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ORIGINAL ARTICLE

Two new stemphol sulfates from the mangrove endophytic fungus *Stemphylium* sp. 33231

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Two new stemphol sulfates, stemphol A (1) and stemphol B (2), along with known compound stemphol (3) were isolated from the EtOAc extract of the fermentation of an endophytic *Stemphylium* sp. 33231. The structures of these compounds were elucidated on the basis of spectroscopic analysis. The isolated compounds exhibited potent antibacterial activities against six terrestrial pathogenic bacteria with MIC values of 0.6–10 µg ml^{-1}. The inhibitory activities of all compounds against five cancer cell lines were evaluated.
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INTRODUCTION

Endophytic fungi of the genus *Stemphylium* are a rich source of bioactive secondary metabolites with diverse structures.[1–4] Our group recently reported the isolation of two new α-pyrone derivatives, infectopyrones A and B,[5] as well as four new anthraquinone derivatives, auxarthrol C, 2-*O*-acetyl-altersolanol B, 2-*O*-acetyl-alter-solanol L and macrosporin-2-*O*-(6′-acetyl)-*α*-ᴅ-glucoside. Four new alterporriol-type anthranoid dimers, alterporriols T–W from the *Stemphylium* sp. 33231, were also reported.[6] In our ongoing investigation of natural antibacterial and cytotoxic products, two new unusual phenolic sulfates, stemphol A (1) and stemphol B (2), along with known compound stemphol (3) (Figure 1) were isolated from the potato glucose liquid fermentation broth of *Stemphylium* sp. 33231. Phenolic sulfate derivatives isolated from microorganisms are uncommon. Only panosialin as a phenolic sulfate derivative was isolated from *Streptomyces*.[7,8] Herein we report the isolation, structure elucidation and biological activities of these compounds (1–3).

RESULTS AND DISCUSSION

Isolation and identification of compounds

Compound **1** was isolated as a white powder. The high-resolution electrospray ionization mass spectrometry (HRESIMS) exhibited ion peaks at *m/z* 361.1066 [M+Na]$^+$ and 315.1263 [M − Na]$^-$, indicating a molecular formula of $C_{15}H_{23}O_5SNa$. The presence of sulfate was confirmed by the characteristic loss of 102 amu at *m/z* 259.1673 [M +Na−SO$_3$Na+H]$^+$. In the ^{1}H NMR (Table 1) spectrum, one hydroxy group at δ_H 8.02 (s), two meta-coupled aromatic proton groups at δ_H 6.94 (d, *J* = 1.2 Hz) and 6.48 (d, *J* = 1.2 Hz), seven methylene groups and two methyl groups were observed. A combined analysis of the ^{13}C NMR (Table 1) and DEPT spectra revealed 15 resonances including six olefinic carbons (δ_C 156.3, 153.2, 141.0, 119.9, 113.6 and 111.6),

seven methylene carbons (δ_C 36.4, 32.4, 32.3, 31.8, 24.2, 23.6 and 23.2) and two methyl carbons (δ_C 14.4 and 14.3). These ^{1}H and ^{13}C NMR data indicated that a hydroxy group in resorcinol with two short unbranched chains was replaced by a sulfate group for **1**. This assignment was confirmed by the following HMBC correlation data. The HMBC correlations from H-8 to C-7, C-9 and C-10 (Figure 2) indicated that the short branched chain consists of three methylene carbons and one methyl carbon. Therefore, the other short branched chain consists of four methylene carbons and one methyl carbon. Next, we found that the structure of **1** was very similar to **3**, except for the extra signals (δ_C 153.2, 111.6 and δ_H 6.94) in **1**. These characteristics implied that a hydroxy group in **3** was replaced by a sulfate group for **1**, generating the extra two olefinic carbon signals in **1**. Furthermore, the sulfate group at this position was confirmed by the upfield-shifted resonances of C-1 (δ_C 153.2) relative to C-3 (δ_C 156.3). Thus, the structure of **1** was identified as shown in Figure 1. Compound **1** was named as stemphol A.

Compound **2** was also isolated as a white powder. The HRESIMS exhibited ion peaks at *m/z* 403.1165 [M+Na]$^+$ and 357.1375 [M − Na]$^-$, indicating a molecular formula of $C_{17}H_{25}O_6SNa$. The ^{1}H and ^{13}C NMR data (Table 1) of **2** resembled those of **1**, except for the disappearance of a hydroxy signal at δ_H 8.02 (s) in **1** and the presence of an acetoxy group (δ_C 169.7, 20.8 and δ_H 2.26, s) in **2**. These characteristics implied that the hydroxy group in **1** was replaced by an acetoxy group in **2**. Further confirmation was achieved by the observed HMBC correlations of H-Ac to C-3 (this is a weak signal) (Figure 2). Detailed analysis of 2D NMR (HSQC, ^{1}H–^{1}H COSY and HMBC) spectra confirmed that the other parts of the molecule were the same as those of **1**. Thus, the structure of **2** was identified as stemphol B.
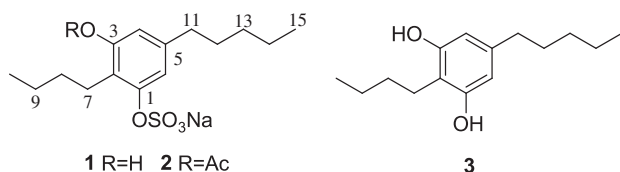
Figure 1 The structure of compounds 1–3.

Table 1 NMR data for compounds 1, 2 and 3

Position	1 (in Acetone-d_6) δ_H	1 (in Acetone-d_6) δ_C	2 (in Acetone-d_6) δ_H	2 (in Acetone-d_6) δ_C	3 (in Acetone-d_6) δ_H	3 (in Acetone-d_6) δ_C
1	—	153.2, C	—	153.4, C	—	156.7, C
2	—	119.9, C	—	125.6, C	—	113.8, C
3	—	156.3, C	—	150.4, C	—	156.7, C
4	6.48 d (1.2)	111.6, CH	6.25 d (1.6)	118.4, CH	6.22 s	107.6, CH
5	—	141.0, C	—	141.5, C	—	141.7, C
6	6.94 d (1.2)	113.6, CH	7.32 d (1.6)	119.7, CH	6.22 s	107.6, CH
7	2.68 t (7.6)	24.2, CH$_2$	2.57 t (7.6)	24.8, CH$_2$	2.61 t (8.0)	23.5, CH$_2$
8	1.50 m	32.3, CH$_2$	1.48 m	32.2, CH$_2$	1.49 m	32.2, CH$_2$
9	1.33 m	23.6, CH$_2$	1.33 m	23.5, CH$_2$	1.37 m	23.4, CH$_2$
10	0.88 t (7.2)	14.3, CH$_3$	0.89 t (7.2)	14.2, CH$_3$	0.90 t (7.2)	14.3, CH$_3$
11	2.42 t (7.6)	36.4, CH$_2$	2.53 t (7.6)	36.1, CH$_2$	2.38 t (8.0)	36.2, CH$_2$
12	1.55 m	31.8, CH$_2$	1.58 m	31.7, CH$_2$	1.54 m	31.7, CH$_2$
13	1.30 m	32.4, CH$_2$	1.33 m	32.3, CH$_2$	1.33 m	32.3, CH$_2$
14	1.28 m	23.1, CH$_2$	1.30 m	23.2, CH$_2$	1.28 m	23.2, CH$_2$
15	0.88 t (7.2)	14.4, CH$_3$	0.87 t (7.2)	14.3, CH$_3$	0.87 t (7.2)	14.4, CH$_3$
Ac-Me	—	—	2.26 s	20.8, CH$_3$		
Ac-C=O	—	—	—	169.7, C	—	
3-OH	8.02 s					
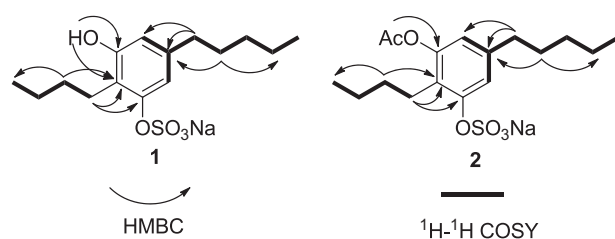
Figure 2 Key partial structures of compounds 1 and 2 from HMBC data and ^{1}H–^{1}H COSY.

The structure of known **3** was identified by comparison of its ^{1}H/^{13}C NMR spectra with those in the literature.[9–12]

Biological properties of stemphol A and B

The antibacterial activities of all the compounds were determined against six terrestrial pathogenic bacteria *Microccus tetragenus* (ATCC 13623), *Escherichia coli* (ATCC 25922), *Bacillus cereus* (ATCC

Table 2 Antibacterial activity for compounds 1, 2 and 3

Compound	MIC ($\mu g\,ml^{-1}$) S. aureus	E. coli	B. subtilis	M. tetragenus	K. rhizophila	B. cereus
1	>10.0	5.0	>10.0	10.0	5.0	5.0
2	1.25	0.6	2.5	1.25	1.25	0.6
3	5.0	0.6	2.5	1.25	2.5	5.0
Ciprofloxacin[a]	0.6	0.3	0.6	0.3	0.3	0.3

[a]Ciprofloxacin was used as a positive control.

14579), *Staphylococcus aureus* (ATCC 8799), *Kocuria rhizophila* (ATCC 9341) and *Bacillus subtilis* (ATCC 6633). Compounds **1–3** exhibited broad-spectrum antibacterial activites against these bacterias (Table 2). Compound **2** exhibited especially potent antibacterial activites against *E. coli* and *B. cereus* with MIC values of 0.6 µg mlL^{-1}. All compounds were evaluated for cytotoxic activities against the human promyelo-cytic leukemia cell line (HL-60), the human hepatocarcinoma cell line (SMMC-7721), human lung carcinoma cell line (A-549), human breast adenocarcinoma cell line (MCF-7) and human colorectal cancer cell line (SW480). However, no significant cytotoxic activities against the five cancer cell lines were observed at 40 µM.

METHODS

Fungal materials

The fungal strain *Stemphylium* sp. 33231 was isolated from the fresh healthy leaf of *Brguiera sexangula var. rhynchopetala* collected in the South China Sea in August, 2012. The fungal strain was deposited in the Key Laboratory of Tropical Medicinal Plant Chemistry of Ministry of Education, College of Chemistry and Chemical Engineering, Hainan Normal University of PR China, Hainan with an accession number KF479349.[5] The strain was grown in 30l of liquid potato glucose liquid medium (15 g of glucose and 30 g of sea salt in 1 l of potato infusion, in 1-l Erlenmeyer flasks each containing 300 ml of culture broth) at room temperature under static conditions for 28 days.

General experimental procedures

Octadecylsilyl silica gel (YMC, Kyoto, Japan; 12 nm–50 µm), silica gel (Qing Dao Hai Yang Chemical Group Co., Qing Dao, China; 200–300 mesh) and Sephadex LH-20 (GE) were used for column chromatography (CC). Precoated silica gel plates (Yan Tai Zi Fu Chemical Group Co., Yantai, China; G60, F-254) were used for TLC. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker AV spectrometer (Bruker, Zurich, Switzerland) at 400 and 100 MHz in acetone-d_6. Chemical shifts δ are reported in ppm, using tetramethylsilane (TMS) as internal standard, and coupling constants (J) are in Hz. HRESIMS spectra were measured on a Q-TOF Ultima Global GAA076 LC mass spectrometer (Waters Asia, Ltd, Singapore). UV spectra were recorded on a Beckman DU 640 spectrophotometer (Brea, CA, USA). IR spectra were recorded on a Nicolet 6700 spectrophotometer (Thermo Fisher Scientific Co., Waltham, MA, USA).

Extraction and isolation

The fungal cultures were filtered through cheesecloth, and the filtrate was extracted with EtOAc (3 × 30 l, 10 h each). The EtOAc extracts were concentrated *in vacuo to* yield an oily residue (25.2 g), which was subjected to silica-gel CC (petroleum ether/EtOAc v/v, 100:0–0:100) to generate five fractions (Fr. 1–Fr. 5). Fr. 1 was isolated by CC on silica gel eluted with petroleum ether:EtOAc (20:1) and further purified by using Sephadex LH-20 CC, eluting with mixtures of petroleum CHCl$_3$:MeOH (1:1) to obtain compound **3** (10.2 mg). Fr. 5 was isolated by CC on silica gel eluted with petroleum MeOH:EtOAc (1:10) and then subjected to Sephadex LH-20 CC, eluting with mixtures of petroleum

CHCl$_3$:MeOH (1:1), and further purified by using octadecylsilyl silica gel eluted with 30% MeOH/H2O to obtain compounds **1** (25.0 mg) and **2** (11.2 mg).

Physical properties of compounds 1 and 2

Stemphol A (**1**): white power; UV (MeOH) λ_{max} (log ε) 273 (2.22), 239 (1.26) nm; IR (KBr) ν_{max} 3324, 2927, 1627, 1435 and 1208 cm^{-1}; ^{1}H and ^{13}C NMR: see Table 1; HRESIMS *m/z* 361.1066 [M+Na]$^+$ (calcd for C$_{15}$H$_{23}$O$_5$SNa$_2$, 361.1062), 315.1263 [M–Na]$^-$ (calcd for C$_{15}$H$_{23}$O$_5$S, 315.1266).

Stemphol B (**2**): white power; UV (MeOH) λ_{max} (log ε) 278 (2.82), 249 (1.44) nm; IR (KBr) ν_{max} 3468, 2930, 1732, 1624, 1398 and 1168 cm^{-1}; ^{1}H and ^{13}C NMR: see Table 1; HRESIMS *m/z* 403.1165 [M+Na]$^+$ (calcd for C$_{17}$H$_{25}$O$_6$SNa$_2$, 403.1167), 357.1375 [M–Na]$^-$ (calcd for C$_{17}$H$_{25}$O$_6$S, 357.1372).

Biological assays

Cytotoxic activity was tested by the MTT method as described previously.[13] Five cancer cell lines, the human promyelocytic leukemia cell line (HL-60), the human hepatocarcinoma cell line (SMMC-7721), human lung carcinoma cell line (A-549), human breast adenocarcinoma cell line (MCF-7) and human colorectal cancer cell line (SW480) were used. Cisplatin (MW300) was used as a positive control. Antibacterial activity was determined by the conventional broth dilution assay.[14] Six terrestrial pathogenic bacteria *M. tetragenus, E. coli, B. cereus, S. aureus, K. rhizophila* and *B. subtilis* were used, and ciprofloxacin was used as a positive control.

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