Induced Production of Methyl Bromodihydroxyphenyl Acetates by the Marine-Derived Fungus *Aspergillus* sp.

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The addition of metal bromides (NaBr and CaBr₂) during fermentation of a marine isolate of the fungus *Aspergillus* sp. induced production of two new brominated dihydroxyphenylacetic acid derivatives, methyl 2-(6-bromo-3,4-dihydroxyphenyl)acetate (**1**) and methyl 2-(2,5-dibromo-3,4-dihydroxyphenyl)acetate (**2**), and a known compound, 2-(3,4-dihydroxyphenyl)acetic acid (**3**). The structures of the two new compounds (**1**, **2**) were assigned through the combination of spectroscopic data analyses and comparison with the spectral data of compound **3**. Compounds **1**–**3** exhibited potent radical-scavenging activity against 1,1-diphenyl-2-picrylhydrazyl (DPPH) with IC₅₀ values (14.2, 12.1, 11.0 μM, respectively) demonstrating greater activity than the positive control (L-ascorbic acid; IC₅₀, 20.0 μM).

Key words *Aspergillus*; methyl 2-(bromodihydroxyphenyl)acetate; dihydroxyphenylacetic acid

Marine-derived microorganisms continue to attract attention as rich sources of structurally novel bioactive metabolites that are potential lead compounds for the development of new drugs.^{1,2} When marine-derived microorganisms are cultured under saline conditions, they rarely produce interesting biological halogenated metabolites (e.g., salinosporamide A,³ a highly potent inhibitor of the 20S proteasome, and its halogenated derivatives⁴); cytotoxic halogenated polyenyl pyrroles, isorumbin and bromisorumbin⁵); the nematocidal and antimicrobial lachnumon and mycorrhizin A derivatives⁶); bromomyrothenone B⁷); and the antibacterial chlorohydroaspirones A and B⁸). Encouraged by the detection of bioactive halogenated marine analogs, and in an effort to gain access to a wider cross-section of halogenated secondary metabolites, we manipulated fermentation of the marine-derived fungi *Phoma herbarum*, *Penicillium chrysogenum*, *Fusarium tricinctum*, and *Dothideomycete* sp. by the addition of halide salts to their respective culture mediums. We detected the production of radical scavenging haloquinones (bromochlorogentisylquinones A and B),⁹ halodiphenyl ethers,¹⁰ antibacterial bromomethylchlamydsoporols A and B,¹¹ and antibacterial and radical scavenging 5-bromotoluhydroquinone.¹² This report describes the production, isolation, identification, and radical scavenging activity of two new brominated dihydroxyphenylacetic acid derivatives (compounds **1**, **2**) and a known compound, 2-(3,4-dihydroxyphenyl)acetic acid (**3**).¹³ TLC analysis showed that the composition of the extract derived from fermentation in the halide-enriched culture medium differed from that derived during halide-free culture fermentation. In addition to compound **3**, in the former extract two other spots were detected by TLC analysis; these were subsequently purified by repeated silica gel flash chromatography (*n*-hexane in ethyl acetate) and HPLC (octadecyl silica (ODS)-A, MeOH–H₂O) to yield the two new compounds **1** and **2**.

Methyl 2-(6-bromo-3,4-dihydroxyphenyl)acetate (**1**) was isolated as a slightly yellow solid having the molecular formula C₉H₉BrO₄, based on high resolution electron impact mass spectrometry (HR-EI-MS) and ¹³C-NMR analyses. The compound had isotopic clusters at *m/z* 260 [M(⁷⁹Br)]⁺ (**17**) and

262 [M(⁸¹Br)]⁺ (**17**) with a ratio of 1:1 in the EI-MS, suggesting the presence of a single bromine atom. The IR spectrum of compound **1** exhibited bands characteristic of hydroxyl (3307 cm^{−1}) and ester (1732, 1168, 1010 cm^{−1}) functionalities. Detailed analyses of the ¹H- and ¹³C-NMR spectra of compound **1**, including the distortionless enhancement by polarization transfer (DEPT) and 2D-NMR spectra, correlation spectroscopy (COSY), heteronuclear multiple quantum correlation (HMBC), and heteronuclear multiple bond coherence (HMBC), revealed signals attributable to a methyl ester [δ_{H} 3.79 (3H, s, 1-OCH₃), δ_{C} 52.9 (q, 1-OCH₃), and 173.8 (s, C-1)], a methylene [δ_{H} 3.67 (2H, s, H₂-2) and δ_{C} 40.9 (t, C-2)], and 1,2,4,5-tetrasubstituted phenyl groups (Table 1 and supplementary information). The connection among the functional groups of compound **1** was achieved on the basis of HMBC. The key HMBC correlations (from 1-OCH₃ to C-1; from H₂-2 to C-1, C-1', 2', and C-6'; from H-2' to C-2, C-3', C-4', and C-6'; and from H-5' to C-1', C-3', C-4', and C-6') showed the connection between C-2 and C-1' as well as the positions of the 6'-bromo and 3',4'-dihydroxyl groups on the phenyl ring (Fig. S5 of supplementary information). These spectroscopic features revealed that compound **1** had the general structural features of 2-(3,4-dihydroxyphenyl)acetic acid (**3**).¹³ The NMR data for both compounds showed similar patterns, except for the appearance of additional proton and carbon signals corresponding to an oxymethyl group [δ_{H} 3.79 (3H, s, 1-OMe) and δ_{C} 52.9 (q, 1-OMe)], and the disappearance of a proton signal at C-6', which was changed from the *sp*²-methine [δ_{H} 6.63 (1H,

Fig. 1. Structures of Compounds **1**–**3**

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d, $J=7.5$ Hz, H-6') and δ_C 120.0 (d, C-6')] in compound **3** to the sp^2 -quaternary carbon [δ_C 114.4 (s, C-6')] in compound **1** (see Table 1 and supplementary information). Thus, compound **1** was identified as methyl 2-(6-bromo-3,4-dihydroxyphenyl)-acetate.

Methyl 2-(2,5-dibromo-3,4-dihydroxyphenyl)acetate (**2**) was obtained in the form of a slightly yellow solid. It showed isotopic clusters at m/z 338 [$M(^{79}Br_2)^+$ (13), 340 [$M(^{79}Br, ^{81}Br)^+$ (24), and 342 [$M(^{81}Br_2)^+$ (12) with a ratio of 1:2:1 in the EI-MS, suggesting the presence of two bromine atoms. A molecular formula of $C_9H_8Br_2O_4$, which gave five degrees of unsaturation, was established by HR-EI-MS and ^{13}C -NMR. The IR spectrum of compound **2** exhibited band characteristics of hydroxyl (3429 cm^{-1}) and ester (1736 , 1137 , 1027 cm^{-1}) functionalities. The general features of the UV, IR, and NMR spectra closely resembled those of compound **1**, except that one sp^2 -methine proton in compound **1** was replaced with a bromine. The positions of the two bromines in compound **2** were determined by HMBC. The key HMBC correlations (from 1-OCH₃ to C-1; from H₂-2 to C-1, C-1', 2', and C-6'; and from H-6' to C-2, C-1', C-2', C-4', and C-5') showed the positions of the 2',5'-dibromo and 3',4'-dihydroxyl groups on the phenyl ring (Fig. S6 of supplementary information). Detailed analyses of the 1H - and ^{13}C -NMR spectra of compound **2** (including the results from DEPT, COSY, HMQC, and HMBC experiments) enabled its structure to be assigned as methyl 2-(2,5-dibromo-3,4-dihydroxyphenyl)acetate (see Table 1 and supplementary information).

The known 2-(3,4-dihydroxyphenyl)acetic acid (**3**) was also found during this study. It was identified from NMR spectra and from comparison with published data values.¹³⁾

There is considerable recent evidence that free radicals induce oxidative damage to biomolecules. This oxidative damage is considered to play a causative role in aging and several degenerative diseases such as Alzheimer's disease, rheumatoid arthritis, cancer, and atherosclerosis.¹⁴⁾ The antioxidant activity was assessed on the basis of the radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical. Compounds **1–3** exhibited significant radical scavenging activity against DPPH with IC_{50} values (14.2, 12.1, $11.0\mu\text{M}$, respectively) demonstrating greater activity than the positive control (L-ascorbic acid; IC_{50} , $20.0\mu\text{M}$).

We plan to assess the inhibitory activity of compounds **1–3** against the epidermal growth factor receptor (EGFR),^{15,16)} and the results will be reported in due course.

Experimental

General UV/visible spectra were measured on a Hitachi U-2001 UV/Vis spectrometer. IR spectra were recorded on a Bruker FT-IR model IFS-88 spectrometer. 1H - (400 MHz) and ^{13}C -NMR (100 MHz) spectra were obtained on a JEOL JNM-ECP 400 NMR spectrometer, using TMS or solvent peaks [DMSO- d_6 : 1H (δ 2.50) and ^{13}C (δ 39.5)] as reference standard. MS spectra were obtained on a JEOL JMS-700 spectrometer. HPLC was performed on a Young Lin ACME HPLC system using a reversed-phase analytical column (Gemini C18, $4.6\times 250\text{ mm}$, $5\mu\text{m}$) with UV detection. Incubations of microorganisms and feeding experiment were performed on an Incubator Shaker JS-FS-2500 (Johnsam Co., Inchon, Korea).

Fungal Isolation and Feeding Halide Salt The fungal strain was isolated from the surface of brown alga *Ishige okamurae* (Korean name: Pae) collected at Uljin, Korea in 2002, and identified to be *Aspergillus* sp. (gb/EU301661.1) on the basis of morphology and 18S ribosomal RNA (rRNA) analysis (SolGent Co., Ltd., Daejeon, Korea), identity of 98%. A voucher specimen is deposited at Pukyong National University with the code MFB151. The fungus was cultured (1L $\times$ 20) in SS medium consisting of soytone (0.1%), soluble starch (1.0%), and distilled water (100%). The cultures (1L $\times$ 20) were incubated at 29°C for 10d on a rotary shaker (120rpm), and NaBr and CaBr₂ (each 50mM)^{17,18)} was subsequently added to the each culture (1L). All the cultures were incubated for a further 19d under the same conditions. The culture control was incubated in the absence of metal halides (NaBr and CaBr₂) in the same manner as described above. TLC analysis showed that the composition of the extract differed from the extract derived from bromide-free SS medium.

Extraction and Isolation The mycelium and broth were separated by filtration through the cheesecloth, and the whole broth was extracted with EtOAc (20L) to afford broth extract (1.2g). A portion of this extract (1.0g) was subjected to Si gel flash chromatography. Elution was performed with *n*-hexane–EtOAc (stepwise, 0–100% EtOAc) to yield twenty collections (50mL each). These collections were pooled on the basis of their TLC profiles to give five combined fractions. Fractions 2 and 4 were separated by medium pressure liquid chromatography (MPLC) (ODS) using a H₂O–MeOH gradient elution to afford crude compounds **1**, **2**, and **3**, respectively. These were further purified by HPLC (Gemini C18, $4.6\times 250\text{ mm}$, 1mL/min) utilizing a 30min gradient program of 50 to 100% MeOH in H₂O to furnish compounds **1** (9mg), **2** (7.5mg), and **3** (8mg), respectively.

Table 1. NMR Spectral Data for Methyl 2-(6-Bromo-3,4-dihydroxyphenyl)acetate (**1**) and Methyl 2-(2,5-Dibromo-3,4-dihydroxyphenyl)acetate (**2**)^{a)}

Carbon No.	1		2	
	δ_H (mult., J in Hz)	δ_C (mult.)	δ_H (mult., J in Hz)	δ_C (mult.)
1		173.8 (qC)		171.2 (qC)
2	3.67 (s)	40.9 (CH ₂)	3.70 (s)	40.8 (CH ₂)
1'		125.2 (qC)		127.2 (qC)
2'	6.68 (s)	118.0 (CH)		111.6 (qC)
3'		144.1 (qC)		141.9 (qC)
4'		143.8 (qC)		140.8 (qC)
5'	6.85 (s)	119.4 (CH)		108.2 (qC)
6'		114.4 (qC)	7.01 (s)	125.5 (CH)
1-OMe	3.79 (s)	52.9 (CH ₃)	3.73 (s)	52.6 (CH ₃)

a) Recorded in CDCl₃ at 400MHz (1H) and 100MHz (^{13}C).

Methyl 2-(6-Bromo-3,4-dihydroxyphenyl)acetate (**1**): A slightly yellow solid; IR (KBr) $\nu_{\max}$ 3307, 1732, 1621, 1513, 1452, 1334, 1168, 1010, 868, 823 cm^{-1} ; UV $\lambda_{\max}$ (MeOH) (log ϵ) 290 (3.44) nm; $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): see the Table 1; LR-EI-MS m/z : 262 $[\text{M}^{(81}\text{Br})]^+$ (17), 260 $[\text{M}^{(79}\text{Br})]^+$ (17), 203 $[\text{M}^{(81}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (87), 201 $[\text{M}^{(79}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (88), 181 $[\text{M}^{(79}\text{Br})-^{79}\text{Br}]^+$ (100), 153 (26); HR-EI-MS m/z 259.9685 $[\text{M}^{(79}\text{Br})]^+$ (Calcd for $\text{C}_9\text{H}_9\text{O}_4^{79}\text{Br}$, 259.9684) (+0.4 ppm); m/z 261.9663 $[\text{M}^{(81}\text{Br})]^+$ (Calcd for $\text{C}_9\text{H}_9\text{O}_4^{81}\text{Br}$, 261.9664) (-0.4 ppm).

Methyl 2-(2,5-Dibromo-3,4-dihydroxyphenyl)acetate (**2**): A slightly yellow solid; IR (KBr) $\nu_{\max}$ 3429, 3234, 2920, 1736, 1566, 1472, 1289, 1137, 1027, 981, 815, 748 cm^{-1} ; UV $\lambda_{\max}$ (MeOH) (log ϵ) 289.0 (3.38) nm; $^1\text{H-NMR}$ (CDCl_3 , 400 MHz) and $^{13}\text{C-NMR}$ (CDCl_3 , 100 MHz): see the Table 1; LR-EI-MS m/z : 342 $[\text{M}^{(81}\text{Br}_2)]^+$ (12), 340 $[\text{M}^{(79}\text{Br}, ^{81}\text{Br})]^+$ (24), 338 $[\text{M}^{(79}\text{Br}_2)]^+$ (13), 283 $[\text{M}^{(79}\text{Br}, ^{81}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (46), 281 $[\text{M}^{(79}\text{Br}, ^{81}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (100), 279 $[\text{M}^{(79}\text{Br}, ^{81}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (50), 261 $[\text{M}^{(81}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (68), 259 $[\text{M}^{(79}\text{Br})-\text{CO}_2\text{CH}_3]^+$ (81), 199 (8), 203 (2), 201 (10), 180 (16); HR-EI-MS m/z 337.8792 $[\text{M}^{(79}\text{Br}_2)]^+$ (Calcd for $\text{C}_9\text{H}_8\text{O}_4^{79}\text{Br}_2$, 337.8790) (+0.6 ppm), m/z 339.8767 $[\text{M}^{(79}\text{Br}, ^{81}\text{Br})]^+$ (Calcd for $\text{C}_9\text{H}_8\text{O}_4^{79}\text{Br}^{81}\text{Br}$, 339.8769) (-0.5 ppm), m/z 341.8746 $[\text{M}^{(81}\text{Br}_2)]^+$ (Calcd for $\text{C}_9\text{H}_8\text{O}_4^{81}\text{Br}_2$, 341.8748) (+0.7 ppm).

2-(3,4-Dihydroxyphenyl)acetic Acid (**3**): Spectroscopic data were virtually identical to those reported in the literature.¹³⁾

Radical Scavenging Activity against DPPH¹⁹⁾ Samples (compounds **1–3**, and positive control, L-ascorbic acid) to be tested were dissolved in MeOH and the solution (160 μL) was dispensed into wells of a 96-well microtiter tray. Forty microliter of the DPPH solution in MeOH ($1.5 \times 10^{-4}\text{M}$) 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 microplate reader (Packard Co., Spectra CountTM). The scavenging activity on 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)].

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Supporting Information Available ^1H - and ^{13}C -NMR and HMBC spectra of **1** and **2**.

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