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Two New Cytotoxic Quinone Type Compounds from the Halotolerant Fungus *Aspergillus variecolor*

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Abstract Two new quinone type compounds, variecolorquinones A (**1**) and B (**2**) together with eleven known related compounds **3**∼**13** have been isolated from the metabolites produced by the halotolerant fungal strain *Aspergillus variecolor* B-17. The structures of **1** and **2** were determined by spectroscopic methods. **1** exhibited selective cytotoxicity against A-549 cells with the IC_{50} values of 3.0 μM. **2** showed cytotoxicity against HL60 and P388 cells with the IC_{50} values of 1.3 and 3.7 μM, respectively.

Keywords variecolorquinone, cytotoxic, metabolite, quinone, halotolerant fungi

Introduction

It demonstrated that halotolerant marine fungal species have evolved unique metabolic mechanisms that are responsive to salt concentrations and marine-derived fungal metabolite production could have implications for drug discovery [1]. Besides marine samples, halotolerant fungal species had been isolated from other hypersaline environment such as salterns, salines and dried fish. But, to the best of our knowledge, there were only a few reports on secondary metabolites of halotolerant fungus in the literature [2∼4]. In order to screen "talented strains" [5] to find structural novel and bioactive secondary metabolites, we continue to isolate and identify halotolerant microbes from both marine [6∼13] and other salt environments. As a result, a halotolerant fungal strain, *Aspergillus variecolor* B-17, was isolated from the sediments collected in Jilantai salt field, Alashan, Inner Mongolia, China.

The crude extract of the mycelia of *A. variecolor* B-17 showed significant cytotoxicity against the mouse *cdc2* mutant cell line (tsFT210) and was subjected to flash column chromatography over silica gel, RP-18 and HPLC separation to afford two new quinone type compounds variecolorquinones A (**1**) and B (**2**), together with eleven known compounds, (2*S*)-2,3-dihydroxypropyl 1,6,8-trihydroxy-3-methyl-9,10-dioxoanthracene-2-carboxylate (**3**) [14], emodin (**4**) [15], physcion (**5**) [15], questin (**6**) [15], questinol (**7**) [15], catenarin (**8**) [15], erythroglaucin (**9**) [16], rubrocristin (**10**) [16], fallacinol (**11**) [15], eurotinone (**12**) [17] and 2-methyleurotinone (**13**) [17] (Fig. 1), by a bioassay-guided isolation procedure.

Materials and Methods

Strain

The working strain *Aspergillus variecolor* B-17 was isolated from the sediments collected in Jilantai salt field, Alashan, Inner Mongolia, China. It was identified by Prof. Li Tian, the First Institute of Oceanography, SOA, Qingdao, China. Working strain was prepared on Potato Dextrose agar slants containing 10% NaCl and stored at 4°C.

Fermentation

The fungus was grown under static conditions at 30°C for 28 days in thirty 1000-ml conical flasks containing the liquid medium (300 ml/flask) composed of (g/liter): glucose

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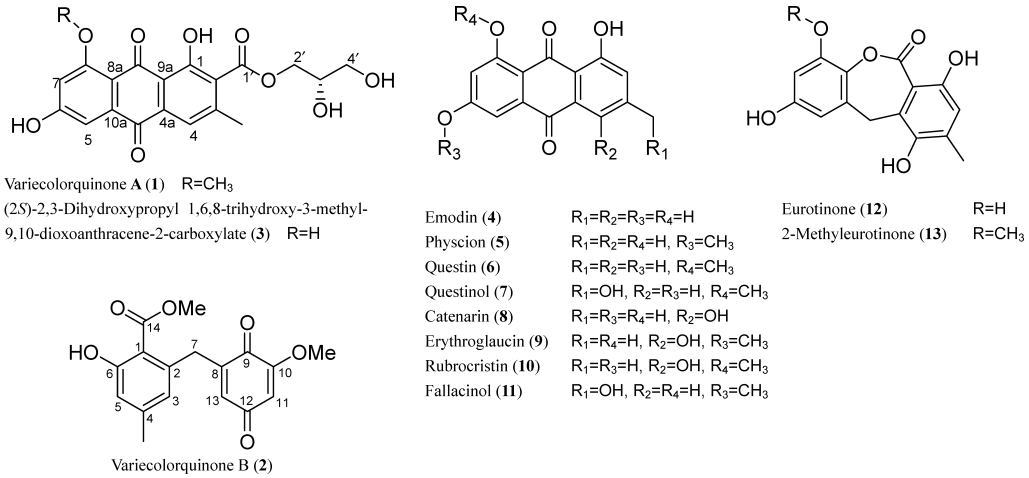

Fig. 1 Structures of variecolorquinones A (**1**) and B (**2**), eleven known compounds **3**～**13**.

(10), maltose (20), mannitol (20), malt extract (3.0), monosodium glutamate (10), NaCl (120), NH_4Cl (10), $MgSO_4 \cdot 7H_2O$ (5.0) and KCl (5.0) after adjusting its pH to 7.0.

Extraction and Isolation

The fermented whole broth (9.0 liters) was filtered through cheese cloth to separate into supernatant and mycelia. The mycelia was extracted three times with Me_2CO. The Me_2CO solution was concentrated under reduced pressure to afford crude extract (7.8 g), showing strong cytotoxicity against the mouse *cdc2* mutant cell line (tsFT210, IC_{50} 68 μg/ml) and antioxidation against 1,1-diphenyl-2-pierylhydrazyl (DPPH, IC_{50} 98 μg/ml). The crude extract was separated into 4 fractions on a silica gel column using a step gradient elution of $CHCl_3$ - MeOH. The fraction 1 (1.9 g) was separated into 5 subfractions on a silica gel column using a step gradient elution of petroleum ether : Me_2CO. Subfraction 1-4 (73 mg) was crystallized from $CHCl_3$/MeOH (v/v 9 : 1) to yield **5** (39 mg). **9** (11 mg) was isolated from the mother liquid of subfraction 1-4 by Sephadex LH-20 column (v/v 2 : 1 $CHCl_3$/MeOH). The fraction 3 (0.8 g) was subjected to column chromatography over silica gel (v/v 93 : 7 $CHCl_3$/MeOH) to afford 16 subfrations. Subfraction 3-2 (72 mg) was crystallized from $CHCl_3$/MeOH (v/v 1 : 1) to yield **8** (59 mg). The mother liquid of subfraction 3-2, togather with the Fr. 3-3 and 3-4 were combined and subjected to HPLC separation (gradient elution of 70～100% MeOH) to yield **4** (5.0 mg), **6** (7.0 mg), **7** (9.0 mg), **10** (7.0 mg) and **11** (6.0 mg). The Fr. 3-7 (59 mg) was further separated by HPLC to yield **2** (31 mg). Subfractions 3-14 (29 mg) and 3-15 (83 mg) were further separated by HPLC to yield **1** (15 mg) and **3** (45 mg), respectively. Subfractions 3-5 and 3-6 were combined and subjected to HPLC to yield **12** (31 mg) and **13** (23 mg).

Physico-chemical Analyses

Optical rotations were obtained on a JASCO P-1020 digital polarimeter. UV spectra were recorded on Beckmen DU® 640 spectrophotometer. IR spectra were taken on a NICOLET NEXUS 470 spectrophotometer in KBr discs. ^{1}H-, ^{13}C-NMR and DEPT spectra and 2D-NMR were recorded on a JEOL JNM-ECP 600 spectrometer using TMS as internal standard and chemical shifts were recorded as δ values. ESI-MS was measured on a Q-TOF ULTIMA GLOBAL GAA076 LC mass spectrometer. Semipreparative HPLC was performed on a SHIMADZU LC-6AD Liquid Chromatograph with SPD-M10A *vp* Diode Array Detector.

Biological Assay

Cell lines were grown in RPMI-1640 supplemented with 10% FBS under a humidified atmosphere of 5.0% CO_2 and 95% air at 37°C. Two hundred microliters of those cell suspensions at a density of 5×10^4 cell/ml was plated in 96 well microtiter plates and incubated for 24 hours at the above conditions. Then 2.0 μl of the test compound solutions (in MeOH) at different concentrations was added to each well and further incubated for 72 hours in the same conditions. Twenty microliters of the MTT solution (5.0 mg/ml in RPMI-1640 medium) was added to each well and incubated for 4 hours. An old medium (150 μl) containing MTT was then gently replaced by DMSO and pipetted to dissolve any formazan crystals formed. Absorbance was then determined on a SPECTRA MAX PLUS plate reader at 540 nm.

In SRB assay, 200 μl of the cell suspensions were plated

Table 1 ^{1}H- and ^{13}C-NMR data for variecolorquinone A (**1**) in DMSO-d_6

No.	δ_H (*J* in Hz)	δ_C	No.	δ_H (*J* in Hz)	δ_C
1		158.8s	10		182.2s
2		129.4s	10a		137.1s
3		143.3s	1-OH	13.6 (s)	
4	7.48 (s)	119.6d	6-OH	11.3 (s)	
4a		132.6s	3-CH_3	2.37 (3H, s)	19.9q
5	7.19 (br s)	107.1d	8-OCH_3	3.91 (3H, s)	56.8q
6		166.3s	1′		164.0s
7	6.83 (br s)	105.0d	2′	4.38 (dd, 10.9, 3.9)	67.3t
				4.19 (dd, 10.9, 6.4)	
8		165.2s	3′	3.76 (m)	69.7d
8a		113.0s	4′	3.43 (dd, 11.0, 5.5)	63.0t
				3.38 (dd, 11.0, 6.2)	
9		186.5s	3′-OH	5.05 (br s)	
9a		115.1s	4′-OH	4.75 (br s)	

Table 2 ^{1}H- and ^{13}C-NMR data for variecolorquinone B (**2**) in $CDCl_3$

No.	δ_H (*J* in Hz)	δ_C	HMBC (H–C)	^{1}H-^{1}H COSY
1		109.1s		
2		138.4s		
3	6.53 (d, 1.3)	125.4d	1, 2, 5	5
4		146.1s		
5	6.78 (br s)	117.7d	1, 3, 6, 4-Me	3
6		163.6s		
7	4.00 (2H, d, 1.8)	34.9 t	1,2, 3, 8, 9, 13	13
8		147.7s		
9		181.7s		
10		158.6s		
11	5.87 (d, 2.2)	107.1d	9, 10, 12, 13	13
12		187.4s		
13	5.99 (dt, 1.8, 2.2)	132.7d	7, 9, 11	7, 11
14		170.8s		
4-Me	2.32 (3H, s)	21.6q	3, 4, 5	
10-OMe	3.85 (3H, s)	56.4q	10	
14-OMe	3.76 (3H, s)	52.2q	14	
6-OH	11.29 (s)		1, 5, 6	

in 96-cell plates at a density of 2×10^5 cell/ml. Then 2.0 μl of the test compound solutions (in MeOH) at different concentrations was added to each well and the culture was further incubated for 24 hours. Following drug exposure, the cells were fixed with 12% TCA and the cell layer was stained with 0.4% SRB. The absorbance of SRB solution was measured at 515 nm. Dose response curves were generated and the IC_{50} values, the concentration of compound required to inhibit cell proliferation by 50%, were calculated from the linear portion of log dose response curves.

In DPPH scavenging assay, 160 μl of reaction mixtures containing test samples and 40 μM DPPH (Sigma) dissolved in methanol were plated in 96-cell plates and kept in dark for 30 minutes. After the reaction, absorbance was then measured at 517 nm, and percent inhibition was

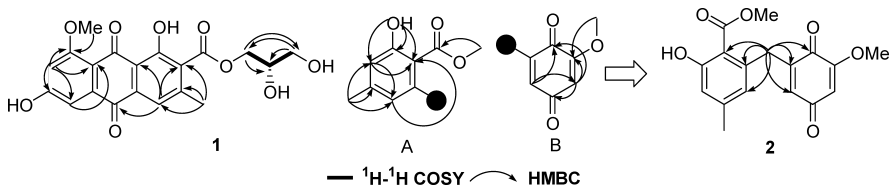

Fig. 2 The key HMBC and ^{1}H-^{1}H COSY correlations of **1** and **2**.

calculated. IC_{50} values denote the concentration of sample required to scavenge 50% of the DPPH free radicals.

Results and Discussion

Physico-chemical Properties

1: yellow amorphous powder; $[\alpha]_D^{25}$ −18 (*c* 0.03 MeOH); HRESI-MS *m/z* 401.0871 (calcd for $C_{20}H_{17}O_9$, 401.0873); UV λ_{max}^{MeOH} (log ε) nm 223 (4.1), 251 (3.8), 286 (3.9), 440 (3.5); IR ν_{max} cm^{-1} (KBr) 3425, 2941, 1718, 1672, 1635, 1598, 1574, 1436, 1343, 1245, 1203, 1146, 1114, 1072, 1047, 962, 843. ^{1}H (DMSO-d_6, 600 MHz) and ^{13}C (DMSO-d_6, 150 MHz) see Table 1.

2: yellow amorphous powder; HRESI-MS *m/z* 315.0880 (calcd for $C_{17}H_{15}O_6$, 315.0869); UV λ_{max}^{MeOH} (log ε) nm 197 (4.2), 242 (4.1), 297 (3.9), 352 (3.7); IR ν_{max} cm^{-1} (KBr) 3068, 1652, 1602, 1445, 1326, 1238, 1177. ^{1}H ($CDCl_3$, 600 MHz) and ^{13}C ($CDCl_3$, 150 MHz) see Table 2.

Structure Determination

1 was obtained as a yellow amorphous powder. Its molecular formula was determined as $C_{20}H_{18}O_9$ based on HRESI-MS at *m/z* 401.0871 $[M-H]^-$ (calcd 401.0873). The diagnostic IR peaks were observed for hydroxyl and carbonyl at 3425 and 1718 cm^{-1}, respectively. The UV spectra absorptions at λ_{max} (log ε) 223 (4.1), 251 (3.8), and 286 (3.9), 440 (3.5) suggested the presence of emodin skeleton in **1** [15]. Analysis of the 1D NMR spectra of **1** displayed three carbonyls, nine sp^2 quarternary carbons, three sp^2 methines, one oxygenated methine, two oxygenated methylenes, one methoxyl and one methyl (Table 1). Except for a methoxyl signal (δ_H 3.91 and δ_C 56.8) instead of hydroxyl signal (δ 12.72), the ^{1}H- and ^{13}C-NMR spectra of **1** were similar to those of **3** [14], suggesting their structures were very similar. Further comparison of the ^{13}C-NMR spectra of **1** with those of **3** revealed −2.0, −3.1, −1.3, −4.9, +3.9 and +2.0 ppm of chemical shift effects for C-5, C-7, C-8, C-9, C-8a and C-10a, respectively, showing that **1** is 8-*O*-methyl derivative of **3**. This conclusion was further confirmed by key ^{1}H with ^{13}C long range correlations of –OCH_3 with C-8, H-5 with C-7 and C-8a, H-7 with C-5 and C-8a. The absolute configuration of C-2′ was assigned as *S* by comparing $[\alpha]_D$ (−18°) with those of **3** ($[\alpha]_D$ −23°) [14]. Thus, the structure of **1** was elucidated as (2*S*)-2,3-dihydroxypropyl-1,6-dihydroxy-8-methoxy-3-methyl-9,10-dioxoanthracene-2-carboxylate.

2 was obtained as yellow amorphous powder. Its molecular formula was determined as $C_{17}H_{16}O_6$ based on HRESI-MS at *m/z* 315.0880 $[M-H]^-$ (calcd 315.0869). Its ^{1}H spectral data revealed the signals of a hydrogen-bonded hydroxyl (1-OH, δ_H 11.29), two pairs of meta-coupled aromatic protons (δ_H 6.53 and 6.78; 5.87 and 5.99), two methoxyls (δ_H 3.76 and 3.85), an sp^3 methyl (δ_H 2.32) and an sp^3 methylene (δ_H 4.00). The ^{13}C-NMR spectra of **2** contained three carbonyls, six sp^2 quaternary carbons including two oxygenated ones, four sp^2 methines, an sp^3 methylene, two methoxyls and a methyl (Table 1). According to 2D NMR spectrum, these protons and carbons were linked into two structural moieties, *i.e.* methyl 2-substituted-4-methyl-6-hydroxybenzoate and 2-substituted-6-methoxybenzoquinone. These two moieties were further connected together *via* a methylene which was confirmed by HMBC correlations between H-7 and C-1, C-2, C-3, C-8, C-9 and C-13, and between H-13 and C-7. These data are sufficient to assign the structure of **2** as methyl 2-hydroxy-6-[(5-methoxy-3,6-dioxocyclohexa-1,4-dienyl)methyl]-4-methylbenzoate (Fig. 2).

Cytotoxity and Radical Scavenging Activity

The cytotoxicities of new compounds **1** and **2** were assayed *in vitro* against the P388 and HL-60 cell lines by the MTT method [18], and BEL-7402 and A-549 cell lines by the SRB method [19]. The IC_{50} values of **1** and **2** against P388, HL-60, BEL-7402 and A-549 cells were 266, 309, 114, 3.0 and 3.7, 1.3, 29 and 56 μM, respectively. **1** selectively inhibited the proliferation of A-549 cell and **2** inhibited the proliferation of P388 and HL-60 cells. **1**, **2**, **12** and **13** were also evaluated for their radical scavenging activity against DPPH [20]. **1** showed moderate activity with IC_{50} values of 28 μM, while **2** was inactive (IC_{50} >100 μM). **12** and **13** which were strong KDR kinase inhibitors [17] exhibited strong antioxidative activity against DPPH with IC_{50} values

of 6.0 μM and 11 μM, respectively (ascorbic acid as positive control, IC_{50} 22 μM).

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