FR65814, A NOVEL IMMUNOSUPPRESSANT ISOLATED FROM A *PENICILLIUM* STRAIN

TAXONOMY, FERMENTATION, ISOLATION, PHYSICO-CHEMICAL AND BIOLOGICAL CHARACTERISTICS AND STRUCTURE ASSIGNMENT

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FR65814, a novel immunosuppressant, was isolated from the cultured broth of *Penicillium jensenii* F-2883. The structure was assigned to be 5,6-dihydroxy-4-(1,2-epoxy-1,5-dimethyl-4-hexenyl)-1-oxaspiro[2,5]octane by spectroscopic analyses. The compound suppressed the immune response at low concentration.

In addition, a structually related component fumagillol, a known carcinolytic agent, was also isolated and found to show immunosuppressive activity.

In the course of our screening program for novel immunosuppressants in the cultured broths of microorganisms, we have found that FR65814 was produced by *Penicillium jensenii* F-2883 which organism was isolated from a soil sample collected in the State of New York, U.S.A.

Another component, isolated from the crude mixture, was identified as fumagillol[1] based on spectroscopic data and mp.

The present paper describes the taxonomy and fermentation of F-2883 and the isolation, physico-chemical and biological properties and structure assignment of FR65814. Furthermore the immunosuppressive effect of fumagillol, published as a carcinolytic agent[2], is also reported.

Characteristics of Strain F-2883

Strain F-2883 was originally isolated from a soil sample collected in the State of New York, U.S.A. This organism grew rather rapidly on various agar media, and formed white to olivaceous gray colonies. We observed its anamorph, conidial structures consisting of penicillate conidiophores and dry chains of conidia (Fig. 1).

Colonies on malt extract agar grew rather restrictedly, attaining 3.5 cm in diameter after 2 weeks at 25°C. The colony surface was raised, floccose and yellowish white to light olivaceous gray. The conidial structures were abundantly produced (Fig. 2). The reverse was light brown, and pale yellow orange pigments were spread in the media.

The conidiophores were straight, slightly roughened, hyaline, borne from vegetative hyphae as short branches, 15~75 μm long and 2~2.5 μm thick. The penicilli were biverticillate and divaricate, and consisted of 2~4 metulae. The metulae were 10~20×2.5~3.5 μm in size, with whorls of 4~8 phialides. The phialides were smooth, hyaline, lecythiform, 7~10 μm long and 2~2.5 μm thick. The conidia were produced in basipetal chains. They were unicellular, hyaline, smooth to slightly

Fig. 1. Micrograph of conidial structures of *Penicillium jensenii* F-2883.

Scale: 20 μm.

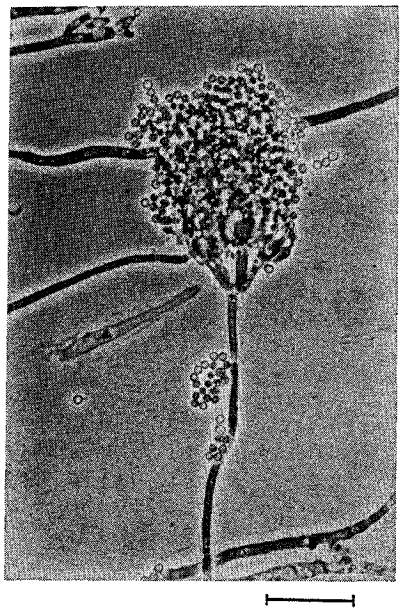
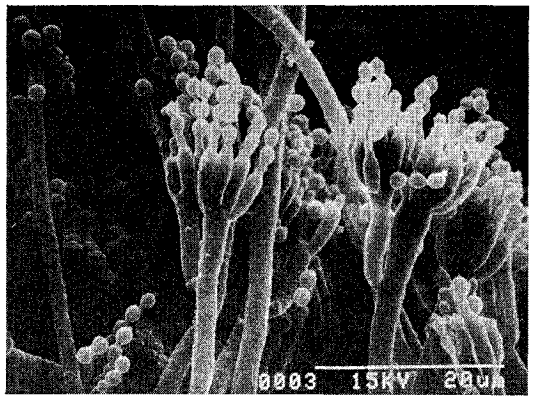


Fig. 2. Scanning electron micrograph of anamorph of *Penicillium jensenii* F-2883 on malt extract agar.



roughened, 2~3 μm in diameter, globose to subglobose with one small projection at the both ends. The vegetative hyphae were septate, hyaline, slightly roughened, and branched. The chlamydospores were absent.

From the above-mentioned characteristics, the strain F-2883 was identified as one strain of *P. jensenii* Zaleski[3,4], and named *P. jensenii* F-2883. We deposited it at the Fermentation Research Institute, Agency of Industrial Science and Technology, Japan, as FERM-P 7645.

Assay Method

The mixed lymphocyte reaction was used as an approach to the screening for novel immunosuppressants. The detailed method is described in the Biological Properties section.

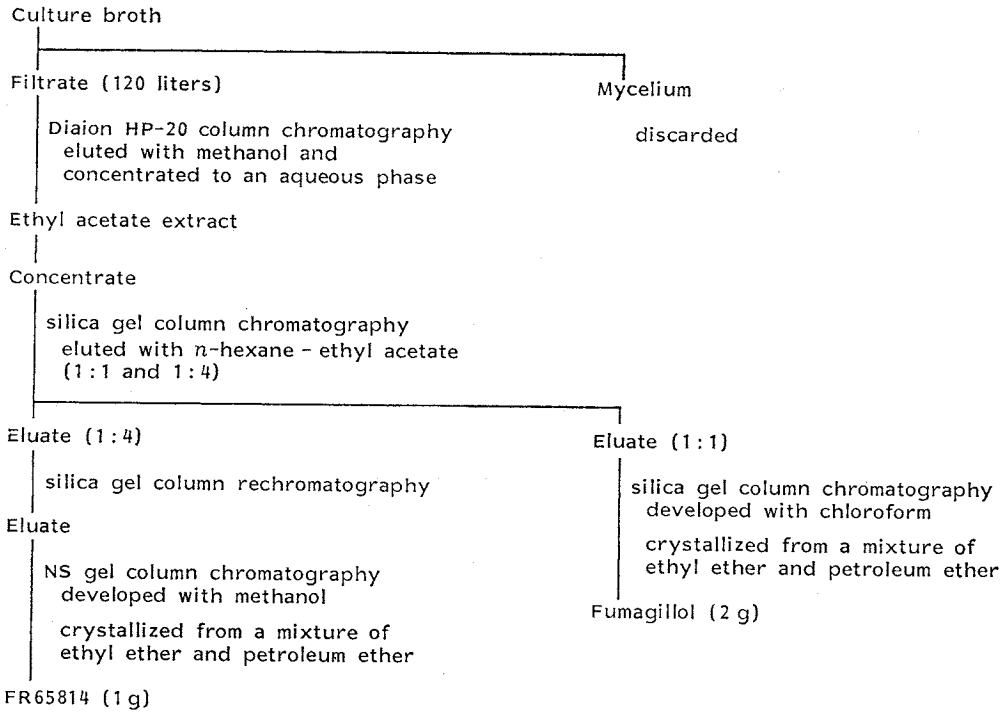
Fermentation

A loopful of the slant culture of *P. jensenii* F-2883 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of a sterile seed medium and incubated on a rotary shaker at 25°C for 4 days. The seed medium (pH 6.0) was composed of soluble starch 1%, corn starch 1%, glucose 1%, Parmamedia 0.5%, dried yeast 0.5%, corn steep liquor 0.5% and calcium carbonate 0.2%. Two liters of the incubated seed culture was transferred to 150 liters of a production medium (pH 6.5) containing soluble starch 2%, glucose 2%, corn steep liquor 2%, peanut powder 0.5%, peptone 0.5%, dried yeast 0.5%, and calcium carbonate 0.2% in a 200-liter jar fermentor. The fermentation was carried out at 25°C for 4 days with agitation at 300 rpm and air flow of 150 liters per minute. FR65814 production began at 40 hours and reached a maximum potency after a 90-hour incubation period.

Isolation

The isolation scheme is shown in Fig. 3. The cultured broth was filtered with the aid of diatomaceous earth (5 kg). The filtrate (120 liters) was adsorbed on a column of Diaion HP-20 (Mitsubishi Chemical Industries Limited, Japan) (12 liters). The column was washed with water and 50% aqueous methanol, and then eluted with methanol. The eluate (30 liters) was concentrated *in vacuo* to 1 liter. The aqueous solution was extracted with ethyl acetate (1 liter). The extract was concen-

Fig. 3. Isolation and purification procedure.



trated *in vacuo* to give an oily residue. The residue was mixed with silica gel (70~230 mesh, Merck Co., Ltd., U.S.A.) and applied to a silica gel column (1 liter) packed with *n*-hexane. The column was developed with *n*-hexane (3 liters) and a mixture of *n*-hexane and ethyl acetate (1 : 1, 3 liters and 1 : 4, 3 liters). FR65814, following the elution of fumagillol, was eluted with a mixture of *n*-hexane and ethyl acetate (1 : 4, 2 liters). The eluate was rechromatographed on silica gel (1 liter) and the active fraction was concentrated *in vacuo*. The residue, dissolved in a small amount of methanol, was further purified by column chromatography on NS gel (400 ml) developed with methanol. Fractions containing FR65814 were concentrated *in vacuo* to give an oily residue. The oil was dissolved in a small amount of ethyl ether, and then petroleum ether was added to the solution to give FR65814 (1 g) as colorless prisms.

As shown in Fig. 3, fumagillol (2 g) was crystallized from a mixture of ethyl ether and petroleum ether, following rechromatography on silica gel developed with chloroform.

Physico-chemical Properties

The physico-chemical properties of FR65814 are summarized in Table 1. Its Rf value on silica gel TLC developed with a mixture of chloroform and methanol (10 : 1) was 0.47.

Biological Properties

Antimicrobial Activities

The antimicrobial activities were tested by a conventional agar dilution method, using a nutrient agar for bacteria and a SABOURAUD's agar for fungi. FR65814 had no inhibitory activity at 100 μg/ml against six microorganisms: *Staphylococcus aureus*, *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Candida albicans* and *Aureobasidium pullulans*.

Table 1. Physico-chemical properties of FR65814.

Appearance	Colorless prisms
MP	46~47 °C
Molecular formula	$C_{15}H_{24}O_4$
MW	268
Optical rotation	$[\alpha]_D^{23}$ −38.4° (*c* 2.4, MeOH)
Elemental analysis (%)	
Calcd for $C_{15}H_{24}O_4$:	C 67.13, H 9.02.
Found:	C 67.38, H 8.76.
UV spectrum	End absorption
Color reaction	
Positive:	Sulfuric acid, potassium permanganate, iodine vapor, Ehrlich
Negative:	Ferric chloride, Dragendorff, ninhydrin, Molisch
Solubility	
Soluble:	MeOH, EtOH, Me_2CO, EtOAc, benzene, $CHCl_3$, ethyl ether
Sparingly soluble:	*n*-Hexane
Insoluble:	Water

Table 2. Effect of FR65814 and fumagillol on mixed lymphocyte reaction (MLR).

Drug concentration (ng/ml)	Radioactivity (mean cpm±SE)		MLR suppression (%)	
	FR65814	Fumagillol	FR65814	Fumagillol
5,000	3,997±623**	2,183±291**	80.7	89.4
500	4,539±296**	4,758±417**	78.1	77.0
250	5,140±296**	6,660±599**	75.1	67.8
125	5,512±321**	8,318±460**	73.3	59.8
63	6,345±574**	10,809±642**	69.3	47.7
31	7,150±298**	14,586±235**	65.4	29.5
16	8,436±439**	18,131±644*	59.2	12.3
8	10,932±447**	19,640±1,183*	47.1	5.0
4	16,023±248*	—	22.5	—
2	19,562±184*	—	5.4	—
0	20,682±573	20,682±573	0	0

* $P<0.01$ by Student's t-test. ** $P<0.001$ by Student's t-test.

Suppression of Mixed Lymphocyte Reaction (MLR)

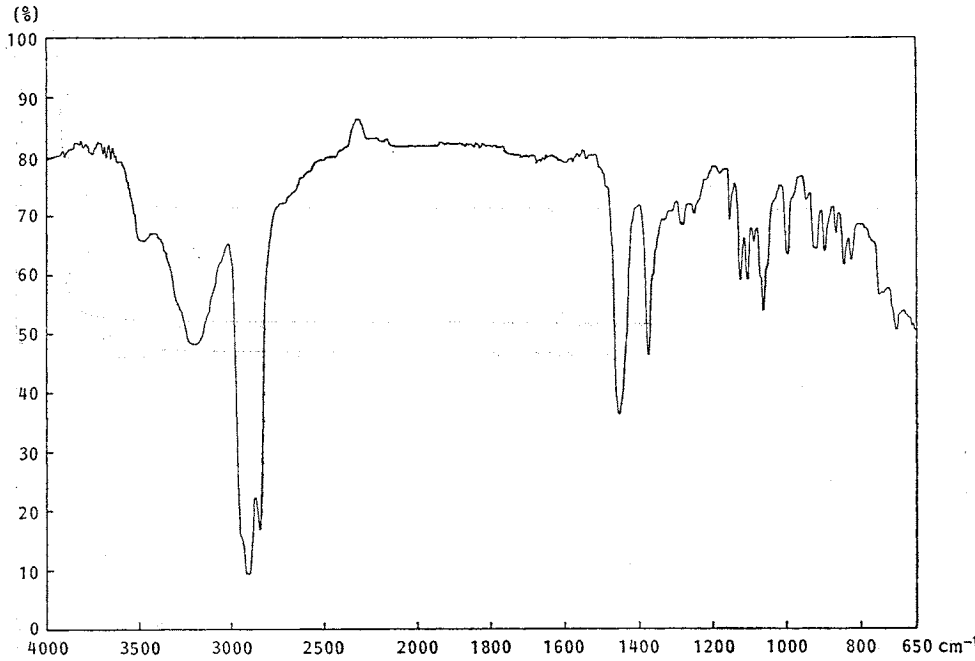
The MLR test was performed in microtiter plates, with each well containing 5×10^5 C57BL/6 responder cells (H-2^b), 5×10^5 mitomycin C treated (25 μg/ml mitomycin C at 37°C for 30 minutes and washed three times with RPMI 1640 medium) BALB/C stimulated cells (H-2^d) in 0.2 ml RPMI 1640 medium supplemented 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% CO_2: 95% of air for 68 hours and pulsed with [^{3}H]thymidine (0.5 μCi) 4 hours before the cells were collected. FR65814 and fumagillol were dissolved in ethanol and further diluted in RPMI 1640 medium and added to the cultures.

The suppressive effect of FR65814 and fumagillol on the mouse MLR is shown in Table 2. The IC_{50} values were 9 ng/ml (34 nM) and 70 ng/ml (250 nM), respectively. FR65814 is a more potent immunosuppressive agent than fumagillol.

Acute Toxicity in Mice

FR65814 showed no adverse effect when administered intraperitoneally to BALB/C mice at 200 mg/kg.

Fig. 4. IR spectrum of FR65814 (Nujol).



Structure Assignment

The ^1H NMR spectra (400 MHz) of two active compounds with immunosuppressive effect on the mouse MLR were similar to that of fumagillin (**3**). One of them was identified as fumagillol (**2**) by a comparison with an authentic sample prepared from fumagillin[5,6] (**3**).

FR65814 (**1**) has the molecular formula $C_{15}H_{24}O_4$ as established by elemental analysis and by fast atom bombardment (FAB)-MS. Its IR spectrum (Fig. 4) showed a strong band at 3170 cm^{-1}, indicating the presence of hydroxyl groups in **1**. In the ^1H NMR ($CDCl_3$, 400 MHz) (Fig. 5) the signal pattern of **1** was similar to that of fumagillol (**2**) (Fig. 6) except for the absence of a signal at δ 3.42 due to a methoxyl group existing in **2**. It was presumed from this that both substances have the same fundamental carbon skeleton. In the ^{13}C NMR ($CDCl_3$, 100 MHz) (Fig. 7) of **1**, compared with that of **2**, the signal at δ 56.3 attributable to the methoxyl carbon in **2** (Fig. 8) was absent and the signal at δ 80.9 in **2** was shifted upfield (δ 75.2). This indicated that the methoxyl group attached to C-5 in **2** should be replaced by a hydroxyl group in **1** which would be vicinal to the hydroxyl group at C-6. The correlation spectroscopy (COSY) NMR spectrum ($CDCl_3$, 400 MHz) of **1** provided a ^1H scalar coupling relationship (Fig. 9). Groups of coupled protons which were separated by three quaternary carbons could be detected, and the assignments of these groups of protons were similar for **1** and **2**. These facts suggested that **1** was 5,6-dihydroxy-4-(1,2-epoxy-1,5-dimethyl-4-hexenyl)-1-oxaspiro[2,5]octane.

Next, we focused on the stereochemistry of the hydroxyl groups on the cyclohexane ring of **1**. The stereochemistry of fumagillol (**2**) had been determined through the X-ray crystallographic studies on fumagillin[7,8]. This indicated that the hydroxyl group was axial and the methoxyl group was equatorial on the cyclohexane ring in **2**. The results of ^1H NMR experiments (400 MHz) (δ 3.55, dd, J=11.1 and 2.7 Hz, 5-H; δ 4.30, dd, J=5.8 and 2.7 Hz, 6-H) were the same as that of X-ray crystal-

Fig. 5. ^1H NMR spectrum of FR65814 ($CDCl_3$).

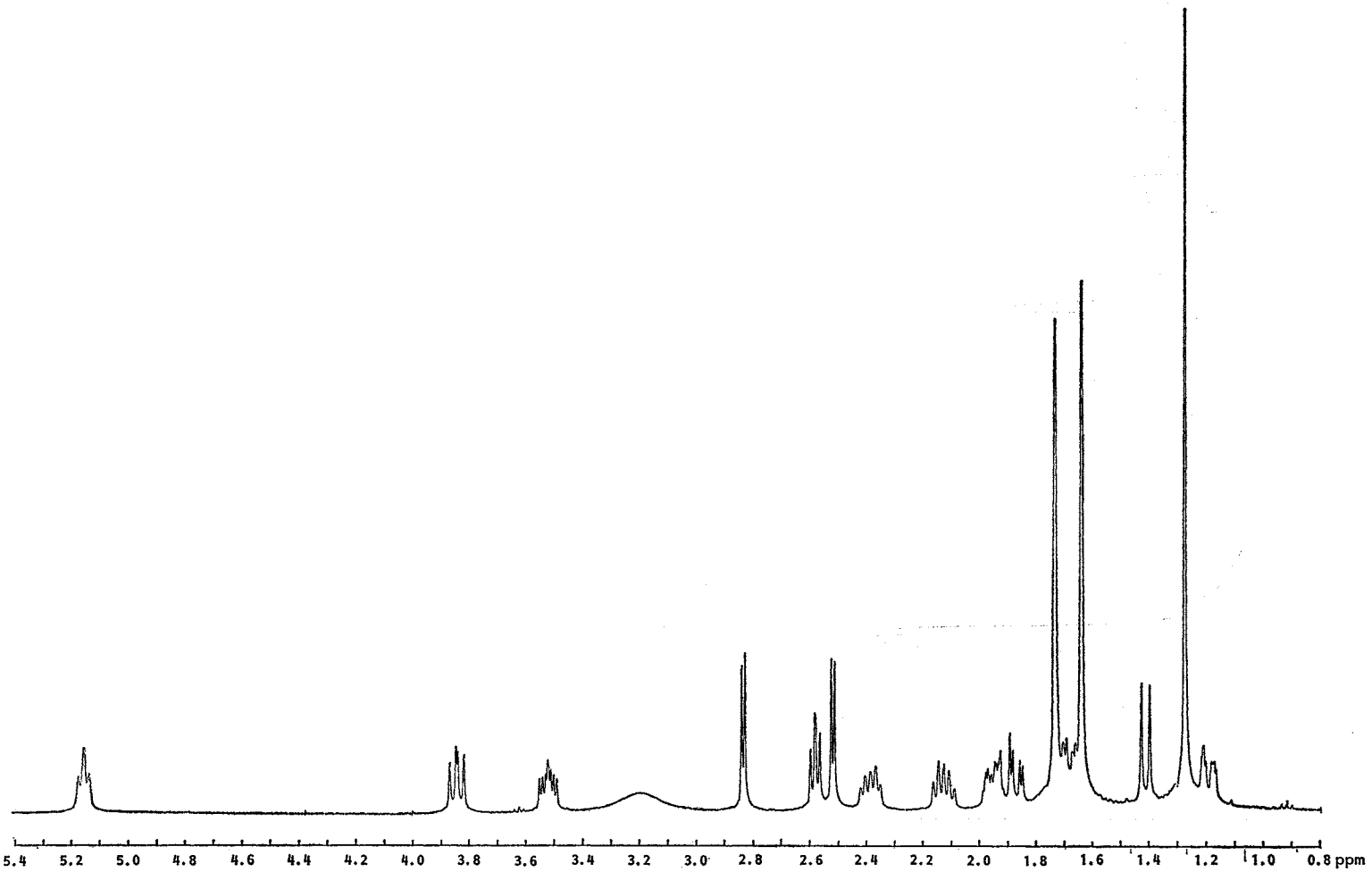


Fig. 6. ^1H NMR spectrum of fumagillol ($CDCl_3$).

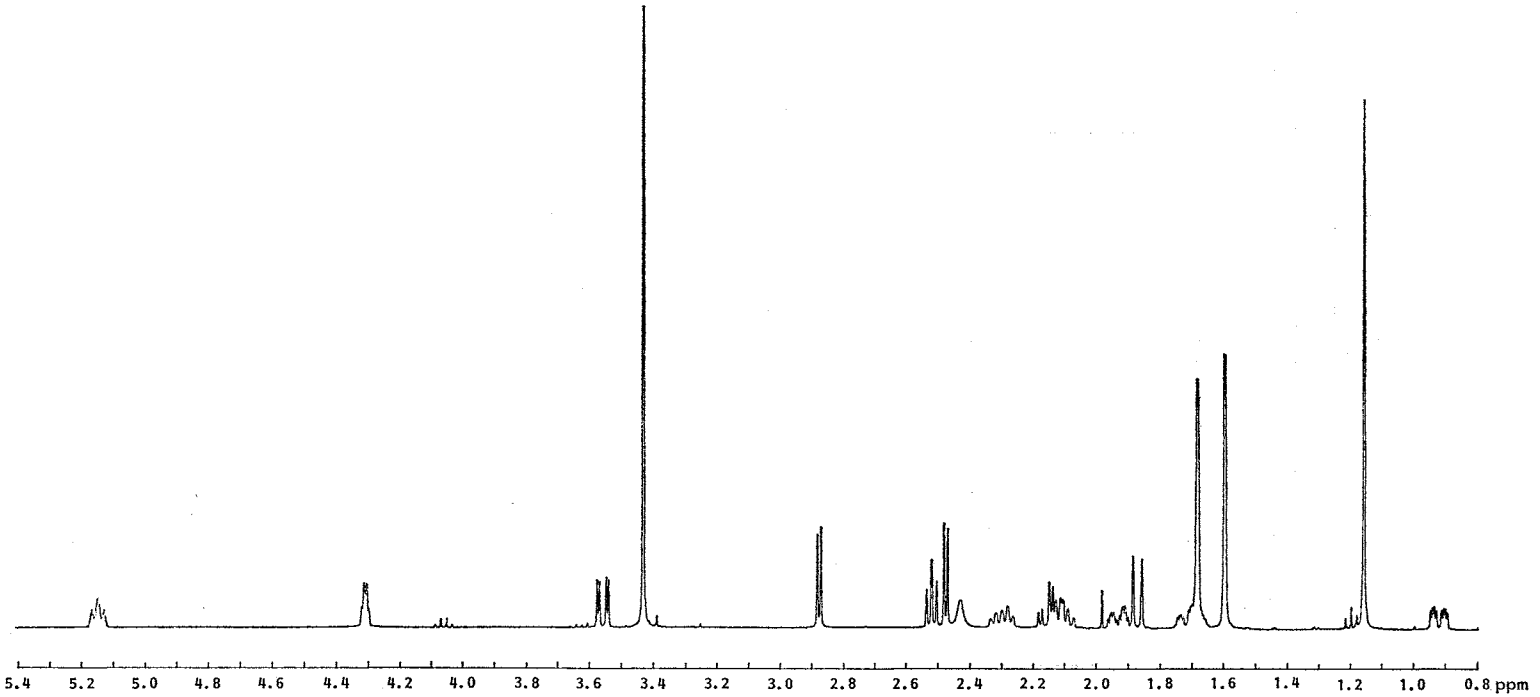


Fig. 7. ^{13}C NMR spectrum of FR65814 ($CDCl_3$).

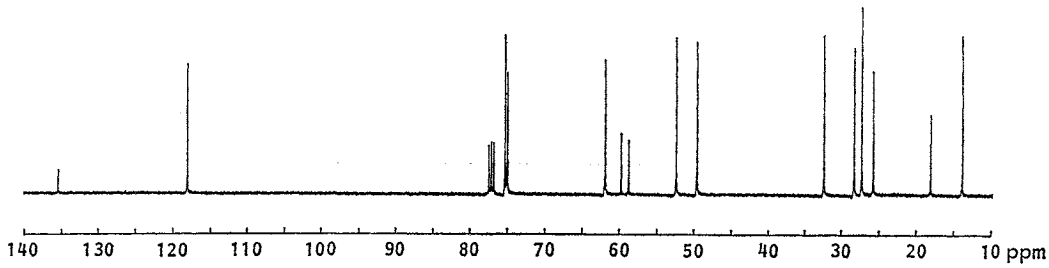
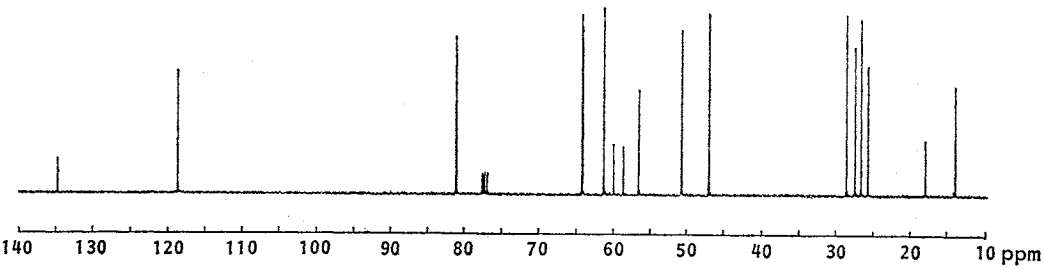
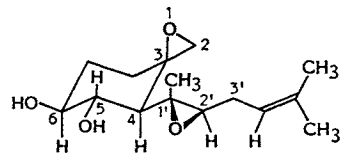
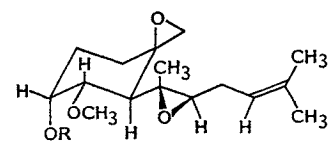


Fig. 8. ^{13}C NMR spectrum of fumagillol ($CDCl_3$).





FR65814 (**1**)



Fumagillol (**2**) R = H

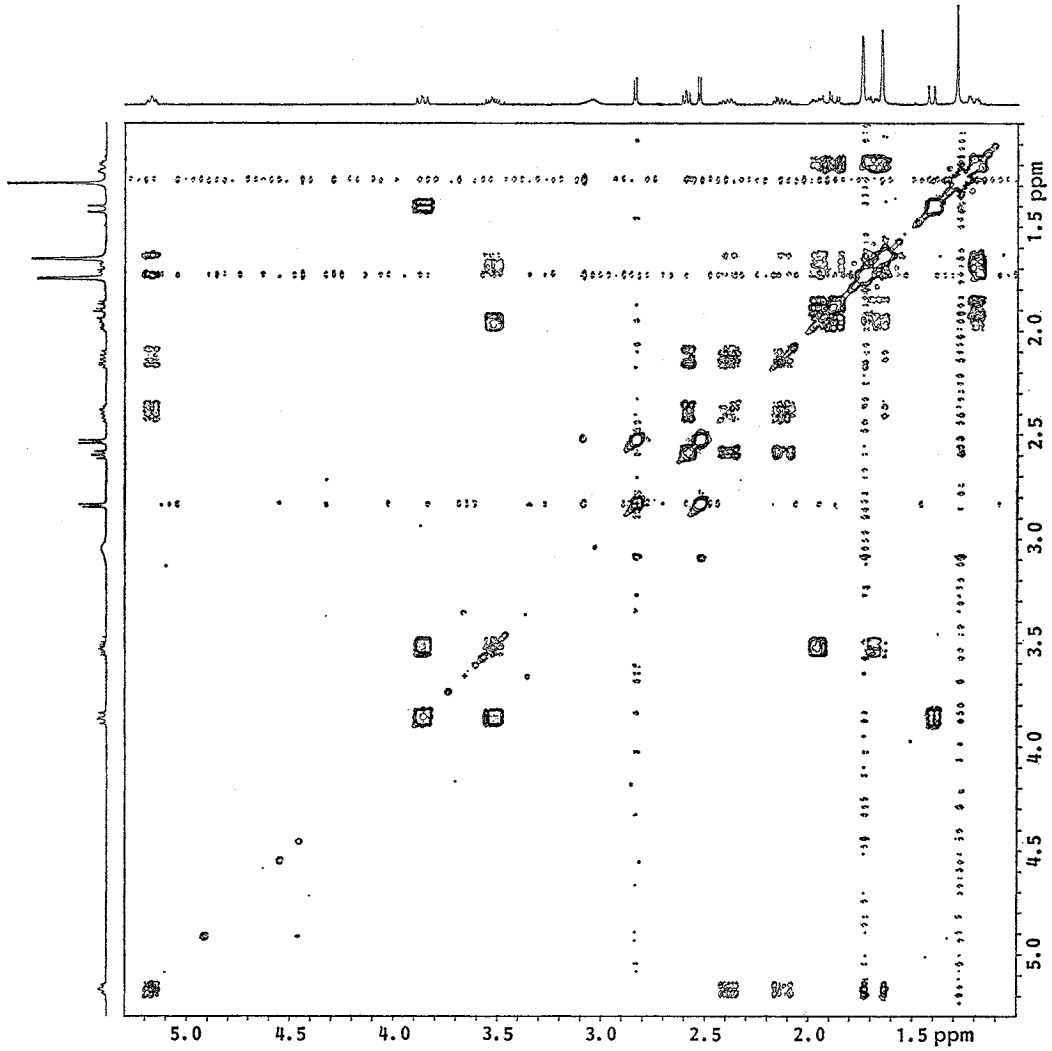
Fumagillin (**3**) R = $CO(CH{=}CH)_4COOH$ (*E*)

lographic analysis in **2**. The ^1H NMR spectrum of **1** showed signals at δ 3.52 (ddd, J=8.7, 4.3 and 11.4 Hz) and δ 3.84 (dd, J=8.7 and 11.1 Hz) which were assigned to the protons attached to carbons bearing hydroxyl groups. The coupling constants suggested that both protons were axial, so the configuration of the hydroxyl group at C-6 was equatorial in **1** rather than axial as in fumagillol. These findings are consistent with assignment of the structure of FR65814, including the stereochemistry, to be **1**.

Discussion

The MLR has been thought to be an *in vitro* correlative model[9] of delayed-type hypersensitivity (DTH) and allograft rejection, and a representative reaction of interleukin 2 (IL-2) dependent T cell proliferation. Therefore, we have searched for novel metabolites from microorganisms which would show specific suppressive effects on the mouse MLR, and not on the growth of IL-2 independent EL4 lymphoma. As the result of extensive studies, FR65814[10] and fumagillol[11] were isolated from the cultured broth of *P. jensenii* F-2883. FR65814 is a new de-methyl derivative of epifumagillol. Fumagillol can be prepared synthetically from fumagillin which has antiparasitic[12] and carcinolytic[2] activity. The IC_{50} values of FR65814 and fumagillol for the mouse MLR were 34 nM and 250 nM, respectively. Moreover, in our screening system *Penicillium nigricans* F-5261 has been found to pro-

Fig. 9. COSY NMR spectrum of FR65814 ($CDCl_3$).



duce fumagillin[11]. Hydrolysis of fumagillin for removal of the polyene moiety gave fumagillol, indicating that the polyene moiety is not necessary for the immunosuppressive activity.

In our previous papers[13,14], we have reported that FK-506 is a novel 23-membered macrolide extracted from *Streptomyces tsukubaensis* which has remarkable immunosuppressive properties *in vitro* and *in vivo*. FK-506 shows a specific inhibitory effect on the mouse MLR without entirely affecting EL4 growth. On the other hand, FR65814 and fumagillol have less specifity with weak inhibition of EL4 growth at pharmacologic concentrations (data not shown). In addition, preliminary results have revealed that these compounds do not inhibit IL-2 production (unpublished data), suggesting that they are immunosuppressants with a different site of action as compared to FK-506. However, we believe that they will fulfill the role of immunosuppressive agents to treat and cure transplantation and autoimmune deceases, because of their low toxicity and suppression of DTH *in vivo* (data not shown).

Acknowledgments

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