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Antibacterial anthraquinone dimers from marine derived fungus *Aspergillus* sp

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ABSTRACT

As a continue investigation of the bioactive secondary metabolites from marine derived fungi, two new anthraquinone dimers (**1**, **2**), along with three known anthraquinones (**3–5**) and two known xanthones (**6**, **7**) were isolated from the marine-derived fungus *Aspergillus versicolor*. Their structures, including the absolute configurations, were elucidated by NMR, HRMS, and comparison with reported ones. Among them, compounds **1** and **2** were identified as anthraquinone dimers which dimerized by a rare C-O-C ether linkage, and both of them showed selective antibacterial activity against Gram-positive *Staphylococcus aureus*; whilst compound **6** exhibited moderate cytotoxicity against human cancer cell lines.

1. Introduction

Endozoic microorganisms inhabit the inner tissues of animals as specific microbial biota. It always produces unique secondary metabolites in a special ecological niche, which possess a variety of antibacterial, antifungal, antifouling, antiviral, anti-inflammatory, cytotoxic, and insecticidal activities and are widely used in medicine and agriculture [1–6].

Recently, marine-derived microorganisms have proven to be important sources of prodrug. As part of our program to discover novel biological natural products from marine-derived microorganisms originating from the East China Sea, we isolated natural rarely dimeric indole derivatives from the marine derived actinomycete *Rubrobacter radiotolerans* [7,8], anti-chlamydial phenazine derivatives from marine bacteria *Pseudomonas aeruginosa* [9], and cytotoxic peptides from the marine-derived *Penicillium citrinum* [10]. Recently, the fungus *Aspergillus versicolor* was isolated from a marine clam sample. Metabolomic and subsequent chemical investigations of this strain cultivated in potato-based medium supplemented with 3% sea salt led to the isolation and identification of two new natural rarely anthraquinones (**1–2**). In addition, five known components (**3–7**) were also obtained from this fungus. We report herein the fermentation, isolation, structure elucidation, and biological activities of these marine-derived fungal compounds.

2. Experimental section

2.1. General experimental procedures

The optical rotations were measured using a Jacso DIP-370 digital polarmeter. 1D and 2D NMR spectra were recorded on Varian INOVA 500 spectrometers. Chemical shifts are reported with reference to the respective residual solvent or deuterated solvent peaks (δ_H 3.30 and δ_C 49.0 for CD_3OD). HRESIMS and ESIMS data were obtained on a Finnigan LCQ^{DECA} instrument (Thermo Finnigan, San Jose, CA, USA). HPLC was performed on a Shimadzu Liquid Chromatograph LC-20AD with an YMC packed *J*'sphere ODS-H80 column (250 × 10 mm, 4 μm, 80 Å) using a Shimadzu UV/VIS detector SPD-20A.

2.2. Fungal strain

The fungus strain was isolated from a marine clam unidentified. Following a rinse with sterile water, small pieces of the inner tissue of the clam were homogenized and then inoculated. The sterilized potato-based medium contained agar (15 g/L), glucose (20 g/L), and antibiotics (10,000 Units/mL penicillin and 10 mg/mL streptomycin, 5 mL/

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L). Emerging colonies were transferred to the same media in a petri dish and incubated at 28 °C for 10–14 days to allow colony development. The pure fungus strain, designated as INF16–17, was identified as *Aspergillus versicolor* by a morphological and biochemical analysis by Professor Guangtong Chen, A voucher sample was deposited at the Department of Pharmacy, Nantong University.

2.3. Extraction and isolation

Fermentation was performed in 100 mL potato-based medium in 300 mL Erlenmeyer flasks for seed broth. For the production culture, 100 mL of subculture was transferred into a 1 L Erlenmeyer flask each containing 0.5 L potato-based medium, and fermentation was carried out at 30 °C for 40 days under stilling. The cultured broth (20*L*) was extracted with 10 L of ethyl acetate, filtered and concentrated to remove ethyl acetate to yield a red brown extract (11.2 g). The organic extract was subjected to vacuum liquid chromatography over a silica gel column, using a gradient elution with *n*-haxane-CH_2Cl_2-MeOH to give 24 fractions (fractions 1–15). Guided by LC-MS-UV data, fraction 5 (1.7 g) was purified by semi-preparative HPLC (MeCN-H_2O (85:15)), to afford compound **7** (3.4 mg). Fraction 10 was also purified by semi-preparative HPLC, eluted by MeCN-H_2O ((82:18), 0.1% formic acid, 2.0 mL/min), to afford compounds **1** (4.7 mg), the neighboring fraction 11 was subjected to semi-preparative HPLC, eluted with MeCN-H_2O ((82:18), 0.1% formic acid, 2.0 mL/min), to afford compounds **2** (3.9 mg), **3** (24.3 mg), **4** (6.3 mg), and **5** (2.9 mg), for compound **6** (121.7 mg), it was yield from fraction 9 by repeated crystallization.

*Compound **1***: red, amorphous powder; $[\alpha]_D^{23}$ − 72.4 (c 0.13, MeOH); UV (MeOH) λ_{max} (log ε) 220 (3.01), 257 (2.80), 314 (3.07), 465 (2.34) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); (+)-ESIMS *m/z* 753 [M - H]$^-$; (+)-HRESIMS m/z 753.2558 (calcd for $C_{42}H_{41}O_{13}$, 753.2547).

*Compound **2***: red, amorphous powder; $[\alpha]_D^{23}$ − 51.4 (c 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 222 (3.03), 264 (2.80), 320 (3.09), 455 (2.38) nm; ^{1}H and ^{13}C NMR (see Tables 1 and 2); (+)-ESIMS m/z 725 [M - H]$^-$; (+)-HRESIMS m/z 725.2240 (calcd for $C_{40}H_{37}O_{13}$, 725.2234).

2.4. Cytotoxic activity (MTT assay)

Compounds **1–7** were tested for cytotoxic activity against a small panel of human solid tumor cell lines (HCT115, DU-145, PC-3, XF498, and HT29), which were kindly provided by Pro. Chen, Nantong University. By the MTT assay, briefly, the cells were seeded into a 96-well plate and kept in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. After 24 h, and different concentrations of compounds **1–7** was added and the incubation continued for another 48 h. Then, the cell proliferation reagent MTT was added to evaluate the cell viability by measuring the optical density of the color produced by MTT dye reduction with an ELISA plate reader at 570 nm.

Table 1
^{1}H NMR data for compounds **1–3** in MeOD-d_4.[a]

Position	Compound **1**	Compound **3** (1′-*O*-methylaverantin)	Compound **2**
2/2′	7.04, d (2.0)	7.10, d, (1.8)	7.05, d (2.1)
4/4′	6.38, d (2.0)	6.48, d, (1.8)	6.37, d (2.1)
10/10′	7.09, s	7.13, s	7.00, s
11/11′	4.94, dd (5.9, 7.9)	4.95, dd (5.9, 8.3)	5.15, dd (5.4, 7.7)
12a/12a'	1.93, m	1.94, m	1.85, m
12b/12b'	1.88, m	1.87, m	1.77, m
13a/13a'	1.49, m	1.49, m	1.53, m
13b/13b'	1.34, m	1.34, m	1.33, m
14/14′	1.33, m	1.32, m	1.34, m
15/15′	1.33, m	1.32, m	1.34, m
16/16′	0.88, t (6.5)	0.89, t (6.9)	0.89, t (6.8)
-OCH_3	3.37, s	3.37, s	

[a] ^{1}H NMR data were measured at 500 MHz.

Table 2
^{13}C NMR data for compounds **1–3** in MeOD-d_4.[a]

Position	Compound **1**	Compound **3** (1′-*O*-methylaverantin)	Compound **2**
1/1′	189.7	190.9	189.0
1a/1a'	136.3	136.6	136.5
2/2′	111.9	109.9	111.7
3/3′	170.8	167.2	170.3[c]
4/4′	109.3	109.2	109.6
5/5′	166.7	166.4	166.4
5a/5a'	108.5	110.1	109.0
6/6′	183.6	182.9	184.6
6a/6a'	109.6	110.0	107.9
7/7′	163.6[b]	163.8[b]	162.4[b]
8/8′	120.3	120.1	123.2
9/9′	165.0[b]	164.6[b]	165.7[b]
10/10′	109.7	110.2	113.1
10a/10a'	135.3	135.4	134.7
11/11′	78.9	78.3	70.1
12/12′	34.9	34.9	37.8
13/13′	26.5	26.4	26.5
14/14′	32.8	32.8	33.0
15/15′	23.6	23.6	23.7
16/16′	14.4	14.4	14.4
-OCH_3	57.8	57.9	

[a] ^{13}C NMR data were measured at 125 MHz.
[b] Signals were exchangeable.
[c] Chemical shift was not observed in ^{13}C spectrum, it obtained from HMBC data.

2.5. Antibacterial activity (radial diffusion assay)

The antibacterial activity was tested by the radial diffusion assay with some modifications. Bacteria were grown overnight at 37 °C in LB media and diluted to 1/100. A gel solution containing 2.5% (*w/v*) of powdered LB medium, and 1.5% agar was prepared and autoclaved. Then, 0.15 mL of the diluted bacterial culture was added to 15 mL of the gel solution at 40–50 °C. Once the bacteria were adequately dispersed, the gel was poured into a Petri dish (90 × 15 mm). After solidification, wells were made using a 2 mm punch. Ten μL of the each sample (3 mg/mL for tested compounds and positive control) were added to each well, and the plates were incubated for 18 h at 37 °C. Tetracycline (≥ 98%) was used as a positive control. The diameters of the inhibition zones surrounding the wells were measured in millimeters.

3. Results and discussion

Compounds **1** was obtained as a red, amorphous powder, with a formula of $C_{42}H_{42}O_{13}$, as deduced from its HRESIMS peak at *m/z* 753.2558 [M-H]$^-$. In the ^{13}C NMR spectrum, the conspicuous signals were that of two carbonyl carbons at δ_C 189.7 and 183.6, and twelve olefinic carbons containing 4 oxygen-bearing ones at δ_C 170.8–108.5 were observed. Meanwhile, two oxygen-bearing sp^3 carbons at δ_C 78.9 and 57.8 were observed (Table 2). The remaining five carbons were also categorized by ^{13}C and DEPT spectrum (4 × CH_2, and 1 × CH_3). Given this information, totally 21 carbons were observed in the ^{13}C NMR data, in conjunction with the molecular formula analysis, revealed that compound **1** was symmetrical dimers.

The ^{1}H NMR spectrum of **1** (Table 1) revealed resonances of two phenyl spin system at δ_H 7.04 (1H, d, 2.0 Hz, H-2)/6.37 (1H, d, 2.0 Hz, H-4), and δ_H 7.09 (1H, s, H-10), attributable to a 1,2,3,5-tetra-substituted phenyl moiety, and a pentasubstituted phenyl moiety, respectively. In conjunction with the twelve olefinic and two carbonyl carbons, suggesting that compound **1** shared the carbon skeleton of anthraquinone. Whilst, 4 oxygen-bearing olefinic carbons indicating that compound **1** was a highly oxygenated anthraquinone derivate. Comparison of the spectroscopic data with those reported

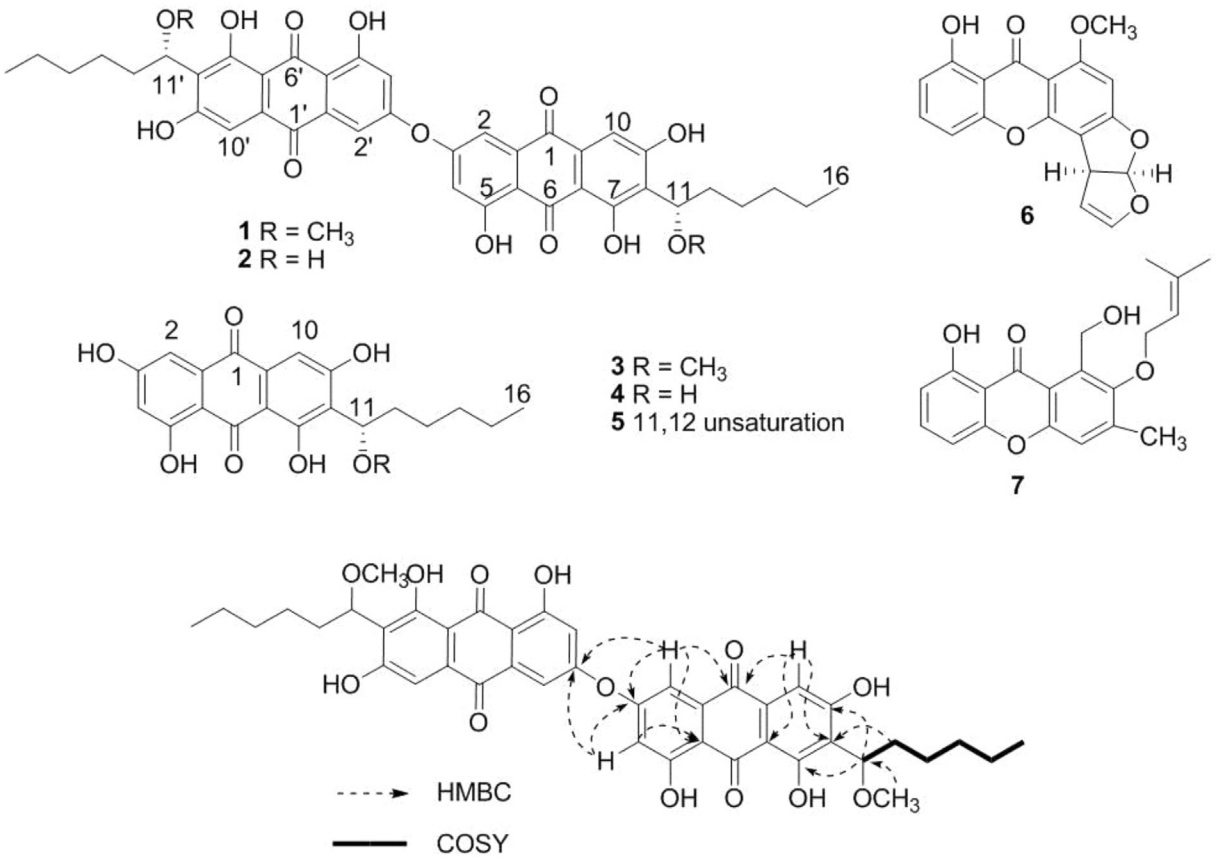

Fig. 1. COSY and key HMBC correlations for compound **1**.

anthraquinone derivatives and the typical chemical shift of the quaternary carbons, indicated that positions C-3, C-5, C-7, and C-9 were substituted with a hydroxyl group. Besides the aforementioned highly oxygenated anthraquinone moiety, proton COSY and HSQC data revealed the presence of another spin system, successive COSY connections from 11-CH to 16-CH_3 indicated the existence of a haxane moiety, and the final one protons were observed as one methoxyl group at $\delta_{H/C}$ 3.37/57.8.

Correlation observed in the HMBC spectrum (Fig. 1) for the methoxyl protons and the methine carbon (δ_C 78.9, C-11) was consistent with the presence of a 1-methoxyhexane group in compound **1**. Meanwhile, the methine proton (δ_H 4.93, H-11) in turn showed correlations with the carbons C-7∼C-9 anthraquinone moiety, indicating that these two fragments should be linked between C-11 and C-8 via a C—C linkage. Thus, the anthraquinone moiety of compound **1** was determined to be 1′-*O*-methylaverantin, which component also has been yield in our experiment (compound **3**). Compound **1** give almost same ^{1}H and ^{13}C NMR data to those of compound **3** except for slightly chemical shift difference at H-2, and H-4. The chemical shifts of H-2 (δ_H 7.04, δ_H 7.10 for compound **3**) and H-4 (δ_H 6.38, δ_H 6.48 for compound **3**) were significantly shifted highfiled. Meanwhlie, the molecular formula analysis revealed that compound **1** was symmetrical dimers. From all the above results, it was deduced that those two 1′-*O*-methylaverantin moieties should be joined via an ether linkage at C-3 and C-3′. The absolute configurations of the stereogenic center at C-11 and C-11′ were assigned as *S* on the basis of the reported NMR data and specific rotation data of related compounds [11], as well as the shared biosynthetic pathway. Ultimately, the structure of compound **1** was elucidated as 6,6′-oxybis(1,3,8-trihydroxy-2-((*S*)-1-methoxyhexyl)anthracene-9,10-dione).

Compound **2** was obtained as a red, amorphous powder. It showed a quasimolecular ion peak at *m/z* 725.2240 $[M\text{-}H]^-$ in the HRESIMS spectrum, representing a 28 mass unit loss relative to compound **1** and a molecular formula of $C_{40}H_{38}O_{13}$, The ^{1}H and ^{13}C NMR spectroscopic data of compound 2 were very similar to those of 1 (Tables 1 and 2), except the methoxyl signals were disappeared in **2**. Additionally, ^{13}C NMR data revealed the oxygen-bearing methane changed from δ_C 78.9 (C-11/11′) in **1** to δ_C 70.1 in **2**, suggesting C-11/11′ connectivity to a hydroxyl group in **2** instead of a methoxyl moiety encountered at the same position in compound **1**. On the basis of these data and comparative analyses relative to compound **1**, compound **2** was identified as 6,6′-oxybis(1,3,8-trihydroxy-2-((*S*)-1-hydroxyhexyl) anthracene-9,10-dione).

In addition, five known compounds, 1′-*O*-methylaverantin (**3**), averantin (**4**), averythrin (**5**), stergmatocystin (**6**), and variecoxanthone A (**7**) were identified on the basis of comparison of their 1D NMR data with previously reported ones [11–13]. Anthraquinones dimers are widespread, structurally-diverse family of natural products frequently found in plants, fungi and lichens. They feature an intriguing variety of linkages between the component anthraquinones. To the best of our knowledge, one of the more common ways in which dimerisation is found in anthraquninones is through the biaryl C–C linkage, and this is the first example of anthraquinone dimers by a rare ether C-O-C linkage from nature. The oxidative dimerisation is thus most likely mediated by some unknown enzymes, which also might be shared the same biosynthesis pathway (Fig. S16) with some reported xanthone dimerisation derivatives [14–16].

Both anthraquninones and xanthones comprise a group of structurally diverse, biosynthetically intriguing, biologically active and synthetically challenging natural products. Many anthraquninones and xanthones have been found to exhibit pronounced biological activities, for example anti-tumor effects, antibacterial activity [17–19]. In this study, all the isolated compounds were evaluated for their antibacterial activities against selected human pathogens, *Staphylococcus aureus*,

Escherichia coli TEM, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Klebsiella aerogenes*, and *Enterobacter cloacae* using a disk diffusion method, and compounds **1** and **2** exhibited selective antibacterial activity against Gram-positive *Staphylococcus aureus* at a concentration of 30 μg/well (Table S1),. Compounds **1–7** were also tested for their cytotoxic activities against human lung cancer A-549; human ovarian cancer SK-OV-3; human skin cancer SK-MEL-2; human CNS cancer XF-498; and human colon cancer HCT-15 using the previously reported MTT method. Among these compounds, only stergmatocystin (**6**) exhibited moderate cytotoxic activity with IC_{50} values from 11.25–17.36 μg/mL (Table S2).

Conflict of interest

There is no conflict of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fitote.2018.11.015.

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