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TWO NEW ANTHRAQUINONES FROM THE CIGAR TOBACCO-DERIVED FUNGUS *Aspergillus versicolor* AND THEIR BIOACTIVITIES

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*Two new anthraquinones, 2′,6-dimethyl-7-methoxy-[2,3-*b*]furan-anthraquinone (**1**) and 1,7-dimethoxy-2′,6-dimethyl-[2,3-*b*]furan-anthraquinone (**2**), were isolated from the fermentation products of the cigar tobacco-derived endophytic fungus* Aspergillus versicolor. *Their structures were elucidated by spectroscopic methods, including extensive 1D and 2D NMR techniques. Compounds **1** and **2** were evaluated for their antibacterial and antioxidant activities. The results showed that compounds **1** and **2** demonstrated strong anti-methicillin-resistant* Staphylococcus aureus *(anti-MRSA) activities with an inhibition zone diameter of 16.4 ± 2.2 and 18.5 ± 2.5 mm, respectively. Compounds **1** and **2** also showed good antioxidant activities, with IC_{50} values of 4.05 and 3.94 μg/mL, respectively.*

Keywords: anthraquinones, endophytic fungus *A. versicolor*, antibacterial activity, antioxidant activity.

Natural compounds possess a variety of antibacterial, antifungal, and antiviral activity, etc., and are widely used in medicine and agriculture [1–3]. Among them, the microorganisms gradually took an important role because they appear to be prolific producers of a wide diversity of secondary metabolites [4, 5]. Anthraquinone with the basic structure of 9,10-anthracenedione, also called 9,10-dioxoanthracene, characterized by two carbonyl groups of the skeleton, is the most important quinone derivative of anthracene. Anthraquinone derivatives, containing more than 700 molecules described of quinoid compounds with chemical diversity and biological activity, attracted significant attention from industries in such fields as pharmaceuticals, clothes colouration, and food colorants [6–9].

Endophytes are a unique type of microorganisms colonizing plants without causing apparent diseases [10]. Extensive research into endophytes during the past decades has demonstrated their tremendous capability to produce structurally and biologically interesting molecules. Among them, the *Aspergillus* is an important fungal genus containing economically important species, as well as pathogenic species for animals and plants [11, 12]. They produce a number of structurally complicated molecules with various biological activity, including antimicrobial, antioxidant, and antiviral [13, 14]. A growing number of novel anthraquinones with important biological functions from this genus have been reported recently, and they have drawn widespread attention as potential drug candidates [15–18]. As our ongoing research into *Aspergillus* species and aimed at structurally diverse and biologically significant compounds, the fermented extracts of the fungus *Aspergillus versicolor* were investigated and resulted in the yield of two new (**1** and **2**) and five known (**3–7**) anthraquinones. Herein, we describe the isolation, structure elucidation, and biological activity of these compounds.

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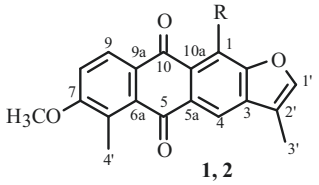
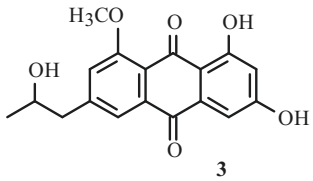
TABLE 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds **1** and **2** ($CDCl_3$, δ, ppm, J/Hz)

C atom	**1**		**2**	
	δ_C	δ_H	δ_C	δ_H
1	112.4 (CH)	7.90 (s)	146.9 (C)	–
2	156.7 (C)	–	149.2 (C)	–
3	132.9 (C)	–	136.9 (C)	–
4	123.6 (CH)	8.08 (s)	114.3 (CH)	7.92 (s)
5	179.7 (C)	–	179.4 (C)	–
6	126.6 (C)	–	126.3 (C)	–
7	161.9 (C)	–	161.6 (C)	–
8	118.3 (CH)	6.95 (d, J = 7.8)	118.4 (CH)	6.97 (d, J = 7.8)
9	127.9 (CH)	7.73 (d, J = 7.8)	128.1 (CH)	7.76 (d, J = 7.8)
10	181.0 (C)	–	180.9 (C)	–
5a	134.1 (C)	–	135.0 (C)	–
6a	131.6 (C)	–	135.8 (C)	–
9a	125.0 (C)	–	125.5 (C)	–
10a	136.2 (C)	–	120.4 (C)	–
1′	143.8 (CH)	7.38 (s)	142.8 (CH)	7.39 (s)
2′	117.6 (C)	–	116.7 (C)	–
3′	13.9 (CH_3)	1.98 (s)	14.0 (CH_3)	1.98 (s)
4′	9.69 (CH_3)	2.13 (s)	9.86 (CH_3)	2.17 (s)
MeO-1			60.8 (CH_3)	3.81 (s)
MeO-7	56.1 (CH_3)	3.83 (s)	56.3 (CH_3)	3.84 (s)

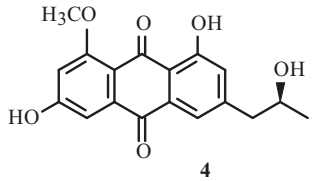


1, 2

1: R = H; **2:** R = OCH_3



3



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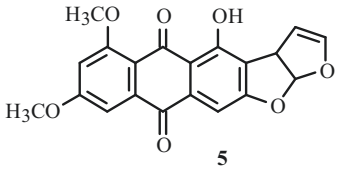

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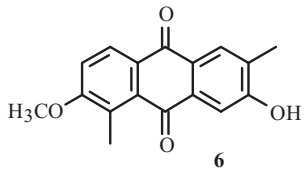

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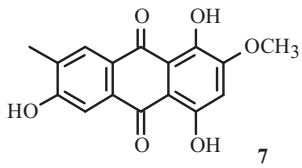

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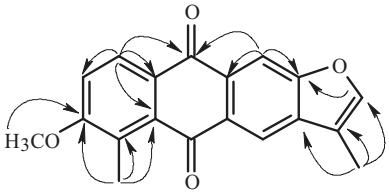
The fermented substrate was extracted using EtOH, and then the extract was subjected repeatedly to column chromatography on silica gel, RP-18, and semi-preparative RP-HPLC separation to afford two new anthraquinones, 2′,6-dimethy-7-methoxy-[2,3-*b*]furan-anthraquinone (**1**) and 2′,6-dimethyl-1,7-dimethoxy-[2,3-*b*]furan-anthraquinone (**2**), together with five known anthraquinone derivatives (**3–7**). The NMR data of **1** and **2** were listed in Table 1. The known compounds, isorhodoptilometrin-1-methyl ether (**3**) [19], penipurdin A (**4**) [20], 6,8-di-*O*-methylversicolorin A (**5**) [21], 7-hydroxy-2-methoxy-1,6-dimethylanthraquinone (**6**) [22], and 1,4,6-trihydroxy-2-methoxy-7-methylanthraquinone (**7**) [23] were identified by the comparison of their spectroscopic data with the literature.

Compound **1**, a reddish gum, was assigned the molecular formula $C_{19}H_{14}O_4$ according to the sodium adduct ion at *m/z* 329.0795 $[M + Na]^+$ in the (+)-HR-ESI-MS spectrum, indicating 13 degrees of unsaturation. The UV spectrum of **1** exhibited absorption bands at 408, 285, 262, and 215 nm, suggesting the existence of aromatic chromophores. Strong absorption bands accounting for carbonyl (1668 cm^{-1}), and aromatic groups (1612, 1588, and 1432 cm^{-1}) could also be observed in its IR spectrum. The ^{1}H and ^{13}C NMR spectrum of **1** (Table 1) showed the presence of two phenyl rings (C1–C4, C6–C9, C-5a, C-6a, C-9a, and C-10a; H-1, H-4, H-8, and H-9), two carbonyl groups (C-5 and C-10), one methyl group (C-4′ and H_3-4′), one methoxy group (δ_C 56.1 q and δ_H 3.83 s), and one -O-CH=C(CH_3)- moiety (C-1′–C-3′, H-1′, and H_3-3′).



1

Fig. 1. Key HMBC correlations of **1**.

According to the above NMR data, two phenyl rings and two carbonyl groups could form an anthraquinone core [22], and the -O-CH=C(CH_3)- moiety should be incorporated with a phenyl ring to form a 2-methylbenzofuran ring [24], supporting the 13 degrees of unsaturation in compound **1**. In addition, the existence of an anthraquinone core was supported by the HMBC correlations (Fig. 1) from H-1 to C-10, C-5a, and C-10a, from H-4 to C-5, C-5a, and C-10a, and from H-9 to C-10, C-6a, and C-9a, and the existence of the 2-methylfuran moiety was also supported by the HMBC correlations from H-4 to C-2′, from H-1′ to C-2 and C-3, and from H_3-3′ to C-1′, C-2′, and C-3.

As the anthraquinone skeleton was determined, the positions of substituents (one methyl, one methoxy, and one -O-CH=C(CH_3)- moiety) can also be determined by further analysis of its HMBC data (Fig. 1). The HMBC correlations from the methyl singlet (δ_H 2.13) to C-6, C-7, and C-6a established that the methyl group was located at C-6. The methoxy group location at C-7 was clearly indicated by the HMBC correlations from methoxy proton (δ_H 3.83 s) to C-7. Furthermore, the -O-CH=C(CH_3)- moiety incorporated with a phenyl ring to form a 2-methylbenzofuran at C-2 and C-3, and C-2′ linked to C-3 was supported by the HMBC correlations from H-4 to C-2′ and from H-1′ to C-2 and C-3. On the basis of the above evidence, the structure of **1** was established as shown and named 2′,6-dimethyl-7-methoxy-[2,3-*b*]furan-anthraquinone.

For compound **2**, a reddish gum, the molecular formula was established as $C_{20}H_{16}O_5$ by the $[M + Na]^+$ ion at *m/z* 359.0890 (calcd for $C_{20}H_{16}NaO_5$, 359.0895) in HR-ESI-MS. The ^1H and ^{13}C NMR data (Table 1) of **1** and **2** showed great similarity. The obvious chemical shift differences resulted from an aromatic proton (δ_H 7.90 s) being replaced by a methoxy group (δ_C 60.8 q, δ_H 3.81 s) in **2**. This methoxy group attached to C-1 was supported by the HMBC correlation from the methoxy protons (δ_H 3.81) to C-1. In addition, the positions of the methyl group, 2-methylfuran moiety, and the other methoxy group can also be determined by further analysis of its HMBC correlations. Thus, the structure of 1,7-dimethoxy-2′,6-dimethyl-[2,3-*b*]furan-anthraquinone (**2**) was determined as shown.

As certain of the anthraquinone derivatives from fungi exhibit potential antibacterial and antioxidant activities, compounds **1** and **2** were evaluated for those activities.

The anti-methicillin-resistant *Staphylococcus aureus* (anti-MRSA) activities were screened according to an arbitrary criterion [25] with an inhibition zone diameter (IZD) as follows: very weak inhibition (with IZD of 6–8 mm), weak inhibition (with IZD of 8–12 mm), good inhibition (with IZD of 12–16 mm), and strong inhibition (with IZD of > 16 mm) activity, respectively. The IZD of the positive control (vancomycin) was 32 mm and that of the negative control was zero. The results revealed that compounds **1** and **2** showed strong inhibition with an IZD of 16.4 ± 2.2 and 18.5 ± 2.5 mm, respectively.

Compounds **1** and **2** were also tested for antioxidant activity by the detection of the oxidative products with the 2′,7′-dichlorofluorescin diacetate (DCFH) method reported previously [26]. The results revealed that compounds **1** and **2** show good antioxidant activity with an IC_{50} value of 4.05 and 3.94 μg/mL, respectively.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. 1D and 2D NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as the internal standard. ESI-MS and HR-ESI-MS analyses were measured on an Agilent 1290UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semipreparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (21.2 × 250 mm) or Venusil MP C_{18} (20 × 250 mm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), Sephadex LH-20

(Sigma-Aldrich, Inc., USA), or MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). The column fractions were monitored by TLC (silica gel 60 F_{254} on a glass plate), and visualized by UV light at 254 nm and 365 nm, or spraying with 5% H_2SO_4 in ethanol and heating. All solvents used in column chromatography were distilled.

Fungal Material. The culture of *A. versicolor* YNCA1208 was isolated from the leaves of cigar tobacco, collected from Gengma County, Lincang Prefecture, Yunnan Province, in 2018. The strain was identified by one of authors (Dr. Qi-Li Mi) based on the analysis of the ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28°C. Agar plugs were inoculated into 250-mL Erlenmeyer flasks, each containing 100 mL potato dextrose broth, and cultured at 28°C on a rotary shaker at 180 rpm for 5 days. Large-scale fermentation was carried out in 20 Fernbach flasks (5.0 L), each containing 1.0 kg of rice and 120 mL of distilled H_2O. Each flask was inoculated with 50 mL of cultured broth and incubated at 25°C for 45 days.

Extraction and Isolation. The fermented substrate was extracted four times with ethanol (4 × 25 L) at room temperature and filtered. The filtered solution was concentrated under reduced pressure to yield a crude extract, which was suspended in water and partitioned with EtOAc. The EtOAc extract (285 g) was applied to silica gel (200–300 mesh) column chromatography, eluted with $CHCl_3$–MeOH (20:1, 9:1, 8:2, 7:3, 6:4, 5:5), to give six fractions (A–F). The further separation of fraction B (9:1, 28.5 g) by silica gel column chromatography, eluted with $CHCl_3$–Me_2CO (9:1, 8:2, 7:3, 6:4, 1:1), afforded five subfractions (B1–B5). The further separation of subfraction B2 (8:2) by silica gel column chromatography, eluted with petroleum ether–EtOAc, and preparative HPLC (with 62% aqueous MeOH as a mobile phase, at a flow rate of 20 mL/min) afforded **1** (18.5 mg), **2** (22.6 mg), and **5** (16.4 mg). The further separation of subfraction B3 (7:3) by silica gel column chromatography, eluted with petroleum ether–EtOAc, and preparative HPLC (with 54% aqueous MeOH as a mobile phase, at a flow rate of 20 mL/min) afforded **3** (25.4 mg), **4** (13.8 mg), **6** (16.9 mg), and **7** (23.6 mg).

Anti-MRSA Agar Disk Diffusion Assay. The MRSA strain ZR11 was clinically isolated from infectious samples of critically ill patients in the Clinical Laboratory of the First People′s Hospital of Yunnan Province and confirmed by standard cefoxitin disk diffusion test following CLSI standard procedures [25]. The anti-MRSA activity of the compounds was evaluated via the disk diffusion method. The ZR11 strain was inoculated in Mueller Hinton Broth and was incubated at 37°C for 24 h. The turbidity of bacterial suspension was adjusted to a 0.5 McFarland standard, which is equal to 1.5×10^8 colony-forming units (CFU)/mL. Sterile filter paper disks (6 mm) were impregnated with 20 μL (50 μg) of each compound and placed on inoculated Mueller–Hinton agar containing bacterial suspension, which was adjusted to a 0.5 McFarland standard. The commercially available disks containing 30 μg vancomycin were used as a positive control whereas disks without samples (5% DMSO) acted as a negative control. The inhibition zones including the diameter of the disk (mm) were measured and compared after incubation at 37°C for 24 h. The tests were carried out in triplicate for each sample.

Antioxidant Activity Assay. The antioxidant activity was tested using a 2′,7′-dichlorofluorescin diacetate (DCFH) method reported previously [26]. Myelomonocytic HL-60 cells (1×10^6 cells/mL, ATCC) were suspended in RPMI 1640 medium with 10% FBS and antibiotics at 37°C in 5% CO_2: 95% air. 125 μL of the cell suspension was added to each well of a 96-well plate. After treatment with a different concentration of the test material for 30 min, the cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 min. Then the cells were incubated for 15 min after the addition of 5 μg/mL DCFH-DA (Molecular Probes). DCFH-DA is a nonfluorescent probe that diffuses into cells. Cytoplasmic esterases hydrolyze DCFH-DA to 2′,7′-dichlorofluorescin (DCFH), and the reactive oxygen species (ROS) generated within HL-60 cells oxidize DCFH to the fluorescent dye 2′,7′-dichlorofluorescein (DCF). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalyzed oxidation of DCFH in HL-60 cells was measured by PMA-treated control incubations with and without the test materials. The levels of DCF were measured using a CytoFluor 2350 fluorescence measurement system (Millipore) with an excitation wavelength at 485 nm (bandwidth 20 nm) and emission at 530 nm (bandwidth 25 nm).

2′,6-Dimethyl-7-methoxy-[2,3-*b*]furan-anthraquinone (1), $C_{19}H_{14}O_4$, obtained as a reddish gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.26), 262 (3.38), 285 (3.57), 408 (3.40). IR (KBr, ν_{max}, cm^{-1}): 3034, 2965, 2816, 1668, 1612, 1588, 1432, 1328, 1147, 1015, 758. For ^1H and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$), see Table 1. ESI-MS *m/z* 329 $[M + Na]^+$; HR-ESI-MS *m/z* 329.0795 $[M + Na]^+$ (calcd for $C_{19}H_{14}O_4Na$, 329.0790).

1,7-Dimethoxy-2′,6-dimethyl-[2,3-*b*]furan-anthraquinone (2), $C_{20}H_{16}O_5$, obtained as a reddish gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.21), 265 (3.42), 288 (3.62), 412 (3.36). IR (KBr, ν_{max}, cm^{-1}): 3041, 2962, 2823, 1665, 1616, 1574, 1438, 1323, 1152, 1018, 764. For ^1H and ^{13}C NMR data (500 and 125 MHz, $CDCl_3$), see Table 1. ESI-MS *m/z* 359 $[M + Na]^+$; HR-ESI-MS *m/z* 359.0890 $[M + Na]^+$ (calcd for $C_{20}H_{16}O_5Na$, 359.0895).

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