Chlorohydroaspyrones A and B, Antibacterial Aspyrone Derivatives from the Marine-Derived Fungus *Exophiala* sp.

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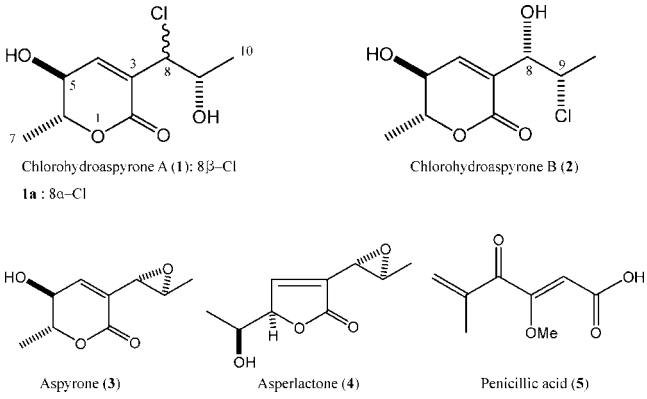
Chlorohydroaspyrones A (**1**) and B (**2**), antibacterial aspyrone derivatives, and the previously described aspyrone (**3**), asperlactone (**4**), and penicillic acid (**5**) have been isolated from the broth of a marine isolate of the fungus *Exophiala*. The structure and absolute stereochemistry of the compounds were determined on the basis of the physicochemical data analysis and chemical reactions. Compounds **1** and **2** exhibited a mild antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus*. The MIC values of each strain are as follows: compound **1** showed 62.5 μg/mL for *S. aureus* and 125 μg/mL for methicillin-resistant *S. aureus* and multidrug-resistant *S. aureus*, and compound **2**, 62.5 μg/mL for *S. aureus* and methicillin-resistant *S. aureus* and 125 μg/mL for multidrug-resistant *S. aureus*.

Exploitation of the marine environment has been intriguingly successful in recent years in the search for structurally unusual and biologically active natural products.[1] We are studying fungi isolated from marine sources for their potential to provide new natural products.[2] As part of an ongoing effort to discover biologically active natural products from marine microorganisms,[3] a marine-derived fungus, *Exophiala* sp., was selected from our screening program for further studies to examine the antimicrobial activity of its extract against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* strains. This paper describes the isolation and characterization of chlorohydroaspyrones A (**1**) and B (**2**), as well as three known polyketides, aspyrone (**3**),[4] asperlactone (**4**),[4] and penicillic acid (**5**).[5] Compounds **1** and **2** displayed moderate antibacterial effects against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* strains.



Chlorohydroaspyrone A (**1**) was obtained in the form of a colorless oil. It showed an isotopic cluster at *m/z* 243 [M (^{35}Cl) + Na]$^+$ (20) and 245 [M (^{37}Cl) + Na]$^+$ (7) with a 3:1 ratio in the FABMS, suggesting the presence of a chlorine atom. A molecular formula of $C_9H_{13}ClO_4$, which gave three degrees of unsaturation, was established by HRESIMS and ^{13}C NMR methods. The IR absorptions at 3344 and 1709 cm^{-1} indicated the presence of a hydroxyl and α,β-unsaturated δ-lactonyl functionality in **1**, respectively. The UV spectrum of **1** revealed the presence of a conjugated enone chromophore [272 nm (log ε 4.10)]. In the ^{1}H NMR spectrum, two protons were exchanged by D_2O, suggesting that **1** had two hydroxyl protons [5.87 (1H, d, J = 5.9 Hz, 5-OH); 5.16 (1H, d, J = 5.5 Hz, 9-OH)]. Detailed analyses of the ^{1}H and ^{13}C NMR spectra of **1**, including DEPT, COSY, HMQC, and HMBC experiments, revealed signals ascribable to an α,β-unsaturated δ-lactone having 1-chloro-2-hydroxypropanyl, hydroxyl, and methyl substituents (Table 1). The connection of functional groups in **1** was achieved on the basis of COSY and HMBC. The key COSY correlations between H-5 and 5-OH, between H-9 and 9-OH, and between H-6 and H_3-7 were critical in establishing the positions of 5-OH, 9-OH, and 6-CH_3. The diagnostic HMBC correlations, from 5-OH to C-4, C-5, and C-6, from H-4 to C-2, C-5, and C-8, from H_3-7 to C-5 and C-6, from H-8 to C-2, C-3, C-4, C-9, and C-10, and from 9-OH to C-8, C-9, and C-10 showed the connections of C3−C8 as well as the positions of 5,9-dihydroxy and 6-methyl groups. These spectroscopic features revealed that compound **1** had the general structural features of aspyrone (**3**).[4] The NMR data of both compounds showed similar patterns, except for the appearance of one hydroxyl proton and the downfield shift of not only H-8 [δ_H 4.65 (1H, d, J = 6.5 Hz); $\Delta\delta$ = 1.30 ppm] and C-8 [δ_C 62.5; $\Delta\delta$ = 8.4 ppm] but also of H-9 [δ_H 4.02 (1H, ddq, J = 5.5, 6.5, 6.5 Hz); $\Delta\delta$ = 1.14 ppm] and C-9 [δ_C 67.9: $\Delta\delta$ = 10.3 ppm] in **1**. Thus, compound **1** was characterized as the 8-chloro-9-hydroxyl derivative of aspyrone (**3**), and a direct comparison of NMR data of **1** with those of **3** provided additional support in justifying the planar structure shown for **1**.

The relative stereochemistry of **1** was assigned by NOE and by an analysis of vicinal proton−proton coupling constants. The NOE correlation between H-5 and H_3-7 suggested that H-5 and H-6 were *trans* oriented, which was further supported by comparing the coupling constant between H-5 and H-6 in **1** (J_{H5-H6} = 8.5 Hz) with values reported for the *trans* stereoisomers of aspyrone (**3**) (J_{H5-H6} = 8.5 Hz),[4] dihydroaspyrone (J_{H5-H6} = 8.0 Hz),[4] and 9-chloro-8-hydroxy-8,9-deoxyaspyrone (J_{H5-H6} = 9.0 Hz)[6] and the *cis* stereoisomer of (+)- goniotriol (J_{H5-H6} = 3.2 Hz).[7] From the magnitude of the ^3J vicinal coupling constants (6.5 Hz) between H-8 and H-9, these two protons were assigned an *erythro* C8−C9 configuration.[7,8] This is similar to the data for side chains of α-pyrone and γ-butyrolactone derivatives, in which the ^3J vicinal coupling constants for the *syn* and *anti* configurations are 2.0−4.5 and 7.5−9.0 Hz, respectively.[7−9] To establish the stereochemistry

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Table 1. NMR Spectroscopic Data (400 MHz, DMSO-d_6) for Chlorohydroaspyrones A (**1**) and B (**2**)

	chlorohydroaspyrone A (**1**)		chlorohydroaspyrone B (**2**)	
position	δ_C, mult.	δ_H, (J in Hz)	δ_C, mult.	δ_H, (J in Hz)
2	161.9, qC		162.9, qC	
3	128.1, qC		130.4, qC	
4	148.8, CH	6.96, d (1.7)	146.1, CH	6.87, dd-like (2.0,1.1)
5	66.4, CH	4.16, ddd (8.5, 6.0, 2.0)	66.0, CH	4.19, ddd (7.5, 6.0, 2.0)
6	78.2, CH	4.20, dq (8.5, 5.9)	78.7, CH	4.23, dq (7.5, 6.0)
7	17.6, CH_3	1.33, d (5.9)	17.9, CH_3	1.32, d (6.0)
8	62.5, CH	4.65, d (6.5)	71.2, CH	4.46, ddd (5.5, 4.5, 1.1)
9	67.9, CH	4.02, ddq (5.5, 6.5, 6.5)	59.8, CH	4.35, dq (4.5, 7.0)
10	19.4, CH_3	1.13, d (6.5)	18.2, CH_3	1.31, d (7.0)
5-OH		5.87, d (5.9)		5.82, d (6.0)
8-OH				5.73, d (5.5)
9-OH		5.16, d (5.5)		

of chlorohydroaspyrone A (**1**), a chemical synthesis of **1** was carried out from aspyrone (**3**). Treatment of **3** with 37% HCl gave not only **1** and its 8-epimer (**1a**) as main products but also **2** as one of the minor products (see Experimental Section and Figure S1 in the Supporting Information). The two products (**1** and **1a**) showed coupling constants of $J_{H8-H9} = 6.5$ Hz in **1** and $J_{H8-H9} = 4.5$ Hz in **1a**, indicating *trans* and *cis* conformation between H-8 and H-9, respectively.[7,8] On the basis of the evidence described above, we suppose that the two main products (**1**, **1a**) were formed from the nucleophilic substitution of the 8,9-epoxide by a chloride ion through an S_N1 reaction. The spectroscopic data, TLC, and HPLC behavior of the synthetic compound (**1**) proved to be identical to those observed for the natural product chlorohydroaspyrone A (see Figure S1 in the Supporting Information). Accordingly, the absolute stereostructure of chlorohydroaspyrone A was determined as (8*R*,9*S*)-8-chloro-9-hydroxy-8,9-deoxyaspyrone (**1**).

Chlorohydroaspyrone B (**2**) was also obtained in the form of a colorless oil. The general features of its MS, UV, IR, and NMR spectra (Table 1) closely resembled those of chlorohydroaspyrone A (**1**), except that the NMR signals including chemical shift and coupling constants assigned to H-8 and C-8 were changed from δ_H 4.65 (1H, d, $J = 6.5$ Hz, H-8) and δ_C 62.5 (C-8) for compound **1** to δ_H 4.46 (1H, ddd, $J = 5.5$, 4.5, 1.1 Hz, H-8) and δ_C 71.2 (C-8) for compound **2** (Table 1). Detailed analyses of the ^{1}H and ^{13}C NMR spectra of **2**, including the results from DEPT, COSY, HMQC, HMBC, and NOESY experiments, suggested that **2** is a regioisomer of compound **1**, 9-chloro-8-hydroxy-8,9-deoxyaspyrone. The structure of compound **2** was further supported by the HMBC correlations between 8-OH and C-3, C-8, and C-9. Thus, the planar structure of chlorohydroaspyrone B was confidently assigned.

As in **1**, the relative stereochemistry of metabolite **2** was deduced by analyzing the vicinal proton−proton coupling constant and by interpreting the NOE difference spectroscopic data. The relative configuration of H-5 and H-6 was assigned *trans* on the basis of a large ^3J vicinal coupling constant (7.5 Hz)[4,6,7] between H-5 and H-6 and also on the basis of an NOE correlation between H-5 and H_3-7. The relative configuration of H-8 and H-9 was assigned a *syn* configuration on the basis of the classical *cis* vicinal coupling constant (4.5 Hz) between H-8 and H-9.[8,9] Because the carbon backbones of compounds **1** and **2** are likely to be formed by the same biosynthetic pathway, the orientation of the hydroxyl group of 2-chloro-1-hydroxypropanyl side chain is assumed to be the same. This assumption was further supported by **2** derived from the treatment of aspyrone (**3**) with 37% HCl (see Figure S1 in the Supporting Information). In considering the stereochemistry of natural product **2**, we propose that the minor product **2** is also formed through an S_N1 reaction. Hence, an absolute configuration of 5*S*, 6*R*, 8*S*, and 9*S* is proposed for **2**.

To rule out the possibility of the two new metabolites (**1**, **2**) being artifacts formed as a result of the extraction and purification process, a careful HPLC analysis of a new extract and purified fractions was carried out. Both **1** and **2** were detected in the original organic crude extract and the polar fraction obtained from Si gel column chromatography.

Compound **2** appears to be identical in its planar structure to the chlorine-containing compound 9-chloro-8-hydroxy-8,9-deoxyaspyrone reported by Namikoshi et al.[6] The two compounds have significantly different $^3J_{H8-H9}$ vicinal coupling constants (4.5 Hz for **2** as compared to 12.5 Hz for the compound reported by Namikoshi et al.[6]), as well as optical rotations (+70 for **2** as compared to +17.2 for the compound reported by Namikoshi et al.[6]). The configurations at C-8 and C-9 in the latter compound were suggested to be 8*S** and 9*R** from consideration of its hypothetical biogenetic pathway, which is similar to that of aspyrone; however, their absolute configurations remain unsolved.

Polyketides **3**−**5** were also obtained in this investigation. They were identified by inspecting their NMR spectra and comparing these data to literature values.[4,5]

Compounds **1** and **2** exhibited mild antibacterial activity against *Staphylococcus aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus*. The MIC values to each strain are as follows: compound **1** showed 62.5 μg/mL for *S. aureus* and 125 μg/mL for methicillin-resistant *S. aureus* and multidrug-resistant *S. aureus*, and compound **2**, 62.5 μg/mL for *S. aureus* and methicillin-resistant *S. aureus* and 125 μg/mL for multidrug-resistant *S. aureus*.

Experimental Section

General Experimental Procedures. Optical rotation was determined on a Perkin-Elmer model 341 polarimeter. UV/visible spectra were measured on a Hitachi U-2001 UV/vis spectrometer. IR spectra were recorded on a Bruker FT-IR model IFS-88 spectrometer. ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using TMS or solvent peaks [DMSO-d_6: ^{1}H (δ 2.50) and ^{13}C (δ 39.5)] as reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer. HPLC was performed on a JASCO LC-2000 Plus system using a reversed-phase analytical column (Alltec Appolo C18, 4.6 × 250 mm, 5 μm) with UV detection.

Fungal Isolation and Culture. The fungal strain *Exophiala* sp. (family Herpotrichiellaceae) was isolated from the surface of the marine sponge *Halichondria panicea* collected on Bogil Island, Jeonnam Province, Korea, in 2006 and identified on the basis of morphological evaluation and fatty acid methyl ester analysis (Korean Culture Center of Microorganism, Seoul, Korea, a similarity index of 0.828). A voucher specimen is deposited at Pukyong National University with the code MFC353. The fungus was cultured (10 L) for 3 weeks (static) at 29 °C in SWS medium consisting of soytone (0.1%), soluble starch (1.0%), and seawater (100%).

Extraction and Isolation. The mycelium and broth were separated by filtration using cheeesecloth. A crude extract from a small-scale (100 mL) culture of an *Exophiala* sp. displayed antimicrobial activity in a primary screen using *S. aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus*. This prompted the growth of a larger-scale culture (20 L) to facilitate the purification of metabolites from the broth extract. The filtered broth was extracted with EtOAc to afford

broth extract (1.5 g), which was subjected to Si gel flash chromatography. Elution was performed with *n*-hexane−EtOAc (stepwise, 0−100% EtOAc) to yield four fractions. Fractions 3 and 4 were separated by medium-pressure liquid chromatography (MPLC) (ODS) using a H_2O−MeOH gradient elution to afford crude compounds **3**−**5** and **1/2**, respectively. These were further purified by HPLC (YMC, ODS-A) utilizing a 30 min gradient program of 50% to 100% MeOH in H_2O to furnish **1** (5.2 mg), **2** (5.4 mg), **3** (60 mg), **4** (70 mg), and **5** (80 mg), respectively.

Chlorohydroaspyrone A (1): colorless oil; $[\alpha]_D^{20}$ −110 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 272 (4.10); IR (neat) ν_{max} 3344, 2980, 1709, 1639, 1448, 1379, 1219, 1144, 1060 cm^{-1}; ^1H and ^{13}C NMR, see Table 1; EIMS 223 [M (^{37}Cl) + 1]$^+$ (0.18), 221 [M (^{35}Cl) + 1]$^+$ (0.5), 203 [(M + 1) − H_2O]$^+$ (0.3), 178 [M (^{37}Cl) − CH_3CHO]$^+$ (4.4), 176 [M (^{35}Cl) − CH_3CHO]$^+$ (15), 160 [176 (^{37}Cl) − H_2O]$^+$ (12), 158 [176 (^{35}Cl) − H_2O]$^+$ (37), 141 (30), 123 (100), 95 (46); FABMS *m*/*z* 245 [M (^{37}Cl) + Na]$^+$ (7), 243 [M (^{35}Cl) + Na]$^+$ (20), 223 [M (^{37}Cl) + H]$^+$ (3), 221 [M (^{35}Cl) + H]$^+$ (9), 178 [M (^{37}Cl) − CH_3CHO]$^+$ (6), 176 [M (^{35}Cl) − CH_3CHO]$^+$ (36), 156 (7), 154 (26); HRESIMS *m*/*z* 243.0406 (calcd for $C_9H_{13}ClO_4Na$, 243.0400).

Chlorohydroaspyrone B (2): colorless oil; $[\alpha]_D^{20}$ +70 (*c* 0.1, $CHCl_3$); UV (MeOH−$CHCl_3$, 9:1) λ_{max} (log ε) 272 (4.40); IR (neat) ν_{max} 3379, 2981, 1706, 1647, 1541, 1379, 1214, 1048 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6), see Table 1; ^1H NMR (C_6D_6, 400 MHz) δ 6.43 (1H, deformed-d, $J \approx 2.0$ Hz, H-4), 3.47 (1H, ddd, $J = 8.6$, 6.5, ∼2.0 Hz, H-5), 3.89 (1H, dq, $J = 8.6$, 6.5 Hz, H-6), 1.05 (3H, d, $J = 6.5$ Hz, H_3-7), 4.34 (1H, dd, $J = 6.5$, 5.9 Hz, H-8), 4.41 (1H, dq, $J = 6.5$, 6.5 Hz, H-9), 1.36 (3H, d, $J = 6.5$ Hz, H_3-10), 3.26 (1H, d, $J = 6.5$ Hz, 5-OH), 1.62 (1H, d, $J = 5.9$ Hz, 8-OH); ^{13}C NMR (C_6D_6, 100 MHz) δ 164.1 (qC, C-2), 130.4 (qC, C-3), 146.3 (CH, C-4), 68.0 (CH, C-5), 79.2 (CH, C-6), 18.1(CH_3, C-7), 75.9 (CH, C-8), 59.7 (CH, C-9), 20.1 (CH_3, C-10); EIMS *m*/*z* 223 [M (^{37}Cl) + 1]$^+$ (0.3), 221 [M (^{35}Cl) + 1]$^+$ (0.6), 203 [(M + 1) − H_2O]$^+$ (0.6), 178 [M (^{37}Cl) − CH_3CHO]$^+$ (9), 176 [M (^{35}Cl) − CH_3CHO]$^+$ (29), 157 (100), 139 (57), 113 (84), 95 (40), 85 (70); HRESIMS *m*/*z* 243.0404 (calcd for $C_9H_{13}ClO_4Na$, 243.0400).

Aspyrone (3), Asperlactone (4), and Penicillic Acid (5). Spectroscopic data were virtually identical to those reported in the literature.[4,5]

Treatment of Aspyrone (3) with 37% HCl and HPLC Analysis of the Reaction Mixture. Hydrochloric acid (37%, 0.1 mL) was added to a solution of aspyrone (**3**) (5.0 mg) (0.02 mmol) in $CHCl_3$ (1.0 mL) at 0 °C, and then the mixture was stirred for 30 min. The reaction mixture was then poured into H_2O and extracted with EtOAc. The EtOAc extract was washed with brine, then dried over $MgSO_4$. After removal of the solvent under reduced pressure, the residue was divided into two samples for purification of reaction product and HPLC analysis (∼1 mg less). The former was purified by silica gel column chromatography (*n*-hexane−EtOAc, 1:5), followed by HPLC (ODS, MeOH−H_2O, 2:3) to furnish chlorohydroaspyrone A (**1**, 2.0 mg) and its isomer, (8*S*,9*S*)-8-chloro-9-hydroxy-8,9-deoxyaspyrone (**1a**, 1.0 mg). The latter was dissolved in MeOH (1 mL), and an aliquot (10 μL) of the sample solution was analyzed by HPLC [Alltech, Apollo C18, 10 × 250 mm, MeOH−H_2O, 2:3, UV detector (254 nm)] (see Figure S1 in the Supporting Information).

(8S,9S)-8-Chloro-9-hydroxy-8,9-deoxyaspyrone (1a): ^1H NMR (DMSO-d_6, 400 MHz) δ 7.02 (1H, d, $J = 2.7$ Hz, H-4), 4.18 (1H, m, H-5), 4.23 (1H, m, H-6), 1.32 (3H, d, $J = 6.5$ Hz, H_3-7), 4.66 (1H, d, $J = 4.3$ Hz, H-8), 4.00 (1H, ddq, $J = 6.5$, 5.9, 4.3 Hz, H-9), 1.12 (3H, d, $J = 5.9$ Hz, H_3-10), 5.88 (1H, d, $J = 5.9$ Hz, 5-OH), 5.06 (1H, d, $J = 5.9$ Hz, 9-OH).

Antibacterial Assay. The *in vitro* antibacterial activity of the fermentation broth and purified samples was evaluated by a conventional 2-fold serial dilution method using *S. aureus*, methicillin-resistant *S. aureus*, and multidrug-resistant *S. aureus* as indicator strains. A 5 mL suspension containing 10^5 cells per mL was used as inoculum of the test organism. The MIC values were determined after the inoculation for 18 h at 37 °C.[2]

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Supporting Information Available: ^1H and ^{13}C NMR spectra of **1** and **2** in DMSO-d_6 and of **2** in C_6D_6, ^1H NMR spectrum of **1a** (in DMSO-d_6), and comparison of HPLC chromatograms of the reaction mixture with those of **1** and **2**. These materials are available free of charge via the Internet at http://pubs.ac.org.

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