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Brasilinolide A, New Immunosuppressive Macrolide from Actinomycete *Nocardia brasiliensis*

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Abstract: A new 32-membered macrolide, brasilinolide A (**1**), with potent immunosuppressive and antifungal activity was isolated from the cultured broth of the actinomycete *Nocardia brasiliensis* IFM 0406 and the structure elucidated on the basis of spectroscopic data and chemical means. Copyright © 1996 Elsevier Science Ltd

In our continuing search for bioactive substances from microorganisms,[1] we isolated a new 32-membered macrolide, brasilinolide A (**1**), possessing potent immunosuppressive and antifungal activity from the cultured broth of the actinomycete *Nocardia brasiliensis* IFM 0406. In this paper we describe the isolation and structure elucidation of **1**.

The supernatant of the fermentation broth of *N. brasiliensis* IFM 0406 was subjected to a Diaion HP-20 column and antifungal fractions against *Aspergillus niger* were purified by a silica gel column ($CHCl_3$/MeOH) followed by reversed-phase HPLC (MeCN/H_2O, 1:1) to give brasilinolide A (**1**).

HRFABMS analysis of brasilinolide A (**1**), a colorless amorphous solid ($[\alpha]^{28}_D$ -27.4°), revealed the molecular formula to be $C_{57}H_{98}O_{24}$ [*m/z* 1189.6403 $(M+Na)^+$, Δ -5.7 mmu]. IR absorptions at 3400, 1730, and 1700 cm^{-1} indicated the presence of hydroxy, ester, and α,β-unsaturated ester groups.

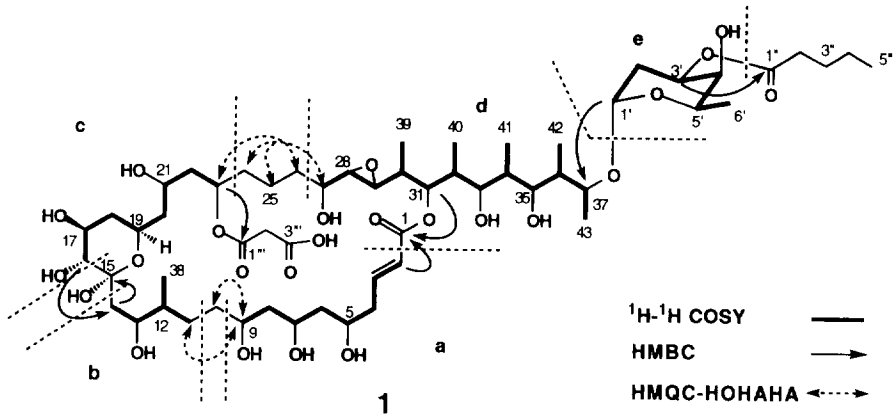

Fig. 1 Structure of brasilinolide A (**1**) and partial structural units (**a** - **e**) based on the ^{1}H-^{1}H-COSYand selected HMBC and HMQC-HOHAHA correlations

Table 1. ^{1}H and ^{13}C NMR Data of Brasilinolide A (**1**) in CD_3OD

position	^{1}H[a]	*J*(Hz)	^{13}C[a]	HMBC (^{1}H)	position	^{1}H	*J*(Hz)	^{13}C	HMBC (^{1}H)
1			169.29 s	2,3,31	29	2.78 dd	6.3, 2.0	61.21 d	31, 39
2	6.05 d	15.6	125.44 d	4	30	1.64 m		41.22 d	29, 31, 39
3	7.11 dt	15.6, 7.3	148.99 d	2	31	5.36 d	10.3	76.01 d	33, 39, 40
4	2.52 m		42.01 t	2, 3	32	1.94 m		39.34 d	31, 33, 40
	2.45 m				33	3.42 m		78.57 d	31, 32, 40, 41
5	4.00 m		70.52 d	3, 4, 6	34	1.88 m		36.82 d	33, 35, 41
6	1.67 m		46.75 t		35	3.51 m		80.40 d	33, 41, 42
7	4.05 m		68.95 d	6, 8	36	2.01 m		41.22 d	35, 42, 43
8	1.56 m		45.89 t		37	4.09 m		75.20 d	35, 42, 43, 1'
9	3.82 m		70.20 d	8, 10	38	0.96 d	7.0	15.27 q	
10	1.54 m		37.00 t		39	1.11 d	7.0	15.22 q	29, 31
11	1.67 m		31.71 t	9, 13, 38	40	0.89 d	7.0	10.23 q	31, 32
12	1.64 m		41.22 d	38	41	0.94 d	7.0	5.89 q	34
13	3.82 m		71.98 d	14b, 38	42	0.86 d	7.0	10.94 q	36, 37
14(a)	1.88 m		45.74 t	16	43	1.16 d	7.0	16.46 q	
(b)	2.00 m				1'	5.04 s		98.36 d	5'
15			100.59 s	14a, 14b, 19	2'(a)	1.76 m		31.71 t	4'
16	3.39 d	9.3	77.89 d	17	(b)	2.14 m			
17	3.93 m		70.52 d	16, 18a, 18b	3'	5.15 m		72.59 d	1', 2'a, 2'b, 4'
18(a)	1.22 m		41.83 t		4'	3.77 m		69.90 d	2'a, 5', 6'
(b)	1.90 m				5'	4.07 m		68.14 d	1', 4'
19	4.20 m		66.79 d	18b, 20	6'	1.24 d	7.0	17.85 q	5'
20	1.57 m		41.13 t		1"			172.44 s	3'
21	4.17 m		66.92 d	20, 22	2"	1.91 m		30.93 t	
22	1.94 m		45.59 t		3"	1.41 m		33.54 t	
23	3.82 m		70.20 d	22	4"	1.36 m		24.40 t	5"
24	1.50 m		39.78 t		5"	0.95 t	7.0	15.22 q	
25	1.69 m		23.48 t		1"'			173.03 s	23
26	1.45 m		34.71 t		2"'	3.39[b] s		46.31 t	
27	3.44 m		71.82 d	28	3"'			175.62 s	
28	2.83 brd	2.0	64.63 d						

a) δ in ppm b) in DMSO-d_6

UV absorption at 214 (ε 14900) nm also supported the presence of α,β-unsaturated ester group. Interpretation of ^{1}H and ^{13}C NMR data (Table 1) of **1** in CD_3OD indicated the presence of one carboxyl, three ester carbonyls, one disubstituted olefin, one hemiketal carbon, one hemiacetal carbon, nineteen oxymethines, five methines, seventeen sp^3 methylenes, and eight methyls. Since five out of nine unsaturations were thus accounted for, compound **1** was inferred to contain four rings (viz., lactone, epoxide, ether ring, and carbohydrate ring each). The presence of a malonyl group was elucidated from fragmentation ions at *m/z* 1122 $(M\text{-}CO_2)^-$, 1080 $(M\text{-}COCH_2CO_2)^-$, and 1064 $(M\text{-}O_2CCH_2CO_2)^-$ in the negative FABMS/MS and ^{13}C NMR data [δ_C 175.62 (C-3"') and 173.03 (C-1"')]. Detailed analysis of the ^{1}H-^{1}H COSY spectrum of **1** revealed connectivities of C-2 ~ C-9 (**a**), C-11 ~ C-14 (**b**), C-16 ~ C-23 (**c**), C-27 ~ C-37 (**d**), and C-1' ~ C-6' (**e**) (Fig. 1). ^{13}C NMR signals for sp^3 methylene carbons located between hydroxy-bearing methines (viz., 2-position of 1,3-diol) were observed at δ_C 41 ~ 47, whereas sp^3 methylene carbons between a hydroxy-bearing methine and another sp^3 methylene resonated at δ_C 34 ~ 40. The location of secondary hydroxyls and methyl groups was elucidated mainly by the HMBC and HMQC-HOHAHA[2] correlations (Fig. 1). In the ^{13}C NMR spectrum of **1** measured in CD_3OH, eleven oxymethine

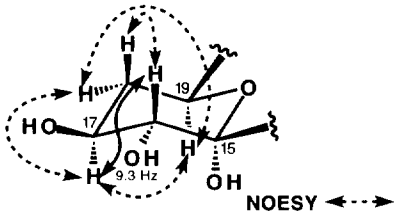

Fig. 2 NOESY correlations and proton coupling of tetrahydropyran ring (C-15-C-19) in **1**

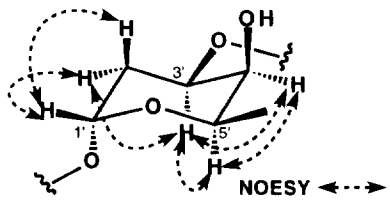

Fig. 3 NOESY correlations of 2-deoxy-fucopyranose moiety (C-1'-C-6') in **1**

carbon resonances (C-5, C-7, C-9, C-13, C-16, C-17, C-21, C-27, C-33, C-35, and C-4') were slightly shifted (0.1 ~ 0.2 ppm) as compared with those in CD_3OD, indicating that hydroxy groups were attached at these carbons.

The presence of an α,β-unsaturated ester was revealed by the proton signals at δ_H 6.05 (H-2) and 7.11 (H-3) (J = 15.6 Hz, *trans*-oriented) and HMBC correlations for H-2/C-1 (δ_C 169.29) and H-3/C-1. Units **a** and **b** were connected through an sp^3 methylene (C-10) by HMQC-HOHAHA correlations for H-9/C-10 and H-9/C-11. HMBC correlations of H_3-38 to C-11, C-12, and C-13 indicated that a methyl group was attached at C-12. The presence of a hydroxy group at C-13 (δ_C 71.98) was assignable from a HMBC cross-peak for H-14b to C-13. The hemiketal carbon (C-15) was elucidated to be adjacent to C-14 by HMBC correlations for H-14a/C-15 and H-14b/C-15, and was connected to C-16 (**c**) by an HMBC cross-peak for H-16/C-14. The HMBC correlation of H-19 to C-15 revealed the presence of a tetrahydropyran ring bearing a hydroxyl group at C-16 (δ_C 77.89) and C-17 (δ_C 70.52). A chair form of the tetrahydropyran ring was assignable from NOESY cross-peaks for H-16/H-18a, H-17/H-18b, H-17/H-19, and H-18b/H-19 (Fig. 2). The hydroxy groups at C-16 and C-17 were assigned to be equatorial on the basis of the coupling constant ($J_{16,17}$ = 9.3 Hz). A NOESY correlation between H-13 and H-16 indicated that the hydroxy group at C-15 was assigned to be axial. HMBC correlations of H-19 and H-23 to C-21 (δ_C 66.92) revealed that a hydroxy group was attached at C-21. The malonyl group was attached at C-23 based on HMBC correlations of H-23/C-1'''. Connection between units **c** and **d** was substantiated by HMQC-HOHAHA correlations of both H-23 and H-27 to C-24, C-25, and C-26. The large C-H couplings observed for the two oxymethine carbons [δ_C 64.63 (C-28, J_{CH} = 172 Hz) and 61.21 (C-29, J_{CH} = 174 Hz)] indicated the presence of an epoxide ring. The configuration of the 28,29-epoxide was elucidated to be *trans* by the ^1H-^1H coupling constant ($J_{28,29}$ = 2.0 Hz). The oxymethine proton (δ_H 5.36) on C-31 showed an HMBC correlation to the ester carbonyl carbon (δ_C 169.29, C-1), which in turn showed HMBC correlations to H-2 and H-3. Thus, C-31 (**d**) and C-2 (**a**) were connected through the C-1 carbonyl to construct a 32-membered macrocyclic lactone ring.[3] ^1H-^1H COSY and HMBC data implied that methyl groups were attached at C-30, C-32, C-34, C-36, and C-37. The sugar part (**e**) was connected to C-37 of the aglycone moiety by an HMBC correlation for H-1'/C-37 and NOESY correlations for H-1'/H-36 and H-1'/H-37. The carbohydrate moiety was elucidated to be 2-deoxyfucopyranose by NOESY correlations of H-1'/H-2'a, H-1'/H-2'b, H-2'a/H-3', H-3'/H-4', H-3'/H-5', and H-4'/H-5' (Fig. 3), and was firmly identified as 2-deoxyfucose by GC analysis of trimethylsilyl derivative of the methanolysis product of **1**. The pentanoyl group (C-1'' ~ C-5'') was attached at C-3' based on a HMBC correlation of H-3'/C-1''. The

α-glycosyl bond at C-1' was suggested from a broad singlet proton signal for H-1' in the ^{1}H NMR spectrum. Thus the structure of brasilinolide A was concluded to be **1**.

Brasilinolide A (**1**) is a new 32-membered macrolide with a tetrahydropyran ring and a 2-deoxyfucopyranose from the broth of *N. brasiliensis* IFM 0406. Compound **1** exhibited potent immunosuppressive activity in mouse mixed lymphocyte assay and antifungal activity against *Aspergillus niger* (MIC, 3.13 μg/mL). Detailed biological activities of **1** will be described elsewhere.

Experimental Section

General Methods. Optical rotations were determined on a JASCO DIP-370 polarimeter. UV and IR spectra were obtained on JASCO Ubest-35 and JASCO IR report-100 spectrometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on JEOL EX-400 and Bruker ARX-500 and AMX-600 spectrometers. The 3.35 and 2.49 ppm resonances of residual CD_2HOD and DMSO-d_5, respectively, and 49.8 and 39.5 ppm of CD_3OD and DMSO-d_6, respectively, were used as internal references. FABMS spectra were obtained on a VG70-4SE spectrometer.

Cultivation. The voucher specimen (*Nocardia brasiliensis* IFM 0406) was deposited at the Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University. Cultures of *N. brasiliensis* IFM 0406 were grown in the broth [glycerol (2.0%), polypepton (1.0%), and meat extract (0.5%) in H_2O, pH 7.0]. Cultures were incubated in a 150 L - jar fermentor at 32 °C for 4 days with stirring at 250 rpm and 150 L/min aeration rate and was centrifuged.

Extraction and Separation. The supernatant of the fermentation broth (150 L) was applied on a Diaion HP-20 column (15 x 100 cm) and washed with 2M NaCl aq. (40 L) and H_2O (40 L) and then eluted batchwise with MeOH/H_2O (1:4, 20 L) and MeOH (20 L). The active fractions against *Aspergillus niger* were lyophilized and was chromatographed on a silica gel column (5 x 50 cm) eluted with $CHCl_3$/MeOH (4:1). Active fractions were further purified by reversed-phase HPLC [Capcell pack C_{18} SG120, Shiseido Co. Ltd., 5 x 25 cm, flow rate: 20 mL/min, MeCN/H_2O (1:1)] to give brasilinolide A (**1**, 100 mg).

Brasilinolide A (1). A colorless amorphous solid; $[\alpha]^{28}_D$ -27.4° (*c* 1.0, MeOH); IR (KBr) ν_{max} 3400, 1740 (sh), 1700, 1640, and 1580 cm^{-1}; UV (MeOH) λ_{max} 214 (ε 14900) nm; ^{1}H and ^{13}C NMR (see Table 1); FABMS (positive, *m*-nitrobenzyl alcohol matrix) *m/z* 1189 $(M+Na)^+$; HRFABMS *m/z* 1189.6403 $(M+Na)^+$, calcd for $C_{57}H_{98}O_{24}Na$, 1189.6460; HMBC correlations (see Table 1); NOESY correlations (CD_3OD, H/H): 2/4a, 2/4b, 3/4b, 3/5, 4a/5, 4b/6, 5/6, 6/7, 13/16, 16/18a, 17/18b, 17/19, 18a/18b, 18b/19, 27/28, 27/29, 29/30, 29/31, 29/39, 30/39, 31/32, 31/33, 31/39, 31/40, 32/33, 32/34, 33/34, 34/40, 34/35, 34/42, 35/36, 35/42, 35/43, 36/37, 36/1', 37/43, 37/1', 1'/2'a, 1'/2'b, 2'a/2'b, 2'a/3', 3'/4', 3'/5', and 4'/5'.

Analysis of Carbohydrate Moiety by GC. Brasilinolide A (**1**, 1.0 mg) was dissolved in 0.5 M HCl/MeOH (0.5 mL) and heated in a sealed tube at 65°C for 15h. After evaporation of the solvent by a stream of nitrogen, the residue was dissolved in pyridine (50 μL) and treated with hexamethyldisilazane (10 μL) and trimethylsilyl chloride (5 μL) at room temperature for 30 min. Solvent was removed by a nitrogen stream and the residue dissolved in hexane was used for GC analysis [1.5% OV-17 glass column (3 mm x 2 m); N_2 as a carrier gas; the program rate: 90 - 200°C at 0.5°C/min] showing a peak at t_R 7.9 min, which corresponded to that of 2-deoxyfucose (7.9 min). The TMS/Me derivative of 2-deoxyfucopyranose[4] showed the same retention time as that derived from **1**.

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