CYTOTOXIC CONSTITUENTS FROM THE ENDOLICHENIC FUNGUS *Hypoxylon polyporus*

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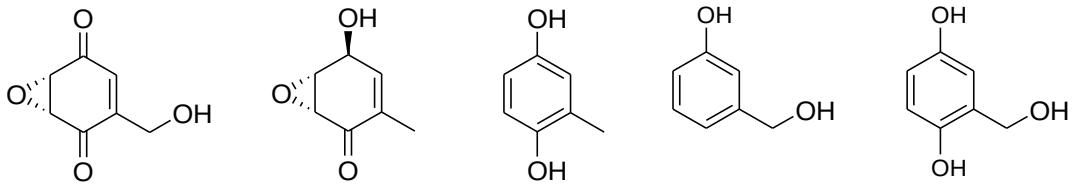
INTRODUCTION

Recent studies have demonstrated that endolichenic fungi are rich sources of structurally diverse small-molecule natural products, some with interesting biological activities (Wijeratne *et al*., 2012). In our continuing search for bioactive and/or novel metabolites of plant- and lichen-associated fungi we investigated the endolichenic fungi, *Hypoxylon polyporus* inhabiting in the thallus of the lichen *Cladonia leporina* (Lecanorales) collected at the Archbold research Station Florida, USA. Although few chemotaxonomic evaluations have been carried out (Bitzer *et al*., 2008; Hsieh *et al*., 2005) this constitutes the first report of bioactivity directed chemical investigation of *H. polyporus*. Herein we report the bioactivity directed fractionation, isolation of cytotoxic (-)-phyllostine (**1**) and toluhydroquinone (**3**) and other constituents **2, 4**, and **5** from ethyl acetate (EtOAc) extract of a culture broth of *H. polyporus*.



(**1**) (**2**) (**3**) (**4**) (**5**)

METHODOLOGY

General Experimental Procedures. 1D and 2D NMR spectra were recorded with a Bruker Avance III 400 spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR using residual solvent as an internal reference. Low-resolution MS were recorded on Shimadzu LCMS-QP8000α. Analytical and preparative thin layer chromatography (TLC) were performed on pre-coated 0.25 mm thick plates of silica gel 60 F_{254}.

Cultivation and Isolation of Metabolites of Hypoxylon polyporus. A seed culture of the fungus *H. polyporus* (FL1289) grown on PDA for two weeks was used for inoculation. Mycelia were scraped out, mixed with sterile water, and filtered through a 100 μm filter to separate spores from the mycelia. Absorbance of the spore solution was measured (at 600 nm) and adjusted to between 0.3 and 0.5. This spore solution was used to inoculate 2.0 L Erlenmeyer flask holding 1.0 L of the potato dextrose broth (PDB) medium containing 0.25 mM $CuSO_4$ and incubated at 160 rpm and 28 °C until the glucose in the medium was completely consumed (glucose level was monitored using URISCAN glucose strips). Mycelia were then separated by filtration, and the filtrate was neutralized (pH 7.0) and extracted with EtOAc (3 × 0.5 L). The combined EtOAc layer was washed with water, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure to give the crude EtOAc extract (521.0 mg), which showed cytotoxic activity. A portion of this extract (455.0 mg) was dissolved in 80% aq. MeOH and partitioned with hexanes to give hexanes fraction (1.7 mg). The 80% aq. MeOH fraction was diluted with water to make it to 50% aq. MeOH and partitioned with $CHCl_3$ to yield the $CHCl_3$ fraction (90.4 mg) and 50% aq. MeOH fraction (326.0 mg). A portion of the cytotoxic 50% aq. MeOH fraction (292.0 mg) was subjected to chromatography

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over a column of SiO_2 made up in CH_2Cl_2 and eluted with CH_2Cl_2 and CH_2Cl_2 containing increasing amounts of MeOH and the resulted column fractions were pooled according to their TLC patterns to yield five major fractions, $F_1 – F_5$. The fraction F_2 which showed strong cytotoxicity against all five cancer cell lines used at 5μg/mL was subjected to prep TLC (eluent; 6% MeOH in CH_2Cl_2) to isolate (-)-phyllostine (**1**), (+)-epiepoformin (**2**) and 2-methylbenzene-1,4-diol (toluhydroquinone) (**3**). Fraction F_4 was found to be a single compound by TLC and the analysis of spectroscopic data revealed that it is 3-hydroxymethylphenol (**4**). Fraction F_5 yielded 2-hydroxymethylbenzene-1,4-diol (gentisyl alcohol) (**5**) on crystallization with $MeOH/CH_2Cl_2$.

Cytotoxicity Assay. Relative cell growth and survival were measured in 96-well microplate format using the fluorescent detection of resazurin (AlamarBlue) dye reduction as an end point. Extracts, fractions and compounds were tested against human non-small-cell lung cancer (NCI-H460), metastatic prostate adenocarcinoma (PC-3M), CNS glioma (SF-268), breast cancer (MCF-7), and human metastatic breast adenocarcinoma (MDA-MB-231). Serial dilutions of test compounds or vehicle control (DMSO) were added to triplicate wells. After 72 h incubation, dye solution was added to each well (1:10 dilution). After brief agitation, incubation was continued for 4 h at 37 °C before data acquisition using a microplate fluorometer (Ex/Em: 560/590). Mean fluorescence intensity per well as a measure of relative viable cell number in compound-treated wells was compared to that of DMSO treated wells. The conventional chemotherapeutic drug doxorubicin served as a positive control.

RESULTS AND DISCUSSION

Compound **1** was obtained as a colorless oil. ^{1}H and ^{13}C NMR (DMSO-d_6) together with HSQC data of **1** revealed that it contained a hydroxymethylene group [δ_H 4.17 (1H, ddd, J = 18.6, 5.5, 2.1 Hz), 4.29 (1H, ddd, J = 18.6, 5.5, 2.3 Hz); δ_C 57.2], three methines of which one was vinylic, [δ_H 6.95 (m); δ_C 129.6] and other two were oxygenated, [δ_H 3.94 (dd, J = 4.0, 2.5); δ_C 53.9 and δ_H 3.97 (d, J = 4.0); δ_C 54.0] and three quaternary carbons of which one was vinylic, (δ_C 149.7) and other two were carbonyls (δ_C 191.9 and 192.1). The OH protons of hydroxymethylene appeared at δ 5.41 (t, J = 5.5 Hz). These data accounted for $C_7H_6O_3$. The low resolution APCI-MS, m/z 155 $[M+H]^+$ indicated the presence of an additional oxygen atom in the molecule suggesting an epoxide ring incorporating the two oxygenated methines at δ_H 3.94 and 3.97. The hydroxymethylene protons at δ 4.17 and 4.29 showed HMBC correlations to both vinylic carbons at δ 129.6 and 149.7 and to carbonyl carbon at δ 191.7 placing the CH_2OH group on the quaternary vinylic carbon. Thus the planar structure of **1** was determined as 3-(hydroxymethyl)-7-oxa-bicyclo[4.1.0]hept-3-ene-2,5-dione. Comparison of spectral data with those of reported (Okamura *et al*., 2003) confirmed its identity as (-)-phyllostine (**1**).

Compound 2 was obtained as a colorless film. Its 1H NMR data (CDCl3) showed a close resemblance to 1. The major difference was found to be the absence of a CH2OH group in 2; instead it showed the presence of a CH3 group at δ 1.83 (t, J = 1.2 Hz) and an additional hydroxymethine group [δ 4.66 (brs)]. Thus the planar structure of compound 2 was determined as 5-hydroxy-3-methyl-7-oxa-bicyclo[4.1.0]hept-3-en-2-one. Comparison of spectral data of 2 with those reported (Okamura et al., 2003) confirmed its identity as (+)-epiepoformin (2).

Compound **3** was obtained as a colorless oil. ^{1}H NMR spectrum of **3** showed the presence of a 1,2,4-trisubstituted benzene ring [δ 6.55 (1H, d, J = 8.6 Hz), 6.53 (1H, d, J = 3.0 Hz), 6.44 (1H, dd, J = 8.6, 3.0 Hz)] and a methyl group attached to a benzene ring (δ 2.11, s). ^{13}C NMR spectrum showed three aromatic quaternary carbons of which two are oxygenated (δ 149.6 and 147.9) indicating the presence of two OH groups while the signal at δ 125.5 accounted for the quaternary C bearing the CH_3. The remaining aromatic methines appeared at δ 117.6, 115.5 and 113.0. The signal at δ 16.2 indicated the presence of aromatic CH_3 group.

Comparison of the NMR data of this compound with those of reported (Biorad NMR database) led to the identification of **3** as 2-methylbenzene-1,4-diol (toluhydroquinone).

Compound **4** was obtained as off white oil. ^{1}H and ^{13}C NMR spectra (DMSO-d_6) together with HSQC data indicated the presence of a phenolic OH [δ_H 9.31 (1H, s); δ_C 157.3], 1,3-disubstituted benzene ring {[δ_H 7.08 (1H, t, J = 7.8 Hz); δ_C 129.0], [δ_H 6.74 (1H, m); δ_C 113.2], [δ_H 6.70 (1H, br d, J = 7.5 Hz); δ_C 116.9], [δ_H 6.60 (1H, br dd, J = 8.0, 2.5 Hz); δ_C 113.5]} and a hydroxymethyl group [δ 4.40 (2H, d, J = 5.8 Hz, CH_2), 5.10 (1H, t, J = 5.8 Hz, O*H*); δ_C 62.9]. The aromatic quaternary carbon bearing hydroxymethyl group appeared at δ_C 144.3. Thus compound **4** was identified as 3-hydroxymethyl phenol. Comparison of ^{13}C NMR data of **4** with those of reported (Mikami *et al*., 1996) confirmed its identity.

Compound **5** was obtained as white crystalline solid. ^{1}H NMR spectrum (DMSO-d_6) of **5** indicated that it consisted of two phenolic OH groups [δ 8.55 and 8.54 (1H each, s)] 1,2,4-trisubstituted benzene ring [δ 6.73 (1H, d, J = 3.0 Hz), 6.53 (1H, d, J = 8.5 Hz), 6.40 (1H, dd, J = 8.5, 3.0 Hz)], OH group [δ 4.89 (1H, br)] and hydroxymethylene group [δ 4.39 (2H, d, J = 4.2 Hz)]. Thus the structure of **5** was elucidated as 2-hydroxymethylbenzene-1,4-diol (gentisyl alcohol). Comparison of ^{1}H NMR data with those reported (Sakamura *et al*., 1971) confirmed its identity.

Compounds **1**, **2,** and **3** were evaluated for cytotoxicity against five cancer cell lines and the results are summarized in Table 1. Doxorubicin served as the positive control.

Table 1. Cytotoxicity (AlamarBlue®) assay data of compounds **1**–**3**.

Compound	Concentration		% Inhibition				
	(μg/mL)	(μM)	PC-3M	NCI-H460	SF-268	MCF-7	MDA-MB-231
1	1.25	0.008	90.4	99.9	94.8	99.3	79.4
2	1.25	0.009	3.6	-4.2	-5.8	-1.6	-14.3
3	1.25	0.010	-14.2	-10.6	78.2	20.4	25.0
Doxorubicin		1.000	77.4	96.7	78.8	84.9	63.6

It was found that (-)-phyllostine (**1**) showed strong cytotoxic activity against all five cell lines while toluhydroquinone (**3)** showed selective cytotoxicity against SF-268.

CONCLUSIONS

Bioactivity-guided fractionation of *H. polyporus* resulted in the isolation of two cytotoxic constituents, (-)-phyllostine (**1**) and toluhydoquinone (**3**). (-)-Phyllostine (**1**) showed strong cytotoxic activity against all five cancer cell lines while toluhydoquinone (**3**) exhibited moderately weak but selective activity against CNS glioma (SF-268).

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