Notes

Antimalarial Benzoquinones from an Endophytic Fungus, *Xylaria* sp.

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Two novel benzoquinone metabolites, 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**1**) and xylariaquinone A (**2**) together with two known compounds were isolated from an endophytic fungus, *Xylaria* sp. Their structures were assigned by analysis of spectroscopic data, and the structures of **1** and **3** were also confirmed by single-crystal X-ray data. Compounds **1** and **2** showed *in vitro* activity against *Plasmodium falciparum*, K1 strain, with IC_{50} values of 1.84 and 6.68 μM and cytotoxicity against African green monkey kidney fibroblasts (Vero cells) with IC_{50} values of 1.35 and >184 μM, respectively.

Endophytic fungi are often found living in apparently healthy plants and cause no apparent symptoms of disease for the host plant.[1] Endophytic fungi show high potential as sources of novel antiviral, anticancer, antioxidant, and insecticidal compounds.[2] The Xylariaceae is a large family of fungi that includes endophytes found in a variety of plants.[3] These fungi produce metabolites such as dihydroisocoumarins,[4] succinic acid and derivatives,[5] xanthones,[6,7] lactones,[8] cytochalasins,[9,10] sesquiterpene alcohols,[11] and griseofulvin.[12] Our research group has been interested in studies of bioactive compounds produced by endophytic fungi of Thai medicinal plants. In the course of our investigations we have identified novel metabolites **1** and **2** together with two known compounds, **3** and **4**, from an endophytic fungus, *Xylaria* sp. PBR-30. Herein we describe the isolation and structural elucidation of these compounds and their *in vitro* antimalarial activity against *Plasmodium falciparum* (K1 strain). The culture broth of *Xylaria* sp. PB-30 was extracted with EtOAc, and this extract was subjected to silica gel column chromatography followed by crystallization. Compounds **1** and **2** were obtained by elution with a mixture of CH_2Cl_2/hexane (70:30). Elution with a mixture of CH_2Cl_2/MeOH (90:10) yielded 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**3**) and 4-hydroxymellein (**4**).

Compound **1** crystallized from hexane/$CHCl_3$ as yellow crystals, and the molecular formula $C_8H_7O_3Cl$ was assigned on the basis of HRESIMS analysis. The ^{1}H NMR spectrum of **1** had a methyl signal at δ_H 2.14, a methoxy signal at δ_H 3.80, and an aromatic proton signal at δ_H 5.97. The ^{13}C and HSQC NMR spectra showed eight carbon signals comprising three quaternary sp^2-carbons, a methine sp^2-carbon, two carbonyl carbons, a methoxy carbon, and a methyl carbon. On the basis of HMBC correlations (Figure 1) and X-ray crystallography analysis (Figure 2), compound **1** was identified as 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione. Compound **1** is a new terrestrial-derived organohalogen natural product. In general, organohalogens are found more often in marine organisms compared to terrestrial organisms;[13] however, halogenated natural products have previously been reported as *Xylaria* metabolites.[14,15]

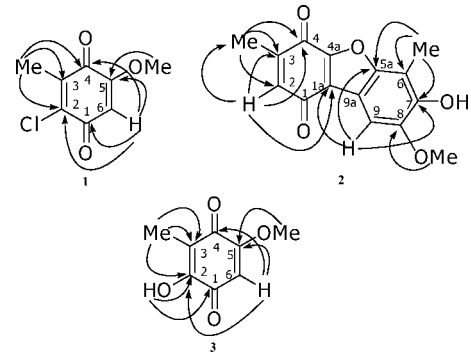
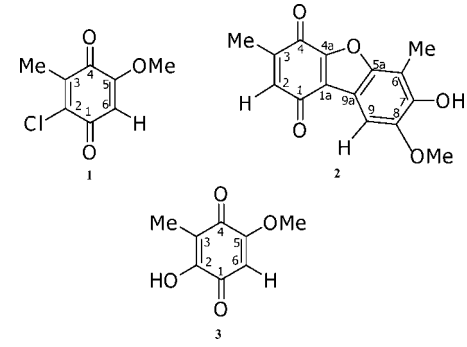


Figure 1. HMBC correlations of **1**–**3**.



Compound **2** was obtained as dark red crystals and had the composition $C_{15}H_{12}O_5$ on the basis of HRESIMS analysis. The ^{1}H NMR spectrum of **2** displayed methyl signals at δ_H 2.16 (d, J = 1.6 Hz) and at δ_H 2.45 (s), a hydroxyl proton signal at δ_H 3.49, a methoxy signal at δ_H 4.00, and two aromatic signals at δ_H 6.53 and 7.31. The ^{13}C NMR spectrum and HSQC experiment of **2** indicated two methyl carbons, a methoxy carbon, two methine sp^2-carbons, eight quaternary sp^2-carbons, and two carbonyl carbons. The connectivities of **2** were established by HMBC correlations,

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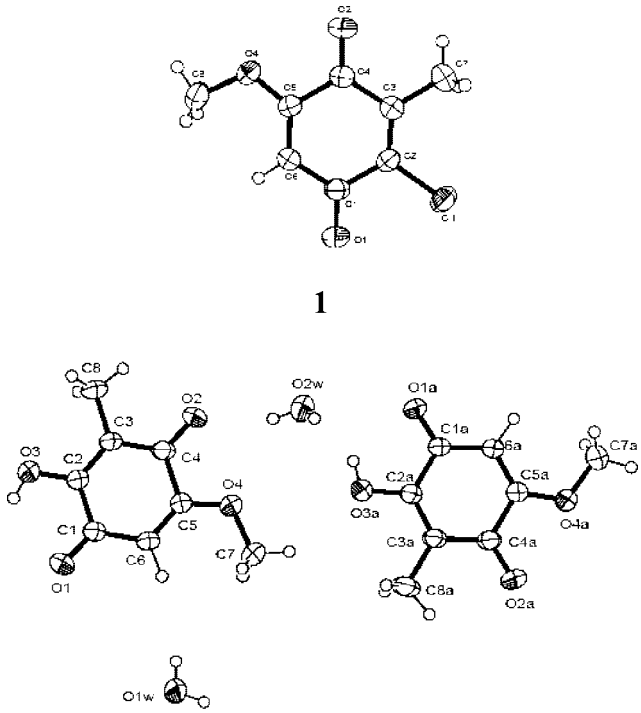

Figure 2. ORTEP views for compounds **1** and **3** (displacement ellipsoids are drawn at the 50% probability level).

shown in Figure 1. HMBC correlations from the methyl group (δ_H 2.16) of the quinone ring to the quaternary C-3, the methine C-2, and carbonyl carbon C-4 suggested that this methyl group was located at C-3. The HMBC of the other methyl group (δ_H 2.45), correlated with the sp^2-hybridized carbons C-5a, C-6, and C-7, revealed that this methyl group was located at C-6. The position of the methoxyl group was established by the HMBC correlation of the methoxyl to C-8. HMBC correlations from H-9 to the sp^2-hybridized carbons C-1a, C-5a, and C-7 and from H-2 to C-1a indicated that C-1a of the quinone ring was connected to C-9a of the aromatic ring and that C-9 was adjacent to C-9a and C-8. Since seven of the 10 unsaturations were accounted for, it was implied that **2** should contain three rings. The chemical shifts of C-4a (δ 150.8) and C-5a (δ 152.0) suggested that both were oxygenated sp^2-carbons and that C-4a must attach to C-5a through an oxygen bridge, thus forming a ring and accounting for the one remaining degree of unsaturation. On the basis of the spectroscopic data, xylariaquinone A (**2**) was identified as 7-hydroxy-8-methoxy-3,6-dimethyldibenzofuran-1,4-dione.

Compound **3** was obtained as red crystals and determined to be $C_8H_8O_4$ on the basis of HRESIMS analysis. The NMR data of **3** were similar to those of **1**, except that the chlorine atom of **1** was replaced by a hydroxyl group. HMBC correlations of **3** (Figure 1) led to the establishment of the structure as shown, and this structure was confirmed by X-ray crystallographic analysis. Compound **3** is a known compound, which has been synthesized by reacting 2,5-dihydroxy-3-methylbenzoquinone with boiling MeOH.[16] This is the first report of compound **3** found as a natural product.

From spectroscopic data analysis compound **4** was identified as 4-hydroxymellein, an isocoumarin metabolite produced by microorganisms such as *Aspergillus ochraceus*,[17,18] *Cercospora taiwanensis*,[19,20] and *Microsphaeropsis* sp.[21] and by endophytic fungi including *Xylaria longiana* (Rehm.),[4] *Phomopsis* sp.,[22] and *Diplodia corticola*.[23]

ORTEP views of **1** and **3** are shown in Figure 2. The X-ray data showed that both molecules were planar. The crystal packing of **1** revealed that there were no intermolecular interactions other than van der Waals. Compound **3** was crystallized in the triclinic form containing two molecules of **3** and two water molecules in an asymmetric unit. The quinone oxygen atom (O2) forms intermolecular H-bonds with the water molecules (O2W). The hydroxyl group participates in the strong hydrogen bond with a water molecule [O3a···O2W; 2.596(2) Å].

Compounds **1**–**4** were examined for *in vitro* antimalarial activity against *P. falciparum* (K1, multidrug-resistant strain). Dihydroartemisinine was used as a positive control and exhibited antimalarial activity with an IC_{50} value of 3.3 nM. Compounds **1** and **2** exhibited antiplasmodal activity with IC_{50} values of 1.84 and 6.68 μM, respectively, while compounds **3** and **4** were inactive. The cytotoxic activity of compounds **1** and **2** against African green monkey kidney fibroblasts (Vero cells) was also examined using a colorimetric method.[24] Elipticine was used as positive control, with an IC_{50} value of 2.03 μM. Compounds **1** and **2** exhibited cytotoxicity with IC_{50} values of 1.35 and >184 μM, respectively. Although the *in vitro* antiplasmodal activity of **2** was much weaker than standard drugs, its relative noncytotoxicity may be worthy of further study.

Experimental Section

General Experimental Procedures. ^1H and ^{13}C NMR spectra were recorded on a Varian Mercury +400 MHz NMR spectrometer (^1H at 400 MHz and ^{13}C at 100 MHz). Chloroform-*d* ($CDCl_3$) was used for NMR experiments, and chemical shifts (δ) are referenced to residual solvent signals at 7.26 ppm (^1H) and 77.0 ppm (^{13}C). HRESIMS spectra were recorded on a Micromass LCT (LC/MS). Optical rotations were measured on a Perkin Elmer 341 polarimeter, using a sodium lamp at wavelength 589 nm. FT-IR spectra were recorded on a Perkin-Elmer model 1760X Fourier transform infrared spectrophotometer. Melting points were measured using a Fisher-Johns melting point apparatus. All solvents used for column chromatography were commercial grade and were distilled prior to use. TLC was carried out on precoated silica gel 60 F_{254} (Merck), and spots were detected under UV (254 and 365 nm) and visualized by spraying with a vanillin/sulfuric acid solution followed by heating. Isolations were carried out using column chromatography (CC) [silica gel 60 (Merck, 0.040–0.063 mm)]. Fungal culture media were purchased from Himedia.

Isolation of the Endophytic Fungus. Healthy leaves of *Sandoricum koetjape*, collected in September 2002 from Prachinburi Province, were cleaned with running tap water and cut into a small pieces (0.5 cm of diameter) followed by surface sterilization in 90% EtOH (30 s), 10% NaOCl (1 min), and 90% EtOH (30 s) and immersing twice in sterile distilled water (1 min), respectively. The sterile samples were placed on malt extract agar (MEA) plates and incubated at 30 °C. The germinating hypha tips were observed by stereomicroscopy and transferred to new MEA plates and then subcultured until pure cultures were obtained. The endophytic fungi were deposited at the culture collection of Research Centre for Bioorganic Chemistry (RCBC), Chulalongkorn University, Bangkok, Thailand. During purification procedures the endophytic fungus PB-30 was identified as a *Xylaria* sp. by means of morphology, including microscopic and macroscopic features, and molecular identification. Genomic DNA was prepared from fungal mycelium and extracted with cetyltrimethylammonium bromide (CTAB)[25] and by the ribosomal internal transcribed spacer (ITS) analysis.[26,27] The nucleotide sequence data of ITS1-5.8S-ITS2 of *Xylaria* sp. PB-30 reported in this paper will appear in the DDBL/EMBL/GenBank nucleotide sequence databases with accession number AB285482. A GenBank search for sequences similar to its ITS region revealed *Xylaria* sp. NR-2006-A59 and *Xylaria longipes* as the closest match, with 91% sequence identity. These results suggested that the endophytic fungus PBR-30 belongs to the genus *Xylaria*.

Fermentation, Extraction, and Isolation. The fungus was cultured in 250 mL Erlenmeyer flasks (× 100) containing 100 mL of malt extract broth (MEB) at 30 °C for 5 weeks under static conditions. The culture broth was filtered through filter paper (Whatman no. 1). The filtrate was concentrated under reduced pressure to 500 mL and then extracted with EtOAc (500 mL). Evaporation of the solvent afforded a dark brown crude extract (10.69 g), which was subjected to silica gel 60 column chromatography (∅ 5 cm × 60 cm) eluted with hexane, CH_2Cl_2, and MeOH in a stepwise fashion. Similar fractions were combined on the

basis of TLC. Fraction 14, eluted by 70% CH_2Cl_2 in hexane, was crystallized from hexane/$CHCl_3$ to afford 2-chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**1**) as yellow crystals (76 mg). Fraction 15, eluted by 70% CH_2Cl_2 in hexane, was crystallized from hexane/$CHCl_3$ in the presence of a small amount of MeOH to afford xylariaquinone A (**2**) as a red powder (16 mg). Fraction 24, eluted with a mixture of CH_2Cl_2/MeOH (90:10), was crystallized from hexane/$CHCl_3$ to afford 2-hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (**3**) as red crystals (45 mg), and fraction 22 was crystallized from hexane/$CHCl_3$ to afford 4-hydroxymellein (**4**) as a white powder (12 mg).

2-Chloro-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (1): yellow crystals; mp 133–135 °C; UV (MeOH) λ_{max} (log ε) 273 (4.16) nm; IR (KBr) ν_{max} 3061, 2978, 2917, 2848, 1679, 1644, 1592, 1231, 1075, 992 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.14 (3H, s, Me-3), 3.80 (3H, s, OMe), 5.97 (1H, s, H-6); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 13.4 (Me-3), 56.6 (OMe), 106.6 (C-6), 140.1 (C-2), 141.5 (C-3), 158.8 (C-5), 179.1 (C-1), 179.6 (C-4); ESITOFMS *m/z* 187.0156 $[M + H]^+$ (calcd for $C_8H_8O_3Cl$, 187.0162).

Xylariaquinone A (2): dark red crystals; mp 132–134 °C; UV (MeOH) λ_{max} (log ε) 253 (3.53), 209 (3.73) nm; IR (KBr) ν_{max} 3426br, 3013, 2961, 2922, 2848, 1658, 1566, 1284, 1084 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 2.16 (3H, d, J = 1.6 Hz, Me-3), 2.45 (3H, s, Me-6), 3.49 (1H, s, OH-7), 4.00 (3H, s, OMe), 6.53 (1H, q, J = 1.6 Hz, H-2), 7.31 (1H, s, H-9); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 8.6 (Me-6), 15.7 (Me-3), 56.5 (MeO-8), 99.2 (C-9), 108.5 (C-6), 113.4 (C-9a), 123.2 (C-1a), 133.1 (C-2), 145.4 (C-3), 146.4 (C-7), 146.7 (C-8), 150.8 (C-4a), 152.0 (C-5a), 176.5 (C-4), 184.5 (C-1); ESITOFMS *m/z* 273.0741 $[M + H]^+$ (calcd for $C_{15}H_{13}O_5$ 273.0763).

2-Hydroxy-5-methoxy-3-methylcyclohexa-2,5-diene-1,4-dione (3): red crystals; mp 149–151 °C; UV (MeOH) λ_{max} (log ε) 278 (4.12), 206 (3.87) nm; IR (KBr) ν_{max} 3552br, 3078, 2987, 2939, 1643, 1613, 1522, 1456, 1396, 1226, 1065, 1021 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.94 (3H, s, Me-3), 3.86 (3H, s, OMe), 5.84 (1H, s, H-6), 7.27 (1H, s, OH); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 7.8 (Me-3), 56.8 (OMe), 102.1 (C-6), 114.8 (C-3), 151.6 (C-2), 161.2 (C-5), 182.0 (C-4), 184.5 (C-1); ESITOFMS *m/z* 169.0548 $[M + H]^+$ (calcd for $C_8H_9O_4$ 169.0501).

X-ray Crystallography of 1. Crystal data of **1**[28] were obtained using a Bruker SMART CCD diffractometer; Mo Kα radiation (λ = 0.71073 Å); graphite monochromator; $C_8H_7ClO_3$; triclinic; space group $P\bar{1}$; unit cell dimensions a = 7.3122(9) Å, b = 7.3839(9) Å, c = 8.5878(10) Å, α = 112.135(2)°, β = 97.002(2)°, γ = 103.759(2)°; volume = 405.42(8) $Å^3$; D_{calc} = 1.528 g/cm^3; Z = 2; $F(000)$ = 192; μ = 0.430 mm^{-1}. Data were collected at 293 (2) K using ω–2θ scans in the range θ = 2.63–28.32°. A total of 4793 reflections were collected, 1913 were unique (R_{int} = 0.0212). The structure was refined by full-matrix least-squares on F^2 using SHELXL-97 package software.[29] The non-hydrogen atoms were refined anisotropically. The hydrogen atoms were placed in the idealized positions and refined using a riding model. The final refinement [$I > 2\sigma(I)$] gave R_1 = 0.0498, wR_2 = 0.1174.

X-ray Crystallography of 3. Crystal data of **3**[28] were obtained with a Bruker SMART CCD diffractometer; Mo Kα radiation (λ = 0.71073 Å); graphite monochromator; $C_8H_8O_4 \cdot H_2O$; triclinic; space group $P\bar{1}$; unit cell dimensions a = 7.5959(2) Å, b = 9.2871(3) Å, c = 12.5007(3) Å; α = 95.0380(10)°, β = 93.2420(10)°, γ = 95.787(2)°; volume = 872.06(4) $Å^3$; D_{calc} = 1.418 g/cm^3; Z = 4; $F(000)$ = 392; μ = 0.120 mm^{-1}. Data were collected at 293(2) K using ω–2θ scans in the range θ = 1.64–30.47°. A total of 6433 reflections were collected, 4689 were unique (R_{int} = 0.0179). The structure was refined by full-matrix least-squares on F^2 using SHELXL-97 package software.[33] Nonhydrogen atoms were refined anisotropically. The hydrogen atoms were placed in the idealized positions and refined using a riding model. The final refinement [$I > 2\sigma(I)$] gave R_1 = 0.0536, wR_2 = 0.1396.

Antiplasmodal Activity. *P. falciparum* was cultivated *in vitro*[30] in RPMI 1640 medium containing 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), 32 mM $NaHCO_3$, and 10% heat-activated human serum with 3% erythrocytes and incubated at 37 °C in an incubator with 3% CO_2. Cultures were diluted with fresh medium and erythrocytes every day according to cell growth. Quantitative assessment of *in vitro* antimalarial (antiplasmodal) activity was determined by microculture radioisotope techniques.[31] Briefly, a mixture of 200 μL of 1.5% erythrocytes with 1% parasitemia at the early ring stage was pre-exposed to 25 μL of the medium containing a test sample dissolved in 1% DMSO (0.1% final concentration) for 24 h employing the incubation conditions described above. Subsequently, 25 μL of [^{3}H] hypoxanthine (Amersham) in culture medium (0.5 μCi) was added to each well, and plates were incubated for an additional 24 h. Levels of incorporated radioactively labeled hypoxanthine indicating parasite growth were determined using the TopCount microplate scintillation counter (Packard). Inhibition concentration (IC_{50}) represents the concentration that indicates 50% reduction in parasite growth.

Cytotoxicity against Vero Cells. Compounds were tested for their cytotoxic activity against Vero cells (African green monkey kidney fibroblasts) in 96-well tissue culture plates. Vero cell suspension (190 μL) containing 1 × 10^5 cells/mL and 10 μL of test compound was added to each well in triplicate. Ellipticine and 10% DMSO were used as positive and negative controls, respectively. The cells were incubated at 37 °C for 72 h in 5% CO_2 and determined by colorimetric method. The cytotoxicity was expressed as 50% inhibitory concentration (IC_{50}), i.e., the concentration of compound that inhibited cell growth by 50%, compared with untreated cell.

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(28) Crystallographic data (excluding structure factors) of **1** and **3** have been deposited with the Cambridge Crystallographic Data Centre as supplementary publication numbers CCDC 616296 and 616297, respectively. Copies of the information may be obtained free of charge from the Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge CB2 1EZ, UK (fax: +44 1223 336 033; e-mail: deposit@ccdc.cam.ac.uk or http://www.ccdc.cam.ac.uk).

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