A NEW IMMUNOMODULATOR, FR-901235

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FR-901235 is a new type of immunoactive substance produced by an imperfect fungus, *Paecilomyces carneus* F-4882.

The mitogen-induced lymphocyte proliferation which had been suppressed by addition of immunosuppressive factor, was restored to a normal level by the addition of FR-901235. Furthermore, the subsequent administration of FR-901235 partially restored the impaired delayed-type hypersensitivity to sheep red blood cells in tumor-bearing mice.

In the course of searching for new types of immunoactive substances from microorganisms[1~4], FR-901235 was discovered in the fermentation broth of an imperfect fungus, *Paecilomyces carneus* F-4882 which was isolated from a soil sample.

FR-901235 has the capacity to restore the depression of mitogenic responses of mouse spleen cells by the immunosuppressive factor[1] in the serum of tumor-bearing mice.

In this paper we describe the taxonomy of the producing strain, the production, isolation, physicochemical properties and biological activities of FR-901235.

Materials and Methods

Culture and Medium Conditions

The microorganism used in this study, *P. carneus* F-4882 was obtained from soil.

The seed medium contained soluble starch 2%, glucose 2%, Polypeptone 1%, dried yeast 1%, corn steep liquor 2% and $CaCO_3$ 0.2%, pH 5.0. A loopful of slant culture of *P. carneus* F-4882 was inoculated to each of thirty five 250-ml Erlenmeyer flasks containing 80 ml of the seed medium and cultured at 25°C for 72 hours on a rotary shaker with 7.6 cm-throw at 200 rpm. The resultant culture was inoculated into the same seed medium (150 liters) in a 200-liter jar fermenter, and cultured at 25°C for 96 hours under aeration of 150 liters/minute and agitation of 300 rpm.

Animals

Female mice ICR/JCL, *dd*Y and Balb/c were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animal (Hamamatsu, Japan).

Preparation of Immunosuppressive Factor from Tumor-bearing Mouse Serum

Sarcoma 180 cells maintained *in vivo* in ICR/JCL mouse (8 weeks of age) in ascites form, were used. Immunosuppressive factor was partially purified from the serum of sarcoma 180 tumor-bearing mice by the method of OH and MOOLTEN[5].

Assay of Mitogen Induced Mouse Spleen Cell Proliferation

Spleens were removed under sterile conditions from Balb/c mice (8 weeks of age). Mitogen induced spleen cell proliferation was assayed according to the method described previously with a slight modification[1]. Briefly, the cells were suspended in RPMI-1640 medium to contain 5×10^5 cells/ml. In each well of a microtitration plate (Falcon No. 3040) was poured 0.1 ml of the above cell suspension and 0.05 ml of the prescribed concentrate of the compound and this was incubated for

1 hour. Then the immunosuppressive factor and concanavalin A (Con A) (1 μg/ml) were added to the culture (final volume, 0.2 ml). The culture was incubated in triplicate at 37°C in a humidified atomosphere (95% air, 5% CO_2) for 48 hours.

Con A-induced mouse spleen cell proliferation was assayed by [^{3}H]thymidine incorporation. Twenty μl of 10 μCi/ml of [^{3}H]thymidine was added to each well of the culture. After a further 24-hour incubation, the radioactivity incorporated into DNA was measured.

Delayed-type Hypersensitivity (DTH) to Sheep Red Blood Cells (SRBC)

For active immunization *dd*Y mice (10 weeks of age) were sensitized by injection of 10^8 SRBC in 0.05 ml of saline in the left footpad and 4 days later challenged with 10^8 SRBC in the right footpad. Twenty-four hours thereafter, the resulting footpad swelling was measured with a caliper. The DTH response was expressed by the difference in the thickness of the left footpad from that of the right footpad.

Antimicrobial Activity

The antimicrobial activity of FR-901235 was determined by a serial broth dilution method in bouillon medium for bacteria and 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.

Results

Identification of Producing Strain

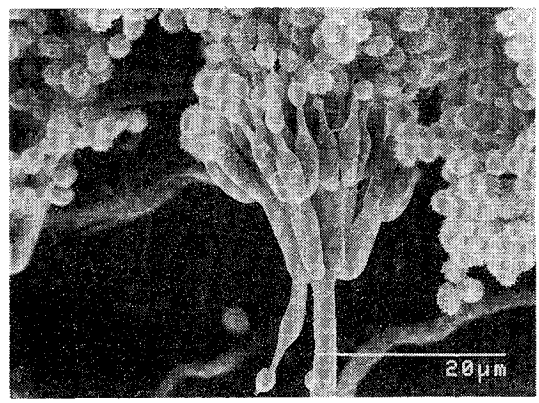
The producing strain F-4882 was freshly isolated from a soil sample, collected at Nagasaki-city, Nagasaki Prefecture, Japan. Its mycological characteristics are as follows.

On potato - glucose agar, this strain grew restrictedly to form pale pink and powdery colonies, attaining 1.5 cm in diameter within 2 weeks at 25°C. The colony surface was plain or somewhat raised, powdery or felty, and pale pink to pale yellowish orange. The reverse was dark olivaceous green or yellow brown to dark yellowish orange. Conidial structures were abundantly produced. They consisted of penicillate conidiophores and conidial dry chains (Fig. 1). The conidiogenesis was phialidic.

The conidiophores were mononematous, erect, straight, hyaline, smooth, 60~150 μm long and 2~3 μm thick. They consisted of monoverticillate or biverticillate branches, with whorls of 2 to 8 phialides. The phialides were hyaline, smooth, lageniform or obclavate, 10~20×2~3 μm, with a tapering neck measuring 4~7×1~2 μm. The conidia, produced in basipetal chains, were hyaline, spiny, one-celled, ellipsoidal to subglobose, and 3~4(~4.5)×2~2.5(~3.5) μm.

In consequence of comparing the above characteristics with species descriptions[6,7], we identified the producing strain as *P. carneus* (Duché et Heim) Brown et Smith 1957. The strain was named to *P. carneus* F-4882 and deposited in the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as No. FERM P-10566.

Fig. 1. Scanning electron micrograph of anamorph of *Paecilomyces carneus* F-4882.



Isolation of FR-901235

The fermentation broth (150 liters) was filtered with the aid of diatomaceous earth (20 kg). The mycelial cake was extracted with 100 liters of ethyl acetate. The ethyl acetate layer was separated and concentrated *in vacuo* to a volume of 20 ml. The concentrated fraction was applied to a silica gel chromatographic column (1.5 liters). After developing with 5 liters of chloroform, the column was eluted with a mixture of chloroform - methanol (50 : 1, 3.5 liters). The active fractions were concentrated to dryness under reduced pressure. Pale yellow crystals (250 mg) were obtained from methanol[8].

Physico-chemical Properties of FR-901235

FR-901235 is an acidic substance and soluble in dimethyl sulfoxide, sparingly soluble in chloroform and methanol, and insoluble in water.

It is positive to ferric chloride and iodine vapor color reactions, though negative to ninhydrin and Molisch color reactions.

The IR, ^{1}H and ^{13}C NMR spectra of FR-901235 are shown in Figs. 2, 3 and 4, respectively. The other physico-chemical properties of FR-901235 are summarized in Table 1. The structure of FR-901235 was determined as 2-acetonyl-2,4,9-trihydroxy-6-methoxy-7-methyl-1*H*-phenalene-1,3(2*H*)-dione (Fig. 5), on the basis of chemical and spectral evidence, and the details will be reported elsewhere.

Table 1. Physico-chemical properties of FR-901235.

Appearance	Pale yellow prisms
Molecular formula	$C_{18}H_{16}O_7$
FAB-MS (*m/z*)	345 (M^++1)
Elementary analysis	
Calcd for $C_{18}H_{16}O_7$:	C 62.79, H 4.68.
Found:	C 62.44, H 5.12.
MP	168~170°C
$[\alpha]_D^{23}$	−0.6° (*c* 0.2, $CHCl_3$)
UV λ_{max}^{MeOH} nm(ε)	215(37,500), 253(36,100), 330(20,300)
$\lambda_{max}^{MeOH-HCl}$ nm	215, 253, 330
$\lambda_{max}^{MeOH-NaOH}$ nm	207, 230, 260, 347
IR ν_{max}^{KBr} cm^{-1}	3400, 3100, 2920, 1695, 1595, 1460, 1385, 1350, 1330, 1210, 1170, 1050, 980, 930, 900
TLC (Silica gel plate)	
Rf[a]	0.4
Rf[b]	0.2

FAB-MS: Fast atom bombardment MS.
[a] Solvent system: $CHCl_3$ - MeOH (30 : 1).
[b] Solvent system: Benzene - EtOAc (1 : 1).

Fig. 2. IR spectrum of FR-901235 (KBr disk).

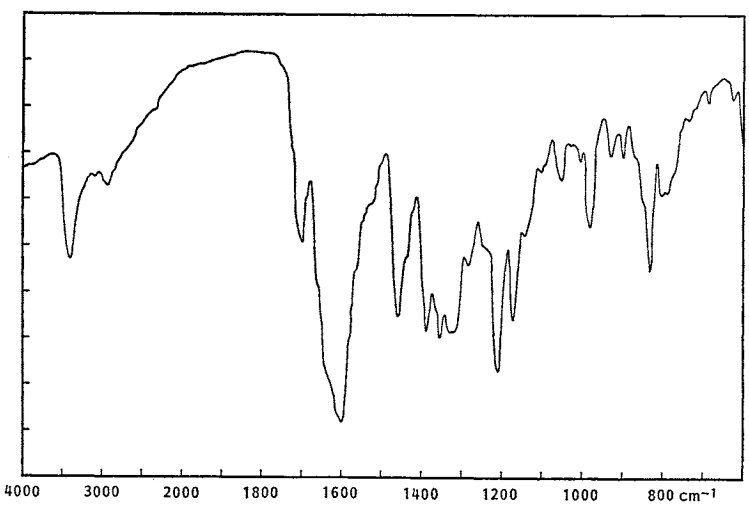


Fig. 3. 400 MHz ^{1}H NMR spectrum of FR-901235 in $CDCl_3$ - CD_3OD (10 : 1).

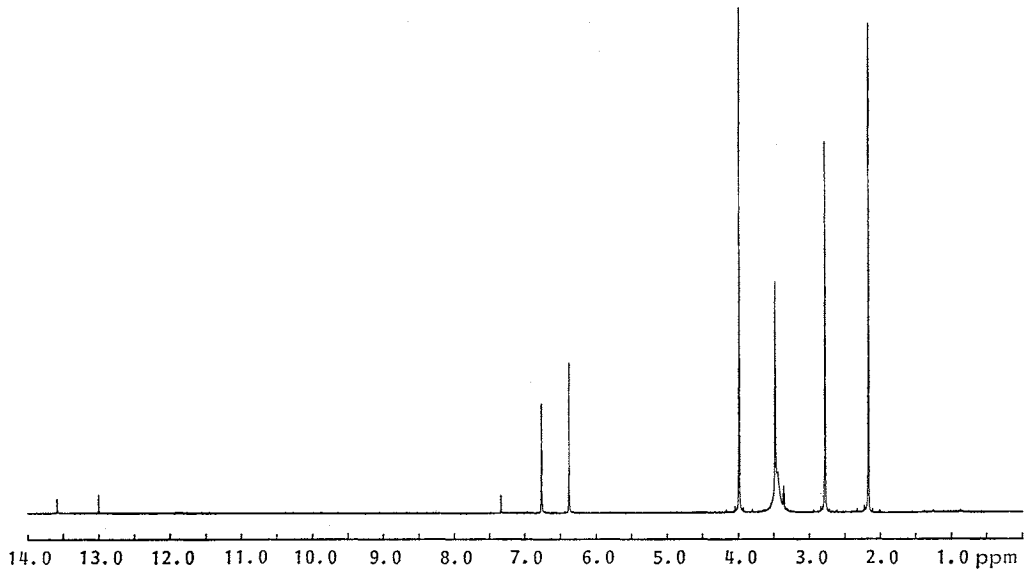
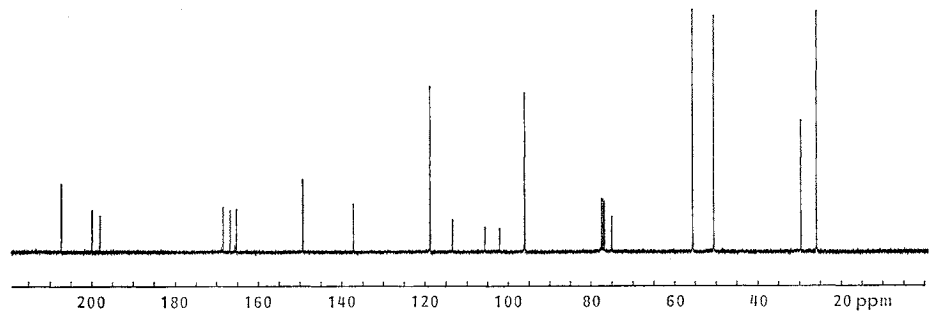


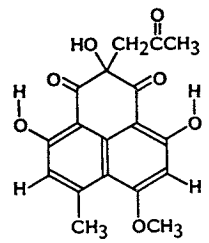
Fig. 4. 100 MHz ^{13}C NMR spectrum of FR-901235 in $CDCl_3$ - CD_3OD (10 : 1).



The Suppressive Effect of Tumor-bearing Mouse Serum on Mouse Spleen Cell Proliferation and Its Restoration by FR-901235

Immunosuppressive factor from tumor-bearing mice serum suppressed the Con A-induced stimulation of [^{3}H]thymidine incorporation by the mouse spleen cells.

Fig. 5. Chemical structure of FR-901235.



The addition of FR-901235 to the culture containing spleen cells partially prevented the suppression (Table 2).

Effect of FR-901235 on DTH Response to SRBC in Tumor-bearing Mice

As shown in Table 3, compared to normal mice, DTH response was markedly suppressed in sarcoma 180 tumor-bearing mice.

Table 2. Suppression of Con A-induced mouse spleen cell proliferation by immunosuppressive factor and its partial restoration by FR-901235.

Treatment of spleen cells		[^{3}H]Thymidine uptake (cpm)[a]
Non-treated control		1,590±411
Con A (3 μg/well)		107,863±4,814
(1 μg/well)		46,529±4,438
Immunosuppressive factor		
(8 μl/well)		267±24
(6 μl/well)		628±29
FR-901235 (μg/well)	300	287±56
	100	766±88
	30	1,425±179
	10	1,374±245
	3	1,195±208
Con A (1 μg/well)+immunosuppressive factor (8 μl/well)		2,158±471
(6 μl/well)		3,862±598
Con A (1 μg/well)		
+FR-901235 (μg/well)	300	9,685±328
	30	35,829±1,528
	3	47,796±1,828
Con A (1 μg/well)+immunosuppressive factor (6 μl/well)		
+FR-901235 (μg/well)	100	12,249±359
	30	24,438±1,571
	10	32,585±4,244
	3	35,724±3,317
	1	26,372±1,452

[a] Mean±SE (n=4).

Table 3. Effect of FR-901235 on DTH response to SRBC in tumor-bearing mice[a].

Treatment		Increase of foot pad thickness (0.1 mm) mean±SE
Control (0.5% methyl cellulose solution)		7.6±0.5
Tumor-bearing mouse		1.3±0.4
Tumor-bearing mouse		
+FR-901235 (mg/kg)	100	2.9±0.1[b]
	10	3.5±0.1[b]
	1	3.2±0.4[b]
FR-901235 (mg/kg)	100	7.4±0.2[b]
	10	7.7±0.1[b]
	1	6.8±0.4[b]

[a] 2×10^6 cells of sarcoma 180 ascites tumor cells were inoculated on day-0. Four days thereafter, mice were immunized by subcutaneous injection of SRBC to right foot pad. FR-901235 was given subcutaneously to mice once a day (days 2~6). Seven mice were used for each group.

[b] Significantly different from tumor-bearing control at $P<0.01$ (Student's t-test).

The administration of FR-901235 reversed this impaired DTH to SRBC in the tumor-bearing mice.

Antimicrobial Activity

FR-901235 was devoid of antimicrobial activity when tested versus the following microorganisms at 100 μg/ml: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Staphylococcus aureus*, *Candida albicans*, *Aureobasidium pullulans* and *Aspergillus niger*.

Discussion

The present work shows that FR-901235 has the capacity to restore the depression of mitogenic response of mouse spleen cells caused by immunosuppressive factor. Furthermore, the administration of FR-901235 reversed the suppressed DTH response in tumor-bearing mice. It has been reported that in Ehrlich ascites tumor-bearing mice the administration of bestatin restored their impaired DTH to SRBC[9]. OHTA *et al.*, also reported that thymosin accelerated the natural recovery of DTH response suppressed by injection of 5-fluorouracil in mice[10]. These facts indicate that FR-901235 restores the suppressive functions of cell mediated immune responses.

In addition, we have found that FR-901235 exhibited potent antioxidative activity (unpublished data). Further studies on biological activities of FR-901235 are now in progress.

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