FR-900130, A NOVEL AMINO ACID ANTIBIOTIC

I. DISCOVERY, TAXONOMY, ISOLATION, AND PROPERTIES

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A new antibiotic, designated FR-900130, has been discovered in the culture filtrate of *Streptomyces*. The producing organism has been identified as *Streptomyces catenulae*. The antibiotic was purified by adsorption onto Duolite C-20, passage through Amberlite IRC-50, final purification and desalting on Sephadex G-25. It shows antimicrobial activity against Gram-positive bacteria and synergy with D-cycloserine. The antibiotic is very labile in alkaline solution above pH 8.0 and decomposed during lyophilization or solvent precipitation. It was converted to an acetyl derivative and the biological properties of the derivative were examined.

FR-900130 has been discovered in the course of a screening program of soil microorganisms for production of substances exhibiting synergy with D-cycloserine. Taxonomic studies of the producing strain WS-4707, resulted in its identification as *Streptomyces catenulae*[1,2].

FR-900130 was distinguished from the previously known antibiotic, O-carbamyl-D-serine, by the fact that it is very labile and that its activity is not reversed by D-alanine.

This paper describes taxonomic studies, production by fermentation, purification, properties, acetylation of the antibiotic and properties of the acetyl derivative.

Materials and Methods

(1) Cycloserine-agar plate

FR-900130 content in broth and partially purified samples was determined by disc-agar diffusion assay using *Staphylococcus aureus* 279 on Mueller-Hinton agar plates (Eiken Kagaku Co., Japan) supplemented with 5 μg/ml of D-cycloserine.

(2) Urinary excretion

Acetyl derivative of FR-900130 was given subcutaneously or orally to mice in a single dose of 3 mg/mouse. Urine was collected at 30 and 60 minutes after subcutaneous injection and 1 and 2 hours after oral administration.

(3) Standard curve for the assay of the antibiotic.

The most purified solution of the antibiotic was divided into two parts. One part was diluted two-fold decrementally immediately after purification until the inhibition zone of the antibiotic failed to appear. The other part was lyophilized and the weight of the powder was determined. The theoretical MIC value was expressed as the division of the weight by the end point volume.

(4) Tissue distribution

The acetyl derivative of FR-900130 was administered subcutaneously in single dose of 3 mg/mouse. Mice were bled to death 30 minutes after injection. The lung, heart, liver, spleen, and kidney were removed. Tissue from each of the organs was homogenized in the same volume of 1/15 M phosphate buffer (pH 7.0) as that of the tissue. The antibiotic concentration was bioassayed with the super-

natant of each tissue obtained by centrifugation. Serum level of the antibiotic was also assayed with the serum separated from the blood by centrifugation.

(5) Deacetylase-cycloserine-agar plate

Cycloserine-agar plate supplemented with the supernatant of rat kidney homogenate was used to determine the concentration of the acetyl derivative of FR-900130. Thirty g of rat kidney was homogenized in 30 ml of saline for 30 minutes at 4°C. The homogenate was centrifuged for 30 minutes at 4°C (3,000 rpm). One ml of the resulting supernatant was added as supplement to each of the above-described cycloserine-agar plate (10 ml).

Results

Taxonomy of the Producing Strain

FR-900130 producing strain (No. 4707) was isolated from a soil sample collected at Tatsuno City, Hyogo Prefecture, Japan. The microorganism was identified as a strain of *Streptomyces catenulae*[1,2]. It has the fundamental characteristics of the organism namely, straight spore chains are generally short with 3~20 spores per chain. The spore chain morphology is classified in Rectiflexibilis Section (Fig. 1). The spores are oval to cylindrical, averaging 0.6~0.8×1.2~1.6 μm in size, with smooth surface (Fig. 2).

Mature aerial mass color is in the Gray color-series on most agar media commonly used in taxonomic studies. Reverse side of colony does not show distinctive pigment on most media. Greenish brown to olive green pigment is produced in some media (Table 1). Physiological properties are presented in Table 2. Melanoid pigment is not found in peptone-yeast iron agar and tyrosine agar. Starch is not hydrolyzed by this strain. The hydrolytic activity on gelatin or milk is weak. Glucose, raffinose, D-mannitol, mannose, galactose and maltose are utilized for growth of the organism.

From the above-mentioned informations, strain No. 4707 is considered a strain of *Streptomyces catenulae*. This strain has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan as FERM-P 4365.

Fig. 1. Aerial mycelium of strain No. 4707.
Yeast-malt extract agar, 7 days at 30°C.

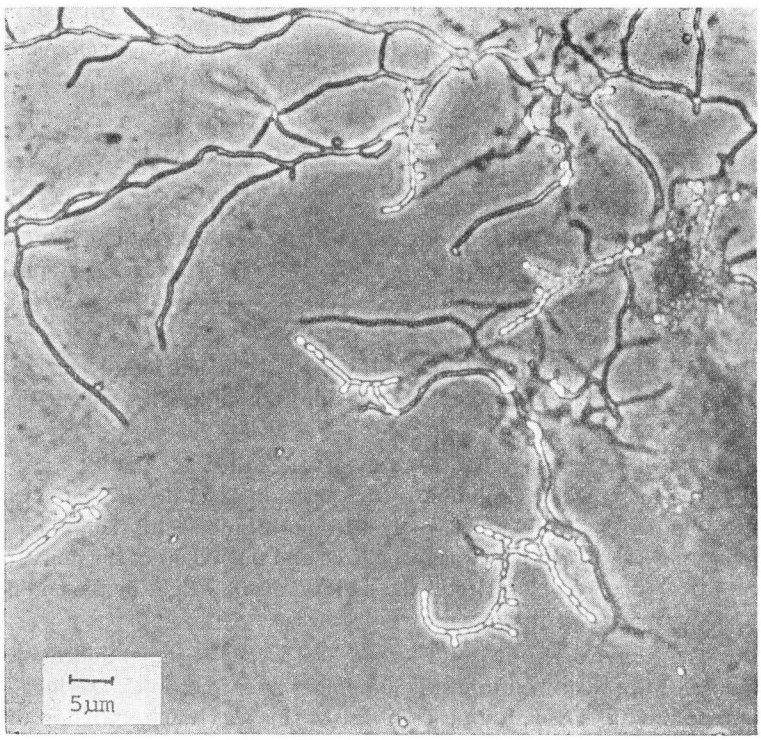


Fig. 2. Spores of strain No. 4707.
Yeast-malt extract agar, 10 days at 30°C.

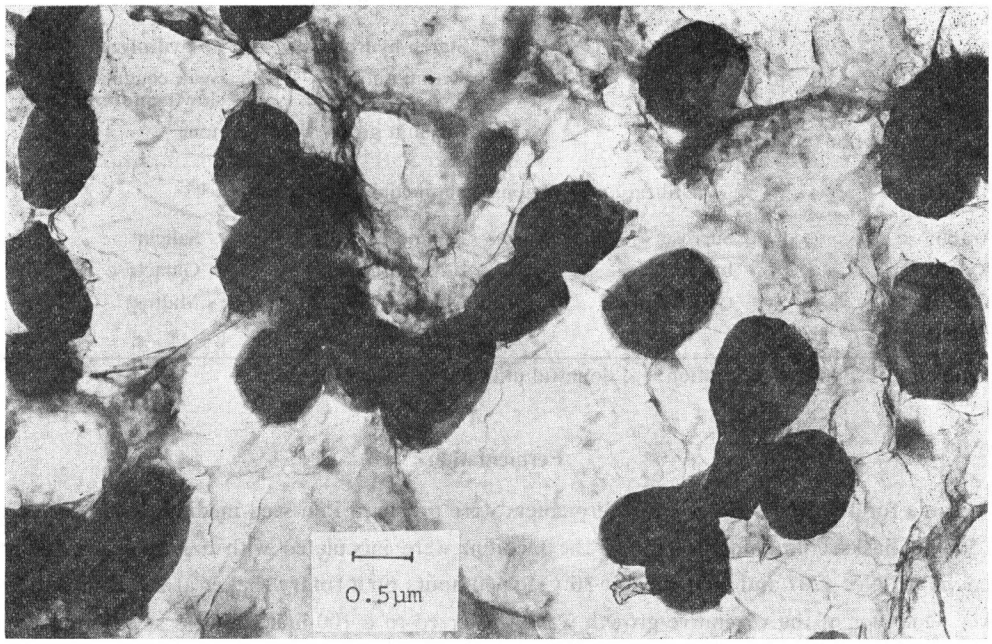


Table 1. Cultural characteristics of strain No. 4707.

Medium	Characteristics	Medium	Characteristics
Sucrose-nitrate agar	AM: very thin, powdery VG: scant growth, small colonies SP: none	Yeast-malt extract agar	AM: light gray, powdery VG: dark yellowish brown, small colonies SP: trace of greenish brown
Glucose-asparagine agar	AM: thin powdery, light greenish gray VG: colorless, small colonies SP: none	Oatmeal agar	AM: light gray, powdery VG: colorless to pale yellow, small colonies SP: trace of yellow
Glycerin-asparagine agar	AM: light olive gray, powdery VG: colorless, small colonies SP: yellow	Glucose-peptone gelatin stab	AM: none VG: colorless SP: none
Starch-inorganic salts agar	AM: light olive gray, powdery VG: yellowish brown, small colonies SP: dark olive green	Milk	AM: white, powdery VG: pale yellow, growth on surface ring SP: none
Tyrosine agar	AM: light gray, powdery VG: colorless to cream, flat, small colonies SP: none	Peptone-yeast iron agar	AM: none VG: cream colored, flat SP: none
Nutrient agar	AM: none VG: colorless to pale yellow, small colonies SP: none		

Symbols: AM, aerial mycelium; VG, vegetative growth; SP, soluble pigment.

Table 2. Physiological properties of strain No. 4707.

Property observed	Characteristics	Property observed	Characteristics
Temperature requirements	growth from 16°C to 37°C sporulation good from 21°C to 34°C opt. 28°C	Starch hydrolysis	no hydrolyzed
		Action on milk	weak coagulation, slow peptonization
Gelatin liquefaction	liquefied	Melanin production	none

Utilization of various carbon compounds.

L-Arabinose	−	Sucrose	−	Raffinose	+	Salicin	−
D-Xylose	−	Inositol	−	D-Mannitol	+	Galactose	+
D-Glucose	+	L-Rhamnose	−	Mannose	+	Maltose	+
D-Fructose	±						

Symbols: +, positive utilization; ±, doubtful utilization; − no utilization.

Fermentation

Inocula for 100-liter stainless steel fermenters were prepared in a seed medium shown in Table 3. Ten 500-ml flasks containing 100 ml of the medium were inoculated with a lyophilized culture or frozen vial of No. 4707 and incubated at 30°C for 60 hours on a rotary shaker at 200 rpm with 3-inch stroke. One liter of the vegetative growth was transferred to a 100-liter stainless steel fermenter containing 80 liters of production medium shown in Table 3. The culture was incubated at 30°C for 60 hours, agitated at 200 rpm and aerated at 80 liters per minute.

Table 3. Media used for production of FR-900130.

Seed medium		Production medium			
Potato starch	1%	Soluble starch	2.00%	Corn steep liquor	0.25%
Cotton seed meal	1	Cotton seed meal	0.50	KH_2PO_4	0.50
Dried yeast	1	Wheat germ	0.50	$Na_2HPO_4 \cdot 12H_2O$	0.50
		Dried yeast	0.25		

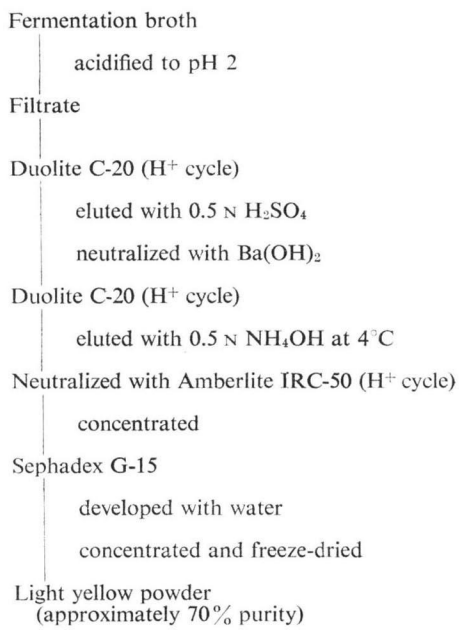
Purification

The diagram of the isolation method described below is shown in Fig. 3. The broth was acidified with sulfuric acid to pH 2.0 and filtered with filter aid (Radiolite) on a filter press. The filtrate was passed through a column of Duolite C-20 (H^+ cycle, 10 liters). The column was washed with water and eluted with 0.5 N sulfuric acid. The active fractions were combined and adjusted to pH 6.0 with saturated barium hydroxide solution, and the precipitates formed were discarded. After concentration to a volume of 4 liters, the concentrate was again passed through a column of Duolite C-20 (H^+ cycle, 3 liters). The column, after washing with water, was eluted with 0.5 N ammonium hydroxide. The antibiotic was very labile in alkaline solution above pH 8.0. Therefore, this elution process was conducted at 4°C and in as short a time as possible. The ammonical eluate was neutralized with Amberlite IRC-50 (H^+ cycle, 4 liters) right after the elution. These operations, however, resulted in low yield.

Active fractions, after concentration to 100 ml, were applied to a column of Sephadex G-15 (2

liters) and developed with water. Concentration of the active fractions gave an essentially pure antibiotic solution, freeze-drying of which yielded 320 mg of FR-900130 (70% purity).

Fig. 3. Purification process of FR-900130.



Properties of FR-900130

FR-900130 is a light yellow hygroscopic powder, freely soluble in water and sparingly soluble in methanol. The mobility of the antibiotic when subjected to thin-layer chromatography is shown in Table 4. Localization was achieved by bioautography or spraying with potassium permanganate solution. In addition, a positive reaction was observed with the ninhydrin test (a unique yellowish orange color).

The antibacterial spectrum of FR-900130 was examined by the disc-agar method. Results are listed in Table 5. The antibiotic is active against Gram-positive bacteria but has no effect on Gram-negative bacteria. The antibiotic is ineffective on *Mycoplasma gallisepticum* PG-1 and *Acholeplasma laidlawii* A LD-1, which lack cell wall. The activity against *Staphylococcus aureus* 279 is 16-fold potentiated by addition of 5 μg/ml D-cycloserine.

Table 4. Chromatographic behavior of FR-900130.

T.L.C.	Solvent system	Rf
Cellulose	*n*-Butanol saturated with water	0.03
	n-Propanol - water (7: 3)	0.30
	n-Butanol - acetic acid - water (8: 2: 3)	0.45

Table 5. Antibacterial activity of FR-900130.

Conc. of FR-900130	Diameter of inhibitory zone (mm)[a]						
	St. aureus FDA-209P	*St. aureus* No. 279	*St. aureus*[c] No. 279 + C.S.	*B. subtilis* ATCC-6633	*E. coli* NIHJ-JC-2	*Pr. vulgaris* IAM-1025	*Ps. aeruginosa* NCTC-10490
2,500 μg/ml	22	28	38	27	(28)[b]	—	—
625	17	24	32	24	(23)	—	—
156	11	19	28	17	(16)	—	—
39	—	13	23	12	(+)	—	—
9.8	—	+	18	—	—	—	—
2.5	—	—	13	—	—	—	—
0.6	—	—	+	—	—	—	—

a) Paper-disc (8 mm) assay on MUELLER-HINTON agar plate.
b) Parenthesis represents the hazy inhibition zone.
c) C.S. = 5 μg/ml of cycloserine was supplemented in *S. aureus* No. 279 agar plate.

Acute toxicity of the antibiotic was tested with ICR strain mice. No mice died after a single intravenous injection of a dose of 250 mg/kg.

Thin-layer chromatography of the purified antibiotic solution gave a single spot when examined immediately after the purification process. But the TLC of the freeze-dried powder revealed that the purity was approximately 70%. Stability of the antibiotic solution at various pH and susceptibility to inactivation by various concentration processes were examined. As shown in Table 6, it is stable at pH below 7.0. Concentrating and drying procedures caused loss of its activity. Cold trap experiment indicated that the antibiotic did not distill or sublime under reduced pressure at 50°C in an aqueous solution at pH 7.0.

Table 6. Stability of FR-900130.

Procedure	Residual activity (%)
pH 2~7 at 4°C for 60 min. (aqueous soln.)	100
pH 8 〃	70
pH 10 〃	40
Liophilized at 20°C	25
Precipitated with acetone	25
Stocked at 20°C for 12 hours (powder)	0

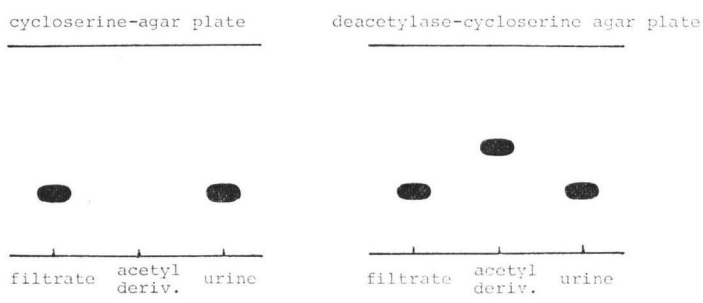
Chemical properties of particular utility in resolving the above-mentioned problems were its stability at pH below 7.0 and its positive reaction to the ninhydrin test, which suggested that the primary amino function of FR-900130 participates in its stability by forming the hydrochloride salt. Therefore, it was acetylated by dropwise addition of acetic anhydride to a vigorously stirred aqueous solution of purified FR-900130.

Biological Properties of the Acetyl Derivatives of FR-900130

The acetyl derivatives of FR-900130 showed no activity against various bacteria tested. It is, however, deacetylated and converted to the original active antibiotic when it is administered in mice subcutaneously or orally, as shown in Fig. 4.

Urinary excretion and tissue distribution after the injection of the acetyl derivative are listed in Tables 7 and 8. Incubation of the acetyl derivative with the supernatant of tissue homogenate from various organs at 37°C for 2 hours revealed that deacetylase activity was localized mostly in kidney and liver. Subsequently, cycloserine-agar plate supplemented with supernatant of kidney homogenate was prepared as described in Materials and Methods. The standard curve for the acetyl derivative is

Fig. 4. Bioautogram of the urinary excretion.



1) *Staphylococcus aureus* No. 279 was used as test organism.
2) Bioautography was conducted as follows:
T.L.C.: Cellulose. Solvent system: *n*-Propanol - water (7: 3).

Table 7. Urinary excretion of FR-900130[1].

Substance	Route	Minute	St.[2]	CS+St.[2]
FR-900130[3]	oral	60	—	—
		120	—	—
	s.c.	30	22[4]	30
		60	20	28
FR-900130 acetyl derivative	oral	60	16	26
		120	10	19
	s.c.	30	19	28
		60	18	26

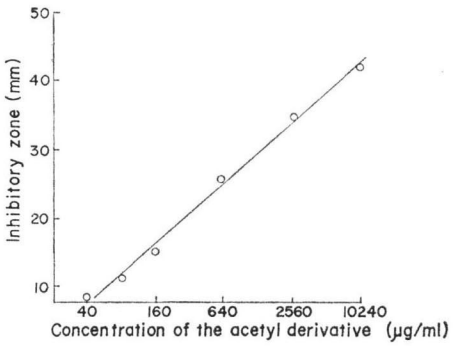
1) The antibiotics were administered at a dose of 3 mg/mouse.
2) St.=*Staphylococcus aureus* No. 279, CS+St.=*Staphylococcus aureus* No. 279 agar plate supplemented with D-cycloserine.
3) FR-900130 (70% purity).
4) The diameter of the inhibitory zone by paper-disc assay of the urine collected at the time described (mm).

Table 8. Tissue distribution of FR-900130[1].

Organ	St.[2]	CS+St.[2]
Serum	—	—
Heart	—	—
Lung	—	—
Spleen	—	—
Liver	—	9[3]
Kidney	—	16

1) FR-900130 acetyl derivative was administered at a dose of 3 mg/mouse.
2) St.,CS+St.: See the foot-note 2) in Table 7.
3) The diameter of the inhibitory zone by paper-disc assay of the homogenate of each organ (mm).

Fig. 5. Standard curve of FR-900130 acetyl derivative.
Assay was conducted using deacetylase-cycloserine agar plate.



shown in Fig. 5. This bioassay plate was successfully used for the isolation of the acetyl derivative of FR-900130 as described in the succeeding paper.

Discussion

Potentiation of D-cycloserine activity by 1-aminoethylphosphonic acid[3] or O-carbamyl-D-serine[4] has been reported. The substances which show synergy with cycloserine are considered possibly to be cell wall synthesis-inhibitors according to the theory of sequential blockade of enzymatic reactions in cell wall biosynthesis. We undertook, therefore, a screening program using a cycloserine-containing plate. Actually, FR-900130 caused spheroplast formation in a hypertonic medium by susceptible cells. The antibiotic has been found to inhibit alanine racemase; the details will be reported elsewhere.

References

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