

FR-900520 AND FR-900523, NOVEL IMMUNOSUPPRESSANTS
ISOLATED FROM A *STREPTOMYCES*

II. FERMENTATION, ISOLATION AND PHYSICO-CHEMICAL
AND BIOLOGICAL CHARACTERISTICS

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(Received for publication June 3, 1988)

FR-900520 and FR-900523, novel neutral macrolide immunosuppressants, were isolated from the cultured broth of *Streptomyces hygroscopicus* subsp. *yakushimaensis* No. 7238. Their molecular formulae were determined as $C_{43}H_{89}NO_{12}$ and $C_{43}H_{87}NO_{12}$, respectively. The compounds suppressed immune response *in vitro*. IC_{50} values of FR-900520 and FR-900523 for mouse mixed lymphocyte reaction were 0.55 nM and 1.6 nM, respectively. FR-900520, the major component, clearly prolonged skin allograft survival in rats.

The detection of immunosuppressants which have the ability to prevent rejection of transplanted organs has been of major interest in our laboratories.

A mixed lymphocyte reaction (MLR) has been thought to be the *in vitro* correlative model for allograft rejection and a representative reaction of interleukin-2 (IL-2) dependent T cell proliferation. On the basis of this, we have searched for efficient immunosuppressants with specific suppression of MLR in cultured broths of microorganisms isolated from soil samples.

Previously we reported the discovery of FK-506¹⁾ which has been proven to be potentially more potent as an antirejection drug²⁻⁷⁾ than cyclosporine (CsA)⁸⁻¹²⁾, the most widely used immunosuppressant at this time.

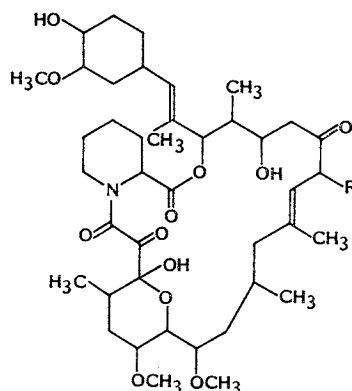
We isolated new compounds FR-900520 and FR-900523 (Fig. 1), both structurally related to FK-506¹³⁾, from cultured broth of *Streptomyces hygroscopicus* subsp. *yakushimaensis* No. 7238¹⁴⁾. The present paper describes the fermentation of strain No. 7238 and the isolation, physico-chemical and biological properties of FR-900520 and FR-900523. The determination of their chemical structures will be reported in a later paper.

Materials and Methods

Fermentation

A loopful of the slant culture of the produc-

Fig. 1. Structures of FR-900520, FR-900523 and FK-506.



FR-900520	R = CH ₂ CH ₃
FR-900523	R = CH ₃
FK-506	R = CH ₂ CH=CH ₂

ing strain No. 7238 was inoculated into a 500-ml Erlenmeyer flask containing 160 ml of a sterile seed medium and incubated at 30°C for 4 days on a rotary shaker. The seed medium (adjusted to pH 6.5) was composed of glycerol (1%), corn starch (1%), glucose (0.5%), cotton seed meal (1%), dried yeast (0.5%), corn steep liquor (0.5%) and calcium carbonate (0.2%). 3.2 liters of the seed culture were transferred to 150 liters of a production medium (adjusted to pH 6.8) containing glucose (4.5%), corn steep liquor (1%), dried yeast (1%), gluten meal (1%), wheat germ (0.5%), calcium carbonate (0.1%) and Adekanol (defoaming agent, Asahi Denka Co., Ltd.) (0.1%) in a 200-liter jar fermentor. Fermentation was carried out at 30°C for 4 days under aeration of 150 liters/minute and agitation at 250 rpm.

Suppression of *In Vitro* MLR

The MLR tests were performed in microtiter plates, with each well containing 5×10^5 C57BL/6 (female, 6~7 weeks old) responder cells (H-2^b), 5×10^5 mitomycin C treated (25 µg/ml mitomycin C at 37°C for 30 minutes and washed three times RPMI 1640 medium) BALB/C (female, 6~7 weeks old) stimulator cells (H-2^d) in 0.2 ml RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM sodium bicarbonate, benzylpenicillin (50 U/ml) and streptomycin (50 µg/ml). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂: 95% air for 68 hours and pulsed with [³H]-thymidine ([³H]TdR) (0.5 µCi) 4 hours before the cells were collected. The test compounds were dissolved in ethanol and further diluted in RPMI 1640 medium before adding to the cultures.

Suppression of *In Vitro* EL-4 Growth

Cytotoxicity for EL-4 lymphoma was measured by a 4-hour [³H]TdR incorporation in microtiter plates with 1×10^4 cells per well cultured for 68 hours at 37°C in 0.1 ml RPMI 1640 complete medium.

Antimicrobial Activities

Antimicrobial activities were determined by a conventional agar dilution method, using a nutrient agar for antibacterial tests and a SABOURAUD's agar for antifungal tests.

Skin Grafting

Full-thickness ear skin grafts from donor rats, F344 (RT-1¹⁷¹ male, 8~10 weeks old) were transplanted on to the lateral thorax of recipient rats, WKA (RT-1^k male, 8~10 weeks old) and covered with sterile bactericidal gauze. The entire chest was then wrapped with an elastic bandage. The dressings were removed on day 5. The grafts were inspected daily until rejection which was defined as more than 90% necrosis of the graft epithelium. FR-900520 was dissolved in olive oil and administered intramuscularly 5 days a week, for 2 weeks, beginning on the day of transplantation.

Results

Fermentation

A typical time course for the fermentation is presented in Fig. 2. The activities in the fermentation broth were assayed by the suppression of MLR as a complex of FR-900520 and FR-900523. The compounds production began at 40 hours, and reached a maximum potency after a 90-hour incubation period.

Isolation and Purification

FR-900520 and FR-900523 were isolated as shown in Fig. 3. The cultured broth was filtered with the aid of diatomaceous earth (5 kg). The mycelial cake was extracted with acetone (50 liters). The acetone extract diluted with water (200 liters), following the filtrate (135 liters), was adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Industries Ltd.) (10 liters). After washing with 50% aqueous acetone (30 liters), elution was carried out with 75% aqueous acetone. The eluate (30 liters) was evaporated *in vacuo* to 2 liters. The aqueous solution was extracted with ethyl acetate

(2 liters) twice and concentrated *in vacuo* to give an oily residue. This oily residue was mixed with twice its weight of acidic silica gel (special silica gel grade 12, maker Fuji Devison Co., Ltd.), and this dry mixture was applied to a column of the same acidic silica gel (1,000 ml) packed with *n*-hexane. The column was developed with *n*-hexane (3 liters), a mixture of *n*-hexane and ethyl acetate (4:1, 3 liters) and ethyl acetate (3 liters). The fractions containing the object compounds (*n*-hexane and *n*-hexane-ethyl acetate, 4:1) were collected and concentrated *in vacuo*. The oily residue was dissolved in a mixture of *n*-hexane and ethyl acetate (1:1, 50 ml) and subjected to column chromatography of silica gel (70~230 mesh, Merck Co., Ltd.) (1,000 ml) containing the same two solvents. The column was washed with the solvents (3 liters), and then eluted with a mixture of *n*-hexane and ethyl acetate (1:2, 3 liters) and ethyl acetate (3 liters). The concentration of active fractions gave a crude yellowish powder (4.5 g) in which FR-900520 and FR-900523 were contained in the ratio of four to one. The powder was dissolved in methanol (20 ml) and mixed with water (10 ml). This mixture was chromatographed on YMC gel (ODS, 60~200 mesh made by Yamamura Chemical Institute) (500 ml) packed and developed with 80% aqueous methanol. Fractions were monitored by HPLC analysis (Fig. 4). FR-900523 and FR-900520 were eluted at elution volumes from 950 ml to 1,350 ml (Fraction I) and 1,670 ml to 2,750 ml (Fraction II), respectively.

Fraction II was concentrated *in vacuo* to remove methanol. After extraction with ethyl acetate, the product was completely dried and the resulting pale yellowish powder (1.8 g) was dissolved in a small amount of diethyl ether. After standing overnight, plates were obtained. Recrystallization from the same solvent gave 600 mg of purified FR-900520 in the form of colorless plates.

The ethyl acetate extract of Fraction I was concentrated *in vacuo* to give a pale yellowish powder (510 mg). This crude FR-900523 contaminated with FR-900520 was dissolved in acetonitrile (3 ml) and applied to YMC gel (70 ml), which was packed and developed with a mixture of acetonitrile, tetrahydrofuran and 50 mM phosphate buffer (pH 2.0) (3:2:5). Active fractions were extracted with ethyl acetate and evaporated *in vacuo* to dryness (190 mg). The rechromatography on YMC gel gave FR-900523 (80 mg) separated from FR-900520. This white product, dissolved in a small amount of diethyl ether, was kept at room temperature overnight, whereupon needles (56 mg) were obtained. 34 mg of FR-900523 were recrystallized from the same solvent as colorless needles.

Fig. 2. Time course of FR-900520 and FR-900523 production.

Potency ($\mu\text{g/ml}$) is estimated as FR-900520.
PMV: Packed mycelium volume.

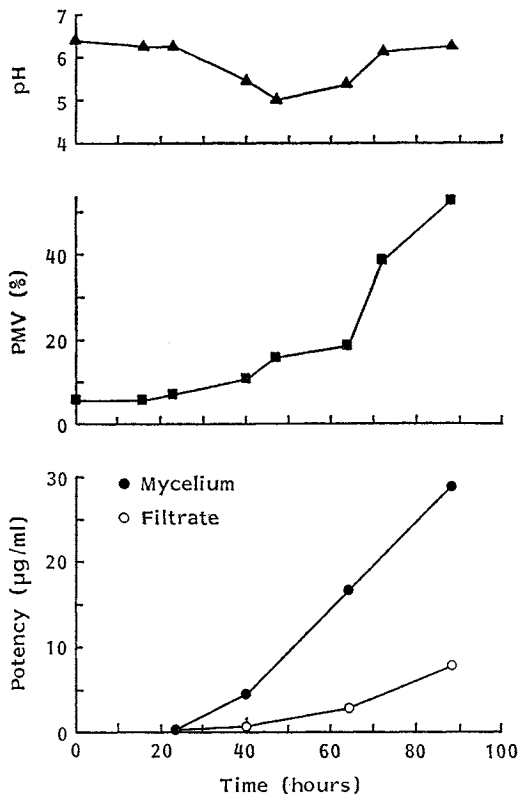
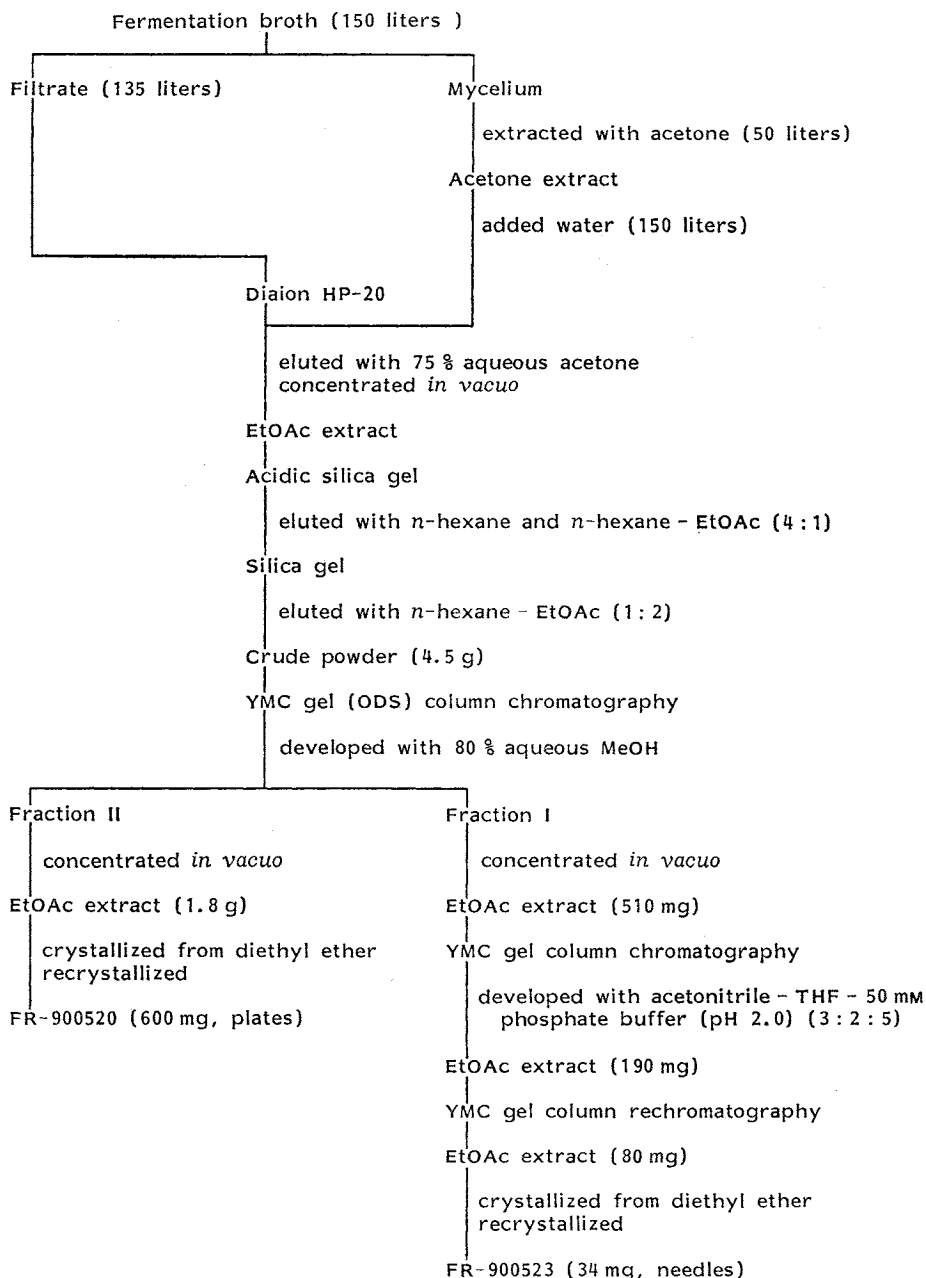


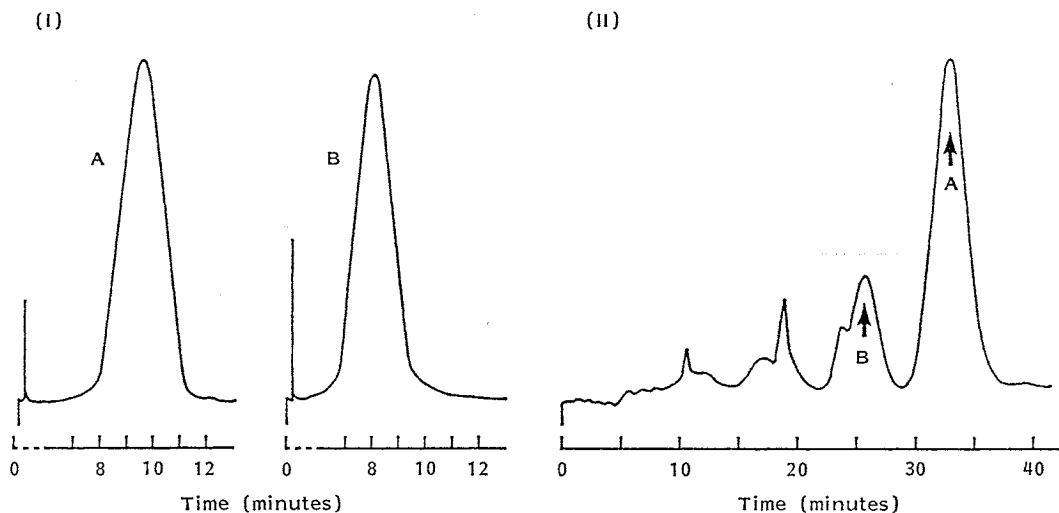
Fig. 3. Isolation and purification of FR-900520 and FR-900523.



Physico-chemical Properties

The physico-chemical properties of FR-900520 and FR-900523 are summarized in Table 1. ^1H and ^{13}C NMR spectra of FR-900520 and FR-900523 are shown in Figs. 5~8, respectively. Their R_f values on silica gel TLC developed with various solvent systems were the same, *e.g.* 0.51 in ethyl acetate. Retention times (minutes) for FR-900520 and FR-900523 using reversed-phase HPLC (Fig. 4) were approximately 9.7 and 8.1 in condition I, and 33.3 and 25.7 in condition II, respectively.

Fig. 4. HPLC chromatograms of FR-900520 and FR-900523.



Condition I: Column; YMC A-302 (ODS, $6\phi \times 150$ mm), mobile phase; MeOH - CH₃CN - 0.5% aq TFA (4:4:2), flow rate; 1 ml/minute, detection; UV at 210 nm, sample A; FR-900520 (plates), sample B; FR-900523 (needles).

Condition II: Column; YMC A-303 (ODS, $4.6\phi \times 250$ mm), mobile phase; CH₃CN - THF - 50 mM phosphate buffer (pH 2.0) (3:2:5), flow rate; 1 ml/minute, detection; UV at 210 nm, sample; crude powder.

Table 1. Physico-chemical properties of FR-900520 and FR-900523.

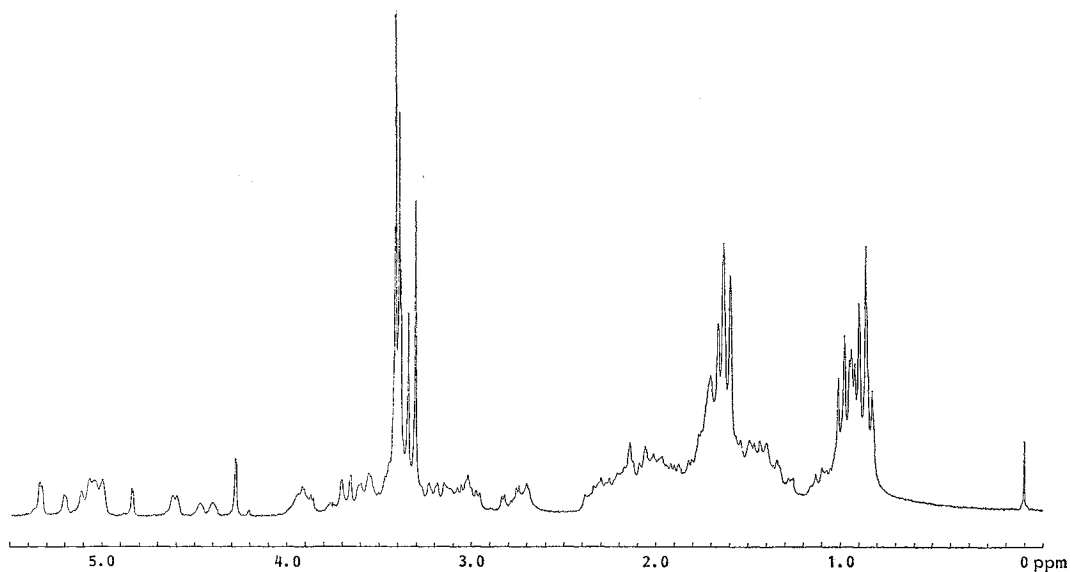
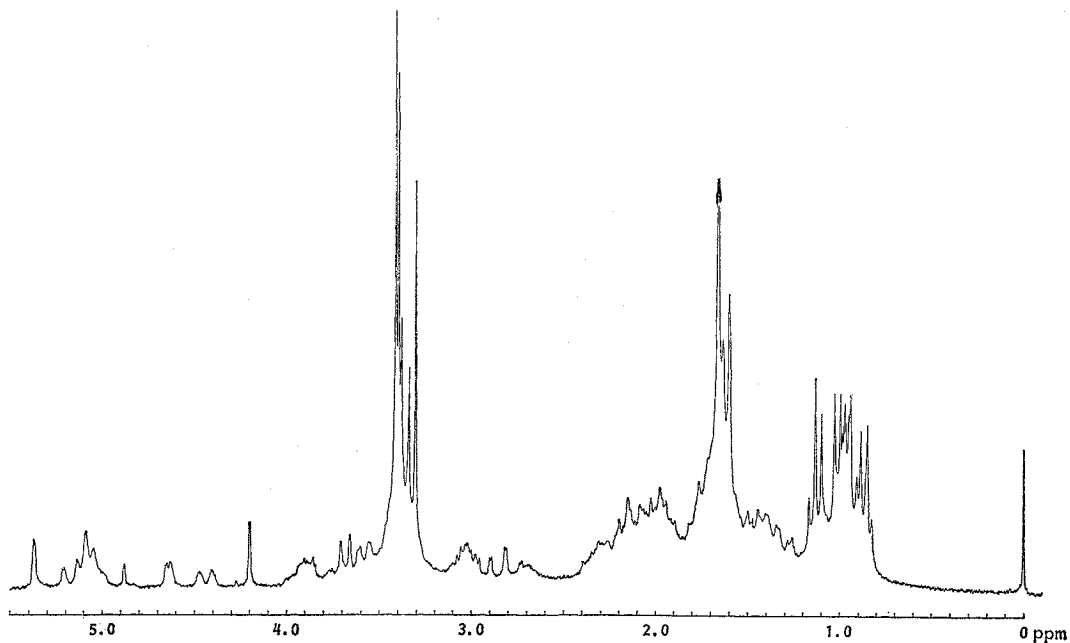
	FR-900520	FR-900523
Appearance	Colorless plates	Colorless needles
MP (°C)	163~165	152~154
Molecular formula	C ₄₃ H ₆₉ NO ₁₂	C ₄₂ H ₆₇ NO ₁₂
SI-MS (<i>m/z</i>)	792 (M+1)	778 (M+1)
Optical rotation	$[\alpha]_D^{25} -84.1^\circ$ (<i>c</i> 1.0, CHCl ₃)	$[\alpha]_D^{25} -73.0^\circ$ (<i>c</i> 0.65, CHCl ₃)
Elemental analysis (%)	Calcd for C ₄₃ H ₆₉ NO ₁₂ : C 65.21, H 8.78, N 1.77. Found: C 64.81, H 8.82, N 1.55.	Calcd for C ₄₂ H ₆₇ NO ₁₂ : C 64.84, H 8.68, N 1.80. Found: C 64.57, H 8.84, N 1.81.
UV spectrum	End absorption	End absorption
IR $\nu_{\max}^{\text{CHCl}_3}$ cm ⁻¹	3575, 3520, 1745, 1725, 1700, 1647, 1090	3580, 3510, 1745, 1722, 1700, 1647, 1090
Color reaction		
Positive:	Cerium sulfate, sulfuric acid, Ehrlich, Dragendorff	
Negative:	Ferric chloride, ninhydrin, Molisch	
Solubility		
Soluble:	MeOH, EtOH, Me ₂ CO, EtOAc, CHCl ₃ , diethyl ether, benzene	
Sparingly soluble:	<i>n</i> -Hexane, petroleum ether	
Insoluble:	Water	

SI-MS: Secondary ion mass spectra.

Biological Properties

Suppression of MLR

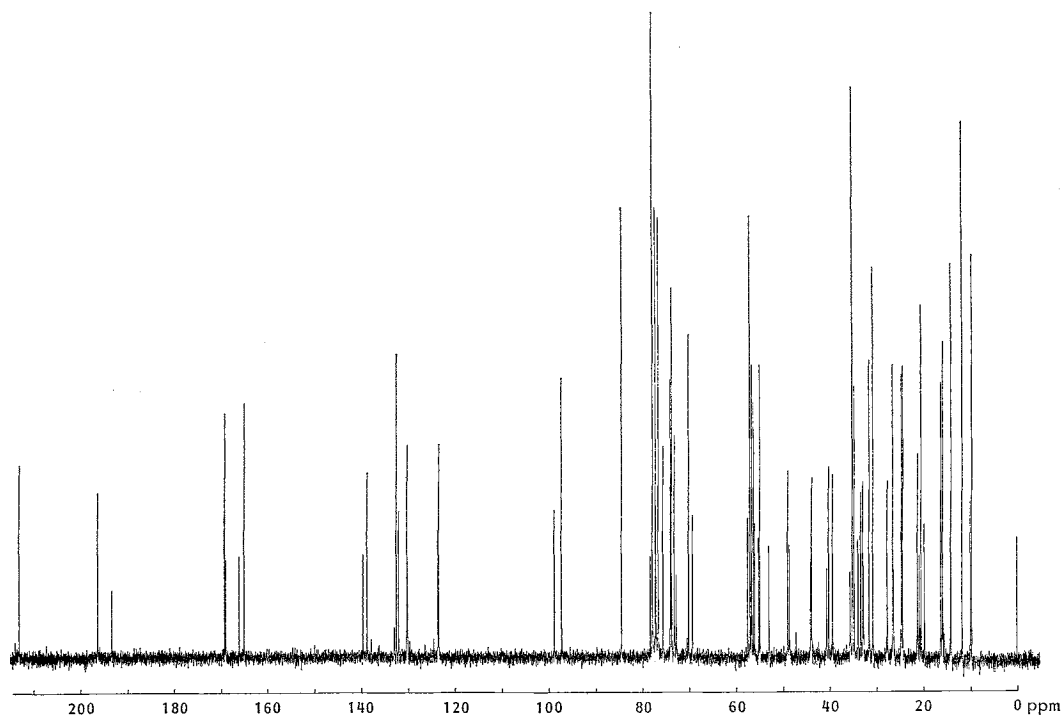
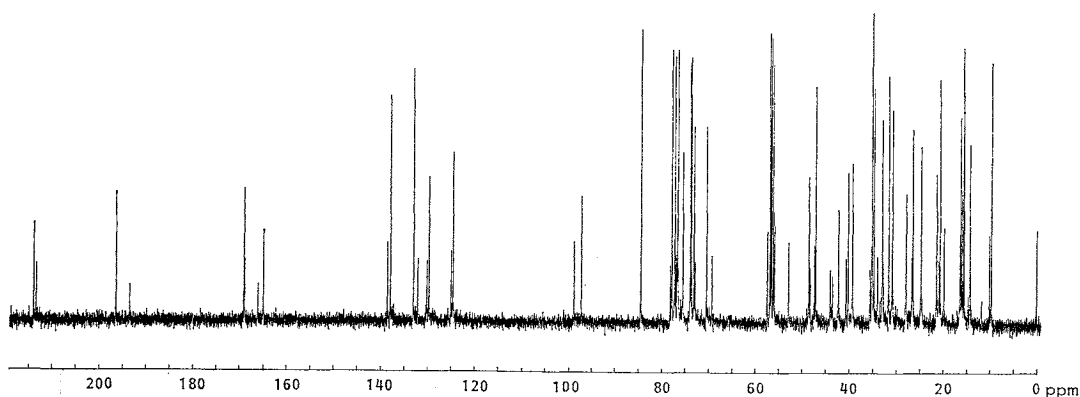
A typical result for suppressive effect of FR-900520 and FR-900523 on mouse MLR is shown in Fig. 9. Both compounds suppressed lymphocyte reaction in a dose dependent fashion. In four

Fig. 5. ^1H NMR spectrum of FR-900520 (CDCl_3 , 200 MHz).Fig. 6. ^1H NMR spectrum of FR-900523 (CDCl_3 , 200 MHz).

experiments, the IC_{50} values were 0.55 μM and 1.6 μM , respectively. FR-900520 had a inhibitory activity at a lower concentration than FR-900523. In addition, the IC_{50} value of FK-506 under the conditions used was 0.23 μM .

Suppression of *In Vitro* EL-4 Growth

A representative inhibition curve for EL-4 growth is presented in Fig. 10. FR-900520 and FR-

Fig. 7. ^{13}C NMR spectrum of FR-900520 (CDCl_3 , 50 MHz).Fig. 8. ^{13}C NMR spectrum of FR-900523 (CDCl_3 , 50 MHz).

900523 were non-toxic at concentrations less than 3,200 nm, at which the percent inhibition was 18.9% and 3.5%, respectively.

Antimicrobial Activities

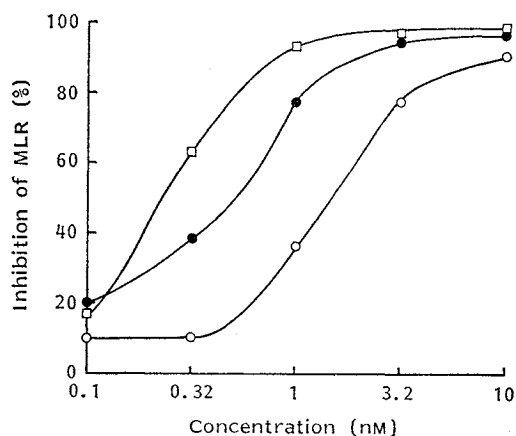
FR-900520 and FR-900523 showed antifungal activity against *Aspergillus fumigatus* IFO 5840 as described in Table 2. The MIC values were 0.03 $\mu\text{g}/\text{ml}$ and 0.3 $\mu\text{g}/\text{ml}$, respectively. They had no inhibitory effect on bacteria or yeast at 100 $\mu\text{g}/\text{ml}$.

Effect of FR-900520 on Skin Allograft Survival in Rats

As shown in Table 3, FR-900520 was dose-dependently effective and clearly prolonged skin allo-

Fig. 9. Effect of FR-900520, FR-900523 and FK-506 on mouse MLR.

FR-900520 (●), FR-900523 (○) and FK-506 (□) were added directly throughout the assay.

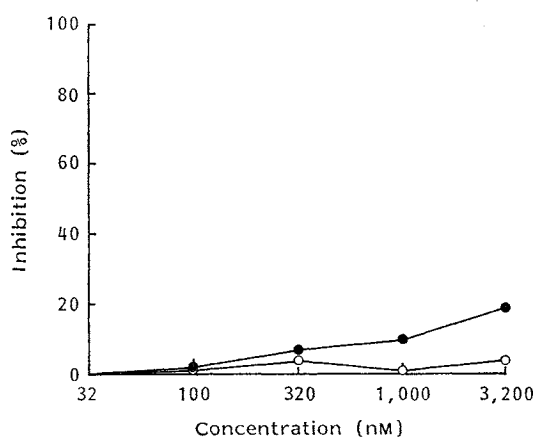


The data are presented as the percentage of inhibition based on response in the control diluent. Mean cpm of [³H]thymidine ([³H]TdR) uptake for mouse MLR was 35,533 ± 1,749.

Unstimulated lymphocyte average 2,396 cpm of [³H]TdR.

Fig. 10. Effect of FR-900520 and FR-900523 on EL-4 growth.

FR-900520 (●) and FR-900523 (○) were added directly throughout the assay.



The data are presented as the percentage of inhibition based on response in the control diluent. Mean cpm of [³H]thymidine uptake for EL-4 was 114,709 ± 7,208.

Table 2. Antimicrobial activity of FR-900520 and FR-900523.

Organisms	MIC (μg/ml)	
	FR-900520	FR-900523
<i>Staphylococcus aureus</i>	> 100	> 100
<i>Bacillus subtilis</i>	> 100	> 100
<i>Proteus vulgaris</i>	> 100	> 100
<i>Pseudomonas aeruginosa</i>	> 100	> 100
<i>Escherichia coli</i>	> 100	> 100
<i>Candida albicans</i>	> 100	> 100
<i>Aspergillus fumigatus</i> IFO 5840	0.03	0.3
<i>Mucor hiemalis</i> FZ-035	> 100	> 100
<i>Phialophora verrucosa</i> FD-146	> 100	> 100
<i>Sporotrichum schenckii</i> FD-158	> 100	> 100

Table 3. Effect of FR-900520 on skin allograft survival in rats^a.

Agent	Dose (mg/kg)	No. of rats	Skin allograft survival day	MST (range)
Control (olive oil)	—	8	6, 6, 6, 6, 6, 6, 7, 7	6.0 (6~7)
FR-900520	0.32	8	6, 7, 7, 7, 7, 11, 11, 11	7.0 (6~11)
	1.0	8	6, 7, 11, 11, 11, 13, 13, 39	11.0 (6~39)
	3.2	8	11, 11, 17, 18, 18, 21, 39, 43	18.0 (11~43) ^b
	10	8	11, 84, 89, 103, 105, 125, 125, 126	104 (11~126) ^b
	32	8	137, 137, 139, 140, 174, 218, 218, 223	157 (137~223) ^b

^a Recipient WKA rats transplanted with F344 skin allografts were given im FR-900520 5 days a week for 2 weeks.

^b $P < 0.01$ calculated by Mann-Whitney U test. MST: Median survival time (days).

graft survival at 3.2 mg/kg or more, though all skin allografts were rejected within 7 days in rats treated intramuscularly with olive oil.

Acute Toxicity in Mice

FR-900520 and FR-900523, dissolved in olive oil, showed no adverse effect when administered intraperitoneally to *ddY* mice (male, 8 weeks old) at 100 mg/kg.

Discussion

CsA, a fungal metabolite, is a potent immunosuppressant with low myelotoxicity. It is widely used clinically in organ transplantation. Furthermore, CsA has a potential as a useful drug for autoimmune diseases due to its specific mode of action. However, its clinical use is limited by the occurrence of CsA-induced nephrotoxicity.

We have searched for safer, more efficient immunosuppressants and isolated two novel compounds from the fermentation broth of *Streptomyces hygroscopicus* subsp. *yakushimaensis* No. 7238. The major and minor components were designated as FR-900520 and FR-900523, respectively. Reversed-phase chromatography led to their separation. They displayed remarkably broad chromatograms in reversed-phase HPLC (Fig. 4). Their chromatograms and Rf values on silica gel TLC suggested that FR-900520 and FR-900523 were very similar to FK-506 produced by *Streptomyces tsukubaensis* No. 9993. On the basis of spectroscopic analyses, the structures of FR-900520 and FR-900523 were found to be new classes of 23-membered macrolide lactones related to FK-506. As shown in Fig. 1, R in FR-900520 and FR-900523 are ethyl and methyl groups, respectively, instead of the allyl group in FK-506. Further structural information will be reported separately. FR-900520 and FR-900523 show a suppressive effect on mouse MLR, which has been thought to be a representative reaction of IL-2 dependent T cell growth, at low concentration. In multiple experiments, the IC_{50} values were 0.55 nM and 1.6 nM, respectively. FR-900520 suppressed at about three times lower concentration than FR-900523. Their toxic concentrations against T cell proliferation such as EL-4 lymphoma were more than 3,200 nM. Unpublished observations have revealed that they also inhibit IL-2 production. These results indicate that the two compounds are more specific and lower-toxic immunosuppressants with the same type of action as FK-506. Fig. 9 shows that FR-900520 and FR-900523 have rather lower potency than FK-506. Of these compounds FR-900523, in which R is a methyl group, has the lowest potency, but much higher than that of CsA. In our previous paper¹⁾, we reported that the IC_{50} value of CsA was 27 nM. In addition, FR075451¹⁵⁾, which can be prepared by catalytic reduction of the allyl group in FK-506 to a propyl group, suppressed mouse MLR in a similar or lower concentration range than that of FR-900520 under the same experimental conditions. Furthermore, the same result was observed in rat MLR (data not shown). These results suggest that the carbon chains in R, especially the double bond (Fig. 1) may affect the immunosuppressive activity. Table 3 shows that 10 and 32 mg/kg of FR-900520 prolong skin allograft survival in rats to 104 and 157 days, respectively. These data indicate that grafts continue to survive after the termination of treatment. INAMURA *et al.*⁹⁾ reported that the effective dose of FK-506 on skin allograft survival was 0.32 mg/kg or more, and 3.2 mg/kg prolonged survival up to 43 days. Comparison of these results indicates that though FR-900520 has a lower immunosuppressive potency than FK-506, FR-900520 may achieve a longer survival time when administered intramuscularly at the highest dose. Thus, FR-900520 and FR-900523 have effective immunosuppressive properties *in vitro* and *in vivo*. This leads us to an expectation that they will prove useful as immunosuppressants in clinical organ transplantation as well as FK-506.

Acknowledgment

The authors thank members of the Nagoya Pilot, Fujisawa Pharmaceutical Co., Ltd., for large scale fermentation.

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