Characterization of 6-*epi*-3-Anhydroophiobolin B from *Cochliobolus heterostrophus*

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The new sesterterpenoid 6-*epi*-3-anhydroophiobolin B (**1**) and six known ophiobolins were isolated from the extracts of the fungus *Cochliobolus heterostrophus* race O. The structure of 6-*epi*-3-anhydroophiobolin B was deduced from analysis of spectral data and the structural characterization of dehydration and dimerization products. Ophiobolin A (**2**) showed potent activity in cytotoxicity assays and marginal activity in antimalarial assays.

Several fungal species, most notably the economically important pathogens of the genus *Cochliobolus*, produce the ophiobolin sesterterpenes.[1] Analogues have also been reported from a scale-insect *Ceroplastes albolineatus*.[2] Ophiobolins have an unusual tricyclic or tetracyclic sesterterpenoid structure and a broad spectrum of biological activity against nematodes, fungi, and bacteria.[3,4] These toxic effects have been attributed to variety of mechanisms, the most fundamental of which is irreversible inhibition of calmodulin.[5]

During studies of bioactive natural products as leads against opportunistic infections (OI) of AIDS, we became interested in ophiobolins because of the previously reported of antifungal activity.[1] Seven ophiobolins were investigated from the methylene chloride extracts of *Cochliobolus heterostrophus*. We report here the isolation and structure elucidation of 6-*epi*-3-anhydroophiobolin B (**1**) and the potent antitumor activity and marginal antimalarial activity of ophiobolin A (**2**) (Chart 1).

Purification of the CH_2Cl_2 extracts of cultural filtrates from *C. heterostrophus* was accomplished by reversed and normal phase HPLC to obtain **1** and six known ophiobolins; ophiobolin A, ophiobolin B, 6-*epi*-ophiobolin A, 3-anhydroophiobolin A, 6-*epi*-3-anhydroophiobolin A, and ophiobolin I.[1,6,7]

The ^{1}H NMR spectrum of **1** was nearly identical to that of ophiobolin B except for an α,β-unsaturated ketone functional group, and ambiguous C-6 configuration.[3,5] The ^{1}H NMR spectrum of **1** differed from that of ophiobolin B by a resonance at 6.04 ppm (*s*, H-4), and a methyl shifted downfield to 2.21 ppm (*s*, Me-20). The *trans*-ring fusion at C-2 and C-6 is atypical, but 6-*epi*-ophiobolins have been previously reported.[1] The key to establishing relative configuration at C-6 in **1** was comparison of δ values with earlier reports. Unfortunately, compound **1** changed during HMBC spectrum acquisition in CH_2Cl_2, during which two structural changes appeared. This mixture of two spontaneously produced degradation products was purified by reversed-phase HPLC; the dimer **3** and dehydration product **4** were obtained.

The molecular formula of compound **3** ($C_{50}H_{70}O_5$) was established by high resolution ESI FT-ICR MS (ionic cyclotronic resonance). The spin system from H-8 to H-10 was clearly identified by a DQF−COSY NMR experiment (δ 6.78, H-8; δ 2.20 and 2.80, H_2-9; δ 2.53, H-10) with the corresponding carbons (δ 155.4, 31.0, 55.0) provided by an HMQC spectrum. The 3-anhydro partial structure of **3** was clearly revealed by HMBC spectral data: Me-20 (δ 2.21) was correlated to 49.6 (C-2), 177.7 (C-3), and 130.2 (C-4), and supported by an NOE correlation between Me-20 and H-4 (s, δ 6.04). The strong HMBC correlation between Me-23 (d, δ 0.95) and C-14 (δ 88.6) indicated that the C-14 position is substituted by an oxygenated group.[7] Comparison of ^{1}H NMR signals of **3** with those of **1** indicated that H-9 and H-10 were downshifted, which confirmed that compound **1** was a symmetrical dimer (**3**). The stereochemistry at C-6 in **3** was elucidated by NOESY spectral data. The H-6 resonance (δ 3.41) correlated significantly to H-10 (δ 2.53), indicating that structure **3** is indeed a 6-*epi*-ophiobolin.

To better understand the relative stereochemistry at C-14 and C-14′ in dimer **3**, we began with a review of likely reaction mechanisms for the formation of **3** and **4** from **1**. Since a carbocation intermediate should be formed under acidic conditions, the dehydration to form **4** would undergo an E1 mechanism. Meanwhile, a substitution (S_N1) would occur in competition with an E1 reaction. This would explain the observed, dimerization which takes place to form **3** through an S_N1 mechanism. Steric constraints from Me-22 and methylene C-9 should direct the dimerization by compound **1** as a nucleophile from the side opposite Me-22. As a result, the relative stereochemistry at C-14 and C-14′ in **3** is the same as **1**. The generation of completely overlapped proton and carbon data indicates that each monomer has identical relative stereochemistry.

The molecular formula of **4** ($C_{25}H_{34}O_2$) was determined by high-resolution ESI FT-ICR MS, suggesting that **4** is an anhydro product of **1**. The spin system from H-8 to H-9 was confirmed by DQF−COSY NMR data: (δ 6.88, H-8, δ 3.68 and 3.15, H_2-9). The olefinic carbon positions at $\Delta^{10(14)}$ was determined by long-range correlations from Me-22 (δ 1.08) to C-10 (δ 139.1) and Me-23 (δ 1.01, d) to C-14 (δ 145.6), respectively, in the HMBC experiment. The characterization of products **3** and **4** allowed the unambiguous structural assignment of **1** as 6-*epi*-3-anhydroophiobolin B.

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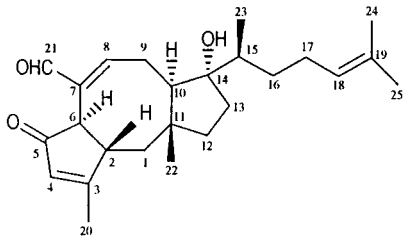
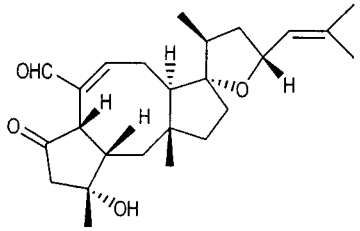
Chart 1



1



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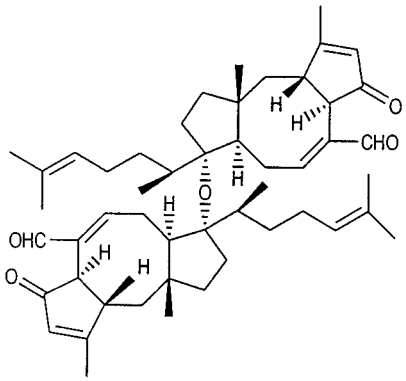
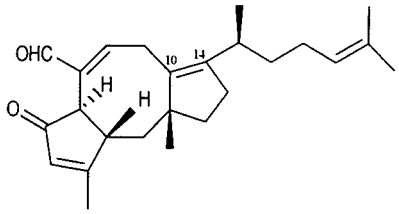


3



4

Ophiobolin A (**2**) showed activity against cancer cell-lines A-549, HT-29, and Mel-20 at IC_{50} (50 ng/mL) and cell-line P-388 at IC_{50} (25 ng/mL) in cytotoxicity assays. Ophiobolin A (**2**) showed marginal activity against *P. falciparum* at IC_{50} (ng/mL) <528.8 (D6 Clone, SI >2.1), 580 (W2 Clone, SI 1.9) in an antimalarial assay. The potent antitumor activity suggests that ophiobolins may provide promising lead chemistries that could be optimized by mutant-producing strains, bioconversions, or semisyntheses to provide clinically useful compounds.

Experimental Section

General Experimental Procedures. IR and UV spectra were obtained using an AATI Mattson Genesis Series FTIR and a Perkin-Elmer Lambda 3B UV/vis spectrophotometer. Optical rotations were measured with a JASCO DIP-370 digital Polarimeter. NMR spectra were recorded on a Bruker DRX 500 spectrometer using the solvent peak as the internal standard. ESI-FT-ICR MS spectra were measured on a Bruker-Magnex BioAPEX 30es ion cyclotron high-resolution HPLC-FT-ICR MS spectrometer by direct injection into an electrospray interface. Semipreparative HPLC was carried out on a Waters system with a gradient programmer.

Fungal Material, Extraction, and Isolation. *C. heterostrophus* (race O, strain (5)) was grown in Complete Medium.[8] Methylene chloride extracts of culture broth were fractionated by repetitive preparative reversed-phase HPLC on an IB-SIL 5 micron C18 column (10 × 250 mm, Phenomenex) using a mobile phase of CH_3CN-H_2O (3:2). Further purification was carried out on normal phase HPLC column (Spherex 5 micron DIOL, 10 × 250 mm, Phenomenex) using hexanes−EtOAc (1:1) as an eluent (flow rate of 1.5 mL/min and UV detection at 240 nm), followed by reversed-phase HPLC (Prodigy 5 micron ODS 3 100A, 10 × 250 mm, Phenomenex) using CH_3CN-H_2O (4:1) as an eluent (flow rate of 2 mL/min and UV detection at 235 nm). Ophiobolins were obtained with the following yields: **1** (0.9 mg), **2** (4.2 mg), ophiobolin B (0.7 mg), 6-*epi*-ophiobolin A (3.1 mg), 3-anhydroophiobolin A (3.1 mg), 6-*epi*-3-anhydroophiobolin A (1.2 mg), and ophiobolin I (1.2 mg). The ^{1}H NMR data of known compounds are consistent with previously reported data.[1,3,6,7]

6-*epi*-3-Anhydroophiobolin B (1): ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 0.76 (3H, s, Me-22), 0.87 (3H, d, J = 6.5 Hz, Me-23), 1.61 and 1.68 (each 3H, s, Me-24 and 25), 2.21 (3H, s, Me-20), 2.43 and 2.48 (each 1H, dd, J = 13.3, 7.6 Hz, H_2-9), 2.66 (1H, br d, J = 14.5, Hz, H-2), 3.17 (1H, br s, H-6), 4.03 (1H, br s, OH-14), 5.14 (1H, t, J = 8.1 Hz, H-18), 6.04 (1H, s, H-4), 7.06 (1H, dd, J = 7.0 and 3.5 Hz, H-8), and 9.38 (1H, s, H-21).

Purification of 3 and 4. Compounds **3** and **4** were purified from 0.9 mg of the degradation product mixture of **1** by reversed-phase HPLC (Prodigy 5 micron ODS 3100A, 10 × 250 mm, Phenomenex) using CH_3CN-H_2O (4:1) as an eluent (flow rate of 1.5 mL/min and UV detection at 235 nm). Compounds **3** (0.3 mg) and **4** (0.2 mg) were obtained.

Di-6-*epi*-3-anhydroophiobolin B (3): $[\alpha]_D$ 0° (c 3.33 × 10^{-3}, $CDCl_3$); IR (neat film) ν_{max} 2925.4, 2868.3, 1698.4, 1615.9, 1456.8, 1375.5 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ϵ) 226 (2.37), 230 (2.35), 242 (2.82) nm; ^{1}H NMR ($CDCl_3$, 500 MHz) δ 0.87 (3H, s, Me-22), 0.95 (3H, d, J = 6.5 Hz, Me-23), 1.32 (1H, dd, J = 13.5, 13.2 Hz, H-1α), 1.61 and 1.69 (each 3H, s, Me-24 and 25), 2.05 (3H, s, Me-20), 2.09 (1H, dd, J = 13.2, 3.8 Hz, H-1β), 2.20 (1H, ddd, J = 20.6, 15.1, 5.8 Hz, H-9β), 2.53 (1H, d, J = 15.1 Hz, H-10), 2.65 (1H, d, J = 13.2 Hz, H-2), 2.83 (1H, d, J = 20.6 Hz, H-9α), 3.40 (1H, br s, H-6), 5.16 (1H, t, J = 6.4 Hz, H-18), 5.94 (1H, s, H-4), 6.78 (1H, d, J = 5.8 Hz, H-8), and 9.26 (1H, s, H-21); ^{13}C NMR (125 MHz, the assignment from HMBC spectrum of mixture **3** and **4** in CD_2Cl_2 and HMQC spectrum of **3** in $CDCl_3$) δ38.1 (C-1), 49.6 (C-2), 177.7 (C-3), 130.2 (C-4), obsc. (C-5), 48.3 (C-6), 130.7 (C-7), 155.4 (C-8), 31.0 (C-9), 55.0 (C-10), 42.9 (C-11), 41.0 (C-12), 33.0 (C-13), 88.6 (C-14), 39.1 (C-15), 33.1 (C-16), 26.5 (C-17), 125.1 (C-18), 133.0 (C-19), 26.1 (C-20), obsc. (C-21), 18.6 (C-22), 16.2 (C-23), 18.0 (C-24) and 22.4 (C-25); ESI FT-ICR MS m/z 751.5947 $[MH]^+$ (calcd for $C_{50}H_{71}O_5$, 751.5301), 789.5694 $[M + K]^+$ (calcd for $C_{50}H_{70}O_5K$, 789.4890).

6-*epi*-3-Anhydro-$\Delta^{10(14)}$-ophiobolin B (4): $[\alpha]_D$ −90° (c 1.33 × 10^{-3}, $CDCl_3$); IR (neat film) ν_{max} 2925.0, 2853.2, 1616.3, 1456.9, 1376.0 cm^{-1}; UV ($CHCl_3$) λ_{max} (log ϵ) 226 (2.56), 244 (3.06) nm; ^{1}H NMR (CD_2Cl_2, 500 MHz) δ 1.01 (3H, d, J = 8.3 Hz, Me-23), 1.08 (3H, s, Me-22), 1.56 and 1.68 (each 3H, s, Me-24 and 25), 2.09 (3H, s, Me-20), 2.60 (1H, br d, J = 13.0, H-2), 3.05 (each 1H, br d, J = 19.0 Hz, H-9α), 3.28 (1H, d, J = 4.0 Hz, H-6), 3.69 (1H, dd, J = 19.0, 6.8 Hz, H-9β), 5.09 (1H, t, J = 6.2 Hz, H-18), 5.95 (1H, s, H-4), 6.88 (1H, dd, J = 6.8 and 3.4 Hz, H-8) and 9.34 (1H, s, H-21); ^{13}C NMR (CD_2Cl_2, 125 MHz, the assignment from HMBC spectrum of mixture **3** and **4**) δ38.2 (C-1), 49.6 (C-2), 177.2 (C-3), 130.2 (C-4), obsc. (C-5), 48.5 (C-6), 130.6 (C-7), 155.2 (C-8), 30.8 (C-9), 139.1 (C-10), 51.0 (C-11), 44.1 (C-12), obsc. (C-13), 145.6 (C-14), 37.0 (C-15), 31.5 (C-16), obsc. (C-17), 125.1 (C-18), 131.2 (C-19), 26.0 (C-20), obsc. (C-21), 17.3 (C-22), 15.6 (C-23), 18.5 (C-24) and

22.6 (C-25); ESI FT-ICR MS *m*/*z* 367.2788 $[M + H]^+$ (calcd for $C_{25}H_{35}O_2$, 367.2637) and 389.2927 $[M + Na]^+$ (calcd for $C_{25}H_{34}O_2Na$, 389.2456).

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