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NOTE

New production of haloquinones, bromochlorogentisylquinones A and B, by a halide salt from a marine isolate of the fungus *Phoma herbarum*

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Marine-derived microorganisms continue to attract attention as a rich source of structurally novel bioactive metabolites that are potential lead compounds for the development of new drugs.[1,2] When the marine-derived microorganisms were cultured under saline condition, they rarely produced interesting biological halogenated metabolites (for example, salinosporamide A[3] of a highly potent inhibitor of the 20S proteasome and its halogenated derivatives,[4] cytotoxic halogenated polyenyl pyrroles, isorumbrin and bromoisorumbrin,[5] nematicidal and antimicrobial lachnumon and mycorrhizin A derivatives,[6] bromomyrothenone B,[7] and antibacterial chlorohydroaspyrones A and B[8]). Encouraged by the detection of halogenated marine analogs, we manipulated the fermentation of *Phoma herbarum* by the addition of halide salts in an effort to gain access to a wider cross-section of halogenated secondary metabolites. This report describes the production, isolation, and identification of halogenated benzoquinones (**1**, **2**), as well as the radical-scavenging activity of these compounds.

The fungal strain, *P. herbarum*, was isolated from the edible marine red alga *Gloiopeitis tenax* (Korean Name: ChamGasari) collected in Cheokpo, Tongnyeong, Korea and identified based on 18S rRNA analyses (SolGent, Daejeon, Korea), identity of 99%. A voucher specimen is deposited at Pukyong National University with the code MFA301. The fungus was cultured (1 liter×10) in SWS medium consisting of soytone (0.1%), soluble starch (1.0%) and seawater (100%). The cultures were incubated at 29 °C for 10 days on a rotary shaker (120 r.p.m.), and $CaBr_2$ (50 mM)[6,9] was subsequently added. Incubation was further continued for 10 days under the same condition. The culture control was incubated in the absence of $CaBr_2$ in the same manner as described above.

The mycelium and broth were separated by filtration using cheesecloth. The broth was extracted with EtOAc to afford a crude extract (0.8 g). The extract was subjected to silica gel flash chromatography. Elution was performed with *n*-hexane-EtOAc (stepwise, 0–100% EtOAc) to yield five fractions. Fraction 2, which was active in 1, 1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay, was separated by medium-pressure liquid chromatography (ODS-A; YMC Co. Ltd, Kyoto, Japan) using an H_2O-MeOH gradient elution to afford crude compounds **1** and **2**. These were further purified by HPLC (ODS-A, 10×250 mm, 1 ml min^{-1}; YMC Co. Ltd) using a 30-min gradient program of 50–100% MeOH in H_2O to furnish **1** (6.5 mg) and **2** (6.0 mg). Compounds **3** (15 mg) and **4** (5.0 mg) were isolated from fraction 3 by the same chromatographic method as described above.

Bromochlorogentisylquinone A (**1**): a yellow solid; IR (KBr) ν_{max} 3399, 3064, 2927, 2853, 1670, 1588, 1428, 1316, 1269, 1232, 1204, 1103, 1025, 941, 876, 803 cm^{-1}; UV λ_{max} (MeOH) (log ε) 204 (4.8), 276 (4.4) nm; MS (EI) *m/z* 250 $[M]^+$(48), 252 $[M+2]^+$(65), 254 $[M+4]^+$(16), 234 (11), 224 (6), 206 (11), 167 (2), 153 (7), 142 (20), 115 (13), 97 (6), 79 (40), 61 (25), 53 (100); HRMS (EI) *m/z* 249.9032 $[M]^+$ (calcd for $C_7H_4O_3{}^{79}Br$, ^{35}Cl, 249.9032)(+0.0 p.p.m./+0.0 m.m.u.); see Table 1 for NMR spectral data.

Bromochlorogentisylquinone B (**2**): a yellow solid, IR (KBr) ν_{max} 3401, 3058, 2921, 1673, 1587, 1316, 1232, 1103, 1025, 941, 875 cm^{-1}; UV λ_{max} (MeOH) (log ε) 204 (4.9), 276 (4.9) nm; MS (EI) *m/z* 250 $[M]^+$(82), 252 $[M+2]^+$(100), 254 $[M+4]^+$(27), 234 (12), 224 (5), 206 (16), 195 (5), 167 (9), 159 (2), 131 (7), 113 (9), 97 (4), 87 (31), 79 (23), 53 (50); HRMS (EI) *m/z* 249.9034 $[M]^+$ (calcd for $C_7H_4O_3{}^{79}Br$, ^{35}Cl, 249.9032)(+0.7 p.p.m./+0.2 m.m.u.); see Table 1 for NMR spectral data.

Chlorogentisyl alcohol (**3**) and gentisyl alcohol (**4**) were isolated as colorless solids, and showed spectral data that were virtually identical to those reported in the literature.[10]

Samples to be tested were dissolved in MeOH and the solution (160 µl) was dispensed into the wells of a 96-well microtiter tray.[10] In all, 40 µl of

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Table 1 NMR spectral data for bromochlorogentisylquinones A (1) and B (2)[a]

	Bromochlorogentisylquinone A (1)			*Bromochlorogentisylquinone B (2)*		
Carbon position	δ_H *(mult., J in Hz)*	δ_C *(mult.)*	*HMBC (H → C)*	δ_H *(mult., J in Hz)*	δ_C *(mult.)*	*HMBC (H → C)*
1		177.9 (s)			176.5 (s)[b]	
2		145.5 (s)			149.8 (s)	
3		136.8 (s)		6.84 (t, 2.0)	129.0 (d)	1, 5, 7
4		176.1 (s)			177.8 (s)[b]	
5	7.55 (s)	133.0 (d)	1, 3, 6		134.9 (s)	
6		142.5 (s)			143.5 (s)	
7	4.45 (s)	58.1 (t)	1, 2, 3, 4[c]	4.36 (d, 2.0)	57.4 (t)	1, 2, 3, 4
7-OH	5.32 (br s)			5.54 (br s)		

[a]Recorded in DMSO-d_6 at 400 MHz (^{1}H) and 100 MHz (^{13}C).
[b]Interchangeable.
[c]Weak correlations.

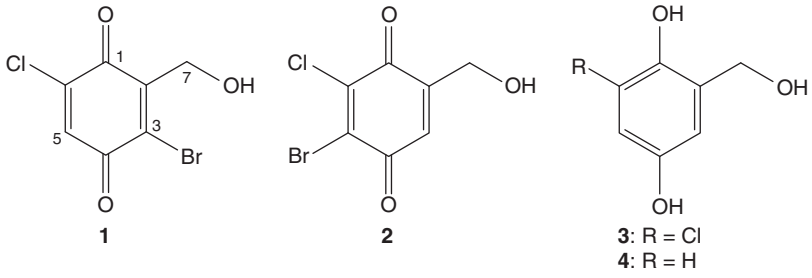

Figure 1 Chemical structures of bromochlorogentisylquinones A (**1**), B (**2**), chlorogentisyl alcohol (**3**), and gentisyl alcohol (**4**).

the DPPH solution in MeOH (1.5×10^{-4} M) was added to each well. The mixture was shaken and left to stand for 30 min, and the absorbance of the resulting solution was measured at 520 nm with a microplate reader (VERSAmax; Molecular Devices, Sunnyvale, CA, USA). The scavenging activity on the DPPH radical was expressed as IC_{50}, which is the concentration of the tested compound required to give a 50% decrease of the absorbance from that of the blank solution (consisting of MeOH (160 µl) and DPPH solution (40 µl)).

TLC analysis showed that the composition of the extract differed from the extract derived from bromide-free SWS medium. Aside from compounds **3** and **4**, two new spots corresponding to **1** and **2** were detected by TLC analysis and purified by repeated silica gel flash chromatography (*n*-hexane in ethyl acetate) and HPLC (ODS-A) to yield compounds **1** and **2**.

Bromochlorogentisylquinone A (**1**) was isolated as a yellow solid, which yielded a molecular formula of $C_7H_4BrClO_3$ by HR-EI-MS and ^{13}C NMR methods. It showed an isotopic cluster at *m/z* 250 [*M* (^{35}Cl, ^{79}Br)]$^+$ (48), 252 [*M* (^{37}Cl ^{79}Br)]$^+$ (65) and 254 [*M* (^{37}Cl ^{81}Br)]$^+$ (16) with a ratio of 100:135:33 in the EI-MS, suggesting the presence of one bromine and one chlorine atom. The IR spectrum of **1** exhibited bands characteristic for hydroxyl (3399 cm^{-1}) and quinone (1670 cm^{-1})[11] functionalities. Detailed analyses of the ^{1}H and ^{13}C NMR spectra of **1**, including DEPT, TOCSY, HMQC and HMBC experiments, revealed signals ascribable to hydroxymethyl and 2,2′,3-trisubstituted benzoquinone groups (Table 1, Figure 1). The latter was further supported by UV spectral data (204 nm (log ε 4.8), 276 (4.4)).[11] The connection of the functional groups in **1** was achieved on the basis of HMBC. The key HMBC correlations, from 5-H to C-1, C-3 and C-6 and from 7-H_2 to C-1, C-2 and C-3, showed the connection of C7–C2 as well as the positions of 2-hydroxymethyl, 3-bromo and 6-chloro (δ_C 142.5 (s, C-6))[12,13] groups. As the carbon backbones of **1** and **3** are likely to be formed via the same biosynthetic pathway, the position of the 6-chloride group of **1** is assumed to be the same as in **3**. This is also supported by the chemical shift of carbon-6 (δ_C 142.5 (s, C-6) in **1**; 145.0 (s, C-6) in **5**].[13] The general features of its NMR spectra closely resembled those of chlorogentisylquinone (**5**)[13], except for the NMR signal at C-3, which was changed from an sp^2-methine (δ_H 6.78 (1H, dd, *J*=2.3, 2.5 Hz, 3-H) and δ_C 131.5 (d, C-3)) in chlorogentisylquinone (**5**) to an sp^2-quaternary carbon (δ_C 136.8 (s, C-3)) in **1** (see Supplementary Information). Thus, compound **1** was characterized as 3-bromo-6-chloro-2-hydroxymethyl-1,4-benzoquinone.

Bromochlorogentisylquinone B (**2**) was also obtained in the form of a yellow solid. The general features of its MS, UV, IR and NMR spectra closely resembled those of bromochlorogentisylquinone A (**1**), except that the NMR signals including chemical shift and coupling constants assigned to H-5 and C-5 as well as H-3 and C-3 were changed from δ_H 7.55 (1H, s, 5-H) and δ_C 133.0 (d, C-5) for compound **1** to δ_C 134.9 (s, C-5) for compound **2**, and from δ_C 136.8 (s, C-3) for compound **1** to δ_H 6.84 (1H, t, *J*=2.0 Hz, 3-H) and δ_C 129.0 (d, C-3) for compound **2** (Table 1). The position of one sp^2 methine was supposed to be C-3 from not only allylic coupling (J_{3H-7H2}=2.0 Hz) between 3-H and 7-H_2 but also HMBC correlation from H-3 to C-7 and from 7-H_2 to C-3. Detailed analyses of the ^{1}H and ^{13}C NMR spectra of **2**, including the results from DEPT, TOCSY, HMQC, and HMBC experiments, suggested that **2** is a regioisomer of compound **1**, 5-bromo-6-chloro-2-hydroxymethyl-1,4-benzoquinone. Direct comparison of the foregoing data for **2** with those for **1** supported the gross structure shown for **2** (Figure 1, see Supplementary Information).

Compounds **3** and **4** were identified as the known compounds, chlorogentisyl alcohol (**3**) and gentisyl alcohol (**4**), respectively, by comparison of their spectroscopic data with the published data.[10]

There is considerable recent evidence that free radicals induce oxidative damage to biomolecules. This oxidative damage is considered to have a causative role in aging and several degenerative diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, and atherosclerosis.[14]

Antioxidant activity was assessed on the basis of the radical-scavenging effect on the DPPH free radical. Compounds **1–4** exhibited significant radical-scavenging activity against DPPH, with IC_{50} values of 3.8, 3.9, 1.0, and 7.0 μM, respectively, which indicated more activity than that of the positive control, L-ascorbic acid (IC_{50}, 20 μM).

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)