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Brasilicardins B–D, new tricyclic terpernoids from actinomycete *Nocardia brasiliensis*

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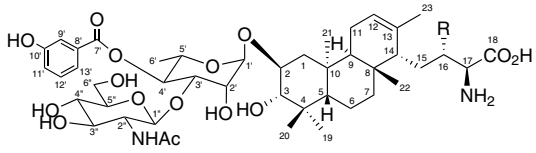
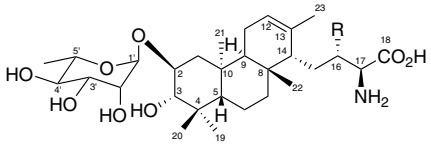
Abstract—Three new tricyclic terpenoids, brasilicardins B–D (**2**–**4**), were isolated together with brasilicardin A (**1**), a potent immunosuppressive compound, from the cultured broth of a pathogenic actinomycete *Nocardia brasiliensis* IFM0406, and the structures and stereochemistry were determined by spectroscopic data and a single crystal X-ray diffraction analysis. The immunosuppressive and cytotoxic activities of **2**–**4** were examined in the comparison with **1**.

1. Introduction

During our search for bioactive substances from pathogenic actinomycetes of the genus *Nocardia*,[1] we have previously isolated brasilicardin A (**1**), a novel tricyclic terpenoid consisting of an *anti*/*syn*/*anti*-perhydrophenanthrene skeleton with two sugars and an amino acid side-chain, from *Nocardia brasiliensis* IFM-0406.[2] This unique terpenoid moiety was shown to be biosynthesized from glucose via the nonmevalonate pathway.[3] Brasilicardin A (**1**) exhibits potent immunosuppressive activity in mouse mixed lymphocyte reaction (MLR) assay and cytotoxic activity against adriamycin-resistant murine lymphoma cells. Its immunosuppressive potency is compatible to those of cyclosporin A or ascomycin.[4] Further investigation on the extract of this strain resulted in the isolation of three new congeners, brasilicardins B–D (**2**–**4**), and the structures and stereochemistry were determined by spectroscopic data and a single crystal X-ray diffraction analysis. In this paper we describe the isolation and structure elucidation of **2**–**4** and their immunosuppressive activities.



brasilicardin A (**1**) : R = OCH_3
brasilicardin B (**2**) : R = H



brasilicardin C (**3**) : R = OCH_3
brasilicardin D (**4**) : R = H

2. Results and discussion

The supernatant of the fermentation broth (80 L) was subjected to a Diaion HP-20 column (50% MeOH aq → MeOH), in which fractions eluted with MeOH were separated on a silica gel and C_{18} columns, and centrifugal partition chromatography followed by C_{18} HPLC (MeOH/H_2O and MeOH/H_2O/CF_3CO_2H) to afford brasilicardins B (**2**, 6.6 mg), C (**3**, 55 mg), and D (**4**, 23 mg) together with a known related compound, brasilicardin A (**1**, 428 mg).

Keywords: Terpenoid; Actinomycete; *Nocardia brasiliensis*; Immunosuppressive.
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Table 1. ^{1}H and ^{13}C NMR data of brasilicardins B–D (**2**–**4**) in MeOH-d_4

Position	**2**			HMBC	**3**			**4**		
	δ_H	m, *J* (Hz)	δ_C		δ_H	m, *J* (Hz)	δ_C	δ_H	m, *J* (Hz)	δ_C
1 (α)	1.85	m	44.76	21	1.82	dd, 12.8, 4.4	44.78	1.84	dd, 12.5, 3.7	44.91
1 (β)	1.49	m			1.46	m		1.43	m	
2	3.76	m	80.60	3	3.70	m	79.83	3.70	m	80.00
3	3.06	d, 9.3	84.15		3.03	d, 9.4	84.08	3.02	d, 9.3	84.24
4			41.89	3, 5, 19, 20			41.84			41.89
5	1.67	m	45.49		1.66	m	44.99	1.64	m	45.53
6 (a)	1.77	m	19.62		1.78	m	19.40	1.77	m	19.66
6 (b)	1.67	m			1.65	m		1.64	m	
7 (a)	1.90	m	32.25	22	1.78	m	31.98	1.89	m	32.35
7 (b)	1.38	m			1.38	m		1.37	m	
8			38.89	15b, 22			39.19			38.93
9	1.43	m	48.15	11, 12, 21	1.31	m	48.02	1.43	m	48.23
10			38.56	21			38.20			38.55
11 (a)	1.94[a]	m	27.71		1.92[a]		27.74	1.93	m	27.73
11 (b)								1.89	m	
12	5.38	m	124.05	11, 23	5.38	m	124.22	5.37	m	124.06
13			139.06	11, 23			139.26			139.09
14	1.33	m	57.50	12, 22, 23	1.61		53.00	1.32	m	57.66
15a	1.67		28.26		1.57		32.71	1.64	m	28.31
15b	1.46				1.46			1.45	m	
16	1.99		34.64		3.82	dd, 10.8, 3.0	81.26	1.97	m	34.86
OCH_3					3.53[b]	s	59.29		—	
17	3.54	t, 5.6	57.60		4.48	d, 3.5	55.63	3.53	t, 5.0	57.60
18			175.13	17			170.88			175.48
19	0.96[b]	s	18.19	3, 20	0.95[b]	m	18.11	0.95[b]	s	18.21
20	1.04[b]	s	30.16	3, 19	1.02[b]	m	30.01	1.02[b]	s	30.15
21	1.18[b]	s	29.52	1b, 5, 9	1.13[b]	m	29.64	1.18[b]	s	29.56
22	1.05[b]	s	24.22		1.08[b]	m	23.70	1.04[b]	s	24.21
23	1.72[b]	s	24.19	12	1.70[b]	s	23.36	1.72[b]	s	24.16
1′	5.08	brs	103.79		4.99	brs	104.18	4.99	brs	104.31
2′	4.39	brd, 3.0	72.71		3.99	m	72.93	3.98	brd, 3.1	73.03
3′	4.13	dd, 9.6, 3.0	80.80	1′, 2′, 1″	3.70	m	73.22	3.70	dd, 9.3, 3.1	73.32
4′	5.31	t, 9.6	74.83	2′, 3′,	3.42	t, 9.4	74.78	3.42	dd, 9.3, 9.3	74.91
5′	4.06	dq 9.6, 6.0	68.73	1′, 6′	3.75	dq, 9.4, 6.4	70.58	3.75	dq, 9.3, 6.2	70.64
6′	1.17[b]	d, 6.0	18.59	4′	1.28[b]	d, 6.4	18.62	1.29[b]	d, 6.2	18.68
7′			168.12	4′, 9′, 13′						
8′			132.94	12′						
9′	7.52	d, 1.9	118.31	11′, 13′						
10′			159.69	9′, 12′						
11′	7.11	dd, 8.1, 1.9	122.56	9′, 13′						
12′	7.38	t, 8.1	131.69							
13′	7.59	d, 8.1	122.84	9′, 11′						
1″	4.57	d, 8.1	105.00	3′, 2″						
2″	3.61	dd, 8.1, 9.3	57.86							
CH$_3$CO	1.52[b]	s	23.41							
CH$_3$*C*O			175.00	2″, *CH*$_3$CO						
3″	3.43	dd, 8.1, 9.3	76.04	2″						
4″	3.36	m	72.71	3″, 6″ a						
5″	3.36	m	78.35							
6′ (a)	3.93	d, 11.8	63.04							
6″ (b)	3.74	m								

[a] 2H.
[b] 3H.

Brasilicardin B {**2**, $[\alpha]_D^{23}$ +17 (*c* 1.0, MeOH)} was obtained as a colorless amorphous solid. HRFABMS data [*m*/*z* 863.4554, $(M+H)^+$, +1.2 mmu] of **2** revealed the molecular formula to be $C_{44}H_{66}N_2O_{15}$, which was smaller than that of brasilicardin A (**1**) by 30 amu. The ^{13}C NMR (Table 1) spectrum of **2** disclosed total 44 signals including one carboxyl, one ester, and one amide carbonyl, three sp^2 quaternary carbons, five sp^2 methines, two hemiacetal carbons, nine oxymethines, three sp^2 quaternary carbons, one oxymethylene, five methylenes, and seven methyls, which were similar to those of brasilicardin A (**1**) except for the absence of a methoxy group and an oxymethine and the presence of an additional methylene. Detailed analysis of 2D NMR data of **2** implied the existence of the same tricyclic skeleton (C-1–C-14) with two sugar moieties (C-1′–C-6′ and C-1″–C-6″) and a 3-hydroxybenzoyl unit (C-7′–C-13′) as that of **1** (Fig. 1). One (C-1′–C-6′) of the two sugar moieties in

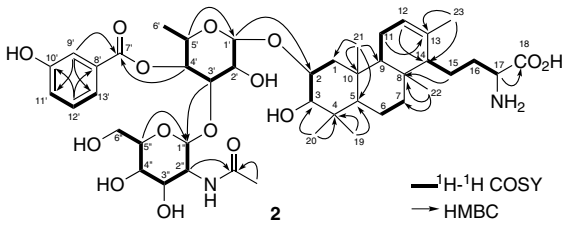


Figure 1. Selected 2D NMR correlations for brasilicardin B (**2**).

2 was elucidated to be α-rhamnose by ^{1}H–^{1}H coupling constants and ROESY data, while the presence of β-*N*-acetylgulcosamine as another sugar unit (C-1″–C-6″) was deduced from the ^{13}C chemical shifts and HMBC correlations for H-2″/$\underline{CO}CH_3$ (Fig. 1). The rhamnose unit was attached to C-2 of aglycone, while *N*-acetylglucosamine (C-1″–C-6″) and 3-hydroxybenzoyl unit (C-7′–C-13′) were connected at C-3′ and C-4′ of the rhamnose, respectively. The relative stereochemistry of the tricyclic core was implied to be the same as that of **1** by analysis of NOESY data (Fig. 2). The β-aminobutyric acid side chain (C-15–C-18) attached to C-14 was revealed by ^{1}H–^{1}H COSY cross-peaks for H-14/H_2-15, H_2-15/H_2-16, and H_2-16/H-17 and HMBC correlations for H-12/C-14, H-15/C-8, H-17/C-18, H_3-23/C-14. Thus the structure and relative stereochemistry of brasilicardin B were elucidated to be **2**.

Hydrolysis of **2** with HCl/MeOH afforded its aglycone **5**, methyl α-rhamnopyranoside (**6**), and methyl α-glucosamine (**7**). The absolute configurations of the sugar units **6** and **7** were determined as L and D, respectively, on the basis of its optical rotations (**6**: $[\alpha]_D^{22} = -39$, **7**: $[\alpha]_D^{22}$ +35).[2] To determine the absolute configuration of the tricyclic core of **2**, the CD exciton chirality method[5] was applied for the dihydroxyl group at C-2 and C-3. The aglycone **5** was treated with *p*-bromobenzoyl chloride to afford a 2-*O*, 3-*O*, 17-*N*-trisbenzoate (**8**), of which the structure was assigned on the basis of ^{1}H–^{1}H COSY, NOESY, and HRFABMS data [*m*/*z* 742.3689 $(M+Na)^+$, calcd for $C_{45}H_{53}NO_7Na$, Δ −1.4 mmu]. The *J*(H-2,H-3) value (10.1 Hz) indicated that the two benzoyl groups at C-2 and C-3 were *trans* diequatrially disposed, and the CD spectrum of **8** showed positive Cotton effect [λ_{ext} 237 nm (Δε +18.4), 223 nm (Δε −10.7)], indicating that the benzoyl groups at C-2 and C-3 should be clockwise. Thus, the absolute configurations at C-2 and C-3 were both *S*. To determine the absolute configuration at C-17, the modified Mosher' s method[6] was employed.

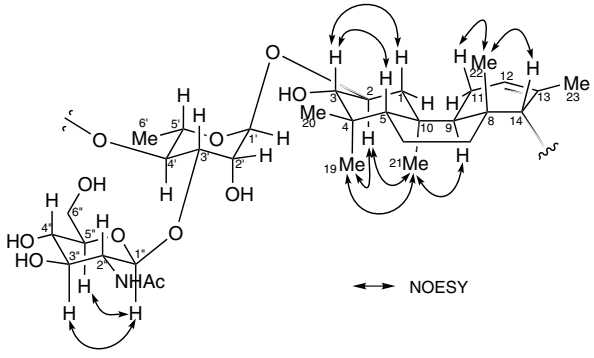
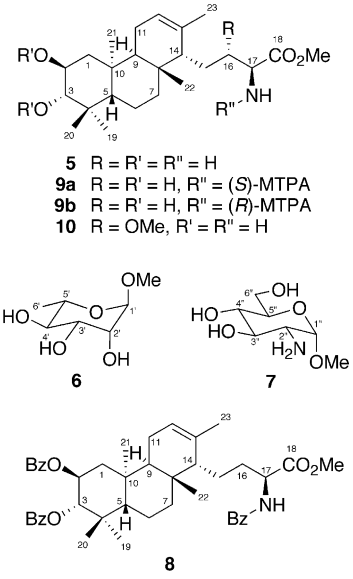


Figure 2. NOESY correlations and relative stereochemistry for brasilicardin B (**2**). *J* in hertz (H/H): H-2/H-3: 9.3, H-1′/H-2′: <1, H-2′/H-3′: 3.0, H-3′/H-4′: 9.6, H-4′/H-5′: 9.6, H-1″/H-2″: 8.1, H-2″/H-3″: 9.3, H-3″/H-4″: 8.1.



Compound **5** was converted into (*S*)- and (*R*)-2-methoxy-2-trifluoromethyl-2-phenylacetyl (MTPA) amides (**9a** and **9b**, respectively). The Δδ values [δ(**9a**) − δ(**9b**)] suggested the *S*-configuration at C-17 (Fig. 3). Therefore, the structure of brasilicardin B (**2**) was concluded to be the desmethoxy form at C-16 of brasilicardin A (**1**).

The molecular formula, $C_{30}H_{51}NO_9$, of brasilicardin C {**3**, $[\alpha]_D^{23}$ +65 (*c* 1.0, MeOH)} was established by HRFABMS data [*m*/*z* 570.3636 $(M+H)^+$, Δ −0.6 mmu]. ^{1}H and ^{13}C NMR data (Table 1) showed the signals due to the same tricyclic aglycone as brasicardin A (**1**) and a rhamnose, while signals due to an *N*-acetylglucosamine and a 3-hydroxybenzoate were not observed for **3**. The absolute stereochemistry of rhamnose moiety was assigned as L on the basis of the optical rotation of methyl α-rhamnopyranoside {**6**, $[\alpha]_D^{20}$ −48} obtained by methanolysis of **3**. Brasilicardin C (**3**) was crystallized from H_2O–MeOH as colorless needles, mp 219–221 °C.

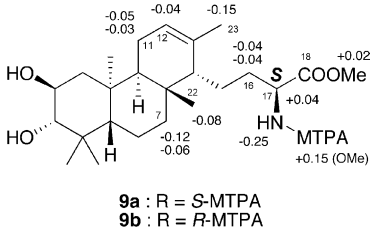


Figure 3. Proton chemical differences [$\Delta\delta$ (in ppm) = $\delta_S - \delta_R$] obtained for (*S*)- and (*R*)-MTPA amides (**9a** and **9b**) of aglycone (**5**) for brasilicardin B (**2**).

The stereostructure of brasilicardin C (**3**) was established by a single crystal X-ray diffraction analysis as shown in Figure 4a. The X-ray analysis revealed the presence of a tetragonal nitrogen atom [N(1)] and a carboxylate [C(18)], suggesting that **3** presented as a zwitter ions. Figure 4b represented association of two molecules of **3**, in which two intermolecular hydrogen bonds were observed at O(8)–HO(17) and O(17)–HO(2). A unit cell of crystal was composed of eight molecules of **3** together with eight molecules of H_2O, and 12 molecules of MeOH, four of which disordered like the MeOH for C(63)–O25 as shown in Figure 4b.

Brasilicardin D {**4**, $[\alpha]_D^{20}$ +79 (*c* 0.5, MeOH)} was suggested to possess the molecular formula, $C_{29}H_{49}NO_8$, by HRFABMS data [*m/z* 540.3358 $(M+H)^+$, Δ +0.2 mmu]. ^1H and ^{13}C NMR data (Table 1) were similar to those of brasilicardin C (**3**), except for the absence of a methoxy signal observed for **3**. The structure of **4** including relative stereochemistry was elucidated by detail analysis of 2D NMR data to be the desmethoxy form of **3**. The absolute stereochemistries of the aglycone and a rhamnopyranoside in **4** were assigned on the basis of spectral data of its aglycone **5** and methyl-α-rhamnopyranoside {**6**, $[\alpha]_D^{20}$ −46} obtained by methanolysis of **4**.

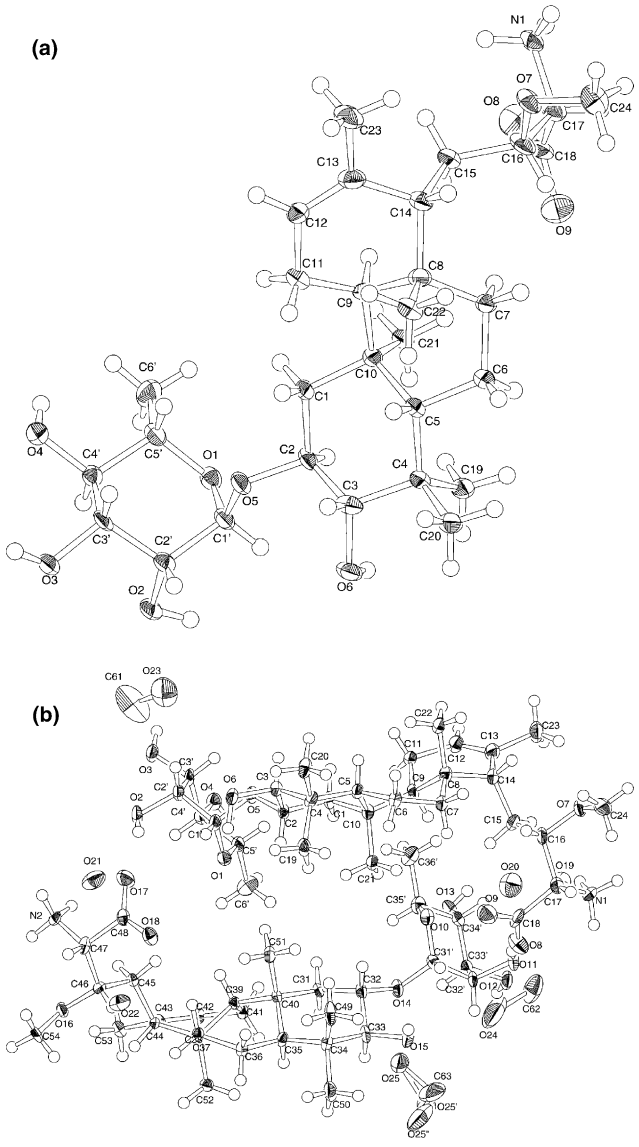


Figure 4. (a) Molecular structure of brasilicardin C (**3**) obtained from X-ray analysis (ORTEP drawing; ellipsoids are drawn at 30% probability level) and (b) contact model of two molecules of brasilicardin C (**3**).

Table 2. MLR inhibitory and cytotoxic activities of brasilicardins A–D (**1**–**4**)

Compd.	IC_{50} (μg/mL)	
	MLR	L1210
1	0.057	1.2
2	2.5	>10
3	2.5	7.8
4	>10	>10
Cyclosporin A	0.15	[a]

[a] Not tested.

Brasilicardins B–D (**2**–**4**) are new congeners of brasilicardin A (**1**) with immunosuppressive activity and cytotoxicity. Effects of brasilicardins B–D (**2**–**4**) on mouse MLR were examined in comparison with that of brasilicardin A and cyclosporin A. Suppressive activities of brasilicardins B (**2**) and C (**3**) on the proliferative response of mouse lymphocytes to alloantigen stimulation were 50 times less potent than that of **1**. On the other hand, brasilicardin D (**4**) showed no such suppressive activity. Cytotoxic activities of **2**–**4** were tested against murine lymphoma L1210 cells in vitro. The IC_{50} value of brasilicardin C (**3**) was 7.8 μg/mL, which was less potent than that of **1**, while **2** and **4** showed no cytotoxicity (IC_{50} > 10 μg/mL). These results suggest that the presence of a methoxy group at C-16 as well as a glucosamine unit and/or a benzoyl group are important for the immunosuppressive and cytotoxic activities for brasilicardin A (**1**) (Table 2).

3. Experimental

3.1. General experimental procedure

The 3.35 ppm resonance of residual CH_3OH and 49.8 ppm of CD_3OD were used as internal references for ^1H and ^{13}C NMR spectra, respectively. FAB mass spectra were obtained using nitrobenzylamine as a matrix.

3.2. Cultivation

The voucher specimen of *Nocardia brasiliensis* (strain IFM 0406) was deposited at the Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University (deposit No. FERM BP-5498).[4] This actinomycete was 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 were centrifuged.

3.3. Extraction and isolation

The supernatant of the fermentation broth (80 L) was passed through a Diaion HP-20 column and washed with 2 M NaCl aq (20 L) and H_2O (20 L) and then eluted batchwise with MeOH/H_2O (1:1, 20 L) and MeOH (20 L). The fraction eluted with MeOH was chromatographed on a silica gel column eluted with stepwise gradient of $CHCl_3$/MeOH to yield a fraction (13.7 g), which was separated by a C_{18} column with stepwise gradient of MeOH/H_2O. The fraction eluted with 50–70% MeOH/H_2O was further separated by C_{18} HPLC (YMC-Pack ODS R&D, YMC Co., Ltd, 2 × 25 cm; MeOH/H_2O, 70:30; flow rate 10 mL/min; UV detection at 205 nm) and then C_{18} HPLC [YMC-Pack ODS R&D; MeOH/H_2O (65:35 → 67:33) containing CF_3CO_2H (25 ppm); flow rate 10 mL/min; UV detection at 205 nm] to afford brasilicardins B (**2**, 6.6 mg) and C (**3**, 11.8 mg).

Parts (50 g) of the fraction eluted with MeOH/H_2O (1:1) in the previous HP-20 column were partitioned between EtOAc/H_2O and then *n*-BuOH/H_2O. The *n*-BuOH-soluble materials were subjected to silica gel column chromatography ($CHCl_3$/MeOH/H_2O, 7:2:0.2–6:3:0.5) to yield a fraction (1.1 g), which was purified by a C_{18} column (MeOH/H_2O), centrifuged partition chromatography (descending mode, *n*-BuOH/MeOH/H_2O, 4:5:1), and then C_{18} HPLC (YMC-Pack ODS R&D, 2 × 25 cm; MeOH/H_2O 70:30; flow rate 10 mL/min; UV detection at 205 nm) to afford brasilicardins C (**3**, 45 mg) and D (**4**, 23.1 mg).

3.3.1. Brasilicardin B (2). Colorless amorphous solid; $[\alpha]_D^{23}$ +17 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3428, 1679, and 1633 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 863 $(M+H)^+$; HRFABMS *m/z* 863.4554 $(M+H)^+$ (calcd for $C_{44}H_{67}N_2O_{15}$, 863.4542).

3.3.2. Brasilicardin C (3). Colorless amorphous solid; $[\alpha]_D^{23}$ +65 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3433, 1679, and 1633 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 570 $(M+H)^+$; HRFABMS *m/z* 570.3636 $(M+H)^+$ (calcd for $C_{30}H_{52}NO_9$, 570.3642).

3.3.3. Brasilicardin D (4). Colorless amorphous solid; $[\alpha]_D^{20}$ +79 (*c* 0.5, MeOH); IR (KBr) ν_{max} 3420, and 1631 cm^{-1}; ^1H and ^{13}C NMR (Table 1); FABMS *m/z* 540 $(M+H)^+$; HRFABMS *m/z* 540.3358 $(M+H)^+$ (calcd for $C_{29}H_{50}NO_8$, 540.3356).

3.4. Mathanolysis of brasilicardins B–D (2–4)

Generally, each brasilicardins B–D (**2**–**4**, 1.0 mg each) was treated with 5% HCl/MeOH (30 μL) at 100 °C for 16 h. After evaporation of the solvent using nitrogen stream, the residue was subjected to silica gel column chromatography ($CHCl_3$/MeOH, 9:1). From **2**, aglycone **5** (0.5 mg), methyl α-L-rhamnopyranoside {**6**, 0.14 mg; $[\alpha]_D^{22}$ −39 (*c* 0.02, MeOH), HRFABMS *m/z* 179.0922 $(M+H)^+$ (calcd for $C_7H_{15}O_5$, 179.0920)}, and methyl α-D-glucosamine {**7**, 0.22 mg; $[\alpha]_D^{22}$ +35 (*c* 0.04, H_2O); HRFABMS *m/z* 193.1032 $(M+H)^+$ (calcd for $C_7H_{16}NO_5$, 193.1029)} were obtained. Methanolysis of brasilicardin C (**3**) under the same condition as **2** afforded aglycone **10**[2] {0.34 mg; $[\alpha]_D^{22}$ +56 (*c* 0.06, MeOH); HRFABMS *m/z* 437.3135 $(M+H)^+$ (calcd for $C_{25}H_{44}NO_5$, 438.3219)} and methyl α-L-rhamnopyranoside {**6**, 0.17 mg; $[\alpha]_D^{20}$ −48 (*c* 0.05, MeOH); HRFABMS *m/z* 179.0918 $(M+H)^+$ (calcd for $C_7H_{15}O_5$, 179.0920)} were yielded. Methanolysis of brasilicardin D (**4**, 1.2 mg) gave an aglycone **5** {0.9 mg, $[\alpha]_D^{25}$ +107 (*c* 0.10, MeOH); HRFABMS *m/z* 408.3119 $(M+H)^+$ (calcd for $C_{24}H_{42}NO_4$, 408.3114)} and methyl α-L-rhamnopyranoside {**6**, 0.20 mg; $[\alpha]_D^{20}$ −46 (*c* 0.05, MeOH); HRFABMS *m/z* 179.0923 $(M+H)^+$ (calcd for $C_7H_{15}O_5$, 179.0920)}.

3.4.1. Compound 5. Colorless amorphous solid; $[\alpha]_D^{25}$ +107 (*c* 0.1, MeOH); IR(KBr) ν_{max} 3428, 2924, 1737, 1633, and 1061 cm^{-1}; ^1H NMR (CD_3OD) δ 0.94 (3H, s, H_3-19), 1.02 (3H, s, H_3-20), 1.04 (3H, s, H_3-22), 1.15 (3H, s, H_3-21), 1.30 (1H, m, H-14), 1.36 (1H, m, H-7), 1.37 (1H, m, H-15), 1.39 (1H, m, H-1), 1.40 (1H, m, H-9), 1.53 (1H, m, H-15), 1.66 (1H, m, H-5), 1.68 (1H, m, H-6), 1.69 (3H, s, H_3-23), 1.73 (1H, dd, J = 4.3 and 12.5 Hz, H-1), 1.78 (1H, m, H-6), 1.80 (1H, m, H-7), 1.83 (2H, m, H_2-16), 1.90 (1H, m, H-11), 1.96 (1H, m, H-11), 2.91 (1H, d, J = 9.5 Hz, H-3), 3.53 (1H, t, J = 6.0 Hz, H-17), 3.68 (1H, ddd J = 4.3, 9.5, and 11.4 Hz, H-2), 3.78 (3H, s, 18-OCH_3), and 5.37 (1H, m, H-12); ^{13}C NMR (CD_3OD) δ 18.1 (C-19), 19.1 (C-6), 24.0 (C-23), 24.2 (C-22), 27.7 (C-11), 28.3 (C-15), 29.6 (C-21), 30.1 (C-20), 32.3 (C-7), 37.4 (C-16), 38.6 (C-10), 39.0 (C-8), 41.7 (C-4), 45.6 (C-9), 45.6 (C-1), 48.3 (C-14), 53.3 ($COOCH_3$), 56.2 (C-17), 57.6 (C-5), 71.0 (C-2), 85.3 (C-3), 124.2 (C-12), 139.0 (C-13), and 177.3 (C-18); FABMS *m/z* 408 $(M+H)^+$; HRFABMS *m/z* 408.3128 $(M+H)^+$ (calcd for $C_{24}H_{42}NO_4$, 408.3114).

3.5. Tris-benzoate (8) of aglycone 5

Compound **5** (0.4 mg), DMAP (0.23 mg) and benzoyl chloride (1.1 mg) in dry pyridine (30 μL) was heated at 80 °C for 10 h. The mixture was diluted with satd NH_4Cl aq and extracted with EtOAc. The organic layer was washed with water and brine, and then evaporated. The residue was separated by a silica gel column (hexane/EtOAc, 85:15) and C_{18} HPLC (Develosil ODS UG-5, Nomura Chemical Co., Ltd, 10 × 250 mm; eluent MeOH/H_2O, 90:10; flow rate, 2.5 mL/min; UV detection at 230 nm) to afford a tris-benzoate (**8**, 0.2 mg t_R 26 min): UV (MeOH) λ_{max} 228 nm (ε 39000); CD (MeOH) λ_{ext} 237 ($\Delta\varepsilon$ + 18.4) and 223 nm (−10.7); ^1H NMR ($CDCl_3$) δ 0.97 (3H, s, H_3-20), 1.01 (3H, s, H_3-22), 1.13 (3H, s, H_3-19), 1.21 (3H, s, H_3-21), 1.35 (1H, m, H-7), 1.39 (1H, m, H-15), 1.40 (1H, m, H-9), 1.60 (3H, s, H_2-22), 1.64 (1H, m, H-6), 1.71 (1H, m, H-1), 1.73 (1H, m, H-15), 1.78 (1H, m, H-6), 1.84 (1H, m, H-14), 1.86 (2H, m, H_2-11), 1.90 (1H, m, H-16), 1.99 (1H, m, H-1), 2.01 (1H, m, H-7), 2.08 (1H, m, H-16), 2.26 (1H, m, H-5), 3.81 (3H, s, $COOCH_3$), 4.85 (1H, m, H-17), 5.16 (1H, d, J = 10.1 Hz, H-3), 5.29 (1H, s, H-12), 5.47 (1H, m, H-2), 6.67 (1H, d, J = 6.7 Hz, 17-NH), 7.30–7.36 (4H, m, Ph), 7.44–7.47 (4H, m, Ph), 7.53 (1H, t, J = 7.7 Hz, Ph), 7.81 (2H, d, J = 7.7 Hz, Ph), 7.88 (2H, d, J = 7.7 Hz, Ph) and 7.96 (2H, d, J = 7.8 Hz, Ph); ESIMS

m/z 742 $(M+Na)^+$; HRFABMS *m/z* 742.3689 [$(M+Na)^+$, calcd for $C_{45}H_{53}NO_7Na$, 742.3703].

3.6. (*S*)- and (*R*)-MTPA amide (9a and 9b) of aglycone 5

A solution of compound **5** (0.2 mg) in CH_2Cl_2 (20 μL) were added to DCC (0.13 mg) and (*S*)-(+)-MTPA (0.13 mg), and the mixture was stirred at room temperature for 1 h. The residue was passed through a silica gel column (hexane/EtOAc, 1:1) and purified by C_{18} HPLC (Develosil ODS UG-5, 10 × 250 mm; eluent MeOH/H_2O, 82:12; flow rate, 2.5 mL/min; UV detection at 230 nm) to give the (*S*)-MTPA amide (**9a**, 0.1 mg, t_R 17 min) of compound **5**: ^{1}H NMR ($CDCl_3$) δ 0.84 (3H, s), 0.87 (3H, s), 0.92 (3H, s), 0.95 (3H, s), 1.21 (1H, m, H-9), 1.23 (1H, m, H-14), 1.25 (1H, m, H-7), 1.32 (1H, m, H-1), 1.41 (3H, s, H_3-22), 1.45 (1H, m, H-5), 1.54 (1H, m, H-7), 1.54 (1H, m, H-6), 1.67 (1H, m, H-6), 1.71 (1H, m, H-1), 1.79 (1H, m, H-11), 1.81 (2H, m, H_2-16), 1.86 (1H, m, H-11), 1.95 (2H, m, H_2-15), 2.89 (1H, d, J = 10.0 Hz, H-3), 3.46 (3H, s, OCH_3 of MTPA), 3.70 (1H, m, H-2), 3.71 (3H, s, 18-OCH_3), 4.60 (1H, m, H-17), 5.24 (1H, m, H-12), 7.13 (1H, br s, 17-NH), 7.32 (3H, m, Ph), and 7.49 (2H, m, Ph); HRESIMS *m/z* 646.3326 $(M+Na)^+$ (calcd for $C_{34}H_{48}NO_6F_3Na$, 646.3332).

The (*R*)-MTPA amide (**9b**, 0.1 mg) of compound **5** was prepared from the same procedure as described above. Compound **9b**: ^{1}H NMR ($CDCl_3$) δ 0.92 (3H, s), 0.97 (3H, s), 1.00 (3H, s), 1.09 (3H, s), 1.31 (1H, m, H-9), 1.31 (1H, m, H-7), 1.34 (1H, m, H-1), 1.41 (1H, m, H-14), 1.53 (1H, m, H-5), 1.62 (1H, m, H-6), 1.62 (3H, s, H_3-22), 1.66 (1H, m, H-7), 1.74 (1H, m, H-1), 1.82 (1H, m, H-11), 1.83 (1H, m, H-6), 1.85 (2H, m, H_2-16), 1.91 (1H, m, H-11), 2.03 (2H, m, H_2-15), 2.97 (1H, d, J = 10.0 Hz, H-16), 3.37 (3H, s, OCH_3 of MTPA), 3.76 (3H, s, 18-OCH_3), 4.63 (1H, m, H-17), 5.33 (1H, m, H-12), 7.41 (3H, m, Ph), 7.46 (1H, brs, 17-NH), and 7.54 (2H, m, Ph); HRESIMS 646.3328 $(M+Na)^+$ (calcd for $C_{34}H_{48}NO_6F_3Na$, 646.3331).

3.7. X-ray crystallography of brasilicardin C (3)

Brasilicardin C (**3**) was crystallized from H_2O–MeOH as colorless needles, mp 219–221 °C. Crystal data: $C_{31.5}H_{61}NO_{12.5}$ [$C_{30}H_{51}NO_9$, 1.5(CH_4O), 2(H_2O), M_r = 653.83, crystal dimensions 0.400 × 0.100 × 0.050 mm, C-centered monoclinic, space group C_2 (no. 5), a = 32.27(1) Å, b = 8.243(4) Å, c = 27.83(1) Å, β = 111.18(3)°, V = 6873(4) Å^3, Z = 8, D_{calc} = 1.264 g/cm^{-1}. All measurements were made on Rigaku RAXIS-RAPID Imaging Plate diffractometer with graphite monochromated Mo-Kα radiation (λ = 0.71075 Å) at a temperature of −175 ± 1 °C to a maximum 2θ value of 60.1°. A total of 70 images, corresponding to 210.0° oscillation angles, were collected with two different goniometer settings. Exposure time was 5.50 min per degree. The camera radius was 127.40 mm. Readout was performed in the 0.100 mm pixel mode. Data were processed by the PROCESS-AUTO program package. Of the 37,433 reflections, which were collected, 10,630 were unique (R_{int} = 7.7%); equivalent reflections were merged. The linear absorption coefficient, μ, for Mo-Kα radiation was 1.0 cm^{-1}. An absorption correction using the program ABSCOR5 was applied, which resulted in transmission factors ranging from 0.88 to 1.00. The data were corrected for Lorentz and polarization effects.

The structure was solved by direct methods (SHELXS86)[7] and expanded using the Fourier technique (DIRDIF94).[8] The nonhydrogen atoms were refined anisotropically. Hydrogen atoms were included but not refined. The final cycle of full matrix least-squares refinement was based on 10,630 observed reflections ($I > -3.00\sigma(I)$, $2\theta < 60.07$) and 828 variable parameters and converged with unweighted and weighted agreement factors of RI = 0.0825, R_w = 0.2016. The standard deviation of an observation of unit weight was 1.02. The weighting scheme was based on counting statistics and included a factor (p = 0.062) to downweight the intense reflections. Plots of $\Sigma\omega(Fo^2 - Fc^2)^2$ versus Fo^2 reflection order in data collection, sin θ/λ and various classes of indices showed no unusual trends. The maximum and minimum peaks on the final difference Fourier map corresponded to 1.37 and −0.71 e^-/Å^3, respectively. All calculations were performed using the teXsan crystallographic software package of Molecular Structure Corporation. Crystallographic data for brasilicardin C (**3**) have been deposited at the Cambridge Crystallographic Data Center (deposition number CCDC 244063).

3.8. Mouse MLR assay

Mouse MLR assay was performed as described by Hatanaka et al.[9] The spleens obtained from BALB/C and C57BL/6 mice (female, 6–7 weeks old) were homogenized into single cell suspensions. Ammonium chloride buffer (0.15 M $KHCO_3$, 0.1 mM Na_2 EDTA, pH 7.2) was added to the cell suspension in order to lyse erythrocytes followed by washing three times with RPMI1640 medium. The erythrocyte-free cell preparation was then resuspended in RPMI1640 complete medium (supplemented with 10% FCS and 50 μM 2-mercaptoethanol). The mouse MLR was performed in a 96-well round-bottom microtest plate with each well containing 5×10^5 C57BL/6 spleen cells (responder cells, H-2^b) and 5×10^5 mitomycin C-treated (25 μg/mL of mitomycin C at 37 °C for 30 min and washed three times with RPMI1640 medium) BALB/C spleen cells (stimulator cells, H-2^d), and various amounts of test compound in 0.2 mL RPMI1640 complete medium. The cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2–95% air. After 92 h of cultivation, cells were pulse-labeled with 0.5 μCi of [3H]thymidine for 4 h at 37 °C and harvested using a multiple cell harvester. The radioactivity incorporated into the cells was measured with a liquid scintillation counter. Results were expressed as IC_{50} values.

3.9. Cytotoxicity assay

Cytotoxicity assay was performed in 96-well flat-bottom microtest plate; each well contained 104 cells and a var-

iable amount of test compound in 0.2 mL of RPMI1460 medium. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2–95% air for 72 h. The cell growth was measured by MTT colorimetric assay.[10]

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