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Antioxidant bisabolane-type sesquiterpenoids from algal-derived fungus *Aspergillus sydowii* EN-434*

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Abstract A new aromatic bisabolene-type sesquiterpenoid (7*S*,8*S*)-8-hydroxysydowic acid (**1**), together with a pair of new bisabolene derivative (±)-(7*R**,10*R**)-10-hydroxysydowic acid (**2**), as well as two known analogues hydroxysydonic acid (**3**) and sydowic acid (**4**) were isolated and identified from the endophytic fungal strain *Aspergillus sydowii* EN-434 that was obtained from the marine red alga *Symphyocladia latiuscula*. Their structures were elucidated by nuclear magnetic resonance (NMR), high resolution electrospray ionization mass spectroscopy (HRESIMS), and quantum chemical electronic circular dichroism (ECD) calculations as well as comparison of the data with literature reports. Compound **1** exhibited potent DPPH free radical scavenging activity with IC_{50} value of 113.5 μmol/L.

Keyword: *Aspergillus sydowii*; algal-derived fungus; bisabolene-type sesquiterpenoid; antioxidant activity

1 INTRODUCTION

Marine-derived fungi are important part of marine microbiological environments and can be found in all habitats (Tasdemir, 2017). Following the discovery of penicillin, fungi hold great potential to discover novel bioactive agents for drug development (Schinke et al., 2017; Barzkar et al., 2019). Since the mid of 1990s, the popularity of the Ascomycota, especially species from the genus *Aspergillus*, displayed a very rapid growth (Blunt et al., 2015). *Aspergillus sydowii* has attracted much attention due to the discovery of various bioactive secondary metabolites, such as cyclopentanoids (Teuscher et al., 2006), tris-pyrogallol ethers (Liu et al., 2013), diphenyl ethers (Wang et al., 2018), sesquiterpenes (Trisuwan et al., 2011; Xu et al., 2017), xanthones (Trisuwan et al., 2011; Wang et al., 2018), diketopiperazine dimer (Cho et al., 2018), and alkaloids (Li et al., 2018). Some of them showed significant antibiotic (Li et al., 2018) and antioxidant (Trisuwan et al., 2011) activities as well as mild cytotoxicity (Liu et al., 2017).

Our study started with the purpose of finding new bioactive metabolites from *A. sydowii* and four compounds (Fig.1), including a new aromatic bisabolene-type sesquiterpenoid (7*S*,8*S*)-8-hydroxysydowic acid (**1**), a pair of new bisabolene derivative (±)-(7*R**,10*R**)-10-hydroxysydowic acid (**2**), and two known bisabolene analogues (**3** and **4**) (Hamasaki et al., 1975; Hamasaki et al., 1978) , were obtained from the culture extract of *A. sydowii* EN-434, which was obtained from the marine red alga *Symphyocladia latiuscula*. Their structures were determined by NMR and HRESIMS spectra data. The absolute configuration of compound **1** was identified by quantum chemical ECD calculations. All new compounds were examined for antioxidant activities. Details of isolation, structural elucidation, and bio-activity evaluation are discussed below.

2 MATERIAL AND METHOD

2.1 General experimental procedure

Optical rotations were evaluated using an Optical

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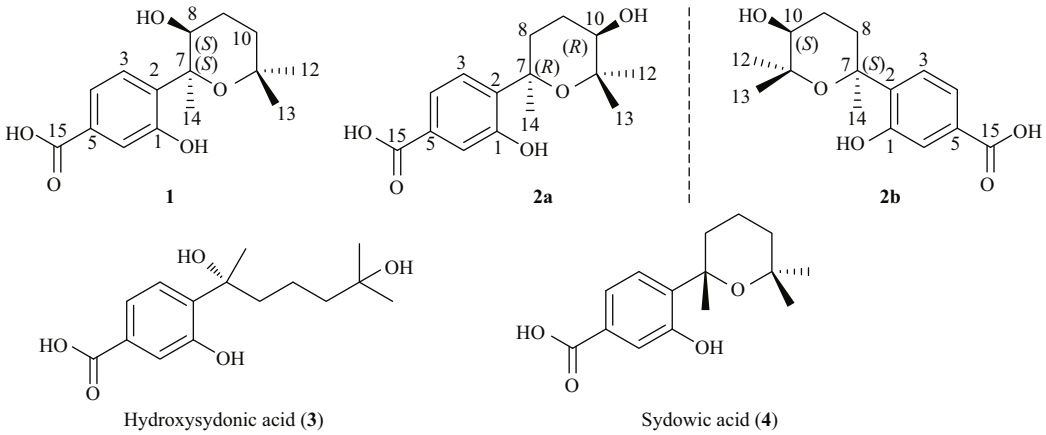

Fig.1 The structures of compounds 1–4 isolated from *A. sydowii*

Activity AA-55 polarimeter (Optical Activity Ltd., Cambridgeshire, UK). UV spectra were recorded using a Lengguang Gold S54 spectrophotometer (Shanghai Lengguang Technology Co. Ltd., Shanghai, China). ECD spectra were measured on a JASCO J-715 spectropolarimeter (JASCO, Tokyo, Japan). A Bruker Avance 500 spectrometer (Bruker Biospin Group, Karlsruhe, Germany) was used to acquire 1D and 2D NMR spectra. HRESIMS were measured with a VG Autospec 3000 or an API QSTAR Pulsar 1 mass spectrometer. Chiral HPLC separations were performed using a Dionex HPLC system on a Chiralpak AD-H column (5 μm, 250 mm, 4.6 mm) with hexane/isopropanol (80∶20) as eluent. Column chromatography (CC) was carried out with silica gel (200–300 mesh, Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (18–110 μm, Merck), and Lobar LiChroprep RP-18 (40–60 μm, Merck, Darmstadt, Germany).

2.2 Fungal material

The fungal strain EN-434 was obtained from the fresh tissue of marine red alga *Symphyocladia latiuscula*, which was collected from Qingdao coastline (36°N, 120°E) in March 2014. The strain was identified as *Aspergillus sydowii* based on sequence analysis of ITS region of its 18S rDNA as described previously (Wang et al., 2006). The sequenced data have been deposited in GenBank (accession no. MN809362). The strain was kept in store at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences (IOCAS).

2.3 Fermentation and extraction

The fresh mycelia of *A. sydowii* EN-434 (1 cm×1 cm agar plugs) were inoculated into 60×1-L Erlenmeyer flasks with sterilized rice solid medium which consisted of 70 g of rice, 0.1 g of corn flour, 0.3 g of peptone, 0.1 g of sodium glutamate, and 100 mL of naturally sourced and filtered seawater (obtained from the Huiquan Gulf of the Yellow Sea near the campus of the IOCAS, pH 6.5–7.0), and statically cultured for 30 days at room temperature. After incubation, the fermented cultures were exhaustively extracted with EtOAc for three times. The final extract (69.1 g) was obtained after concentrating the combined EtOAc solution under reduced pressure.

2.4 Isolation

The organic extract was subjected to vacuum liquid chromatography (VLC) eluting with different solvents of growing polarity from petroleum ether (PE) to MeOH to obtain 9 fractions (Frs. 1–9). Fr. 6 (8.8 g), eluted with PE-EtOAc (1∶1), was disposed by reverse-phase column chromatography (CC) containing Lobar LiChroprep RP-18 (with a MeOH-H_2O gradient from 10∶90 to 100∶0) to obtain three subfractions (Fr.6-1–Fr.6-3). Fr. 6-1 was further purified by CC on silica gel (eluted with CH_2Cl_2-MeOH, 150∶1 to 40∶1) to get compound **1** (7.8 mg). Fr. 6-2 (eluted with MeOH-H_2O (40∶60)) was further purified by prep. TLC (plate: 20 cm×20 cm, developing solvents: CH_2Cl_2-MeOH, 40∶1) to obtain compounds (±)-**2** (11.4 mg) and **3** (23.6 mg). Fr. 6-3 (eluted with MeOH-H_2O (60∶40)) was further purified by CC on silica gel (eluted with CH_2Cl_2-MeOH, 200∶1 to 20∶1) to get compound **4** (130 mg).

2.5 Radical scavenging assay

The radical scavenging activity of compounds **1** and (±)-**2,** on the *α*,*α*-diphenyl-*β*-picrylhydrazyl (DPPH) free radical was measured. A reaction mixture containing 100 μL of DPPH (0.16 mmol/L in MeOH) and 100 μL samples in MeOH diluted to give final

Table 1 ^{1}H and ^{13}C NMR spectroscopic data of compounds 1 and (±)-2

No.	1		(±)-2	
	δ_H (*J* in Hz) [a]	δ_C, Type [b]	δ_H (*J* in Hz) [a]	δ_C, Type [b]
1	–	155.0, qC	–	154.5, qC
2	–	137.1, qC	–	135.4, qC
3	7.47, d (8.2)	126.7, CH	7.30, overlap	124.6, CH
4	7.35, dd (8.2, 1.6)	119.8, CH	7.25, d (7.7)	124.6, CH
5		130.6, qC	–	120.1, qC
6	7.23, d (1.6)	117.5, CH	7.30, overlap	117.4, CH
7	–	80.2, qC	–	76.9, qC
8α	3.91, m	69.2, CH	2.25, m	30.8, CH_2
8β	–	–	1.93, m	–
9α	1.78 m	25.0, CH_2	1.75, m	24.5, CH_2
9β	1.47, m	–		–
10	1.78, m	33.6, CH_2	3.27, dd (7.8, 3.9)	70.7, CH
11	–	73.5, qC	–	76.4, qC
12	1.31,s	28.6, CH_3	1.22, s	24.4, CH_3
13	1.12, s	29.8, CH_3	1.07, s	27.6, CH_3
14	1.50, s	22.3, CH_3	1.46, s	28.3, CH_3
15	–	167.0, qC	–	162.8, qC
1–OH	9.83, s			
8–OH	5.58, d (4.2)			

[a] measured at 500 MHz; [b] measured at 125 MHz. Both a and b measured in Dimethyl Sulfoxide (DMSO)-d_6. – means no data.

concentrations of 100, 50, 25, 12.5, 6.25 and 3.13 μg/mL were placed in 96 cell plates incubated in the dark for 30 min (Wang et al., 2007). After the reaction, absorbance was measured at 517 nm and percent inhibition was calculated. The mixture of 100-μL MeOH and 100-μL of DPPH was measured as a blank control group. IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radical. Butylated hydroxy-toluene (BHT) was used as a positive control.

2.6 ECD calculation

Conformational searches were carried out via molecular mechanics using the MMFF method in Macromodel software, and the geometries were reoptimized at B3LYP/6-31G(d) PCM/MeOH level by means of Gaussian 09 software (Frisch et al., 2013) to generare the energy-minimized conformers. After this, the optimized conformers were used to calculate of ECD spectra using TDDFT at BH and HLYP/TZVP; solvent effects of the MeOH solution were estimated at the same DFT level using the SCRF/PCM method.

2.7 Spectral data

(7*S*,8*S*)-8-hydroxysydowic acid (**1**): Colorless oily liquid; $[\alpha]_D^{20}$=+6.67 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.62), 245 (4.15), 300 (3.75) nm; ECD (1.0 mg/mL, MeOH) λ_{max} ($\Delta\varepsilon$) 212 (+0.99), 220 (-0.28) nm; ^{1}H and ^{13}C NMR data (Table 1); HRESIMS *m*/*z* 281.1387 $[M+H]^+$ (calcd for $C_{15}H_{21}O_5$, 281.138 4).

(±)-(7*R**,10*R**)-10-hydroxysydowic acid (**2**): Colorless oily liquid; $[\alpha]_D^{20}$=0 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 225 (5.30), 276 (4.52) nm; ^{1}H and ^{13}C NMR data (Table 1); HRESIMS *m*/*z* 281.139 1 $[M+H]^+$ (calcd for $C_{15}H_{21}O_5$, 281.138 4).

3 RESULT AND DISCUSSION

Compound **1** was obtained as colorless oil and its molecular formula was decided as $C_{15}H_{20}O_5$ based on the positive HRESIMS data (Supplementary Fig.S1), indicating six degrees of unsaturation. The ^{1}H NMR spectrum (Supplementary Fig.S2) showed three aromatic proton signals at δ_H 7.47 (H-3, 1H, d, *J*=8.2 Hz), 7.35 (H-4, 1H, dd, *J*=8.2, 1.6 Hz) and 7.23 (H-6, 1H, d, *J*=1.6 Hz), one oxygenated methine signal at δ_H 3.91 (H-8, 1H, m), two methylenes at δ_H 1.78 (H_α-9, 1H, m) and δ_H 1.47 (H_β-9, 1H, m) as well as δ_H 1.78 (H-10, 2H, m); and three methyl signals at δ_H 1.50 (H-14, 3H, s), 1.31 (H-12, 3H, s) and 1.12 (H-13, 3H, s). Its ^{13}C NMR and DEPT spectroscopic data (Supplementary Fig.S3) presented 15 carbon atoms, including three methyls, two methylenes, four methines (with one oxygenated and three aromatic), and six non-protonated carbons (with one carbonyl, two oxygenated and three aromatic) (Table 1). The structure of compound **1** was determined to be similar to sydowic acid (**4**) by the detailed analysis of the 1D and 2D NMR spectra (Hamasaki et al., 1975), revealing that **1** belongs to the family of phenolic bisabolanes. The NMR shifts of C-8 in compound **1** was changed from a methylene signal at $\delta_{H/C}$ 2.06, 1.64/30.8 in **4** to a methine signal at $\delta_{H/C}$ 3.91/69.2, indicating the hydroxylation of C-8. Further COSY correlation (Supplementary Fig.S4) between H-8 and H-9, as well as HMBC correlation (Supplementary Fig.S5) from H-14 to C-8 also indicated that the hydroxylation linked at C-8 (Fig.2). Based on the above spectroscopic evidence, the planar structure of **1** was determined.

Based on the analysis of NOESY data (Supplementary Fig.S6), the relative configuration of **1** was assigned (Fig.3). The crucial NOE correlation between H_3-14 and H-8 indicated that they were on the identical side of the molecule. The electronic circular dichroism (ECD) spectrum of **1** was measured

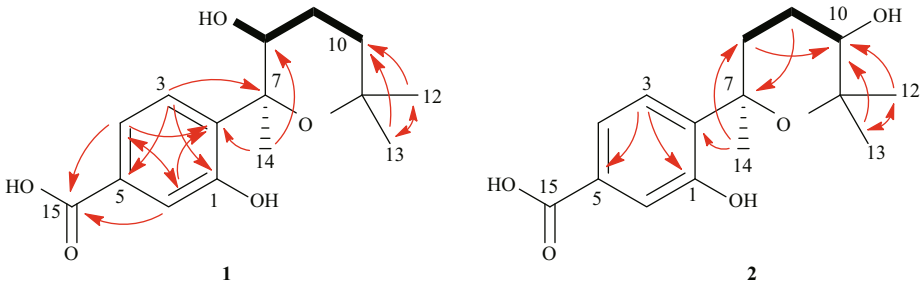

Fig.2 Key COSY (bold lines) and HMBC (red arrows) correlations for 1 and (±)-2

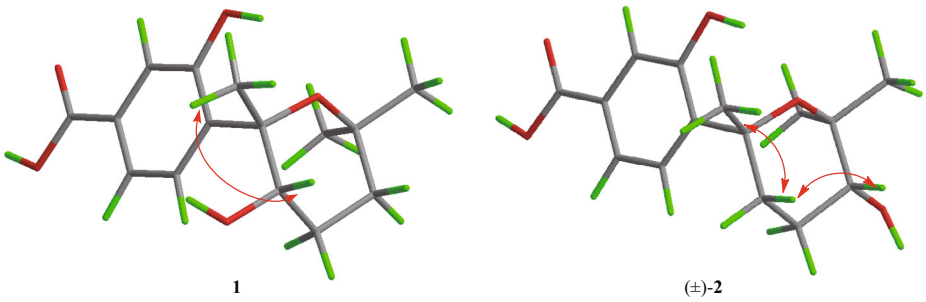

Fig.3 Key NOESY (blue arrows) correlations for 1 and (±)-2

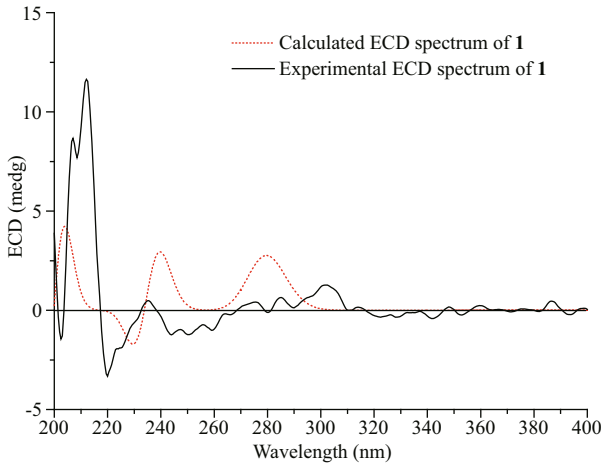

Fig.4 Experimental and calculated ECD for 1

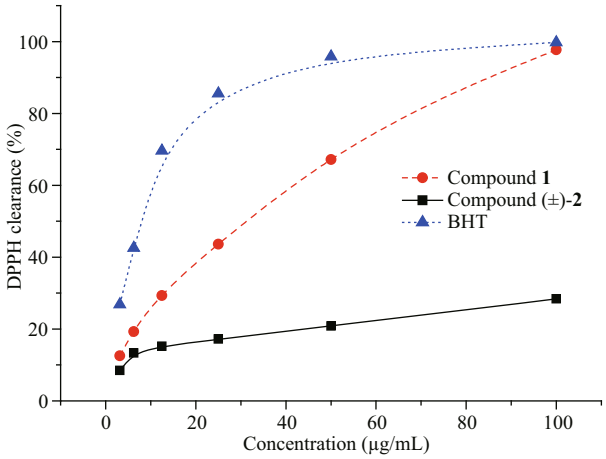

Fig.5 DPPH free radical scavenging activity for 1, (±)-2 and BHT (positive control)

and then calculated with the time-dependent density function theory (TDDFT) method at the B3LYP/6-31G (d) PCM/MeOH level to determine its absolute configuration. The experimental ECD spectrum for **1** matched well with the calculated spectrum for (7*S*,8*S*)-**1** (Fig.4). Therefore, the 7*S*, 8*S* configuration of **1** was established, and the structure of compound **1** was determined as (7*S*,8*S*)-8-hydroxysydowic acid.

Compound (±)-**2** was obtained as colorless oil. The molecular formula of (±)-**2** was also determined to be $C_{15}H_{20}O_5$ by HREIMS (Supplementary Fig.S7), showing that it is the isomer of compound **1**. The difference in ^{1}H NMR spectrum (Supplementary Fig. S8–S9) between compounds **1** and (±)-**2** is that the oxygenated methine signal changed from δ_H 3.91 in **1** to δ_H 3.27 in (±)-**2** (Table 1). Further COSY correlation (Supplementary Fig.S10) between H_2-9 and H-10, as well as HMBC correlations (Supplementary Fig.S11) from H_3-12 and H_3-13 to C-10 confirmed the hydroxylation linked at C-10 (Fig.2).

The key NOE correlations (Supplementary Fig. S12) from H-8α to H-10 and H_3-14 indicated the relative configuration of (±)-**2** is 7*R**, 10*R** (Fig.3). The baseline ECD curve and zero specific rotation value ($[\alpha]_D^{20}$=0 (*c* 0.1, MeOH)) suggested that it was a racemic mixture of two enantiomers with identical NMR data. The chiral HPLC analysis of (±)-**2** showed the separation of the two enantiomers (Supplementary Fig.S13). However, we cannot obtain single enantiomer because both of them will change to each other in dynamic balance after separation.

The isolated new Compounds **1** and (±)-**2** were

evaluated for DPPH free radical scavenging potency. Compound **1** showed significant activity with IC_{50} value of 113.5 μmol/L, while compound (±)-**2** had no activity (IC_{50} value >300 μmol/L). BHT was used as a positive control with IC_{50} value of 30.8 μmol/L (Fig.5). Racemization and the position of the hydroxyl group may affect the DPPH free radical scavenging activity.

4 CONCLUSION

Two new bisabolene sesquiterpenoids along with two known analogues had been isolated from the culture extract of *A*. *sydowii* EN-434, the marine red algal-derived fungus, indicating that this type of bisabolene sesquiterpenoids are the main metabolites of *A*. *sydowii*. The structures of these compounds were elucidated using NMR and HRESIMS as well as quantum chemical ECD calculations. Compound **1** showed a potent DPPH free radical scavenging activity.

5 DATA AVAILABILITY STATEMENT

All the data supporting the results of this study are available within the article and the supplementary material.

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Electronic supplementary material

Supplementary material (Supplementary Figs.S1–S13) is available in the online version of this article at https://doi.org/10.1007/s00343-020-0049-y.