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Metabolites from *Aspergillus versicolor*, an endolichenic fungus from the lichen *Lobaria retigera*

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Summary **Three new anthraquinone derivatives (1-3) and one new artifact (4) were isolated, along with six known anthraquinone derivatives (5-10) and three xanthones (11-13), from a culture of an endolichenic fungus, *Aspergillus versicolor*, that was isolated from the lichen *Lobaria retigera*. The structures of these substances were determined on the basis of 1D and 2D (COSY, HMQC, and HMBC) NMR and MS analyses. The substances 1-4 were also tested for their cytotoxic activity.**

Keywords: Endolichenic fungus, *Aspergillus versicolor*, *Lobaria retigera*, anthraquinone

1. Introduction

Lichens are composite organisms of a fungus (the mycobiont) and an algal partner (the photobiont or phycobiont) growing together in a symbiotic relationship. Endolichenic fungi, an emerging group of endosymbiotic microorganisms, consist of fungal strains that live within asymptomatic lichen thalli, much in the same way as endophytic fungi live within healthy plant tissues (*1*). In contrast to endophytic fungi that have been studied chemically a number of times (*2-4*), only a limited number of endolichenic fungi have thus far been investigated for their secondary metabolites (*5,6*), but studies have shown that endolichenic fungi are also rich sources of structurally diverse and biologically active small-molecule metabolites. In the course of the current authors' ongoing efforts to discover bioactive compounds from endophytic and endolichenic microorganisms, four new anthraquinone derivatives (**1-4**) (Figure 1) were obtained, along with six known anthraquinones (**5-10**) and three xanthones (**11-13**) (Supplemental data, *http://www.ddtjournal.com/docindex.php?year=2014&kanno=2*), from a culture of *Aspergillus versicolor*, an endolichenic strain of the lichen *Lobaria retigera*. The structures of these substances were elucidated using spectroscopic methods, including 1D and 2D nuclear magnetic resonance (NMR) and high-resolution electrospray ionization mass spectrometry (HRESIMS). Substances **1**-**4** were tested for their cytotoxic activity, with **1** and **2** displaying weak inhibitory activity towards the PC-3 and H460 human cancer cell lines. Reported here are the isolation of these compounds, elucidation of their structures, and determination of their bioactivity.

2. Materials and Methods

2.1. *General experimental procedures*

Optical rotations were measured on a GYROMAT-HP

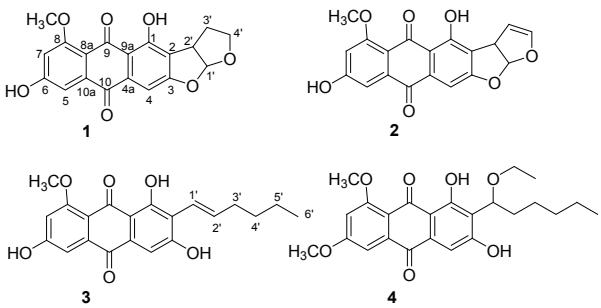

Figure 1. Structures of compounds 1-4

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polarimeter (Shimadzu Corporation, Kyoto, Japan). Infrared spectra (IR) were recorded on a Nicolet iN 10 Micro FTIR spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) in transmission mode. Ultraviolet (UV) spectra were obtained with a Shimadzu UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on a Bruker Avance DRX-600 spectrometer (Bruker BioSpin Group, Billerica, MA, USA) operating at 600 (^{1}H) and 150 (^{13}C) MHz with tetramethylsilane (TMS) as an internal standard. HRESIMS was performed on an LTQ Orbitrap XL (Thermo Fisher Scientific Inc., Waltham, MA, USA). All solvents used were of analytical grade (Laiyang Chemical Reagent Co., Ltd., Shandong, China). High-performance liquid chromatography (HPLC) was performed on an Agilent 1100 G1310A isopump equipped with an Agilent 1100 G1322A degasser, an Agilent 1100 G1314A VWD detector (254 nm), and an Agilent ZORBAX SB-C_{18} column (9.4 mm × 250 mm, 5 μm) (Agilent Technologies, Inc., Santa Clara, CA, USA). Silica gel (200-300 mesh; Qingdao Haiyang Chemical Co., Ltd., Qingdao, Shandong, China), C_{18} reversed-phase silica gel (YMC ODS-A gel, YMC Co., Ltd., Kyoto, Japan), and Sephadex LH-20 (GE Health, Uppsala, Sweden) were used for column chromatography (CC). Thin-layer chromatography (TLC) was performed with high-performance TLC plates precoated with silica gel GF254 (Qingdao Haiyang Chemical Co., Ltd.). Spots of TLC were visualized within iodine vapor or by spraying with H_2SO_4-EtOH (1:9) followed by heating.

2.2. *Microorganism*

The strain of fungus was isolated from the lichen *Lobaria retigera* collected from Mount Laojun, Yunnan Province, China. Nuclear ITS rDNA sequencing revealed that it was *Aspergillus versicolor*. The fungus (accession no. 2011-WX-35b) was deposited in the Key Laboratory of Chemical Biology (Ministry of Education).

2.3. *Fermentation, extraction, and isolation*

The fungal strain was grown on slants of potato dextrose agar (PDA) at 25°C for 15 days. The fungus was then placed in five Erlenmeyer flasks (300 mL) that each contained 120 mL of potato dextrose broth (PDB). These flasks were incubated at 25°C on a rotary shaker (120 rpm) for seven days to obtain the seed culture. The seed broth was added to 40 flasks (500 mL) that each contained an autoclaved culture medium of rice (80 g) and water (120 mL). Afterwards, the flasks were left to stand at room temperature for 40 days until the solid medium had almost disappeared. At harvest, the culture medium containing the mycelium was cut into small pieces, extracted with EtOAc for two days at room temperature, and then filtered. The solvent was evaporated under reduced pressure at 38°C to yield a crude extract (36.0 g) that was separated using CC with silica gel eluted with a gradient of petroleum ether (PE) and acetone from 100:0 to 0:100 (v/v) to yield eleven fractions (Fr. A-Fr. K). Fr. D (771.7 mg) was separated using CC with silica gel (PE/acetone, 80:1 to 2:1) to yield six subfractions (Fr. D1-Fr. D6). Compound **11** (101.5 mg) was obtained from Fr. D2 after recrystallization in CH_2Cl_2. Fr. D4 (230.0 mg) was loaded onto a Sephadex LH-20 column (CH_2Cl_2/MeOH, 1:1) to yield **4** (10.0 mg) and **5** (28.3 mg). Fraction E (80.0 mg) was separated using CC with a Sephadex LH-20 column (CH_2Cl_2/MeOH, 1:1) to yield the primary portion (16.2 mg), which was further purified using HPLC (MeOH-H_2O, 90:10, 1.5 mL/min) to yield **9** (3.0 mg, t_R = 10.9 min). Fraction G (2.0 g) was separated using CC with silica gel eluted with a gradient of CH_2Cl_2-MeOH (60:1 to pure MeOH) to yield seven subfractions (Fr. G1-Fr. G7). After recrystallization in CH_2Cl_2, compounds **6** (8.2 mg) and **7** (10.3 mg) were obtained from Fr. G3 (26.0 mg) and Fr. G2 (29.4 mg), respectively. Fr. G6 (638.7 mg) was subjected to CC with C18 reversed-phase silica gel eluted with a gradient of MeOH-H_2O (50:50 to 100% MeOH) to yield the primary portion, which was further separated using HPLC (MeOH-H_2O, 67:33, 1.5 mL/min) to yield **13** (2.1 mg, t_R = 34.2 min) and **12** (4.7 mg, t_R = 23.4 min). Fraction H (3.6 g) was subjected to CC with silica gel eluted with a gradient of CH_2Cl_2-MeOH (80:1 to 5:1) to yield eight subfractions (Fr. H1-Fr. H8). Fr. H4 (430.9 mg) was first separated on silica gel eluted with a gradient of PE-EtOAc (40:1 to 10:1) to yield the primary portion and then separated on C18 reversed-phase silica gel eluted with a gradient of MeOH-H_2O (50:50 to pure MeOH) to yield six subfractions (Fr. H4-1-Fr. H4-6). Fr. H4-4 (53.1 mg) was purified using HPLC (CH_3CN-H_2O, 50:50, 1.5 mL/min) to yield **1** (3.2 mg, t_R = 19.6 min) and **2** (2.0 mg, t_R = 21.4 min). Fr. H4-5 (38.4 mg) yielded **10** (5.1 mg, t_R = 34.2 min) after HPLC (MeOH-H_2O, 75:25, 1.5 mL/min) purification. Fr. H5 (637.4 mg) was separated on a silica gel column eluted with a gradient of PE-EtOAc (20:1 to 1:1) to yield five subfractions (Fr. H5-1-Fr. H5-5). Fr. H5-2 (44.9 mg) was purified using HPLC (CH_3CN-H_2O, 70:30, 1.5 mL/min) to yield **8** (20.0 mg, t_R = 28.2 min), while Fr. H5-4 (25.0 mg) yielded **3** (4.8 mg, t_R = 24.2 min) after HPLC (CH_3CN-H_2O, 64:36, 1.5 mL/min) purification.

8-*O*-methylversicolorin B (**1**): Orange solid. $[\alpha]_D^{20}$ = –75.76 (*c* 0.132, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (1.65), 223 (2.08), 287 (1.53), 310 (0.96). IR (KBr) ν_{max} cm^{-1}: 3,494, 2,946, 2,851, 1,620, 1,598, 1,242, 949. HRESIMS (negative mode) *m/z* 353.0635 [*M*-H]$^-$ (calcd. for $C_{19}H_{13}O_7$, 353.0661). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

8-*O*-methylversicolorin A (**2**): Orange solid. $[\alpha]_D^{20}$ =

Table 1. ^{1}H-NMR data for compounds 1-4 (at 600 MHz, δ in ppm, J in Hz)

Position	1[a]	2[a]	3[a]	4[b]
4	6.99 (1H, *s*)	7.14 (1H, *s*)	7.26 (1H, *s*)	7.08 (1H, *s*)
5	7.26 (1H, *s*)	7.33 (1H, *s*)	7.31 (1H, *s*)	7.45 (1H, *s*)
7	6.70 (1H, *s*)	6.92 (1H, *s*)	6.91 (1H, *s*)	6.78 (1H, *s*)
1-OH	14.44 (1H, *brs*)	13.89 (1H, *s*)	14.58 (1H, *s*)	13.76 (1H, *s*)
3-OH				9.72 (1H, *s*)
6-OCH_3				4.00 (3H, *s*)
8-OCH_3	3.89 (3H, *s*)	3.97 (3H, *s*)	3.97 (3H, *s*)	4.02 (3H, *s*)
1'	6.45 (1H, *d*, *J* = 4.2)	6.9 (1H, *d*, *J* = 4.2)	6.74 (1H, *d*, *J* = 16.2)	5.03 (1H, *m*)
2'	4.15 (1H, *t*, *J* = 6.6)	4.80 (1H, *m*)	7.02 (1H, *dt*, *J* = 16.2, 7.2)	1.75 (2H, *m*)
3'	2.23 (1H, *m*) (β-H); 2.28 (1H, *m*) (α-H)	5.45 (1H, *m*)	2.28 (1H, *q*, *J* = 7.2)	1.70 (2H, *m*)
4'	3.58 (1H, *m*) (β-H); 4.09 (1H, *t*, *J* = 7.8) (α-H)	6.65 (1H, *s*)	1.49 (2H , *m*)	1.30 (2H, *m*)
5'			1.40 (2H, *m*)	1.25 (2H, *m*)
6'			0.94 (3H, *t*, *J* = 7.2)	0.87 (3H, *m*)
1''				3.60 (2H, *m*)
2''				1.26 (3H, *m*)

[a] Measured in acetone-d_6; [b] Measured in $CDCl_3$

Table 2. ^{13}C-NMR data for compounds 1-4 (at 150 MHz, δ in ppm)

Position	1[a]	2[a]	3[a]	4[b]
1	165.2 (C)	n.o.[c]	161.1 (C)	162.0 (C)
2	120.8 (C)	120.9 (C)	119.2 (C)	119.7 (C)
3	160.7 (C)	164.1 (C)	164.0 (C)	162.4 (C)
4	99.97 (CH)	100.2 (CH)	107.2 (CH)	108.6 (CH)
4a	136.0 (C)	134.7 (C)	132.0 (C)	132.9 (C)
5	110.5 (CH)	107.0 (CH)	107.6 (CH)	103.8 (CH)
6	169.7 (C)	164.8 (C)	163.5 (C)	164.9 (C)
7	106.2 (CH)	104.9 (CH)	105.7 (CH)	104.9 (CH)
8	165.3 (C)	163.2 (C)	164.8 (C)	162.8 (C)
8a	112.0 (C)	n.o.[c]	114.6 (C)	137.6 (C)
9	186.8 (C)	186.6 (C)	187.6 (C)	186.8 (C)
9a	113.7 (C)	n.o.[c]	111.3 (C)	110.2 (C)
10	183.8 (C)	181.9 (C)	182.8 (C)	182.6 (C)
10a	138.0 (C)	137.3 (C)	132.0 (C)	115.4 (C)
6-OCH_3				56.0 (CH_3)
8-OCH_3	56.4 (CH_3)	55.9 (CH_3)	56.8 (CH_3)	56.7 (CH_3)
1'	114.0 (CH)	113.2 (CH)	120.4 (CH)	77.6 (CH)
2'	45.2 (CH)	48.9 (CH)	139.1 (CH)	34.8 (CH_2)
3'	31.5 (CH_2)	101.7 (CH)	35.4 (CH_2)	31.6 (CH_2)
4'	68.1 (CH_2)	145.5 (CH)	32.6 (CH_2)	25.1 (CH_2)
5'			23.1 (CH_2)	22.6 (CH_2)
6'			14.0 (CH_3)	14.1 (CH_3)
1''				66.3 (CH_2)
2''				15.0 (CH_3)

[a] Recorded in acetone-d_6; [b] Measured in $CDCl_3$; [c] These quaternary carbons were not observed.

– 95.24 (*c* 0.063, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (1.16), 223 (1.06), 285 (0.81). IR (KBr) ν_{max} cm^{-1}: 3,366, 2,921, 2,851, 1,738, 1,630, 1,598, 1,347, 1,294, 1,223, 973. HRESIMS (negative mode) *m/z* 351.0503 $[M\text{-H}]^-$ (calcd. for $C_{19}H_{11}O_7$, 351.0505). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

8-*O*-methylaverythin (**3**): Orange solid. UV (MeOH) λ_{max} (logε) nm: 195 (2.30), 225 (2.45), 295 (2.90). IR (KBr) ν_{max} cm^{-1}: 3,529, 3,377, 3,089, 3,027, 2,956, 2,927, 2,856, 1,618, 1,583, 1,336, 1,300, 1,258, 1,061, 978. HRESIMS (negative mode) *m/z* 367.1177 $[M\text{-H}]^-$ (calcd. for $C_{21}H_{19}O_6$, 367.1182). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

1'-*O*-ethyl-6,8-di-*O*-methylaverantin (**4**): Yellow solid. $[\alpha]_D^{20}$ = – 11.63 (*c* 0.086, MeOH). UV (MeOH) λ_{max} (logε) nm: 197 (3.02), 224 (3.79), 286 (3.14). IR (KBr) ν_{max} cm^{-1}: 3,219, 2,926, 2,854, 1,736, 1,670, 1,622, 1,595, 1,333, 1,265, 1,162, 1,063. HRESIMS (positive mode) *m/z* 429.1908 $[M\text{+H}]^+$ (calcd. for $C_{24}H_{29}O_7$, 429.1913). For ^{1}H- and ^{13}C-NMR data, see Tables 1 and 2.

2.4. *Cytotoxicity assay*

A tetrazolium-based colorimetric assay (MTT assay) was used to determine cell viability. PC-3 cells (ATCC CRL-1435 human prostate adenocarcinoma) and NCI-H460 cells (ATCC HTB 177 human lung carcinoma) were used. The cell culture and cytotoxic activity assay followed the same procedures as previously described (*7*). Doxorubicin was used as a positive control, for which the IC_{50} values were 0.2 and 0.3 μM against PC-3 and NCI-H460, respectively.

3. Results and Discussion

Compound **1** was obtained as an orange powder. HRESIMS revealed a pseudo molecular ion $[M\text{-H}]^-$ peak at *m/z* 353.0635 (calcd. 353.0661) compatible with the molecular formula $C_{19}H_{14}O_7$, indicating 13 degrees of unsaturation. Evident in the ^{1}H-NMR spectrum for this compound (Table 1) was the resonance of one hydro-bonded phenolic hydroxyl group at δ_H 14.44 (s), three aromatic protons at δ_H 7.26 (s), 6.99 (s), and 6.70 (s), an oxygenated methine at δ_H 6.45 (d, *J* = 4.2 Hz), a methine at δ_H 4.15 (t, *J* = 6.6 Hz), a pair of oxygen-bearing methylene protons at δ_H 4.09 (t, *J* = 7.8 Hz) and δ_H 3.58 (m), another methylene at δ_H 2.28 (m) and δ_H 2.23 (m), and one oxygenated methyl group at δ_H 3.89 (3H, s). Evident in the ^{13}C-NMR spectrum (Table 2) was resonance of two carbonyls (δ_C 186.8 and 183.8), three aromatic methines, and nine aromatic quarternary

carbons (including four oxygenated) with chemical shifts attributable to a highly substituted anthraquinone scaffold (*8*). In addition, two methylenes (including one oxygenated), two methines, and one oxygenated methyl groups were observed. These data indicated that **1** was similar to versicolorin B (*9*), except that a methoxy group was present at C-6 in **1** instead of the 6-OH group in versicolorin B. The relative configuration of **1** was determined by analysis of NOESY correlations. Therefore, compound **1** was determined to be 8-*O*-methylversicolorin B.

Compound **2** was also an orange amorphous solid. HRESIMS revealed an $[M-H]^-$ ion peak at *m/z* 351.0503 (calcd. 351.0505) corresponding to the molecular formula $C_{19}H_{12}O_7$. The ^{1}H- and ^{13}C-NMR data (Tables 1 and 2) for **2** closely resembled those for **1**, except for the presence of an additional double bond between C-3' (δ_C 101.7) and C-4' (δ_C 145.5) in **2**. This was verified by the HMBC correlations of H-3' (δ_H 5.45)/C-1' and H-1'/C-2', C-3', and C-4' along with the ^{1}H-^{1}H COSY correlations of H-2'/H-3' and H-3'/H-4'. Therefore, compound **2** was identified as 8-*O*-methylversicolorin A.

Compound **3** was also an orange solid. A molecular formula of $C_{21}H_{20}O_6$ was assigned to **3** based on the quasimolecular ion $[M-H]^-$ peak at *m/z* 367.1177 (calcd. 367.1182) according to HRESIMS. The ^{1}H- and ^{13}C-NMR spectra for **3** (Tables 1 and 2) were similar to those previously reported for averythin (**8**) (*8*). The only difference was replacement of 8-OH by a methoxy group (δ_H 3.97, 3H, s). This was confirmed by HMBC correlations from 8-OMe to C-7 (δ_C 105.7) and C-8 (δ_C 164.8). Therefore, compound **3** was determined to be 8-*O*-methylaverythin.

Compound **4** was obtained as a yellow solid. It was assigned a molecular formula of $C_{24}H_{28}O_7$ based on the $[M+H]^+$ ion peak at *m/z* 429.1908 (calcd. 429.1913) according to HRESIMS. The NMR data for **4** (Table 1) were similar to those for **5** (*10*), except that the 1'-methoxy group in **5** was replaced by an ethoxy moiety in **4**. This was verified by the HMBC correlations of H-2" (δ_H 1.26)/C-1" (δ_C 66.3) and H-1" (δ_H 3.60)/C-1'. Hence, compound **4** was determined to be 1'-*O*-ethyl-6,8-di-*O*-methylaverantin. Compound **4** may be an artifact since EtOH was used in the separation process.

The known compounds 6,8,1'-tri-*O*-methylaverantin (**5**) (*10*), aversin (**6**) (*11-13*), 6,8-di-*O*-methylversicolorin A (**7**) (*11,14*), averythin (**8**) (*8,9,15,16*), 6,8-di-*O*-methylaverufin (**9**) (*10*), 6,8-di-*O*-methylnidurufin (**10**) (*10*) sterigmatocystin (**11**) (*13,17*), 5-methoxysterigmatocystin (**12**) (*13*), and dihydrosterigmatocystin (**13**) (*17*) were identified by comparing their NMR data (see Supplemental Data) to those reported in the literature.

The *in vitro* inhibition of cell proliferation of two cancer cell lines, PC-3 (human prostate cancer cells) and H460 (human lung cancer cells), was evaluated using the new compounds **1**–**4**. Compounds **1** and **2** had weak cytotoxic activity, with IC_{50} values of 19.5 and 12.6 μM against PC-3 cells and 27.2 and 17.3 μM against H460 cells. The other compounds had no activity against PC-3 and H460 cells (IC_{50} > 50 μM).

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