

NOTE

Antimicrobial and antiviral sesquiterpenoids from sponge-associated fungus, *Aspergillus sydowii* ZSDS1-F6

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Infectious diseases have seriously threatened the human health worldwide.¹ One of the more alarming recent trends in infectious diseases has been the increasing frequency of antimicrobial resistance among microbial pathogens. Numerous classes of antimicrobial agents have become less effective as a result of the emergence of antimicrobial resistance due to overuse or misuse of existing antimicrobial drugs and have become a global public health problem.² Thus, it is extremely urgent to develop new antibiotics with novel structures and significant bioactivities.

Marine microorganisms are recognized as important sources of pharmacologically active metabolites.³ In particular, a growing number of marine-derived fungi have been reported to produce novel bioactive secondary metabolites.^{4,5} As part of our ongoing efforts to discover structurally novel and bioactive natural compounds from sponge-associated fungi, fungal strain ZSDS1-F6, identified as *Aspergillus sydowii*, was isolated from an unidentified marine sponge, collected from the Xisha Islands of China. The genus *Aspergillus* (Moniliaceae), widely found in nature with over 180 species, has attracted considerable attention as a rich source of alkaloids, terpenoids, xanthenes, polyketides and etc. These compounds exhibited antifungal, antibacterial, anti-HIV and cytotoxic activities.^{6–8}

A. sydowii have been reported mainly from marine sources and produce unique and biologically active secondary metabolites.^{9–12} When fermented in an oligotrophic medium, the EtOAc extract of *A. sydowii* ZSDS1-F6 showed significant antimicrobial activity against *Klebsiella pneumonia* and *Aeromonas hydrophila*, and richer chemodiversity than those from the nutritive media. Chemical studies of this extract resulted in the isolation and identification of one new bisabolane-type sesquiterpenoid, aspergillusene C (1), together with 11 known compounds (Figure 1), 7-deoxy-7,14-didehydrosydnonol

(2),⁹ aspergillusene A (3),¹¹ (Z)-5-(Hydroxymethyl)-2-(6'-methylhept-2'-en-2'-yl)-phenol (4),¹³ anhydrowaraterpol (5),¹⁴ cyclo-waraterpol A (6),¹⁴ sydonic acid (7),¹⁵ (S)-(+)-dehydroxydonic acid (8),¹⁶ (7S,11S)-(+)-12-acetoxysydonic acid (9),¹⁶ diorcinol (10),¹⁷ cordyol C (11)¹⁸ and cyclo-(L-Trp-L-Phe) (12).¹⁹ Their structures were elucidated using extensive spectroscopic techniques. The isolated compounds were evaluated for their antimicrobial, antiviral (H₃N₂), antituberculosis and cytotoxic activities, respectively. We present herein the fermentation, isolation, structure elucidation (Supplementary Figures S1–S7), antimicrobial and antiviral activities of compounds 1–12.

Compound (1) was obtained as a colorless gum, and the molecular formula C₁₅H₂₀O₄ was established by HRESIMS data. The UV spectrum displayed absorption bands at 205 and 253 nm, characteristic of an aromatic chromophore. The IR spectrum exhibited absorption bands for hydroxy and double-bond functionalities at 3375 and 1686 cm⁻¹, respectively (Supplementary Figure S8). The ¹H NMR spectrum (Table 1) showed characteristic signals for three aromatic protons of a 1,2,4-trisubstituted benzene (δ_{H} 7.46 (d, $J=8.0$ Hz), 7.44 (s) and 7.16 (d, $J=8.0$ Hz)), one olefinic proton of a trisubstituted double-bond (δ_{H} 5.63, td, $J=7.0$ and 1.2 Hz) and three methyl groups (δ_{H} 2.03 (s), 0.9942 (d, $J=6.8$ Hz) and 0.9918 (d, $J=6.7$ Hz)). Three aromatic protons resonating at δ_{H} 7.46, 7.44 and 7.16 were assigned as H-4, H-6 and H-3, respectively, on the basis of their multiplicities, coupling constants and 3J HMBC correlations: H-3/C-1 (δ_{C} 155.3) and C-5 (δ_{C} 132.3); H-4/C-2 (δ_{C} 139.1) and C-6 (δ_{C} 117.6); H-6/C-2 and C-4 (δ_{C} 122.1) (Figure 2). The key HMBC correlations between H-4 and C-15 (δ_{C} 170.9), and H-6 and C-15 indicated that the carboxyl unit was located at C-5 of the benzene ring. The cross-peaks between H₂-9 (δ_{H} 2.42, 2.33) and H-8 (δ_{H}

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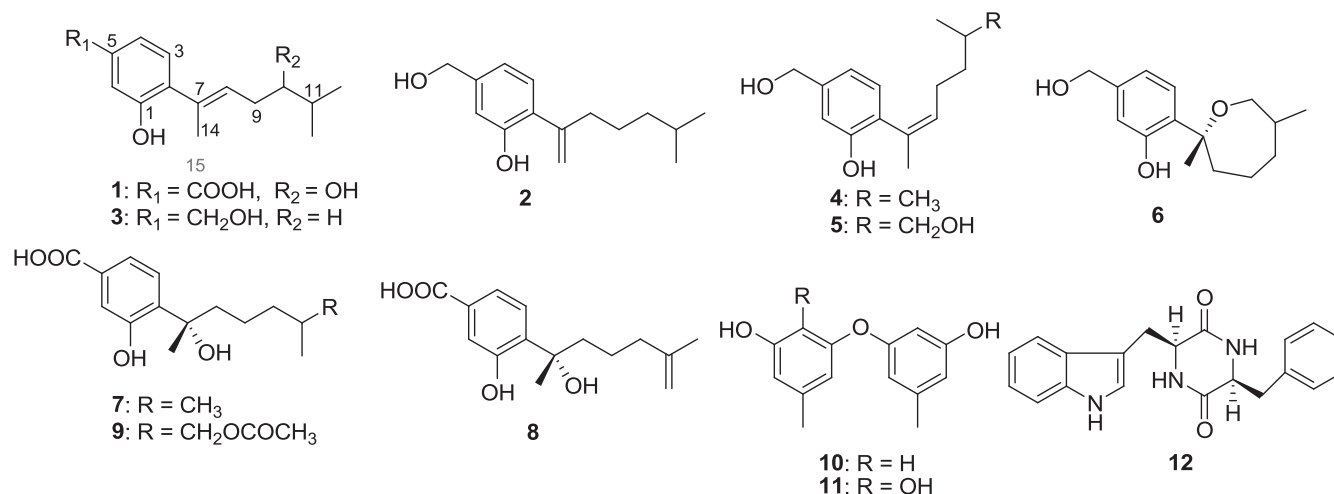


Figure 1 Chemical structures of compounds 1–12. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

5.63)/H-10 (δ_{H} 3.47), H-10 and H₂-9/H-11 (δ_{H} 1.75), and H-11 and H-10/H₃-12 (δ_{H} 0.9942)/H₃-13 (δ_{H} 0.9918) were observed in the ¹H–¹H COSY spectrum (Figure 2). It allowed establishment of the isopentane unit, located at a double-bond group, which was further supported by key HMBC correlations from H₂-9 to C-7 (δ_{C} 136.7) and H-10 to C-8 (δ_{C} 128.8). The key HMBC correlations between H-3 and C-7, H₃-14 (δ_{H} 2.03) and C-2/C-7/C-8, and H-8 (δ_{H} 5.63) and C-2 indicated that the olefinic carbon C-7 was directly connected to the 1,2,4-trisubstituted benzene ring. The substituent at C-1 of the 1,2,4-trisubstituted benzene ring and C-10 (δ_{C} 77.5) of the isopentane unit were identified as a hydroxy group, respectively, according to the chemical shift of C-1 and C-10. Furthermore, the existence of the two hydroxy groups also could be suggested on the basis of the HRESIMS data. In addition, the key NOESY correlation between H₃-14 and H₂-9 clearly indicated an *E*-configuration of Δ^7 double bond (Figure 2). In fact, the modified Mosher's method was tried to determine the absolute configuration of C-10 in **1**; unfortunately, the reaction failed. Owing to the paucity of material, the single crystal of **1** was also not obtained through many attempts with different solvents. Consequently, compound **1**, which is named aspergillusene C, was identified as (*E*)-3-hydroxy-4-(5-hydroxy-6-methylhept-2-*en*-2-yl)-benzoic acid.

The isolated compounds were evaluated for their antimicrobial and antiviral (H₃N₂) activities. Among them, compounds **3**, **4** and **10** showed modest antimicrobial activities against *K. pneumonia* with MIC values of 21.4, 10.7 and 21.7 μM , respectively, and compound **3** exhibited the moderate antimicrobial activity against *A. hydrophila* with MIC value of 4.3 μM , while compound **7** showed modest antimicrobial activity against *E. faecalis* with MIC value of 18.8 μM . In addition, compounds **4**, **10** and **11** displayed weak anti-H₃N₂ activity with IC₅₀ values of 57.4, 66.5 and 78.5 μM , respectively, while none of the compounds exhibited cytotoxic effects on the tested cancer cell lines (IC₅₀ > 100 μM) or any additional antituberculosis activities (MIC > 100 μM).

MATERIALS AND METHODS

General experimental procedures

Optical rotations were measured with a PerkinElmer 341 polarimeter. UV spectra were recorded on a Shimadzu UV-2401PC spectrometer. IR spectra were measured on a JASCO FT/IR-480 plus spectrometer with KBr pellets. ¹H, ¹³C NMR, DEPT and 2D-NMR spectra were recorded on the Bruker DRX-500 spectrometer using TMS as internal standard, and chemical shifts were recorded as δ -values. HRESIMS (including ESIMS) spectra were recorded

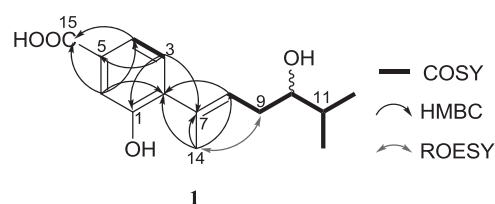


Figure 2 The key ¹H–¹H COSY, HMBC and ROESY correlations of **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

on an Applied Biosystems Mariner 5140 spectrometer. TLC and column chromatography were performed on plates precoated with silica gel GF₂₅₄ (10–40 μm) and over silica gel (200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, China), and Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), respectively. All solvents used were of analytical grade (Tianjin Fuyu Chemical and Industry Factory, Tianjin, China). Semipreparative HPLC was performed using an ODS column (YMC-pack ODS-A, Kyoto, Japan, 10 \times 250 mm, 5 μm , 4 ml min^{−1}).

Fungal strain

A. sydowii ZSDS1-F6 was isolated from an unidentified sponge, which was collected from the Xisha Islands of China in 2012, and stored at −20 °C. The frozen sample was defrosted in sterile distilled water and washed with sterile distilled water. The washed sample (1 g) was ground with sterile distilled water (10 ml) and then was diluted to 10^{−3} g ml^{−1}, 100 μl of which was dispersed across a solid-phase agar plate (Czapek's media) and incubated at 28 °C for 7 days. On the marginal zone, blue-green colonies were observed with white edges. A single colony was transferred onto Czapek's media. It was identified according to its morphological characteristics and ITS gene sequences (GenBank accession no. KF408294). A reference culture is deposited at our laboratory at −80 °C. The producing strain was prepared on potato dextrose agar slants at 3.3% salt concentration and stored at 4 °C.

Fermentation and extraction

A. sydowii ZSDS1-F6 was incubated on a rotary shaker (180 r.p.m.) at 28 °C for 7 days in 500 ml \times 400 conical flasks containing the liquid medium (150 ml per flask) composed of starch soluble (10 g l^{−1}) and polypeptone (1 g l^{−1}), and tap water after adjusting its pH to 7.5. The fermented whole broth (60 liters) was filtered through cheesecloth to separate it into filtrate and mycelia. The filtrate was concentrated under vacuum to about a quarter of original volume and then extracted three times with EtOAc to give an EtOAc solution, while the mycelia were extracted three times with acetone. The acetone solution was evaporated under reduced pressure to afford an aqueous solution. The aqueous

Table 1 ^1H and ^{13}C NMR data of **1** (500 and 125 MHz, CD_3OD , δ in p.p.m.)

1					
Position	δ_{C} mult	δ_{H} (J in Hz)	COSY	HMBC (H \rightarrow C)	NOESY
1	155.3, C				
2	139.1, C				
3	130.4, CH	7.16, d (8.0)	4	1, 5, 7	4, 8
4	122.1, CH	7.46, d (8.0)	3	2, 6, 15	3
5	132.3, C				
6	117.6, CH	7.44, s		1, 2, 4, 15	
7	136.7, C				
8	128.8, CH	5.63, td (7.0, 1.2)	9, 14	2, 14	3, 9, 10
9	34.3, CH_2	2.42, dd (6.7, 4.8); 2.33, q (7.4)	8, 10	7, 8, 10, 11	8, 10, 14
10	77.5, CH	3.47, dt (8.1, 4.9)	9, 11	8, 12, 13	8, 9, 11, 12, 13
11	34.5, CH	1.75, m	10, 12, 13	10, 12, 13	10, 12, 13
12	19.5, CH_3	0.9942, d (6.8)	11	10, 11, 13	10, 11, 13
13	17.9, CH_3	0.9918, d (6.7)	11	10, 11, 12	10, 11, 12
14	17.2, CH_3	2.03, s		2, 7, 8	9
15	170.9, C				

solution was extracted three times with EtOAc to give another EtOAc solution. Both EtOAc solutions were combined and concentrated under vacuum to give an EtOAc extract (19.5 g).

Purification

The EtOAc extract (19.5 g) was subjected to VLC on a silica-gel column using step-gradient elution with $\text{MeOH}/\text{CH}_2\text{Cl}_2$ (0–100%) to separate into eight fractions based on TLC properties. Fraction 4 (1.1 g) was divided into five parts (Frs. 4-1–4-5) followed by Sephadex LH-20 (MeOH). Fr. 4-3 (115 mg) was directly separated by HPLC (80% $\text{MeOH}/\text{H}_2\text{O}$) to yield **2** (3.2 mg, t_{R} 24.7 min), **3** (5.4 mg, t_{R} 26.3 min) and **4** (3.8 mg, t_{R} 22.4 min), respectively. Fr. 4-3-1 (14 mg) was purified by Sephadex LH-20 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 1:1) to yield **5** (1.3 mg) and **6** (1.1 mg). Fr. 5 (320 mg) was purified by Sephadex LH-20 ($\text{CH}_2\text{Cl}_2/\text{MeOH}$, 1:1) followed by HPLC (60% $\text{MeOH}/\text{H}_2\text{O}$) to yield **10** (47.1 mg, t_{R} 24.3 min). Fraction 6 (2.8 g) was divided into four parts (Frs. 6-1–6-4) by Sephadex LH-20 (MeOH). Fr. 6-3 (1.6 g) was further purified by Sephadex LH-20 (MeOH) to separate into two fractions (Frs. 6-3-1 and 6-3-2). Compounds **1** (3.7 mg, t_{R} 21.0 min), **7** (993 mg, t_{R} 29.3 min), **8** (4.7 mg, t_{R} 23.1 min) and **9** (7.3 mg, t_{R} 16.8 min) were obtained from Fr. 6-3-1 (1.3 g) by semipreparative HPLC eluting with 68% MeOH . Similarly, compounds **11** (11.3 mg, t_{R} 13.3 min) and **12** (2.4 mg, t_{R} 10.5 min) were obtained from Fr. 6-3-2 (210 mg) by semipreparative HPLC eluting with 63% MeOH .

Aspergillus **1** (1): colorless gum; $[\alpha]_{\text{D}}^{25}$ –4.1 (c 0.4, MeOH); UV (MeOH) λ_{max} (log ϵ) 205 (3.35), 253 (2.86), 296 (2.66) nm; IR (KBr) ν_{max} 3375, 1686, 1420, 1288, 1261, 1204, 1146, 1018 cm^{-1} ; ^1H NMR and ^{13}C NMR data, see Table 1; HRESIMS m/z 265.1423 $[\text{M} + \text{H}]^+$ (calcd for $\text{C}_{15}\text{H}_{21}\text{O}_4$, 265.1434).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (<http://www.nature.com/ja>)