NEW ANTITUMOR ANTIBIOTICS, FR-900405 AND FR-900406

II. PRODUCTION, ISOLATION, CHARACTERIZATION AND ANTITUMOR ACTIVITY

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The new antitumor antibiotics, FR-900405 and FR-900406, were isolated from the culture broth of *Actinomadura pulveracea* sp. nov. No. 6049. These compounds which contain sulfur in the molecule, represent a novel class of antitumor agents. FR-900405 and FR-900406 are highly active in mice against experimental tumors and exhibit antimicrobial activity against Gram-positive and Gram-negative bacteria and fungi.

An *Actinomadura pulveracea* sp. nov. No. 6049 was found to produce new antitumor antibiotics, which were extracted from the fermentation broth and separated into two major components, named FR-900405 and FR-900406[1)††].

The present paper describes the production and isolation of FR-900405 and FR-900406 as well as physico-chemical properties, antimicrobial and antitumor activities. The taxonomy of FR-900405 and FR-900406 producing strain *A. pulveracea* sp. nov. is reported in a separate paper[2)].

Fermentation

A loopful of a mature slant culture of *A. pulveracea* No. 6049 was inoculated into a seed medium (160 ml) containing starch 2%, glucose 5%, cotton seed flour 1%, dried yeast 1%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 7.0) in a 500-ml Erlenmeyer flask, and cultured at 30°C on a rotary shaker with 7.5-cm throw at 220 rpm at 30°C for 96 hours. The seed culture was then transferred at the rate of 2.5% to 20 liters of the production medium in a 30-liter jar fermentor and cultivation was carried out for 96 hours at 30°C under aeration of 20 liters/minute and agitation of 300 rpm. The production medium contained sucrose 4%, K_2HPO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.1%, NaCl 0.1%, $(NH_4)_2SO_4$ 0.2%, $CaCO_3$ 0.2%, dried yeast 0.5%, $FeSO_4 \cdot 7H_2O$ 0.0001%, $MnCl_2 \cdot 4H_2O$ 0.0001% and NaI 0.00005%.

Both antibiotic levels of FR-900405 and FR-900406 in the fermentation broth and the extracts were assayed by a paper disc-agar diffusion method using *Staphylococcus aureus* 209P as a test organism.

Isolation and Purification

The fermentation broth (20 liters) was filtered with the aid of diatomaceous earth and the activity was found both in the mycelial cake and filtrate. Ten liters of ethyl acetate was added to the mycelial cake and stirred for 10 minutes. This extraction procedure was carried out twice and the extracts were combined. The extracts were washed with 10 liters of 1% sodium bicarbonate, 10 liters of 10%

†† FR-900405 and FR-900406 are identical with WS 6049-A and WS 6049-B[1)].

sodium chloride, and concentrated *in vacuo* to a volume of one liter. After dehydration with anhydrous sodium sulfate, the ethyl acetate solution was further concentrated *in vacuo* and the oily materials were applied to a 70-ml silica gel chromatographic column.

After developing with 200 ml of chloroform, the active substances were eluted with a mixture of chloroform - methanol (40: 1). Active fractions (500 ml) were concentrated *in vacuo* to give a crude powder (140 mg).

The powder was dissolved in 2 ml of chloroform and subjected to chromatography on a 20-ml column of silica gel. After developing with a mixture of chloroform - acetone (8: 1), and most of the FR-900405 was eluted with a mixture of chloroform - acetone (4: 1). The majority of the FR-900406 was eluted with a mixture of chloroform - acetone (2: 1). Each fraction containing FR-900405 or FR-900406 was concentrated *in vacuo* to give a crude powder of FR-900405 (17 mg) and FR-900406 (15 mg), respectively. They were separately analyzed by high performance liquid chromatography (HPLC), monitored at 254 nm. Two antibiotics were separated effectively using a μPorasil column (Waters), 0.79 cm (ID) $\times$ 30 cm and a mixture of *n*-hexane, chloroform and methanol (25: 10: 2). Retention times at a flow rate of 3 ml/minute, for FR-900405 and FR-900406 are 18 and 26 minutes, respectively. Twelve mg of FR-900405 and 8 mg of FR-900406 were obtained, as colorless powder.

Physico-chemical Properties

FR-900405 and FR-900406 show similarities in their solubilities and color reactions. Thus, both are readily soluble in methanol, acetone and chloroform, slightly soluble in diethyl ether, and insoluble in hexane and water. They gave positive reactions to Dragendorff, Ehrlich and ceric sulfate reagents, though negative to ninhydrin test. They show, however, different properties on TLC as shown in Table 1.

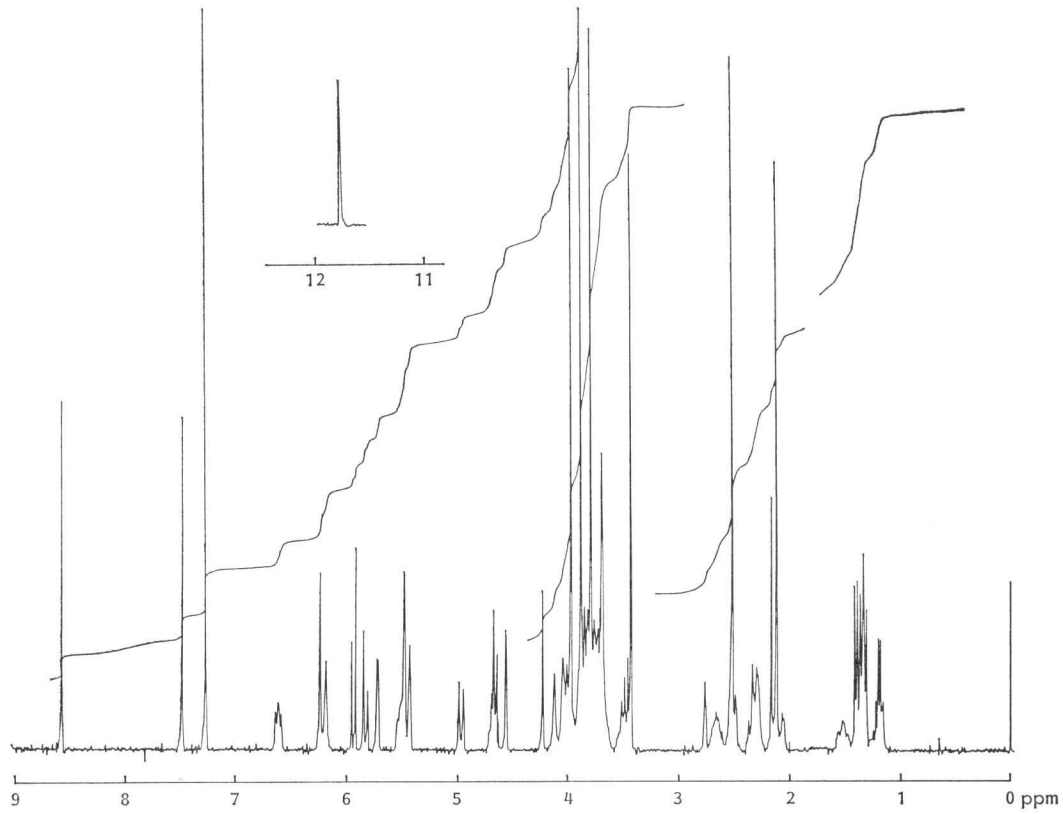
Table 1. Physico-chemical properties of FR-900405 and FR-900406.

	FR-900405	FR-900406
MP (dec)	150°C	145°C
$[\alpha]_D^{25}$ (*c* 1, $CHCl_3$)	−208°	−201°
UV λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$)	252 (270), 280 (160), 320 (100)	253 (250), 280 (150), 320 (100)
$\lambda_{max}^{MeOH+HCl}$ nm	252, 280, 320	253, 280, 320
$\lambda_{max}^{MeOH+NaOH}$ nm	250, 280, 310 (sh)	250, 282, 310 (sh)
IR $\nu_{max}^{CHCl_3}$ cm^{-1}	3450, 3350, 3250, 2960, 2920, 1725, 1675, 1610, 1595, 1520, 1465, 1450, 1405, 1370, 1350, 1310, 1250, 1180, 1155, 1115, 1075, 1020, 985, 955, 905, 850	3450, 3350, 3250, 2990, 2920, 1725, 1675, 1610, 1595, 1520, 1465, 1450, 1405, 1370, 1350, 1310, 1250, 1180, 1155, 1115, 1070, 1020, 985, 955, 905, 880, 850
Elemental analysis (%)	C 52.03, H 5.71, N 4.15, S 9.86	C 51.58, H 5.75, N 4.27, S 9.80
MW (GPC*)	1,100 ~ 1,200	1,100 ~ 1,200
FAB-MS (*m/z*)	1,333, 1,311	1,297
TLC** Rf[a]	0.59	0.53
Rf[b]	0.33	0.18

* Gel permeation chromatography.

** Stationary phase, silica gel sheet (Merck); developing solvent, [a]; $CHCl_3$ - MeOH (10: 1), [b]; $CHCl_3$ - acetone (1: 1).

Fig. 1. ^{1}H NMR spectrum of FR-900405 in $CDCl_3$ (270 MHz).



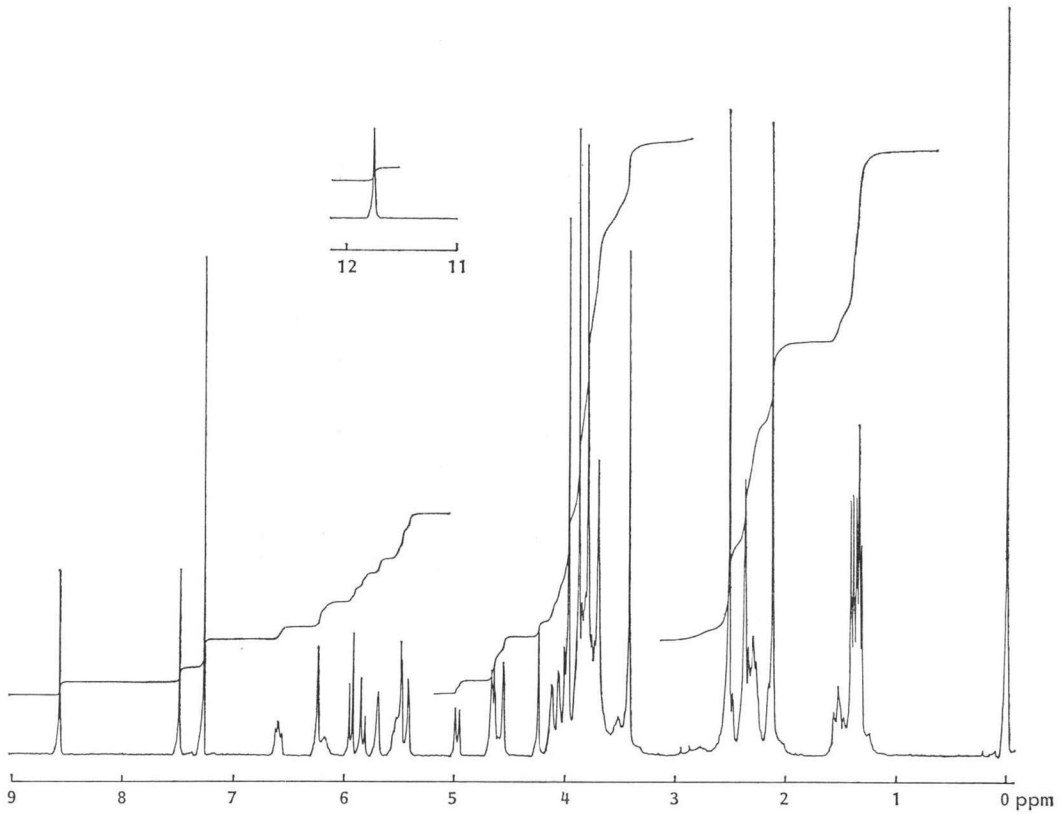
The ^{1}H and ^{13}C NMR spectra of FR-900405 and FR-900406 are shown in Figs. 1~4, respectively. The other physico-chemical properties of the two components are summarized in Table 1. The UV and IR spectra of the two components were almost superimposable, although some differences were observed in their ^{1}H and ^{13}C NMR spectra. FR-900406 lacks the four-proton resonance (multiplet) at δ 1.20 which was observed in the ^{1}H NMR spectrum of FR-900405. Instead, FR-900405 showed a four (or five) fewer proton resonance in the region of δ 2.20 to 2.40 as compared to FR-900406. In the ^{13}C NMR spectra of the two compounds, a major difference was observed in the region of 10 to 50 ppm. Thus, FR-900405 showed eleven carbon signals, while FR-900406 showed ten carbon signals, in this region. Elemental analyses indicated that both FR-900405 and FR-900406 contain nitrogen and sulfur atoms.

Multiple proton resonances at δ 3.5~4.2 and 4.6~6.0 in the ^{1}H NMR spectrum of FR-900405 implied the presence of sugar-like moieties and four 3H singlet signals at δ 3.6~4.0 suggested the presence of methoxy groups in the molecules. FR-900405 showed an absorption at 1725 cm^{-1} in the IR spectrum, indicating that it contains an ester (or lactone) function. These speculations were also the cases for FR-900406.

Treatment of FR-900405 with saturated K_2CO_3 - MeOH at room temperature for 1 hour, gave two degradation products, D-1 and D-2.* D-1 was characterized as $C_{14}H_{17}NO_6$ [M^+, m/z 295.09778

* Experimental detail will be reported, together with results on further experiments for the full structure of FR-900405, in a subsequent paper in this series.

Fig. 2. ^1H NMR spectrum of FR-900406 in $CDCl_3$ (270 MHz).



(Calcd 295.10561)] by high resolution mass spectrum. The ^1H NMR spectrum ($CDCl_3$) of D-1 showed the following protons: =CH– ×2 [δ 8.58 (s) and 7.44 (s)], $=CH_2$ ×1 [δ 5.48 (d, J=2.5 Hz) and 4.56 (d, J=2.5 Hz)], $-OCH_3$ ×4 [δ 3.97 (s), 3.90 (s), 3.88 (s) and 3.79 (s)] and NH (or OH) ×1 [δ 12.00 (br s)]. The ^{13}C NMR spectrum ($CDCl_3$) of D-1 revealed the following functional groups: >C=O ×2 [167.4 (s), 160.5 (s)], =C< ×5 [154.3 (s), 153.6 (s), 143.8 (s), 136.6 (s) and 107.5 (s)], =CH– ×2 [103.7 (d) and 112.3 (d)], $=CH_2$ ×1 [90.4 (t)] and $-OCH_3$ ×4 [56.2 (q)×4]. Based on these spectral data together with NOE and ^{13}C-(^1H) LSPD experimental results, the structure of D-1 was established as shown in Fig. 5. The formation of D-1 and D-2 by alkaline methanolysis of FR-900405 suggested that the substituted anthranylic acid residue (Figs. 5 and 6) is connected to the remaining moiety through an ester linkage. Comparison of the ^1H NMR spectra of FR-900405 and the degradation products revealed that FR-900405 comprises D-1 and D-2 and that one proton signal at δ 5.51 in the parent compound was shifted upfield by 1.38 ppm in D-2. Moreover, ^1H NMR decoupling experiments ascertained the presence of a 2-deoxyfucose moiety in D-2 which contains the upfield-shifted proton (δ 4.13). Taking these results into consideration, the partial structure of FR-900405 has been determined as shown in Fig. 6.

It is assumed from the similarities in the spectra of FR-900406 to those of FR-900405 as described above that the former also contains a similar partial structure in its molecule.

Fig. 3. ^{13}C NMR spectrum of FR-900405 in $CDCl_3$ (67.8 MHz).

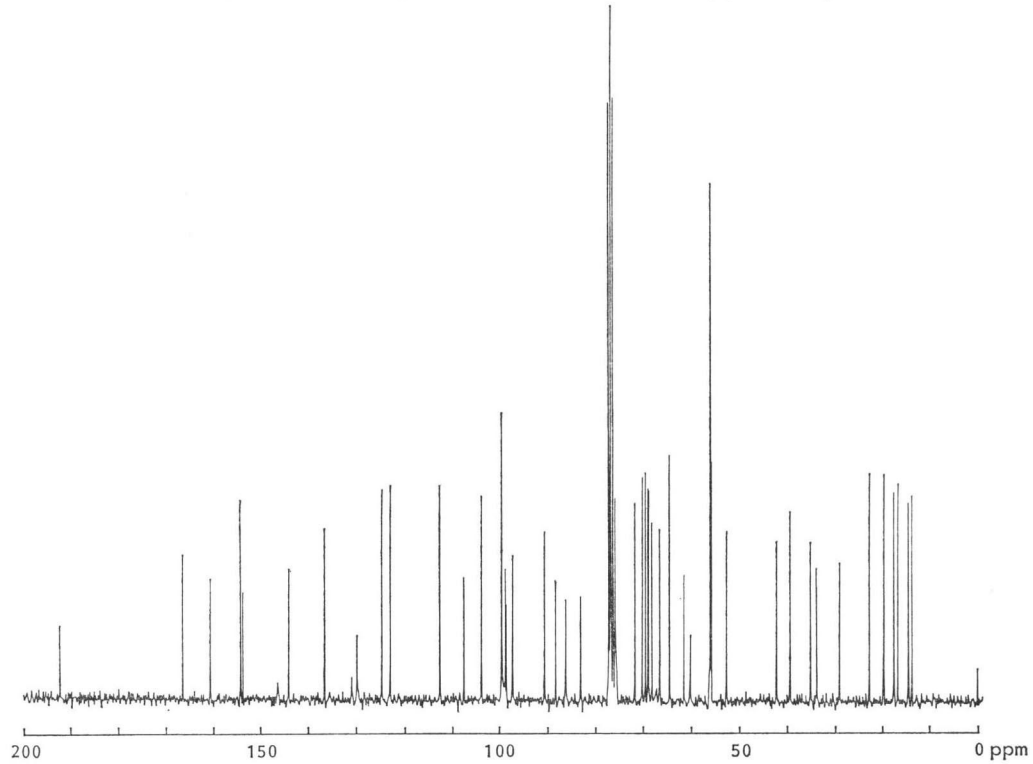


Fig. 4. ^{13}C NMR spectrum of FR-900406 in $CDCl_3$ (67.8 MHz).

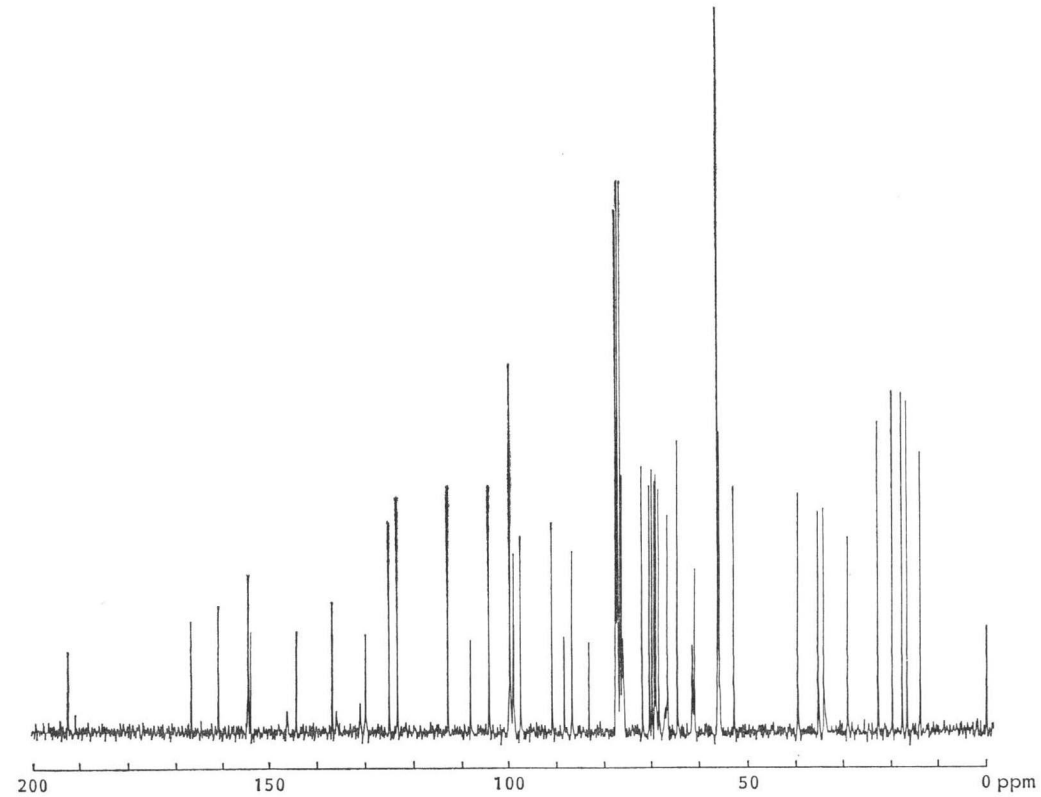


Fig. 5. Structure of D-1.

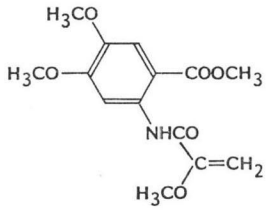


Fig. 6. Partial structure of FR-900405.

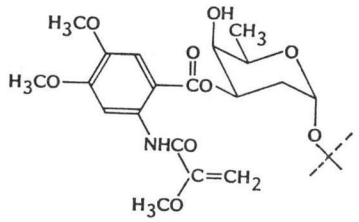


Table 2. Antimicrobial spectrum of FR-900405 and FR-900406.

Microorganism	MIC (μg/ml)	
	FR-900405	FR-900406
Staphylococcus aureus 209 P	0.0001 ~ 0.0002	0.0001 ~ 0.0002
Bacillus subtilis ATCC 6633	0.001	0.001
Escherichia coli NIHJ JC-2	0.6	0.6
Pseudomonas aeruginosa NCTC 10490	0.6	0.6
Proteus vulgaris	1.2	1.2
Candida albicans	5.0	5.0
Aspergillus oryzae	0.6	0.6
Penicillium chrysogenum	0.3	0.3
Aureobasidium pullulans IFO 4466	0.15	0.15

Antimicrobial Activity

The antimicrobial activity of FR-900405 and FR-900406 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. Minimum inhibitory concentrations (MIC) 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 yeasts. The antimicrobial spectra of FR-900405 and FR-900406 are shown in Table 2.

From these results, FR-900405 and FR-900406 have a broad antimicrobial activity and show growth inhibition against *S. aureus* 209P at an extremely low concentration. They may be among the most potent antibiotics ever discovered. The antimicrobial spectrum of FR-900405 is similar to that of FR-900406.

Antitumor Activity

The antitumor activities of FR-900405 and FR-900406 were determined in experimental tumor systems in mice. Lymphocytic leukemia P388, lymphoid leukemia L1210, melanotic melanoma B16 and mastocytoma P815 were implanted intraperitoneally into BDF_1 mice (female, 8 weeks old) at an inoculum size of 1×10^6, 5×10^5, 1×10^6 and 1×10^6 cells per mouse, respectively. Twenty-four hours after the implantation of tumor cells, graded doses of the antibiotics were administered to mice intraperitoneally. Treatments were given on day 1, 2, 3 and 4 (qd 1→4). FR-900405 and FR-900406 were suspended in physiological saline solution (0.9% saline). Control animals received intraperitoneal doses of physiological saline solution. The injection volume was 0.2 ml in all experiments. Doxorubicin hydrochloride (Adriacin-Kyowa) was suspended in physiological saline solution and comparatively tested as a reference compound.

Death or survival of the treated and non-treated animals was recorded daily after the tumor im-

Table 3. Antitumor activity of FR-900405 and FR-900406 against leukemia P388.

Drug	Dose (μg/kg/day)	Weight change (g) Day 0~Day 4	Mean survival time (days)	T/C (%)
FR-900405	10	−2.0	16.4	173 (1/5)*
	3.3	+0.2	21.5	226 (1/5)*
	1.1	+0.2	22.5	237
	0.37	+0.2	21.6	227
	0.12	+0.2	18.6	196
	0.04	+0.1	16.6	175
	0.013	+0.6	12.3	129
FR-900406	10	−1.7		Toxic
	3.3	−0.4	29.7	248
	1.1	−1.2	20.7	178
	0.37	+0.3	22.5	188
	0.12	±0	21.9	183
	0.04	+0.5	24.9	208
	0.013	+0.6	17.7	148
Doxorubicin	1,500	+0.8	25.0	263 (2/5)*
	400	+0.1	20.8	219
	100	+0.1	16.8	177
	25	+0.9	11.3	119
Control	—	+0.1	9.5	100

* Numbers of survivors at Day 30/total mice.

Table 4. Antitumor activity of FR-900405 against L1210 mouse leukemia.

Drug	Dose (μg/kg/day)	Weight change (g) Day 0~Day 4	Mean survival time (days)	T/C (%)
FR-900405	10	+0.2	13.5	155
	2.5	+0.2	19.3	222 (2/5)*
	0.6	+1.5	12.2	140
	0.15	+0.4	11.2	129
	0.04	+0.8	12.2	140
Doxorubicin	3,000	−0.6	13.6	156
	800	+0.8	19.0	218 (2/5)*
	200	+1.3	11.3	130
	50	+1.6	9.4	108
Control	—	+1.3	8.7	100

* Numbers of survivors at Day 30/total mice.

Table 5. Antitumor activity of FR-900405 against melanotic melanoma B16.

Drug	Dose (μg/kg/day)	Weight change (g) Day 0~Day 4	Mean survival time (days)	T/C (%)
FR-900405	10	−0.2	18.1	88
	3.3	+0.6	32.8	160 (1/10)*
	1.1	+1.6	35.1	171 (2/10)*
	0.37	+0.5	33.4	163 (2/10)*
	0.12	+1.8	26.8	131 (1/10)*
Doxorubicin	3,000	+0.8	39.7	194 (8/10)*
	300	+1.4	38.4	187 (6/10)*
	3	+1.3	26.5	129
Control	—	+1.4	20.5	100

* Numbers of survivors at Day 40/total mice.

Table 6. Antitumor activity of FR-900405 against P815 mouse mastocytoma.

Drug	Dose (μg/kg/day)	Weight change (g) Day 0~Day 4	Mean survival time (days)	T/C (%)
FR-900405	10	−2.2	23.3	149
	2.5	−0.5	38.2	245
	0.6	−0.2	35.3	226 (2/5)*
	0.15	+0.3	32.6	209
	0.04	+1.0	20.1	129
Doxorubicin	3,000	−0.3	40.8	262 (3/5)*
	800	+0.9	36.6	235
	200	+1.2	35.5	228
	50	+0.6	23.3	149
	12	+1.8	22.8	146
Control	—	+1.4	15.6	100

* Numbers of survivors at Day 45/total mice.

plantation and the mean survival time was measured. The results were expressed as T/C % [mean survival time of treated group/mean survival time of non-treated (control) group, $\times 100$].

The antitumor activity of FR-900405 and FR-900406 was determined comparatively against P388 leukemia. As shown in Table 3, FR-900405 and FR-900406 are quite active against P388. Doses between 0.013~10 μg/kg on the qd$\times$4 scheduled resulted in significant increase in life span in mice. Doxorubicin is also active against P388 at doses between 0.05~6.0 mg/kg on the same schedule.

The antitumor activity of FR-900405 was further determined against leukemia L1210, melanoma B16 and mastocytoma P815. Doxorubicin was used as a reference compound. As shown in Tables 4, 5 and 6, FR-900405 was quite active in these tumor systems including L1210, B16 and P815 over a wide range of extremely low doses. *In vivo* activity in mice was reproducibly demonstrated at doses less than 1 μg/kg.

Acute Toxicity

The acute toxicity of FR-900405 and FR-900406 was determined in *dd*Y mice (5 weeks old, female) by a single intraperitoneal injection of graded doses of the test compounds in 5 mice. The LD_{50} was 0.05 mg/kg and 0.05 mg/kg, respectively.

Discussion

FR-900405 and FR-900406 are highly potent and unique sulfur containing antibiotics representing a novel class of antitumor agents. *In vivo* activity against P388 and other tested tumors in mice is demonstrated at doses less than 1 μg/kg. The antitumor activity of FR-900405 and FR-900406 was approximately 3-fold more potent than that of doxorubicin. In tissue culture, IC_{50} values against L1210 cells for FR-900405 and FR-900406 are 5×10^{-6} μg/ml and 5×10^{-6} μg/ml. These extraordinarily strong activities *in vitro* indicate potency about twice that of CC-1065, which is the most potent antitumor antibiotic described in the literature[3].

Recently it was reported by BUNGE *et al.*[4] that an *Actinomadura* sp. produced two new antibiotics, PD 114,759 and PD 115,028 which exhibited *in vitro* and *in vivo* antitumor activity at extremely low doses. These antibiotics have a molecular weight of 1,356 and contain four atoms each of nitrogen and sulfur. These data as well as other physico-chemical properties (UV, IR and NMR spectra) show a close resemblance between PD 114,759, PD 115,028 and our compounds. In addition, BBM-1675, a second antibiotic related to FR-900405 and FR-900406 has been isolated from a culture broth

of *Actinomadura verrucosospora* by KONISHI *et al.*[5]. Its physico-chemical properties revealed that BBM-1675 is closely related (structually) to our compounds.

Structual studies on FR-900405 and FR-900406 is in progress and their full structures will be the subject of a future manuscript.

An *in vivo* evaluation of the antitumor activities of these compounds is now in progress in the Division of Cancer Treatment, National Cancer Institute (USA).

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