STUDIES ON NEW PHOSPHONIC ACID ANTIBIOTICS

I. FR-900098, ISOLATION AND CHARACTERIZATION

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A strain of *Streptomyces*, isolated from a soil sample and identified as *Streptomyces rubellomurinus* sp. nov., has been found to produce FR-900098, an interesting new antibiotic containing phosphorus in its molecule. The antibiotic, obtained as colorless crystals, was shown to inhibit a wide variety of Gram-negative bacteria including *Pseudomonas*, *Proteus*, and *Escherichia coli*. Its antibacterial action involves interference with bacterial cell wall synthesis as evidenced by the fact that it causes spheroplast formation by susceptible cells.

During the course of screening program directed toward the isolation and evaluation of new cell wall-inhibitory antibiotics, we isolated from a soil sample collected at Mt. Hira, Shiga Prefecture, a strain of *Streptomyces* designated strain No. 5818, which was found to produce a new antibiotic, FR-900098. The antibiotic was detected in the fermentation broth by the use of nocardicin C-supersensitive mutant of *Pseudomonas aeruginosa* NCTC 10490[1]. This new antibiotic proved to be of considerable interest because of its chemical structure containing phosphorus and its broad antibacterial spectrum against Gram-negative bacteria, and low toxicity in experimental animals.

In this report, we describe fermentation, isolation procedures, and chemical and biological properties of FR-900098.

Characterization of the producing strain will be presented in the succeeding paper.

Fermentation

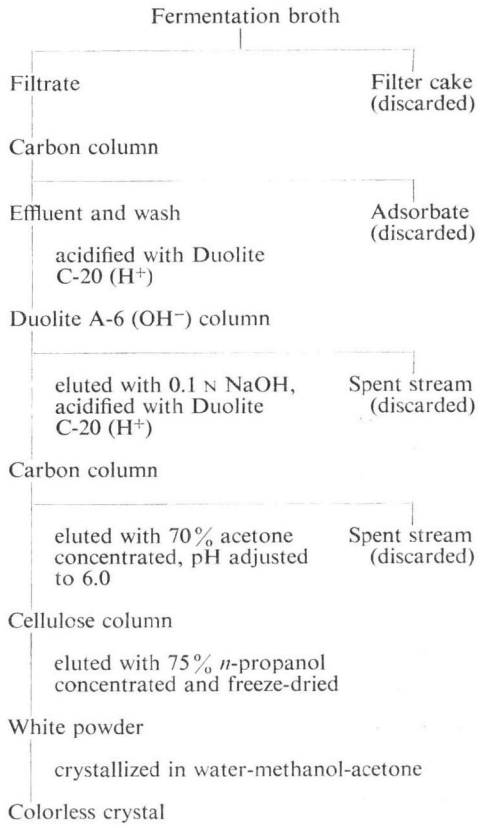
The growth of *Streptomyces rubellomurinus* sp. nov. ATCC 31215[2] on the mature slant culture was used to inoculate four 500-ml flasks containing 100 ml each of sterile growth medium shown in Table 1. The flasks were shaken on a shaker (220 rpm, 2-inch throw) for 3 days. The content of the flasks was used to inoculate 20 liters of fermentation medium in a stainless steel fermentor. The composition of the fermentation medium is shown in Table 1.

Fermentation was allowed to proceed for 3 days at a temperature of 30°C, air flow of 20 liters per minute and with agitation of 300 rpm. Progress of the fermentation was monitored by standard disc-agar diffusion assay of the supernatant fluid from a centrifuged broth sample (3,000 rpm for 10 minutes). *Pseudomonas aeruginosa* NCTC 10490-III, a nocardicin C-supersensitive mutant, was used as a test organism for the bioassay. *Enterobacter cloacae* 10-19C, which has been found to have natural high susceptibility to FR-900098, was also used.

Table 1. Media used for production of FR-900098.

Seed medium		Production medium	
Potato starch	2%	Soluble starch	5%
Cotton seed meal	1	Cotton seed meal	0.5
Dried yeast	1	Gluten meal	2.5
		Dried yeast	0.5
		$MgSO_4 \cdot 7H_2O$	1
		KH_2PO_4	1
		$Na_2HPO_4 \cdot 12H_2O$	0.7

Fig. 1. Purification process of FR-900098.



Isolation Procedures

The diagram of the isolation method described below is shown in Fig. 1. Most of the antibiotic activity was found in the broth filtrate. The culture broth was filtered using filter aid (Radio-lite).

The filtrate (20 liters) was concentrated to one liter under reduced pressure. To this concentrate, 4 liters of methanol was added and the precipitates formed were removed by filtration. After concentration of this filtrate to a volume of one liter, the concentrate was passed through a column of activated charcoal (one liter). The effluent and the wash were combined and adjusted to pH 2.0 with a cation-exchange resin (Duolite C-20, H^+ cycle). The acidified solution was passed through a column of anion exchange resin (Duolite A-6, OH^- cycle, 500 ml). The resin was washed with water and the antibiotic activity was eluted with 0.1 N sodium hydroxide solution. The active fractions were combined and adjusted to pH 2.0 by Duolite C-20 (H^+ cycle) and again adsorbed onto a column of activated charcoal (300 ml). The activity was eluted with 60% aqueous acetone. After adjusting to pH 6.5, the eluate was concentrated to dryness. The residue was applied to a column packed with cellulose powder. The column was washed with propanol, 90% propanol, 80% propanol, successively and eluted with 75% aqueous propanol. The active fractions were combined and evaporated under reduced pressure to give white powder (600 mg). The powder thus obtained was dissolved in a small volume of methanol, and about ten volumes of acetone were added to this solution. FR-900098 crystallized at 4°C as the monosodium salt. From 20 liters of fermentation broth, 450 mg of pure antibiotic was obtained as colorless crystals.

Physico-chemical Properties

FR-900098 is a colorless crystal which melts at 193~194°C. It is soluble in water, methanol, dimethylsulfoxide, slightly soluble in ethanol, substantially insoluble in acetone, ethyl acetate and hexane. The observed specific rotation, $[\alpha]_D^{25}$ is 0 (c 1.0, H_2O). It moves toward the anode with phos-

phate buffer pH 6.5 at 300 volts in a paper electrophoresis for two hours. Potentiometric titration of the monosodium salt shows an equivalent weight of 240, with a pKa′ 2.0, 7.2, 9.4. Elemental analysis gave the following compositions:

Calcd. for $C_5H_{11}NO_5PNa$: C 27.39; H 5.02; N 6.39; P 14.15; Na 10.50
Found: C 27.74; H 5.02; N 6.66; P 12.35; Na 10.31

Mass spectrometry of the monosodium salt did not give a satisfactory result, but the molecular wight of the dimethyl ester of this compound, as determined by mass spectroscopy, was 225, which supported the above molecular weight.

Color reactions are as follows: positive in ferric chloride, iodine, potassium permanganate and molybdate tests, negative in ninhydrin, EHRLICH, DRAGENDORFF and MOLISCH tests. The ultraviolet absorption spectrum (Fig. 2.) in 0.1 N sodium hydroxide solution showed an absorption, λ_{max} at 230 nm, ($E^{1\%}_{1cm}$ 325) and end absorption in acidic and neutral solution. Elemental analysis, titration and color tests suggested the existence of phosphonic acid.

The Rf value in thin-layer chromatography on a cellulose plate is shown in Table 2. The infrared absorption spectrum is shown in Fig. 3, with the following significant absorption maxima (in KBr): 3600~2600, 1615 (–CO–N–), 1160 (P–O), 1040 and 885 cm^{-1}.

Table 2. Chromatographic properties of FR-900098.

System	Rf
T.l.c. cellulose (Eastman Kodak)	
75% Aqueous propanol	0.5
n-Butanol saturated with H_2O	0
70% Aqueous acetonitrile	0.4

Fig. 3. IR spectrum of FR-900098 (KBr).

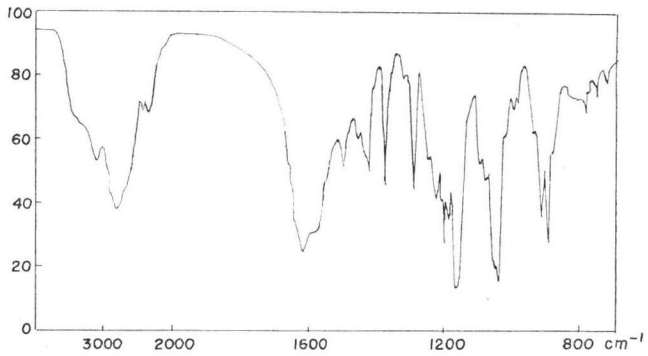


Fig. 2. UV spectra of FR-900098 (H_2O).

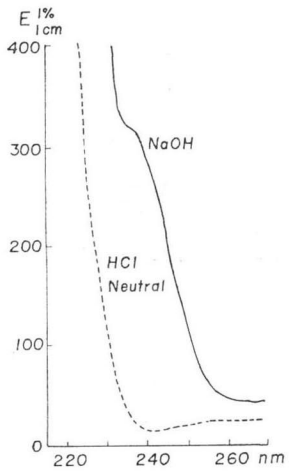
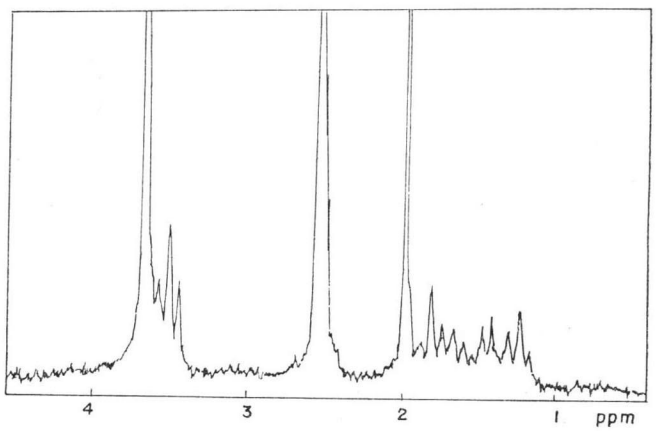


Fig. 4. NMR spectrum of FR-900098 (DMSO/D_2O).



As shown in Fig. 4, the ^{1}H-nmr spectrum has the following characteristics: acetyl protons at δ 1.95 (3H, s), two protons at δ 1.35 (2H, m), two protons at δ 1.75 (2H, m), and two protons at δ 3.50 (2H, t, J=7 Hz). Irradiation of the signals at δ 1.75 changed the triplet signals at δ 3.80 into singlet. The fact that two methylene protons were observed at δ 3.50 indicated the existence of $-CH_2-N-$ (or O).

These suggest a partial structure of this antibiotic as CH_3-, $-CO-$, $-N(OH)-$, $-N-CH_2-$, and $-CH_2-PO_3H$.

Biological Characteristics

The antibacterial activity of FR-900098 is compared with that of cefazolin in Table 3. One loopful of inoculum of approximately 10^6 cells in 1 ml of medium was streaked on agar plates containing two-fold decremental dilutions of the antibiotics. The inoculated plates were incubated for 18~24 hours at 37°C before reading. FR-900098 showed selective antibacterial activity against Gram-negative bacteria and no inhibitory effect on *Staphylococcus aureus*, *Mycobacterium*, yeasts, and fungi.

Cells of *Pseudomonas aeruginosa* were treated with a lethal concentration of FR-900098 in a hypertonic medium. As shown in Plate 1, all cells were transformed into spheroplasts. Thus, it seems likely that the antibiotic inhibits bacterial cell wall synthesis.

FR-900098 is remarkably non-toxic. Each of five ICR mice (20~25 g), was given a single intravenous dose of 100 mg (4~5 g/kg) and all survived. During 14 days of observation after injection, no toxic symptoms were observed.

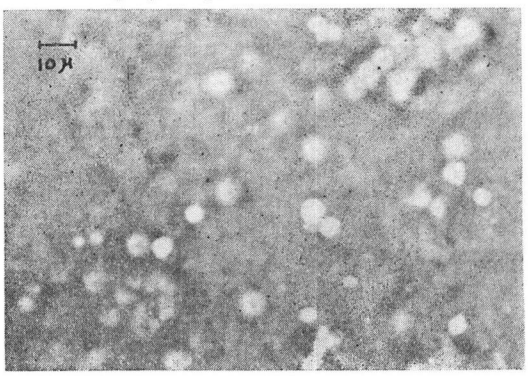
Table 3. Antimicrobial spectrum of FR-900098.

Test microorganisms	MIC (mcg/ml)	
	FR-900098	Cefazolin
Staphylococcus aureus 209P JC-1	>250	0.39
Bacillus subtilis ATCC 6633	125	0.39
Sarcina lutea PCI 1001	8	0.78
Escherichia coli NIHJ JC-2	63	1.56
Klebsiella pneumoniae NCTC 418	>250	1.56
Proteus vulgaris IAM 1025	125	>250
Pseudomonas aeruginosa IAM 1095	250	>250
Salmonella typhi T-287	2	1.56
Shigella flexneri Ia EW 8	8	0.78
Mycobacterium phlei 607	>250	>250
Saccharomyces cerevisiae	>250	>250
Aspergillus niger	>250	>250

MIC test was conducted by the usual serial agar dilution method using a nutrient agar.

Plate 1. Morphology of cells treated with FR-900098.

One tenth ml of logarithmic culture of *Pseudomonas aeruginosa* NCTC 10490 was put on the Difco Antibiotic Medium 3 plate with 30% sucrose. Soft agar (0.7%) containing 500 mcg/ml of FR-900098 and 20% sucrose was overlayed. After overnight incubation, cell morphology was examined under microscope (×240).



Discussion

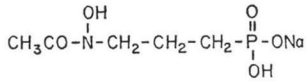
Discovery of nocardicin A by the use of penicillin-supersensitive mutants[1] proved the usefulness of this screening system with sensitive mutants. We undertook, therefore, a screening program using a mutant strain of *Pseudomonas aeruginosa* NCTC 10490 which is supersensitive to nocardicin C, which has only weak antibacterial activity[3]. FR-900098 has been detected by this mutant organism.

The data described in this report characterize FR-900098 as an acidic, water soluble, electrophoretically mobile, cell wall-inhibitory, phosphorus containing substance with unique antimicrobial activity. Several phosphorus-containing antibiotics have been reported[4~6]. FR-900098 is distinct from any of these antibiotics in its chemical and biological properties.

Studies in our laboratories[7] have shown that the antibiotic has the chemical structure shown in Fig. 5.

Its activity against Gram-negative bacteria and low toxicity in experimental animals suggest that the antibiotic has potential as an effective chemotherapeutic agent.

Fig. 5. Chemical structure of FR-900098.



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