Anthraquinone Derivatives and an Orsellinic Acid Ester from the Marine Alga-Derived Endophytic Fungus *Eurotium cristatum* EN-220

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Cultivation of the fungal strain *Eurotium cristatum* EN-220, an endophyte obtained from the marine alga *Sargassum thunbergii*, resulted in the isolation of one new anthraquinone glycoside, 3-*O*-(α-D-ribofuranosyl)questinol (**1**) and one new orsellinic acid ester, cristatumside A (**2**), together with three known aromatic glycosides, 3-*O*-(α-D-ribofuranosyl)questin (**3**), isotorachrysone 6-*O*-α-D-ribofuranoside (**4**), and asperflavin ribofuranoside (**5**), as well as three anthraquinone derivatives, asperflavin (**6**), eurorubrin (**7**), and (+)-variecolorquinone A (**8**). The structures of these compounds were determined by extensive analysis of their spectroscopic data, as well as by their comparison with those in the literature. Each of the isolated compounds was evaluated for its antimicrobial activity and brine shrimp lethality.

Introduction. – Secondary metabolites obtained from marine-derived fungi which possess unique structures and interesting biological properties have attracted considerable attention in recent years. Among the investigated fungal strains, marine algae are the predominant source for fungal diversity, and a number of interesting natural products from alga-derived fungi have been described [1]. The fungal genus *Eurotium*, which is the teleomorph of *Aspergillus*, turned out to be a rich source of aromatic polyketides such as anthraquinone derivatives [2–4]. These compounds were reported to generally possess radical scavenging [3], antimicrobial [4], and cytotoxic activities [4][5]. During our ongoing search for biologically active compounds from marine-derived endophytic fungi [3][4][6–8], we have recently focused on one fungal strain *E. cristatum* EN-220, which was isolated from the marine alga *Sargassum thunbergii*. Investigation of the strain has resulted in the isolation and characterization of several indole alkaloids from the rice fermentation culture [6]. Further work on this fungus has now resulted in the identification of eight aromatic derivatives, **1**–**8**, including four anthraquinones, **1** and **6**–**8**, and two related glycosides, **3** and **5**, as well as two other aromatic compounds, **2** and **4**. The structures of these compounds were identified as one new anthraquinone glycoside, namely, 3-*O*-(α-D-ribofuranosyl)questinol (**1**) and one new orsellinic acid ester, namely, cristatumside A (**2**). The other compounds were identified as 3-*O*-(α-D-ribofuranosyl)questin (**3**) [3], isotorachrysone 6-*O*-α-D-ribofuranoside (**4**) [5], asperflavin ribofuranoside (**5**) [9], asperflavin (**6**) [3], eurorubrin (**7**) [3], and (+)-variecolorquinone A (**8**) [5]. All these compounds were evaluated for their antimicrobial activities and brine shrimp lethalities. Herein, we describe the isolation, structure elucidation, and biological activity of the isolated compounds.

Results and Discussion. – 1. *Isolation and Structure Elucidation*. The AcOEt extract from the rice culture of the fungal strain *E. cristatum* EN-220 was fractionated by silica-gel vacuum liquid chromatography (VLC) to yield twelve fractions. *Frs. 7* and *10* were further purified by a combination of silica-gel, *Sephadex LH-20*, and *Lobar LiChroprep RP-18* column chromatography, as well as by preparative HPLC to yield compounds **1**–**8** (*Fig. 1*).

Compound **1** was obtained as red amorphous powder. Its molecular formula, $C_{21}H_{20}O_{10}$, was deduced from the HR-ESI-MS (*m/z* 455.0959 ($[M + Na]^+$)). The ^{1}H-NMR data revealed the presence of four aromatic H-atoms, one O-bearing CH_2, one MeO, and one phenolic OH group along with a sugar residue (*Table*). In the ^{13}C-NMR and DEPT spectra, signals of 21 C-atoms including those of one MeO, two CH_2, and eight CH groups (four aromatic and four O-bearing), as well as two C=O groups and eight aromatic quaternary C-atoms were observed (*Table*). Detailed comparison of the ^{1}H- and ^{13}C-NMR data of **1** (*Table*) with those of 3-*O*-(α-D-ribofuranosyl)questin (**3**) [3] revealed that their structures were very similar, except for the Me group with a signal at δ(C) 21.9 (C(11)) in **3** being replaced by the CH_2O group (δ(C) 62.0 (C(11))) in **1**. Accordingly, the Me signal at δ(H) 2.42 (*s*, Me(11)) in **3** was replaced by CH_2O signal at δ(H) 4.58 (*d*, $J = 5.6$, CH_2(11)) of **1**. This observation

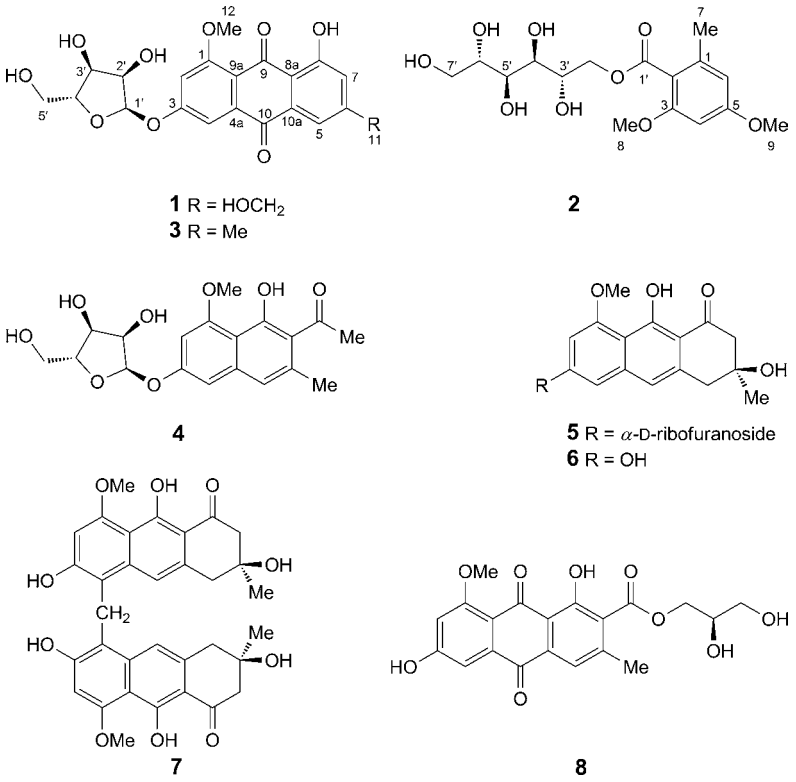

Fig. 1. *Chemical structures of compounds* **1**–**8**

Table. *^1H- and ^{13}C-NMR Data* (500 and 125 MHz, resp., (D_6)DMSO) *of* **1** *and* **2**. δ in ppm, J in Hz. Arbitrary atom numberings as indicated in *Fig. 1*.

Position	**1**		Position	**2**	
	δ(H)	δ(C)		δ(H)	δ(C)
1		162.7 (*s*)	1		137.2 (*s*)
2	7.06 (*d*, *J* = 1.8)	106.6 (*d*)	2		116.6 (*s*)
3		162.9 (*s*)	3		157.5 (*s*)
4	7.44 (*d*, *J* = 1.8)	106.7 (*d*)	4	6.45 (*d*, *J* = 2.0)	96.1 (*d*)
4a		132.0 (*s*)	5		160.8 (*s*)
5	7.57 (*s*)	115.9 (*d*)	6	6.42 (*d*, *J* = 2.0)	106.8 (*d*)
6		151.5 (*s*)	7	2.21 (*s*)	19.3 (*q*)
7	7.20 (*s*)	120.9 (*d*)	8	3.74 (*s*)	55.8 (*q*)
8		161.6 (*s*)	9	3.77 (*s*)	55.2 (*q*)
8a		115.0 (*s*)	1′		167.4 (*s*)
9		186.5 (*s*)	2′	4.48 (*dd*, *J* = 11.2, 2.2),	67.5 (*t*)
9a		114.5 (*s*)		4.12 (*dd*, *J* = 11.2, 6.8)	
10		182.0 (*s*)	3′	3.70 – 3.72 (*m*)	68.5 (*d*)
10a		136.5 (*s*)	4′	3.58 – 4.00 (*m*)	69.3 (*d*)
11	4.58 (*d*, *J* = 5.6)	62.0 (*t*)	5′	3.55 – 3.58 (*m*)	69.5 (*d*)
12	3.93 (*s*)	56.5 (*q*)	6′	3.44 – 3.49 (*m*)	71.2 (*d*)
1′	5.87 (*d*, *J* = 4.5)	100.2 (*d*)	7′	3.61 – 3.64 (*m*),	63.8 (*t*)
2′	4.15 – 4.19 (*m*)	71.6 (*d*)		3.37 – 3.42 (*m*)	
3′	3.97 – 4.00 (*m*)	69.2 (*d*)	3′-OH	4.80 (*d*, *J* = 6.1)	
4′	4.02 – 4.06 (*m*)	87.2 (*d*)	4′-OH	4.30 (*d*, *J* = 7.6)	
5′	3.48 – 3.54 (*m*)	61.4 (*t*)	5′-OH	4.19 (*d*, *J* = 7.6)	
8-OH	13.09 (*s*)		6′-OH	4.43 (*d*, *J* = 5.5)	
11-OH	5.52 (*t*, *J* = 5.6)		7′-OH	4.35 (*t*, *J* = 5.6)	
2′-OH	4.86 (*d*, *J* = 9.2)				
3′-OH	5.01 (*d*, *J* = 5.2)				
5′-OH	4.89 (*t*, *J* = 5.7)				

suggested that the Me group at C(11) of **3** was replaced by a CH_2(11)OH in **1**, and this deduction was strongly supported by the HMBC from CH_2(11) to C(5), C(6), and C(7), as well as by the ^{1}H,^{1}H-COSY cross-peak O*H*–*C*(11)/CH_2(11) (*Fig. 2*).

The ^{1}H- and ^{13}C-NMR data of the sugar residue in **1** (*Table*) were very similar to those of compounds **3**, **4**, and **5**, indicating that they had the same ribose residue [3][5][9]. The sugar moiety was determined as α-D-ribofuranose by comparison of the J(1′,2′) value (4.5 Hz) with those of the methyl α-D-ribofuranoside (J(1,2) = 4.3 Hz) and methyl β-D-ribofuranoside (J(1,2) = 1.2 Hz) [5]. The presence of D-ribose in **1** was further confirmed by acid hydrolysis, followed by the measurement of the optical rotation ($[\alpha]_D^{20} = -17.6$ ($c = 0.07$, H_2O)) as compared with literature report [5]. Based on the above spectral evidence, the structure of **1** was determined and it was named as 3-*O*-(α-D-ribofuranosyl)questinol.

Compound **2**, a colorless amorphous powder, had the molecular formula $C_{16}H_{24}O_9$ on the basis of its HR-ESI-MS data. The ^{13}C-NMR (DEPT) data (*Table*) revealed the presence of 16 C-atoms including two MeO, one Me, two CH_2O, and six CH (two aromatic and four O-bearing) groups, as well as one C=O group and four aromatic quaternary C-atoms. The ^{1}H-NMR spectral data exhibited signals for two *meta*-coupled

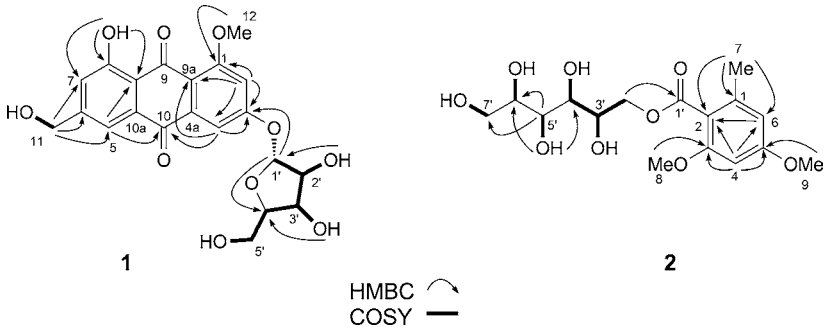

Fig. 2. *Key HMBCs and COSY correlations of compounds* **1** *and* **2**

aromatic H-atoms, two MeO, and one Me groups along with a C_6 monosaccharide residue (*Table*). The HMBCs from Me(7) to C(1), C(2), and C(6), from Me(8) to C(3), and from Me(9) to C(5) evidenced the location of the Me group at C(1), and two MeO groups at C(3) and C(5). In addition, the observed HMBCs from H–C(4) to C(2), C(3), C(5), and C(6), and from H–C(6) to C(2) unambiguously established the substitution mode of the benzene ring. Based on the above evidences, the orsellinic acid moiety was assigned. The key HMBC from $CH_2(2')$ to the C=O C(1′) established the connection of the monosaccharide and the orsellinic acid units [10]. The monosaccharide residue was further confirmed as mannitol by acid hydrolysis, followed by the GC analysis of the Ac derivative [11][12]. Thus, the structure of **2** was determined and named cristatumside A.

2. *Antimicrobial Activity and Brine Shrimp Lethality.* All of the isolated compounds were evaluated for their antibacterial activities against *Staphyloccocus aureus* and *Escherichia coli*, as well as antifungal activities against *Physalospora obtuse*, *Alternaria brassicae*, *Valsa mali*, *Alternaria solania*, and *Sclerotinia miyabeana*. Compounds **3** and **7** showed inhibitory activities against *E. coli* with the *MIC* values of 32 and 64 μg/ml, compared to chloramphenicol with the *MIC* value of 4 μg/ml. This is the first report on the antibacterial activities of compounds **3** and **7**. The antibacterial activity of **3** was probably due to the Me group at C(11) of anthraquinone nucleus, compared with the CH_2O group of **1**. All other compounds had no antibacterial or antifungal activity. As for the lethality against brine shrimp (*Artemia salina*), compound **7** exhibited moderate activity with the lethal rate 41.4% at a concentration of 10 μg/ml, while other compounds were not active.

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Experimental Part

General. Column chromatography (CC): commercial silica gel (SiO_2, 100–200 and 200–300 mesh; *Qingdao Haiyang Chemical Group Co.*), *Lobar LiChroprep RP-18* (40–63 μm; *Merck*), and *Sephadex LH-20* (*Pharmacia*). TLC: Precoated SiO_2 *GF-254* plates (*Qingdao Haiyang Chemical Group Co.*). Semi-prep. HPLC: *Dionex* HPLC system equipped with a *P680* pump, an *ASI-100* automated sample

injector, and a *UVD-340U* multiple-wavelength detector; *ODS* column (*Sinochrom ODS-BP*, 10 × 300 mm, 10 μm) with UV detection. Gas chromatography (GC): *Shimadzu GC-2014C* system equipped with a FID detector; cap. column (*DB-1701*, 0.25 mm × 30 m, 0.25 μm; *Agilent Technologies Co.*). Optical rotations: *AA-55* digital polarimeter (*Optical Activity Ltd.*). UV Spectra: *Gold Spectrumlab-54* UV/VIS spectrophotometer (*Shanghai Lengguang Tech. Co.*); λ_{max} (log ε); in nm. NMR Spectra: *Bruker Avance 500* spectrometer (500 (^{1}H) and 125 MHz (^{13}C)); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. Low- and high-resolution (LR and HR, resp.) ESI-MS: *VG Autospec 3000* mass spectrometer; in *m*/*z*.

Fungal Strain. The endophytic fungus *Eurotium cristatum* EN-220 was isolated from the marine alga *Sargassum thunbergii* collected from the coast of Qingdao, P. R. China, in November 2009. The fungus was identified by analysis of its ITS region of the rDNA, as described in [13]. The sequence data derived from the fungal strain was deposited with *GenBank*, under accession No. JQ743649. A BLAST search result showed that the sequence was the most similar (99%) to the sequence of *Eurotium cristatum* (compared to accession No. GU784865). The strain is preserved at the Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. For chemical investigation, the fungal strain was statically fermented at r.t. for 30 d on sterilized solid medium containing rice (100 g/flask), peptone (0.6 g/flask), and sea water (100 ml/flask) in 1000-ml *Fernbach* flasks (× 100). The rice culture of the fungal strain was exhaustively extracted with AcOEt to give a crude extract, which was dried and fractionated by SiO_2 vacuum liquid chromatography (VLC) with different solvents of increasing polarity from petroleum ether (PE) to MeOH to yield twelve fractions, *Frs. 1–12* based on TLC analysis. *Fr. 7* was subjected to CC (SiO_2; $CHCl_3$/MeOH from 100 : 1 to 10 : 1; then *Sephadex LH-20*; MeOH), and finally prep. HPLC (55% MeOH/H_2O) to yield compounds **5** (t_R 16.41 min; 15.8 mg) and **7** (t_R 24.01 min; 20.7 mg). *Fr. 10* was subjected to CC (SiO_2; $CHCl_3$/MeOH from 15 : 1 – 5 : 1; then *Sephadex LH-20*; MeOH) to yield two subfractions, *Frs. 10-1* and *10-2*. *Fr. 10-1* was purified by prep. HPLC (45% MeOH/H_2O; 3 ml/min) to yield compounds **2** (t_R 18.24 min; 8.7 mg), **6** (t_R 20.35 min; 20.9 mg), and **1** (t_R 23.21 min; 12.7 mg). Further purification of *Fr. 10-2* by prep. HPLC (55% MeOH/H_2O; 3 ml/min) yielded compounds **4** (t_R 14.87 min; 7.0 mg), **8** (t_R 19.34 min; 3.5 mg), and **3** (t_R 24.17 min; 4.7 mg).

*3-*O*-(α-*D*-Ribofuranosyl)questinol* (*= 9,10-Dihydro-5-hydroxy-7-(hydroxymethyl)-4-methoxy-9,10-dioxoanthracen-2-yl α-*D*-Ribofuranoside*; **1**). Red amorphous powder. $[\alpha]_D^{20} = +156.3$ ($c = 0.32$, MeOH). UV (MeOH): 223 (4.54), 266 (4.28), 419 (3.98). ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 455 ([*M* + Na]$^+$). HR-ESI-MS: 455.0959 ([*M* + Na]$^+$, $C_{21}H_{20}NaO_{10}^+$; calc. 455.0954).

Acidic Hydrolysis of **1**. A soln. of **1** (10.2 mg) was reacted in 6M HCl (5 ml) for 10 h at 100°, and then the mixture was extracted with AcOEt repeatedly to remove the aglycone. The H_2O layer was then concentrated to furnish the sugar residue (2.1 mg), which was determined by comparing its specific rotation ($[\alpha]_D^{20} = -17.6$, $c = 0.07$, H_2O) with that reported for D-ribose ($[\alpha]_D^{20} = -18.5$, $c = 0.085$, H_2O) [5].

Cristatumside A (*= 1-*O*-(2,4-Dimethoxy-6-methylbenzoyl)-*L*-mannitol*; **2**). Colorless amorphous powder. $[\alpha]_D^{20} = +2.4$ ($c = 0.42$, MeOH). UV (MeOH): 203 (4.26), 248 (3.58), 281 (3.36). ^{1}H- and ^{13}C-NMR: see the *Table*. ESI-MS: 383 ([*M* + Na]$^+$). HR-ESI-MS: 383.1322 ([*M* + Na]$^+$, $C_{16}H_{24}NaO_9^+$; calc. 383.1313).

Monosaccharide Analysis of **2**. Compound **2** (1.9 mg) was treated with 6M HCl at 100° for 10 h to give an acid hydrolysate, which was dried and then reacted with the mixture of 1 ml of Ac_2O and 1 ml of pyridine for 12 h at 110°. Subsequently, the reaction product was determined by GC (inj. temp., 310°; determination temp., 310°; oven program: started with 3 min at 200°, then increased with 1°/min to 220°, and finally maintained for 5 min). Two authentic monosaccharides including mannitol and glucitol were treated in the same manner. When compared with the standards, the monosaccharide in **2** was confirmed as mannitol with t_R of 23.34 min. Under the above conditions, the t_R values of the authentic mannitol and glucitol were 23.34 and 25.23 min, resp.

Antimicrobial Activity and Brine Shrimp Lethality. The antibacterial assay against *E. coli* and *S. aureus*, and antifungal assay against *A. brassicae*, *V. mali*, *P. obtuse*, *A. solani*, and *S. miyabeana* were carried out as described in [14]. Chloramphenicol was used as positive control of antibacterial assay, and amphotericin B was used for antifungal assay. The brine shrimp lethality was carried out as described in [15].

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