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Natural Anthraquinone Derivatives from a Marine Mangrove Plant-Derived Endophytic Fungus *Eurotium rubrum*: Structural Elucidation and DPPH Radical Scavenging Activity

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There is considerable interest in the isolation of potent radical scavenging compounds from natural resources to treat diseases involving oxidative stress. In this report, four new fungal metabolites including one new bisdihydroanthracenone derivative (1, eurorubrin), two new *seco*-anthraquinone derivatives [3, 2-*O*-methyl-9-dehydroxyeurotinone and 4, 2-*O*-methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone], and one new anthraquinone glycoside [6, 3-*O*-(α-D-ribofuranosyl)-questin], were isolated and identified from *Eurotium rubrum*, an endophytic fungal strain that was isolated from the inner tissue of the stem of the marine mangrove plant *Hibiscus tiliaceus*. In addition, three known compounds including asperflavin (2), 2-*O*-methyleurotinone (5), and questin (7) were also isolated and identified. Their structures were elucidated on the basis of spectroscopic analysis. All of the isolated compounds were evaluated for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

Keywords: Mangrove plant, *Hibiscus tiliaceus*, endophytic fungus, *Eurotium rubrum*, anthraquinone, radical scavenging activity

Oxidative stress contributes a lot to free radical-mediated diseases such as aging, atherosclerosis, cancer, ischemic heart disease, and neurodegenerative diseases [2, 5, 6, 11, 18]. Since the last three decades, there is increasing interest in the isolation of potent radical scavenging compounds from natural resources to treat diseases involving oxidative stress [5, 6, 18].

Anthraquinone and its deoxidized derivatives are important naturally occurring pigments that are commonly distributed in higher plants, lichens, and fungi. These compounds exhibit a range of biological activities including cytotoxic, antifungal and antiviral activities. They also served as laxatives, diuretics, and phytoestrogens [15, 17, 19, 26, 27], or are used in the industry as textile dyes, food colorants, and bug repellents [3, 16]. Many anthraquinones have been characterized from fungi [8–10].

As part of our ongoing investigation directed toward the discovery of structurally new and biologically active natural products from marine endophytic fungi [20, 22–25], we studied the chemical constituents of an endophytic fungus, *Eurotium rubrum*, which was isolated from the inner tissue of the marine mangrove plant *Hibiscus tiliaceus* that was collected from Hainan Island, China, and resulted in the isolation and structural elucidation of one new bisdihydroanthracenone derivative (**1**, eurorubrin), two new *seco*-anthraquinone derivatives [**3**, 2-*O*-methyl-9-dehydroxyeurotinone and **4**, 2-*O*-methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone], and one new anthraquinone glycoside [**6**, 3-*O*-(α-D-ribofuranosyl]-questin]. In addition, three known compounds, asperflavin (**2**) [7], 2-*O*-methyleurotinone (**5**) [C. Eder *et al.* 2004. U.S. Patent 6818667], and questin (**7**) [7], were also identified (Fig.1). All of these compounds were evaluated for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity.

Materials and Methods

General Experimental Procedures

Optical rotations were measured on an ATAGO POLAX-L polarimeter. UV spectra were determined on a Spectrumlab 54 UV–visible spectrophotometer. IR spectra were recorded on a Nicolet NEXUS 470 FT–IR spectrophotometer. 1D and 2D NMR spectra were obtained at 500 and 125 MHz for ^{1}H and ^{13}C, respectively, on a Bruker Avance 500 MHz NMR spectrometer in $CDCl_3$ with TMS as an internal

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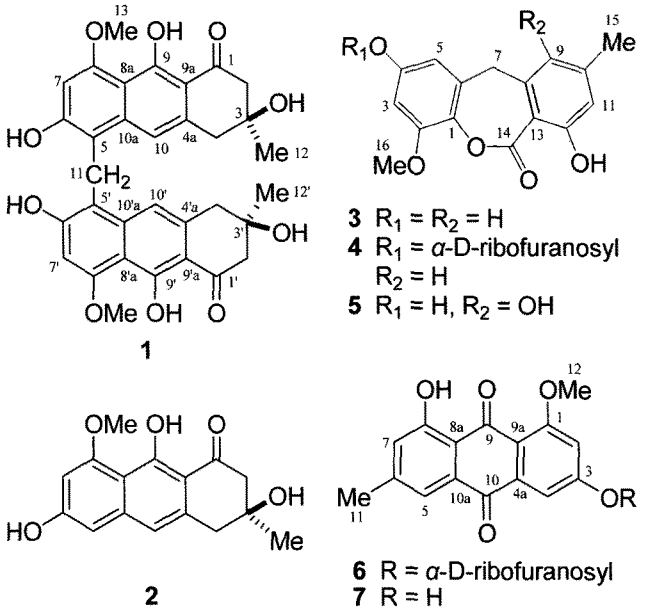


Fig. 1. Structures of compounds **1**–**7**.

standard. Mass spectra were recorded using a VG Autospec 3000 mass spectrometer. Column chromatography (CC) was performed with Si gel (200–300 mesh, Qingdao Haiyang Chemical Co., Qingdao, China) and Sephadex LH-20 (Sigma). TLC was carried out with precoated Si gel plates (GF-254, Qingdao Haiyang Chemical Co., Qingdao, China).

Material

The mangrove plant *Hibiscus tiliaceus* was collected in August 2004 from Hainan Island, China, and was identified by Prof. H. Peng at the Kunming Institute of Botany of the Chinese Academy of Sciences. The endophytic fungus was isolated from the inner tissue of stems of *H. tiliaceus* by using the procedures in our previous report [20]. The strain is preserved at the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences under accession number QEN-0407-G2.

The fungus was identified as *Eurotium rubrum* using a molecular biological protocol by DNA amplification and sequencing of the ITS region as described previously [20]. The sequence data derived from the fungal strain have been submitted and deposited at GenBank under accession number EU001331. The BLAST search result showed that the sequence was the most similar (99%) to the sequence of *E. rubrum* (compared with gb AY373891.1).

Extraction and Purification

For chemical investigations, the fungal strain was static cultivated in potato dextrose broth (PDB) containing 50% (v/v) sea water (glucose

Table 1. ^1H, ^{13}C NMR data of compounds **1** and **6**.

Position	**1**[a]		**6**	
	δ_C (DEPT)	δ_H (mult, *J* in Hz)	δ_C (DEPT)	δ_H (mult, *J* in Hz)
1	203.8 (s)		164.1 (s)	
2	52.7 (t)	2.68 (d, 16.9); 2.76 (d, 16.9)	107.4 (d)	7.08 (d, 2.6)
3	70.7 (s)		164.1 (s)	
4	44.6 (t)	2.94 (d, 5.2)	107.9 (d)	7.49 (d, 2.6)
4a	138.1 (s)		133.4 (s)	
5	111.1 (s)		120.1 (d)	7.43 (br. s)
6	157.8 (s)		147.8 (s)	
7	98.4 (d)	6.64 (s)	125.0 (d)	7.04 (br. s)
8	161.1 (s)		163.5 (s)	
8-OH				13.15 (s)
8a	112.9 (s)		116.2 (s)	
9	166.8 (s)		187.9 (s)	
9-OH		14.78 (s)		
9a	110.0 (s)		115.7 (s)	
10	114.8 (d)	7.46 (s)	183.0 (s)	
10a	141.7 (s)		138.1 (s)	
11	21.1 (t)	4.49 (s)	21.9 (q)	2.42 (s)
12	29.3 (q)	1.31 (s)	57.0 (q)	3.97 (s)
13	56.2 (q)	3.85 (s)		
Sugar moiety				
1'			101.7 (d)	5.89 (d, 4.4)
2'			73.4 (d)	4.36 (m)
3'			71.1 (d)	4.21 (m)
4'			88.7 (d)	4.24 (m)
5'			63.1 (t)	3.71 (m)

Measured in acetone-d_6. [a]Data for monomer.

10 g/l, mannitol 20 g/l, peptone 5 g/l, yeast extract 3 g/l, and monosodium glutamate 3 g/l, pH 6.0) for 30 days at room temperature. Mycelia and culture broth of *E. rubrum* (30 l) were homogenized using a Waring blender and exhaustively extracted with MeOH and EtOAc, respectively. Since the TLC and HPLC profiles of the two extracts were nearly identical, they were combined before further separation. The combined extract (70 g) was subjected to a column chromatography (CC) over silica gel and eluted with different solvents, by increasing polarity, to yield 14 fractions (Fr.1–Fr.14). Fr.10 was further fractionated by CC on silica gel (petroleum ether–acetone, 5:1) and reversed-phase silica gel C_{18} (MeOH) to yield compounds **3** (20.5 mg) and **7** (50.1 mg). Fr.11 was subjected to CC over silica gel (petroleum ether–EtOAc, 2:1) and Sephadex LH-20 ($CHCl_3$–MeOH, 2:1) to give compound **5** (18.7 mg). Fr. 13 was subjected to CC on silica gel ($CHCl_3$–MeOH, 25:1) and Sephadex LH-20 ($CHCl_3$–MeOH, 1:1) to afford compound **2** (57.2 mg). Fr.14 was subjected to CC over silica gel ($CHCl_3$–MeOH, 20:1) and Sephadex LH-20 (MeOH) to obtain compounds **1** (9.5 mg), **4** (13.4 mg), and **6** (20.8 mg).

Eurorubrin (1). Brown amorphous powder; $[\alpha]_D^{25}$+21.1° (*c* 0.3, MeOH); UV (MeOH) λ_{max} (log ε) 396 (3.50), 273 (3.95), 225 (3.91) nm; IR (KBr) ν_{max} 3,375, 2,954, 2,922, 2,852, 1,622, 1,593, 1,545, 1,459, 1,373, 1,339, 1,219, 1,088 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESIMS (positive) *m/z* 589 $[M+H]^+$ (100), 413 (20), 339 (30), 301 (40); HRESIMS (positive) *m/z* 589.2057 $[M+H]^+$ (calcd for $C_{33}H_{33}O_{10}^+$, 589.2074).

2-*O*-Methyl-9-dehydroxyeurotinone (3). Colorless amorphous powder; UV (MeOH) λ_{max} (log ε) 312 (2.91), 256 (3.17), 216 (3.79), 203 (3.90) nm; IR (KBr) ν_{max} 3,390, 2,956, 2,921, 2,851, 1,670, 1,655, 1,621, 1,561, 1,460, 1,439, 1,219, 1,196 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; EIMS *m/z* 286 $[M]^+$ (100), 271 (25), 255 (11), 254 (32), 243 (41), 201 (30); HRESIMS (positive) *m/z* 309.0738 $[M+Na]^+$ (calcd for $C_{16}H_{14}O_5Na^+$, 309.0747).

2-*O*-Methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone (4). Colorless amorphous powder; $[\alpha]_D^{25}$+89.1° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 282 (3.43), 206 (4.33) nm; IR (KBr) ν_{max} 3,394, 2,956, 2,926, 2,854, 1,703, 1,653, 1,607, 1,545, 1,462, 1,220, 1,089, 1,038 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; LRESIMS (negative) *m/z* 417 $[M-H]^-$ (100), 327 (15); HRESIMS (negative) *m/z* 417.1197 $[M-H]^-$ (calcd for $C_{21}H_{21}O_9^-$, 417.1186).

3-*O*-(α-D-Ribofuranosyl)-questin (6). Orange amorphous powder; $[\alpha]_D^{25}$+89.4° (*c* 0.02, MeOH); UV (MeOH) λ_{max} (log ε) 421 (3.39), 268 (3.66), 225 (3.91) nm; IR (KBr) ν_{max} 3,330, 2,954, 2,921, 2,851, 1,628, 1,593, 1,545, 1,461, 1,376, 1,219 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; LRESIMS (negative) *m/z* 415 $[M-H]^-$ (10), 283 (100); HRESIMS (negative) *m/z* 415.1043 $[M-H]^-$ (calcd for $C_{21}H_{19}O_9^-$, 415.1029).

RESULTS AND DISCUSSION

Structure Determination

Compound **1** was obtained as brown amorphous powder. The IR spectrum displayed strong absorption peaks for

Table 2. ^1H, ^{13}C NMR data of compounds **3** and **4**.

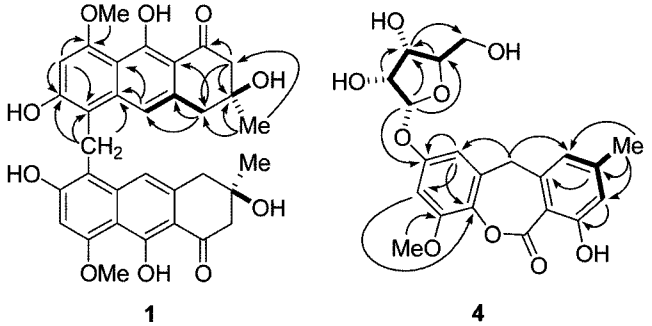
Position	3		4	
	δ_C (DEPT)	δ_H (mult, *J* in Hz)	δ_C (DEPT)	δ_H (mult, *J* in Hz)
1	131.7 (s)		133.6 (s)	
2	150.4 (s)		152.1 (s)	
3	98.7 (d)	6.31 (d, 2.6)	100.1 (d)	6.40 (s)
4	155.2 (s)		156.4 (s)	
4-OH		9.49 (s)		
5	105.4 (d)	6.28 (d, 2.6)	106.5 (d)	6.40 (s)
6	135.1 (s)		136.4 (s)	
7	36.3 (t)	3.72 (s)	37.5 (t)	3.89 (s)
8	144.0 (s)		145.4 (s)	
9	118.7 (d)	6.63 (br. s)	122.3 (d)	6.87 (s)
10	144.2 (s)		145.7 (s)	
11	115.7 (d)	6.63 (br. s)	116.8 (d)	7.01 (s)
12	159.0 (s)		158.3 (s)	
12-OH		10.01 (s)		
13	111.0 (s)		116.8 (s)	
14	164.4 (s)		164.9 (s)	
15	21.0 (q)	2.20 (s)	21.7 (q)	2.32 (s)
16	55.6 (q)	3.72 (s)	56.4 (q)	3.76 (s)
Sugar moiety				
1'			103.0 (d)	5.65 (br. s)
2'			73.8 (d)	4.20 (m)
3'			71.8 (d)	4.04 (m)
4'			88.8 (d)	4.20 (m)
5'			63.2 (t)	3.66 (m)

Measured in DMSO-d_6 for **3** and acetone-d_6 for **4**.

hydroxyl (3,375 cm^{-1}), carbonyl carbon (1,622 cm^{-1}), and aromatic (1,593 and 1,545 cm^{-1}) functional groups. LRESIMS (positive) exhibited a quasimolecular ion-peak at *m/z* 589 $[M+H]^+$ as a base peak. The molecular formula was determined to be $C_{33}H_{32}O_{10}$ by HRESIMS (positive, *m/z* 589.2057, $[M+H]^+$, calcd for $C_{33}H_{33}O_{10}^+$, 589.2074). The ^1H NMR spectrum (Table 1) showed signals for two tertiary methyls at δ 1.31 (6H, s, H-12, and H-12'); five methylenes at δ 2.68 (2H, d, *J*=16.9 Hz, H-2a, and H-2'a), 2.76 (2H, d, *J*=16.9 Hz, H-2b, and H-2'b), 2.94 (4H, d, *J*=5.2 Hz, H-4, and H-4'), and 4.49 (2H, s, H-11); two methoxyl groups at δ 3.85 (6H, s, H-13, and H-13'); four aromatic protons at δ 6.64 (2H, s, H-7, and H-7') and 7.46 (2H, s, H-10, and H-10'); and two phenolic hydroxyl protons at δ 14.78 (2H, s, OH-9, and OH-9'). The ^{13}C NMR spectrum (Table 1) exhibited 17 carbon signals attributable to 2 methyls (with one methoxyl group), 3 methylenes, 2 methines, and 10 quaternary carbon atoms according to the DEPT experiments. Analysis of the above MS and NMR data supported the fact that **1** is a symmetrical dimeric compound composed of two molecules of asperflavin (**2**) through a methylene group. Asperflavin (**2**) is an immunomodulatory constituent previously isolated from *Microascus tardifaciens* (Ascomycete) [7]. In the ^{13}C NMR spectrum, the methine carbon signal at δ 103.5 for C-5 in **2** was replaced by a downfield quaternary carbon signal resonating at δ 111.1 for C-5 and C-5' in **1**. This observation was strongly supported by the fact that the aromatic methine proton signal appearing at δ 6.51 for H-5 in **2** was absent in the ^1H NMR spectrum of **1**. Furthermore, additional methylene signals at δ 4.49 (2H, s, H-11) and δ 21.1 (CH_2, C-11) were present in the ^1H and ^{13}C NMR spectra of **1**, respectively. The observed correlations from H-11 to C-5, C-6, and C-10a in the HMBC spectrum confirmed the above deduction (Fig. 2). The appearance of optical rotation of **1** ($[\alpha]_D^{25}$+21.1°) was similar to that of **2** [7, 14], suggesting the same stereochemistry at C-3 for **1** and **2**. On the basis of the above data, the structure of **1** was established as 5,5'-methylenebisasperflavin, which was named as eurorubrin.

Compound **3** was obtained as colorless amorphous powder. The IR spectrum displayed strong absorptions for hydroxyl (3,390 cm^{-1}), carbonyl carbon (1,670 cm^{-1}), and aromatic (1,655, 1,621, and 1,561 cm^{-1}) functional groups. The EIMS exhibited a molecular ion peak at *m/z* 286 $[M]^+$ as a base peak. The molecular formula was determined to be $C_{16}H_{14}O_5$ on the basis of positive HRESIMS (*m/z* 309.0738, $[M+Na]^+$, calcd for $C_{16}H_{14}O_5Na^+$, 309.0747). The ^1H NMR spectrum (Table 2) showed signals due to one tertiary methyl at δ 2.20 (3H, s, H-15); one methoxyl and one methylene at δ 3.72 (5H, s, H-7, and H-16); four aromatic protons at δ 6.28 (1H, d, *J*=2.6 Hz, H-5), 6.31 (1H, d, *J*=2.6 Hz, H-3), and 6.63 (2H, br.s, H-9, and H-11); and two phenolic hydroxyl protons at δ 9.49 (1H, s, OH-4) and 10.01 (1H, s, OH-12). The ^{13}C NMR spectrum exhibited 16 carbon signals attributable to 2 methyls (with 1 methoxyl), 1 methylene, 4 methines, and 9 quaternary carbon atoms according to the DEPT experiments. Detailed comparison of the ^{13}C NMR spectral data (Table 2) of **3** with that of 2-*O*-methyleurotinone (**5**), a metabolite of *Eurotium echinulatum* Delacroix (DSM 13872) [C. Eder *et al.* 2004. U.S. Patent 6818667], revealed that **3** was a 9-dehydroxyl derivative of **5**. This was indicated by the fact that the quaternary aromatic carbon signal resonating in the lower field at δ 142.4 for C-9 in **5** was replaced by an aromatic methine signal appearing at higher field at δ 118.7 for C-9 in **3**. The additional methine proton signal observed at δ 6.63 for H-9 in the ^1H NMR spectrum of **3** confirmed this deduction. Based on the above spectral evidence, the chemical structure of compound **3** was determined to be 2-*O*-methyl-9-dehydroxyeurotinone.

Compound **4** was obtained as colorless amorphous powder. The IR spectrum displayed strong absorptions for hydroxyl (3,394 cm^{-1}), carbonyl carbon (1,703 cm^{-1}), and aromatic (1,653, 1,607, and 1,545 cm^{-1}) functional groups. LRESIMS (negative) exhibited a quasimolecular ion peak at *m/z* 417 $[M-H]^-$ as a base peak. The molecular formula was determined as $C_{21}H_{22}O_9$ on the basis of negative HRESIMS (*m/z* 417.1197, $[M-H]^-$, calcd for $C_{21}H_{21}O_9^-$, 417.1186). The ^1H and ^{13}C NMR spectral data (Table 2) of **4** revealed the presence of similar proton and carbon signals to those of **3**. In addition, one oxymethylene proton signal at δ 3.66 (2H, m, H-5') and four oxymethine signals at δ 5.65 (1H, br. s, H-1'), 4.20 (2H, m, H-2' and H-4'), and 4.04 (1H, m, H-3') were also observed in the ^1H NMR spectrum of **4**. In accordance, five additional carbon signals including one oxymethylene carbon resonating at δ 63.2 (C-5') and four oxymethine carbons resonating at δ 103.0 (C-1'), 88.8 (C-4'), 73.8 (C-2'), and 71.8 (C-3') were also observed in the ^{13}C NMR spectrum of **4**. These data suggested the presence of a sugar moiety in **4**. Detailed comparison of the ^1H and ^{13}C NMR spectral data with that of asperflavin ribofuranoside, an anthraquinone glycoside previously isolated from a marine isolate of the fungus *Microsporum* sp. [14], revealed the sugar moiety in **4** should be ribofuranosyl. The chemical shift values observed for the sugar moiety in **4** were very similar to that of asperflavin ribofuranoside, suggesting the same stereochemistry for the



1 4

Fig. 2. Key HMBC (arrow) and ^1H-^1H COSY (bold line) correlations of **1** and **4**.

sugar moiety. The observed HMBC correlation from H-1' (δ 5.65) to C-4 (δ 156.4) indicated that the sugar moiety was attached to C-4. From the above evidence, **4** was deduced as 2-*O*-methyl-4-*O*-(α-D-ribofuranosyl)-9-dehydroxyeurotinone.

Compound **6** was obtained as orange amorphous powder. The IR spectrum displayed strong absorptions for hydroxyl groups (3,330 cm^{-1}), carbonyl carbon (1,628 cm^{-1}), and aromatic ring (1,593 and 1,545 cm^{-1}). Negative LRESIMS exhibited a quasimolecular ion peak at *m/z* 415 $[M-H]^-$. The molecular formula was determined to be $C_{21}H_{20}O_9$ on the basis of HRESIMS (negative, *m/z* 415.1043, $[M-H]^-$, calcd for $C_{21}H_{19}O_9^-$, 415.1029). The ^1H NMR spectrum (Table 1) revealed the presence of signals due to one tertiary methyl at δ 2.42 (3H, s, H-11); one methoxyl at δ 3.97 (3H, s, H-12); one oxymethylene at δ 3.71 (2H, m, H-5'); four oxymethines at δ 4.21 (1H, m, H-3'), 4.24 (1H, m, H-4'), 4.36 (1H, m, H-2'), and 5.89 (1H, d, *J*=4.4 Hz, H-1'); two aromatic singlets at δ 7.04 (1H, br.s, H-7) and 7.43 (1H, br.s, H-5); two aromatic doublets at δ 7.08 (1H, d, *J*=2.6 Hz, H-2) and 7.49 (1H, d, *J*=2.6 Hz, H-4); and one phenolic hydroxyl proton at δ 13.15 (1H, s, OH-8). The ^{13}C NMR spectrum (Table 1) exhibited the presence of 21 carbon signals attributable to 2 methyls (with 1 methoxyl), 1 methylenes, 8 methines, and 10 quaternary carbon atoms according to the DEPT and HSQC experiments. Detailed comparison of the ^1H and ^{13}C NMR spectral data of **6** with those of questin (**7**), a constituent of *Microascus tardifaciens* (Ascomycete) and many other fungi [7], revealed that **6** was a glycoside that consisted of **7** as aglycone and one sugar unit. Extensive analysis of 1D and 2D NMR spectral data and comparison with those of **4** and literature report [14] suggested that the sugar unit was also ribofuranosyl. The observed key HMBC correlation from H-1' (δ 5.89) to C-3 (δ 164.1) indicated connection of the sugar moiety to C-3. From the above deduction, the structure of **6** was established as 3-*O*-(α-D-ribofuranosyl)-questin.

Many bisanthraquinones have been isolated from plants and fungi as natural products and some of them showed biological activities [1, 12, 21]. These dimeric compounds are usually generated by direct coupling or by ether linkage of the related monomeric anthraquinones. To the best of our knowledge, compound **1** represents the first example of a symmetrical bisanthraquinone derived from monomeric anthraquinones through a C-CH_2-C linkage.

DPPH Radical Scavenging Activity

All of the isolated compounds were evaluated for their radical scavenging activities by using the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay, as in our previous reports [4, 13]. Compounds **1** and **5** showed strong activities with IC_{50} values of 44.0 and 74.0 μM, respectively, which were stronger than that of the well-known synthetic antioxidant butylated hydroxytoluene (BHT, IC_{50}=82.6 μM). However, compounds **2**–**4** and **6**–**7** only showed weak or moderate activities.

Acknowledgments

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