Activity against Plasmodium falciparum of Lactones Isolated from the Endophytic Fungus Xylaria sp.

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Online Publication Date: 01 October 2008

To cite this Article: Jiménez-Romero, Carlos, Ortega-Barría, Eduardo, Arnold, A. Elizabeth and Cubilla-Rios, Luis (2008)'Activity against Plasmodium falciparum of Lactones Isolated from the Endophytic Fungus Xylaria sp.', Pharmaceutical Biology, 46:10, 700 — 703

To link to this Article: DOI: 10.1080/13880200802215859

URL: http://dx.doi.org/10.1080/13880200802215859
Activity against *Plasmodium falciparum* of Lactones Isolated from the Endophytic Fungus *Xylaria* sp.

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Abstract

Three lactones were isolated from the culture medium of the endophytic fungus *Xylaria* sp. Grev. (Xylariaceae). The major compound, which showed weak activity (13 µg/mL) against a chloroquine-resistant strain of *Plasmodium falciparum*, was identified as (+)-phomalactone (1). The others were 6-(1-propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one (2) and 5-hydroxymellein (3). Compounds 1 and 2 are reported for the first time as constituents of *Xylaria*. Also, this is the first report of the activity of the compounds 1–3 against a chloroquine-resistant *Plasmodium falciparum* strain.

Keywords: Antiplasmodial, endophytic fungus, phomalactone, *Plasmodium falciparum*, *Xylaria* sp.

Introduction

Over half of the world’s population in some 100 countries is at risk from malaria, with about 500 million acute infections and approximately 1 million deaths recorded each year (Gelb & Hol, 2002). As part of the International Cooperative Biodiversity Groups (ICBG) program established in the Republic of Panama, we are carrying out a study of extracts from endophytic fungi isolated from plant species collected in protected areas of Panamanian rainforests and screening for activity against causal agents of diseases such as malaria, Leishmaniasis, Chagas disease, and cancer (Coley et al., 2003). Tropical endophytic fungi represent a largely untapped resource of biological and biochemical diversity (Dreyfus & Chapela, 1994; Cubilla et al., 2006). These highly diverse fungi can be isolated and grown in culture on standard media, with multiple species of endophytes typically recovered from individual leaves (Arnold et al., 2000). We describe herein the isolation of three lactones, two of them 1 and 2 as members of a 6-substituted-5,6-dihydro-2H-pyran-2-one group (Fukushima et al., 1998). Compound 1, (+)-phomalactone (Fig. 1), has been previously reported from ascomycotan fungi including an unidentified *Nigrospora* sp. (Evans et al., 1969), *Phoma* sp. (Yamamoto et al., 1970), *Hirsutella thompsonii* var. *synnematos*a (Krasnoff & Gupta, 1994), and *Nigrospora sacchari* (Fukushima et al., 1998). Compound 2 was isolated for the first time as a natural product and has not been reported previously in the literature. The third compound, 5-hydroxymellein 3, has been previously isolated from the ascomycotan fungi *Septoria nodorum* (Davis et al., 1994), and *Botryosphaeria obtus* (Venkatasubbaiah & Chilton, 1990). Additionally, we report the results of the bioassay of compounds 1–3 against a chloroquine-resistant *P. falciparum* strain and their cytotoxicity in Vero Cells.

Materials and Methods

General experimental procedures

Optical rotations were determined on an Autopol III 6971 Automatic Polarimeter (Rudolph Research Analytical; NJ, USA). IR spectra were measured on a Perkin-Elmer FT-IR Spectrometer Spectrum RXI. The 1H-NMR 300 and 400 MHz spectra (13C NMR 75.5 MHz) were recorded on a Bruker Avance 300 and a JEOL MSRoute spectrometer, respectively. MS and HRCIMS were recorded on a Kratos MS50TC instrument. HPLC and UV spectrum were carried out on a Waters LC system, with a 600 pump and a 996 photodiode array detector.
Fungal Material

The focal isolate of *Xylaria* sp. Grev. (Xylariaceae) was isolated from the interior of a healthy leaf of *Siparuna* sp. (Siparunaceae) collected at Altos Campanas National Park, Panama. Leaf material was surface-sterilized following Arnold et al. (2000) and small fragments cultured on 2% malt extract agar under sterile conditions. The strain was isolated into pure culture and deposited at the Smithsonian Tropical Research Institute (STRI) as 300A7-2. Because the isolate did not produce fruiting structures in culture, it was identified using molecular phylogenetic analyses of the fast-evolving nuclear ribosomal internal transcribed spacer (ITS), a ca. 600bp locus frequently used in fungal systematics at the species level (Arnold et al., 2007). Total genomic DNA was extracted directly from fresh, axenic mycelia using an SDS extraction protocol (Arnold et al., 2007). DNA was diluted 1:10 prior to amplification of the ITS region using the polymerase chain reaction (PCR). PCR cycling reactions, reagents, and reaction volumes followed Arnold and Lutzoni (2007). PCR products were cleaned following amplification, visualized on a 1% agarose gel, and sequenced in two directions using the PCR primers ITS5 and ITS4 on an ABI 3700 Automated Sequencer. Sequences were assembled into contigs and basecalls edited manually. The consensus sequence was (1) subjected to BLAST searches of the NCBI GenBank database for preliminary identification, followed by phylogenetic analyses using neighbor-joining in the context of the 100 top matches; and (2) compared against a phylogenetically referenced database of 3250 ITS sequences for endophytic fungi (Arnold & Lutzoni, 2007). Both analyses positively identified the isolate as a xylariaceous fungus (Xylariaceae, Xylariales, Sordariomycetes, Ascomycota) with close phylogenetic affinity for known species (1–3% sequence divergence). In contrast, when compared against publically available sequences in GenBank, the ITS sequence most closely matched sequences from three unidentified *Xylaria* species (1–3% sequence divergence). It is probable that this strain represents a novel species of *Xylaria*, a common and highly diverse lineage of tropical leaf- and wood-associated fungi. The ITS sequence for this isolate has been submitted to GenBank (accession no. EU016102).

Extraction and isolation

A culture maintained on M-1-D (25°C, 30 days) was inoculated into 20 × 1 L Erlenmeyer flasks each containing 500 mL of autoclaved modified M-1-D medium (Wagenaar et al., 2000). Fungal cells were separated from a broth by filtration, the culture medium was freeze dried under −40°C and subsequently extracted with EtOAc (equal volume, ×3), yielding 147 mg of a crude extract. The crude extract was fractionated using a solid phase extraction (7G, J.T. Baker). The cartridge was packed and equilibrated with 15 mL CHCl3 before sample loading. The main compounds (screened at 1H-NMR) were eluted with CHCl3100% (50 mL) followed by MeOH 100% (30 mL). The MeOH fraction was screened at 1H-NMR showing a major sugar composition, while the CHCl3fraction (80 mg) was further purified by semi-preparative HPLC (YMC-Pack SIL, 5 µm, 10 × 150 mm) using isocratic elution (flow 3 mL/min, CHCl3100%). Five fractions were collected (A–E). Fraction B yielded 2 (2.5 mg, tR: 22 min). Fraction C was subjected to repeated semi-preparative HPLC (Nova Pack C18, 6 µm, 7.8 × 300 mm) using gradient elution (flow 3 mL/min, 9:1 MeCN/H2O to MeCN 100%), to afford compound 1 (3.2 mg, tR: 7 min). Finally, Fraction D was further purified by semi-preparative HPLC (YMC-Pack SIL, 5 µm, 10 × 150 mm) using a gradient elution (flow 1.0 mL/min, 8:2 CHCl3/EtOAc to CHCl3 100%), to yield compound 3 (1.1 mg, tR: 36 min).

6-(1-Propenyl)-3,4,5,6-tetrahydro-5-hydroxy-4H-pyran-2-one 2

Colorless oil; [α]D24 = +36.4° (CHCl3, c 0.14); IR (film) vmax 3418, 2922, 1764, 1372, 1262, 1188, 1034, 806 cm−1; 1H NMR (300 MHz, CDCl3) δ 5.85 (1H, m, H-8); 5.51 (1H, dd, J = 5.7 and 9.7 Hz, H-7); 4.44 (1H, q, H-6); 4.08 (1H, dd, J = 6.3 and 12.8 Hz, H-5); 2.21 (2H, m, H-3); 2.07 (2H, m, H-4); 2.00 (3H, d, J = 6.5 Hz, H-9); 13C NMR
Assay for the inhibition of Plasmodium falciparum

The antiplasmodial activity was evaluated using a fluorometric method based on the detection of parasite DNA with the fluorochrome PicoGreen using a chloroquine-resistant strain (Indocrina W2) of P. falciparum (Corbett et al., 2004). The parasites were maintained in vitro by a modification of the method of Trager and Jensen (1976).

Assay for the inhibition of Vero cell growth

Vero cells, derived from the kidney of the African green monkey, adhering to 96-well plates, were used to evaluate the toxicity of the compounds purified from Xylaria sp. on the basis of reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) (Morel, 1984). After the treatment with the test compound and 4 h incubation at 37°C, cell viability was evaluated in an ELISA reader at 570 nm.

Results and Discussion

The EtOAc crude extract of Xylaria sp showed weak activity (IC₅₀ = 30 µg/mL) against a chloroquine-resistant strain of Plasmodium falciparum. Compounds (1–3) were isolated by solid phase extraction, followed by HPLC.

Compound 1 was obtained as a colorless oil and shown to have a molecular formula of C₈H₁₀O₃ by HRCIMS ([M + H]+ m/z 157.0860 [calcd. for C₈H₁₂O₃, 157.0865]). Copies of the original spectra can be obtained from the corresponding author.

Table 1. Antiplasmodial and Cytotoxic activities of lactones 1–3.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Activity IC₅₀ (µg/mL)</th>
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</thead>
<tbody>
<tr>
<td>Phomalactone (1)</td>
<td>13</td>
</tr>
<tr>
<td>Compound (2)</td>
<td>&gt;50</td>
</tr>
<tr>
<td>5-hydroxymellein (3)</td>
<td>19</td>
</tr>
<tr>
<td>Chloroquine</td>
<td>0.03</td>
</tr>
</tbody>
</table>

a Experiments performed with Vero cells.

b ND = not determined.

The presence of an absorption peak at (1764 cm⁻¹), in the IR spectrum and a signal at δ 176.9 (C-2) in the ¹³C NMR. The ¹H NMR spectrum for 2 revealed signals very similar to those for compound 1, the major difference being the disappearance of the double bond on the pyranone ring. Instead, two new signals appear at δ 2.10 (2H, m) and δ 2.20 (2H, m). The ¹³C NMR spectrum showed 8 resonances: one quaternary, 4 methine, 2 methylene and one methyl group. The presence of two new signals at the ¹H-NMR spectrum (δ 2.21 H-3 and δ 2.07 H-4) in 2 was consistent with the difference in molecular weights of 2 uma between 1 and 2, which suggested the hydrogenation of the pyranone ring. The absolute configuration of 2 was elucidated on the basis of optical rotation and analysis of the ¹H NMR spectrum.

Compound 3 has a molecular formula of C₁₀H₁₀O₄ by HRCIMS ([M + H]+ m/z 195.0638, calcd. for 195.0657). The IR spectrum showed peaks corresponding to an hydroxyl group at (3178 cm⁻¹) and a carbonyl group at (1660 cm⁻¹). The NMR data and optical rotation of 3 were compared with literature values, which confirmed it as 5-hydroxymellein (Venkatasubbaiah & Chilton, 1990; Davys et al., 1994).

The anti-parasitic and cytotoxic activities of compound 1–3 are presented in Table 1. Compounds 1 and 3 showed weak anti-plasmodial activity when tested against a chloroquine-resistant strain of P. falciparum with IC₅₀ values of 13 and 19 µg/mL, respectively. It is likely that the unsaturated moiety present compound 1 is responsible for its greater anti-malarial activity as compared to compound 2. As Table 1 shows those compounds were cytotoxic against Vero cells.

The diverse and widespread fungal genus Xylaria has been known to be a rich source of bioactive secondary metabolites. Some examples include xylarene A and B from Xylaria periscaria (Smith et al., 2002), five unique xylaketals A–E from the mangrove fungus Xylaria sp. (Lin et al., 2001), and multiploides A and B from Xylaria multiplex (Boonphong et al., 2001). This is the first report on the occurrence of (+) phomalactone and 5-hydroxymellein from this genus.

Acknowledgements

The authors extend special thanks to the Smithsonian Tropical Research Institute and the International Cooperative
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Biodiversity Groups, ICBG-Panama program (grant number 1 U01 TWW6634-01), for financial support for this research and the Institute for Advanced Scientific Investigation and Technology Services for NMR services. AEA gratefully acknowledges additional support from NSF DEB-0200413, DEB-0516564, and DEB-0640956. We thank Thomas A. Kursar and Phyllis D. Coley for developing the endophytic fungus program in the ICBG.

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