A NEW ANTITUMOR ANTIBIOTIC, FR900840

I. DISCOVERY, IDENTIFICATION, ISOLATION AND CHARACTERIZATION

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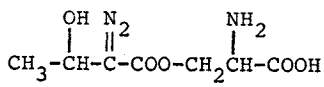
A new antitumor antibiotic, FR900840, was isolated from a culture broth of *Streptomyces* strain No. 8727 as a pale yellowish prism and the molecular formula was determined to be $C_7H_{11}N_3O_5$. The antibiotic exhibited prominent antitumor effects on human tumor cell lines both *in vitro* and *in vivo*.

In the course of our screening program for antitumor compounds from microbial fermentation products, FR900840 was discovered in the culture broth of *Streptomyces* strain No. 8727[1]. The chemical structure of FR900840 was determined on the basis of chemical and spectroscopic evidences as shown in Fig. 1. The structural elucidations of this compound are reported in a separate paper[2].

Fig. 1. Structure of FR900840.



In this paper we describe the taxonomy of the producing strain, fermentation and isolation procedures and some physico-chemical and biological properties of FR900840.

Materials and Methods

Taxonomic Study

The methods described by SHIRLING and GOTTLIEB[3] were employed principally for this taxonomic study.

Morphological observations were made with light and electron microscopes from cultures grown at 30°C for 21 days on yeast extract - malt extract agar, inorganic salts - starch agar, oatmeal agar and glucose - asparagine agar.

Cell wall analysis was performed by the methods of BECKER *et al.*[4], and YAMAGUCHI[5].

Temperature range for growth was determined on yeast extract - malt extract agar using a model TN-3 temperature gradient incubator (Toyo Kagaku Sangyo Co., Ltd.).

Utilization of carbon sources was examined according to the method of PRIDHAM and GOTTLIEB[6]. The results were determined after 14 days incubation at 30°C.

Fermentation

A culture medium 100 ml containing corn starch 1%, glycerol 1%, glucose 0.5%, Pharmamedia 0.5%, dried yeast 0.5%, corn steep liquor 0.5% and calcium carbonate 0.2% at pH 6.5 was poured into a 500-ml Erlenmeyer flask and sterilized at 120°C for 30 minutes. The medium was inoculated with a loopful of slant culture of *Streptomyces* sp. No. 8727 and incubated at 30°C for 3 days on a rotary shaker. The resultant culture was inoculated into 150 liters of a medium containing soluble starch 2%, corn starch 1%, Pharmamedia 1%, corn steep liquor 0.5%, dried yeast 0.1%, NaCl 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $CaCO_3$ 0.2%, Adekanol (defoaming agent; Asahi Denka Co., Ltd.) 0.1%

in a 200-liter jar fermentor, which had been sterilized at 120°C for 30 minutes. This was cultured at 30°C for 4 days with aeration of 100 liters/minute and with agitation at 200 rpm.

Tumor Cell Lines and Culture Conditions

Mouse leukemia P388, human lung adenocarcinoma A549 and human mammary adenocarcinoma MCF-7 were used for the experiments. Tumor cells were cultured at 37°C in a humidified atomosphere of 5% CO_2 - 95% air in each medium. RPMI 1640 medium was used for P388, DULBECCO's minimum essential medium was used for A549 and EAGLE's minimum essential medium supplemented with sodium pyruvate and nonessential amino acids was used for MCF-7. Benzylpenicillin (50 U/ml), streptomycin (50 μg/ml) and 10% fetal calf serum were supplemented in all medium.

Antitumor Activity (*In Vitro*)

In vitro antitumor activities were tested in microtiter plates, with each well containing 3×10^3 tumor cells in 100 μl medium. The cells were incubated at 37°C for 7 days and the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay was performed according to the method described by MOSMANN[7]. Briefly, MTT was dissolved in phosphate buffered saline at 5 mg/ml and filtered to sterilize and remove a small amount of insoluble residue. After the culture was terminated, this MTT solution (10 μl per 100 μl medium) was added to all wells, and the plates were further incubated at 37°C for 4 hours. Acid - 2-propanol (100 μl of 0.04 N HCl in 2-propanol) was added to all wells and mixed thoroughly to dissolve the dark blue crystals. After all crystals were dissolved, the plates were read on a 2-wavelength microplate photometer (Model MTP-22; Corona Electric Co., Ltd.) at 550 nm, a reference wavelength of 660 nm. FR900840 was dissolved in the medium and added to the culture to give final concentration of 100 μg/ml or less.

Subrenal Capsule (SRC) Assay

We established a 2-week SRC assay by using an immunosuppressive agent FK-506 which was developed in our Research Laboratories[8]. A precise report of this SRC assay will be published elsewhere. In brief, a 1-mm^3 tumor fragment was implanted under kidney capsule at day-0. FR900840 was dissolved in phosphate buffered saline and was injected ip at day-1, 5 and 9. Fourteen days after tumor implantation, the length and width of tumors were measured by caliper and tumor volume was calculated by the following formula:

$$\text{Tumor volume } (mm^3)=1/2\times a\times b^2$$

where (a) represents the length and (b) represents the width.

Animals

Female mice of BDF_1 strain were purchased from Charles River Japan Inc., Atsugi-city, Japan and were used for the SRC assay.

Results

Taxonomy of Strain No. 8727

Strain No. 8727 was isolated from a soil sample obtained from Ishioka-city, Ibaragi Prefecture, Japan.

The branching type of sporophores was monopodial; the form of mature sporophores was *Spira* with 20 to 70 spores in each chain. The spores were determined by electron microscopy to be oval and measured $0.6\sim0.7\times0.9\sim1.1$ μm in size. The spore surface was spiny (Fig. 2). Neither fragmentation of hyphae nor formation of spores occurred in the substrate mycelium. Sporangia, sclerotia and zoo-spores were not observed.

Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid. Accordingly the cell wall of this strain is classified as type I.

Summarized physiological properties of strain No. 8727 were shown in Table 1. The tempera-

Fig. 2. Electron micrograph of spore chains of strain No. 8727.

Scale: 5 μm.

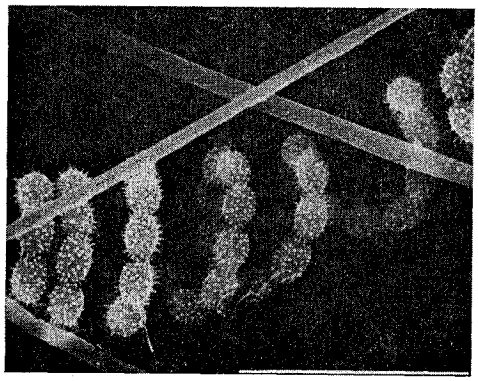
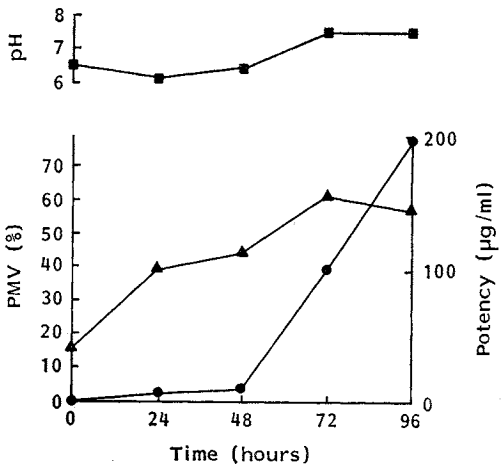


Fig. 3. Time course of FR900840 production.

■ pH, ▲ packed mycelium volume (PMV), ● potency.



Potency of FR900840 was measured by cytotoxic activity against A549 human lung adenocarcinoma cells.

Table 1. Physiological properties of strain No. 8727.

Conditions	Characteristics
Temperature range for growth	13~37°C
Optimum temperature for growth	30~32°C
Starch hydrolysis	Positive
Milk coagulation	Negative
Milk peptonization	Positive
Production of melanoid pigment	Positive
Gelatin liquefaction	Positive
Decomposition of cellulose	Negative

Table 2. Carbon utilization of strain No. 8727.

Compounds	Growth
D-Glucose	+
Sucrose	+
D-Xylose	+
D-Fructose	+
L-Rhamnose	+
Raffinose	+
L-Arabinose	+
Inositol	+
Mannitol	+

+: Utilization.

ture range for growth was from 13 to 37°C with optimum temperature from 30 to 32°C. Milk peptonization and gelatin liquefaction were positive. Production of melanoid pigment was positive on tyrosine agar.

This strain could utilize all carbon sources tested for growth as shown in Table 2.

As a result of BERGEY's Manual and International Streptomyces Project (ISP) reports[9~11] about the results of taxonomic studies presented here, we conclude that strain No. 8727 belongs to the genus *Streptomyces* WAKSMAN and HENRICI 1943, 339. Strain No. 8727 has been deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, under the name *Streptomyces* sp. No. 8727 and the accession No. FERM P-9296.

Fermentation

The time course of FR900840 production by *Streptomyces* sp. No. 8727 in a 200-liter jar fermentor is shown in Fig. 3. The organism reached the stationary phase of growth after a 72-hour incubation. FR900840 production began at 24 hours, and the maximum accumulation was observed after a 96-hour incubation period.

Isolation Procedure

The flow diagram of the isolation procedure described below is shown in Fig. 4. The culture broth

Fig. 4. Isolation procedure of FR900840.

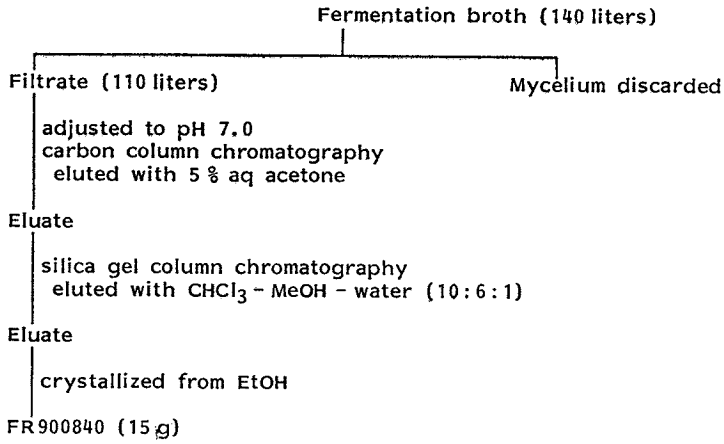


Table 3. Physico-chemical properties of FR900840.

Appearance	Pale yellowish prism
MP	123~125°C (dec)
Molecular formula	$C_7H_{11}N_3O_5 \cdot H_2O$
Mass spectrum	218 ($M^+ + 1$, fast atom bombardment (FAB)-MS)
Optical rotation	$[\alpha]_D^{23}$ +1.5° (*c* 1.0, H_2O)
Elemental analysis (5%)	$C_7H_{11}N_3O_5$
Calcd for:	C 35.74, H 5.57, N 17.87
Found:	C 35.99, H 5.58, N 17.65
UV $\lambda_{max}^{H_2O}$ nm (ε)	257.5 (13,000)
$\lambda_{max}^{H_2O+NaOH}$ nm	257.0
$\lambda_{max}^{H_2O+HCl}$ nm	257.0
IR ν_{max} (Nujol) cm^{-1}	3430, 3270, 3100, 2920, 2160, 1660, 1610, 1600, 1560, 1525, 1460, 1410, 1380, 1350, 1295, 1235, 1160, 1140, 1090, 1070, 1010, 965, 900, 845, 820, 790, 740, 705
^1H NMR (200.13 MHz, D_2O used dioxane as internal standard) δ	4.87 (1H, q, *J*=6.6 Hz), 4.62 (2H, d, *J*=3.8 Hz), 4.10 (1H, t, *J*=3.8 Hz), 1.42 (3H, d, *J*=6.6 Hz)
^{13}C NMR (50.23 MHz, D_2O used dioxane as internal standard) δ	173.55 (s), 169.99 (s), 66.15 (t), 64.74 (d), 56.67 (d), 22.15 (q)

was filtered with the aid of diatomaceous earth (5 kg). The filtrate (110 liters) was adjusted to pH 7.0 and passed through a column of active carbon (10 liters). After washing with water (30 liters), elution was carried out with 5% aqueous acetone (30 liters). The eluate was evaporated under reduced pressure to give an oily residue. The oily residue was mixed with 500 g of silica gel (70~230 mesh, Merck Co., Ltd., U.S.A.), and this mixture was slurried in methanol. After the solvent was evaporated, the resultant dry powder was subjected to column chromatography of the same silica gel (1.5 liters) which was packed with a mixture of chloroform, methanol and water (10 : 6 : 1). The column was developed with the same solvent system. Fractions containing FR900840 were collected and concentrated under reduced pressure to give FR900840 in the form of pale yellowish oil. This oil was dissolved in ethanol and concentrated under reduced pressure. This concentrate was kept at room temperature and purified FR900840 substance (15 g) was obtained as pale yellowish prisms.

Physico-chemical Properties of FR900840

Physico-chemical properties of FR900840 are summarized in Table 3. It is insoluble in acetone,

chloroform, ethyl acetate, diethyl ether and benzene. It is sparingly soluble in methanol, ethanol and soluble in water. Color reactions are as follows: Positive in ceric sulfate, sulfuric acid, ninhydrin, ferric chloride and iodine vapor tests, and negative in Ehrlich, Dragendorff and Molisch tests. Its Rf value on silica gel TLC developed with a mixture of butanol, ethanol, chloroform and aqueous ammonia (2:1:1:2) was 0.44. The determination of the chemical structure of FR900840 will be described in the succeeding paper[2].

Biological Properties of FR900840

Antimicrobial Activities

The antimicrobial activities shown in Table 4. FR900840 showed week antifungal activity against *Geotrichum candidum*. It had no inhibitory effect on bacteria, *Candida albicans*, *Aspergillus niger* and *Aureobasidium pullulans* at 1,000 μg/ml.

Antitumor Activity (*In Vitro*)

In vitro antitumor activities are shown in Table 5. FR900840 had almost equal *in vitro* antitumor activity against human lung adenocarcinoma A549 and human mammary adenocarcinoma MCF-7. The IC_{50} value (the concentration causing 50% inhibition) for the two tumor lines were 0.50 and 0.55 μg/ml, respectively. It showed a comparatively weak growth inhibitory effect on mouse leukemia P388. Its IC_{50} value was 33 μg/ml.

Antitumor Activity in Mice

Antitumor activity of FR900840 was examined against two kinds of human tumors, A549 and MCF-7, implanted under the kidney capsule of immunosuppressed BDF_1 mice (SRC assay). Tumor volume was measured on day-14.

Table 4. Antimicrobial activity of FR900840.

Test strains	MIC (μg/ml)
Bacillus subtilis ATCC 6633	>100
Escherichia coli NIHJ JC-1	>100
Staphylococcus aureus 209P JC-1	>100
Candida albicans	>100
Aspergillus niger	>100
Geotrichum candidum	100
Aureobasidium pullulans	>100

Antimicrobial activity was determined by a serial broth dilution method in bouillon medium for bacteria and in SABOURAUD's medium for fungi and yeasts.

The MIC was expressed in terms of μg/ml after overnight incubation at 37°C for bacteria and 48~72 hours incubation at 28°C for fungi and yeasts.

Table 5. Effects of FR900840 on the growth of tumor cells (*in vitro*).

Dose (μg/ml)	P388		MCF-7		A549	
	OD_{550}	Inhibition (%)	OD_{550}	Inhibition (%)	OD_{550}	Inhibition (%)
0	0.218±0.006	0.0	0.690±0.017	0.0	0.733±0.014	0.0
0.032			0.694±0.012	−0.5	0.718±0.014	2.0
0.1	0.205±0.023	6.1	0.566±0.008	18.0	0.628±0.007	14.3
0.32	0.209±0.003	4.4	0.422±0.011	38.8	0.449±0.008	38.7
1.0	0.222±0.009	−1.5	0.265±0.013	61.6	0.198±0.002	73.0
3.2	0.236±0.001	−7.9	0.237±0.010	65.7	0.167±0.004	77.2
10	0.153±0.005	29.8	0.188±0.005	72.6	0.087±0.002	88.1
32	0.135±0.021	38.0	0.125±0.005	81.8	0.036±0.002	95.1
100	0.044±0.004	79.7				

Tumor cells (P388; 2×10^3/well, MCF-7 and A549; 3×10^3/well) were incubated with FR900840 at 37°C for 7 days, and colorimetric MTT assay was performed. OD_{550} represent the average OD_{550} value± SE of 5 wells.

Table 6. Antitumor activity of FR900840 against human lung adenocarcinoma A549 (SRC assay).

Dose (mg/kg)		Dead/Treated	Body weight change[a] (g)	Tumor volume[b] (mm^3)	Inhibition (%)
Control	—	0/8	1.6	32.3±3.3	—
FR900840	1	0/8	1.8	23.1±3.6	28.5
	3.2	0/8	1.4	16.7±1.1	48.3
	10	0/8	1.6	21.4±3.2	33.7
	32	0/8	−1.1	6.8±1.5	78.9

[a] Body weight change was calculated from the body weights of day-0 and 14.

[b] A 1-mm^3 tumor fragment was implanted under the renal capsule of BDF_1 mouse at day-0 and FR900840 was injected ip at day-1, 5 and 9. The final tumor size was measured at day-14. The tumor volume is indicated by mean±SE.

Table 7. Antitumor activity of FR900840 against human mammary adenocarcinoma MCF-7 (SRC assay).

Dose (mg/kg)		Dead/Treated	Body weight change[a] (g)	Tumor volume[b] (mm^3)	Inhibition (%)
Control	—	0/8	2.7	49.1±2.4	—
FR900840	3.2	0/8	2.6	27.9±2.4	43.1
	10	0/8	1.5	19.0±1.2	61.2
	32	1/8	−0.7	12.4±2.0	74.6

[a,b] Experimental conditions were the same as for Table 6.

As shown in Tables 6 and 7, ip injection of FR900840 inhibited growth of human tumors, A549 and MCF-7. The volume of both tumors decreased by more than 70% at dose of 32 mg/kg.

Acute Toxicity

The LD_{50} values of FR900840 when given intraperitoneally and intravenously to BDF_1 mice were 290 and 320 mg/kg, respectively.

Discussion

P388 mouse leukemia cell was widely used for a long time as a model for screening of antitumor compounds by the National Cancer Institute (NCI) and was believed to give good correlation between animal and clinical effects of known antitumor drugs[12]. However, there is still a need for a more reliable screening method which would predict the clinical effect of novel antitumor compound[13]. For this reason, we have searched for novel products from fermentation broths which would shown a specific inhibitory effect on the growth of human tumor cells, and not on the growth of P388 mouse leukemia cells.

As a result of screening, we isolated a novel antibiotic, FR900840, from the culture broth of *Streptomyces* sp. No. 8727. It was a pale yellowish prism which was soluble in water, and which was found to have a novel structure as shown in Fig. 1. Details on the structural elucidations will be published in the succeeding paper[2]. This compound was found to be unstable in acidic solution (unpublished data). We dissolved it in the culture medium or phosphate buffered saline to examine its biological activities.

FR900840 showed growth inhibitory effects on human solid tumor lines *in vitro*, and IC_{50} values for them were less than 1/50 for mouse leukemia P388. Moreover FR900840 had potent antitumor activities against the two human solid tumors *in vivo*.

Precise antitumor activity of this novel compound will be reported in a separate paper[14].

Addendum in Proof

FR900840 is identical to thrazarine which was isolated independently by T. KAMEYAMA *et al.*, and reported in The Journal of Antibiotics 41: 1561~1567, 1988.

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