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BISABOLANE-TYPE SESQUITERPENOIDS FROM A GORGONIAN-DERIVED *Aspergillus* sp. FUNGUS INDUCED BY DNA METHYLTRANSFERASE INHIBITOR

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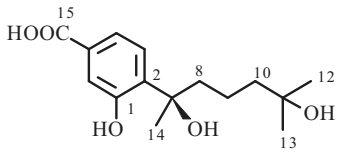
Marine microorganisms, especially marine fungi, are known to be a promising reservoir of chemically diverse and biologically intriguing natural products [1]. However, genomes of filamentous fungi have been shown to possess silent clusters that are inactive under normal environmental conditions. Therefore, activating these clusters by molecular-based techniques and chemical epigenetic manipulations may change their metabolite profiles. The chemical epigenetic perturbation method has proved to be an effective technique for promoting the generation of bioactive secondary metabolites [2].

In our previous ongoing investigation on bioactive natural products from marine-derived fungi collected from the South China Sea [3–8], a series of prenylated indole alkaloids and phenyl ether derivatives were obtained from the gorgonian-derived fungus *Aspergillus* sp. XS-20090066 [9]. In order to discover new types of secondary metabolites, the chemical epigenetic perturbation method was applied to the fermentation of this strain. The culture of fungal strain *Aspergillus* sp. XS-20090066 was grown in rice medium treated with a DNA methyltransferase inhibitor, 5-azacytidine (5-AZA), an epigenetic modifying agent. Interestingly, compared with the control, the extracts of the treatment groups showed a significant change in the HPLC profiles. Consequently, further chemical investigation of the culture treated with 5-AZA led to the isolation of a new type of secondary metabolites, which consists of six bisabolane-type sesquiterpenoids, including (*R*)-(–)-hydroxysydonic acid (**1**) [10], (*S*)-(+)-sydonic acid (**2**) [11], (*S*)-(–)-5-(hydroxymethyl)-2-(2′,6′,6′-trimethyltetrahydro-2*H*-pyran-2-yl)phenol (**3**) [7, 12], (7*S*,11*S*)-(+)-12-hydroxysydonic acid (**4**) [13], (*S*)-(+)-11-dehydrosydonic acid (**5**) [14], and (*S*)-(–)-sydowic acid (**6**) [15]. It has been proposed that 5-azacytidine may suppress DNA methyltransferase and subsequently activate genes that express the bisabolane-type sesquiterpenoids.



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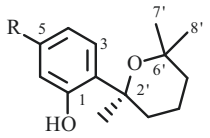

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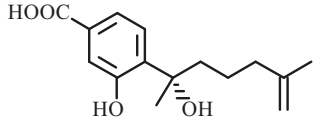
3: R = CH_2OH; **6:** R = COOH



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Compound **1** is a phenolic bisabolane-type sesquiterpene with a tertiary benzylic alcohol moiety as a single stereogenic center. In the ^{1}H NMR spectrum, the proton signals and the coupling constants at δ 7.49 (1H, dd, J = 2.0, 8.5 Hz), 7.29 (1H, d, J = 8.5 Hz), and 7.37 (1H, d, J = 2.0 Hz) indicate the presence of a 1,2,5-trisubstituted benzene system. Three methyl signals at δ 1.10 (3H, s), 1.11 (3H, s), and δ 1.61 (3H, s) were also observed. The ^{13}C NMR and DEPT data implied that **1** has one carbonyl carbon (δ 169.7), six benzene ring carbons, two oxygenated quaternary carbons (δ 78.8 and 71.5), three methyl groups, and three methane carbons. These NMR spectral features were closely similar to those of hydroxysydonic acid isolated from the fungi *Aspergillus sydowi* [10] and *Wicklowia aquatica* [16]. However, this compound was reported previously as a racemic mixture with the specific rotation of 0 (*c* 1, MeOH) [10], the absolute configuration remaining undetermined.

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TABLE 1. Antibacterial Activities of Compounds **1–6** (MIC, μM)*

Compound	*S. aureus*	*B. cereus*	*K. rhizophila*	*P. putida*	*P. aeruginosa*	*S. enterica*	*N. brasiliensis*
1	6.25	12.5	6.25	12.5	12.5	>25.0	> 25.0
2	12.5	6.25	1.56	25.0	12.5	25.0	6.25
3	3.13	6.25	3.13	25.0	25.0	12.5	12.5
4	> 25.0	12.5	6.25	25.0	> 25.0	12.5	> 25.0
5	25.0	> 25.0	12.5	> 25.0	> 25.0	> 25.0	25.0
6	12.5	> 25.0	25.0	> 25.0	25.0	> 25.0	25.0
Ciprofloxacin**	2.5	0.63	0.08	0.31	0.63	5.0	1.25

*Data are expressed in MIC values (μM). **Ciprofloxacin was used as a positive control.

Fortunately, in the present study, one of the enantiomers, (*R*)-(–)-hydroxysydonic acid $[\alpha]_D^{24}$ –8.2° (*c* 1.25, MeOH) (**1**), was first isolated from the marine-derived fungus *Aspergillus* sp. Furthermore, the absolute configuration of **1** was established by comparing its ECD spectrum and optical rotation with the related known analogues for the first time. Since the Cotton effect of the bisabolane-type sesquiterpenoids at about 210 nm depends on the absolute configuration of C-7 [11, 17], the negative diagnostic Cotton effect around 210 nm in the ECD spectrum of **1** indicates the absolute configuration of the C-7 as *R*, which is opposite to that of **5**. In addition, comparison of the specific rotation ($[\alpha]_D$) with that of the known *S*-analogue **5** (+10.1) *vs* **1** (–8.2) might provide additional evidence. Therefore, **1** was elucidated as (*R*)-(–)-hydroxysydonic acid.

All isolated compounds were tested for their antibacterial activities against various pathogenic bacteria strains (Table 1). Compounds **1**–**3** showed broad spectrum activities against five tested bacteria, *S. aureus*, *B. cereus*, *K. rhizophila*, *P. putida,* and *P. aeruginosa*, with MIC ≤ 25 μM, while the others had weak or no antibacterial activities. In particular, **3** showed pronounced antibacterial activity against *S. aureus* with an MIC value of 3.13 μM, which was close to the positive control ciprofloxacin (MIC = 2.5 μM). Interestingly, compound **6**, with a carboxyl group at C-7, exhibited weak activity against *S. aureus*, suggesting that the hydroxymethyl group at C-7 in **3** might be a functional group.

Optical rotations were measured on a JASCO P-1020 digital polarimeter. CD spectra were recorded on a JASCO J-810 circular dichroism spectrometer. NMR spectra were acquired using an Agilent DD2 NMR spectrometer (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR), using TMS as an internal standard. ESI-MS spectra were obtained on a Micromass Q-TOF spectrometer. HPLC separations were performed using a Hitachi L-2000 prepHPLC system coupled with a Hitachi L-2455 photodiode array detector. A Kromasil C_{18} preparative HPLC column (250 × 10 mm, 5 μm) was used. Silica gel (Qing Dao Hai Yang Chemical Group Co.; 200–300 mesh), Sephadex LH-20 (Amersham Biosciences), and octadecylsilyl silica gel (Unicorn; 45–60 μm) were used for column chromatography. Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co.; G60, F-254) were used for thin-layer chromatography.

Fungus Material. The fungus *Aspergillus* sp. XS-20090066 was isolated from the fresh tissue of the inner part of the gorgonian *Dichotella gemmacea*, which was collected from the Xisha Islands coral reef in the South China Sea in December, 2009 and was identified by ITS sequence. The strain was deposited at the Key Laboratory of Marine Drugs, the Ministry of Education of China, School of Medicine and Pharmacy, Ocean University of China, Qingdao, P. R. China, with the GenBank accession number HM535361.

Culture Conditions. The epigenetic modified cultures of the fungal strain *Aspergillus* sp. XS-20090066 were set in small scale in rice medium treated with different concentrations of 5-AZA (filter-sterilized), while the control cultures were treated with the autoclaving H_2O. The EtOAc extracts of the fungal cultures were analyzed by reverse-phase HPLC under the same conditions. Compared to the control, the extracts of the treatment groups exhibited new peaks in the HPLC profiles. In particular, the culture treated with 100 μM 5-AZA showed the most abundant metabolites. Therefore, a scaled-up fermentation was carried out statically with 100 μM 5-AZA in a rice medium (100 mL seawater, 100 g rice, 0.6 g peptone) in 1 L Erlenmeyer flasks. Fifty flasks of the fungal strain were incubated for 28 days at room temperature.

Extraction and Isolation. The fermented solid medium was extracted three times with EtOAc. Then the combined EtOAc layers were evaporated to dryness under reduced pressure to give an EtOAc extract (20.0 g), which was subjected to vacuum liquid chromatography (VLC) on silica gel using step gradient elution with EtOAc–petroleum ether (0–100%) and then with MeOH–EtOAc (0–100%) to afford five fractions (Fr. 1–Fr. 5). Fraction 3 was subjected to Sephadex LH-20 column chromatography (CC) eluting with a mixture of $CHCl_3$–MeOH (1:1, v/v), then further purified by repeated semi-preparative C_{18} HPLC (mobile phase 45% to 80% MeOH in H_2O, 2.0 mL/min) to provide **1** (3 mg, 0.15‰ yield), **6** (5 mg, 0.25‰ yield),

and **5** (4 mg, 0.2‰ yield). Fraction 2 was separated using silica gel CC and then subjected to Sephadex LH-20 CC (MeOH), which was further purified using HPLC (MeCN–H_2O, 20:80, 2.0 mL/min) to yield **2** (12 mg, 0.6‰ yield), **3** (2 mg, 0.1‰ yield), and **4** (3 mg, 0.15‰ yield).

Antibacterial Assay. The antibacterial activity of all isolated compounds was evaluated by the conventional broth dilution assay [18]. Seven pathogenic bacteria strains, including Gram-positive *Staphylococcus aureus*, *Bacillus cereus*, *Kocuria rhizophila*, and Gram-negative *Pseudomonas putida*, *P. aeruginosa*, *Salmonella enterica*, and *Nocardia brasiliensis*, were used; ciprofloxacin was used as a positive control. The results were observed with a Tecan SUNRISE microplate reader at 630 nm.

(*R*)-(–)-Hydroxysydonic Acid (1). $C_{15}H_{22}O_5$. Colorless oil; CD (MeOH, λ_{max}, nm) (Δε): 208 (–2.45), 300 (+1.50). $[\alpha]_D^{24}$ –8.2° (*c* 1.25, MeOH). ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.49 (1H, dd, J = 8.5, 2.0, H-4), 7.37 (1H, d, J = 2.0, H-6), 7.29 (1H, d, J = 8.5, H-3), 1.97 (2H, m, H-8), 1.61 (3H, s, H-14), 1.38 (2H, m, H-10), 1.36 (1H, m, H-9a), 1.24 (1H, t, J = 7.5, 7.0, H-9b), 1.11 (3H, s, H-12 or 13), 1.10 (3H, s, H-12 or 13). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 169.7 (C, 15-COOH), 156.9 (C, C-1), 137.6 (C, C-5), 131.6 (C, C-2), 126.1 (CH, C-3), 121.6 (CH, C-4), 118.6 (CH, C-6), 78.8 (C, C-7), 71.5 (C, C-11), 45.0 (CH_2, C-8), 43.9 (CH_2, C-10), 29.2 (CH_3, C-13), 29.2 (CH_3, C-12), 28.9 (CH_3, C-14), 19.9 (CH_2, C-9). ESI-MS *m/z* 281 $[M - H]^-$.

(*S*)-(+)-Sydonic Acid (2). $C_{15}H_{22}O_4$. Pale yellow oil; $[\alpha]_D^{25}$ +0.19° (*c* 1.47, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.58 (1H, d, J = 1.5, H-6), 7.56 (1H, dd, J = 8.5, 1.5, H-4), 7.09 (1H, d, J = 8.5, H-3), 1.93 (1H, m, H-8a), 1.81 (1H, m, H-8b), 1.68 (3H, s, H-14), 1.51 (2H, m, H-10), 1.50 (1H, m, H-11), 1.30 (2H, m, H-9), 0.84 (6H, d, J = 6.5, H-12/13). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 171.1 (C, 15-COOH), 156.3 (C, C-1), 135.2 (C, C-5), 129.8 (C, C-2), 126.5 (CH, C-6), 121.3 (CH, C-4), 119.5 (CH, C-3), 77.9 (CH, C-7), 43.1 (CH_2, C-8), 39.1 (CH_2, C-10), 29.3 (CH_3, C-14), 27.9 (CH, C-11), 22.7 (CH_3,C-12/13), 22.6 (CH_3, C-12/13), 21.8 (CH_2, C-9). ESI-MS *m/z* 265 $[M - H]^-$.

(*S*)-(–)-5-(Hydroxymethyl)-2-(2′,6′,6′-trimethyltetrahydro-2*H*-pyran-2-yl)phenol (3). $C_{15}H_{22}O_3$. Colorless oil; $[\alpha]_D^{25}$ –0.13° (*c* 0.9, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 9.19 (1H, s, 1-OH), 7.02 (1H, d, J = 8.0, H-3), 6.84 (1H, br.s, H-6), 6.83 (1H, d, J = 8.0, H-4), 4.63 (2H, s, H-7), 2.42 (1H, dt, J = 14.0, 3.0, H-3′a), 1.69 (1H, m, H-3′b), 1.68 (1H, m, H-4′a), 1.63 (1H, m, H-4′b), 1.52 (2H, m, H-5′), 1.49 (3H, s, H-9′), 1.25 (3H, s, H-7′), 0.96 (3H, s, H-8′). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 157.3 (C, C-1), 141.7 (C, C-5), 130.5 (C, C-2), 124.9 (CH, C-3), 117.9 (CH, C-4), 116.0 (CH, C-6), 77.7 (C, C-2′), 75.0 (C, C-6′), 65.2 (CH_2, C-7), 37.1 (CH_2, C-5′), 34.1 (CH_2, C-3′), 32.1 (CH_3, C-7′), 31.9 (CH_3, C-9′), 25.0 (CH_3, C-8′), 16.8 (CH_2, C-4′). ESI-MS *m/z* 249 $[M - H]^-$.

(7*S*,11*S*)-(+)-12-Hydroxysydonic Acid (4). $C_{15}H_{22}O_5$. Colorless oil; $[\alpha]_D^{25}$ +1.35° (*c* 0.50, MeOH). ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.45 (1H, d, J = 8.5, H-3), 7.37 (1H, d, J = 1.5, H-6), 7.28 (1H, dd, J = 8.5, 1.5, H-4), 3.33 (1H, m, H-12a), 3.28 (1H, m, H-12b), 2.02 (1H, m, H-8a), 1.93 (1H, m, H-8b), 1.61 (3H, s, H-14), 1.47 (1H, m, H-11), 1.43 (1H, m, H-9a), 1.28 (1H, m, H-10a), 1.22 (1H, m, H-9b), 0.98 (1H, m, H-10b), 0.82 (3H, d, H-13). ESI-MS *m/z*: 283 $[M + H]^+$, 305 $[M + Na]^+$.

(*S*)-(+)-11-Dehydrosydonic Acid (5). $C_{15}H_{20}O_4$. Colorless oil; CD (MeOH, λ_{max}, nm) (Δε): 210 (+0.80), 300 (+1.10); $[\alpha]_D^{25}$ +10.1° (*c* 0.10, MeOH). ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.56 (1H, br.s, H-6), 7.55 (1H, d, J = 8.0, H-4/3), 7.08 (1H, d, J = 8.0, H-4/3), 4.69 (1H, br.s, H-12a), 4.63 (1H, br.s, H-12b), 1.99 (2H, t, J = 7.0, H-10), 1.81 (2H, m, H-8), 1.69 (3H, s, H-14), 1.65 (3H, s, H-13), 1.46 (2H, m, H-9). ESI-MS *m/z* 263 $[M - H]^-$.

(*S*)-(–)-Sydowic Acid (6). $C_{15}H_{20}O_4$. Colorless oil; $[\alpha]_D^{25}$ –0.71° (*c* 0.27, MeOH). ^{1}H NMR (500 MHz, CD_3OD, δ, ppm, J/Hz): 7.45 (1H, dd, J = 8.0, 1.5, H-4), 7.37 (1H, d, J = 1.5, H-6), 7.23 (1H, d, J = 8.0, H-3), 2.51 (1H, dt, J = 13.5, 3.5, 3.0, H-3′a), 1.75 (1H, m, H-4′a), 1.72 (1H, m, H-3′b), 1.67 (1H, m, H-4′b), 1.55 (2H, m, H-5′), 1.46 (3H, s, H-9′), 1.28 (3H, s, H-7′), 0.94 (3H, s, H-8′). ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 169.8 (C, 7-COOH), 158.1 (C, C-1), 137.9 (C, C-5), 132.2 (C, C-2), 127.8 (CH, C-3), 122.0 (CH, C-4), 119.1 (CH, C-6), 77.9 (C, C-2′), 76.2 (C, C-6′), 37.7 (CH_2, C-5′), 34.8 (CH_2, C-3′), 32.3 (CH_3, C-7′), 31.7 (CH_3, C-9′), 25.2 (CH_3, C-8′), 17.7 (CH_2, C-4′). ESI-MS *m/z* 263 $[M - H]^-$.

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