A NEW IMMUNOMODULATOR, FR-900490

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FR-900490 is a new type of immunoactive substance produced by a fungus *Discosia* sp. F-11809.

The colony forming units in culture (cfu-c) in bone marrow cells, which were suppressed by immunosuppressive factor obtained from the serum of sarcoma 180 tumor bearing mouse, was restored to normal level by the addition of FR-900490 *in vitro*. Furthermore, in mitomycin C (MMC)-treated mice the subsequent administration of FR-900490 caused a significant increase of cfu-c in bone marrow cells depressed by MMC.

In the course of a screening for new types of immunoactive substances from microorganisms[1], FR-900490 was discovered in the fermentation broth of a fungus, *Discosia* sp. F-11809 which was isolated from a soil sample.

FR-900490 is effective in preventing the damages in bone marrow cells by immunosuppressive factor, obtained from the serum of tumor bearing mouse.

This paper describes the taxonomy of the producing strain, the production, isolation, physicochemical properties and biological activities of FR-900490.

Materials and Methods

Culture and Medium Conditions

The fungus *Discosia* sp. F-11809 obtained from soil, was used in this study.

The seed medium contained soluble starch 1%, corn starch 1%, glucose 1%, cotton seed meal 0.5%, dried yeast 0.5%, corn steep liquor 0.5% and $CaCO_3$ 0.2% (pH 6.0). The production medium contained soluble starch 2%, glucose 2%, corn steep liquor 2%, peanut powder 0.5%, peptone 0.5%, dried yeast 0.5% and $CaCO_3$ 0.2% (pH 6.5).

A loopful of mature slant culture of *Discosia* sp. F-11809 was inoculated to each of thirty 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 production medium (160 liters) in a 200-liter jar fermentor, and cultured at 30°C for 96 hours under aeration of 160 liters/minute and agitation of 300 rpm.

Animals

Female ICR/JCL and BDF_1 (8 weeks of age) were purchased from Shizuoka Agricultural Cooperative Association for Laboratory Animal (Hamamatsu, Japan).

Preparation of Bone Marrow Cells

Femurs removed from BDF_1 mice, were placed on petri dishes containing HANKS' solution. The bone marrow cells were flushed out with a syringe with a 22-gauge needle into α-minimum essential medium (α-MEM) supplemented with 5% fetal bovine serum (FBS). A portion of bone marrow cells was stained with 0.2% trypan blue solution, and the viable nucleated cells were counted microscopically in a BURKER's hematocytometer.

Assay of Colony Forming Units in Culture (cfu-c)

The cfu-c were measured according to a modification of the method of PIKE and ROBINSON[2]. Bone marrow cells (1×10^5) were suspended in 1 ml of α-MEM supplemented with methylcellulose 0.88%, FBS 20%, benzylpenicillin 60 μg, streptomycin 20 μg and the conditioned medium from L929 cells 20%. The conditioned medium was used as the source of colony stimulating factor. One ml of culture was placed into a 35-mm Falcon petri dish in triplicate, and incubated at 37°C in a humidified atmosphere (95% air, 5% CO_2). Colonies (more than 50 cells) were counted 7 days later under a dissecting microscope.

Preparation of Immunosuppressive Factor

Immunosuppressive factor was partially purified from the serum of sarcoma 180 tumor bearing mice according to the method described previously[1,3].

Results

Identification and Characterization of the Producing Strain

The fungus strain F-11809 was originally isolated from a soil sample collected at the foot of Mt. Takao, Kyoto Prefecture, Japan. This organism spread broadly on various agar media, and formed olivaceous grey to brownish gray colonies. We observed two anamorphs, hyphal conidiomata on agar media, and pycnidial conidiomata in the leaf segment bedded on media. The conidia produced from these conidiomata were very similar. Their conidiogeneses were holoblastic, and conidia were solitary.

The pycnidia were immersed, separate or aggregated, flattened stromatic, papillate, with one ostiole, 200~400 μm in diameter and 40~80 μm high (Fig. 1). The conidiophores were absent. The conidiogenous cells, formed at the lower layer of inner pycnidial walls, were obpyriform to lageniform, 7~10 μm long and 4~5 μm thick, tapering to 2 μm at the apices. The conidia were subhyaline, smooth, cylindrical to allantoid, 3-septate, truncate at base, and 15~22 (~25)×2.5~3.5 μm in size (Fig. 2). Their apical and basal cells had a setula near the septum, respectively. The setulae were unbranched, filiform, and 12~20 (~30) μm long. The vegetative hyphae were septate, subhyaline to dark brown, smooth and branched. The chlamydospores were absent.

Colonies on corn meal agar grew rapidly, attaining 7.5 cm in diameter after 2 weeks at 25°C. The colony surface was plane, thin, felty and grayish yellow brown to brownish gray. The conidia

Fig. 1. Micrograph of section through pycnidia of *Discosia* sp. F-11809 in the leaf segment.
Scale: 50 μm.

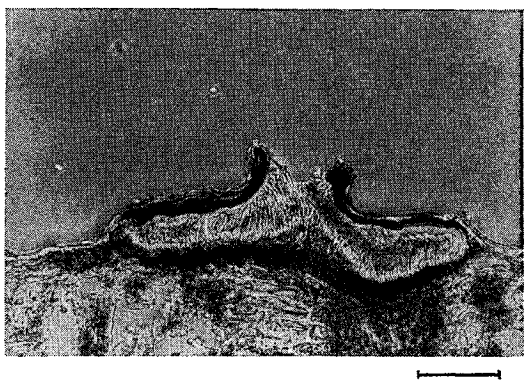
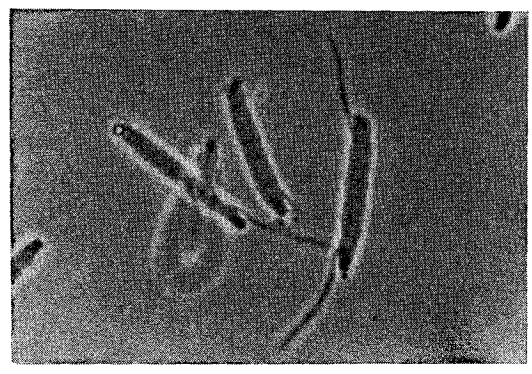
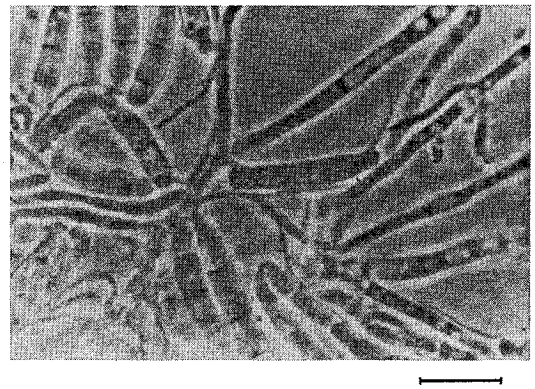


Fig. 2. Micrograph of conidia of *Discosia* sp. F-11809, producing in pycnidia.
Scale: 10 μm.



were abundantly observed at many positions on colony and dark brown hyphal masses formed in or on agar media.

Fig. 3. Micrograph of conidia of *Discosia* sp. F-11809, formed from hyphae on corn - meal agar. Scale: 10 μm.



From the above mentioned characteristics, the strain F-11809 seemed to belong to the coelomycete genus *Discosia* Libert. The genus was divided into five described species plus three other species delimited on the basis of conidial characters by SUBRAMANIAN and CHANDRA-REDDY[4]. According to their criteria, our strain resembled *Discosia strobilina* Libert, but its conidial characteristics (*i.e.*, shape, number of septa and position of the setula *etc.*) were often changed by cultural conditions (Figs. 2 and 3). These differences were very important to the species identification of genus *Discosia*. Further, SUTTON reported the status of *Discosia* species was open to question[5]. Therefore, we determined the strain F-11809 as one strain of the genus *Discosia*, and named to *Discosia* sp. F-11809. We deposited it at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM-P 8070.

Isolation and Purification

The cultured broth thus obtained was filtered with an aid of diatomaceous earth (4 kg). The filtrate was passed through an active carbon column (30 liters). The column was washed with deionized water (90 liters) and eluted with 60% aqueous acetone (90 liters). The eluate was concentrated *in vacuo* to a volume of 60 liters. The active fraction was charged to a column of Dowex 50WX2 (H^+ type) (6 liters). The column was washed with deionized water (18 liters) and eluted with 1.5% ammonia water (18 liters). The eluate was concentrated *in vacuo* to dryness and then subjected to a column chromatography on silica gel (1.8 liters). The column was washed with 80% aqueous isopropyl alcohol (1.8 liters) containing 0.28% ammonia water and 75% aqueous isopropyl alcohol (1.8 liters) containing 0.28% ammonia water, and then the elution was carried out 70% aqueous isopropyl alcohol containing 0.28% ammonia water. The active fractions (2.5 liters) were concentrated *in vacuo* to a volume of 100 ml and subjected to a column chromatography on silica gel (900 ml). The column was washed with a mixture (1.8 liters) of *n*-butyl alcohol, acetic acid and deionized water (3 : 1 : 2) and eluted with a mixture (1.8 liters) of *n*-butyl alcohol, acetic acid and deionized water (2 : 1 : 2). The active eluate was neutralized with 6 N NaOH and concentrated *in vacuo* to a volume of 100 ml. This solution was subjected to a column chromatography on silica gel (600 ml). The column was developed with a mixture of *n*-butyl alcohol, ethyl alcohol, chloroform and 28% ammonia water (2 : 2 : 1 : 2). The active fractions (700 ml) were concentrated *in vacuo* to a volume of 400 ml and then passed through an active carbon column. The column was washed with deionized water (400 ml) and eluted with 60% aqueous acetone (400 ml). The eluate was concentrated *in vacuo* to a volume of 30 ml and lyophilized to give 1.5 g of colorless powder of FR-900490.

Physico-chemical Properties

The physico-chemical properties of FR-900490 are summarized in Table 1. The IR, ^{13}C and

Table 1. Physico-chemical properties of FR-900490.

Appearance	Colorless powder
Nature	Amphoteric
MP	172~174°C (dec)
$[\alpha]_D^{27}$ (c 0.7, H_2O)	+8.5°
SI-MS (m/z)	371 (M^++1)
Molecular formula	$C_{14}H_{22}N_6O_6 \cdot 2H_2O$
Anal:	Calcd: C 41.38, H 6.45, N 20.68.
	Found: C 40.55, H 6.19, N 20.45.
UV	End absorption
Color test:	Positive: Ninhydrin, iodine, $CeSO_4$
	Negative: Molisch, Dragendorff, $FeCl_3$
Solubility:	Soluble: H_2O
	Slightly soluble: MeOH, EtOH
	Insoluble: Acetone, $CHCl_3$, EtOAc

Fig. 4. IR spectrum of FR-900490 in KBr.

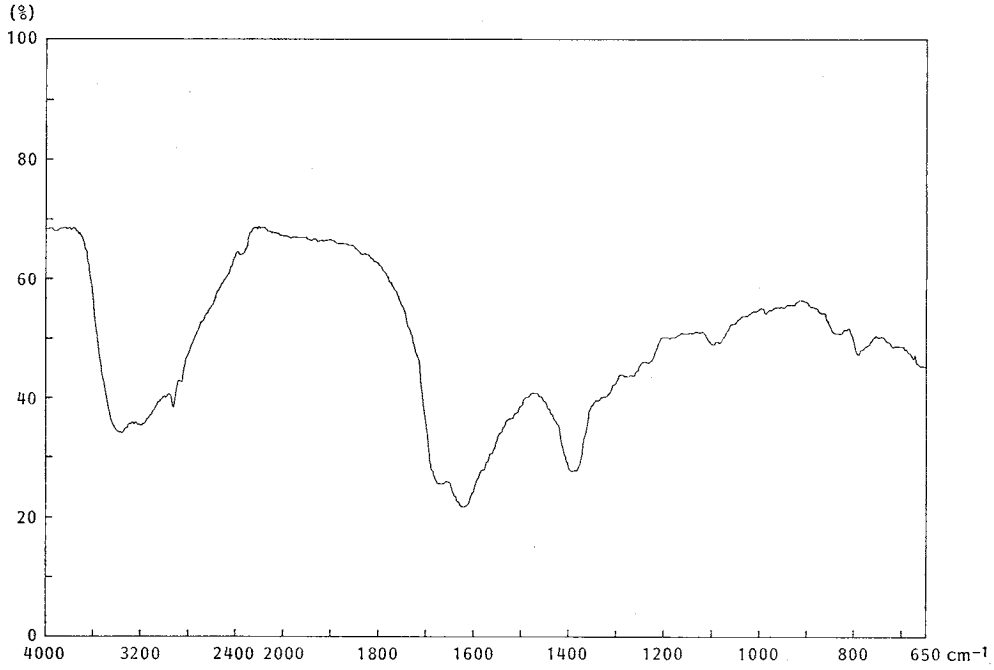


Fig. 5. ^{13}C NMR spectrum of FR-900490 in D_2O (67.5 MHz).

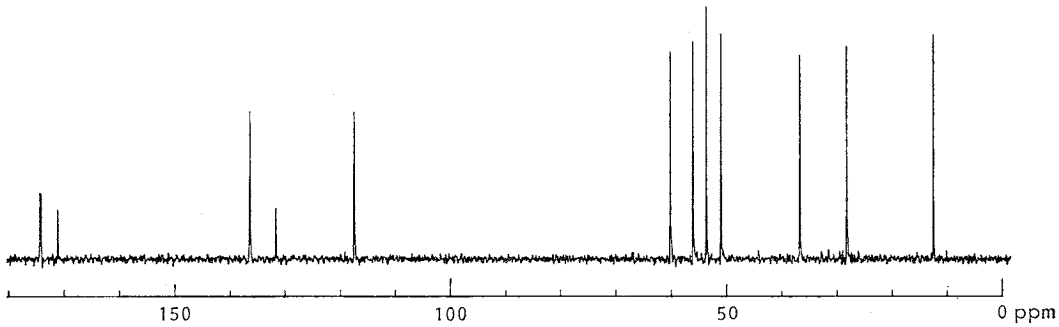
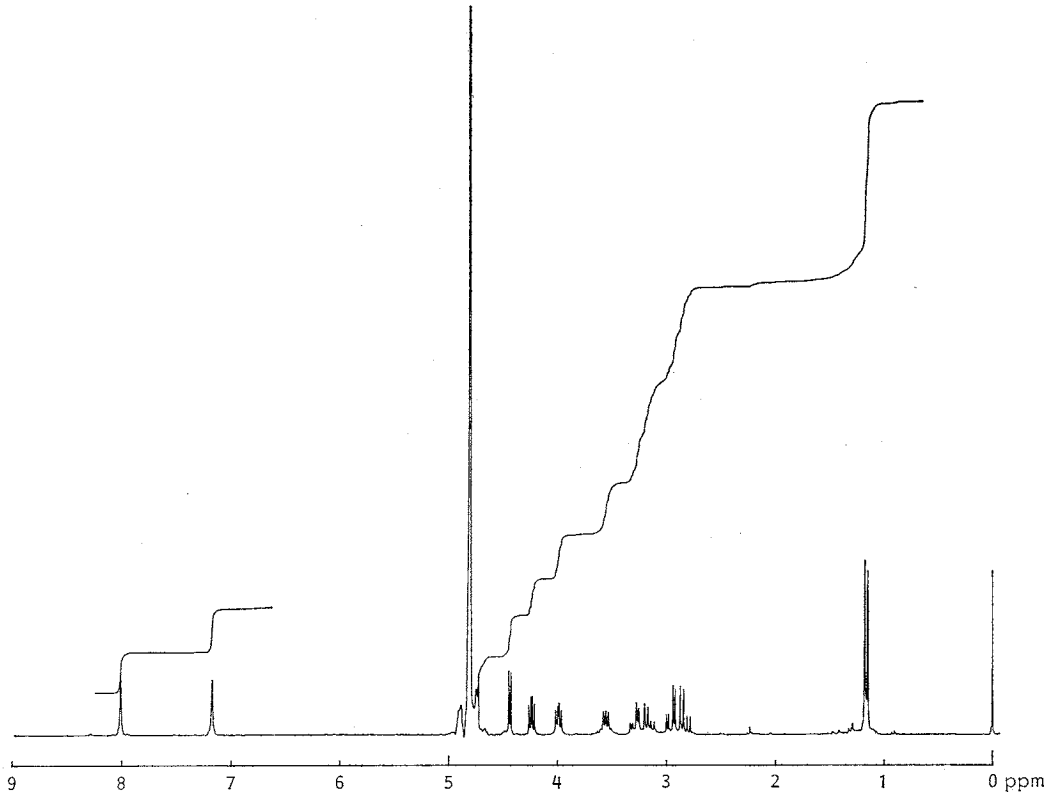


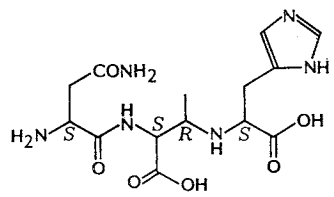
Fig. 6. ^1H NMR spectrum of FR-900490 in D_2O (270 MHz).



^1H NMR spectra are represented in Figs. 4, 5 and 6, respectively.

FR-900490 is a water soluble amphoteric substance and was isolated as colorless powder. The molecular formula of FR-900490 was determined to be $C_{14}H_{22}N_6O_6$ by the elemental analysis and secondary ion (SI)-MS data. The structure of FR-900490 was shown in Fig. 7 on the basis of chemical and spectral evidence, and the details will be reported elsewhere.

Fig. 7. Structure of FR-900490.



Biological Activity

Antimicrobial Activity

The antimicrobial activity of FR-900490 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. FR-900490 had weak antimicrobial activity against *Staphylococcus aureus* 209P (MIC 20 μg/ml).

The Suppressive Effect of Tumor Bearing Mouse Serum on Colony Formation of Bone Marrow Cells and Its Restoration by FR-900490

The immunosuppressive factor from tumor bearing mouse serum reduced the proportion of

Table 2. Depression of cfu-c in bone marrow cells by immunosuppressive factor and its restoration by FR-900490.

Sample No.	Treatment of bone marrow cells added	cfu-c*
1	Non-treated control	144.0± 4.6
2	Non-treated control – CM[a]	11.3± 4.1
3	Immunosuppressive factor	
	10 μl/ml	97.0± 2.6
	20 μl/ml	82.3± 5.5
	40 μl/ml	71.7±10.0
	80 μl/ml	61.3± 2.1
4	FR-900490	
	10 mg/ml	145.7± 7.1
	1 mg/ml	145.7± 4.6
	0.1 mg/ml	142.7± 2.8
5	Immunosuppressive factor (20 μl/ml) +FR-900490	
	10 mg/ml	135.3± 7.2
	1 mg/ml	141.3± 5.2
	0.1 mg/ml	140.0± 5.3
	0.01 mg/ml	147.7± 9.6
	0.001 mg/ml	133.0±10.7

* Mean±SE (n=6).
[a] Conditioned medium from L929 cells.

Table 3. Effect of FR-900490 on cfu-c in mitomycin C-treated mice[a].

Treatment	cfu-c (mean±SE)
Non-treated control	118 ±5.3
Non-treated control – CM	20.1±3.19
MMC (1 mg/kg)	12.6±0.34
FR-900490 (100 mg/kg)	115.8±5.95
MMC (1 mg/kg)	
+FR-900490 100 mg/kg	57.2±2.51[b]
10 mg/kg	70.4±6.9[b]
1 mg/kg	59.3±2.86[b]

[a] Mitomycin C (MMC, Kyowa Hakko Kogyo Co., Ltd.) was injected intraperitoneally at 0 and 1 day. FR-900490 was injected intraperitoneally daily for 5 days starting from the day of MMC injection (day 0~day 4).
[b] Significantly different from MMC treated control at $P<0.01$ (Student's t-test). Six mice per group.

cfu-c in bone marrow cells (Table 2). This reduction was dose dependent.

The addition of FR-900490 to the culture containing immunosuppressive factor restored the proportion of cfu-c. This result suggests that FR-900490 has the capacity to prevent damages in bone marrow cells by immunosuppressive factor.

Further, the addition of FR-900490 to the culture of bone marrow cells did not affect colony forming ability of bone marrow cells or bone marrow cell viability.

Effect of FR-900490 on cfu-c in Bone Marrow Cells in the Immunodeficient Mice

The effect of FR-900490 on cfu-c in bone marrow cells was studied in mitomycin C (MMC)-treated mice. MMC was given intraperitoneally to mice on days 0 and 1. FR-900490 was injected intraperitoneally daily for 5 days (day 0~day 4) starting from the day of the drug injection, and after 5 days its effect on cfu-c was determined by colony forming assay of bone marrow cells.

The results were shown in Table 3. Compared to non-treated mice, the injection of MMC decreased the proportion of cfu-c in bone marrow cells. The subsequent treatment with FR-900490 (3.0~100 mg/kg) caused a significant increase of cfu-c depressed by MMC. FR-900490 did not increase cfu-c of normal mice. Furthermore, the number of bone marrow cells in MMC-treated mice was almost equal to that in non-treated mice in these experiments.

Discussion

The present studies show that the colony formation of bone marrow cells was suppressed by immunosuppressive factor. The addition of FR-900490 prevented the damages caused by the immunosuppressive factor. Furthermore, the administration of MMC caused damages in bone marrow cells *in vivo*. The subsequent treatment with FR-900490 reduced these damages, though only partially. These facts suggest that FR-900490 is useful in combination with cancer chemotherapeutic agents to reduce damages to bone marrow in cancer patients.

Recently, OHTA *et al.* reported that thymosin α prevented the 5-fluorouracil (5-FU) induced bone marrow toxicity in BDF_1 mice, as determined by bone marrow cell counting[6]. We also found that FR-900490 reduced damages in bone marrow cells in 5-FU-treated mice (data not shown).

The mode of action of FR-900490 in the immunodeficient mice is now in progress.

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