A New Antifungal Macrolide Component, Brasilinolide B, Produced by *Nocardia brasiliensis*

YUZURU MIKAMI*, HISAYUKI KOMAKI, TAMAE IMAI and KATSUKIYO YAZAWA

Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, 1-8-1, Inohana, Chuo-ku, Chiba 260-8673, Japan

AKIRA NEMOTO and YASUSHI TANAKA

Research Laboratory, Higeta Shoyu Co., Ltd., Chuo-ku, Choshi, Chiba 288-8680, Japan

UDO GRÄEFE

Hans-Knoell-Institute of Natural Products Research, Beutenbergstrasse 11, Jena, Germany

(Received for publication September 17, 1999)

Our continuing studies on biologically active compounds from pathogenic microorganism[1,2] showed that *Nocardia brasiliensis* IFM 0466 produces a new antifungal brasilinolide group antibiotic[3~5], brasilinolide B (**1**), distinguishable from brasilinolide A[3~5] by its chromatographic properties. This paper describes the fermentation, isolation and structural elucidation of this macrolide (**1**).

The seed broth was prepared by inoculating mycelial elements of the producing strain (*N. brasiliensis* IFM 0466) grown on Sabouraud dextrose agar (Difco) in 10 ml brain heart infusion broth with 2% glucose in 50-ml Erlenmeyer shake flasks. The culture was incubated on a rotary shaker at 250 rpm for 96 hours. Ten percent inoculum was transferred to a 2.0-liter fermenter containing 1.0 liter of the production medium composed of meat extract 0.5%, peptone 0.5%, glucose 2.0% and supplemented with antifoam 0.05%. The pH was adjusted to 7.4. The jar fermenter was stirred at 500 rpm with aeration at 1.0 liter/minute at 30°C for 4 days. After 4 days of incubation, one and one half volume of methanol was added to the culture broth and further incubated for 3 hours to kill the *Nocardia* and to extract the active substance from the mycelia. Thereafter the broth was filtered and evaporated under reduced pressure. The crude residue from 10-liter cultures was then subjected to silica gel chromatography using $CHCl_3$-MeOH (1:0, 10:1, 5:1). Active fractions were obtained with $CHCl_3$-MeOH (5:1) when antifungal activity was tested against *Aspergillus niger* IFM 40406. The combined active fraction was purified by preparative reverse phase HPLC (column; Capcell pal SG120 C18, 3×25 cm, Shiseido Co., Ltd., linear gradient elution by MeCN-MeOH (4:1) from 20% to 100% for 80 minutes at the flow rate of 30 ml/minute). The active fractions eluting at 43~55 minutes were combined and further purified using NH-silica gel column chromatograpy (Chromatorex NH [Fuji Silysia Chemical Ltd.], column size; 1.8×10 cm, elution; $CHCl_3$-MeOH=1:0, 20:0, 10:0, and 5:1, 200 ml each). The active fraction was obtained from 10:1 to 5:1 eluents, and combined. From 10 liter cultures, 19.2 mg of brasilinolide B was obtained.

The structure of brasilinolide B (**1**, relative stereochemistry) as shown in Fig. 1 was settled on the basis of the spectroscopic and mass spectrometric data (Tables 1 and 2). The IR spectrum displayed the presence of a double bond (1645 cm^{-1}), three carbonyl groups (1720, 1726, 1732 cm^{-1}) and hydroxyl groups (3425 cm^{-1}). The molecular weight of 1180 Da and the chemical formula of **1** ($C_{59}H_{104}O_{23}$; see Table 1) were suggested by *m/z* 1203.6890 in the HRESI-MS ($[M+Na]^+$, calcd. 1203.6888). In addition, *m/z* 613.5 ($[M+2Na]^{2+}$) was observed. The appearance of *m/z* 1179.7 in the negative ion-mode ESI-MS ($[M-H]^-$) confirmed the molecular weight of **1**. CID-MS/MS of *m/z* 1198 (30% intensity, $[M+NH_4]^+$) furnished *m/z* 815.3 (100% intensity) which is suggested to represent on olefinic structure which is formed *via* the elimination of both the sugar and the malonyl side chain, and the splitting of oxygen from the epoxide structure as well. The same type of fragment was also observed during ion source-CID of *m/z* 1203.3 using an electrospray ion source coupled to an ion trap mass analyzer. These mass spectrometric results support the contention that **1** contains the same macrolide skeleton as brasilinolide A. MS^n experiments using an ESI-coupled ion-trap analyzer furnished a series of diagnostic fragment ions (positive ion mode: MS^2 of *m/z* 1203: ($[M+Na]^+$): *m/z* 1185.8, 1159.8 and 1129.7, MS^3 of *m/z* 1185.8: *m/z* 1141.7 and 1111.7, MS^4 of *m/z* 1111.7: *m/z* 1067.7, MS^5 of *m/z* 1067.7: *m/z* 805.7, 879.8, 1023.6, MS^6 of *m/z* 1023.6: *m/z* 893.5 and 708.0). Negative ion MS^n experiments resulted in the eliminations of two H_2O fragments. The chemical formula $C_{59}H_{104}O_{23}$ suggested the presence of 8 double bonds. Compared to brasilinolide A, **1** is missing a double bond and oxygen, but has additional two carbons and six protons. Conclusive evidence for the structure of **1** was furnished by the one and two-dimensional ^1H and ^{13}C NMR spectra of **1** (COSY, DEPT,

Fig. 1. Structure (structure and relative stereochemistry) of brasilinolide B (**1**).

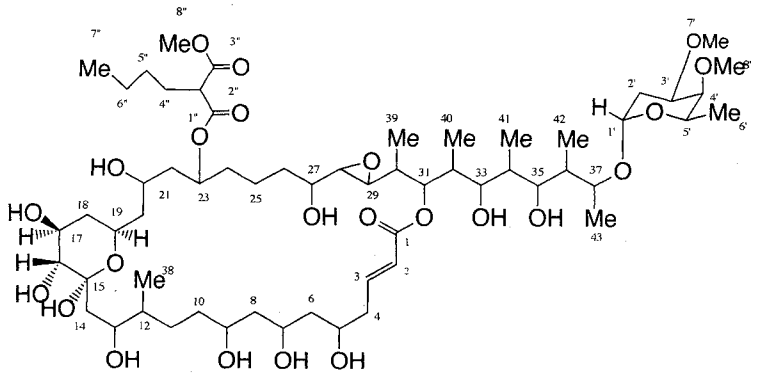


Table 1. Physico-chemical properties of brasilinolides B.

Appearance	white microcrystals
Molecular weight	1180
Formula	$C_{59}H_{104}O_{23}$
HRESI-MS	([M+Na]$^+$: m/z 1203.6890, calcd. 1203.6888)
$IR_{\lambda max}$ (cm^{-1}, in KBr)	963, 985, 1027, 1085, 1092, 1153, 1194, 1230, 1257, 1315, 1350, 1378, 1445, 1453, 1645, 1720, 1726, 1732, 2925, 3425

HSQC, HMBC, NOESY, TOCSY). Comparison with the published data for brasilinolide A[1] was particularly helpful during the assignment of the many overlapping macrolide ring protons and carbons.

Thus, the ^{1}H spectrum of **1** showed obvious similarities with that of brasilinolide A due to the diagnostic signals of the macrolide ring[5]. The protons of the (*E*)-double bond next to the lactone carbonyl (5.9 ppm, d, 14.8 Hz; 6.9 ppm, d, 14.8 Hz; t, 6.8 Hz) and the six doublet methyl signals (0.93, 0.75, 0.73, 0.96, 0.47 and 1.0 ppm) were readily recognized as characteristic features. In addition, a triplet 1H signal at 3.48 ppm (H-2″, 8.0 Hz) coupling with H-4″ (1.78 ppm, COSY) was compatible with the presence of an alkyl-substituted malonic acid ester side chain (see also the singlet methoxyl proton H-8″ at 3.62 ppm). The anomeric proton at 4.9 ppm (triplet, broad), two additional methoxyl proton signals at 3.29 and 3.31 ppm, respectively, and one sugar methyl group (1.08 ppm) were of also diagnostic importance.

The eleven hydroxyl protons appearing with the exception of 15-OH as doublets (5.0 Hz) supported the assignment of the macrolide protons *via* the COSY spectrum and carbons *via* the C, H long-range couplings in the HMBC spectrum. The ^{13}C-NMR spectrum displayed 59 carbon atoms whose multiplicity was readily settled by the DEPT experiment.

Evaluation of the ^{1}H, ^{1}H-COSY and C, H long-range correlated NMR spectra (HMBC) confirmed unambiguously that **1** contains the same substituted macrolide skeleton as brasilinolide A and that differences

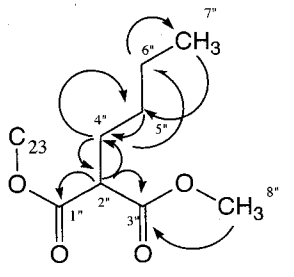
Table 2. ^{1}H and ^{13}C NMR data of brasilinolide B (**1**) in DMSO-d_6 (δ in ppm, I in Hz).

Position	δ_H	δ_C	HMBC (^{1}H)
1	-	165.5	2, 3
2	5.9 d, 14.8	122.6	3
3	6.9 d,t, 14.8, 6.8	146.3	2, 4
4	2.38 m	39.7	2, 3, 5
5	3.80 m	67.4	4, 5-OH
	4.90 br, 5.0 (5-OH)		
6	1.49 m	44.2	4, 7
7	3.81m	65.6	6
	4.70 br, 5.0 (7-OH)	-	
8	1.42 m	43.9	7, 9
9	3.80 m	64.3	9-OH
	4.85 d, 6.0 (9-OH)	-	
10	1.39 m	37.8	9
11	1.49 m	32.4	12, 38
12	1.45 m	44.8	38
13	3.95 m	71.9	13-OH, 38
	4.35 d, 5.0 (13-OH)	-	
14	1.71 m, 1.88 m	43.4	
15	5.15 s (15-OH)	98.2	15-OH, 14, 16
16	3.08 dd, 5, 8	76.0	16-OH,17-OH,17
	4.22 d, 5.0 (16-OH)		
17	3.62 m	67.3	16-OH, 16
	4.45 d, 5.0 (17-OH)	-	
18	1.75 m, 1.11 m	40.6	17-OH
19	3.98 m	64.5	
20	1.52 m	44.3	21-OH
21	3.95 m	66.9	21-OH
	4.26 m (21-OH)	-	-
22	1.38 m	45.0	21-OH, 22
23	4.93 m	73.7	22
24	1.51 m	38.2	25
25	1.50 m	21.9	26
26	1.55 m	33.6	27
27	3.45 m	68.1	28
	4.75 d (27-OH), 5.0	-	-
28	2.61 m	62.4	27, 29
29	2.6 m	57.5	28
30	1.44 m	38.3	29
31	5.25 m	71.3	39
32	1.8 m	36.8	31, 40
33	3.22 m	75.6	33-OH, 40
	4.76 d, 5 (33-OH)	-	
34	1.65 m	34.5	41
35	3.85 m	76.0	35-OH, 41, 42
	4.6 d, 5 (35-OH)	-	
36	1.87 m	38.7	42
37	3.95 m	71.8	43, 1
38	1.0 d, 7	14.4	
39	0.93 d, 7	14.2	
40	0.75 d, 7	9.3	
41	0.73 d, 7	5.0	
42	0.96 d, 7	14.8	
43	0.47 d, 7	9.5	

Table 2. (Continued)

Position	δ_H	δ_C	HMBC (^{1}H)
1'	4.9 t, br	95.6	2'
2'	1.55 m, 1.9 m	29.5	1'
3'	4.98 m	70.5	7'
4'	3.62 m, br	67.0	8'
5'	3.62 m, br	68.6	6'
6'	1.08 m	16.7	-
7'	3.29 s	67.2	-
8'	3.31 s	76.8	-
1''	-	168.5	2''
2''	3.48 t, 8	51.0	
3''	-	169.5	2'', 9''
4''	1.78 m	28.3	-
5''	1.25 m, d	26.3	-
6''	1.23 d, 7	30.9	4'', 5''
7''	0.82 t, 7	13.8	-
8''	3.62 s	57.2	-

Fig. 2. Diagnostic C, H long-range couplings in the HMBC spectrum of the malonic acid ester side chain of brasilinolide B (**1**).



are only in the malonic acid ester side chain and the sugar moiety (Fig. 2). The relative stereochemistry at positions 15, 16, 17 and 19 was suggested to be the same as was described for brasilinolide A[3] due to the comparative ^{1}H, ^{1}H vicinal coupling constants and missing NOE between H-17 and H-19. The chemical shift values of the two malonyl carbonyls (165.5 and 169.5 ppm) suggested that both occur as esters. This suggestion was confirmed further by a methoxyl carbon signal at 57.2 ppm (C-8″). The singlet proton signal at 3.62 ppm (H-8″) displayed a strong C, H long-range correlation to one of the carbonyl groups appearing at 168.5 ppm. Otherwise in the HMBC spectrum the triplet proton signal at 3.48 ppm bound to C-2″ of the malonic acid diester group (51.04 ppm) showed C, H long-range correlations with both carbonyls and, in addition, to C-3″ of the side chain. The structure of the aliphatic side chain was readily assigned due to the observable COSY and HMBC correlations. The anomeric proton of the 2-deoxy-fucopyranose unit displayed cross-peaks in the HMBC spectrum both with C-37 of the aglycone and C-2 of the sugar. COSY and HMBC data were helpful for the identification of the sugar substituents and their relative stereochemistry. Thus, in the NOESY spectrum the NOE's of the anomeric proton with H-37, H_{ax}-2′ and H_{eq}-2′ were visible. Further NOE's (H_{eq}-2′/H_{ox}-3, H_{eq}-2′/H_{ox}-5′) suggested that the sugar skeleton (2-deoxyfucopyranose) is the same as in brasilinolide A. The physico-chemical data thus show **1** as a new representative of the macrolide family of microbial secondary metabolites. Alkyl-substituted malonic acid ester structures belong to the unusual building moieties of natural products but a few representatives have been reported[5].

Brasilinolide A shows narrow antifungal spectrum activity: it was active against *A. niger* IFM 40406, but not against a series of other fungi. However, as shown in Table 3, brasilinolide B was moderately active against all tested fungi in concentrations from 12.5 to 25.0 μg/ml. Although brasilinolide A exerted an immunosuppressive activity[8] in the assay system of MLR with the IC_{50} value of 0.625

Table 3. Antifungal activity of brasilinolide B.

test organism	MIC (μg/ml)
Aspergillus niger	12.5
A. fumigatus IFM 41219	12.5
Paecilomyces variotii	12.5
Candida albicans ATCC 90028	25
C. albicans IFM 40007	12.5
C. albicans 94-2530*	25
C. krusei M 1005	25
C. parapsilosis ATCC 90018	12.5
C. glabrata ATCC 90030	25
Cryptococcus neoformans ATCC 90112	12.5
Cry. neoformans 145A	25

MIC values were determined by microbroth dilution method using RPMI1640 medium supplemented with 0.165 M MOPS, pH7.0.
*: resistant strain to azole-type antifungals.

μg/ml, the related brasilinolide B (**1**) did not show the same effect even at the concentration of 10 μg/ml. Further studies on structure-activity relationships of brasilinolide-type antibiotics are of interest, since it is known that the malonyl side chain in the macrolide antibiotics is not essential for antifungal activity[7].

References

1) TSUDA, M.; H. SATO, Y. TANAKA, K. YAZAWA, Y. MIKAMI, T. SASAKI & J. KOBAYASHI: Brasiliquinones A~C, new cytotoxic benz[*a*]anthraquinones with an ethyl group at C-3 from actinomycete *Nocardia brasiliensis*. J. Chem. Soc. Perkin Trans. 1: 1773~1775, 1996
2) TANAKA, Y.; U. GRAEFE, K. YAZAWA, Y. MIKAMI & M. RITZAU: Nocardicyclins A and B: New anthracycline antibiotics produced by *Nocardia pseudobrasiliensis*. J. Antibiotics 50: 822~827, 1997
3) SHIGEMORI, H.; Y. TANAKA, K. YAZAWA, Y. MIKAMI & J. KOBAYASHI: Brasilinolide A, new immunosuppressive macrolide from actinomycete *Nocardia brasiliensis*. Tetrahedron 52: 9031~9034, 1996
4) TANAKA, Y.; H. KOMAKI, K. YAZAWA, Y. MIKAMI, A. NEMOTO, T. TOJYO, K. KADOWAKI, H. SHIGEMORI & J. KOBAYASHI: Brasilinolide A, a new macrolide antibiotic produced by *Nocardia brasiliensis*. 11. Producing strain, isolation and biological activitiy. J. Antibiotics 50: 1036~1041, 1997
5) SHIGEMORI, H.; H. KOMAKI, K. YAZAWA, Y. MIKAMI, A. NEMOTO, Y. TANAKA, T. SASAKI, Y. IN, T. ISHIDA & J. KOBAYASHI: Brasilicardin A, a novel tricyclic metabolite with potent immunosuppressive activity from actinomycete *Nocardia brasiliensis*. J. Org. Chem. 63: 6900~6904, 1998
6) LAATSCH, H.: AntiBase; Database on Microbial Products-Chemical Concepts, Weinheim, 1998
7) KAZUTOH, T. & T. BEPPU: Demalonyl derivatives of guanidylfungin A and copiamycin: their synthesis and antifungal activity. J. Antibiotics 38: 1363~1370, 1985