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SAR studies of brasilicardin A for immunosuppressive and cytotoxic activities

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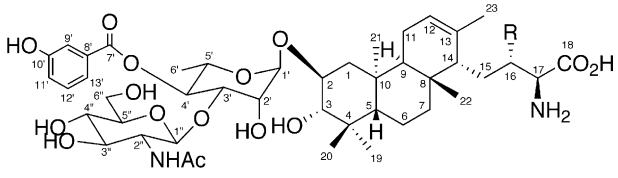
Abstract—Eleven derivatives (**5**–**13**, **15**, and **16**) of an immunosuppressive and cytotoxic tricyclic terpenoid, brasilicardin A (**1**), were prepared and assayed for inhibitory effects to the mouse mixed lymphocyte reaction (MLR) and seven human tumor cell lines. The 17*N*-methyl form (**8**) of **1** showed the most potent immunosuppressive activity in mouse MLR, while induction of more bulky group for N-17 resulted in significant decrease of the activity. Compound **8** also showed potent cytotoxic activity against DLD-1, Lu-65, A549, K562, and MOLT-4 cells, while the benzyl ester (**13**) of **1** exhibited potent cytotoxicity against K562, MOLT-4, and jarkat leukemia cell lines. The 17*N*-acetyl derivative (**11**) of **1** selectively inhibited the cell growth of DLD-1 cells. The methyl ester (**5**) of **1** showed potent cytotoxic activity against K562, MOLT-4, and Ball-1 cell lines, the last of which was resistant to **1**, **8**, and **13**.

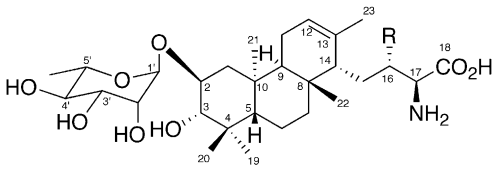
1. Introduction

Brasilicardin A (**1**) is a novel tricyclic terpenoid consisting of an *anti*/*syn*/*anti*-perhydrophenanthrene skeleton with two sugars and an amino acid side chain isolated from the cultured broth of an actinomycete *Nocardia brasiliensis* IFM0406,[1] and exhibits potent immunosuppressive activity in mouse mixed lymphocyte reaction assay and cytotoxic activity against adriamycin-resistant murine lymphoma cells.[2] The mechanism of immunosuppressive activity of **1** was different from those of known immunosuppressive agents such as cyclosporin A[3] or FK-506,[4] which inhibit interleukin-2 production from T helper cells. Furthermore, our preliminary cytotoxic assays for brasilicardin A (**1**) showed effective against DLD-1, Lu65, A549, and K562.

Recently, three new congeners of **1**, brasilicardins B–D (**2**–**4**), were isolated, and the structures were determined by spectroscopic data and a single crystal X-ray diffraction analysis.[5] Immunosuppressive activity of basilicardin B (**2**), the desmethoxy form of **1**, was 50 times less potent than that of **1**, suggesting the presence of the methoxy group at C-16 is important for the immunosuppressive activity of brasilicardin A (**1**). Furthermore, the inhibitory activities of brasilicardins C (**3**) and D (**4**), which were the congeners lacking *N*-acetylglucosamine unit and 3-hydroxybenzoate of **1** and **2**, respectively, in mouse MLR were 50 times less potent than that of **1**.



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



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

Keywords: SAR; Terpenoid; Immunosuppressive; Cytotoxic.
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To investigate the structure–activity relationship (SAR) of brasilicardin A (**1**), 11 derivatives (**5**–**13**, **15**, and **16**) were prepared from **1**, and immunosuppressive and cytotoxic activities of these derivatives were examined. In this paper we describe the SAR results of brasilicardin A (**1**) for immunosuppressive activities in mouse MLR assay and cytotoxicities against seven human tumor cell lines.

2. Results and discussion

2.1. Chemistry

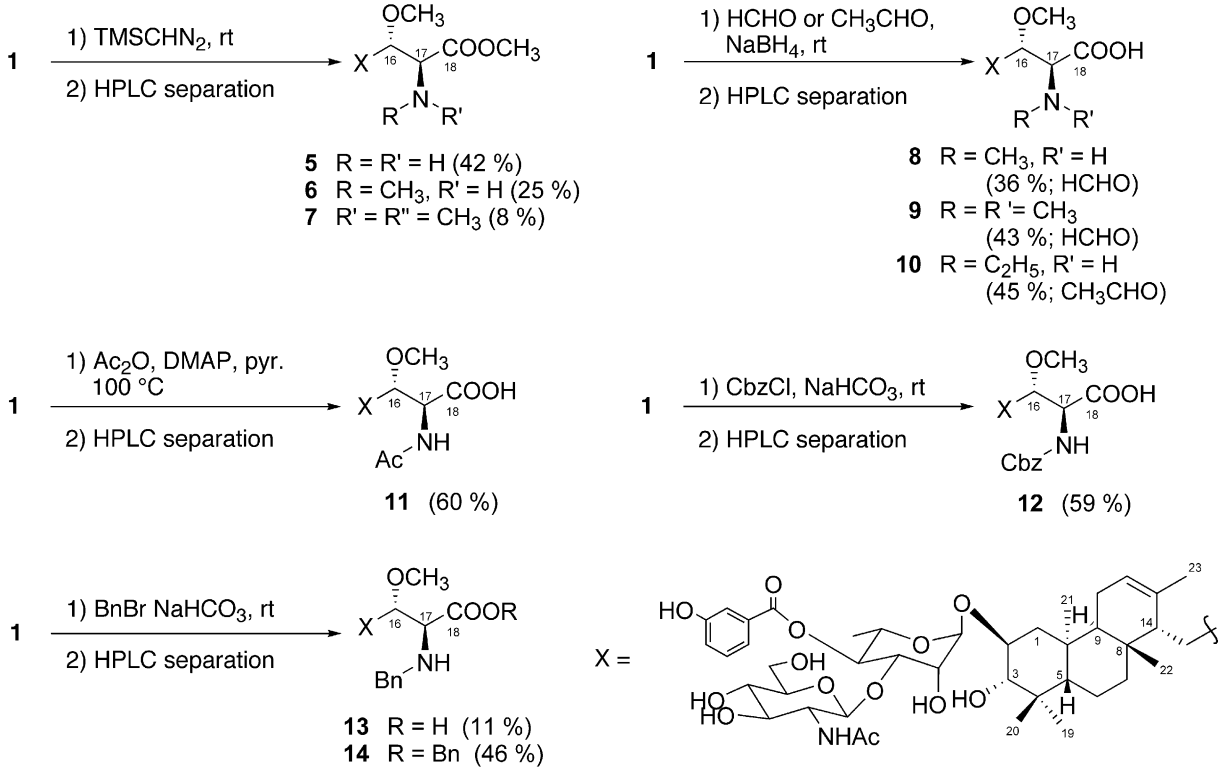
The amino acid side chain attached to the perhydrophenanthrene skeleton seems to be important for the immunosuppressive activity of brasilicardin A (**1**), since its activity was decreased by loss of the methoxy group at C-16.[5] On the other hand, immunosuppressive and cytotoxic activities of brasilicardin C (**3**), lacking the *N*-acetylglucosamine and 3-hydroxylbenzoate from **1**, were less potent than those of brasilicardin A (**1**). Therefore, we planned to estimate influences of the carboxyl and amino groups in the side chain and the (*N*-acetyl)glucosame and (3′-hydroxy)benzoyl moieties for immunosuppressive and cytotoxic activities.

Brasilicardin A (**1**) was treated with trimethylsilyldiazomethane to afford a methyl ester **5** in a 42% yield, together with two *N*-methyl derivatives [**6** and **7** (25% and 8% yield, respectively)] of **5** (Scheme 1). On the other hand, the monomethyl and dimethyl forms (**8** and **9**, respectively), of the 17-nitrogen atom in **1** were obtained in 36% and 43% yields, respectively, by treatment of **1** with formaldehyde and $NaBH_4$. Similarly, treatment of **1** with acetaldehyde and $NaBH_4$ afforded the 17-*N*-ethyl form (**10**, 45%) of **1** and 40% recovery of **1**. Treatment of **1** with 2 mol equiv of Ac_2O gave the 17*N*-acetyl form (**11**) of **1** in 60% yield. The 17*N*-carbobenzoyl (Cbz) derivative (**12**) of **1** was obtained in 59% yield by treatment of **1** with CbzCl. The 18*O*-benzyl ester (**13**) (11% yield) and 17*N*, 18*O*-bisbenzyl derivative (**14**, 46% yield) of **1** were produced by reaction between **1** and benzyl bromide.

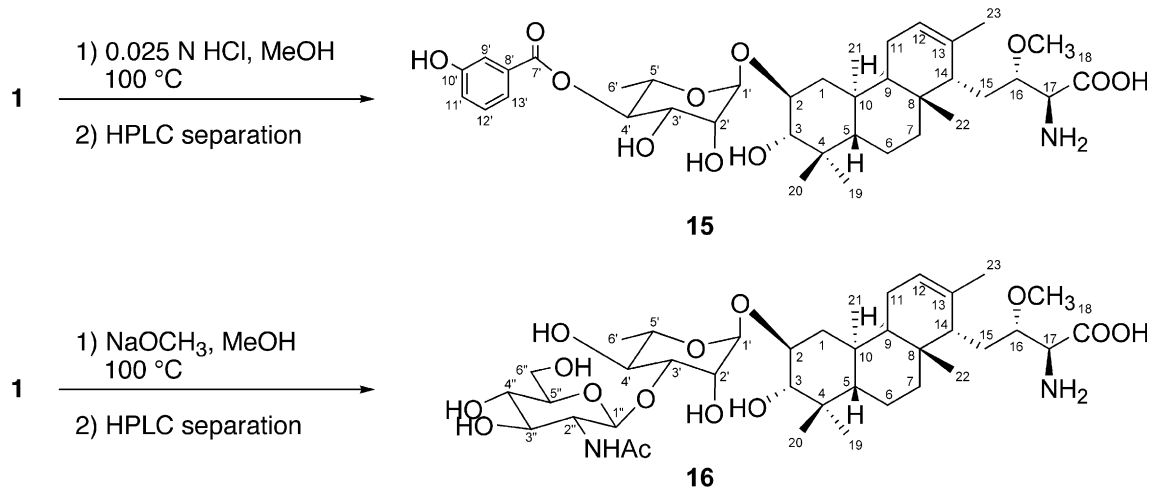
The des-(*N*-acetyl)glucosame and des-(3′-hydroxy)benzoyl forms (**15** and **16**, respectively), of **1** were prepared as shown in Scheme 2. Compound **15** was afforded in 57% yield by partial hydrolysis of **1** with diluted HCl in methanol at 100 °C in a shielded tube. On the other hand, hydrolysis of **1** with sodium methoxide in methanol at 100 °C in a shielded tube gave compound **16** in 57% yield.

2.2. Immunosuppressive activity

The immunosuppressive activities on mouse MLR of 11 derivatives (**5**–**13**, **15**, and **16**) of brasilicardin A (**1**) were examined together with **1** (Table 1). The 17*N*-methyl derivative (**8**) exhibited the most potent immunosuppressive activity, comparable to that of **1**, while that of the *N*,*N*-dimethyl form **9** was five times less potent than that of compound **8**. The IC_{50} values of *N*-ethyl, *N*-acetyl, and *N*-carbobenzoyl forms (**10**, **11**, and **12**, respectively), at 17-position in **1** were 4.28, 16.8, and 13.8 μg/mL, respectively. Thus, the immunosuppressive activity



Scheme 1.



Scheme 2.

Table 1. Immunosuppressive and cytotoxic activities of brasilicardin A (**1**) and 11 derivatives (**5**–**13**, **15**, and **16**)

Compd	IC_{50} (μg/mL)							
	MLR	DLD-1	Lu65	A549	K562	MOLT4	Jurkat	Ball-1
1	0.18	2.48	1.25	0.27	0.02	29	0.78	50
5	1.38	25	25	4.86	0.31	0.24	25	0.68
6	5.24	50	100	19.4	5.24	4.25	100	4.25
7	21.8	50	7.21	18.6	10.2	9.45	9.45	25
8	0.12	0.23	0.85	0.34	0.63	0.02	2.8	25
9	0.68	50	100	2.89	100	2.46	2.46	50
10	4.28	50	17.4	4.56	5.23	1.25	1.25	1.25
11	16.8	0.89	100	100	100	100	100	100
12	13.8	100	100	100	100	100	100	100
13	73.2	50	50	10.4	0.31	0.28	0.28	50
15	100	4.26	4.28	2.48	5.47	1.14	1.14	50
16	100	50	50	50	18.6	7.28	7.28	7.28

DLD-1: human colon adenocarcinoma; Lu65: human lung non-small cell carcinoma; A549: human lung adenocarcinoma; K562: human erythroleukemia, *bcr-abl* (+); MOLT4: human T-cell acute lymphoblastic leukemia, CD4 (+); jurkat: human T-cell leukemia, IL-2 receptor (−); Ball-1: human B-cell lymphoblastic leukemia.

of **1** was increased 40% by monomethylation of N-17, while induction of more bulky group for N-17 resulted in significant decrease of the activity.

The methyl ester (**5**) of **1** and its *N*-methyl form (**6**) were 10 and 50 times less potent than that of **8**, respectively, indicating that the existence of the carboxylic acid at C-18 was important for the immunosuppressive activity. Des-(*N*-acetyl)glucosame and des-(3′-hydroxy)benzoyl forms (**15** and **16**, respectively), of **1** showed no immunosuppressive activity at 100 μg/mL, suggesting that both of *N*-acetylglucosamine unit and 3′-hydroxybenzoyl group are essential for the immunosuppressive activity of brasilicardin A (**1**).

2.3. Cytotoxic activity

Our preliminary cytotoxic assays for brasilicardin A (**1**) showed effective against DLD-1, Lu65, A549, and K562. The cytotoxic activities of 11 derivatives (**5**–**13**, **15**, and **16**) of brasilicardin A (**1**) against four human tumor cell lines, DLD-1, Lu65, A549, and K562, and three human lymphoma cells, MOLT4, jurkat, and Ball-1, as references of cytotoxicity, are summarized in Table 1. Brasilicardin A (**1**) exhibited potent cytotoxicity against human lung adenocarcinoma A549, human erythroleukemia K562, and human T-cell leukemia jurkat cell lines with IC_{50} values of 0.27, 0.02, and 0.78 μg/mL, respectively. On the other hand, the 17*N*-methyl derivative (**8**) of **1** showed a broad cytotoxicity spectrum including human colorectal adenocarcinoma DLD-1 (IC_{50} 0.23 μg/mL), lung non-small cell carcinoma Lu65 (IC_{50} 0.85 μg/mL), human T-acute lymphoblastic leukemia MOLT4 (IC_{50} 0.02 μg/mL), A549 (IC_{50} 0.34 μg/mL), and K562 (IC_{50} 0.63 μg/mL) cell lines, while the *N*,*N*-dimethyl derivative (**9**) of **1** was less cytotoxic than **1** or **5**. The *N*-ethyl form (**10**) of **1** showed modest cytotoxicity against A549, K562, MOLT-4, jurkat, and human B leukeimic Ball cells (IC_{50} 1.25–5.23 μg/mL), of which the profile was different from that of **1** or **5**, although the cytotoxicities were less potent than that of **8**. The *N*-acetyl form (**11**) of **1** showed selective cytotoxicity against DLD-1 cells (IC_{50} 0.89 μg/mL), while the Cbz derivative (**12**) showed no cytotoxicity. Thus, it is suggested that induction of bulky substituents to N-17 results in decrease of the cytotoxicity of brasilicardin A (**1**).

The methyl ester (**5**) of **1** showed cytotoxicity against K562, MOLT4, and Ball-1 cells (IC_{50} 0.31, 0.24, and 0.68 μg/mL, respectively), while compound **5** showed weak cytotoxicity against DLD-1, Lu65, and jurkat cells. Among the tested samples, compound **5** exhibited the most potent cytotoxic activity against Ball-1 cell line, which were tolerant against **1**, **8**, and **13**. The 17*N*-methyl form (**6**) of **5** showed less potent cytotoxicity against K562, MOLT-4, and Ball-1 cells (IC_{50} 4.25–5.24 μg/mL) than **5**. The benzyl ester (**13**) of **1** was potent cytotoxic against leukemia cell lines such as K562, MOLT4, and jurkat cells (IC_{50} 0.28–0.31 μg/mL), while compound **13** showed the most potent cytotoxicity against jurkat cells among tested samples. Therefore, a variety of the alcohol introduced to the 18-carboxyl group seems to affect the cytotoxic profile against leukemia cell lines.

Compound **15** showed a broad cytotoxicity spectrum against six cell lines (IC_{50} 1.14–5.47 μg/mL) except for Ball-1 cells. Cytotoxicity of **15** against jurkat cells was more potent than that of **8**. Compound **16** showed modest cyotoxic activity against four leukemia cell lines. Therefore, it is suggested that the presence of the 3′-hydroxylbeonzoyl group is essential for potent cytotoxic activities against solid tumor cell lines, while the *N*-acetylglucosamine unit at C-3′ is important for the cytotoxicity against tested cell lines except for jurkat cell line.

3. Conclusion

In SAR studies of a series of brasilicardin A derivatives (**5**–**13**, **15**, and **16**) for immunosuppressive and cytotoxic activities, it was revealed that the 17*N*-methyl form (**8**) of **1** showed the most potent immunosuppressive activity in mouse MLR, while induction of more bulky group for N-17 resulted in significant decrease of the activity. Compound **8** also showed potent cytotoxic activity against DLD-1, Lu-65, A549, K562, and MOLT-4 cells, while the benzyl ester (**13**) of **1** exhibited potent cytotoxicity against K562, MOLT-4, and jurkat leukemia cell lines. The 17*N*-acetyl derivative (**11**) of **1** selectively inhibited the cell growth of DLD-1 cells. The methyl ester (**5**) of **1** showed potent cytotoxic activity against K562, MOLT-4, and Ball-1 cell lines, the last of which was resistant to **1**, **8**, and **13**.

4. Experimental

4.1. 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. Mouse MLR assay was performed as described by Hatanaka et al.[6] Cytotoxicty assay was performed in 96-well flat-bottom microtest plate; each well contained 104 cells and a variable 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.[7]

4.2. Reaction of brasilicardin A (1) with trimethylsilyl diazomethane

Brasilicardin A (**1**, 15 mg, 16.8 μmol) in MeOH (1 mL) was added 70 μL of 2 M solution of trimethylsilyl diazomethane in hexane and stirred at rt for 4 h, and then solvent was evaporated. The residue was purified by C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 1.0 × 25 cm; H_2O/MeOH, 19:81) to afford compounds **5** (6.4 mg, 7.1 μmol, 42%, t_R 8 min), **6** (3.8 mg, 4.1 μmol, 25%, t_R 9 min), and **7** (1.2 mg, 1.3 μmol, 8%, t_R 13 min).

4.2.1. Compound 5. $[\alpha]_D^{21}$ +14 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3423, 1713, and 1630 cm^{-1}; ^1H NMR (C_5D_5N) δ 1.05 (3H, s, H_3-20), 1.06 (3H, s, H_3-21), 1.09 (3H, s, H_3-19), 1.14 (3H, s, H_3-22), 1.23 (1H, m, H-15), 1.35 (1H, m, H-7), 1.45 (3H, d, *J* = 5.5 Hz, H-6′), 1.61 (1H, m, H-1), 1.62 (1H, m, H-5), 1.68 (1H, m, H-6), 1.70 (1H, m, H-7), 1.73 (1H, m, H-14), 1.75 (3H, s, NAc), 1.76 (1H, m, H-11), 1.76 (3H, s, H_3-23), 1.78 (1H, m, H-6), 1.79 (1H, m, H-15), 1.81 (1H, m, H-9), 1.83 (1H, m, H-11), 1.98 (1H, m, H-1), 3.37 (1H, d, *J* = 9.1 Hz, H-3), 3.48 (3H, s, OMe), 3.71 (1H, m, H-5″), 3.79 (3H, s, COOMe), 3.81 (1H, m, H-16), 3.96 (1H, t, *J* = 8.9 Hz, H-4″), 4.07 (1H, m, H-2), 4.09 (1H, m, H-6″), 4.17 (1H, dd, *J* = 8.5 and 8.1 Hz, H-2″), 4.32 (1H, m, H-6″), 4.34 (1H, m, H-3″), 4.51 (1H, s, H-17), 4.54 (1H, m, H-5′), 4.85 (1H, d, *J* = 9.1 Hz, H-3′), 5.11 (1H, s, H-2′), 5.28 (1H, s, H-12), 5.40 (1H, d, *J* = 8.1 Hz, H-1″), 5.80 (1H, s, H-1′), 6.08 (1H, t, *J* = 9.4 Hz, H-4′), 7.32 (1H, m, H-13′), 7.32 (1H, m, H-11′), 7.82 (1H, d, *J* = 6.7 Hz, H-12′), 8.15 (1H, s, H-9′); HRESIMS 929.4629 $(M+Na)^+$, calcd for $C_{46}H_{70}N_2O_{16}Na$, 929.4623.

4.2.2. Compound 6. $[\alpha]_D^{21}$ +11 (*c* 1.0, MeOH); IR (KBr) ν_{max} 3421, 1712, and 1630 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.04 (3H, s, H_3-20), 1.08 (3H, s, H_3-22), 1.13 (3H, s, H_3-21), 1.17 (3H, d, *J* = 6.8 Hz, H-6′), 1.27 (1H, m, H-9), 1.39 (1H, m, H-15), 1.41 (1H, m, H-7), 1.41 (1H, m, H-6), 1.47 (1H, m, H-15), 1.49 (1H, m, H-1), 1.51 (1H, m, H-6), 1.59 (1H, m, H-14), 1.66 (1H, m, H-5), 1.66 (3H, s, NAc), 1.69 (3H, s, H_3-23), 1.81 (1H, m, H-7), 1.83 (1H, m, H-1), 1.92 (2H, m, H_2-11), 2.80 (3H, s, NMe), 3.07 (1H, d, *J* = 9.5 Hz, H-3), 3.35 (1H, m, H-5″), 3.44 (1H, m, H-3″), 3.44 (1H, m, H-4″), 3.57 (3H, s, OMe), 3.61 (1H, t, *J* = 9.2 Hz, H-2″), 3.74 (1H, m, H-2), 3.75 (1H, m, H-6″), 3.76 (1H, m, H-16), 3.93 (1H, m, H-6″), 3.93 (3H, s, COOMe), 4.05 (1H, m, H-5′), 4.13 (1H, dd, *J* = 9.7 and 2.8 Hz, H-3′), 4.39 (1H, s, H-2′), 4.50 (1H, d, *J* = 3.4 Hz, H-17), 4.59 (1H, d, *J* = 8.4 Hz, H-1″), 5.07 (1H, s, H-1′), 5.30 (1H, t, *J* = 9.7 Hz, H-4″), 5.39 (1H, br, H-12), 7.11 (1H, d, *J* = 7.8 Hz, H-11′), 7.38 (1H, t, *J* = 7.8 Hz, H-12′), 7.52 (1H, s, H-9′), 7.59 (1H, d, *J* = 7.5 Hz, H-13′); HRESIMS 943.4766 $(M+Na)^+$, calcd for $C_{47}H_{72}O_{16}N_2Na$ 943.4779.

4.2.3. Compound 7. $[\alpha]_D^{25}$ +19 (*c* 0.3, MeOH); IR (KBr) ν_{max} 3427, 1711, and 1630 cm^{-1}; ^1H NMR (CD_3OD) δ

0.97 (3H, s, H_3-19), 1.04 (3H, s, H_3-20), 1.10 (3H, s, H_3-22), 1.17 (3H, s, H_3-21), 1.17 (3H, d, J = 6.8 Hz, H_3-6′), 1.32 (1H, m, H-9), 1.41 (1H, m, H-6), 1.41 (1H, m, H-7), 1.50 (1H, m, H-1), 1.50 (1H, m, H-15), 1.52 (1H, m, H-6), 1.53 (3H, s, NAc), 1.59 (1H, m, H-14), 1.69 (1H, m, H-5), 1.72 (3H, s, H_3-23), 1.81 (1H, m, H-7), 1.86 (1H, m, H-1), 1.90 (1H, m, H-15), 1.95 (2H, m, H_2-11), 3.02 (6H, s, NMe_2), 3.07 (1H, d, J = 9.5 Hz, H-3), 3.34 (1H, m, H-5″), 3.35 (1H, m, H-4″), 3.42 (1H, m, H-3″), 3.58 (3H, s, OMe), 3.61 (1H, m, H-2″), 3.74 (1H, m, H-2), 3.74 (1H, m, H-6″), 3.77 (1H, m, H-16), 3.93 (1H, m, H-6″), 3.94 (3H, s, COOMe), 4.05 (1H, dd, J = 9.7 and 6.5 Hz, H-5′), 4.13 (1H, dd, J = 9.7 and 2.9 Hz, H-3′), 4.39 (1H, s, H-2′), 4.52 (1H, m, H-17), 4.58 (1H, d, J = 8.4 Hz, H-1″), 5.08 (1H, d, J = 1.5 Hz, H-1′), 5.31 (1H, t, J = 10.0 Hz, H-4′), 5.43 (1H, br, H-12), 7.10 (1H, d, J = 7.8 Hz, H-11′), 7.38 (1H, t, J = 7.8 Hz, H-12′), 7.52 (1H, s, H-9′), 7.59 (1H, d, J = 7.6 Hz, H-13′); HRESIMS 957.4921 $(M+Na)^+$, calcd for $C_{48}H_{74}O_{16}N_2Na$ 957.4936.

4.3. Reaction of brasilicardin A (1) with formaldehyde and sodium borohydride

Brasilicardin A (**1**, 2.5 mg, 2.8 μmol) in MeOH (300 μL) was added 4 μL of formaldehyde and stirred at rt for 1 h $NaBH_4$ (0.1 mg) was added, and the mixture was stirred at room temperature for 2 h. After evaporation of the solvent, the residue was applied on a SiO_2 column (ϕ 0.5 × 6 cm; $CHCl_3$/MeOH/H_2O, 7:2:0.2 → 6:3:0.5) and then C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 10 × 250 mm; 0.1% CH_3COONH_4 aq/MeOH, 32:68) to afford compounds **8** (0.9 mg, 1.0 μmol, 36%, t_R 9 min) and **9** (1.1 mg, 1.2 μmol, 43%, t_R 11 min).

4.3.1. Compound 8. $[\alpha]_D^{21}$ +7 (c 0.2, MeOH); IR (KBr) ν_{max} 3408, 1715, and 1637 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.03 (3H, s, H_3-20), 1.06 (3H, s, H_3-22), 1.16 (3H, s, H_3-21), 1.17 (3H, d, J = 6.8 Hz, H-6′), 1.28 (1H, m, H-15), 1.33 (1H, m, H-7), 1.34 (1H, m, H-9), 1.47 (1H, m, H-1), 1.52 (3H, s, NAc), 1.57 (1H, m, H-14), 1.63 (1H, m, H-5), 1.66 (1H, m, H-6), 1.69 (3H, s, H_3-23), 1.73 (1H, m, H-15), 1.73 (1H, m, H-6), 1.82 (1H, dd, J = 11.9 and 3.7 Hz, H-1), 1.83 (1H, m, H-7), 1.90 (2H, m, H_2-11), 2.73 (3H, s, NMe), 3.03 (1H, d, J = 9.3 Hz, H-3), 3.35 (1H, m, H-5″), 3.44 (1H, m, H-4″), 3.44 (1H, m, H-3″), 3.51 (3H, s, MeO), 3.58 (1H, dd, J = 10.1 and 8.2 Hz, H-2″), 3.73 (1H, m, H-6″), 3.74 (1H, m, H-2), 3.76 (1H, m, H-16), 3.86 (1H, br, H-17), 3.92 (1H, dd, J = 11.9 and 1.9 Hz, H-6″), 4.04 (1H, m, H-5′), 4.11 (1H, dd, J = 10.1 and 3.0 Hz, H-3′), 4.38 (1H, s, H-2′), 4.57 (1H, d, J = 8.5 Hz, H-1″), 5.07 (1H, s, H-1′), 5.31 (1H, t, J = 9.3 Hz, H-4′), 5.36 (1H, br, H-12), 7.11 (1H, d, J = 7.6 Hz, H-11′), 7.38 (1H, t, J = 7.6 Hz, H-12′), 7.51 (1H, s, H-9′), 7.59 (1H, d, J = 7.6 Hz, H-13′); HRESIMS 929.4605 $(M+Na)^+$, calcd for $C_{46}H_{70}O_{16}N_2Na$ 929.4623.

4.3.2. Compound 9. $[\alpha]_D^{25}$ +17 (c 0.25, MeOH); IR (KBr) ν_{max} 3427, 1711, and 1630 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.03 (3H, s, H_3-20), 1.07 (3H, s, H_3-22), 1.16 (3H, s, H_3-21), 1.18 (3H, d, J = 6.8 Hz, H-6′), 1.32 (1H, m, H-7), 1.39 (1H, m, H-15), 1.39 (1H, m, H-9), 1.48 (1H, m, H-1), 1.53 (3H, s, NAc), 1.59 (1H, m, H-14), 1.64 (1H, m, H-6), 1.65 (1H, m, H-5), 1.69 (3H, s, H_3-23), 1.74 (1H, m, H-6), 1.81 (1H, m, H-7), 1.84 (1H, m, H-1), 1.92 (2H, m, H_2-11), 2.13 (1H, dd, J = 15.0 and 6.2 Hz, H-15), 2.96 (6H, s, NMe_2), 3.04 (1H, d, J = 10.0 Hz, H-3), 3.35 (1H, m, H-5″), 3.44 (1H, m, H-4″), 3.44 (1H, m, H-3″), 3.53 (3H, s, MeO), 3.58 (1H, t, J = 9.3 Hz, H-2″), 3.73 (1H, m, H-6″), 3.74 (1H, m, H-2), 3.77 (1H, m, H-16), 3.88 (1H, br, H-17), 3.92 (1H, d, J = 11.8 Hz, H-6″), 4.05 (1H, br dd, J = 10.0 and 5.6 Hz, H-5′), 4.12 (1H, dd, J = 10.0 and 3.1 Hz, H-3′), 4.38 (1H, s, H-2′), 4.57 (1H, d, J = 8.1 Hz, H-1″), 5.07 (1H, s, H-1′), 5.31 (1H, t, J = 10.0 Hz, H-4″), 5.38 (1H, br, H-12), 7.10 (1H, d, J = 8.1 and 2.5 Hz, H-11′), 7.38 (1H, t, J = 8.1 Hz, H-12′), 7.51 (1H, br, H-9′), 7.59 (1H, d, J = 8.1 Hz, H-13′); HRESIMS 943.4784 $(M+Na)^+$, calcd for $C_{47}H_{72}O_{16}N_2Na$ 943.4780.

4.4. Reaction of brasilicardin A (1) with acetaldehyde and sodium borohydride

Brasilicardin A (**1**, 2.0 mg, 2.2 μmol) in MeOH (300 μL) was added 90% acetaldehyde (4 mg) and stirred at room temperature for 15 min. After addition of $NaBH_4$ (0.2 mg), the reaction mixture was stirred at rt for 25 min. After addition of H_2O, the mixture was applied on a C_{18} column (ϕ 0.5 × 2.5 cm; H_2O/MeOH, 30:70 → 0:100). The fraction eluted by MeOH was concentrated, and then the residue was separated by C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 10 × 250 mm, 0.1% CH_3COONH_4 aq/MeOH, 42:58) to give compound **10** (0.9 mg, 1.0 μmol, 45%, t_R 15 min), and the starting material (**1**, 0.8 mg, 0.9 μmol, 41%, t_R 14 min) was recovered.

4.4.1. Compound 10. $[\alpha]_D^{25}$ +19 (c 0.1, MeOH); IR (KBr) ν_{max} 3427, 1712, and 1636 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.03 (3H, s, H_3-20), 1.07 (3H, s, H_3-22), 1.16 (3H, s, H_3-21), 1.17 (3H, d, J = 6.8 Hz, H-6′), 1.33 (1H, m, H-7), 1.33 (1H, m, H-15), 1.35 (3H, t, J = 7.3 Hz, NCH_2CH_3), 1.35 (1H, m, H-9), 1.47 (1H, t, J = 12.0, H-1), 1.52 (3H, s, NAc), 1.57 (1H, m, H-14), 1.64 (1H, m, H-5), 1.65 (1H, m, H-6), 1.70 (3H, s, H_3-23), 1.73 (1H, m, H-6), 1.73 (1H, m, H-15), 1.82 (1H, m, H-1), 1.84 (1H, m, H-7), 1.90 (2H, m, H_2-11), 3.03 (1H, d, J = 9.7 Hz, H-3), 3.07 (1H, q, J = 7.3 Hz, NCH_2CH_3), 3.17 (1H, q, J = 7.3 Hz, NCH_2CH_3), 3.32 (1H, m, H-5″), 3.32 (1H, m, H-4″), 3.41 (1H, m, H-3″), 3.51 (3H, s, MeO), 3.58 (1H, dd, J = 9.5 and 8.5 Hz, H-2″), 3.74 (1H, m, H-6″), 3.74 (1H, m, H-2), 3.77 (1H, m, H-16), 3.91 (1H, br, H-17), 3.92 (1H, d, J = 9.4 Hz, H-6″), 4.04 (1H, br dd, J = 9.7 and 6.3 Hz, H-5′), 4.11 (1H, dd, J = 9.7 and 2.9 Hz, H-3′), 4.38 (1H, s, H-2′), 4.57 (1H, d, J = 8.4 Hz, H-1″), 5.07 (1H, s, H-1′), 5.31 (1H, t, J = 9.9 Hz, H-4″), 5.36 (1H, br, H-12), 7.09 (1H, d, J = 7.8 Hz, H-11′), 7.38 (1H, t, J = 7.8 Hz, H-12′), 7.51 (1H, br, H-9′), 7.59 (1H, d, J = 7.8 Hz, H-13′); HRESIMS 943.4774 $(M+Na)^+$, calcd for $C_{47}H_{72}O_{16}N_2Na$ 943.4780.

4.5. Acetylation of brasilicardin A (1)

Brasilicardin A (**1**, 1.3 mg, 1.5 μmol) in pyridine (70 μL) was added Ac_2O (0.23 mg), DMAP (0.02 mg), and pyridine (5 μL), and the mixture was heated at 100 °C for 3 h in a shield tube. After the solvent was removed under N_2 stream, the residue was separated by C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 1.0 × 25 cm; 0.1% CH_3COONH_4 aq/MeOH, 40:60) to yield compound **11** (0.8 mg, 0.9 μmol, 60%, t_R 15 min): $[\alpha]_D^{22}$ +20 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3431 and 1629 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.03 (3H, s, H_3-20), 1.05 (3H, s, H_3-22), 1.16 (3H, s, H_3-21), 1.17 (3H, d, *J* = 6.8 Hz, H_3-6′), 1.32 (1H, m, H-7), 1.37 (1H, m, H-15), 1.38 (1H, m, H-9), 1.47 (1H, m, H-1), 1.52 (3H, s, NAc), 1.62 (1H, m, H-5), 1.65 (1H, m, H-15), 1.66 (3H, s, H_3-23), 1.67 (1H, m, H-6), 1.68 (1H, m, H-14), 1.73 (1H, m, H-6), 1.81 (1H, m, H-1), 1.88 (1H, m, H-7), 1.89 (2H, m, H_2-11), 2.03 (3H, s, 17-NAc), 3.03 (1H, d, *J* = 10.0 Hz, H-3), 3.32 (1H, m, H-5″), 3.33 (1H, m, H-4″), 3.40 (1H, m, H-3″), 3.59 (1H, t, d, *J* = 8.7 Hz, H-2″), 3.68 (3H, s, MeO), 3.69 (1H, m, H-16), 3.74 (1H, m, H-6″), 3.75 (1H, m, H-2), 3.92 (1H, d, *J* = 11.8 Hz, H-6″), 4.05 (1H, dd, *J* = 10.0 and 6.2 Hz, H-5′), 4.11 (1H, br d, *J* = 10.0 Hz, H-3′), 4.38 (1H, s, H-2′), 4.89 (1H, br, H-17), 4.57 (1H, d, *J* = 8.1 Hz, H-1″), 5.08 (1H, s, H-1′), 5.31 (1H, m, H-4″), 5.32 (1H, br, H-12), 7.09 (1H, d, *J* = 8.1 Hz, H-11′), 7.38 (1H, t, *J* = 8.1 Hz, H-12′), 7.51 (1H, s, H-9′), 7.59 (1H, d, *J* = 8.1 Hz, H-13′); HRESIMS 957.4574 $(M+Na)^+$, calcd for $C_{47}H_{70}O_{17}N_2Na$ 957.4572.

4.6. Carbobenzoylation of brasilicardin A (1)

To a solution of brasilicardin A (**1**, 1.5 mg, 1.7 μmol) and $NaHCO_3$ (0.2 mg) in dioxane/H_2O (1:1, 200 μL) was added carbobenzoyl chloride (14.5 μL, 2.5 mg/100 μL in dioxane) and stirred at rt for 70 min. Solvent was removed in vacuo, and the residue was dissolved in 0.1% CH3COONH4 aq/MeOH (1:1) and separated by C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 1.0 × 25 cm; 0.1% CH_3COONH_4 aq/MeOH, 32:68) to afford compound **12** (1.0 mg, 1.0 μmol, 59%, t_R 15 min): $[\alpha]_D^{23}$ +33 (*c* 0.5, MeOH); IR (KBr) ν_{max} 3432, 1715, and 1633 cm^{-1}; ^1H NMR (CD_3OD) δ 0.95 (3H, s, H_3-19), 1.02 (3H, s, H_3-20), 1.05 (3H, s, H_3-22), 1.13 (3H, s, H_3-21), 1.17 (3H, d, *J* = 6.2 Hz, H_3-6′), 1.33 (1H, m, H-7), 1.36 (1H, m, H-9), 1.36 (1H, m, H-15), 1.46 (1H, t, *J* = 11.8, H-1), 1.53 (3H, s, NAc), 1.59 (1H, m, H-15), 1.63 (1H, m, H-6), 1.63 (1H, m, H-14), 1.66 (3H, s, H_3-23), 1.67 (1H, m, H-5), 1.73 (1H, m, H-6), 1.81 (1H, dd, *J* = 11.8 and 3.1 Hz, H-1), 1.84 (1H, m, H-7), 1.90 (2H, m, H_2-11), 3.03 (1H, d, *J* = 9.7 Hz, H-3), 3.33 (1H, m, H-5″), 3.35 (1H, m, H-4″), 3.40 (1H, m, H-3″), 3.48 (3H, s, MeO), 3.59 (1H, t, *J* = 9.3 Hz, H-2″), 3.66 (1H, m, H-16), 3.74 (1H, m, H-2), 3.74 (1H, m, H-6″), 3.92 (1H, d, *J* = 11.8 Hz, H-6″), 4.04 (1H, br dd, *J* = 9.3 and 6.2 Hz, H-5′), 4.11 (1H, dd, *J* = 9.3 and 3.1 Hz, H-3′), 4.37 (1H, s, H-2′), 4.57 (1H, d, *J* = 8.1 Hz, H-1″), 4.62 (1H, m, H-17), 5.08 (1H, s, H-1′), 5.12 (2H, s, Ph*C*H_2CO), 5.31 (1H, t, *J* = 9.7 Hz, H-4″), 5.32 (1H, br, H-12), 7.09 (1H, d, *J* = 7.5 Hz, H-11′), 7.30–7.42 (5H, m, Ph), 7.38 (1H, m, H-12′), 7.51 (1H, br, H-9′), 7.59 (1H, d, *J* = 8.1 Hz, H-13′); HRESIMS 1049.4863 $(M+Na)^+$, calcd for $C_{53}H_{74}O_{18}N_2Na$ 1049.4834.

4.7. Reaction of brasilicardin A (1) with benzyl bromide

To a solution of brasilicardin A (**1**, 5.0 mg, 5.6 mmol) and $NaHCO_3$ (1.9 mg) in DMF (300 μL) was added benzyl bromide (4.2 mg) and stirred at rt for 4 h. After addition of saturated NH_4Cl aq to the reaction solution, and the mixture was extracted with EtOAc. The organic layer was washed with H_2O and then brine, and concentrated in vacuo. The residue was separated by a SiO_2 column (ϕ 0.5 × 7 cm; $CHCl_3$/MeOH = 9:1, 85:15 and 8:2) and then C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 1.0 × 25 cm; 0.1% CH_3COONH_4 aq/MeOH, 25:75) to give compounds **13** (0.6 mg, 0.6 mmol, 11%, t_R 10 min) and 14 (2.8 mg, 2.6 mmol, 46%, i_R 16 min).

4.7.1. Compound 13. $[\alpha]_D^{22}$ +27 (*c* 0.3, MeOH); IR (KBr) ν_{max} 3432, and 1632 cm^{-1}; ^1H NMR (CD_3OD) δ 0.89 (3H, s, H_3-19), 0.99 (3H, s, H_3-20), 1.02 (3H, s, H_3-22), 1.02 (3H, s, H_3-21), 1.17 (3H, d, *J* = 6.2 Hz, H_3-6′), 1.26 (1H, m, H-7), 1.26 (1H, m, H-9), 1.38 (1H, m, H-6), 1.40 (1H, m, H-15), 1.45 (1H, m, H-1), 1.48 (1H, m, H-15), 1.53 (3H, s, NAc), 1.55 (1H, m, H-7), 1.57 (1H, m, H-5), 1.57 (1H, m, H-6), 1.57 (1H, m, H-14), 1.66 (3H, s, H_3-23), 1.80 (1H, dd, *J* = 12.5 and 4.4 Hz, H-1), 1.90 (2H, m, H_2-11), 3.01 (1H, d, *J* = 9.7 Hz, H-3), 3.32 (1H, m, H-5″), 3.35 (1H, m, H-4″), 3.41 (1H, m, H-3″), 3.47 (3H, s, MeO), 3.58 (1H, dd, *J* = 9.9 and 8.6 Hz, H-2″), 3.60 (1H, m, H-16), 3.72 (1H, m, H-2), 3.73 (1H, m, H-6″), 3.92 (1H, dd, *J* = 11.8 and 1.9 Hz, H-6″), 4.03 (1H, br dd, *J* = 9.7 and 6.2 Hz, H-5′), 4.09 (1H, m, H-17), 4.11 (1H, dd, *J* = 10.0 and 2.5 Hz, H-3′), 4.37 (1H, s, H-2′), 4.57 (1H, d, *J* = 8.1 Hz, H-1″), 5.07 (1H, s, H-1′), 5.18 (1H, d, *J* = 12.5 Hz, PhC*H*HO), 5.30 (1H, d, *J* = 12.5 Hz, PhC*H*HO), 5.31 (1H, t, *J* = 9.3 Hz, H-4′), 5.33 (1H, br, H-12), 7.09 (1H, d, *J* = 8.1 Hz, H-11′), 7.39–7.46 (5H, m, Ph), 7.38 (1H, t, *J* = 8.1 Hz, H-12′), 7.51 (1H, br, H-9′), 7.59 (1H, d, *J* = 7.5 Hz, H-13′); HRESIMS 1005.4943 $(M+Na)^+$, calcd for $C_{52}H_{74}O_{16}N_2Na$ 1005.4936.

4.7.2. Compound 14. $[\alpha]_D^{22}$ +62 (*c* 0.2, MeOH); IR (KBr) ν_{max} 3430, and 1635 cm^{-1}; ^1H NMR (CD_3OD) δ 0.91 (3H, s, H_3-19), 0.99 (3H, s, H_3-20), 1.01 (3H, s, H_3-22), 1.06 (3H, s, H_3-21), 1.18 (3H, d, *J* = 6.8 Hz, H_3-6′), 1.21 (1H, m, H-7), 1.28 (1H, m, H-9), 1.43 (1H, m, H-15), 1.45 (1H, t, *J* = 12.0, H-1), 1.50 (1H, m, H-14), 1.53 (3H, s, NAc), 1.55 (1H, m, H-5), 1.55 (1H, m, H-6), 1.60 (1H, m, H-6), 1.60 (1H, m, H-7), 1.65 (3H, s, H_3-23), 1.67 (1H, m, H-15), 1.81 (1H, m, H-1), 1.89 (2H, m, H_2-11), 3.02 (1H, d, *J* = 9.3 Hz, H-3), 3.28 (3H, s, MeO), 3.32 (1H, m, H-5″), 3.32 (1H, m, H-4″), 3.41 (1H, m, H-3″), 3.49 (1H, m, H-16), 3.58 (1H, m, H-2″), 3.61 (1H, m, H-17), 3.67 (1H, d, *J* = 12.0 Hz, Ph*C*H*H*O), 3.72 (1H, m, H-2), 3.73 (1H, dd, *J* = 5.0 and 11.8 Hz, H-6″), 3.92 (1H, m, H-6″), 3.92 (1H, m, Ph*C*H*H*O), 4.04 (1H, br dd, *J* = 10.0 and 6.2 Hz, H-5′), 4.11 (1H, dd, *J* = 10.0 and 3.1 Hz, H-3′), 4.37 (1H, s, H-2′), 4.58 (1H, d, *J* = 8.1 Hz, H-1″), 5.08 (1H, s, H-1′), 5.20 (1H, d, *J* = 12.5 Hz, PhC*H*HO), 5.26 (1H, d,

J = 12.5 Hz, PhC*H*HO), 5.32 (1H, t, J = 10.0 Hz, H-4′), 5.32 (1H, br, H-12), 7.09 (1H, dd, J = 8.1 and 1.9 Hz, H-11′), 7.27–7.47 (10H, m, Ph), 7.38 (1H, t, J = 7.5 Hz, H-12′), 7.51 (1H, br, H-9′), 7.59 (1H, d, J = 7.5 Hz, H-13′); HRESIMS 1095.5234 $(M+Na)^+$, calcd for $C_{59}H_{80}O_{16}N_2Na$ 1095.5201.

4.8. Acidic hydrolysis of brasilicardin A (1)

Brasilicardin A (**1**, 2.5 mg, 2.8 μmol) in MeOH (100 μL) was added 100 μL of MeOH solution of HCl (1 μL 6 M HCl aq/217 μL MeOH), and the mixture was heated at 100 °C for 4.5 h in a shield tube. The mixture diluted with water was applied on a C_{18} column (ϕ 0.5 × 5 cm; H_2O/MeOH, 20:80) and then C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 1.0 × 25 cm; H_2O/MeOH, 40:60) to yield compound **15** (1.1 mg, 1.6 μmol, 57%, t_R 14 min): $[\alpha]_D^{25}$ +24 (c 0.4, MeOH); IR (KBr) ν_{max} 3429, 1711, and 1630 cm^{-1}; ^1H NMR (CD_3OD) δ 0.96 (3H, s, H_3-19), 1.03 (3H, s, H_3-20), 1.07 (3H, s, H_3-22), 1.17 (3H, s, H_3-21), 1.21 (3H, d, J = 6.7 Hz, H-6′), 1.33 (1H, m, H-7), 1.33 (1H, m, H-15), 1.35 (1H, m, H-9), 1.48 (1H, t, J = 11.6 Hz, H-1), 1.59 (1H, m, H-14), 1.63 (1H, m, H-5), 1.64 (1H, m, H-6), 1.65 (1H, m, H-15), 1.69 (3H, s, H_3-23), 1.73 (1H, m, H-6), 1.84 (1H, dd, J = 11.8 and 3.7 Hz, H-1), 1.88 (1H, m, H-7), 1.91 (2H, m, H_2-11), 3.05 (1H, d, J = 9.5 Hz, H-3), 3.49 (3H, s, MeO), 3.74 (1H, m, H-2), 3.79 (1H, m, H-16), 3.99 (1H, m, H-17), 4.00 (1H, m, H-3′), 4.03 (1H, m, H-5′), 4.06 (1H, m, H-2′), 5.07 (1H, s, H-1′), 5.20 (1H, t, J = 9.5 Hz, H-4′), 5.36 (1H, br, H-12), 7.07 (1H, dd, J = 7.9 and 2.1 Hz, H-11′), 7.34 (1H, t, J = 7.9 Hz, H-12′), 7.50 (1H, br, H-9′), 7.56 (1H, d, J = 7.9 Hz, H-13′); HRESIMS 712.3691 $(M+Na)^+$, calcd for $C_{37}H_{55}O_{11}NNa$ 712.3672.

4.9. Alkaline hydrolysis of brasilicardin A (1)

Brasilicardin A (**1**, 1.9 mg, 2.1 μmol) in MeOH (160 μL) was added NaOMe (1.5 mg), and the mixture was heated at 100 °C for 3 h in a shield tube. After addition of saturated NH_4Cl aq, the aqueous solution was applied on an ion exchange column (ϕ 0.5 × 4 cm, water and then MeOH). The fraction eluted by MeOH was evaporated, and the residue was subjected to C_{18} HPLC (DEVELOSIL ODS UG-5, ϕ 10 × 250 mm, H_2O/MeOH, 45:55) to afford compound **16** (0.9 mg, 1.2 μmol, 57%, t_R 9 min): $[\alpha]_D^{23}$ +37 (c 0.4, MeOH); IR (KBr) ν_{max} 3428 and 1635 cm^{-1}; ^1H NMR (CD_3OD) δ 0.95 (3H, s, H_3-19), 1.01 (3H, s, H_3-20), 1.05 (3H, s, H_3-22), 1.15 (3H, s, H_3-21), 1.28 (3H, d, J = 5.9 Hz, H-6′), 1.33 (1H, m, H-7), 1.34 (1H, m, H-15), 1.35 (1H, m, H-9), 1.58 (1H, m, H-14), 1.61 (1H, m, H-5), 1.62 (1H, m, H-6), 1.66 (1H, m, H-15), 1.69 (3H, s, H_3-23), 1.72 (1H, m, H-6), 1.87 (1H, m, H-7), 1.80 (1H, dd, J = 12.0 and 4.1 Hz, H-1), 1.88 (2H, m, H_2-11), 2.04 (3H, s, NAc), 3.00 (1H, d, J = 9.5 Hz, H-3), 3.34 (1H, m, H-5″), 3.40 (1H, m, H-4″), 3.49 (3H, s, MeO), 3.51 (1H, m, H-4″), 3.51 (1H, m, H-3″), 3.70 (1H, m, H-2), 3.71 (1H, m, H-2′), 3.72 (1H, m, H-6″), 3.76 (1H, m, H-5′), 3.76 (1H, m, H-3′), 3.78 (1H, m, H-6″), 3.79 (1H, m, H-16), 3.91 (1H, d, J = 11.5 Hz, H-6″), 3.99 (1H, br, H-17), 4.19 (1H, br, H-2′), 4.69 (1H, d, J = 8.3 Hz, H-1″), 5.00 (1H, s, H-1′), 5.34 (1H, br, H-12); HRESIMS 795.4229 $(M+Na)^+$, calcd for $C_{38}H_{64}O_{14}N_2Na$ 795.4256.

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