A NEW IMMUNOMODULATOR, FR-900483

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FR-900483 is a new immunoactive substance produced by a fungus, *Nectria lucida* F-4490. Concanavalin A-stimulated lymphocyte proliferation, which had been suppressed by addition of immunosuppressive factor, was restored to a normal level by the addition of FR-900483. Furthermore, FR-900483 restored the capacity of immunosuppressed mice to produce antibody against sheep red blood cells.

In our screening program for immunoactive substances from microorganisms, we found that a fungus, *Nectria lucida* F-4490, produced a novel compound FR-900483 which enhanced the activity of the mouse immune system *in vitro*.

This paper describes the taxonomy of the producing strain, isolation, physico-chemical properties and biological activities of FR-900483.

Taxonomy of the Producing Strain

The strain F-4490 was originally isolated from a soil sample collected at Kaizuka City, Osaka Prefecture, Japan. Its mycological characteristics were as follows.

Colonies on malt extract agar spread broadly, attaining 5.0 cm in diameter after 2 weeks at 25°C. The colony surface was raised, floccose and dull reddish purple. The conidial structures were abunduntly formed. The reverse was reddish brown. Cultures on corn meal agar reached 6.0 cm in diameter under the same conditions. They were plane, thin and pale yellow brown. The reverse was the same. The conidia were poor. The perithecia were formed on this medium after 1 month at 25°C.

The ascomata (perithecia) were superficial, globose to subglobose, papillate, without terminal hairs, brown to dark brown, and 320~500 μm in diameter. The peridium was composed of thick-walled cells. The papillae, possessing an ostiole, were 120~150 μm in diameter and 60~90 μm high. The asci were unitunicate, cylindrical to clavate, eight-spored, 80~90 μm long and 7~9 μm thick. The ascospores were obliquely uniseriate, hyaline, smooth, two-celled, ellipsoidal, rounded at the ends and constricted at the septum, 10~13×5.5~7 μm in size.

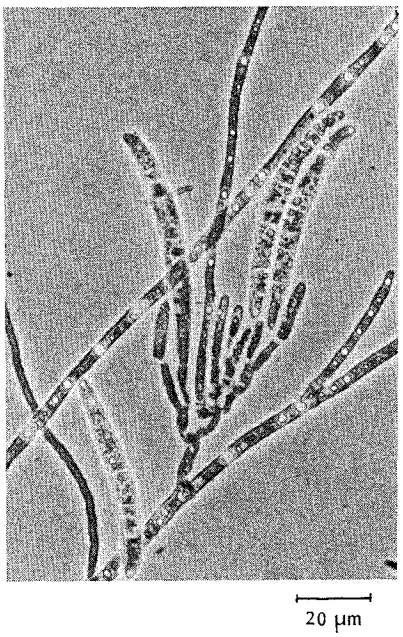
The conidiophores were hyaline, smooth, mononematous, 7~20 μm long and 3~4.5 μm thick (Fig. 1). They were simple or branched a few times, and each of the apical laterals became phialides. The phialides were hyaline, smooth, cylindrical, with marked terminal collar, 12~17 μm long and 3~4 μm thick. They produced subhyaline conidia, which aggregated in slimy heads at the open ends of phialides. The conidia were smooth, cylindrical or curved narrowing towards the apex, with rounded ends, typically three-septate and 50~60 (70)×5.5~7 μm. The microconidia were absent.

The vegetative hyphae were septate, hyaline, smooth and branched. The hyphal cells were cylindrical and 2~6 μm thick. The chlamydospores developed in the cell of conidium, while not in

the hyphae.

From these morphological characteristics, we identified strain F-4490 as one strain of *Nectria lucida* Höhnel[1,2]. This strain was deposited in the American Type Culture Collection (U.S.A.), as ATCC 20722.

Fig. 1. Microphotograph of anamorph of strain F-4490.



Fermentation

A loopful of mature slant culture of *Nectria lucida* F-4490 was inoculated to a seed medium (80 ml) containing soluble starch 1%, glucose 1%, dried yeast 0.5%, cotton seed meal 0.5% and corn steep liquor 0.5% (pH 6.0) in a 250-ml Erlenmeyer flask and cultured at 25°C for 72 hours on a rotary shaker with 7.5 cm throw at 200 rpm.

Sixteen hundred ml of the seed culture were inoculated to the production medium containing the same composition of seed medium in a 200-liter jar fermentor and cultured at 25°C for 72 hours under aeration of 160 liters/minute and agitation of 300 rpm.

FR-900483 was assayed and purified from the culture broth of *N. lucida* F-4490 by measuring its ability to reverse the immunosuppressive factor inhibition[3] of concanavalin A (Con A)-induced proliferation of mouse spleen cells.

Isolation and Purification

The cultured broth (150 liters) was filtered with an aid of diatomaceous earth (4 kg). To the mycelia containing diatomaceous earth, were added 100 liters of deionized water and stirred for 10 minutes. The suspension of mycelia was disrupted by two cycles of freezing and thawing, and then filtered. The filtrate was charged to the column of Dowex 50W-X2 (H^+ form, 14 liters), washed with deionized water (60 liters) and eluted with 1.5% ammonia water (60 liters). The eluate was concentrated *in vacuo* to a volume of 7.5 liters and applied to CM-Sephadex C-25 column (NH_4^+ form, 30 liters). The column was washed with deionized water (30 liters) and eluted with 1.5% ammonia water (10 liters). The active eluate was concentrated *in vacuo* to a volume of 300 ml and applied to DEAE-Sephadex A-25 column (OH^- form, 2.5 liters). This column was washed with deionized water (3 liters) and eluted with 1.5% ammonia water (3 liters). The active fraction was concentrated *in vacuo* to a volume of 30 ml and lyophilized to give 250 mg of colorless powder of FR-900483.

Physico-chemical Properties

The physico-chemical properties of FR-900483 are summarized in Table 1. The IR, ^1H and ^{13}C NMR spectra are shown in Figs. 2, 3 and 4, respectively.

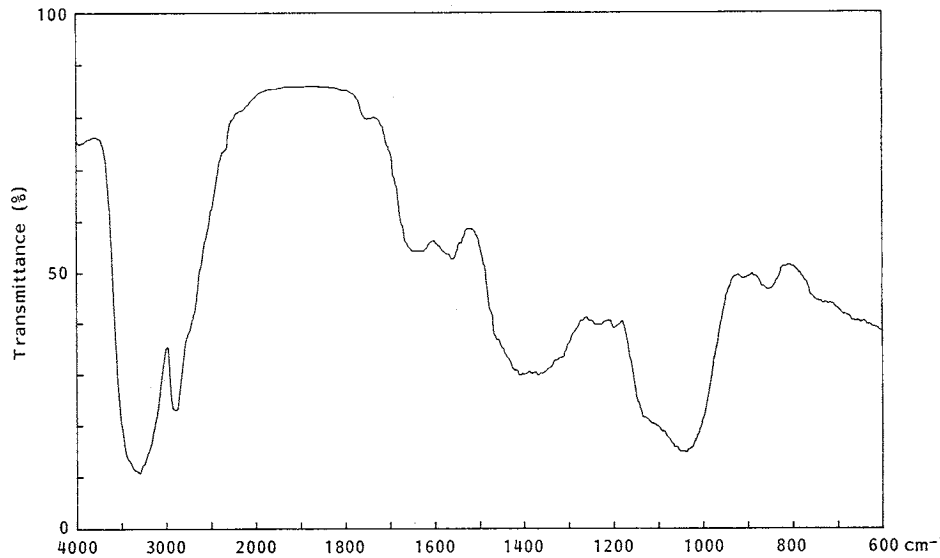
FR-900483 is soluble in water, slightly soluble in methanol and ethanol and substantially insoluble in acetone, chloroform and ethyl acetate. Color reactions are as follows: Positive in ninhy-

Table 1. Physico-chemical properties of FR-900483.

Appearance	A colorless powder
MP (°C, dec)	75
$[\alpha]_D^{23}$	+22° (*c* 0.55, H_2O)
UV $\lambda_{max}^{H_2O}$ nm (ε)	320 (100)
IR ν_{max}^{KBr} cm^{-1}	3300, 2900, 1640, 1560, 1400, 1240, 1200, 1040, 850
Elementary analysis	
Calcd for $C_5H_9NO_3$:	C 45.80, H 6.92, N 10.68.
Found:	C 45.08, H 6.57, N 10.16.
FAB-MS (*m/z*)	132 (M+H)
TLC Rf[a]	0.35
Rf[b]	0.27

Stationary phase: Silica gel sheet (Merck), developing solvent: [a] $CHCl_3$ - MeOH - ammonia water (28%) (5 : 3 : 1), [b] 2-propanol - water (7 : 3).

Fig. 2. IR spectrum of FR-900483 in KBr.



drin, iodine vapor and potassium permanganate tests; negative in sulfuric acid, ferric chloride and Molisch tests.

The structure of FR-900483 was determined as (3*R*,4*R*,5*R*)-3,4-dihydroxy-5-hydroxymethyl-1-pyrroline (**1**), on the basis of its chemical and spectroscopic evidence and finally by a total synthesis starting from D-glucose. The full account of the structure determination will be reported in the succeeding paper.

Biological Activity

Competitive Effect of FR-900483 against Immunosuppressive Factor Obtained from Tumor Bearing Mice Serum

The ability of FR-900483 to reverse the inhibition of proliferation of mouse spleen cells to Con A which can be induced by immunosuppressive factor, was measured by the method described previously[3]. Table 2 shows that the immunosuppressive factor from tumor bearing mouse serum pro-

Fig. 3. ^{1}H NMR spectrum of FR-900483 in D_2O at 270 MHz.

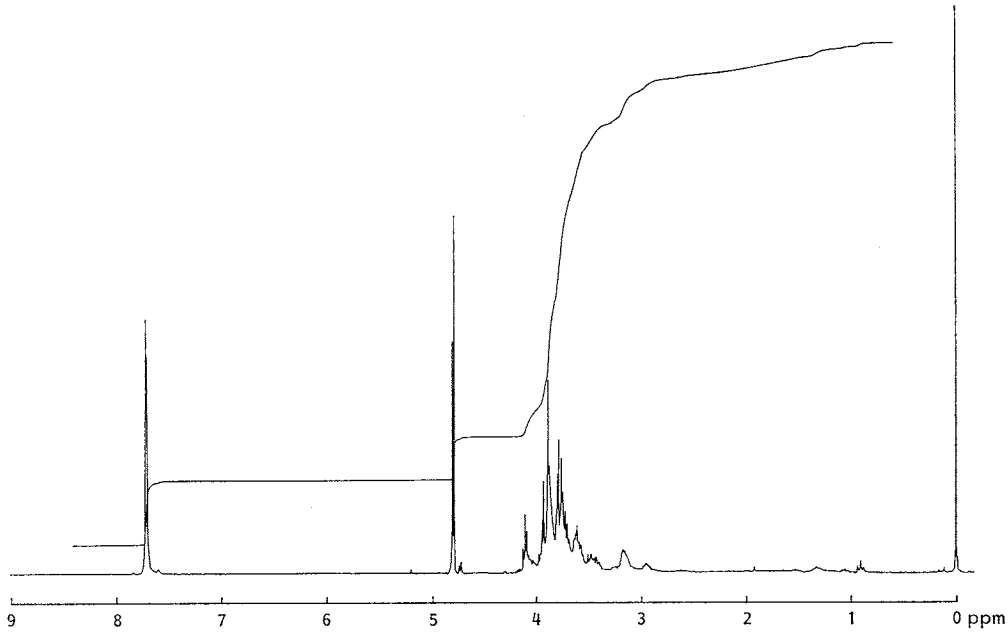
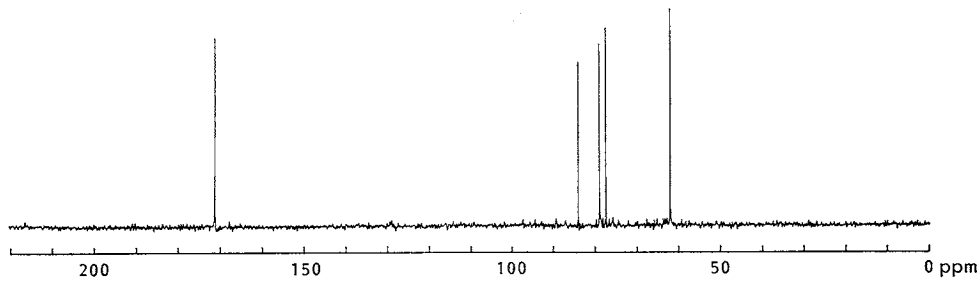
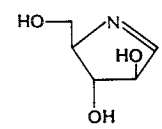


Fig. 4. ^{13}C NMR spectrum of FR-900483 in D_2O at 67.8 MHz.



foundly suppressed the Con A-induced stimulation of [^{3}H]thymidine incorporation by mouse spleen cells. The addition of FR-900483 partially reversed this suppression.



FR-900483 (1)

Effect of FR-900483 on Antibody Formation to SRBC in the Immuno-deficient Mice

Mitomycin C (1 mg/kg, Kyowa) was injected intraperitoneally 4 days before immunization into mice. Four days later they were immunized by intravenous injection with 1×10^{8} sheep red blood cells (SRBC). FR-900483 at doses ranging from 7 mg/kg to 500 mg/kg was administered intraperitoneally for 5 consecutive days starting from the day of the mitomycin C injection, and after 5 days the effect on antibody formation was determined by the hemolytic plaque-forming cells assay[3,4]. As shown in Table 3, the antibody formation to SRBC in mice treated with mitomycin C was markedly suppressed. The administration of FR-900483 reversed the suppressed immune response.

Table 2. Suppression of Con A-induced mouse spleen cell proliferation by immunosuppressive factor and its restoration by FR-900483.

Treatment of spleen cells		[^{3}H]Thymidine uptake (cpm)*
Non-treated control		931±46
Con A (2 μg/well)		103,425±5,285
Immunosuppressive factor (5 μl/well)		307±83
FR-900483 (μg/well)	10	348±86
	1	705±106
	0.1	924±182
Con A (2 μg/well)+immunosuppressive factor (5 μl/well)		5,279±126
Con A (2 μg/well) +FR-900483 (μg/well)	10	75,445±1,979
	3	82,791±2,688
	1	126,457±4,817
	0.3	110,344±4,416
	0.1	108,295±2,941
Con A (2 μg/well)+immunosuppressive factor (5 μl/well) +FR-900483 (μg/well)	3	14,829±1,294
	1	71,624±2,999
	0.3	79,923±2,328
	0.1	84,753±640
	0.03	50,344±441

* Mean±SE (n=4).

Table 3. Effect of FR-900483 on antibody forming capacities in mice treated with mitomycin C[a].

Substance		Anti-SRBC pfc (×10^4 pfc/spleen) Mean±SE
Control (saline)		142±0.55
FR-900483 (500 mg/kg)		150±5.32
Mitomycin C (1.0 mg/kg)		
+Saline		99.6±7.12
+FR-900483 (mg/kg)	7.0	143±8.40[b]
	20.0	145±2.18[b]
	60.0	160±4.22[b]
	170.0	185±7.53[b]
	500.0	208±9.39[b]

[a] BDF_1 mice (female, 8 weeks old) were given ip injection of mitomycin C at 4, 3, 2 and 1 days before immunization (day 0~day 3). FR-900483 was injected intraperitoneally daily for 5 days starting from the day of mitomycin C injection (day 0~day 4).

[b] Significantly different from mitomycin C treated control at $P<0.05$ (Student's t-test), 5 mice per group.

Antimicrobial Activity

Antimicrobial activity of FR-900483 was determined by a serial broth dilution method in bouillon media for Gram-positive and Gram-negative bacteria and Sabouraud media for fungi and yeast. MIC is expressed in terms of μg/ml after overnight at 37°C for bacteria and 48~72 hours incubation at 28°C for fungi and yeast.

FR-900483 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

As reported in this paper, the Con A stimulated lymphocyte proliferation was suppressed by immunosuppressive factor. The addition of FR-900483 restored this to a normal level. Moreover, FR-900483 reversed the depressed immune responses in mitomycin C treated mice. FR-900483 exhibited no effect on the immune responses of normal mice over a wide range of low doses. These facts suggest that the usefulness of FR-900483 in preventing the suppression of immune responses caused by tumor or cancer chemotherapy.

In addition, FR-900483 exhibited potent inhibitory activity against α-glycoside hydrolase isolated from yeast, gastrointestinal tract of rat or porcine small intestine (unpublished data). Further studies on biological activities of FR-900483 are now in progress.

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