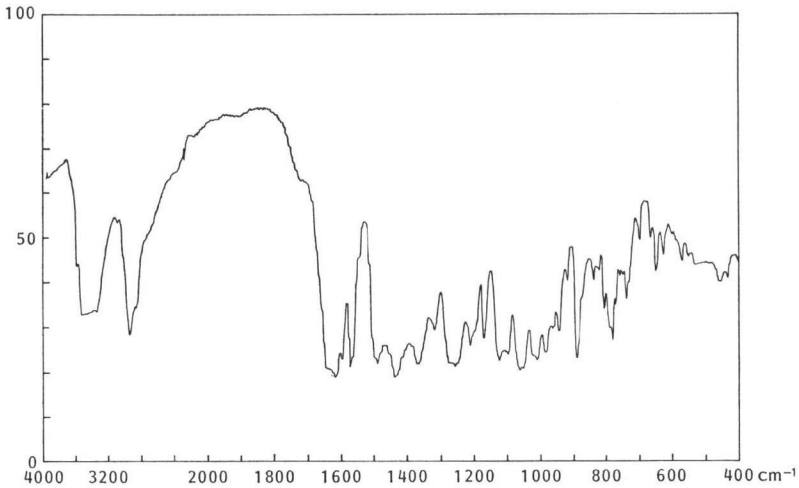


Fig. 5. IR spectrum of FR-900336 in KBr disk.



eluate (2 liters) was concentrated *in vacuo* to 200 ml and extracted twice with 400 ml of 1-butanol. Evaporation of the 1-butanol extracts produced a syrup. The active syrup was loaded on a column of silica gel (100 ml, Merck). The column was washed with chloroform - methanol solution (50: 1, 1 liter) and eluted with chloroform - methanol solution (20: 1, 550 ml). The eluate was concentrated *in vacuo* to dryness, and the residue applied on a column of NS gel (20 ml, Nihon Seimitsu Kagaku Co., Ltd.). The column was developed with methanol in the dark. The active solution (20 ml) was collected and evaporated to dryness to give a yellow solid (30 mg). The powder was crystallized from methanol to give 17 mg of FR-900336.

Physico-chemical Properties of FR-900336

FR-900336 is a light yellow crystalline material, soluble in acetone, chloroform, methanol and ethyl acetate, slightly soluble in ethanol and insoluble in water. It decomposes at 214°C. The optical rotation is $[\alpha]_D^{20}$ +365° (*c* 0.1, chloroform). The antibiotic shows ultraviolet absorption maxima at 216 nm ($E_{1cm}^{1\%}$ 650), 238 (330), 259 (465), 266 (sh, 450) and 314 (390) in methanol: at 213 (1,600), 234 (sh, 330), 258 (355), 316 (sh, 215) and 345 (290) in 0.01 N NaOH: at 220 (sh, 210), 238 (225), 243 (240), 258 (sh, 240), 273 (285), 310 (170) and 351 (210) in 0.01 N HCl as shown in Fig. 4. In the IR spectrum (Fig. 5), characteristic absorptions attributable to alcohol and quinonic carbonyl groups were observed at 3600~3300 and 1640 and 1620 cm^{-1}, respectively. Color reaction is as follows: positive to Dragendorff and $FeCl_3$ reactions, negative to ninhydrin, Ehrlich and Molish reactions.

Elemental analysis gave the following data;

Anal Calcd for $C_{30}H_{30}NO_{13}Cl$: C 55.60, H 4.67, N 2.16, Cl 5.47
Found: C 55.72, H 4.75, N 2.02, Cl 5.59

No molecular ion was visible in the mass spectrum (FD mass). The ^1H NMR and ^{13}C NMR of the antibiotic in $CDCl_3$ are shown in Table 5 and Table 6, respectively. The Rf value on a silica gel 60 F-254 plate (Merck) with chloroform - methanol (20: 1) was 0.40.

Biological Properties of FR-900336

The antibacterial spectrum of FR-900336 is shown Table 7. This test was conducted by the serial agar dilution method.

Table 5. ^{1}H NMR spectrum of FR-900336.

δ (ppm)	
2.62~3.17	3H, m
3.30	3H, s
3.38	3H, s
3.54	3H, s
3.69	3H, s
4.08	3H, s
4.4~5.7	9H, m
7.04	1H, s
7.36	1H, d, J=8 Hz
7.86	1H, d, J=8 Hz

Chemical shifts in δ values (ppm down field from internal TMS). $CDCl_3$ was used as solvent.

Table 6. ^{13}C NMR spectrum of FR-900336.

No.	δ (ppm)	No.	δ (ppm)
1	175.70	16	116.97
2	168.06	17	115.52
3	159.51	18	111.06
4	151.50	19	92.22
5	149.31	20	90.40
6	145.49	21	79.60
7	143.61	22	77.06
8	140.03	23	72.99
9	139.79	24	72.74
10	132.45	25	67.53
11	126.50	26	65.40
12	124.50	27	61.82
13	123.71	28	58.37
14	120.61	29	58.12
15	119.71	30	36.22

$CDCl_3$ solvent with TMS as internal standard.

Table 7. Antimicrobial spectrum of FR-900336.

Test organism	Medium*	MIC (μg/ml)
Staphylococcus aureus 209P JC1	1	8.0
S. aureus 209P JC1	2	0.008
Bacillus subtilis ATCC 6633	1	2.0
Escherichia coli NIHJ JC2	1	>500
Pseudomonas aeruginosa NCTC 10490	1	>500
Candida albicans	3	64
C. albicans	4	0.125
Schizosaccharomyces pombe	3	125
Trichophyton mentagrophytes	5	16
Aureobacidium pullulans	6	250
Mucor hiemalis	6	250
Rhizopus acetorinus	6	250
Fusarium sp. R 2	6	8
Helminthosporium sp. 2-1	6	32

* Medium 1: Mueller-Hinton (pH 7.3), 2: Mueller-Hinton (pH 5.0), 3: glucose 0.5%, Polypeptone 0.175%, yeast extract 0.175% and agar 1% (pH 7.0), 4: glucose 0.5%, Polypeptone 0.175%, yeast extract 0.175% and agar 1% (pH 5.0), 5: Sabouraud, 6: potato - dextrose agar.

FR-900336 shows activity against Gram-positive bacteria, yeasts and filamentous fungi, but it is ineffective against Gram-negative bacteria.

Studies in which the pH of Mueller-Hinton broth was varied from pH 7.0 to pH 5.0, showed that FR-900336's activity is greater against *Staphylococcus aureus* and *C. albicans* at low pH (Table 7).

Intraperitoneal administration of 200 mg/kg of FR-900336 into mice did not result in any toxic symptom for one week after injection.

Discussion

FR-900336 is an antibiotic with activity against Gram-positive bacteria and fungi. Its inhibitory activity is greatly enhanced at low pH. Results obtained with a typical strain are given in Table 8. Increased MIC values are observed with increased pH values.

FR-900336 shows UV absorption maximum at 216, 238, 259, 266 and 314 nm and decomposes at 214°C. Its molecular formula has been determined to be $C_{30}H_{30}NO_{13}Cl$. From these characteristics,

Table 8. Effect of pH on *in vitro* inhibitory activity of FR-900336.

	MIC (μg/ml) at various pH values				
	pH 4	pH 5	pH 6	pH 7	pH 8
*C. albicans**	0.063	0.125	2	62	1,000
S. aureus 209P**	ND	0.008	ND	8	ND

* Medium: 0.1 M Phosphate buffer, glucose 0.5%, Polypeptone 0.175%, yeast extract 0.175% and agar 1%.
** Medium: 0.1 M Phosphate buffer and Mueller-Hinton agar.

FR-900336 can be differentiated from any of antibiotics so far reported and, therefore, can be considered to be a new antibiotics.

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