NEW ANTITUMOR ANTIBIOTIC, FR-900462

II. PRODUCTION, ISOLATION, CHARACTERIZATION AND BIOLOGICAL ACTIVITY

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FR-900462 is a new antitumor antibiotic produced by *Streptomyces tokashikiensis* No. 7124. It was highly active against leukemia P388 and melanoma B16. Furthermore, it has weak antimicrobial activity against some Gram-positive bacteria.

In the course of screening for antitumor substances, we have isolated a new antitumor antibiotic, FR-900462[1], from the fermentation broth of *Streptomyces tokashikiensis* No. 7124. The taxonomy of the producing strain is reported in a separate paper[2].

In this report, we describe the production, isolation, physico-chemical properties and biological activities of FR-900462.

Fermentation

A loopful of slant culture of *S. tokashikiensis* No. 7124 was inoculated to a seed medium (80 ml) containing corn starch 0.5%, glycerol 1%, glucose 0.5%, cotton seed meal 1%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 6.5), poured into 250-ml Erlenmeyer flasks and cultured at 30°C for 96 hours at 200 rpm using a rotary shaker.

A seed culture was transferred at the rate of 2% to 150 liters of a production medium containing soluble starch 3%, sucrose 1%, corn starch 0.5%, dried yeast 0.5%, gluten meal 0.5%, feather meal 0.5%, $MgSO_4 \cdot 7H_2O$ 0.05%, NaCl 0.05%, $CoCl_2 \cdot 6H_2O$ 0.0004%, NaI 0.00005% and Na_2CO_3 0.2% (pH 6.8) in a 200-liter jar fermentor and cultured at 30°C for 3 days under aeration of 20 liters/minute and agitation of 300 rpm.

The level of active metabolites in the fermentation broth was assayed by antimicrobial activity against *Bacillus subtilis* ATCC 6633 and cytotoxic activity against P388 murine leukemia cells in tissue culture.

Isolation and Purification

The cultured broth (150 liters) was filtered with the aid of diatomaceous earth (4 kg). The mycelial cake was treated with 120 liters of acetone and stirred for 30 minutes. The extract was concentrated *in vacuo* to a volume of 30 liters and the concentrate was extracted with butanol (30 liters). The butanol layer was separated and concentrated *in vacuo*. The oily material thus obtained was chromatographed on silica gel (4 liters). After being developed with 6 liters of acetone and then 12 liters of a mixture of acetone - methanol (10 : 1), the column was eluted with a mixture of acetone - methanol (5 : 1). Active fractions (6 liters) were concentrated under a nitrogen stream at reduced pressure to give a crude powder (5 g). The crude powder was dissolved in 20 ml of methanol and

rechromatographed on a column of silica gel (300 ml), and then eluted with a mixture of chloroform - methanol (10 : 1). Fractions containing active substance (500 ml) were concentrated under a nitrogen stream at reduced pressure to give a powder. The powder (0.9 g) was dissolved in methanol and subjected to HPLC. HPLC was carried out on a steel column (1×25 cm) packed with silica gel (Cosmosil $5C_{18}$) and monitored at 370 nm. The mobile phase was a mixture of methanol and distilled water (7 : 3) containing 100 mM ammonium acetate. The active fraction had a retention time of 10.2 minutes at a flow rate of 5 ml/minute. Active fractions were concentrated under a nitrogen stream at reduced pressure to obtain a water suspension. The suspension was passed through a Diaion HP-20 column (20 ml). The column was washed with distilled water (100 ml) and eluted with 50% aqueous acetone (50 ml). The active fraction was concentrated under a nitrogen stream at reduced pressure and kept in cool to obtain pale yellow crystals (100 mg).

Physico-chemical Properties

Physico-chemical properties of FR-900462 are summarized in Table 1. The UV, IR, ^1H and ^{13}C NMR spectra are represented in Figs. 1 to 4, respectively.

FR-900462 was readily soluble in methanol and acetone, slightly soluble in ethanol and chloroform, and insoluble in water, hexane and benzene. Color reactions are as follows: FR-900462 gave positive reactions to Dragendorff, iodine vapor and ninhydrin reagents, though negative to Molisch test.

The IR spectrum (Fig. 2) shows the presence of an amide function which is probably in position of conjugation with a benzene ring or a double bond (1610 cm^{-1}). In the ^1H NMR spectrum (Fig. 3), seven methyl signals are observed

Fig. 1. UV absorption spectrum of FR-900462 in methanol.

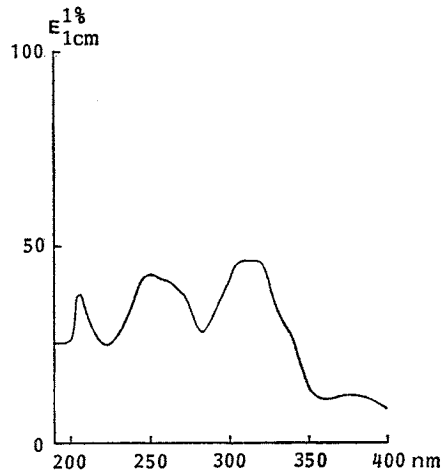


Table 1. Physico-chemical properties of FR-900462.

Appearance	Pale yellow powder
Molecular formula	$C_{27}H_{37}N_3O_6$
MW (*m/z*)	
EI-MS	499 (M^+)
FAB-MS	500 (M^++1)
Elementary analysis	
Calcd for $C_{27}H_{37}N_3O_6 \cdot H_2O$:	C 62.65, H 7.59, N 8.12
Found:	C 62.60, H 7.01, N 8.11
MP	178~182°C
$[\alpha]_D^{23}$	+186.2° (*c* 1.05, MeOH)
TLC (silica gel plate)	
Rf[a]	0.52
Rf[b]	0.27

EI: Electron impact, FAB: fast atom bombardment.

[a] Solvent system: Chloroform - methanol, 3 : 1.

[b] Solvent system: Acetone - methanol, 4 : 1.

Fig. 2. IR spectrum of FR-900462 (in Nujol).

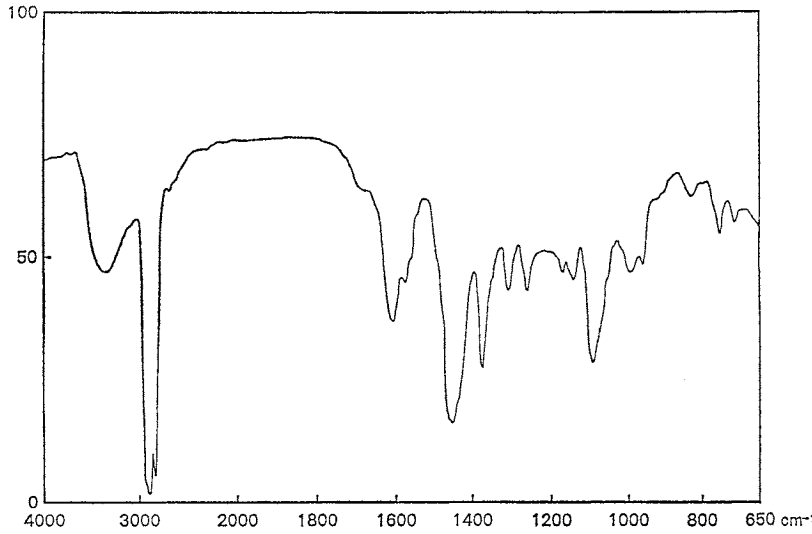
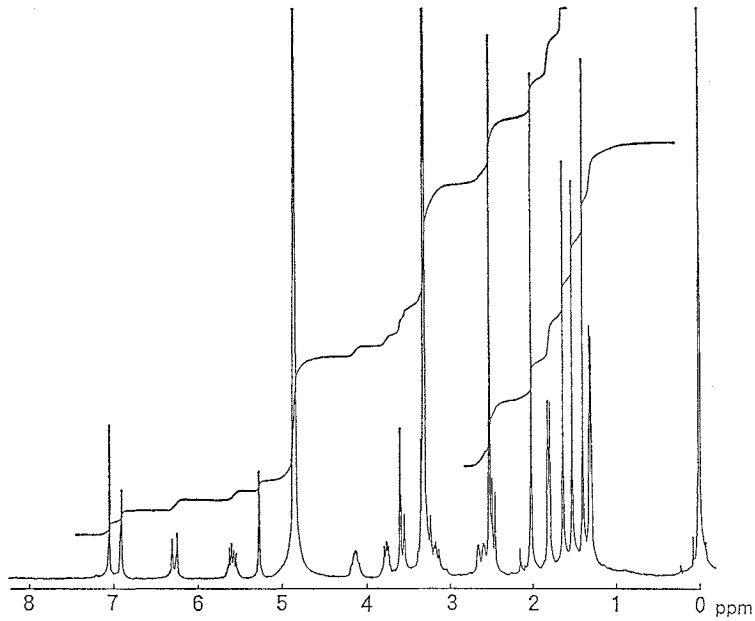
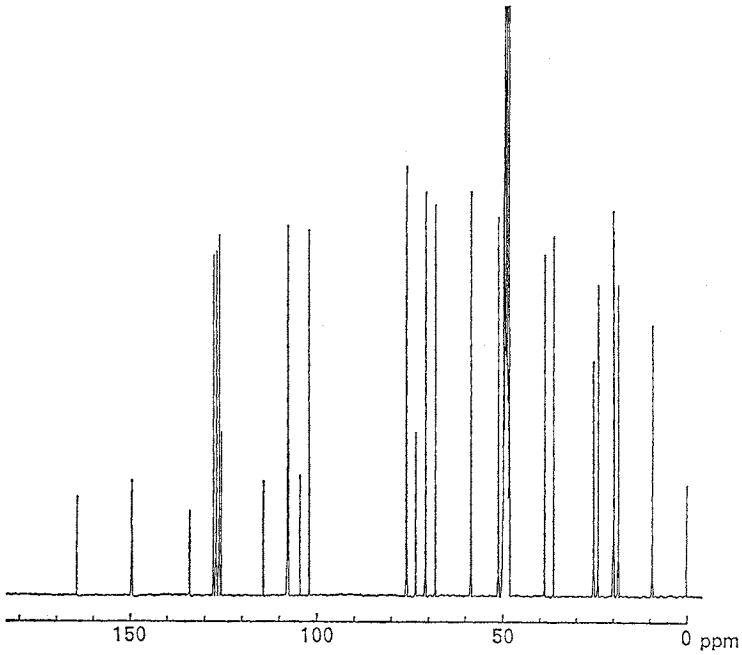


Fig. 3. ^{1}H NMR spectrum of FR-900462 (in CD_3OD, 270 MHz).



at δ 1.30~2.52: two $>CHCH_3$ (δ 1.30 (d, J=6.3 Hz) and 1.81 (d, J=6.3 Hz)), three $-\overset{|}{\underset{|}{C}}CH_3$ (δ 1.39 (s), 1.52 (s), 1.63 (s)), and two $>CCH_3$ (δ 2.01 (s) and 2.52 (s)) groups. These are supported by the carbon signals observed in the ^{13}C NMR spectrum (Fig. 4): δ 9.36, 18.57, 19.75, 19.95, 24.19, 25.29 and 36.19 (or 38.55). One of the two CH_3 groups bonded to the sp^2 carbons is extended to a propenyl (*trans*) group, because two olefinic protons are observed in the ^{1}H NMR spectrum: δ 5.57 (m) and 6.27 (d, J=16 Hz) and the former signal is coupled to the methyl group. The ^{1}H NMR spectrum further shows two aromatic protons, suggesting the presence of a benzene ring in the molecule. Further

Fig. 4. ^{13}C NMR spectrum of FR-900462 (in CD_3OD, 67.8 MHz).



structural studies on FR-900462 are the subject of future works.

Biological Activities

Antimicrobial Activity

Antimicrobial activity of FR-900462 was determined by a serial broth dilution method in bouillon media for bacteria and in Sabouraud media for fungi and yeasts. MICs were 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 yeast.

Table 2. Antimicrobial activity of FR-900462.

Strains	MIC (μg/ml)
Bacillus subtilis ATCC 6633	1.25
Staphylococcus aureus 209P JC-1	2.5
Escherichia coli NIHJ JC-2	>100
Pseudomonas aeruginosa NCTC 10490	>100
Candida albicans	>100
Aureobasidium pullulans IFO 4466	>100

FR-900462 had an antimicrobial activity for *B. subtilis* ATCC 6633 and *Staphylococcus aureus* 209P (Table 2).

Antitumor Activity

The antitumor activity *in vivo* of FR-900462 was determined in mice. Lymphocytic leukemia P388 and melanotic melanoma B16 were implanted intraperitoneally in BDF_1 mice (female, 8 weeks old) at an inoculum size of 1×10^6 cells per mouse. Twenty four hours after the implantation of tumor cells, graded doses of FR-900462 were administered to mice intraperitoneally. Treatments were carried out on day-1, 2 and 3. FR-900462 was solubilized in methanol, concentrated *in vacuo* and then suspended in the sterilized water. Control animals received intraperitoneal doses of physiological saline solution. Five mice were used for each experimental group. Doxorubicin hydrochloride (Adriacin, Kyowa) was used as a reference compound. Antitumor activity was evaluated by the mean survival time of group of mice and also expressed as T/C (%) value (mean survival time

Table 3. Antitumor activity of FR-900462 against leukemia P388.

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
FR-900462	1.25	14.8	129
	0.6	18.1	158
	0.3	17.1	150
	0.15	14.8	129
	0.075	13.8	121
Doxorubicin hydrochloride	0.3	15.5	135
Control		11.4	100

Table 4. Antitumor activity of FR-900462 against melanoma B16.

Drug	Dose (mg/kg/day)	Mean survival time (days)	T/C (%)
FR-900462	0.5	25.4	158
	0.25	26.7	166
	0.125	20.5	127
	0.06	20.5	127
	0.03	19.8	123
Doxorubicin hydrochloride	0.2	28.5	177
Control		16.1	100

of treated group/mean survival time of non-treated group (control) $\times 100$).

The results are shown in Tables 3 and 4. FR-900462 was active against leukemia P388 and melanoma B16. Doses between $0.075 \sim 1.25$ mg/kg/day against P388 and $0.03 \sim 0.5$ mg/kg/day against B16 resulted in significant increase in the life span of mice.

Acute Toxicity

The acute toxicity of FR-900462 was determined in *dd*Y mice (5 weeks old, female) by a single intraperitoneal injection of graded doses of FR-900462 into a group of 5 mice. The LD_{50} was approximately 20 mg/kg.

Discussion

As reported in this paper, FR-900462 produced by a new actinomycete named *S. tokashikiensis* No. 7124[1], is weakly active in Gram-positive bacteria and showed potent antitumor activity against mouse leukemia P388 and melanoma B16. Further, *in vivo* evaluation of the antitumor activity of FR-900462 is in progress.

The chemical structure of FR-900462 has not been clarified, although this compound is assumed to be a new type of antitumor antibiotic from physico-chemical properties, ^{1}H and ^{13}C NMR and IR spectra, suggesting the presence of an amide function which is in position of conjugation with a benzene ring, two $>CHCH_3$, three $-\overset{|}{\underset{|}{C}}CH_3$ and two $>CCH_3$ groups in the molecule.

References

1) NAKAYAMA, O.; Y. HORI, M. IWAMI, M. KOHSAKA & H. TERANO (Fujisawa): FR-900462 substance and its preparation. Jpn. Kokai 192593 ('85), Oct. 1, 1985
2) IWAMI, M.; O. NAKAYAMA, H. TERANO & M. KOHSAKA: New antitumor antibiotic, FR-900462. I. Taxonomy of the producing strain. J. Antibiotics 42: 680~685, 1989