Awajanomycin, a Cytotoxic γ-Lactone-δ-lactam Metabolite from Marine-Derived *Acremonium* sp. AWA16-1

Jae-Hyuk Jang,* Kaneo Kanoh, Kyoko Adachi, and Yoshikazu Shizuri

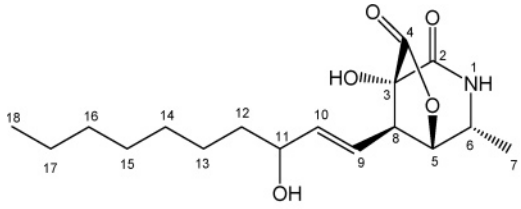
Laboratory of Applied Bioorganic Chemistry, Marine Biotechnology Institute Co., Ltd., 3-75-1 Heita, Kamaishi, Iwate 026-0001, Japan

Received April 18, 2006

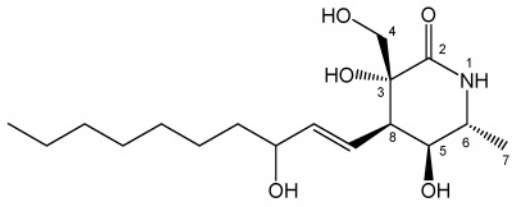
The new fungal metabolite awajanomycin (**1**), which has γ-lactone-δ-lactam rings, was isolated from the marine-derived fungus *Acremonium* sp. AWA16-1, which had been collected from sea mud off Awajishima Island in Japan. The structure of **1** was elucidated by spectroscopic analysis and chemical methods. Awajanomycin (**1**) and its derivative (**2**) inhibited the growth of A549 cells with IC_{50} values of 27.5 and 46.4 μg/mL, respectively.

Marine microorganisms have recently gained attention as important sources of chemically interesting and biologically active secondary metabolites for the development of new pharmaceutical agents.[1] In particular, marine-derived fungi have shown great potential as suggested by the diversity of secondary metabolites, including many that have novel carbon skeletons.[2] We have been screening marine-derived microorganisms for antimicroalgal, antibacterial, and antitumor activities to find novel bioactive substances.[3−5] In the course of screening for antitumor substances, we found an activity in the extract of the marine-derived fungus *Acremonium* sp. AWA16-1. Fungi belonging to the genus *Acremonium* are known to produce many interesting secondary metabolites, including orbuticin,[6] acremolactones A−C,[7−9] acremonidin,[10] virescenosides,[11−13] hydroquinone derivatives,[14] and octapeptides.[15] We have previously reported halymecins D and E from the marine-derived fungus *Acremonium* sp. FK-N30, which had been isolated from a marine alga.[16] We report here the isolation, structure elucidation, and biological activities of awajanomycin (**1**) and of two derivatives (**2** and **3**).

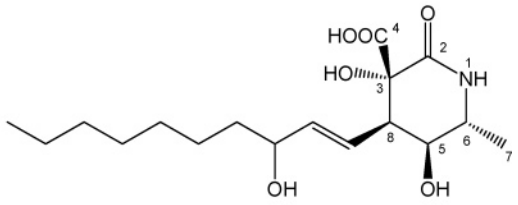
The acetone extract of the filtered culture was concentrated and partitioned between EtOAc and H_2O. The EtOAc-soluble material was purified by silica gel chromatography and ODS flash chromatography, followed by ODS HPLC to afford awajanomycin (**1**). Compound **1** was obtained as a colorless gum, and the molecular formula of $C_{17}H_{27}NO_5$ was determined by HRFABMS data, *m*/*z* 326.1981 $[M + H]^+$. The IR spectrum exhibited hydroxyl groups (3397 cm^{-1}) and two carbonyl groups (1685 and 1793 cm^{-1}). The ^{13}C and ^1H NMR spectra of **1** are summarized in Table 1. The ^1H and ^{13}C NMR and HSQC spectra indicated the presence of two carbonyls and one sp^3 quarternary, four sp^3 methine, two olefinic, six methylene, and two methyl carbons. Additionally, three exchangeable ^1H signals were observed in DMSO-d_6: one amide proton (δ 8.20 s) and two hydroxyl protons (δ 5.92 and 4.70). Interpretation of the COSY spectrum and the unambiguously observed cross-peaks enabled a sequential proton spin system to be constructed (unit A and unit B shown in Figure 1). C-11 was suggested to be a methine having a hydroxyl group on the basis of the carbon chemical shift of 70.2 ppm, this being confirmed by the HMBC correlation of C-11 with an exchangeable proton at δ 4.70 (OH-11). The configuration of the double bond at C-9 and C-10 was assigned as *trans* based on the large vicinal coupling constant, $J_{9,10}$ = 15.7 Hz. The remaining signals, two carbonyl carbons (δ 165.9 and 172.5), a quaternary carbon (δ 77.1), and a hydroxyl proton (δ 5.92), and the degree of unsaturation in unit B suggested the presence of a γ-lactone and δ-lactam bicyclic ring, this being supported by the IR absorptions at 1793 and 1685 cm^{-1}. The δ-lactam ring was assigned according to the following HMBC analysis: the HMBC correlations (H-5/C-3 and C-8, H-8/C-3 and C-4, H-6/C-5, C-7, C-8, and C-4, NH/C-3, C-5, and C-6) and hydroxyl proton (δ 5.92) attached at C-3 were assigned on the basis of the HMBC correlations observed from 3-OH to C-2, C-3, and C-8. Furthermore, the HMBC correlations of OH-3 and H-5 to C-4 (carbonyl carbon) revealed the presence of another ring, in which oxygen linked C-4 and C-5, forming a γ-lactone fused onto the δ-lactam ring. Further structural analysis with ^{15}N HSQC and HMBC experiments showed N-1 to be correlated with H-5 and H-7, thereby completing the gross structure of the 1-hydroxy-4-methyl-6-oxa-3-azabicyclo[3.2.1]octan-2,7-dione ring.



Awajanomycin (**1**)



2



3

To confirm this structure, we attempted to reduce the C-4 carbonyl group of **1** with $NaBH_4$. Insufficient reagent resulted in **1** being hydrolyzed to **3**. However, clean reduction with excess $NaBH_4$ afforded **2**. $^1H-^1H$ COSY showed cross-peaks between a newly formed methylene proton (δ 3.31 and 3.58 d, H_2-4) and 4-OH (δ 4.58) and HMBC correlations of H_2-4 to C-2, C-3, and C-8 in **2**. On the other hand, **3** showed an HMBC cross-peak from 3-OH (δ 4.95) to C-3, C-8, and a carboxyl carbon (C-4). These data confirmed the planar structure of **1**, this being further supported by the IR absorption at 1793 cm^{-1} (the γ-lactone signal) that disappeared in **2** and **3**.

* Corresponding author. Tel: 81-193-26-6581. Fax: 81-193-26-6592. E-mail: jang.jaehyuk@mbio.jp.

10.1021/np060170a CCC: $33.50
Published on Web 09/02/2006

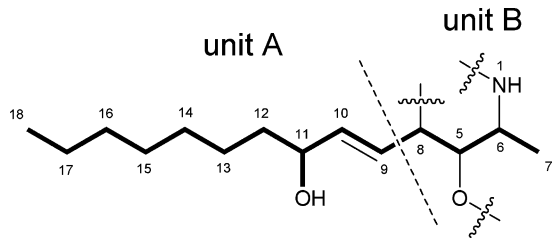
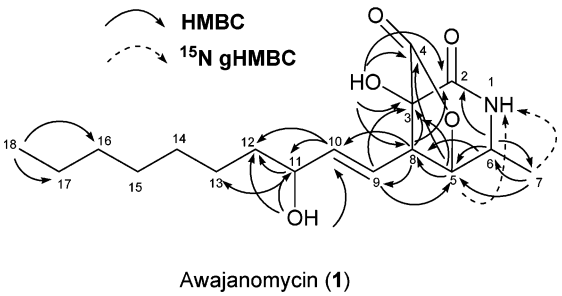
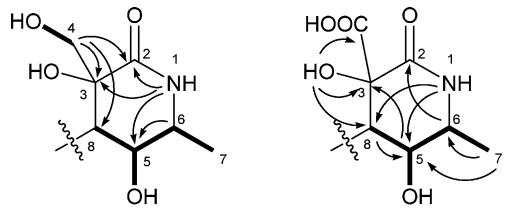


Figure 1. $^1H-^1H$ COSY correlations observed in units A and B of **1**.



Awajanomycin (**1**)

Figure 2. HMBC correlations for **1**.



2 **3**

Figure 3. COSY and HMBC correlations for the bicyclic portion in derivatives **2** and **3**.

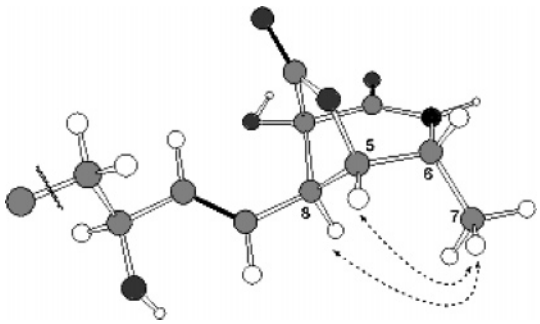


Figure 4. Relative stereochemistry of the bicyclic portion and selected NOESY correlations for **1**.

The relative stereochemistry of the bicyclic portion was elucidated on the basis of NOESY data (Figure 4) in acetone-d_6 because of signal overlap between the methyl proton (H_3-7) and methylene protons (H_2-12−H_2-17) in DMSO-d_6. In the NOESY spectrum of **1**, the methyl group (H_3-7) at C-6 showed correlations to H-5 and H-8. Therefore, the methyl groups on C-6, H-5, and H-8 were all located on the same side, as shown in Figure 4.

The IC_{50} values, for cytotoxic activity against the A549 cells (human lung adenocarcinoma), of awajanomycin (**1**) and its derivative (**2**) were 27.5 and 46.4 μg/mL, respectively. Compound **3** did not show any cytotoxic activity up to 125 μg/mL. These compounds did not show any antimicrobial activity against *Escherichia coli* IFO 3301, *Bacillus subtilis* IFO 3134, *Staphylococcus aureus* IFO 12732, *Salinivibrio costicola* ATCC 33508, *Cytophaga marinoflava* IFO 14170, α-*Proteobacterium* MBIC3368, or *Candida albicans* IFO 1060.

Table 1. ^1H and ^{13}C NMR Spectral Data for **1** in DMSO-d_6

position	δ_C	δ_H (mult., J in Hz)
1-NH	125[a]	8.20 (s)
2	165.9	
3	77.1	
4	172.5	
5	79.6	4.64 (s)
6	51.6	3.68 (d, 6.7)
7	18.1	1.22 (d, 6.7)
8	43.7	3.30 (d, 6.4)
9	121.1	5.52 (dd, 15.5, 6.7)
10	138.4	5.75 (dd, 15.7, 5.4)
11	70.2	3.92 (m)
12	37.2	1.36 (m)
13	24.9	1.23 (m)
14	29.0	1.23 (m)
15	28.6	1.23 (m)
16	31.2	1.23 (m)
17	22.1	1.25 (m)
18	14.0	0.86 (t, 7.07)
3-OH		5.92 (d, 4.1)
11-OH		4.70 (d, 5.1)

[a] By ^{15}N HSQC experiment.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a HORIBA SEPA-300 high-sensitivity polarimeter. UV spectra were recorded on a Beckman DU 640 spectrometer, and IR spectra with a JASCO FT/IR-430 instrument. The ^1H and ^{13}C NMR and all 2D NMR spectra were recorded with a Varian Inova Instrument at 750 and 125 MHz. Chemical shifts are referenced to the solvent peaks of δ_H 2.49 and δ_C 39.5 for DMSO-d_6. FAB mass spectra were measured on a JEOL JMS700 spectrometer.

Fungal Isolation and Cultivation. The fungal strain AWA16-1 was isolated from a sea mud sample collected off Awajishima Island in Japan. Strain AWA16-1 was identified as the genus *Acremonium* and named *Acremonium* sp. AWA16-1, on the basis of morphological studies. This strain has been deposited as NITE P-151 at the National Institute of Bioscience and Human-Technology of the Agency of Industrial Science and Technology, Japan. *Acremonium* sp. AWA16-1 was cultivated without shaking for 2 weeks at 25 °C in a 4 L volume, using a PDA medium (24 g of potato dextrose broth and 15 g of agar in 1 L of 50% natural seawater at pH 6.8 before autoclaving).

Extraction and Isolation. All the mycelia and agar were extracted with acetone for 2 days. After being passed through a Whatman phase-separation filter, the solvent was evaporated in vacuo, leaving an acetone extract. This acetone extract was partitioned between EtOAc and H_2O. The active EtOAc fraction was subjected to Si gel CC with $CHCl_3$, $CHCl_3$/MeOH (95:5, 9:1, and 8:2), and $CHCl_3$/MeOH/H_2O (6:4:1). The $CHCl_3$/MeOH (95:5) layer was separated by ODS flash chromatography with 40, 50, 60, 70, 80, 90, and 100% MeOH and then with $CHCl_3$/MeOH/H_2O (7:3:0.5 and 6:4:1). The active fractions in the 50, 60, and 70% MeOH/H_2O layers were combined and purified by reversed-phase HPLC (TSK gel ODS 80Ts, 2.0 × 25 cm, Tosoh Co.) with a linear gradient from 50% to 100% MeCN containing 0.1% TFA. Final purification of the active fraction was performed by reversed-phase ODS HPLC in the same column with 65% MeOH to afford awajanomycin (**1**; 8.8 mg).

Awajanomycin (1): colorless gum; $[\alpha]^{25}_D$ +78 (*c* 0.1, MeOH); UV λ_{max} (MeOH) (log ϵ) 274 (3.73) nm; IR (KBr) ν_{max} 3408, 2927, 2856, 1793, 1685, 1440, 1357, 1255, 1154 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6), see Table 1; ^1H NMR (750 MHz, acetone-d_6) δ 7.50 (br s, 1H, NH-1), δ 5.88(dd, 1H, H-10), δ 5.70 (dd, 1H, H-9), δ 4.82 (br s, 1H, OH-3), δ 4.67 (d, 1H, H-5), δ 4.10 (br s, 1H, H-11), δ 3.95 (dd, 1H, H-6), δ 3.80 (br s, 1H, OH-11), δ 3.35 (d, H-8), δ 1.50 (m, 2H, H-12), δ 1.40 (d, 3H, H-7), δ 0.88 (t, 3H, H-18); FABMS (positive mode) *m/z* 326 $[M+H]^+$; HRFABMS *m/z* 326.1981 (calcd for $[C_{17}H_{27}NO_5 + H]^+$ 326.1967).

Preparation of 2 and 3. A solution of **1** (3 mg) in MeOH (1 mL) was added to $NaBH_4$ (10 mg) at RT. The mixture was stirred for 4 h at RT before being quenched with ice and evaporated. The residue was purified by ODS HPLC (gradient elution with 60−100% MeOH) to afford **2** and **3**.

2: colorless gum; $[\alpha]^{25}_D$ +15 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3435, 2924, 1655 cm^{-1}; ^1H NMR (750 MHz, DMSO-d_6) δ 7.51 (br s, 1H, NH-1), 5.55 (dd, 1H, H-10), 5.38 (dd, 1H, H-9), 5.05 (br s, 1H, OH-3), 4.95 (d, 1H, OH-5), 4.58 (br s, 1H, OH-5), 4.50 (br s, 1H, OH-11), 3.88 (1H, H-11), 3.78 (1H, H-5), 3.58 (d, 1H, H-4a), 3.31 (d, 1H, H-4b), 3.16 (H-6), 2.58 (1H, H-8), 1.36 (m, 2H, H-12), 1.26 (m, 2H, H-17), 1.23 (m, 2H, H-13), 1.23 (m, 2H, H-14), 1.23 (m, 2H, H-15), 1.23 (m, 2H, H-16), 1.13 (d, 3H, H-7), 0.86 (t, 3H, H-18); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.6 (C-2), 138.7 (C-10), 122.8 (C-9), 72.8 (C-3), 70.6 (C-11), 69.0 (C-5), 64.3 (C-4), 51.4 (C-6), 48.9 (C-8), 37.3 (C-12), 31.2 (C-16), 29.0 (C-14), 28.7 (C-15), 24.8 (C-13), 22.0 (C-17), 20.1 (C-7), 14.0 (C-18); HRFABMS *m*/*z* 330.2269 $[M + H]^+$ (calcd for $[C_{17}H_{31}NO_5 + H]^+$ 330.2280).

3: colorless gum; $[\alpha]^{25}_D$ −20 (*c* 0.1, MeOH); IR (KBr) ν_{max} 3435, 2926, 1655 cm^{-1}; ^1H NMR (750 MHz, DMSO-d_6) δ 8.96 (br s, 1H, OH-5), 7.23 (br s, 1H, NH-1), 5.47 (dd, 1H, H-10), 5.45 (dd, 1H, H-9), 4.95 (br s, 1H, OH-3), 4.43 (br s, 1H, OH-11), 3.76 (1H, H-11), 3.32 (1H, H-5), 3.26 (H-6), 2.59 (1H, H-8), 1.34 (m, 2H, H-12), 1.26 (m, 2H, H-17), 1.23 (m, 2H, H-13), 1.23 (m, 2H, H-14), 1.23 (m, 2H, H-15), 1.23 (m, 2H, H-16), 1.07 (d, 3H, H-7), 0.84 (t, 3H, H-18); ^{13}C NMR (125 MHz, DMSO-d_6) δ 171.6 (C-4), 170.9 (C-2), 135.9 (C-10), 127.0 (C-9), 75.9 (C-3), 71.7 (C-5), 70.8 (C-11), 55.3 (C-6), 44.8 (C-8), 37.5-(C-12), 31.2 (C-16), 29.0 (C-14), 28.7 (C-15), 24.9 (C-13), 22.0 (C-17), 21.6 (C-7), 13.9 (C-18); HR FABMS *m*/*z* 366.1927 $[M + Na]^+$ (calcd for $[C_{17}H_{29}NO_6 + Na]^+$ 366.1893).

Cytotoxicity Assay. A549 cells (human lung adenocarcinoma) were cultured in a DMEM medium supplemented with 10% fetal bovine serum. The cells were seeded (8×10^3 cells/well in 200 μL) in 96-well microplates and then incubated for 14 h at 37 °C (in a 5% CO_2/air). Serially diluted samples were added to each well, and the cells were cultured for a further 72 h. After 72 h, 10 μL of Alamar Blue was added and the mixture was incubated for 4 h. The viability of the cells was estimated by the absorbance at a wavelength of 590 nm.

Antimicrobial Assay. The antimicrobial activity against seven species of microorganisms was measured by the paper disk method. Nutrient broth agar (Difco) was used as the medium for growing *Escherichia coli* IFO 3301, *Bacillus subtilis* IFO 3134, *Staphylococcus aureus* IFO 12732, and *Candida albicans* IFO 1060, while marine broth agar (Difco) was used for *Salinivibrio costicola* ATCC 33508, *Cytophaga marinoflava* IFO 14170, and α-*Proteobacterium* MBIC3368. Each sample was applied to a paper disk (6 mm) at a dose of 50 μg/disk, and the paper disk was then air-dried. The impregnated paper disk was placed on the surface of an agar plate seeded with one of the microbial strains. The growth inhibition zone was measured after 48 h of incubation at 30 °C.

Acknowledgment. We thank Assistant Professor R. Sakai of Kitasato University for the HRFABMS measurements. This work was performed as part of the project entitled "Constructing the Genetic Resource Library of Unidentified Microbes Based on Genome Information" supported by New Energy and Industrial Technology Development Organization (NEDO).

Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Blunt, J. W.; Copp, B. R.; Murano, M. H.G.; Northcote, P. T.; Prisep, M. R. *Nat. Prod. Rep.* **2004**, *21*, 1−49.
(2) Bugni, T. S.; Ireland, C. M. *Nat. Prod. Rep.* **2004**, *21*, 143−163.
(3) Kanoh, K.; Matsuo, Y.; Adachi, K.; Imagawa, H.; Nishizawa, M.; Shizuri, Y. *J. Antibiot.* **2005**, *58*, 289−292.
(4) Adachi, K.; Kanoh, K.; Wisespongp, P.; Nishijima, M.; Shizuri, Y. *J. Antibiot.* **2005**, *58*, 145−150.
(5) Yoshikawa, K.; Adachi, K.; Nishida, F.; Mochida, K. *J. Antibiot.* **2003**, *56*, 866−870.
(6) Roy, K.; Chatterjee, S.; Deshmukh, S. K.; Vijayakumar, K. S. E.; Ganguli, N. B.; Fehlhaber, H. W. *J. Antibiot.* **1996**, *49*, 1186−1187.
(7) Sassa, T.; Kinoshita, H.; Nukina, M.; Sugiyama, T. *J. Antibiot.* **1998**, *51*, 967−969.
(8) Sassa, T.; Ooi, T.; Kinoshita, H.; Okada, K. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2201−2204.
(9) Sassa, T.; Ooi, T.; Kinoshita, H. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 2633−2636.
(10) He, H.; Bigelis, R.; Solum, E. H.; Greenstein, M.; Carter, G. T. *J. Antibiot.* **2003**, *56*, 923−930.
(11) Afiyatullov, S. S.; Kuznetsova, T. A., Isakov, V. V.; Pivkin, M. V.; Prokof'eva, N. G.; Elyakov, G. B. *J. Nat. Prod.* **2000**, *63*, 848−850.
(12) Afiyatullov, S. S.; Kalinovsky, A. I.; Kuznetsova, T. A.; Isakov, V. V.; Pivkin, M. V.; Dmitrenok, P. S.; Elyakov, G. B. *J. Nat. Prod.* **2002**, *65*, 641−644.
(13) Afiyatullov, S. S.; Kalinovsky, A. I.; Kuznetsova, T. A.; Pivkin, M. V.; Pfrkof'eva, N. G.; Dmitrenok, P. S.; Elyakov, G. B. *J. Nat. Prod.* **2004**, *67*, 1047−1051.
(14) Abdel-Lateff, A.; Konig, G. M.; Fisch, K. M.; Holler, U.; Jones, P. G.; Wright, A. D. *J. Nat. Prod.* **2002**, *65*, 1605−1611.
(15) Boot, C. M.; Tenney K.; Valeriote F. A.; Crews P. *J. Nat. Prod.* **2006**, *69*, 83−92.
(16) Chen, C.; Imamura, N.; Nishijima, M.; Adachi, K.; Sakai, M.; Sano, H. J. *J. Antibiot.* **1996**, *49*, 998-1005.

NP060170A