BU-4794F, A NEW β-1,3-GLUCAN SYNTHASE INHIBITOR

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New β-1,3-glucan synthase inhibitor (BU-4794F) was isolated from the culture broth of *Gilmaniella* sp. FA4459. Structural studies indicated that it was a novel member of the papulacandin group of antibiotics.

The fungal cell wall contains a variety of glucans whose types are known to be species-specific. Since *Candida albicans* is a major pathogen in human systemic fungal infections, candida-specific beta-linked glucans which are produced by β-1,3-glucan synthase were assumed to be suitable targets for screening of new anticandidal compounds. In mass screening, *Candida albicans* (target pathogenic yeast) and *Rhodotorula glutinis* (negative control yeast containing no β-glucan) were used in parallel as test strains for primary differential assay, and primarily selected compounds which showed significant differential activity against these two strains were further subjected to the enzyme inhibition assay using β-1,3-glucan synthase prepared from *Candida albicans*.

In our search for β-1,3-glucan synthase inhibitors, a fungal strain identified as *Gilmaniella* sp. FA4459 was found to produce a novel antibiotic designated BU-4794F. This paper presents the taxonomy of the producing organism, fermentation, isolation, structure and biological activities of BU-4794F.

Taxonomy

Strain FA4459 was isolated from a soil sample collected in Yokohama City, Kanagawa Prefecture, Japan.

The color names and codes were determined by directly comparing the culture with the color chips (numbers in parentheses) of the Manual of Color Names (Japan Enterprise Co., Ltd., 1987).

Morphology

Morphological observations were made under a light microscope using the culture grown at 25°C on Potato-dextrose agar.

Submerged hyphae were smooth and hyaline, thin-walled, septate, and $1.5 \sim 4\,\mu m$ in width. Aerial hyphae were hyaline, turning to slightly brown, smooth or verrucose, and septate. Conidiogenous cells arose from both submerged and aerial hyphae, and formed laterally at right angles or intercalarily vegetative hyphae. They were smooth, thin-walled, cylindrical or clavate to pyriform in shape and $4 \sim 15\,\mu m \times 1.5 \sim 3.5\,\mu m$ in size, sometimes produced septate and elongated cells which were straight or flexuous. They were monoblastic or polyblastic, and usually formed solitary conidia at the tip. Lateral pairs or triplets of conidia arose occasionally in the apical region. The conidia were mostly spherical, dry, dark brown, smooth, thick-walled, and $7 \sim 12\,\mu m$ in diameter. They were one-celled with small but

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distinct apical spores of approximately 1 μm in diameter, and occurred singly or occasionally in short chains of longitudinal two or three spores. Teleomorph was not observed.

Cultural Characteristics

For cultural characterization, strain FA4459 was incubated for 7 days at 25°C using three agar media as described below.

Colonies on Bacto potato dextrose agar (Difco Laboratories) grew rapidly, attaining 65~70 mm in diameter and were thin and velvety. The surface color of colony was yellowish gray (402) and quickly turned into grayish olive (170) and finally olive black (429). The margin of colony was white. The reverse color was dark grayish green (244). Exudate and odor were absent.

Colonies on Bacto malt extract agar (Difco) reached at 38~40 mm in diameter, and were thin and velvety. The surface was grayish yellow (158). The margin was white. The reverse color was grayish olive (170). Exudate and odor were absent.

Colonies on Bacto Czapek solution agar (Difco) reached at 68~70 mm in diameter, were very loose-textured and colorless, and showed poor sporulation. Exudate and odor were absent.

Growth Conditions

This strain showed an optimum growth temperature of 34~36°C on a half strength malt extract agar slant, but did not grow at 40°C or higher.

The morphology and cultural characteristics mentioned above indicate that strain FA4459 belongs to the Hyphomycetes and is classified as a species of the genus *Gilmaniella* Barron based on the description of BARRON.[1)]

Fermentation

A well-grown slant culture of *Gilmaniella* sp. FA4459 was inoculated into a 500-ml Erlenmeyer flask containing 100 ml of seed medium composed of soluble starch (Nichiden Kagaku Co.) 2%, glucose 0.5%, NZ-case (Scheffield) 0.3%, yeast extract (Oriental Yeast Co.) 0.2%, Fish extract D30X (Banyu Eiyoh Co.) 0.5%, and $CaCO_3$ 0.3% (pH 7.0). The seed culture was incubated at 28°C for 1 day on a rotary shaker (200 rpm). A 5-ml portion of the seed culture was transferred into a 500-ml Erlenmeyer flask containing 100 ml of production medium composed of wheat bran (Nisshin Seifun Co.) 3%, glucose 0.2%, Pharmamedia (Traders Protein) 1.0%, NaCl 0.3%, K_2HPO_4 0.1%, and $CoCl_2$, $6H_2O$ 0.001% (pH 7.0). Fermentation was carried out at 28°C for 1 day on a rotary shaker (200 rpm). The antibiotic production in the fermentation broth was monitored by the broth microdilution assay using *Candida albicans* A9540.

Isolation and Purification

The mycelial cake which was separated from 18 liters of the fermented broth by filtration was extracted twice with methanol (10 liters each) and the methanolic extracts were concentrated to an aqueous solution. The solution was extracted three times with ethyl acetate (600 ml each) and the extracts were evaporated to dryness to afford a crude solid (6.7 g). The solid dissolved in methanol was applied on a column of silica gel (30 i.d. × 450 mm, Wako-gel C-200), which was washed with dichloromethane (500 ml) and then eluted gradiently with mixtures of dichloromethane - methanol (98 : 2~90 : 10, v/v). The eluates were monitored by the broth microdilution assay against *Candida albicans* and color reaction on TLC (SiO_2, CH_2Cl_2 - MeOH, 5 : 1). Active fractions were pooled and concentrated to yield a solid (100 mg) which was charged on a column of silica gel (15 i.d. × 200 mm, Wako-gel C-200). Gradient elution was performed

with ethyl acetate-methanol (98:2~95:5, v/v) and active eluate fractions were evaporated *in vacuo* to yield a semi-pure solid (50 mg). This solid was purified by reverse phase column chromatography (YMC ODS A, YMC Co., 22 i.d. × 420 mm), which was washed with acetonitrile-water (30:70, v/v) and then developed with acetonitrile-water (50:50, v/v) (Table 1). Active fractions were pooled, and concentrated *in vacuo*, and the aqueous concentrate was extracted with ethyl acetate. Evaporation of the solvent afforded a pure solid of BU-4794F (30.5 mg).

Physico-chemical Properties

BU-4794F was isolated as a white amorphous

Table 1. Physico-chemical properties of BU-4794F.

Nature	White amorphous powder
MP	205~210°C (dec)
$[\alpha]_D^{26}$	+62.6°±1 (*c* 0.5 MeOH)
Molecular formula	$C_{45}H_{58}O_{16}$
FAB-MS (*m/z*)	855 $(M+H)^+$, 877 $(M+Na)^+$
HRFAB-MS (*m/z*)	
Obs.	877.3636
Calc.	877.3623 ($C_{45}H_{58}O_{16}Na$)
UV λ_{max} nm (ε)	311 (sh, 24,700), 281 (51,900), 273 (52,800), 259 (sh, 41,900)
IR ν_{max}^{KBr} (cm^{-1})	3425, 2970, 2930, 2870, 1700, 1640, 1620, 1150, 1080, 1040, 1000
TLC (SiO_2, Merck F_{254})	$CHCl_3$-MeOH (5:1) Rf 0.37 EtOAc-$(CH_3)_2CO$-H_2O (72:24:4) Rf 0.29
HPLC[a]	Rt 10.9 minutes

[a] YMC ODS A-301-3, CH_3CN-H_2O (1:1) 1.0 ml/minute, UV 254 nm.

Fig. 1. Conidiogenous cells and conidia of strain FA4459 on Bacto potato dextrose agar.

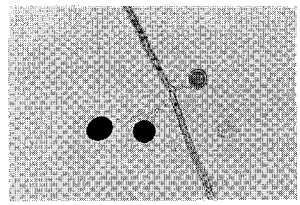


Fig. 2. UV spectrum of BU-4794F.

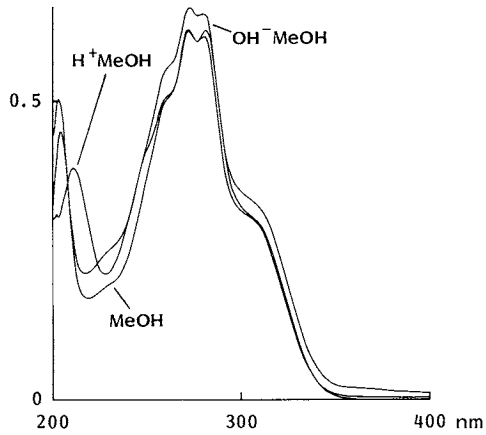


Fig. 3. IR spectrum of BU-4794F.

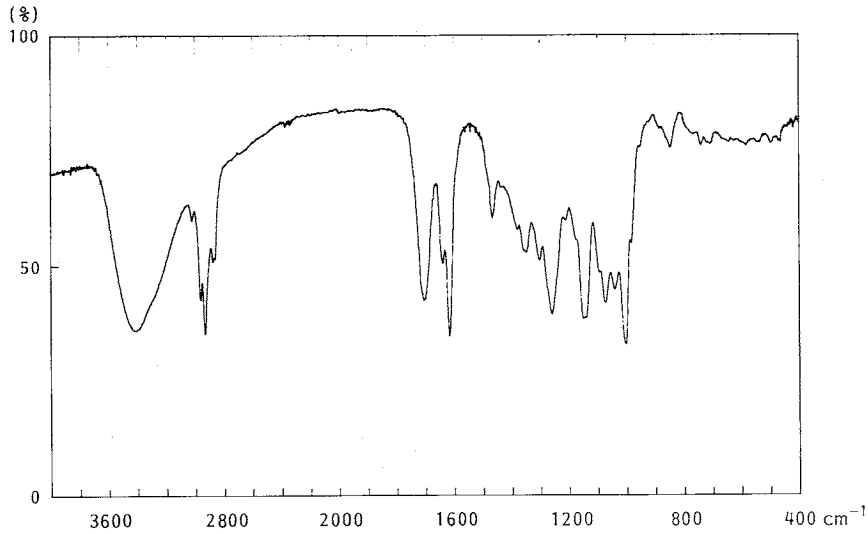


Fig. 4. ^{1}H NMR spectrum of BU-4794F (400 MHz in CD_3OD).

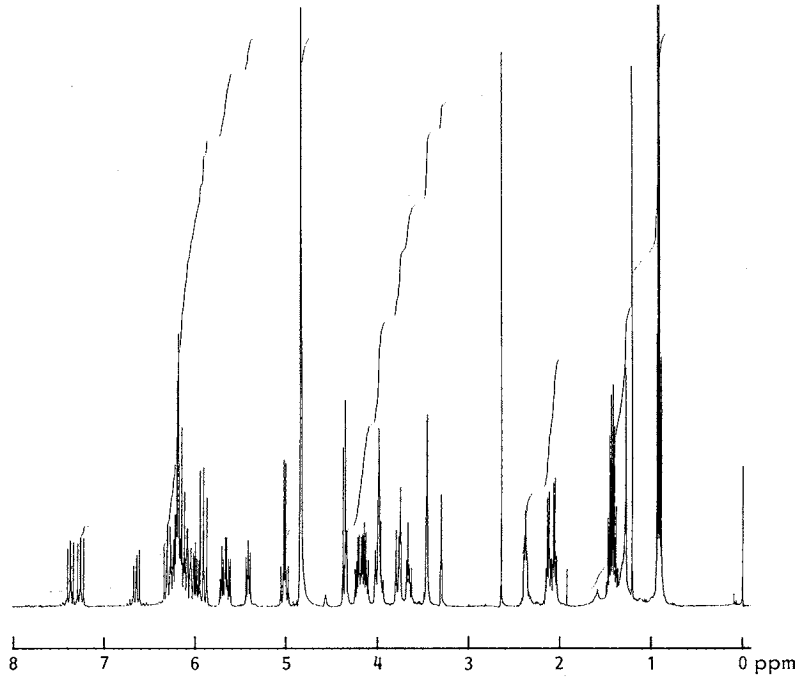
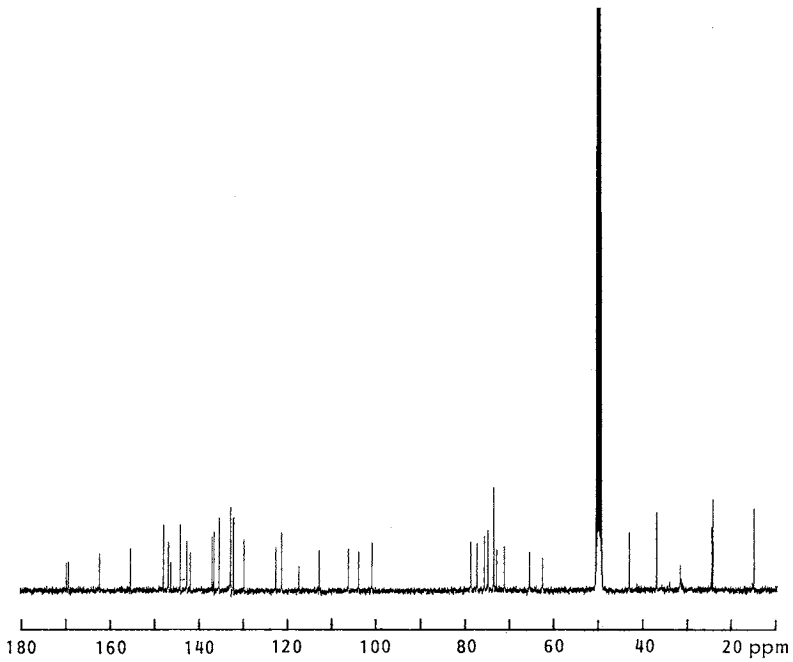


Fig. 5. ^{13}C NMR spectrum of BU-4794F (100 MHz in CD_3OD).



powder. It was soluble in lower alcohols and dimethyl sulfoxide, slightly soluble in ethyl acetate, benzene and dichloromethane, but practically insoluble in hexane and water. It showed positive responses to iodine vapor, ferric chloride, ammonium molybdate-sulfuric acid and anthrone but negative to ninhydrin

Table 2. Comparison of ^{13}C NMR data for BU-4794F and L-687,781[2] (CD_3OD, δ in ppm, mult).

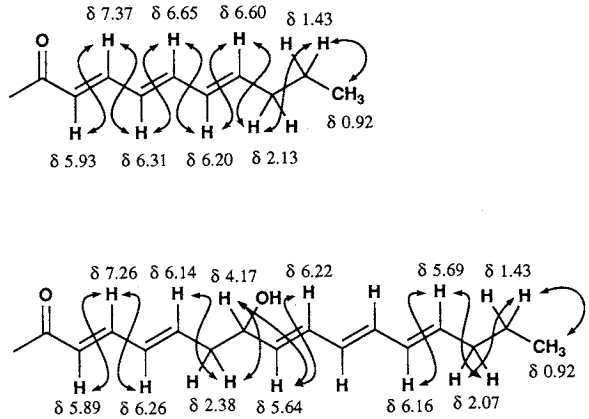
Carbon No.	L-687,781	BU-4794F	Carbon No.	L-687,781	BU-4794F
1	111.9	112.8 s	6″	40.0	43.0 t
2	71.8	72.8 d	7″	77.6	73.4 d
3	76.3	77.2 d	8″	137.5	136.6 d
4	77.7	78.6 d	9″	143.0	135.4 d
5	74.8	75.6 d	10″	131.5	132.8 d[a]
6	61.5	62.4 t	11″	136.2	132.9 d[a]
7	73.9	74.7 t	12″	31.6	132.0 d
8	145.5	146.3 s	13″	30.4	136.9 d
9	116.4	117.4 s	14″	35.2	36.7 t
10	161.6	162.4 s	15″	37.5	24.4 t
11	100.0	100.9 d	16″	11.7	14.8 q
12	154.5	155.4 s	17″	12.2	None
13	103.0	103.9 d	18″	19.5	None
1′	105.3	106.2 d	1‴	168.4	169.4 s
2′	72.5[a]	73.4 d	2‴	121.9	121.2 d
3′	70.4	71.0 d	3‴	141.3	147.9 d
4′	74.6[a]	75.5 d	4‴	127.6	129.7 d
5′	74.0	74.8 d	5‴	127.1	144.1 d
6′	64.9	65.4 t	6‴	25.9	132.2 d
1″	169.0	169.8 s	7‴	37.5	142.7 d
2″	121.6	122.5 d	8‴	73.2[a]	36.9 t
3″	146.0	146.8 d	9‴	31.1	24.1 t
4″	127.1	132.9 d	10‴	10.4	14.8 q
5″	141.6	141.9 d			

[a] Signals are interchangeable.

Table 3. ^1H NMR data of BU-4794F and L-687,781[2] (CD_3OD, δ in ppm, mult, J in Hz).

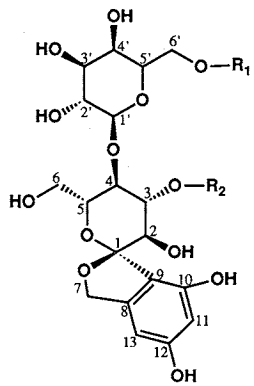
Carbon No.	L-687,781	BU-4794F	Carbon No.	L-687,781	BU-4794F
2	4.37 d 11	4.38 d 10	8″	None	5.64 dd 15, 11
3	5.43 dd 10, 11	5.42 dd 10, 11	9″	5.95	6.22
4	3.94	3.95	10″	6.25	6.06 *ca.* 15
5	4.00	3.95	11″	5.66 dt 17, 8	6.06
6	4.00, 3.78	3.95, 3.75	12″	2.12	6.16
7	5.03 ABq	5.03 ABq	13″	1.12	5.69 dt 15, 7
11	6.20	6.19	14″	1.38	2.07
13	6.22	6.20	15″	1.55	1.43 m 7
1′	4.34 d 8	4.33 d 8	16″	0.88 t 8	0.92 t 7
2′	3.46	3.45	17″	1.72 br s	—
3′	3.75 br, d 5	3.75	18″	0.88 d 7	—
4′	3.46	3.45	2‴	5.98 d 16	5.93 d 15
5′	3.68 ddd 8, 5, 2	3.66	3‴	7.78 dd 17, 13	7.37 dd 15, 12
6′	4.19 dd 12, 8	4.23 dd 11, 7	4‴	6.22	6.31 dd 15, 11
	4.12 dd 12, 5	4.13 dd 11, 6	5‴	5.95	6.65 dd 15, 11
2″	5.92 d 17	5.89 d 15	6‴	2.48 m	6.20
3″	7.27 dd 17, 13	7.26 dd 15, 11	7‴	1.55, 1.20	6.60
4″	6.25	6.26	8‴	3.43	2.13 m 8
5″	6.07 dt 17, 8	6.14 *ca.* 15	9‴	1.55, 1.12	1.43
6″	2.41 t 8	2.38 t 9	10‴	0.91 t 8	0.92 t 8
7″	4.05	4.17			

Fig. 6. ^{1}H-^{1}H COSY data of acyl side chains.

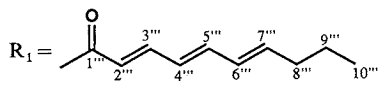


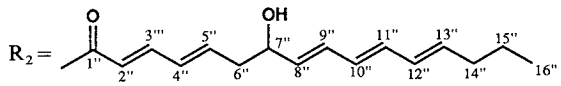
^{13}C-^{1}H COSY 8 Hz.

Fig. 7. Structures of BU-4794F and L-687,781.

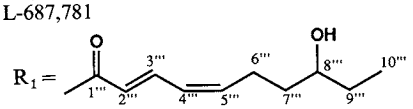


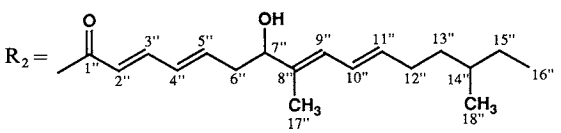
BU-4794F





L-687,781





and Rydon-Smith reagents. Physico-chemical properties are summarized in Table 1. The UV spectrum of BU-4794F (Fig. 2) shows absorption maxima at 273 and 281 nm which suggests the presence of a triene group in the molecule. Its IR spectrum (Fig. 3) was very similar to those of papulacandin analogs which showed strong absorbance at 1700 cm^{-1} (unsaturated ester carbonyl group). The molecular formula of BU-4794F was determined to be $C_{45}H_{58}O_{16}$ on the basis of the HRFAB-MS spectrometric data (*m/z* 877.3636 $(M+Na)^+$). The ^1H and ^{13}C NMR spectra are shown in Figs. 4 and 5, respectively.

Structural Studies

The IR, ^1H and ^{13}C NMR spectra of BU-4794F suggested its close resemblance to those of the papulacandin group of antibiotics, whereas its UV spectrum was clearly different from other papulacandin compounds in the presence of strong triene absorption at 273 and 281 nm. FAB-MS spectrometry gave a pseudomolecular ion peak at *m/z* 877 $(M+Na)^+$, and the molecular formula of $C_{45}H_{58}O_{16}$ was assigned to BU-4794F by the HRFAB-MS spectral analysis. ^{13}C NMR spectrometry exhibits 45 carbon signals which are comparatively assigned with those of L-687,781[2], a related papulacandin antibiotic (Table 2). Both compounds give very similar carbon signals in the spirocyclic core and two sugars moieties. In the ^1H NMR spectrum, the signals corresponding to the core and sugars of BU-4794F (2-H ~ 6′-H) also well coincide with those of L-687,781 (Table 3).

Major differences between those antibiotics were observed in the two acyl side chains which are composed of the remaining structural units ($C_{26}H_{34}O_3$) of BU-4794F. The detailed ^1H-^1H COSY spectral analysis elucidated the structures of these side chains as shown in Fig. 6. More particularly, the C_{10} side chain unit contains a triene carbonyl group and all the double bonds are estimated to have *Z* configurations by the large coupling constants ($J=15$ Hz) of the corresponding olefinic protons. This unit is probably linked at C-6′ position, as the abundant fragment ion which probably occurred from cleavage of the glycoside bond of the sugar moiety was observed at *m/z* 331 ($C_{16}H_{23}O_6$) in the FAB-MS spectrum. The C_{16} side chain has a diene carbonyl group in addition to the triene function. The geometrical configurations of all the double bonds were shown to be *Z*. This unit was considered to be attached to C-3 position of the core structures of papulacandins. All protons and carbons of BU-4794F were assigned as shown in Tables 2 and 3 by the aid of 2D NMR techniques such as ^1H-^{13}C COSY and ^1H-^{13}C long range COSY, and the structure of BU-4794F was established as shown in Fig. 7. In comparison with the structure of L-687,781, BU-4794F is novel in the two acyl side chains. It has the additional double bond at C-6‴

Table 4. Antifungal activity of BU-4794F.

Organism	BU-4794F	Papulacandin B	Papulacandin M	Amphotericin B	Ketoconazole
Saccharomyces cerevisiae ATCC 9763	0.4	6.3	1.6	0.4	50
Candida albicans IAM 4888	0.2	1.6	0.4	0.2	25
C. albicans A9540	0.2	1.6	0.8	0.2	25
C. albicans ATCC 32354 (B311)	0.2	1.6	0.8	0.2	50
C. albicans 83-2-14	0.1	1.6	0.4	0.2	25
C. tropicalis 85-5	0.2	3.1	0.8	0.4	25
C. tropicalis IFO 10241	0.2	3.1	0.8	0.2	50
Cryptococcus neoformans D49	>100	>100	>100	0.2	25
C. neoformans IAM 4514	>100	>100	>100	0.2	50
Aspergillus fumigatus IAM 2034	>100	>100	>100	0.4	3.1

MICs were determined using the broth micro-dilution assay: Medium, glucose - yeast nitrogen base (1% glucose, pH 7.0); inoculum size, 10^5 cells/ml; incubation conditions, 37°C, 24 hours.

position of the C_{10} side chain and no hydroxy group at C-8‴ position. Furthermore, BU-4794F has no methyl group at C-14″ position but possesses an additional double bond at C-12″ position of the C_{16} side chain, resulting in the triene functional group which showed its characteristic UV absorption.

Antifungal Activity

Minimum inhibitory concentrations (MICs) were determined by the two fold serial broth dilution method. Yeast nitrogen base +1% glucose (YNBG, pH 7.0) was used as the assay medium, and the inoculum size was adjusted to 10^5 cfu/ml. Incubation was carried out at 37°C for 24 hours without agitation. Papulacandins B and M, amphotericin B and ketokonazole were used as the reference compounds. Papulacandin M has also been studied as BU-4785F in our institute and recently identified so by direct comparison with the authentic sample.

As summarized in Table 4, BU-4794F exhibits potent inhibitory activity against yeasts but is inactive against filamentous fungi and *Cryptococcus neoformans* in contrast to other members of the papulacandin family. The MIC values of BU-4794F against *Candida* strains are comparable to those of amphotericin B, but lower than those of papulacandins B and M.

Inhibition of β-1,3-Glucan Synthase

β-1,3-Glucan synthetase activity was determined by analysis of the incorporation of ^{3}H-UDP-glucose into digitonin-treated *C. albicans* protoplasts.

C. albicans A9540 (OD_{660} 0.1～0.3 in early log phase) was cultivated at 28°C for 18 hours in YPD medium (1% yeast extract, 2% Polypepton and 2% glucose) and then harvested by centrifugation for 5 minutes at 4°C and 3,000 rpm. Intact *C. albicans* cells were transformed to protoplasts by the method of ARIMA and TAKANO[3]. The protoplasts (1×10^8 protoplasts/ml) were collected and suspended in 50 mM Tris-HCl buffer, pH 7.5, containing 0.2% digitonin and 1 mM ethylenediaminetetraacetic acid (EDTA) and homogenized with a tissue grinder (Kontes Co., Ltd.). This digitonin-treated protoplast suspension was employed for the β-1,3-glucan synthetase assay. Twenty-five microliters of the digitonin-treated suspension (crude enzyme) were distributed into each microplate well and then mixed with 25 μl of a test sample solution. The incorporation of radio-labeled UDP-glucose into glucan by β-1,3-glucan synthetase was started by adding 50 μl of 50 mM Tris-HCl buffer, pH 7.5, containing ^{3}H-UDP-glucose (3 KBq/well: TBK. 385 from Amersham Co., Ltd.), 0.5 mM UDP-glucose (cold), 2 mM EDTA, 0.16 mM guanosine 5′-triphosphate and 10 mM adenosine 5′-triphosphate. After incubation at 30°C for 2 hours, the reaction was terminated by adding 100 μl of 10% trichloroacetic acid (TCA). The precipitated products were harvested from each microplate well on a glass fiber Beta-platemat, and then dried at 80°C for 30 minutes before counting. The radio-activity was measured in a Pharmacia-Wallac 1205 Betaplate liquid scintillation counter system.

Under such assay conditions, the IC_{50} value of BU-4794F was determined to be 0.25 μg/ml.

Discussion

Gilmaniella sp. FA4459 produced BU-4794F as a new β-1,3-glucan synthase inhibitor. Structural studies revealed that it is a new member of the papulacandin family of antibiotics. Among them, BU-4974F is similar to papulacandins C[4,5] and M[6,7] in the presence of a triene group at the C_{10} acyl side chain, but is differentiated from other papulacandin members in having a unique C_{16} side chain with another triene group which yields a characteristic UV absorption band. BU-4794F showed potent *in vitro* antifungal

activity and strongly inhibited β-1,3-glucan synthase prepared from *C. albicans*. Recently, β-1,3-glucan synthesis has been identified as an effective target for *Pneumocystis carinii* pneumonia which is a common opportunistic infection in AIDS patients. It is interesting to note that L-687,781[8], another papulacandin analog, has been reported to show anti-pneumocystis activity in a rat model; and so BU-4794F is also to be expected as a new therapeutic agent effective against *Pneumocystis*.

Acknowledgment

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