BE-29602, a New Member of the Papulacandin Family

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(Received for publication September 11, 1995)

In the course of our screening program for new antifungal antibiotics, a fungal strain F29602 was found to produce a new antifungal antibiotic, BE-29602 (**1**), which belonged to the family of Papulacandin. This strain, F29602, was isolated from a soil sample collected in Saitama Prefecture, Japan. Based on the cultural and morphological characteristics, strain F29602 was identified as belonging to the genus *Fusarium*. The strain F29602 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Japan (No. FERM P-12503). This paper describes the fermentation, isolation, structure elucidation and biological properties of **1**.

Spores of strain F29602 grown on agar slant medium were inoculated into four 500-ml conical flasks, each containing 110 ml of a medium (pH 6.0) comprising of glucose 1.0%, maltose 3.0%, polypeptone 0.3%, wheat germ 1.0%, corn gluten meal 0.5%, malt extract 0.3%, NaCl 0.2%, $NaNO_3$ 0.1%, KH_2PO_4 0.1%, $MgSO_4 \cdot 7H_2O$ 0.05%, $FeSO_4 \cdot 7H_2O$ 0.0002%, $CuCl_2 \cdot 2H_2O$ 0.0004%, $MnCl_2 \cdot 4H_2O$ 0.0004%, $CoCl_2 \cdot 6H_2O$ 0.0004%, $ZnSO_4 \cdot 7H_2O$ 0.00008%, $Na_2B_4O_7 \cdot 10H_2O$ 0.00008% and $(NH_4)_6Mo_7O_{24} \cdot 10H_2O$ 0.00024%. The flasks were shaken on a rotary shaker (180 rpm) at 28°C for 72 hours. Two ml each of the seed culture were inoculated into one hundred 500-ml conical flasks containing 110 ml of the above medium and cultured on a rotary shaker at 28°C for 72 hours.

The mycelial cake obtained by filtration of the cultural broth (*ca.* 10 liters) was extracted twice with methanol (4 liters × 2). The methanolic extracts were concentrated to about 600 ml, and then the aqueous solution was extracted twice with ethyl acetate (500 ml × 2). The ethyl acetate layer was concentrated under reduced pressure and applied to a column of silica gel (3 i.d. × 45 cm, Kieselgel 60, Merck). After washing the column with chloroform (500 ml), the anticandidal active material was eluted with chloroform-methanol (9:1, 1 liter) and chloroform-methanol (4:1, 1 liter). The active eluate was concentrated to yield 550 mg of crude material, which was applied on a second column of silica gel (2 i.d. × 45 cm). BE-29602 containing fractions were obtained by eluting with chloroform-methanol (9:1, 1 liter) and chloroform-methanol (4:1, 1 liter) and evaporated *in vacuo* to obtain 220 mg of active residue. This active residue was finally purified by Sephadex LH-20 column chromatography (1.5 i.d. × 90 cm, methanol). The fractions containing **1** were evaporated *in vacuo* to obtain 16.0 mg of pure **1** as white amorphous powder.

Physico-chemical properties of **1** are summarized in Table 1. BE-29602 was soluble in methanol and dimethyl sulfoxide (DMSO), but insoluble in chloroform and water. The molecular formula of **1** was established as $C_{45}H_{62}O_{16}$ from the HRFAB-MS and NMR spectral analyses. The IR spectrum of **1** showed the presence of α,β-unsaturated ester at 1710 cm^{-1}. NMR data of **1** are shown in Table 2 in comparison with those of L-687,781 (**2**)[1]. In the ^1H NMR spectrum of **1**, fourteen olefinic and aromatic protons were observed in the range between 5.5 and 7.7 ppm, and there was evidence for two sugar moieties. These data suggested that **1** belonged to papulacandin family antibiotics[2,3]. Indeed, the ^1H NMR data for **1** in the two sugar moieties and spirocyclic core were almost identical to those for **2**[1]. Fatty acid moieties A and B in Fig. 1 were elucidated by analyzing the ^1H-^1H COSY, 2D-homonuclear Hartmann-Hahn (HOHAHA) and heteronuclear multiple quantum coherence (HMQC) spectra of **1**. The geometry of C-2″, C-2‴, C-4″, C-8″ and C-10″ were confirmed to be all *E* by the coupling constants of $J_{2''\sim3''}$ = 15.6 Hz, $J_{2'''\sim3'''}$ = 15.6 Hz, $J_{4''\sim5''}$ = 15.6 Hz, $J_{8''\sim9''}$ = 15.2 Hz and $J_{10''\sim11''}$ = 15.0 Hz. The coupling constant of the 4‴-H–5‴-H double bond (10.0 Hz) was determined by decoupling at 6‴-H (2.33 ppm) and the geometry of C-4‴ was confirmed to be *Z*. The connectivity of fatty acids to the two sugar moieties was confirmed from the heteronuclear multiple-bond correlations (HMBC) spectrum of **1**. The carbonyl carbon at δ_C 169.1 was coupled to the 3‴-H (7.70 ppm) and 6′-H (4.24 ppm) protons, and also the carbonyl carbon at δ_C 169.5 was coupled to the 3″-H (7.28 ppm) and 3-H (5.42 ppm) protons. These observa-

Table 1. Physico-chemical properties of BE-29602 (**1**).

Appearance	White amorphous powder
Molecular formula	$C_{45}H_{62}O_{16}$
HRFAB-MS(m/z)	
Found:	859.4147 $(M+H)^+$
Calcd:	859.4111
UV (MeOH) λ nm(ε)	225(20,700), 231(20,200) sh, 264(31,800)
IR ν_{max}(KBr) cm^{-1}	3418, 2932, 2860, 1710, 1641, 1620, 1344, 1269, 1149, 1071, 1038, 1002
TLC (Rf)[a]	0.37
HPLC (Rt, Minutes)[b]	14.5

[a] Kieselgel 60 (F_{254}), Merck; solvent: $CHCl_3$-MeOH (4:1).

[b] Column: Chromatorex ODS (250 × 4.6 mm i.d.); mobile phase: CH_3CN-0.04% TFA (3:2); flow rate: 1.0 ml/minute; detection: 265 nm.

Table 2. ^{13}C and ^{1}H NMR for BE-29602 and L-687,781 (CD_3OD, δ in ppm).

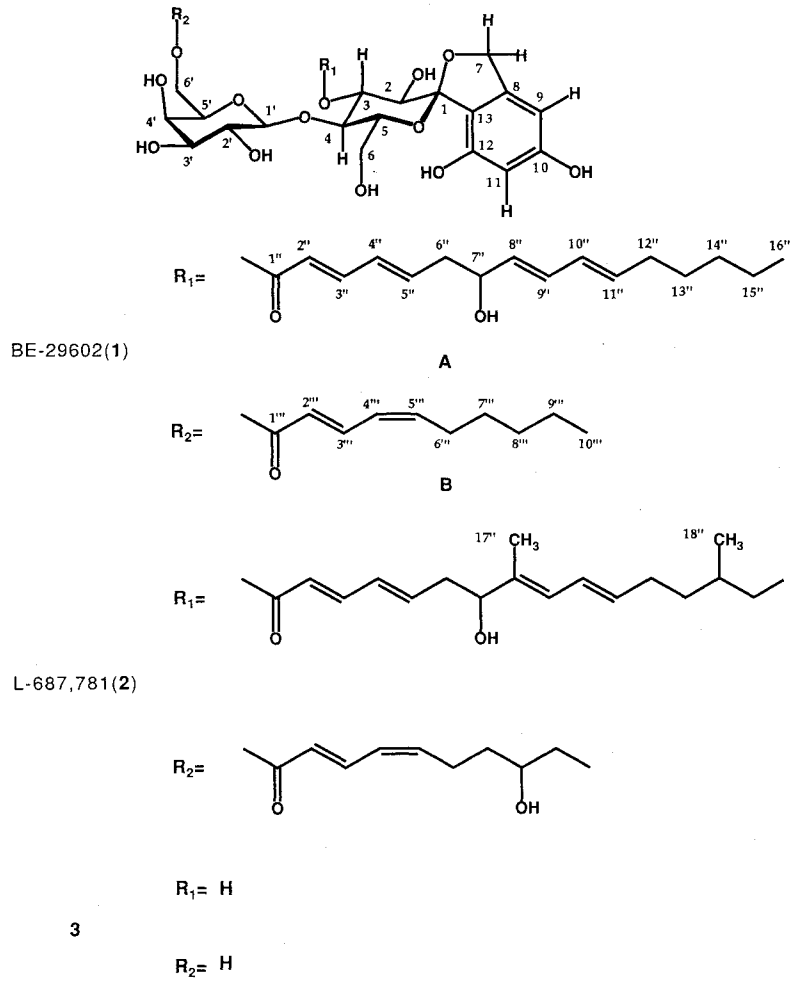
	BE-29602			L-687,781[a]		
	^{13}C NMR (100 MHz)	^{1}H NMR (400 MHz)		^{13}C NMR	^{1}H NMR	
1	112.4	–		111.9	–	
2	72.4	4.36	d, 10.2[b]	71.8	4.37	d, 11[b]
3	76.9	5.42	dd, 10.2, 8.2	76.3	5.43	dd, 10, 11
4	78.3	3.94		77.7	3.94	
5	75.3	3.98	m	74.8	4.00	
6	62.1	3.78, 4.01	m	61.5	4.00, 3.78	
7	74.4	5.00, 5.04	d, 12.8	73.9	5.03	AB q
8	146.0	–		145.5	–	
9	117.0	–		116.4	–	
10	155.1	–		161.6	–	
11	103.5	6.20	brs	100.0	6.20	
12	162.1	–		154.5	–	
13	100.5	6.18	brs	103.0	6.22	
1'	106.0	4.34	d, 7.4	105.3	4.34	d, 8
2'	75.2*	3.46	m	72.5*	3.46	
3'	73.1*	3.46	m	70.4	3.75	brd, 5
4'	70.8	3.75	brs	74.6*	3.46	
5'	74.4	3.68	t, 6.2	74.0	3.68	ddd, 8, 5, 2
6'	65.2	4.15	m	64.9	4.29	dd, 12, 8
		4.24	dd, 11.6, 6.2		4.12	dd, 12, 5
1''	169.5	–		169.0	–	
2''	122.2	5.90	d, 15.6	121.6	5.92	d, 17
3''	146.5	7.28	dd, 15.6, 10.8	146.0	7.27	dd, 17, 13
4''	132.5	6.27	dd, 15.6, 10.8	127.1	6.25	
5''	141.7	6.16[c]		141.6	6.07	dt, 17, 8
6''	42.8	2.38	m	40.0	2.41	t, 8
7''	73.1	4.15	m	77.6	4.05	
8''	134.5	5.56	dd, 15.2, 6.7	137.5	–	
9''	132.6	6.18[c]		143.0	5.95	
10''	131.6	6.01	dd, 15.0, 10.8	131.5	6.25	
11''	136.6	5.67	dt, 15.0, 6.9	136.2	5.66	dt, 17, 8
12''	34.1	2.06	m	31.6	2.12	
13''	30.6	1.22~1.50	m	30.4	1.12	
14''	33.1	1.22~1.50	m	35.2	1.38	
15''	24.1	1.22~1.50	m	37.5	1.55	
16''	14.9	0.90	t, 6.8	11.7	0.88	t, 8
17''				12.2	1.72	bs
18''				19.5	0.88	d, 7
1'''	169.1	–		168.4	–	
2'''	122.2	5.98	d, 15.6	121.9	5.98	d, 16
3'''	142.0	7.70	dd, 15.6, 11.8	141.3	7.78	dd, 17, 13
4'''	128.1	6.22[c]		127.6	6.22	
5'''	143.8	5.95	m	127.1	5.95	
6'''	29.7	2.33	m	25.9	2.48	m
7'''	30.6	1.22~1.50	m	37.5	1.55, 1.20	
8'''	33.0	1.22~1.50	m	73.2*	3.43	
9'''	24.1	1.22~1.50	m	31.1	1.55, 1.12	
10'''	14.9	0.91	t, 6.8	10.4	0.91	t, 8

[a] Data in ref. 1.
[b] Multiplicity, J in Hz.
[c] Overlapping signals.
* Signals are interchangeable.

tion implies that C_{16} side chain should connect to C-3 position and C_{10} side chain should connect to the C-6' position as well as papulacandins[3].

The ^{1}H and ^{13}C NMR assignments for two sugar moieties and aromatic group of **1** were performed by the ^{1}H-^{1}H COSY, 2D-HOHAHA, rotating frame NOESY (ROESY), HMQC and HMBC spectra. In the HMBC spectrum of **1**, the C-12 carbon at δ_C 162.1 was correlated with the protons at δ_H 6.18 and 6.20, and the C-10 carbon at δ_C 155.1 was correlated with only one proton at δ_H 6.20. The NOE signal between the methylene protons and the aromatic proton at δ_H 6.18 was observed from the ROESY spectrum of **1**, and the methylene protons were coupled to the carbon at δ_C 100.5 (C-13). From these data, the assignments for an aromatic group were unambiguous. In the 2D-HOHAHA spectrum of **1**, the signal at δ_H 3.68 (5'-H) was correlated with δ_H 3.75 (4'-H) besides δ_H 4.15 and 4.24 (6'-H), and furthermore 6'-H proton at δ_H 4.24 was coupled to the carbon at δ_C 70.8 (C-4') in the HMBC spectrum of **1**. From these data, the

Fig. 1. Structures of BE-29602 (**1**), L-687,781 (**2**) and **3**.



assignments for galactose moiety could be established except for positions C-2′ and C-3′. Therefore, we consider that the assignments of **2** published earlier (Table 2)[1] are incorrect.

BE-29602 was hydrolyzed by the same method reported previously[3]. Hydrolysis of **1** with sodium hydroxide gave **3** which showed identical ^{1}H and ^{13}C data to the hydrolysis product from **2**[1]. From the optical rotation value of **3** ($[\alpha]_D = +28.4°$, C=0.578, MeOH), the absolute configuration of **3** was assigned to be identical to that of papulacandin B[3].

From the data mentioned above, the structure of **1** was determined as shown in Fig. 1.

Papulacandins were first isolated by TRAXLER *et al.* and the structures of papulacandins A, B, C and D were determined. Thereafter, several papulacandin family compounds such as **2**[1,4], Mer-WF3010[5,6] and Bu-4794F[7] were reported. These compounds, except papulacandin D[3] and Bu-4794F, have identical spirocyclic diglycosides and a C_{16} side chain at the C-3 position. Papulacandin D lacks the galactose and C_{10} side chain[3]. Bu-4794F has no methyl groups at C-8″ and C-14″ positions but possesses a triene functional group in C_{16} side chain. A related compound, chaetiacandin, has a benzyl alcohol group instead of a spirocycle[8,9]. The two side chains of chaetiacandin are distinct from those of papulacandins. BE-29602 has a spirocyclic diglycoside, but its C_{10} side chain at C-6′ position is identical to that of chaetiacandin. Furthermore, **1** has no methyl groups at C-8″ and C-14″ positions, so that the structure between C1″ and C14″ is identical to that of the C_{14} side chain of chaetiacandin at C-3 position.

Minimum inhibitory concentrations (MICs) of **1** were determined by the two-fold serial dilution method with medium agar. BE-29602 showed potent growth inhibition against several yeasts such as *Saccharomyces cerevisiae* and *Candida albicans* (Table 3). Although the growth of *Trichophyton mentagrophytes* and *Staphylococcus aureus* was partly restricted by **1** at the dose of 12.5 μg/ml, they were not inhibited completely by **1** at the dose of 100 μg/ml.

Cytotoxicity of **1** was measured using the P388 mouse leukemic cell line. RPMI 1640 medium containing 10% fetal calf serum and 20 μM 2-mercaptoethanol was used for the culture of P388 cells (1×10^4 cells/ml). After incubation under 5% CO_2 at 37°C for 24 hours, the test

Table 3. Antimicrobial activity of BE-29602 (1).

Test organism	MIC (μg/ml)
Candida albicans Yu 1	0.39
Candida albicans IFO 1270	0.39
Candida albicans IFO 1385	0.20
Saccharomyces cerevisiae IFO 0283	1.56
Shizosaccharomyces pombe IAM 4863	0.78
Trichophyton mentagrophytes TIMM 1189	>100
Aspergillus niger IFO 31012	>100
Penicillium chrysogenum IFO 16223	3.13
Bacillus subtilis ATCC 6633	6.25
Bacillus cereus IFO 3001	12.5
Staphylococcus aureus Smith	>100
Micrococcus luteus ATCC 9341	12.5
Esherichia coli NIHJ JC-2	>100
Serratia marcescens IFO 3736	>100
Pseudomonas aeruginosa IFO 3445	>100

sample was added in DMSO at a final concentration of 0.5%. This mixture was further incubated for 72 hours. The viable cells were detected by measuring the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide (MTT) at OD 550 nm. The concentration of **1** required to inhibit growth of the P388 mouse leukemia cell lines by 50% (IC_{50}%) was 21 μg/ml.

The papulacandins are thought to kill fungi by inhibiting β-1,3-glucan synthesis[4,7,10]. Recent studies have revealed the possibility of β-1,3-glucan synthesis as an effective target for *Pneumocystis carinii* pneumonia which is reported to be a major cause of death in AIDS patients[11]. BE-29602 is also expected to inhibit β-1,3-glucan synthesis, and may be effective against fungal infections and *P. carinii* pneumonia.

References

1) VANMIDDLESWORTH, F.; C. DUFRESNE, J. SMITH & K. E. WILSON: Structure elucidation of L-687,781, A new β-1,3-D-glucan synthesis inhibitor. Tetrahedron 47: 7563～7568, 1991
2) TRAXLER, P.; J. GRUNER & J. A. L. AUDEN: Papulacandins, a new family of antibiotics with antifungal activity. I. Fermentation, isolation, chemical and biological characterization of papulacandins A, B, C, D and E. J. Antibiotics 30: 289～296, 1977
3) TRAXLER, P.; H. FRITZ, H. FUHRER & W. J. RICHTER: Papulacandins, a new family of antibiotics with antifungal activity. Structures of papulacandins A, B, C, and D. J. Antibiotics 33: 967～978, 1980
4) VANMIDDLESWORTH, F.; M. N. OMSTEAD, D. SCHMATZ, K. BARTIZAL, R. FROMTLING, G. BILLS, K. NOLLSTADT, S. HONEYCUTT, M. ZWEERINK, G. GARRITY & K. WILSON: L-687,781, a new member of the papulacandin family of β-1,3-glucan synthesis inhibitors. I. Fermentation, isolation, and biological activity. J. Antibiotics 44: 45～51, 1991
5) KANETO, R.; H. CHIBA, H. AGEMATU, N. SHIBAMOTO, T. YOSHIOKA, H. NISHIDA & R. OKAMOTO: Mer-WF3010, A new member of the papulacandin family. I. Fermentation, isolation and characterization. J. Antibiotics 46: 247～250, 1993
6) CHIBA, H.; R. KANETO, H. AGEMATU, N. SHIBAMOTO, T. YOSHIOKA, H. NISHIDA & R. OKAMOTO: Mer-WF3010, A new member of the papulacandin family. II. Structure determination. J. Antibiotics 46: 356～358, 1993
7) AOKI, M.; T. ANDOH, T. UEKI, S. MASUYOSHI, K. SUGAWARA & T. OKI: BU-4794F, A New β-1,3-glucan synthesis inhibitor. J. Antibiotics 46: 952～960, 1993
8) KOMORI, T.; M. YAMASHITA, Y. TSURUMI & M. KOHSAKA: Chaetiacandin, a novel papulacandin. I. Fermentation, isolation and characterization. J. Antibiotics 38: 455～459, 1985
9) KOMORI, T. & Y. ITOH: Chaetiacandin, a novel papulacandin. II. Structure determination. J. Antibiotics 38: 544～546, 1985
10) PEREZ, P.; R. VARONA, I. GARCIA-ACHA & A. DURAN: Effect of papulacandin B and aculeacin A on β-(1,3)glucan-synthase from *Geotrichum lactis*. FEBS Lett. 129: 249～252, 1981
11) SCHMATZ, D. M.; M. A. ROMANCHECK, L. A. PITTARELLI, R. E. SCHWARTZ, R. A. FROMTLING, K. H. NOLLSTADT, F. L. VANMIDDLESWORTH, K. E. WILSON & M. J. TURNER: Treatment of Pneumocystis carinii pneumonia with 1,3-β-glucan synthesis inhibitors. Proc. Natl. Acad. Sci. U.S.A. 87: 5950～5954, 1990