Brasilibactin A, a Cytotoxic Compound from Actinomycete *Nocardia brasiliensis*

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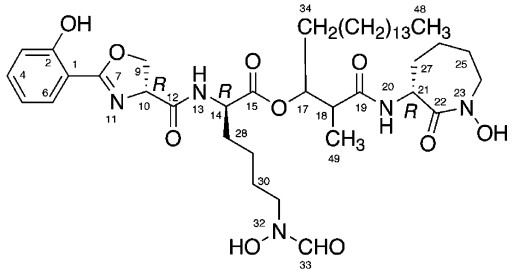
A new cytotoxic compound, brasilibactin A (**1**), has been isolated from the actinomycete *Nocardia brasiliensis* IFM 0995, and the structure was elucidated on the basis of spectroscopic data and chemical means.

During our search for bioactive substances from actinomycetes of the genus *Nocardia*, we previously isolated a 32-membered macrolide possessing immunosupressive and antifungal activity,[1] three biogenetically unique benz[*a*]-anthraquinones with cytotoxic and antibacterial activity,[2] a cytotoxic indole alkaloid with an isonitrile group,[3] a tricyclic terpenoid with immunosuppressive and cytotoxic acitivity,[4] and three cytotoxic benzenoid metabolites.[5] Our recent investigation on extracts of *Nocardia brasiliensis* IFM 0995 resulted in the isolation of brasilibactin A (**1**), a new cytotoxic compound. Here we describe the isolation and structure elucidation of **1**.



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The mycelium of *N. brasiliensis* IFM 0995 obtained from 2 L of the culture broth was extracted with MeOH, and the diethyl ether-soluble parts of the extract were subjected to C_{18} HPLC (CH_3CN/H_2O, 80:20) to yield brasilibactin A (**1**, 33 mg) as a colorless amorphous solid.

The molecular formula of **1** was established to be $C_{42}H_{67}N_5O_{10}$ by HRFABMS [*m/z* 802.4953, $(M + H)^+$, Δ −1.3 mmu]. IR absorptions of **1** indicated the presence of OH and/or NH (3298 cm^{-1}), ester (1738 cm^{-1}), and amide carbonyl (1644 cm^{-1}) groups. UV absorptions [λ_{max} (MeOH) 306 (ϵ 3000), 260 (sh 2500), 250 (sh 6000), and 244 nm (7200)] of **1** were indicative of a substituted benzenoid chromophore. The ^1H and ^{13}C NMR spectra of **1** in DMSO-d_6 (see Experimental Section) revealed the presence of signals due to seven sp^2 quaternary carbons including four carbonyls, one *N*-formyl, four sp^2 methines, five sp^3 methines, three sp^3 methylenes adjacent to heteroatoms, two methyls, and several methylenes in a long alkyl chain. Since nine out of 12 unsaturations were accounted for, **1** was inferred to possess three rings. In the ^{13}C NMR spectrum, the *N*-formyl (C-33: δ 161.5, 156.9) and four methylene carbons (C-28: δ 30.12, 30.13; C-29: δ 22.5, 22.3, C-30: δ 26.45, 26.44; C-31: δ 45.4, 48.9) were observed as a set of signals in the ratio of 6:4, indicating the presence of two rotation isomers of the N−CHO bond.

$^1H-^1H$ COSY, HSQC, CH- and CH_2-selected editing-HSQC, and CH- and CH_2-selected editing-HSQC-TOCSY spectra revealed six $^1H-^1H$ connectivies from H-3 to H-6, from H_2-9 to H-10, from NH-13 to H_2-31, from H_3-49 to H_2-36, from H_2-46 to H_3-48, and from NH-20 to H_2-24 (Figure 1). The coupling patterns of H-3 (δ 7.00, d, $J = 8.3$ Hz), H-4 (δ 7.46, brt, $J = 8.0$ Hz), H-5 (δ 6.94, brt, $J = 8.0$ Hz), and H-6 (δ 7.63, d, $J = 7.7$ Hz) and their ^{13}C chemical shifts (C-3, δ 116.6; C-4 δ 134.0; C-5 δ_C 119.0; C-6 δ 128.0) indicated the presence of a 1,2-disubstituted benzene ring. HMBC correlations for H-3/C-1 (δ 109.8), H-4/C-2 (δ 159.1), and H-5/C-1 suggested that the benzene ring possessed a phenolic hydroxyl group at C-2. HMBC correlations of H-6/C-7 (δ 165.9), H_2-9 (δ 4.65 and 4.47)/C-7, and H-10 (δ 5.02)/C-7 implied that the benzenoid ring (C-1 to C-6) was connected to C-9 and C-10 through C-7 and some heteroatoms. The chemical shifts of C-7, C-9 (δ 69.2), and C-10 (δ 67.0) corresponded well to those of a dihydrooxazole ring rather than those of a dihydrothioxazole ring.[6] The HMBC correlation for H_2-31/C-33 and the NOESY cross-peak for H_2-31/H-33 suggested that the *N*-formyl group was attached to C-31 through N-32. The existence of an ϵ-aminocaprolactam ring was deduced from HMBC correlations from H-21 and H_2-24 to C-22 (δ 168.9). HMBC correlations for H-10/C-12 (δ 169.9), NH-13/C-12, NH-20/C-19 (δ 172.1), and H_3-49/C-19 indicated that C-10 and C-18 were connected to N-13 and N-20, respectively, through amide carbonyls (C-12 and C-19, respectively), while the presence of an ester bond between C-14 and C-17 was suggested by HMBC correlations for H-14/C-15 (δ_C 171.5) and H-17/C-15. A long alkyl chain was assigned as a linear C_{15} chain by FABMS/MS fragment ions as shown in Figure 2. The presence of two hydroxyl groups on N-23 and N-32 were indicated by FABMS/MS fragment ions at *m/z* 145 and 273. The relative stereochemistry at C-17−C-18 was assigned as *erythro* on the basis of *J*-based

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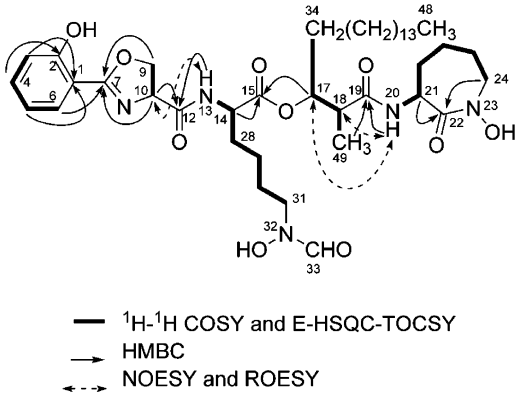

Figure 1. Selected 2D NMR correlations for brasilibactin A (**1**).

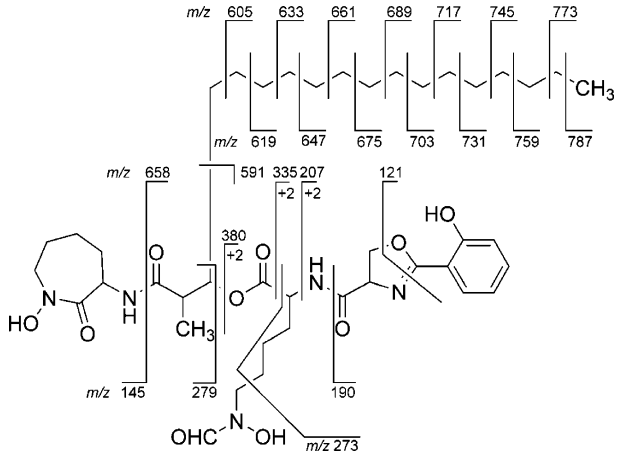

Figure 2. Fragmentation patterns observed in the positive FABMS/MS spectrum of brasilibactin A (**1**) (precursor ion, *m/z* 802).

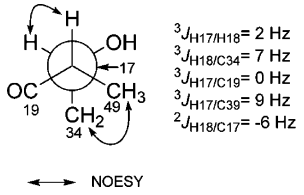

Figure 3. Rotation model for C-17−C-18 bond of brasilibactin A (**1**).

configuration analysis[7] as shown in Figure 3. Thus, the structure of brasilibactin A was elucidated to be **1**.

Brasilibactin A (**1**) was hydrolyzed with 6 N aqueous HCl to afford 1 mol of serine (Ser) and 2 mol of lysine (Lys). Chiral HPLC analyses of the hydrolysate revealed that the Ser residue was D-form, indicating that C-10 had an *R*-configuration. Chiral HPLC analyses of the two Lys residues detected D-Lys. Hence, the absolute configurations at C-14 and C-21 were both assigned as *R*. Therefore, the absolute stereochemistry of brasilibactin A (**1**) was concluded to be 10*R*, 14*R*, and 21*R*.

Brasilibactin A (**1**) is a new compound possessing a 3-hydroxy-2-methyloctadecanoic acid from the actinomycete of *N. brasiliensis* IFM 0955, although many mycobactin-type siderophores similar to **1** have been isolated from actinomycetes of the genera *Mycobacterium*[8] and *Nocardia*.[9−13] The structure of **1** was closely related to that of A77543[13] isolated from *Nocardia* sp. Brasilibactin A (**1**) showed antibacterial activity against *Micrococcus luteus* and *Staphylococcus aureus* (MIC 0.73 and 4.5 mg/mL, respectively), while these activities of **1** were reduced by treatment with $FeCl_3$. Brasilbactin A (**1**) exhibited potent cytotoxicity against murine leukemia L1210 and human epidermoid carcinoma KB cells (IC_{50}, 0.02 and 0.04 μg/mL, respectively) and caused a concentration-dependent increase (0.3−3 μM) in the caspase-3 activity in HL60 cells. Caspase-3 is well-known to play an important role in the final step of a signal transduction pathway of apoptosis.[14]

Experimental Section

General Experimental Procedures. Optical rotations were recorded on a JASCO DIP-1000 polarimeter. The IR and UV spectra were taken on JASCO FT/IR-5300 and JASCO Ubest-35 spectrophotometers, respectively. ^{1}H and ^{13}C NMR spectra were recorded on Bruker AMX-600 and ARX-500 spectrometers, respectively. FAB mass spectra were obtained on JEOL 700TZ and JEOL HX-110 spectrometers. Antimicrobial activities were determined by microbroth dilution method using BHI medium. Cytotoxic activities were determined by the method described.[15] Adriamycin was included as a positive drug control: IC_{50} 0.01 (HL-60), 0.09 (L1210), and 0.97 μg/mL (KB). Caspase-3 activity was measured with a fluorace apopain assay kit (Bio-Rad) that used the substrate DEVD-AFC according to the manufacture's directions.

Fermentation, Extraction, and Isolation. The voucher specimen (*Nocardia brasiliensis* IFM 0955) was deposited at the Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University. *N. brasiliensis* IFM 0955 strain was cultivated at 32 °C for 4 days in 2 L nutrient broth medium [glucose (2.0%), glycerol (2.0%), polypeptone (1.0%), and meat extract (0.5%) in H_2O at pH 7.0]. The cultured broth (2 L) was filtered, and the mycelial cake was extracted with MeOH (500 mL × 3). The ether-soluble materials (4.87 g) of the MeOH extract were subjected to C_{18} HPLC (YMC Pack AM323, YMC Co., Ltd., 20 × 250 mm; eluent, CH_3CN/H_2O, 80:20; flow rate, 10 mL/min; UV detection, 230 nm) to afford brasilibactin A (**1**, 33 mg, t_R 21.2 min).

Brasilibactin A (1): colorless amorphous solid; $[\alpha]^{22}_D$ −31° (*c* 1.0, $CHCl_3$); IR (KBr) ν_{max} 3298, 1738, and 1644 cm^{-1}; UV (EtOH) λ_{max} 306 (ϵ 3000), 260 (sh, 2500), 250 (6000), and 244 nm (7200); ^{1}H NMR (DMSO-d_6, 600 MHz) δ 11.79 (1H, brs, 2-OH), 9.95 (1H, brs, 32-OH), 9.70 (1H, brs, 23-OH), 8.71 (1H, d, J = 11.0 Hz, H-13), 8.22 (0.6H, s, H-33), 8.11 (1H, d, J = 6.9 Hz, H-20), 7.94 (1H, d, J = 7.7 Hz, H-6), 7.87 (0.4H, s, H-33), 7.46 (1H, brt, J = 8.0 Hz), 7.00 (1H, d, J = 8.3 Hz, H-3), 6.94 (1H, brt, J = 8.0 Hz, H-5), 5.02 (1H, dd, J = 7.7, 9.9 Hz, H-10), 4.90 (1H, dt, J = 2.0, 9.0 Hz, H-17), 4.65 (1H, dd, J = 8.8, 9.9 Hz, H-9), 4.47 (1H, dd, J = 7.7, 8.8 Hz, H-9), 4.44 (1H, m, H-21), 4.25 (1H, m, H-14), 3.87 (1H, dd, J = 11.6, 15.8 Hz, H-24), 3.48 (1H, dd, J = 4.4, 15.8 Hz, H-24), 3.42 (1H, m, H-31), 3.35 (1H, m, H-31), 2.62 (1H, m, H-18), 1.82 (2H, m, H_2-26), 1.72 (1H, m, H-28), 1.68−1.60 (2H, m, H-30, H-27), 1.60−1.52 (4H, m, H-30, H-29, H-35), 1.48−1.40 (4H, m, H-27, H-28, H-29, H-35), 1.35−1.20 (27H, m, H-25, H_2-34, H_2-36−H_2-47), 1.09 (1H, m, H-25), 0.95 (3H, d, J = 7.7 Hz, H_3-49), 0.85 (3H, t, J = 6.5 Hz, H_3-48); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 172.1 (C, C-19), 171.5 (C, C-15), 169.9 (C, C-12), 168.9 (C, C-22), 165.9 (C, C-7), 161.5 (C, C-33 major), 159.1 (C, C-2), 156.9 (C, C-33, minor), 134.0 (CH, C-4), 128.0 (CH, C-6), 119.0 (CH, C-5), 116.6 (CH, C-3), 109.8 (C, C-1), 75.6 (CH, C-17), 69.2 (CH_2, C-9), 67.0 (CH, C-10), 52.6 (CH, C-14), 52.5 (CH_2, C-24), 50.6 (CH, C-21), 48.9, 45.4 (CH_2, C-31), 43.6 (CH, C-18), 31.8 (CH_2, C-27), 31.2 (CH_2, C-46), 30.13, 30.12 (CH_2, C-28), 29.0−28.6 (10C, CH_2, C-36−C-45), 26.9 (CH_2, C-26), 26.45, 26.44 (CH_2, C-30), 25.6 (CH_2, C-35) 24.7 (CH_2, C-25), 22.5, 22.3 (CH_2, C-29), 22.0 (CH_2, C-47), 14.4 (CH_3, C-49), 13.9 (CH_3, C-48); FABMS *m/z* 802 $(M + H)^+$; HRFABMS *m/z* 802.4953 $(M + H)^+$, calcd for $C_{42}H_{68}N_5O_{10}$, 802.4966.

Chiral HPLC Analyses of Ser and Lys Residues in Hydrolydate of 1. Brasilibactin A (**1**, 100 μg) was treated with 6 M aqueous HCl (100 μL) at 110 °C for 24 h. Chiral HPLC was carried out using SUMICHIRAL OA-5000 [Sumitomo Chemical Industry, 4 × 150 mm, eluent: 1 mM aqueous $CuSO_4$, flow rate: 0.2 mL/min, UV detection at 254 nm]. Retention times (min) of authentic Ser and Lys were as follows: L-Lys (10.4), D-Lys (12.4) L-Ser (25.6), and D-Ser (27.6). The retention time of the hydrolysate of **1** was as follows: D-Lys (12.4) and D-Ser (27.6).

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