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Halogenated Compounds from Directed Fermentation of *Penicillium concentricum*, an Endophytic Fungus of the Liverwort *Trichocolea tomentella*

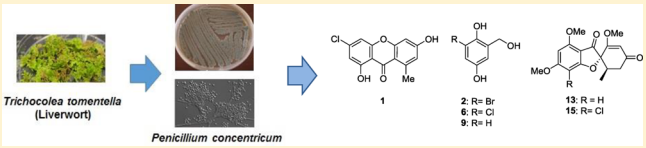
Tehane Ali,[†] Masanori Inagaki,[†] Hee-byung Chai,[†] Thomas Wieboldt,[‡] Chad Rapplye,[§] and L. Harinantenaina Rakotondraibe*[,†]

[†]Division of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, and [§]Department of Microbiology, The Ohio State University, Columbus, Ohio 43210, United States

[‡]Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, Virginia 24061, United States

Supporting Information

ABSTRACT: One new chlorinated xanthone, 6-chloro-3,8-dihydroxy-1-methylxanthone (**1**), a new 2-bromo-gentisyl alcohol (**2**), and a mixture of 6-epimers of 6-dehydroxy-6-bromogabosine C (**3a** and **3b**), together with 19 previously identified compounds, epoxydon (**4**), norlichexanthone (**5**), 2-chlorogentisyl alcohol (**6**), hydroxychlorogentisyl quinone (**7**), 6-dehydroxy-6*α*-chlorogabosine C (**8a**), 6-dehydroxy-6*β*-chlorogabosine C (**8b**), gentisyl alcohol (**9**), gentisyl quinone (**10**), (*R*,*S*)-1-phenyl-1,2-ethanediol (**11**), dehydrodechlorogriseofulvin (**12**), dechlorogriseofulvin (**13**), dehydrogriseofulvin (**14**), griseofulvin (**15**), ethylene glycol benzoate (**16**), alternariol (**17**), griseoxanthone C (**18**), drimiopsin H (**19**), griseophenone C (**20**), and griseophenone B (**21**), were isolated from cultures of *Penicillium concentricum*, a fungal endophyte of the liverwort *Trichocolea tomentella*. The structures of the new compounds (**1**, **2**, **3a**, and **3b**) were elucidated by interpretation of spectroscopic data including one- and two-dimensional NMR techniques. Among these, compounds **2**−**4** displayed modest cytotoxicity to the MCF-7 hormone-dependent breast cancer cell line with IC_{50} values of 8.4, 9.7, and 5.7 μM, respectively, whereas compound **9** exhibited selective cytotoxicity against the HT-29 colon cancer cell line with an IC_{50} value of 6.4 μM. During this study we confirmed that the brominated gentisyl alcohol (**2**) was formed by chemical conversion of **4** during bromide salt addition to culture media.



Liverworts are one of the three phyla (mosses: about 14 000 species, liverworts: about 6000 species, and hornworts: about 300 species) of bryophytes (Hepaticeae), which are taxonomically located between algae and pteridophytes (ferns). Our interest in the chemistry of liverworts started with the isolation of bioactive compounds from Marchantiales.[1] Liverworts grow in contact with microbial-rich environments such as soil, rotten logs, and wet rocks. There is thus a high chance that liverworts are exposed to microorganisms and host them as epiphytes and endophytes. In fact our previous study showed that HM-1 (**22**), a cuparene-type sesquiterpene isolated from the Basidiomycoteous fungus *Helicobasidium mompa*, has also been isolated from *Bazzania*, a liverwort collected from Madagascar.[2] This showed that epiphytic and endophytic microorganisms of liverworts play a very important role not only in the biosynthesis of plant secondary metabolites but also in the protection of their host plant. Most liverworts are not consumed by insects, worms, and other predators. This is obviously linked to their ability to produce bioactive compounds.

In the course of our ongoing systematic search of microbial endophytes of liverworts that contribute to the production of bioactive compounds, we isolated an endophytic fungus, *Penicillium concentricum*, from the liverwort *Trochocolea tomentella* (Trichocoleaceae). The genus *Penicillium* is well-known to produce a variety of bioactive compounds including the clinically used antibiotic penicillin and the antifungal agent griseofulvin (**15**). The ethyl acetate extract from a fermentation of the fungal strain on potato dextrose agar (PDA) showed significant antiproliferative activity against HT-29 cells with an IC_{50} value of 2.2 μg/mL. Scale-up fermentation on PDA led to the isolation of epoxydon (**4**) as the major bioactive compound. To isolate epoxydon-related and unrelated new and bioactive compounds, *P. concentricum* was cultured on rice and on halogen salt (KCl or KBr)-supplemented rice media for 14 days. Successive normal, reversed-phase open, and flash silica gel (C_{18}) and high-performance liquid chromatography (HPLC) separation of the ethyl acetate fraction of the defatted methanol extract of *P. concentricum* fermented on rice medium afforded a new chlorinated xanthone, 6-chloro-3,8-dihydroxy-1-methylxanthone (**1**), together with 15 known compounds, identified as epoxydon (**4**),[3−7] norlichexanthone (**5**),[8−10] gentisyl alcohol (**9**),[11] gentisyl quinone (**10**),[12] (*R*,*S*)-1-phenyl-1,2-ethanediol (**11**),[13] dehydrodechlorogriseofulvin (**12**),[9] dechlorogriseofulvin (**13**),[9] dehydrogriseofulvin (**14**),[9]

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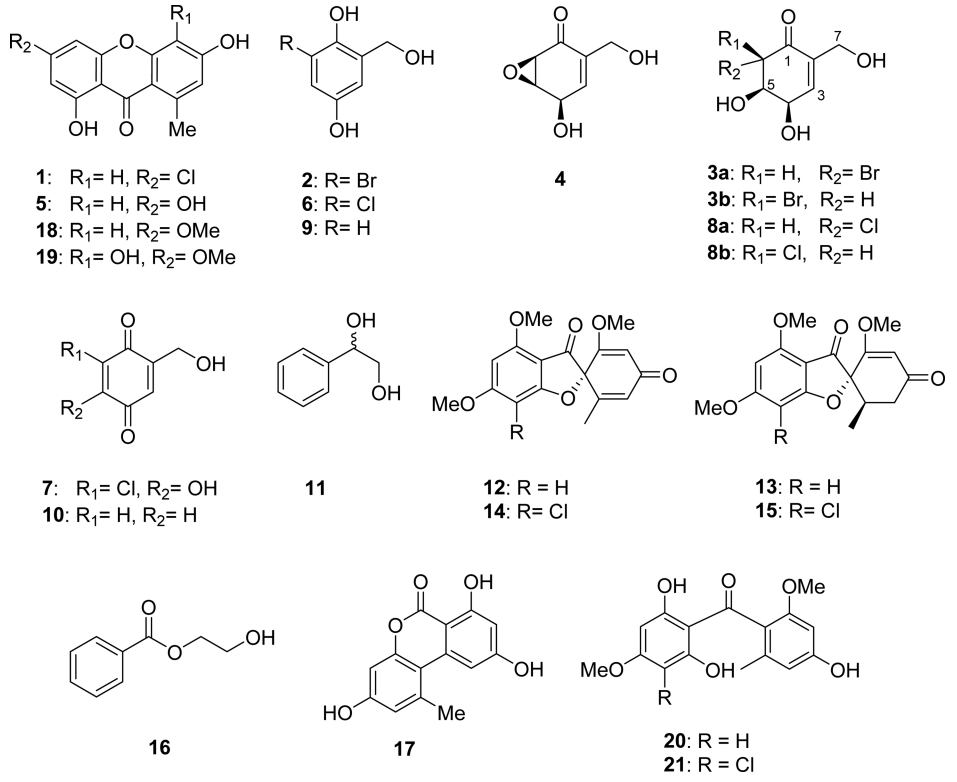
Chart 1



griseofulvin (**15**),[9] ethylene glycol benzoate (**16**),[14] alternariol (**17**),[15,16] griseoxanthone C (**18**),[10,17] drimiopsin H (**19**),[8] griseophenone C (**20**),[9] and griseophenone B (**21**).[9] Ethyl acetate extracts of the same strain fermented on KBr and KCl salt supplemented rice media afforded 2-bromogentisyl alcohol (**2**), 2-chlorogentisyl alcohol (**6**),[18,19] an epimeric mixture of *cis*- and *trans*-bromohydrins (**3a**, **3b**), and *trans*- and *cis*-chlorohydrins (**8a**, **8b**),[19,20] together with the four known compounds identified as **4**, **5**, **13**, and **15**. Herein, we report the isolation, structure elucidation, and antiproliferative activity evaluation of these compounds of *P. concentricum* and discuss the *in media* synthesis of halogenated compounds in halogen salt-rich media.

RESULTS AND DISCUSSION

Structure Elucidation of the New Compound 1 Isolated from *P. concentricum* Cultured on Rice Medium. Silica gel column chromatography and HPLC on octadecyl silyl silica gel (ODS) of the *P. concentricum* rice medium fermentation extract led to the isolation of one new chlorinated methylxanthone (**1**). Negative high-resolution electrospray ionization (HRESI) mass analysis of compound **1** exhibited two deprotonated molecular ion peaks at *m/z* 275.0119 $[M - H]^-$ (100%) and *m/z* 277.0086 $[M - H]^-$ (32%), with the ratio 3:1, indicating the presence of one chlorine atom in the molecule and corresponding to a molecular formula of $C_{14}H_8ClO_4^-$. The IR spectrum showed conjugated ketone stretching at ν_{max} 1616 cm^{-1} and absorption bands corresponding to aromatic (ν_{max} 1586, 1424 cm^{-1}) and hydroxy (ν_{max} 3402 cm^{-1}) functions. Its UV maxima (λ_{max} 240 and 310 nm) were very similar to those of methylxanthones previously isolated from *Penicillium* species.[7,8,21−23] The ^{1}H NMR spectroscopic data of **1** displayed resonances due to two sets of *meta*-coupled aromatic protons at δ 6.05 (d, J = 2.2 Hz, H-7), 6.15 (d, J = 2.2 Hz, H-5), 6.50 (d, J = 2.7 Hz, H-4), and 6.51 (d, J = 2.7 Hz, H-2) and one methyl group attached to an aromatic ring (δ 2.75, s, 3H, CH_3-10). Inspection of the ^{13}C NMR and HSQC spectra revealed the presence of signals for four aromatic methines (δ 94.8, C-5; 99.4, C-7; 102.2, C-4; 118.6, C-2), four oxygen-bearing aromatic carbons (δ 164.8, C-8; 164.9, C-3; 161.2, C-4a; 158.8, C-5a), four additional aromatic carbons (δ 144.2, C-1; 111.7, C-1a; 103.4, C-6 and C-8a), and one polyconjugated ketone carbonyl (δ 183.2) of a xanthone and suggested that compound **1** was a 1,3,6,8-tetrasubstituted methylxanthone. The presence of a hydroxy group at C-8 was deduced based on observation of the downfield hydrogen-bonded ^{1}H NMR (DMSO-d_6) signal at δ 14.1 ppm. The aromatic methyl group was attached to C-1 due to a long-range correlation observed from the methyl group at δ 2.75 to C-1, C-1a, C-2, C-4, C-4a, and C-9. Moreover, the cross-peak from the doublet aromatic proton at δ 6.51 ppm (H-2) to CH_3-10 (δ 23.7), C-3 (δ 164.9), C-4 (δ 102.2), and C-1a (δ 111.7) and the splitting pattern and coupling constant of H-2 and H-4 (both d, J = 2.7 Hz) confirmed the location of the hydroxy group at C-3. The second set of *meta*-coupled aromatic methines at H-5 and H-7 were assigned based on the observation of HMBC cross-peaks from the proton signal at δ 6.05 (H-7) to C-8, C-6 (δ 103.4), and C-5. In addition, comparison of the ^{13}C NMR spectroscopic data of **1** with those of 3,6,8-trihydroxy-1-methylxanthone (**5**) revealed that the downfield aromatic carbon signal at C-6 in δ 165.4 was replaced by a chlorine-bearing carbon signal at δ 103.4. From this evidence, the structure of **1** was concluded to be 6-chloro-3,8-dihydroxy-1-methylxanthone.

Structure Elucidation of Compounds Isolated from *P. concentricum* Cultured on Halogen Salt-Supplemented Rice Medium. Bioactive halogenated secondary metabolites such as griseofulvin, sclerotioramine (**23**),[24] and

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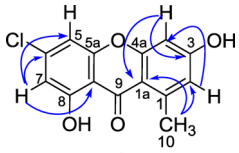

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Figure 1. Key HMBC correlations observed in compound **1**.

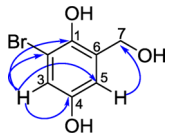

Figure 2. Key HMBC correlations observed in compound **2**.

bromoroquefortine C (**24**)[25] have been reported from *Penicillium* species. The isolation of the two chlorinated compounds (**1**, **15**) from the *Penicillium* fermented in rice medium suggested that the present strain produces a halogenase capable of introducing halogens in the secondary metabolites. In the aim to produce more halogenated compounds, we carried out fermentations in media using two halogen salts (KBr and KCl, respectively). From the culture containing KCl, we isolated the known chlorogentisyl alcohol (**6**) together with an epimeric mixture of chlorohydrin derivatives of epoxydon (**8a**, **8b**). A mixture of two bromohydrin derivatives, 6-dehydroxy-6α-bromogabosine C (**3a**, major) and 6-dehydroxy-6β-bromogabosine C (**3b**, minor), was isolated from KBr-supplemented medium along with the new bromogentisyl alcohol (**2**). Interestingly, griseofulvin was not produced when KBr was used (Figure S21, Supporting Information). This suggested that KBr could have inhibited the biosynthesis of **15**. In addition, mass spectroscopy analysis of the ethyl acetate extract obtained from the KBr salt-supplemented fermentation did not show any bromo derivatives of griseofulvin (data not shown).

Negative HRESI-MS data of compound **2** displayed two deprotonated molecular ion peaks at m/z 216.9503 $[M - H]^-$ (100%) and 218.9482 $[M - H]^-$ (97%), corresponding to a molecular formula of $C_7H_6BrO_3^-$. The IR spectrum exhibited absorption bands characteristic for hydroxy groups (3389 cm^{-1}) and an aromatic ring (1591, 1446, 1107, 1022 cm^{-1}), while the UV spectrum of **2** showed the presence of a substituted aromatic ring. The ^1H NMR spectrum of **2** exhibited signals due to two *meta*-coupled aromatic protons at δ 6.75 (d, J = 2.9 Hz, H-5) and δ 6.82 (d, J = 2.9 Hz, H-3) and an isolated oxygen-bearing methylene signal at δ 4.62 (s, H-7_{ab}). The ^{13}C NMR spectrum displayed resonances due to two aromatic methines at δ 115.2 (C-5) and 118.6 (C-3), four aromatic carbons at δ 111.8 (C-2), 132.1 (C-6), 145.5 (C-1), and 152.3 (C-4), and an oxygen-bearing methylene at δ 61.7 (C-7). The ^1H and ^{13}C NMR data of **2** are very similar to those of gentisyl alcohol (**9**) except for the presence of an AB aromatic system instead of the ABX system in **9**. The hydroxymethylene, bromine, and the two hydroxylated carbons were assigned to be at C-6, C-2, C-1, and C-4, respectively, due to the HMBC long-range correlations from the aromatic methine proton at δ 6.75 (H-5) to C-1, C-3, C-4, and C-7 together with the cross-peaks from δ 6.82 (H-3) to C-1, C-2, C-4, and C-5. Moreover, the upfield shift of the chemical shift of the aromatic carbon at δ 111.8 (C-2) confirmed that C-2 was brominated. Therefore, the structure of **2** was deduced to be 2-bromogentisyl alcohol.

Compounds **3a** and **3b** had the molecular formula $C_7H_9BrO_4$ as determined by positive HRESIMS, which showed typical brominated molecular ion peak patterns at m/z 258.9583 (100%) and m/z 260.9562 (97%), corresponding to [M + Na] and $[M + 2 + Na]^+$, respectively. Their structures were elucidated to be 6-dehydroxy-6α-bromogabosine C (**3a**, major) and 6-dehydroxy-6β-bromogabosine C (**3b**, minor) by analyses of their 1D- (including ^1H and TOCSY) and 2D-NMR spectroscopic data together with comparison of the data of the known chlorohydrins reported in the literature.[19,20] The ^1H signals of the major compound (**3a**) was assigned by the irradiation of the proton signal of H-6 (δ 4.33) in the 1D-TOCSY experiment, which led to the identification of the spin network H-3, H-4, H-5, and H-6. The corresponding carbon signals were identified by HSQC experiment. The attachment of the bromine group at C-6 was deduced by the ^1H and ^{13}C chemical shift values of H-6 (δ_H 4.33) and C-6 (δ_C 49.9). The HMBC correlation from the H-7 methylene group at δ 4.21 (dt, J = 2.69, 3.96 Hz, H-7) to the olefinic methine at δ_C143.0 (C-3), the carbonyl at C-1 (δ_C192.1), and the olefinic carbon at C-2 (δ_C137.3) confirmed the proposed planar structure. The assignment of the minor epimer was deduced by the same manner. The alpha (*trans*) and beta (*cis*) orientations of the bromine in **3a** and **3b**, respectively, were deduced from the coupling constant values of the proton resonances at C-6. As observed in the previously reported halohydrins,[20] the *trans* epimer (**3a**) displayed a wider coupling constant (4.9 Hz) than the *cis* counterpart (2.2 Hz).

There is an increased interest in *in culture* halogenation of microbial secondary metabolites. Recently, chlorinated epidithiodiketopiperazine (**25**) has been isolated from a marine-derived *Trichoderma* sp. TPU199 cultured in various concentrations of seawater media, while the brominated analogue (**26**) was detected during the utilization of NaBr salt dissolved in fresh water in the culture broth of TPU199.[26] It is obvious that the presence of the chemically reactive epoxide ring in the structure of the parent compound (**27**) facilitated non-enzymatic (pure chemical) halogenations. Interestingly, a similar chlorination pattern has been observed in the chlorinated epidithiodiketopiperazines penicisulfuranols A and D produced by the mangrove endophytic fungus *Penicillium janthinellum* HDN13-309 cultured in liquid medium containing seawater. This suggested that an epoxide-bearing molecule (not isolated during the study) could have facilitated the chlorination of one of the epoxide-bearing carbons.[27] Furthermore, Nabeta and co-workers reported that the formation of chlorinated gentisyl alcohol is a nonenzymatic process.[19] Similar processes have been performed during the present study to confirm the nonenzymatic production of **2**, **3a**, **3b**, **8a**, and **8b** (Scheme S1, Supporting Information). Although formation of halohydrins has been observed during the present chemical conversion study of epoxydon, regeneration of **4** via an intramolecular S_N2 attack by the nonbonding electron pair on the bromohydrin oxygen to the adjacent electrophilic carbon has been observed (Scheme S2, Supporting Information). Excess KBr in the solution prevented the regeneration of epoxydon. Halogen-initiated epoxide ring opening has been reported to be a stereoselective process that yields the corresponding *trans*-halohydrin as the major product.[28−30] During the present study, the favored *trans* chloro- and *trans*-bromohydrins were detected as major stereoisomers of the

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Chart 2

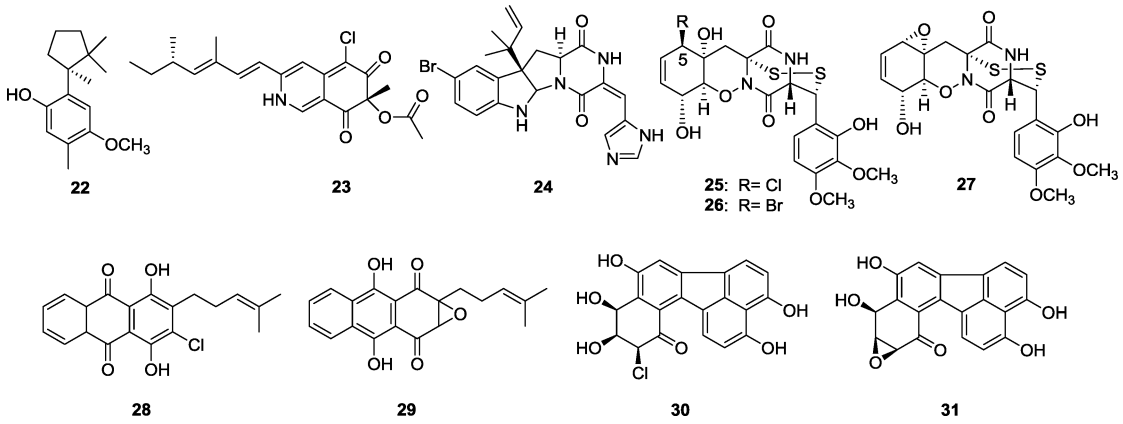

Table 1. NMR Spectroscopic Data for Compounds 1 and Norlichexanthone (5)

	1[a]				5[b]		
position	δ_C, type	δ_H	(J in Hz)	HMBC	δ_C, type	δ_H	(J in Hz)
1	144.2, C				144.8		
1a	111.6, C				112.6		
2	118.6, CH	6.51	d (2.7)	1a, 4, 4a, 9, 10	117.6	6.59	br
3	164.9, C				164.2		
4	102.2, CH	6.50	d (2.7)	1a, 2, 3, 4a, 9, 10	101.8	6.59	br
4a	161.2, C				161.0		
5	94.8, CH	6.15	d (2.2)	5a, 7, 8, 8a, 9	94.5	6.22	d (2.0)
5a	158.8, C				158.7		
6	103.4, C				165.4		
7	99.4, CH	6.05	d (2.2)	5, 6, 8	99.1	6.11	d (2.0)
8	164.8, C				164.9		
8a	103.4, C				103.9		
9	183.2, C				183.5		
10-Me	23.7, CH_3	2.75	s	1, 1a, 2, 4, 4a, 9	23.7	2.77	s

[a]Measured in methanol-d_4 (^{1}H: 700 MHz and ^{13}C: 175 MHz). [b]Measured in acetone-d_6 (^{1}H: 500 MHz and ^{13}C: 125 MHz).

chemical conversion, confirming the reported favored nonenzymatic reaction.[30] Similarly, abiotic synthesis of anthrasesamone C (**28**) from its epoxide-containing precursor 2,3-epoxyanthrasesamone B (**29**) via a nucleophilic addition reaction of chloride anion has been previously studied by Furumoto and Hoshikuma.[31] This pathway was in agreement with the previously reported nonenzymatic synthesis of chlorogentisyl alcohol from epoxydon using chloride ion-rich media and the present results on brominated gentisyl alcohol (**2**).[19]

Halogen salts have been widely used in the fermentation of microorganisms in order to obtain halogenated compounds. It is worth noting that attention should be paid when the strain has been known to produce epoxide-containing compounds or the halogen in the isolated metabolite is alpha-attached to an oxygen-bearing carbon. All of the halogen groups present in the compounds isolated during the halogen incorporation[26,27] are attached at one of the carbons that bears the epoxide.

Most of the isolated compounds in this study (except **7**, **10**, and **14**) have been evaluated for cytotoxicity against HT-29 (colon) and/or MCF-7 (breast) cancer cell lines (Table 3). Among the compounds tested, epoxydon (**4**) was the most active compound against MCF-7 cells (IC_{50} 5.7 μM), while gentisyl alcohol (**9**) showed moderate and weak antiproliferative activities against HT-29 and MCF-7, with IC_{50} values of 6.4 and 17.1 μM, respectively. Brominated gentisyl alcohol (**2**) was more active against the MCF-7 cell line (IC_{50} 8.4 μM) than its chlorine counterpart (**6**, IC_{50} 14.9 μM).

Table 2. NMR Spectroscopic Data for Compound 2

position	δ_C, type	δ_H	(J in Hz)	HMBC
1	145.5, C			
2	111.8, C			
3	118.6, CH	6.82	d (2.9)	1, 2, 4, 5
4	152.3, C			
5	115.2, CH	6.75	d (2.9)	1, 3, 4, 7
6	132.1, C			
7	61.7, CH_2	4.62	s	1, 5, 6

[a]Measured in methanol-d_4 (^{1}H: 400 MHz and ^{13}C: 100 MHz).

EXPERIMENTAL SECTION

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded at 25 °C with a Bruker Avance III 400 HD NMR spectrometer and Bruker Avance III HD Ascend 700 MHz. High-resolution mass spectra were acquired with a Thermo LTQ Orbitrap (analyzer: ITMS and FTMS, mass range: 50−4000 m/z, resolution: 7500−100 000). High-performance liquid chromatography was performed with a Hitachi Primaide HPLC (Hitachi High-Technologies Corporation, Tokyo, Japan) equipped with a Primaide 1430 diode array detector, Primaide 1210 autosampler, and Primaide 1110 pump with a degasser and a semipreparative C_{18} column (Dynamax C_{18} HPLC column 10 × 250 mm). A Shimadzu HPLC system with an LC-

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Table 3. Cytotoxicity Data of the Compounds Isolated from *Penicillium concentricum* on Human Breast (MCF-7) and Colon (HT-29) Cancer Cell Lines

compound	MCF-7[a]	HT-29[a]
1	>20	NT[b]
2	8.4	NT
3	9.7	NT
4	5.7	14.1
5	>20	>20
6	14.9	NT
7	NT	NT
8	>20	NT
9	17.1	6.4
10	NT	NT
11	>20	>20
12	>20	>20
13	>20	>20
14	NT	NT
15	13.9	>20
16	>20	>20
17	>20	>20
18	>20	>20
19	>20	>20
20	>20	>20
21	>20	>20
camptotecin	0.073	NT
paclitaxel	NT	0.0014

[a]IC_{50} in μM. [b]NT: Not tested.

10AD vp oump, an SCL-10A vp system controller, an SPD- M10A vp UV/vis detector, and a DGU-14A degasser were used.

Fungal Source. The endophytic fungus (strain 4E-4) was isolated from the healthy liverwort *Trichocolea tomentella* collected in Newport, Virginia (N 37.27279; W 80.52425). Leaves of *T. tomentella* were washed with running tap water and investigated microscopically for surface damage and disease symptoms. Ten healthy leaves were selected for surface disinfection by stepwise soaking in 2% sodium hypochlorite solution for 1 min, followed by 70% ethanol for 30 s, and two rinses with sterile distilled water. To confirm successful surface disinfection, disinfected leaves were pressed onto PDA plates, the final rinse was plated onto the same medium, and the plates were examined for growth after incubation at room temperature for 3, 7, and 14 days. Surface-disinfected leaves were cut into small pieces (about 1 mm × 1 mm) with a sterilized blade and placed on PDA plates. Incubation was carried out at room temperature for 3 to 14 days to allow growth of microbial endophytes. *Penicllium* (which appeared on the seventh day of incubation) was purified by streaking single colonies onto PDA agar. The fungus was identified by morphological inspection (data not shown) and sequencing of the ITS regions of the rRNA locus. The ITS sequences of the liverwort-derived fungus showed 100% identity to that of *P. concentricum* (GenBank accession KM023345.1 and DQ339561.1) and *P. coprophilium* (GenBank accession AJ608952.1). The isolated fungus also showed high similarity to *P. griseofulvum* (99% identity to GenBank accession KR703615.1) and *P. chrysogenum* (99% identity to GenBank accession KU925907.1). A voucher specimen (4E-4) was deposited in the Division of Medicinal Chemistry and Pharmacognosy of the College of Pharmacy, OSU.

Fermentation, Extraction, and Isolation of Secondary Metabolites from Rice Culture Medium. *P. concentricum* was first cultured on PDA medium for 5 days at room temperature, and then two 0.5 × 0.5 cm of agar containing fungal hyphae were added to a sterilized 500 mL Erlenmeyer flask containing potato dextrose broth for 3 days in a shaker (150 rpm) at room temperature. One milliliter of the fungal culture was then seeded to a 500 mL Erlenmeyer flask containing serilized rice medium. The cultivation was left static at room temperature for another 14 days and stopped by adding EtOAc. The fungal culture was then soaked with 1 L of EtOAc and left for 1 day at room temperature. The procedure was repeated three times, yielding about 3 L of EtOAc extract. The resulting EtOAc extract was evaporated under vacuum using a rotary evaporator to yield a brownish residue (3.5 g). A portion of the concentrated extract was suspended in methanol and partitioned with hexane (3 × 200 mL) to afford 1.6 g of hexanes and 589 mg of methanol fractions. The methanol fraction exhibited an ED_{50} value of 7 μg/mL against the human breast adenocarcinoma cell line (MCF-7). This bioactive MeOH fraction was subsequently fractionated on reversed-phase silica gel liquid chromatography and eluted with 70% aqueous methanol (2 × 150 mL) and MeOH (2 × 150 mL) to yield four fractions, F-1 (360.85 mg), F-2 (31.71 mg), F-3 (56.41 mg), and F-4 (7.47 mg), of which two fractions (F-2 and F-3) were active against MCF-7 cells with ED_{50} values of 17.5 and 15.1 μg/mL, respectively. F-1 was subjected to silica gel column chromatography [2.2 cm × 28 cm; solvent system: hexane−EtOAc (3:2)] to give seven subfractions (F-1a through F-1g). Cytotoxicity evaluation of these fractions revealed that F-1c and F-1g were active, with ED_{50} values of 11 and 12.1 μg/mL against MCF-7 cells, respectively. Fraction F-1c (43.63 mg) was subjected to a semipreparative RP-18 HPLC on a Dynamax C_{18} HPLC column (10 × 250 mm; flow rate: 2 mL/min) with a gradient mixture of H_2O−MeOH from 60:40 to 0:100 for 42 min to give compounds **1** (1.8 mg), **17** (2.4 mg), **20** (6.8 mg), and **21** (4.9 mg). HPLC using an octadecyl silyl silica gel column (ADV5013 C_{18}, 5 μm 4.6 × 250 cm; flow rate: 1 mL/min) with a H_2O−MeOH gradient (from 90:10 to 0:100 for 35 min) of the bioactive fraction F-1f (ED_{50} 15.4 μg/mL, MCF-7) led to the isolation of gentisyl alcohol (**9**) (3.8 mg), gentisyl quinone (**10**) (0.8 mg), and (*R,S*)-1-phenyl-1,2-ethanediol (**11**) (2.2 mg). Silica gel column chromatography of F-1g [182.8 mg, column size: 1.5 cm × 25 cm; solvent system: EtOAc−hexane (3:2)] yielded five fractions (F-1g-I to F-1g-V). HPLC analysis of the five fractions showed that fraction F-1g-III contained four compounds (**12**−**15**), identified as dehydro-dechlorogriseofulvin (**12**) (2.6 mg), dechlorogriseofulvin (**13**) (4.1 mg), dehydrogriseofulvin (**14**), and griseofulvin (**15**). Compounds **14** and **15** were obtained as a mixture (6.8 mg, **14**/**15** in 1:4 ratio) exhibiting an IC_{50} value of 4.9 μg/mL against MCF-7 cells. To get some idea about the activity of dehydrogriseofulvin (**14**), the standard griseofulvin (**15**) was tested against the two cell lines. HPLC of fraction F-2 yielded compound **5** (norlichexanthone, 4.4 mg) and **16** (ethylene glycol benzoate, 3.7 mg). Fraction F-3 was subjected to semipreparative RP-18 HPLC (Dynamax C18 HPLC column 10 × 250 mm; flow rate: 2 mL/min) using a H_2O−MeOH gradient (from 50:50 to 0:100 for 40 min) to afford drimiopsin H (**19**, 8.4 mg). HPLC on RP C_{18} of the hexanes-soluble fraction gave compounds **4** (0.7 mg), **5** (1.7 mg), **9** (0.9 mg), **12** (2.3 mg), **13** (1.2 mg), **14**, **15** (7.2 mg), **19** (1.9 mg), **20** (1.3 mg), and **21** (1.1 mg).

*6-Chloro-3,8-dihydroxy-1-methylxanthone (**1**):* yellow, amorphous semisolid; UV (MeOH) λ_{max} (log ε) 242 (4.26), 310 (3.97) nm; IR (film) ν_{max} 3402, 1616, 1586, 1424 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 275.0119 and 277.0086 (calcd for $C_{14}H_8ClO_4^-$ $[M - H]^-$, 275.0116 and 277.0087).

Fermentation, Extraction, and Isolation of Chlorinated Compounds from KCl-Supplemented Rice Culture Medium. The production of chlorinated compounds and other secondary metabolites was accomplished by incubation of two 0.5 × 0.5 cm of agar containing *P. concentricum* hyphae in a 500 mL Erlenmeyer flasks containing rice medium (250 mL) and KCl at rt for 14 days. The whole rice culture of the fungal strain was extracted three times with EtOAc, and the solution was evaporated under reduced pressure. The obtained crude ethyl acetate extract (2.1 g) was subjected to HPLC analysis using a Dynamax C18 HPLC column (10 × 250 mm; flow rate: 2 mL/min) with a gradient of H_2O−MeOH (from 95:5 to 0:100 for 38 min) as eluting system to afford **4** (0.9 mg), **5** (2.1 mg), **6** (3.2 mg), **8a** and **8b** mixture (1.2 mg), **13** (1.9 mg), and **15** (4.9 mg) (Figure S19).

Fermentation, Extraction, and Isolation of Brominated Compounds from KBr-Supplemented Rice Culture Medium. The fermentation of the fungal strain with KBr was carried out in a

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similar manner to the fermentation described with KCl. The fermented rice material with KBr was extracted repeatedly with EtOAc, and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (1.7 g). The extract was separated by semipreparative C18 reversed-phase HPLC with H2O−MeOH gradient (from 95:5 to 0:100 for 38 min) to yield four compounds, determined as: **2** (5.6 mg), **3a** and **3b** mixture (3.8 mg), **4** (1.1 mg), and **13** (0.8 mg) (Figure S21).

*2-Bromogentisyl alcohol (**2**):* brown, amorphous solid powder; UV (MeOH) λ_{max} (log ε) 298 (3.42) nm; IR (film) ν_{max} 3389, 1591, 1446, 1107, 1022 cm^{-1}; ^1H and ^{13}C NMR data, see Table 2; HRESIMS *m/z* 216.9503 and 218.9482 (calcd for $C_7H_6BrO_3^-$, 216.9505 and 218.9485).

*6-Dehydroxy-6α-bromogabosine C (**3a**, major):* ^1H NMR (400 MHz, CD_3OD) δ 4.21 (2H, dt, *J* = 2.69, 3.96 Hz, H-7), 4.22 (1H, dd, *J* = 1.9, 2.0 Hz, H-5), 4.33 (1H, d, *J* = 4.9 Hz, H-6), 4.78 (1H, ddd, *J* = 2.0, 2.7, 4.9 Hz, H-4), 6.78 (1H, ddd, *J* = 1.9, 2.5, 2.7 Hz, H-3); ^{13}C NMR (100 MHz, CD_3OD) 49.9 (C-6), 59.9 (C-7), 67.0 (C-4), 75.0 (C-5), 137.3 (C-2), 143.0 (C-3), 192.19 (C-1); HRESIMS *m/z* 258.9583 and 260.9562 (calcd for $C_7H_9BrO_4Na^+$, 258.9576 and 260.9556).

*6-Dehydroxy-6β-bromogabosine C (**3b**, minor):* ^1H NMR (400 MHz, CD_3OD) δ 4.21 (2H, t, *J* = 2.5 Hz, H-7), 4.46 (1H, dt, *J* = 2.3, 3.6 Hz, H-5), 4.72 (1H, ddd, *J* = 2.2, 2.3, 2.5 Hz, H-4), 5.17 (1H, d, *J* = 2.2 Hz, H-6), 6.74 (1H, ddd, *J* = 2.2, 2.5, 3.6 Hz, H-3); ^{13}C NMR (100 MHz, CD_3OD) 59.0 (C-7), 59.6 (C-6), 70.2 (C-4), 78.7 (C-5), 137.3 (C-2), 143.0 (C-3), 191.13 (C-1); HRESIMS *m/z* $[M + Na]^+$ 258.9583 and 260.9562 (calcd for $C_7H_9BrO_4Na^+$, 258.9576 and 260.9556).

Antiproliferative Bioassay. The HT-29 (colon cancer) and MCF-7 (breast cancer) cell line antiproliferative bioassays were performed as previously reported, while the MCF-7 breast cancer cell line antiproliferative assay was carried out according to a published protocol.[32,33] Paclitaxel (IC_{50} 0.0014 μM) and camptotecin (IC_{50} 0.073 μM) were used as the positive controls for inhibition of HT-29 and MCF-7 cells, respectively.

Preparation of Halogenated Derivatives of Epoxydon. Epoxydon (**4**, 26 mg) isolated from a 3-day fermentation of *P. concentricum* on rice medium was dissolved in methanol (5 mL) and allowed to stand under UV light for 2 h. The mixture was then stirred at room temperature, and the reaction was monitored periodically at intervals of 1, 3, 12, and 24 h using thin-layer chromatography. The crude reaction mixture was filtered and evaporated under vacuum. The residue was then purified by silica gel chromatography to give the halogenated derivatives.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b01069.

Mass and 1D- and 2D-NMR spectra of compounds **1**−**3**, as well as additional experimental protocols (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +1-614-292-4733. Fax: +1-614-292-4733. E-mail: Rakotondraibe.1@osu.edu.

ORCID

L. Harinantenaina Rakotondraibe: 0000-0003-2166-4992

Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Asakawa, Y.; Ludwiczuk, A.; Nagashima, F.; Toyota, M.; Hashimoto, T.; Tori, M.; Fukuyama, Y.; Harinantenaina, L. *Heterocycles* **2009**, *77*, 99−150.

(2) Harinantenaina, L.; Kurata, R.; Asakawa, Y. *Chem. Pharm. Bull.* **2005**, *53*, 515−518.

(3) Klemke, C.; Kehraus, S.; Wright, A. D.; Konig, G. M. *J. Nat. Prod.* **2004**, *67*, 1058−1063.

(4) Hussain, H.; Root, N.; Jabeen, F.; Al-Harrasi, A.; Al-Rawahi, A.; Ahmad, M.; Hassan, Z.; Abbas, G.; Mabood, F.; Shah, A.; Badshah, A.; Khan, A.; Ahmad, R.; Green, I. R.; Draeger, S.; Schulz, B.; Krohn, K. *Chin. Chem. Lett.* **2014**, *25*, 1577−1579.

(5) Sekiguchi, J.; Gaucher, G. M. *Biochem. J.* **1979**, *182*, 445−453.

(6) Deshmukh, S. K.; Verekar, S. A.; Bhave, S. V. *Front. Microbiol.* **2014**, *5*, 715.

(7) Venkatasubbaiah, P.; Van Dyke, C. G.; Chilton, W. S. *Mycologia* **1992**, *84*, 715−723.

(8) Zhuang, Y. B.; Yin, H.; Zhang, X. W.; Zhou, W.; Liu, T. *Helv. Chim. Acta* **2015**, *98*, 699−703.

(9) Cacho, R. A.; Chooi, Y. H.; Zhou, H.; Tang, Y. *ACS Chem. Biol.* **2013**, *8*, 2322−2330.

(10) Mutanyatta, J.; Matapa, B. G.; Shushu, D. D.; Abegaza, B. M. *Phytochemistry* **2003**, *62*, 797−804.

(11) Chen, L.; Fang, Y. C.; Zhu, T. J.; Gu, Q. Q.; Zhu, W. M. *J. Nat. Prod.* **2008**, *71*, 66−70.

(12) Uchida, R.; Tomoda, H.; Arai, M.; Omura, S. *J. Antibiot.* **2001**, *54*, 882−889.

(13) Chen, X.; Mei, T.; Cui, Y. F.; Chen, Q. J.; Liu, X. T.; Feng, J. H.; Wu, Q. Q.; Zhu, D. M. *ChemistryOpen* **2015**, *4*, 483−488.

(14) Wakita, N.; Hara, S. *Tetrahedron* **2010**, *66*, 7939−7945.

(15) Sun, J. Y.; Awakawa, T.; Noguchi, H.; Abe, I. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 6397−6400.

(16) Koch, K.; Podlech, J.; Pfeiffer, E.; Metzler, M. *J. Org. Chem.* **2005**, *70*, 3275−3276.

(17) Mulholland, D. A.; Koorbanally, C.; Crouch, N. R.; Sandor, P. *J. Nat. Prod.* **2004**, *67*, 1726−1728.

(18) Yun, K.; Kondempudi, C. M.; Choi, H. D.; Kang, J. S.; Son, B. W. *Chem. Pharm. Bull.* **2011**, *59*, 499−501.

(19) Nabeta, K.; Ichihara, A.; Sakamura, S. *Agric. Biol. Chem.* **1975**, *39*, 409−413.

(20) Evidente, A.; Sparapano, L.; Andolfi, A.; Bruno, G.; Giordano, F. *Phytopathol. Mediterr.* **2000**, *39*, 299−309.

(21) Belofsky, G. N.; Gloer, K. B.; Gloer, J. B.; Wicklow, D. T.; Dowd, P. F. *J. Nat. Prod.* **1998**, *61*, 1115−1119.

(22) Broadbent, D.; Mabelis, R. P.; Spencer, H. *Phytochemistry* **1975**, *14*, 2082−2083.

(23) Rezanka, T.; Rezanka, P.; Sigler, K. *J. Nat. Prod.* **2008**, *71*, 820−823.

(24) Yuan, W. H.; Wei, Z. W.; Dai, P.; Wu, H.; Zhao, Y. X.; Zhang, M. M.; Jiang, N.; Zheng, W. F. *Chem. Biodiversity* **2014**, *11*, 1078−1087.

(25) Da Silva, J. V.; Fill, T. P.; Lotufo, L. V.; Pessoa, C. D.; Rodrigues, E. *Helv. Chim. Acta* **2014**, *97*, 1345−1353.

(26) Yamazaki, H.; Rotinsulu, H.; Narita, R.; Takahashi, R.; Namikoshi, M.; Rotinsulu, H. *J. Nat. Prod.* **2015**, *78*, 2319−2321.

(27) Zhu, M.; Zhang, X.; Feng, H.; Dai, J.; Li, J.; Che, Q.; Gu, Q.; Zhu, T.; Li, D. *J. Nat. Prod.* **2017**, *80*, 71−75.

(28) Evidente, A.; Maddau, L.; Scanu, B.; Andolfi, A.; Masi, M.; Motta, A.; Tuzi, A. *J. Nat. Prod.* **2011**, *74*, 757−763.

(29) Dawe, R. D.; Molinski, T. F.; Turner, J. V. *Tetrahedron Lett.* **1984**, *25*, 2061−2064.

DOI: 10.1021/acs.jnatprod.6b01069
J. Nat. Prod. XXXX, XXX, XXX−XXX

(30) Du, L.; King, J. B.; Cichewicz, R. H. *J. Nat. Prod.* **2014**, *77*, 2454−2458.
(31) Furumoto, T.; Hoshikuma, A. *Biosci., Biotechnol., Biochem.* **2012**, *76*, 305−308.
(32) Addo, E. M.; Chai, H.-B.; Hymete, A.; Yeshak, M. Y.; Slebodnick, C.; Kingston, D. G. I.; Rakotondraibe, L. H. *J. Nat. Prod.* **2015**, *78*, 827−835.
(33) Vichai, V.; Kirtikara, K. *Nat. Protoc.* **2006**, *1*, 1112−1116.

DOI: 10.1021/acs.jnatprod.6b01069
J. Nat. Prod. XXXX, XXX, XXX−XXX