A NOVEL ANTIFUNGAL ANTIBIOTIC, FR-900848

I. PRODUCTION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES

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Streptoverticillium fervens produced a new antibiotic, FR-900848, which has a high specific activity against filamentous fungi. Purified by solvent extraction and column chromatography, the compound appears as colorless crystals. Its structure is $C_{32}H_{43}N_3O_6$, which consists of 5'-amino-5'-deoxy-5,6-dihydrouridine with an unsaturated fatty acid having unprecedented four serial and one isolated cyclopropane rings.

During the course of screening for antifungal compounds, FR-900848 was discovered in the fermentation broth of *Streptoverticillium fervens* HP-891. This organism was isolated from the soil in Tsukuba city, Japan.

The compound suppressed the growth of fungi, but had no activity against Gram-positive or Gram-negative bacteria. The structure was determined (Fig. 1) by a chemical study which will be reported in a forthcoming paper.

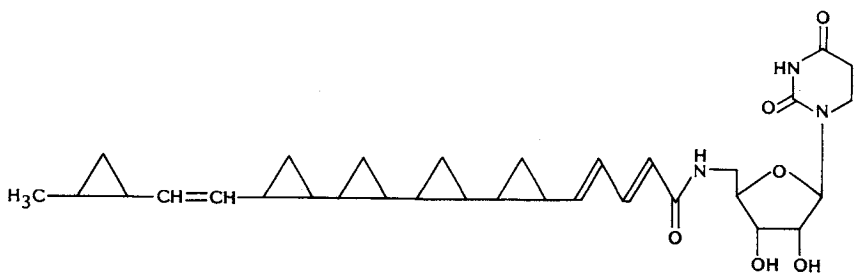
This paper describes the taxonomy and fermentation of the producing strain, and the isolation and physico-chemical and biological properties of the yielded substance, FR-900848.

Materials and Methods

Taxonomy of the Producing Strain

The cultures for all studies were grown at 30°C for 14 days. The procedure described by SHIRLING

Fig. 1. Structure of FR-900848.



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and GOTTLIEB[1] was used for morphological observations. The producing strain was grown on yeast extract-malt extract agar, oatmeal agar and inorganic salts-starch agar and examined under light and electron microscope.

The characteristics of each culture were observed on seven kinds of media described by SHIRLING and GOTTLIEB[1], and WAKSMAN[2].

The color names used in this study were based on Methuen Handbook of Colour[3]. Whole cell analysis was performed by the methods of BECKER *et al.*[4] and YAMAGUCHI[5]. The range of temperature that allowed growth and the optimum temperature were determined on yeast extract-malt extract agar using a temperature gradient incubator (Toyo Kagaku Co., Ltd.).

Ability to utilize carbon sources was examined by the methods of PRIDHAM and GOTTLIEB[6].

Antimicrobial Activity of FR-900848 *In Vitro*

The antimicrobial activity of FR-900848 against bacteria was determined on nutrient agar, and against yeasts and filamentous fungi on potato-glucose agar. A conventional agar dilution method was used.

The MIC was expressed in term of μg/ml after incubation overnight at 37°C for bacteria and 48~72 hours at 28°C for filamentous fungi and yeasts.

Therapeutic Activity of FR-900848 *In Vivo*

The chemotherapeutic activity of FR-900848 on experimental infection with *Trichophyton* induced in guinea pigs was determined by the method of SAKAI[7].

Briefly, guinea pigs in groups of 6, weighing 350~400 g were depilated at four sites on the back with a sharp blade, and inoculated with a spore suspension of *Trichophyton asteroides* at each site. FR-900848 (0.5 g) was dissolved in 100 ml of geraniol-1,3-butyleneglycol (56.2:24.7, w/w).

Three days after the inoculation, FR-900848 0.5% solution, pyrrolnitrin 0.5% and placebo, were applied to respective lesions of each animal; the untreated lesion served as the control. The animals were treated twice daily for 5 days. After 2 days interval, each drug was applied to each lesion twice daily for 2 days. To determine viable organisms, 6 skin specimens taken from each of the lesions were transferred on SABOURAUD's glucose agar containing 20 μg/ml chloramphenicol and cultured for 7 days at 30°C.

Results

Taxonomy of the Producing Strain

The branching type of sporophores was verticillate and the mature sporophores were monoverticillate with 3 to 10 spores in each chain (Fig. 2). The spores were shown by electron microscopy to be cylindrical and measure 0.4~0.6 × 1.0~1.6 μm in size. The surfaces were smooth. There was no fragmentation of hyphae or formation of spores in the substrate mycelium, and no sporangia, sclerotia or zoospores were seen. Aerial mycelia were formed on yeast extract-malt extract agar, inorganic salts-starch agar and glycerol-asparagine agar. It was in the red series.

Fig. 2. Scanning electron micrograph of spores of strain HP-891 on oatmeal agar, 21 days (×4,000).

The cultural characteristics are shown in Table 1. Analysis of whole cell hydrolysates showed the presence of LL-diaminopimelic acid, and the cell wall of the strain was classified accordingly as type I. The physiological properties of the strain are shown in Table 2.

The temperature range for growth was from 10 to 40°C with an optimum temperature of 32°C. The

Table 1. Cultural characteristics of strain HP-891.

Medium	Growth	Aerial mycelium	Reverse side color	Soluble pigment
Yeast extract-malt extract agar	Good	Abundant; pale red	Red	None
Oatmeal agar	Moderate	Moderate; brownish red	Brownish red	None
Inorganic salts-starch agar	Good	Moderate; pale red	Red	None
Glycerol-asparagine agar	Moderate	Moderate; reddish white	Postal red	None
Peptone-yeast extract-iron agar	Good	None	Orange	Brown
Tyrosine agar	Moderate	Poor; pale red	Grayish red	None
Sucrose-nitrate agar	Poor	Thin; reddish white	Reddish white	None

Table 2. Physiological properties of strain HP-891.

Temperature range for growth	10~40°C	Carbon utilization of	
Optimum temperature	32°C	D-Glucose	+
Starch hydrolysis	Positive	Sucrose	−
Milk coagulation	Positive	D-Xylose	−
Milk peptonization	Positive	D-Fructose	+
Melanin production	Positive	L-Rhamnose	−
Gelatin liquefaction	Negative	Raffinose	−
Cellulose hydrolysis	Negative	L-Arabinose	−
		Inositol	+
		Mannitol	−

+: Utilization, −: no utilization.

strain was positive for milk peptonization and coagulation, and for production of melanoid pigment on peptone-yeast extract-iron agar. This strain could utilize D-glucose, D-fructose and inositol.

We concluded from these microscopic studies and cell wall composition analyses that strain HP-891 belongs to the genus *Streptoverticillium*, and that it fits the description of *S. fervens*. This strain HP-891 was designated as *S. fervens* HP-891 (FERM BP-1805)[8] according to BERGEY's Manual and International Streptomyces Project (ISP) reports[9~12]. This strain has been deposited at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM BP-1805.

Fermentation

Culture medium in volumes of 160 ml and consisting of corn starch 1%, glycerol 1%, glucose 0.5%, cotton seed meal 1%, dried yeast 0.5%, corn steep liquor 0.5%, and $CaCO_3$ 0.2% at pH 6.5 was poured into twenty five 500-ml Erlenmeyer flasks and sterilized at 120°C for 30 minutes. Onto each medium was inoculated a well-grown slant culture of strain HP-891. The flasks were shaken on a rotary shaker (250 rpm, 5.0 cm-throw) at 30°C for 4 days.

The resultant cultures were inoculated to 180 liters of a fermentation medium containing soluble starch 4%, corn starch 1%, cotton seed meal 0.5%, dried yeast 0.5%, gluten meal 1%, $MgSO_4 \cdot 7H_2O$ 0.1%, KH_2PO_4 2.0% and $Na_2HPO_4 \cdot 12H_2O$ 1.5%, which had been sterilized at 120°C for 30 minutes. Fermentation was carried out at 30°C for 5 days under aeration at 150 liters/minute and agitation at 300 rpm. The progress of fermentation was monitored by judging from the size of the inhibition zone of

Table 3. Physico-chemical properties of FR-900848.

Appearance	Colorless needles	Solubility:	Soluble: DMSO
Nature	Neutral		Slightly soluble: MeOH, EtOAc, $CHCl_3$
MP	198~201°C (dec)		Insoluble: H_2O
$[\alpha]_D^{20}$ (*c* 0.5, DMSO)	−36.22°	TLC (Rf value)	0.55[a]
Molecular formula	$C_{32}H_{43}N_3O_6$		0.21[b]
Elementary analysis	Calcd for $C_{32}H_{43}N_3O_6$:	HPLC[c] (Rt)	4.42 minutes
	C 67.94, H 7.60, N 7.43	UV λ_{max}^{MeOH} nm (ε)	280 (31,000)
	Found: C 68.57, H 7.51, N 7.53	$\lambda_{max}^{0.1\,N\ HCl\text{-}MeOH}$ nm (ε)	280 (30,000)
FAB-MS (*m/z*)	566 (M+H)	$\lambda_{max}^{0.1\,N\ NaOH\text{-}MeOH}$ nm (ε)	280 (30,500)
Color reaction	Positive: Dragendorff, Ehrlich, $Ce(SO_4)_2$		
	Negative: Ninhydrin, Molisch, $FeCl_3$		

[a] Silica gel plate 60 F_{254} (Merck); $CHCl_3$-MeOH (5:1).
[b] HPTLC plate RP-18 F_{254} S (Merck); MeOH-10% AcOH (9:1).
[c] YMC-Pack A-302 (5 μm, 4.5×150 mm; Yamamura Chemical Inst.), UV 280 nm.

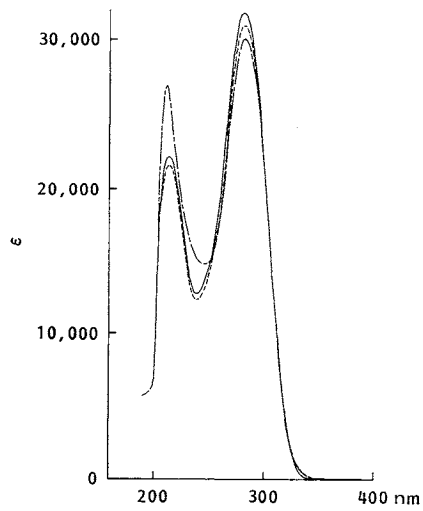
Aspergillus niger, the reference organism.

Isolation

The culture broth (175 liters) thus obtained was filtered with a filter aid (Radiorite No. 600, trade mark, made by Showa Chemical Industry, 5 kg). The mycelia cake was extracted with acetone (150 liters), and yielded 50 liters of the extract. The acetone extract from the mycelia was concentrated to 18 liters of aqueous solution under reduced pressure at 20°C. The concentrate was extracted with ethyl acetate three times, and evaporated under reduced pressure at 20°C. The residue was dissolved in 75% aqueous methanol (500 ml) and passed through a column of reverse phase silica gel (YMC-gel ODS-A60 (60~200 mesh), Yamamura Chemical Institute, 1.5 liters).

Fig. 3. UV spectra of FR-900848.

—— MeOH, ——— 0.1 N HCl-MeOH, ------ 0.1 N NaOH-MeOH.



The column was washed with 75% aqueous methanol (6 liters) and 80% aqueous methanol (4 liters) and then eluted with 90% aqueous methanol (2 liters). The eluate was evaporated to crystals under reduced pressure at 20°C, the residue was dissolved in methanol, and the crystals were left to stand overnight at 4°C. They were then collected by filtration, washed with cooled methanol and dried under reduced pressure. After recrystallization from methanol, colorless needle crystals of FR-900848 substance (328 mg) were produced.

Physico-chemical Properties

Physico-chemical properties of FR-900848 are summarized in Table 3.

FR-900848 is a neutral substance which decomposes at 198~201°C. It is soluble in dimethyl sulfoxide, slightly soluble in methanol, ethyl acetate and chloroform, and insoluble in water.

Its color reactions were positive to Dragendorff, Ehrlich and $Ce(SO_4)_2$, and negative to ninhydrin,

Fig. 4. IR spectrum of FR-900848 (Nujol).

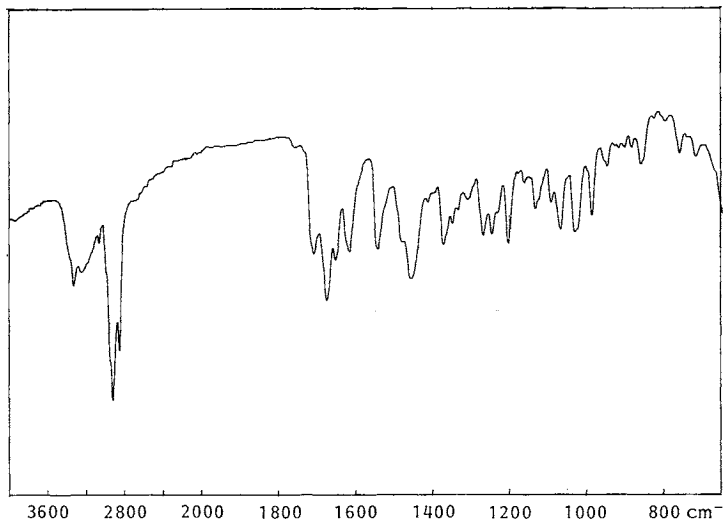
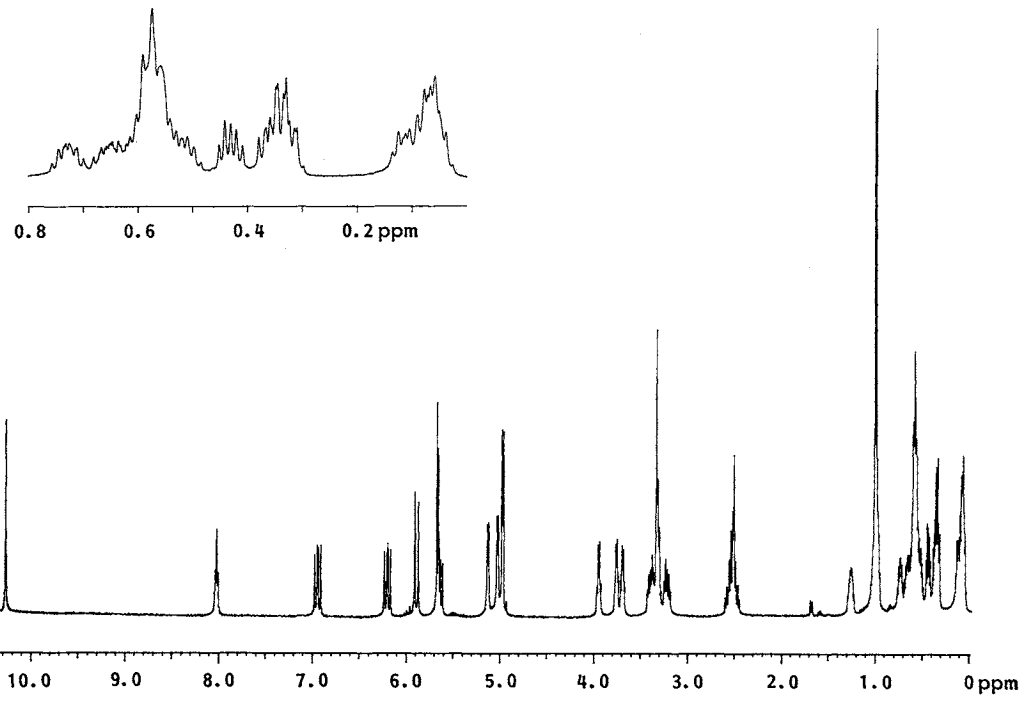


Fig. 5. ^{1}H NMR spectrum of FR-900848 (400 MHz, DMSO-d_6).



Molisch and $FeCl_3$.

The molecular formula was determined to be $C_{32}H_{43}N_3O_6$ by elementary analysis and FAB-MS.

The UV, IR, ^{1}H and ^{13}C NMR spectra of FR-900848 are shown in Figs. 3~6, respectively.

Rf values on TLC and Rt value on HPLC are shown in Table 3.

As shown in Fig. 1, the structure of FR-900848 consists of 5'-amino-5'-deoxy-5,6-dihydrouridine,

Fig. 6. ^{13}C NMR spectrum of FR-900848 (100 MHz, DMSO-d_6).

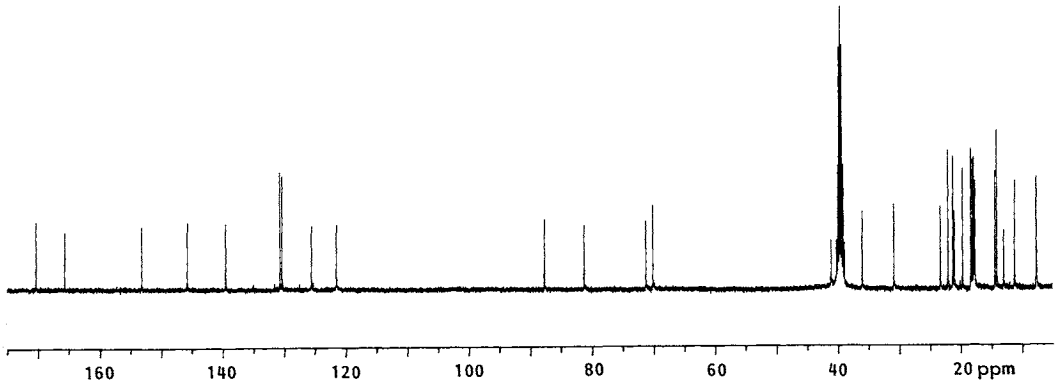


Table 4. Antimicrobial spectrum of FR-900848.

Test organisms	MIC (μg/ml)
Aspergillus niger	0.05
Mucor rouxianus	0.05
Aureobasidium pullulans	0.05
Penicillium chrysogenum	0.1
Trichophyton metagrophytes	0.2
T. asteroides	0.5
T. rubrum	0.5
Fusarium oxysporum	0.1
Sclerotinia arachidis	0.1
Candida albicans	100
C. tropicalis	100
C. guilliermondii	0.2
Cryptococcus albidus	100
Saccharomyces cerevisiae	100
Staphylococcus aureus 209 P	100
Escherichia coli NIHJ	100

Table 5. Therapeutic effect of FR-900848 on animals with fungal infection[a].

Drugs	Culture results[b]			
	0	1	2	3
No treatment			4/36	32/36
Placebo			4/36	32/36
0.5% FR-900848	6/36	4/36	19/36	7/36
0.5% Pyrrolnitrin	2/36	10/36	8/36	16/36

[a] Therapeutic activity of FR-900848 on experimentally-induced infection with *Trichophyton asteroides* FP594 in guinea pigs was determined as described in the text. For the determination of viable organisms, 6 skin specimens taken from each of the lesions were cultured on SABOURAUD's agar for 7 days at 30°C.

[b] Culture results: Numbers of specimen with viable organisms per total numbers of specimen after 7-day culture; 0: No growth, 1: growth on the skin only, 2: growth on the skin and slightly growth on the surrounding agar, 3: skin and agar completely covered by microorganism.

which carries an unsaturated fatty acid side chain having an unprecedented number of five cyclopropane rings.

The details of the studies on the structure of FR-900848 will be described in the subsequent paper.

Biological Properties

The antimicrobial spectrum of FR-900848 is shown in Table 4. FR-900848 had a high specific activity against filamentous fungi in concentrations of 0.05~0.5 μg/ml, suppressing the growth of *A. niger*, *Mucor rouxianus*, *Penicillium chrysogenum*, *Aureobasidium pullulans*, *Trichophyton* species, *Fusarium oxysporum* and *Sclerotinia arachidis*. This compound caused the hyphae of fungi to branch frequently and the filaments to swell. But it had poor activity against *Staphylococcus aureus*, *Escherichia coli* and *Candida albicans*.

As shown in Table 5, the activity of FR-900848 was of a therapeutically effective potency, which was almost as strong as that of pyrrolnitrin.

Acute Toxicity

The 50% lethal dose of FR-900848 by intraperitoneal injection to mice was more than 1 g/kg.

Discussion

FR-900848 was sufficiently active to inhibit filamentous fungi. Therapeutically, the compound had some effect against *Trichophyton* in guinea pigs. This compound was also effective against pathogenic fungi in plants (unpublished data).

FR-900848 has an unusual structure (Fig. 1), which consists of 5'-amino-5'-deoxy-5,6-dihydrouridine with an unsaturated fatty acid having unprecedented four serial and one isolated cyclopropane rings. To our knowledge, such a compound has not been discovered in nature. The mode of action of FR-900848 is now under study.

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