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New Production of 5-Bromotoluhydroquinone and 4-*O*-Methyltoluhydroquinone from the Marine-Derived Fungus *Dothideomycete* sp.

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The addition of NaBr to the fermentation medium of a marine isolate of the fungus *Dothideomycete* sp. resulted in induced production of two toluhydroquinone derivatives, 5-bromotoluhydroquinone (1) and 4-*O*-methyltoluhydroquinone (2), and two known compounds, toluhydroquinone (3) and gentisyl alcohol (4). The structures of 1 and 2 were assigned through the spectroscopic data analyses. Compounds 1–4 showed a potent antibacterial activity against the methicillin-resistant and multidrug-resistant *Staphylococcus aureus* (MRSA and MDRSA) with MIC (minimum inhibitory concentration) values of 6.2, 12.5, 6.2, and 12.5 μg/ml, respectively. Compounds 1–4 also exhibited a moderate radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC_{50} values of 11.0, 17.0, 12.0, and 7.0 μM, respectively, which were more active than the positive control, L-ascorbic acid (IC_{50}, 20.0 μM).

Keywords: 5-Bromotoluhydroquinone, 4-*O*-methyl-toluhydroquinone, toluhydroquinone, gentisyl alcohol, *Dothideomycete* sp.

The exploitation of marine environments in the search for structurally unusual and biologically highly active natural products has been intriguingly successful in recent years [1]. To avoid the depletion of marine resources and to enable access to large quantities of interesting compounds, there is particular interest in culturable marine microorganisms [4]. Thus, we have studied microbial fungi isolated from marine sources to test their potential for providing new natural products [14]. When the marine-derived microorganisms were cultured under saline condition, they often produced interesting biological halogenated metabolites (*e.g.*, salinosporamide A [3] of a highly potent inhibitor of the 20S proteasome and its halogenated derivatives [5], cytotoxic halogenated polyenyl pyrroles, isorumbrin and bromoisorumbrin [2], nematicidal and antimicrobial lachnumon and mycorrhizin A derivatives [12], bromomyrothenone B [6], and antibacterial chlorohydroaspyrones A and B [15]). Encouraged by the detection of halogenated marine analogs, we manipulated the fermentation of marine-derived fungi, *Phoma herbarum*, *Penicillium chrysogenum*, and *Fusarium tricinctum*, by the addition of halide salt to the culture medium in an effort to gain access to a wider cross-section of halogenated secondary metabolites, and found new production of radical scavenging haloquinones, bromochlorogentisylquinones A and B [10], halodiphenyl ethers [13], and antibacterial bromomethylchlamydosporols A and B [9], respectively. This paper describes the production, isolation, identification, and antibacterial and radical scavenging activities of two toluhydroquinone derivatives, 5-bromotoluhydroquinone (**1**) and 4-*O*-methyltoluhydroquinone (**2**), and two known compounds, toluhydroquinone (**3**) and gentisyl alcohol (**4**) [7].

Isolation of the Marine-Derived Fungus *Dothideomycete* sp. and Feeding Halide Salt

The fungal strain was isolated from the surface of the edible marine red alga *Chondria crassicualis* (Korean name: Seosil), collected at Yokji Island, Gyeongnam Province, Korea in 2008, and was identified to be *Dothideomycete* sp. on the basis of morphology and 18S rRNA analysis (SolGent Co., Ltd., Daejeon, Korea), with identity of 99%. A voucher specimen is deposited at Pukyong National University with the code MFA292-1Y. The fungus was cultured (1 l × 20) in SS medium consisting of soytone (0.1%), soluble starch (1.0%), and distilled water (100%). The cultures (1 l × 20) were incubated at 29°C for 10 days on a rotary shaker (120 rpm), and NaBr (50 mM) was

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subsequently added. The culture was incubated for a further 10 days under the same conditions.

Extraction and Isolation

The mycelium and broth were separated by filtration through cheesecloth, and the whole broth was extracted with EtOAc (20 l) to afford crude extract (1.2 g). A portion of this extract (1.0 g) was subjected to silica gel flash chromatography. Elution was performed with *n*-hexane–ethyl acetate (stepwise, 0–100% ethyl acetate) to yield 20 collections (50 ml each). These collections were pooled on the basis of their TLC profiles to give five combined fractions. Fractions 2 and 4 on medium pressure liquid chromatography (MPLC) (ODS) by elution with H_2O–MeOH (stepwise, 0–100% MeOH) afforded crude compounds **1**–**3** and **4**, respectively, which were further purified by HPLC (ODS-A, MeOH-H_2O = 5:1) to yield compounds **1** (11 mg), **2** (7 mg), **3** (12 mg), and **4** (17 mg), respectively.

5-Bromotoluhydroquinone (**1**): colorless amorphous solid; UV (MeOH) λ_{max} (log ε) 302 (3.4) nm; IR (neat) ν_{max} 3,247 (br), 1,626, 1,520, 1,418, 1,191, 976, 878, 803 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6), see Table 1; EI-MS *m/z* 204 [M, $^{81}Br]^+$ (93), 202 [M, $^{79}Br]^+$ (100), 123 [M-Br]$^+$ (86), 105 [M-Br-$H_20]^+$ (12), 95 (23), 94 (25), 67 (40), 66 (39).

4-*O*-Methyltoluhydroquinone (**2**): colorless amorphous solid; UV (MeOH) λ_{max} (log ε) 291 (3.1) nm; IR (neat) ν_{max} 3,454, 1,633, 1,511, 1,461, 1,437, 1,211, 1,048, 862 cm^{-1}; ^1H and ^{13}C NMR (DMSO-d_6), see Table 1; EI-MS *m/z* 138 [M]$^+$ (98), 123 [M-CH_3]$^+$ (100), 105 [M-CH_3-$H_2$0]$^+$ (5), 95 (62), 77 (67), 67 (82).

Structural Determination of 5-Bromotoluhydroquinone (1) and 4-*O*-Methyltoluhydroquinone (2)

Toluhydroquinone (**3**) was isolated from a marine isolate of the fungus *Aspergillus* and exhibited a potent antibacterial activity against the methicillin-resistant *Staphylococcus aureus* (MRSA) and multidrug-resistant *S. aureus* (MDRSA), as well as a mild radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) [7]. In this context, we are interested in the chemical and biological aspects of the derivatives of compound **3** and induced production of them. TLC analysis showed that the composition of the extract differed from the extract derived from bromine-free SS medium. Aside from the two known compounds **3** and **4**, two new spots corresponding to 5-bromotoluhydroquinone (**1**) and 4-*O*-methyltoluhydroquinone (**2**) were detected by TLC analysis and purified by repeated silica gel flash chromatography and HPLC.

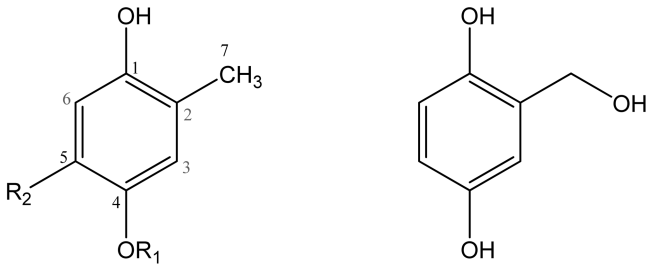
5-Bromotoluhydroquinone (**1**) was isolated as a colorless amorphous solid. It showed an isotopic cluster at *m/z* 202 [M (^{79}Br)]$^+$ (100) and 204 [M (^{81}Br)]$^+$ (93) with a ratio of about 1:1 in the EI-MS, suggesting the presence of a bromine atom. The IR spectrum of **1** exhibited bands characteristic for the hydroxyl (3,247 cm^{-1}) group. The general features of the UV, IR, and NMR spectra of **1** closely resembled those of toluhydroquinone (**3**) [7], except that the NMR signal at C-5 indicated a change from the sp^2-methine [δ 6.36 (1H, dd, *J* = 8.5, 2.8 Hz, H-5), 116.2 (CH, C-5)] of **3** to an sp^2-quaternary carbon [δ 105.2 (qC, C-5)] bearing a bromo group in **1**. Detailed analysis of the ^1H and ^{13}C NMR spectra of **1**, including the results of DEPT, COSY, HMQC, and HMBC experiments, suggested that the induced product **1** is the 5-bromide of **3** (Table 1, Fig. 1). The key HMBC correlations from 1-OH to C-1, C-2, and C-6; from H-3 to C-1, C-5, and C-7; from 4-OH to C-3, C-4, and C-5; from H-6 to C-2, C-4, and C-5; and from H_3-7 to C-1, C-2 and C-3 were critical in establishing the location of the 5-bromo group, as shown in Fig. 1. On the basis of these data, the structure of the metabolite is proposed to be 5-bromotoluhydroquinone (**1**).

4-*O*-Methyltoluhydroquinone (**2**) was isolated as a colorless amorphous solid. The IR spectrum of **2** exhibited bands characteristic for the hydroxyl (3,454 cm^{-1}) group.

Table 1. NMR spectral data for 5-bromotoluhydroquinone (**1**) and 4-*O*-methyltoluhydroquinone (**2**).

Carbon no.	5-Bromotoluhydroquinone (**1**)[a]		4-*O*-Methyltoluhydroquinone (**2**)[b]	
	δ_H (mult., *J* in Hz)	δ_C (mult.)	δ_H (mult., *J* in Hz)	δ_C (mult.)
1		148.5 (qC)		147.9 (qC)
2		124.4 (qC)		125.1 (qC)
3	6.66 (s)	118.1 (CH)	6.69 (d, 3.5)	116.7 (CH)
4		146.2 (qC)		153.7 (qC)
5		105.2 (qC)	6.62 (dd, 8.5, 3.5)	112.0 (CH)
6	6.83 (s)	117.9 (CH)	6.70 (d, 8.5)	115.7 (CH)
7	2.00 (s)	15.8 (CH_3)	2.23 (s)	16.3 (CH_3)
1-OH	8.96 (s)			
4-OH	9.25 (s)			
4-OMe			3.75 (s)	55.9 (CH_3)

[a]Recorded in DMSO-d_6 and [b]recorded in $CDCl_3$ at 400 MHz (^1H) and 100 MHz (^{13}C).



1: R_1 = H, R_2 = Br
2: R_1 = Me, R_2 = H
3: R_1, R_2 = H

gentisyl alcohol (**4**)

Fig. 1. The structures of 5-bromotoluhydroquinone (**1**), 4-*O*-methyltoluhydroquinone (**2**), toluhydroquinone (**3**), and gentisyl alcohol (**4**).

The general features of the UV, IR, and NMR spectra of **2** closely resembled those of toluhydroquinone (**3**) [7], except that the NMR signals of a new methoxyl group [δ 3.75 (3H, s, 4-OMe), 55.9 (CH_3, 4-OMe)] have appeared as a result of the methylation of one hydroxyl group of compound **3**. Detailed analysis of the ^{1}H and ^{13}C NMR spectra of **2**, including the results of DEPT, COSY, HMQC, and HMBC experiments, suggested that the induced product **2** is the 4-methoxide of **3** (Table 1, Fig. 1). The key HMBC correlations from H-3 to C-1, C-5, and C-7; from 4-OMe to C-4; from H-5 to C-1, C-3, and C-4; from H-6 to C-1, C-2, and C-4; and from H_3-7 to C-1, C-2 and C-3 were critical in establishing the location of the 4-OMe group, as shown in Fig. 1. Thus, the structure of 4-*O*-methyltoluhydroquinone (**2**) was confidently determined.

To rule out the possibility of **2** being artifacts, formed as a result of the extraction and purification process, a careful TLC analysis of the extract and purified fractions was carried out. Compound **2** was detected in the fresh original organic crude extract and the fractions obtained after chromatography. In additon, compound **3** was dissolved separately in MeOH and the solution was exposed to air for 3 days, but the TLC of the solution did not reveal the presence of **2**. These experiments provided enough evidence to establish the natural origin of 4-*O*-methyltoluhydroquinone (**2**).

Compound **1** was reported as a synthetic intermediate of triprenylated toluquinone and toluhydroquinone, the fungal metabolites with potent radical scavenging and cytotoxic activities [11]. Compound **2** was also reported as a synthetic intermediate of cyclic phosphazenes bearing the dihydrobenzoxazinoxy group, the synthetic resin [8]. However, to the best of our knowledge, **1** and **2** are the first examples of compounds isolated from a natural source. Toluhydroquinone (**3**) and gentisyl alcohol (**4**) were also identified by inspection and comparison of the physicochemical data including NMR data with those in the literature [7].

Biological Activity

Compounds **1**–**4** showed potent antibacterial activity against MRSA and MDRSA with MIC values of 6.2, 12.5, 6.2, and 12.5 μg/ml, respectively. Compounds **1**–**4** also exhibited mild radical scavenging activity against DPPH with IC_{50} values of 11.0, 17.0, 12.0, and 7.0 μM, respectively, which were more active than the positive control, L-ascorbic acid (IC_{50}, 20.0 μM).

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