A NEW ANTITUMOR ANTIBIOTIC, FR-900482

II. PRODUCTION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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FR-900482 is a new antitumor antibiotic produced by a new actinomyces named *Streptomyces sandaensis* No. 6897. It exhibits potent cytotoxic activity against various tumor cells *in vitro*. Furthermore, it has weak antimicrobial activity against some Gram-positive or Gram-negative bacteria.

In our screening program for antitumor compounds, a new antitumor antibiotic FR-900482 has been isolated from the fermentation broth of *Streptomyces sandaensis* No. 6897. The taxonomy of the producing strain and the antitumor activity of FR-900482 are reported in separate papers[1,2].

The present paper describes the production, isolation, physico-chemical properties and biological activities of FR-900482.

Fermentation

A loopful of mature slant culture of *Streptomyces sandaensis* No. 6897 was inoculated into a seed medium (160 ml) containing soluble starch 2%, glucose 0.5%, cotton seed meal 1%, dried yeast 1%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 7.0) in a 500-ml Erlenmeyer flasks and cultured at 30°C for 72 hours on a rotary shaker with 7.62 cm throw at 200 rpm.

Fermentation studies were carried out in tank fermentors. A seed culture was shaken in the above mentioned Erlenmeyer flasks and then transferred at the rate of 0.7% to 20 liters of the same seed medium in a 30-liter jar fermentor, which was cultured at 30°C for 48 hours under aeration of 20 liters/minute and agitation of 200 rpm. Eighteen liters of the seed culture were inoculated into 1,760 liters of production medium containing soluble starch 8%, dried yeast 1%, peanut powder 3% and soybean meal 0.5% (pH 6.2) in a 2,000-liter stainless steel fermentor which was cultured at 31°C for 96 hours under aeration of 880 liters/minute and agitation of 130 rpm.

Antibiotic levels in the fermentation broth and the extracts of FR-900482 were assayed by paper disc agar diffusion method using *Bacillus stearothermophilus* as a test organism and cytotoxic activity against P388 murine leukemia cells in tissue culture.

A typical time course of the fermentation is presented in Fig. 1.

Isolation and Purification

The cultured broth thus obtained was filtered with an aid of diatomaseous earth (125 kg). The filtrate (1,600 liters) was passed through a column of Diaion HP-20 (400 liters). This column was

Fig. 1. Time course of the fermentation of FR-900482.
PMV: Packed mycelium volume.

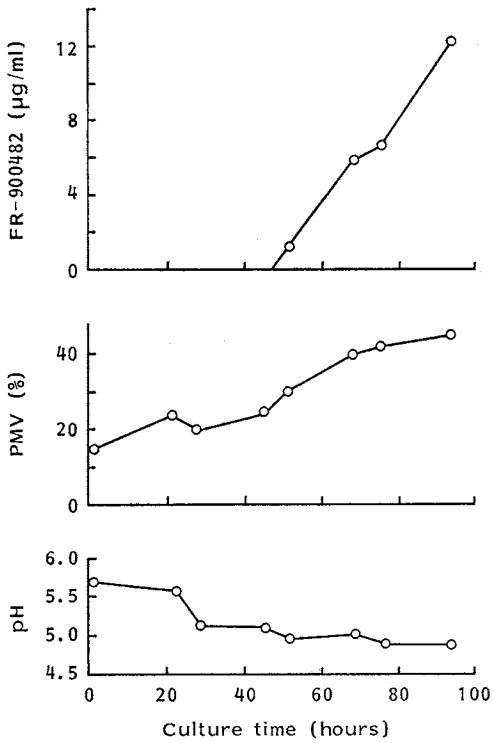
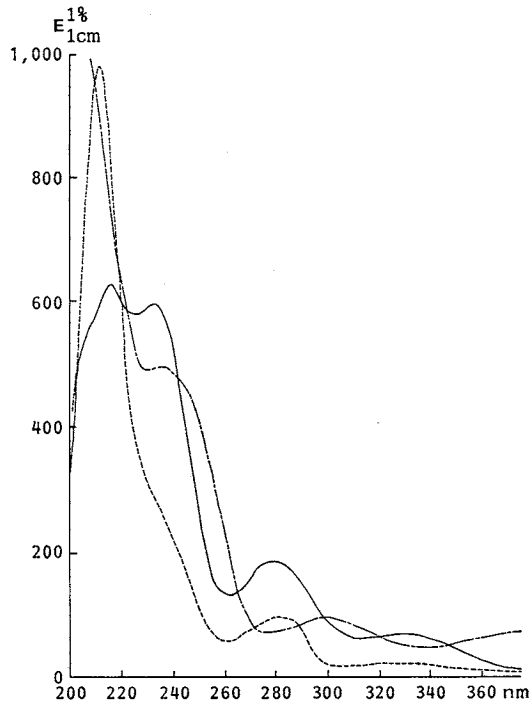


Fig. 2. UV spectrum of FR-900482 (MeOH).
—— MeOH, ------ MeOH - HCl, -·- MeOH - NaOH.



washed with water (400 liters) and eluted with 50% aqueous methanol (1,200 liters). The active eluate was concentrated *in vacuo* to a volume of 300 liters. The active fraction was charged with a column of Amberlite IRC-50 (H^+ form) (300 liters). The column was washed with deionized water (600 liters) and eluted with 0.1 N hydrochloric acid (1,200 liters). The eluate was neutralized with 12 N aqueous sodium hydroxide, and then passed through a column of Diaion HP-20 (200 liters). The Diaion HP-20 column was washed with deionized water (200 liters) and eluted with 50% aqueous methanol (600 liters). The active eluate was concentrated *in vacuo* to a volume of 10 liters, and thereto was added 15 liters of butanol and then stirred for 10 minutes. This extraction procedure was carried out four times and the obtained extracts were combined (60 liters). Then, to the combined butanol extract was added 240 liters of *n*-hexane and stirred for 10 minutes. The aqueous layer was separated and to the butanol - *n*-hexane layer 15 liters of deionized water was added and stirred for 10 minutes. The aqueous layers were combined and concentrated *in vacuo* to a volume of 6 liters, and applied to a column of Alumina Oxide AC-11 (100 liters). The column was developed with 80% aqueous isopropyl alcohol and the active eluate was concentrated *in vacuo* to a volumn of 300 ml. The active fraction was further purified on a column of Toyopearl (HW-40 Fine) (7 liters) using deionized water as an eluent. Fractions containing active material (30 liters) were concentrated *in vacuo* to 500 ml and then lyophilized to give a crude powder (3 g). The crude powder was dissolved in deionized water so as to make a final concentration of 50 mg/ml and subjected to high performance liquid chromatography (HPLC) purification. HPLC was carried out on C_{18} μBondapack column, 0.79 cm (i.d.)×

Table 1. Physico-chemical properties of FR-900482.

Appearance	White powder
Molecular formula	$C_{14}H_{15}N_3O_6$
Molecular weight (*m/z*)	321
Elementary analysis	
Calcd for $C_{14}H_{15}N_3O_6 \cdot H_2O$:	C 49.56, H 5.05, N 12.38.
Found:	C 49.73, H 4.83, N 12.52, S 0.00.
SI-MS (*m/z*)	322 $(M+H)^+$
*p*Ka	$pKa_1=4.1$, $pKa_2=9.0$
MP	175°C (dec)
$[\alpha]_D^{23}$	+8° (*c* 1.0, H_2O), −26.5° (*c* 1.0, 0.1 N HCl)
UV λ_{max}^{MeOH} nm (ε)	236 (19,200), 281 (6,100), 330 (2,200)
$\lambda_{max}^{MeOH+HCl}$ nm	240 (sh), 282, 331
$\lambda_{max}^{MeOH+NaOH}$ nm	238, 302, 374
IR ν_{max}^{KBr} cm^{-1}	3600~3000, 1690, 1580, 1400, 1340, 1080
TLC (Silica gel plate)	
Rf[a]	0.20 and 0.45
Rf[b]	0.55 and 0.65
Rf[c]	0.40

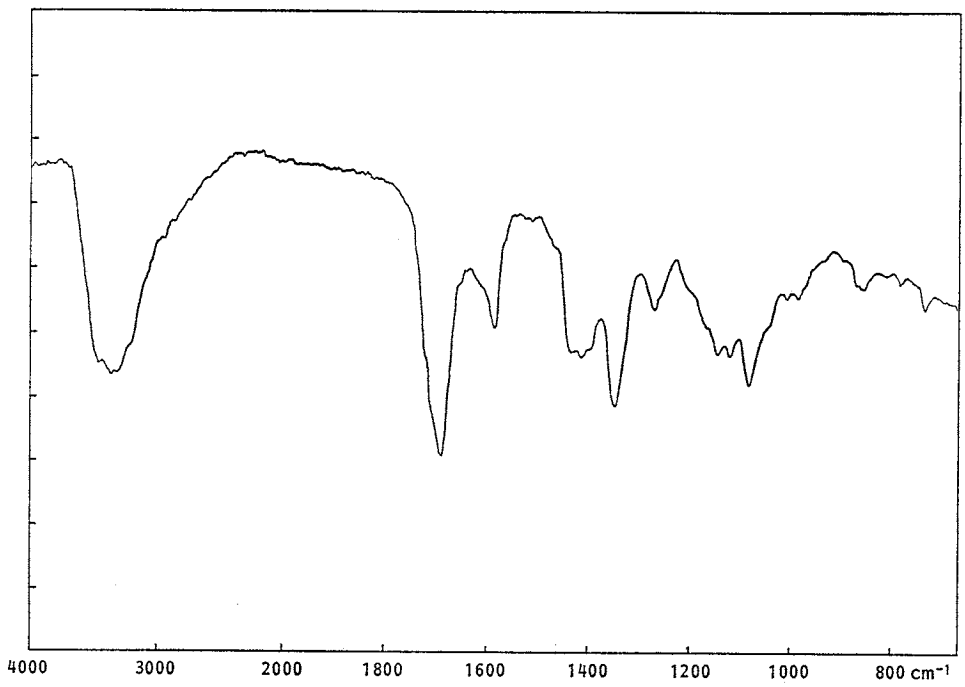
SI-MS: Secondary ion mass spectrum.

[a] Solvent system: Chloroform - methanol, 4 : 1.

[b] Solvent system: 2-Propanol - H_2O, 9 : 1.

[c] Solvent system: Butanol - acetic acid - H_2O, 20 : 1 : 2.

Fig. 3. IR spectrum of FR-900482 (KBr disk).



30 cm and monitored at 254 nm. Mobil phase was a mixture of methanol and distilled water (1 : 9). The active fraction had a retention time of 8 minutes at a flow rate of 6 ml/minute. One g of colorless powder of FR-900482 was obtained.

Fig. 4. 400 MHz ^{1}H NMR spectrum of FR-900482 in D_2O, pD=7.

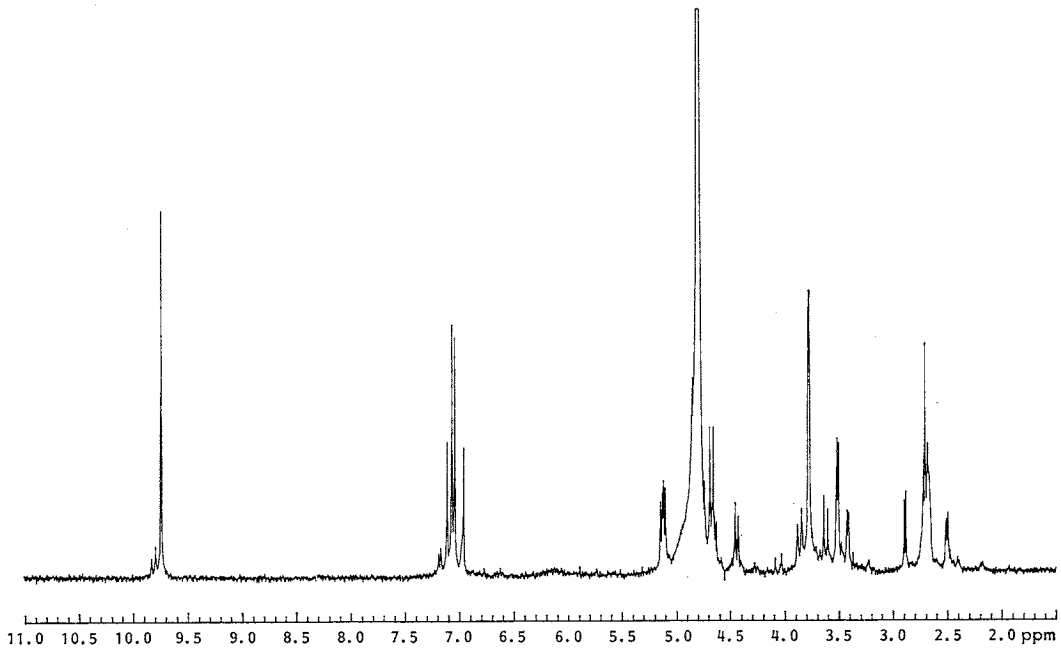
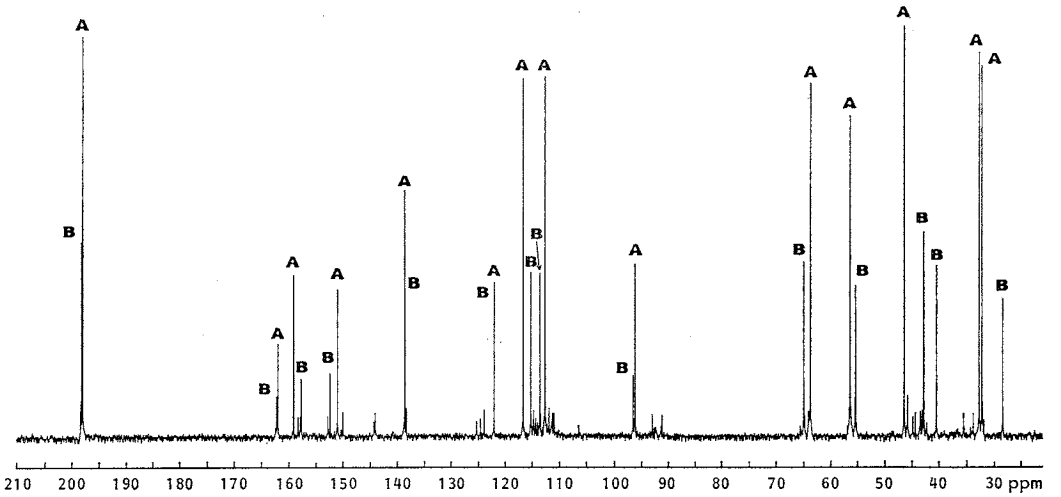


Fig. 5. 100 MHz ^{13}C NMR spectrum of FR-900482 in D_2O, pD=7.



Physico-chemical Properties

The physico-chemical properties of FR-900482 are summarized in Table 1. The UV, IR and ^{1}H NMR spectra are represented in Figs. 2, 3 and 4, respectively.

The molecular formula of FR-900482 was determined to be $C_{14}H_{15}N_3O_6$ by the elemental

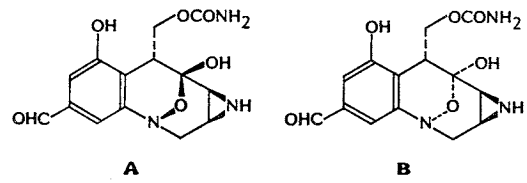


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Table 2. Antimicrobial activity of FR-900482.

Strains	MIC (μg/ml)
Bacillus stearothermophilus var. *calidolactis* C 953	0.03
B. subtilis ATCC 6633	50
Staphylococcus aureus 209P JC-1	100
Escherichia coli NIHJ JC-2	50
Pseudomonas aeruginosa NCTC 10490	25
Candida albicans	1,000
Aureobasidium pullulans IFO 4466	1,000

analysis and mass spectral data. The amphoteric substance of FR-900482 is soluble in water and methanol, and insoluble in acetone, ethyl acetate and chloroform. Color reactions are as follows: FR-900482 gave positive reactions to sulfuric acid, potassium permanganate, 2,4-dinitrophenylhydrazine, iodine vapor and ferric chloride-potassium ferricyanide reagents, though negative to ferric chloride and Sakaguchi reactions.

Table 3. Cytotoxicity of FR-900482.

Cell lines	MIC (μg/ml)
P388 leukemia	0.4
B16 melanoma	0.4
EL4 lymphoma	0.4
FM3A mammary carcinoma	0.4
L1210 leukemia	0.8
3LL lung carcinoma	3.1
PC-3 prostate adenocarcinoma	6.2
BHK-21 kidney	0.8
Bone marrow	3.1
CPAE endotherium	25.0

The structure of FR-900482 was established as **1** on the basis of chemical evidence of **1** and X-ray analysis of its derivative. As can be seen in TLC behavior (see Table 1), FR-900482 exists as an equilibrium mixture of two isomers (**A** and **B**), which was shown to be attributable to tautomerism of the anomeric hydroxyl group.

Biological Activities

Antimicrobial Activity

Antimicrobial activity of FR-900482 was determined by a serial broth dilution method in bouillon media for Gram-positive and Gram-negative bacteria and in Sabouraud media for fungi and yeasts. Minimun inhibitory concentration (MIC) is expressed in terms of μg/ml after overnight at 37°C for all the bacteria except for *Bacillus stearothermophilus* at 55°C and 48~72 hours incubation at 28°C for fungi and yeasts. The antimicrobial spectra of FR-900482 are shown in Table 2.

From the result the antibiotic FR-900482 shows a growth inhibition against *B. stearothermophilus* var. *calidolactis* C 953 at an extremely low concentration (0.03 μg/ml). However, FR-900482 has weak antimicrobial activities against other bacteria, fungi and yeast.

Antitumor Activity

Cytotoxic activity of FR-900482 was determined as follows. Concentration of the compound required for 50% inhibition of cell growth (IC_{50}; μg/ml) was examined by plotting the logarithms of the concentration *versus* the growth rate (percentage of control) of the treated cells. The result is shown in Table 3.

FR-900482 is effective against leukemia P388, melanoma B16, lymphoma EL4, mammary carcinoma FM3A, leukemia L1210 and baby hamster kidney (BHK-21) cells at low concentrations.

Acute Toxicity

The acute toxicity of FR-900482 was determined in *dd*Y mice (5 weeks old, female) by a single

intraperitoneal or intravenous injection of graded doses of FR-900482 into 5 mice. The LD_{50} was approximately 32 mg/kg.

Discussion

The characteristic feature of FR-900482 is that FR-900482 is colorless and fairly soluble in water, despite other traditional antitumor antibiotics are mostly colored and slightly soluble in water. FR-900482 was found to have unique structure **1**, which is in tautomeric equilibrium of **A** and **B**. Details on the structure and the tautomerism of **1** will be published elsewhere.

FR-900482 was quite active in murine tumor systems *in vitro* and *in vivo*[2,3]. Further the structural relationship of the antitumor activity to the tautomeric equilibrium of FR-900482 is now progress.

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