Isolation of phomopsolide A as the antimycobacterial constituent of an unidentified endophyte of the Canadian medicinal plant Geum macrophyllum

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ABSTRACT

An extract of an unidentified green filamentous fungus isolated as an endophyte of the Canadian medicinal plant *Geum macrophyllum* exhibited significant antimycobacterial activity and a distinct ^{1}H NMR profile. Bioassay guided fractionation indicated that the natural product phomopsolide A (**1**) was responsible for the distinguishing characteristics of the extract.

INTRODUCTION

There is an urgent need for new antibiotics as the number of infections caused by resistant microbes continues to increase at an alarming rate (World Health Organization, 2015). Tuberculosis (TB) is particularly problematic given its global prevalence, the widespread emergence of drug–resistant strains of *Mycobacterium tuberculosis* and the burden that the disease places on healthcare systems worldwide (World Health Organization, 2014). Endophytic fungi, particularly those from medicinal plants, are an excellent source of potential drug leads that produce natural products with significant antimicrobial properties (Demain and Sanchez, 2009; Kaul et al. 2012; Deshmukh, Verekar, and Bhave, 2015). In previous work, Ellsworth et al (2013) isolated 81 endophytes from the leaves of plants traditionally used as medicines by the First Nations of the Canadian Atlantic Provinces. Extracts of the fungi were screened for antibiotic activity (Ellsworth et al., 2013) and chemically unique extracts were identified using NMR-based metabolomic analyses (Clark et al., 2014). One of the extracts highlighted in the metabolomic screening displayed significant antimycobacterial activity and was derived from an unidentified green filamentous fungus (isolate TC2-085) isolated from the large-leaved avens, *Geum macrophyllum*. This report describes the bioassay guided fractionation of the TC2-085 extract with the objective of isolating the constituents responsible for its antimycobacterial activity.

RESULTS

Fractionation of the TC2-085 extract by liquid-liquid partition followed by normal phase HPLC yielded compound **1**, which had an IC_{50} of 35 μM against *M. tuberculosis* H37Ra (Figure 1). The molecular formula of **1** was determined to be $C_{15}H_{18}O_6$ by high-resolution electrospray mass spectrometry and indicated seven degrees of unsaturation in the molecule. Examination of the ^{13}C NMR spectrum of **1** allowed six degrees of unsaturation to be assigned to the presence of three carbonyl groups (δ_C 202.2, 166.6 and 162.1) and three carbon – carbon double bonds (δ_C 143.0, 141.1, 139.7, 127.6, 124.6 and 124.3), implying that **1** must also contain one ring. The ^{1}H NMR revealed the presence of two vinylic methyl groups [δ_H 1.78 (d, J = 6.3 Hz) and 1.77(bs)], a methyl group coupled to an oxymethine [δ_H 1.38 (d, J = 7.1 Hz) and 4.34 (q, J = 7.1 Hz)], two mutually coupled oxymethines [δ_H 5.97 (dd, J = 5.9, 2.8 Hz) and 5.64 (dd, J = 5.4, 2.8 Hz)] and five vinylic protons [δ_H 7.08 (dd, J = 9.7, 5.9 Hz), 6.84 (m), 6.41 (2H, m) and 6.23 (d, J = 9.7 Hz)]. Based on the observed molecular formula and the structural features highlighted by

NMR spectroscopy, compound **1** was determined to be phomopsolide A (Figure 2) and its identity was confirmed by comparison with literature data (Grove, 1985; Stierle et al., 1997).

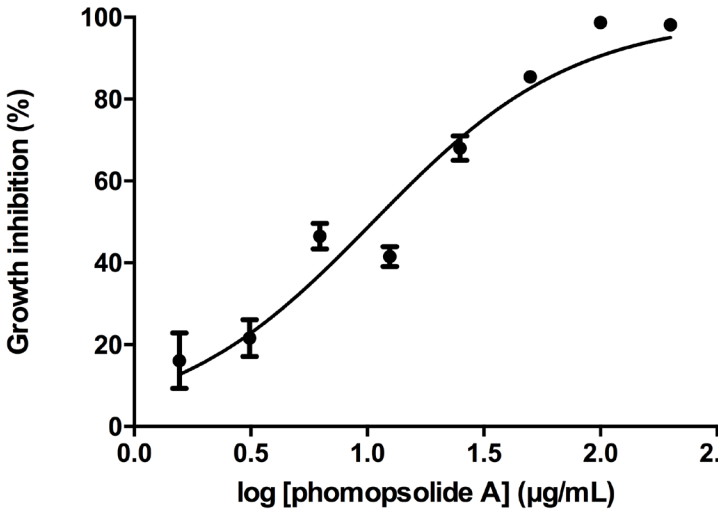

Figure 1. The effect of Phomopsolide A (**1**) on the growth of Mycobacterium tuberculosis H37Ra. Growth inhibition was assessed *in vitro* using a microplate resazurin assay at eight concentrations ranging from 200–1.625 µg/mL in triplicate. Data is shown as the mean percentage inhibition and error bars represent the 95% confidence intervals of the mean.

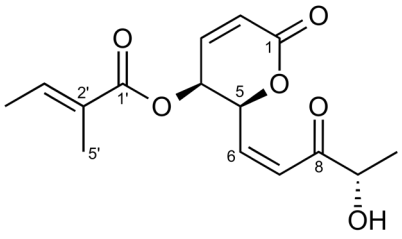

Figure 2. The structure of phomopsolide A (**1**)

DISCUSSION

Phomopsolide A was first described as a natural product of the fungus *Phomopsis oblonga* isolated from elm trees (Grove, 1985) that prevented beetles from eating the tree (O'Callaghan, Atkins and Fairhurst, 1984; Claydon, Grove, and Pople, 1985). It has since been reported from a *Penicillium* sp. isolated from the bark of the pacific yew *Taxus brevifolia* (Stierle, Stierle, and Ganser, 1997), from *Penicillium clavigerum* isolated from the alga *Chlorella vulgaris* (Stierle et al., 2014), and from an unidentified fungus isolated from the cow parsnip *Heracleum maximum* (Clark et al., 2015). Although this phomopsolide A exhibits antimycobacterial activity, it also toxic to human HEK293 cells (Clark et al., 2015) making it an unlikely candidate for further research and development. Future research will therefore focus on exploring other isolates in our fungal library in an effort to discover novel bioactive compounds.

METHODS

All solvents were ACS reagent grade or HPLC grade. Normal phase HPLC was performed using a Waters® 510 pump, a Phenomenex® Luna® silica column (10µm, 100 Å, 250 10 mm) and a Waters® R401 refractive index detector at a flow rate of 4 ml/min. NMR spectra were recorded at 25 °C on an Agilent 400-MR DD2 NMR spectrometer with chemical shifts reported in ppm and were referenced to residual protonated solvent resonances using MestReNova® 6 (Mestrelab Research® S.L.). HRESIMS data were recorded on a Thermo Scientific® LTQ Exactive Orbitrap mass spectrometer. Optical rotations were determined on an Optical Activity Ltd. AA-10 polarimeter and IR spectra were recorded on a Perkin Elmer Spectrum Two FT-IR spectrometer as thin films on sodium chloride disks.

Antimycobacterial assays and median inhibitory concentration determinations were performed in triplicate against *M. tuberculosis* H37Ra as described by O'Neill et al. (2014) using rifampin (0.1 µg/mL) and 2% DMSO as the positive and negative controls, respectively. Fractions that inhibited the growth of *M. tuberculosis* by more than 50% when tested at 100 µg/mL were considered to be bioactive. MICs and IC_{50} were determined on dilution series comprising 8 concentrations (200–1.625 µg/mL) in triplicate. The MIC of a compound was considered to be the lowest concentration at which it inhibited mycobacterial growth by more than a mean value of 90% (Collins & Franzblau, 1997), and the corresponding IC_{50} was estimated by fitting a four parameter logistic curve (Sebaugh, 2011) to the mycobacterial growth data using GraphPad Prism version 6 (GraphPad Software, California, USA).

The isolation TC2-085 from *Geum macrophyllum* and attempts to identify the fungus have been previously reported (Ellsworth et al. 2013). TC2-085 was fermented in 2% malt extract broth (200

ml; 2 × 100 mL batches in 250 mL Erlenmeyer flasks closed with foam stoppers) with shaking (150 rpm) at room temperature under ambient light for 2 weeks. The fungal material and spent fermentation broth was sonicated for thirty seconds before being filtered through cotton wool, extracted with EtOAc (3 × 60 ml) and the combined organic phases concentrated in *vacuo* to give a fungal extract (34 mg). The extract was dissolved in 9:1 MeOH/H_2O (5 ml), extracted with hexanes (3 × 2 ml), the aqueous fraction further diluted with H_2O (2.5 ml) and the resulting 3:2 MeOH/H_2O suspension extracted with CH_2Cl_2 (3 × 2 ml) before being concentrated *in vacuo,* taken up in H_2O (5 ml), and extracted with EtOAc (3 × 2ml) and finally n-BuOH (3 × 2 ml). All five of the resulting fractions were concentrated *in vacuo*, and assayed for antimycobacterial activity. The CH_2Cl_2 fraction exhibited significant bioactivity and was further purified by normal phase HPLC (60:40 Hexane:EtOAc) resulting in the isolation of compound **1** (18 mg).

Spectroscopic and spectrometric data obtained for **1**: $[\alpha]_D^{22}$: +313 (c 0.24, MeOH); IR (NaCl): 3451, 2928, 1715, 1647, 1252, 1130, 1067 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 7.08 (1H, dd, *J* = 9.7, 5.9 Hz, H-3), 6.84 (1H, m, H-3′), 6.41 (2H, m, H-6 and H-7), 6.23 (1H, d, *J* = 9.7 Hz, H-2), 5.97 (1H, dd, *J* = 5.4, 2.8 Hz, H-5), 5.64 (1H, dd, *J* = 5.9, 2.8 Hz, H-4), 4.34 (1H, q, *J* = 7.1 Hz, H-9), 1.78 (3H, d, *J* = 6.3 Hz, H-4′), 1.77 (3H, bs, H-5′), 1.38 (3H, d, *J* = 7.1 Hz, H-10); ^{13}C NMR (100 MHz, $CDCl_3$) δ 202.2 (s, C-8), 166.6 (s, C-1′), 162.1 (s, C-1), 143.0 (d, C-6), 141.1 (d, C-3), 139.7 (d, C-3′), 127.6 (s, C-2′), 124.6 (d, C-2), 124.3 (d, C-7), 77.0 (d, C-5), 73.3 (d, C-9), 63.3 (d, C-4), 19.7 (q, C-10), 14.7 (q, C-4′), 12.1 (q, C-5′); HRESIMS *m/z* 317.1000 [M + Na^+] (calculated for $C_{15}H_{18}O_6$: 317.0996; Δ ppm 1.26).

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