Isolation of an antimicrobial racemic phenalenone derivative from a marine-derived *Penicillium* sp. fungus

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Abstract

A new phenalenone derivative, 2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (**1**), was isolated from the ethyl acetate extract of a marine derived *Penicillium* sp. fungus that exhibited a unique antimicrobial activity profile. The planar structure of **1** was determined through a combination of 1D and 2D NMR experiments, and circular dichroism and polarimetry indicated that it was isolated as a racemic mixture of enantiomers. The antimicrobial activity of **1** was assessed against a panel of Gram-positive and Gram-negative bacteria and fungal strains and it was found to selectively inhibit the growth of *Staphylococcus aureus* and *Mycobacterium tuberculosis*.

Keywords: Phenalenone, *Penicillium*, Natural product, Antimicrobial

Introduction

The kingdom Fungi represents an extraordinarily diverse and extensively distributed taxon with recent estimates of total number of species ranging from 2.2-3.8 million to 11.7-13.2 million.[1–4] Despite only about 145,000 (i.e. between 1 and 5%) of these species being so far described, fungi continue to be a prolific source of structurally diverse and biologically active natural products.[5–8] Although most well-known for the production of antibiotics such as penicillin from *Penicillium chrysogenum*[9] and griseofulvin from *Penicillium griseofulvum*,[10] the genus *Penicillium* is remarkably biosynthetically adept, has attracted intense scrutiny from natural products chemists, and is the source of approximately 10% of all fungal natural products discovered so far.[11,12] *Penicillium* spp. are the preeminent fungal producers of phenalenone natural products, although examples have also been isolated from the genera *Coniothyrium*, *Aspergillus*, and *Talaromyces*.[13,14] Phenalenones are a group of peri-fused tricyclic aromatic natural products that are biosynthesized from a polyketide precursor in fungi and shikimate-derived natural products in some higher plants, notably from the family Haemodoraceae.[15–17]

In continuation of our search for bioactive natural products from fungi,[18–23] the ethyl acetate extract of a *Penicillium* sp. isolate (SC1-079G) was found to possess a unique bioactivity profile within our collection of fungal extracts. Fractionation of the ethyl acetate extract by solid-phase extraction, reversed-phase flash chromatography, and reversed-phase HPLC led to the isolation of the new phenalenone derivative, 2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (**1**, Figure 1). Herein, we report the isolation, structure elucidation, and antimicrobial activity of **1**.

Experimental

General Experimental Procedures: Optical rotations were recorded in MeOH on an Optical Activity Ltd. AA-10 polarimeter at 589 nm. UV spectra were measured in MeOH on a Shimadzu UV-1280 spectrophotometer. CD spectra were measured in MeOH on a JASCO J-810 spectropolarimeter. NMR spectra were recorded on a Bruker AVII 700 instrument equipped with a QNP cryoprobe in deuterated DMSO and were calibrated to residual protonated solvent resonances (DMSO: ^{1}H, 2.500 ppm; ^{13}C, 39.52 ppm). HR-MS data were recorded on a Thermo LTQ Exactive instrument with an electrospray ionization (ESI) source. Solvents for extraction and isolation were purchased from Fisher Scientific (Ottawa, ON, Canada) and deuterated solvents for NMR spectroscopy were purchased from Sigma-Aldrich (Oakville, ON, Canada). Flash chromatography was performed using a Biotage Flash+ chromatography system fitted with a C_{18} (reversed-phase) SiliaSep cartridge (40-63 μm, 60 Å, 25 g; SiliCycle, QC, Canada). Semi-preparative reversed-phase HPLC was performed on a Phenomenex Luna C_{18} column (250 × 10 mm, 10 μm, 100 Å) using an Agilent 1100 HPLC system comprising a G1311A binary pump and a G1315C diode array detector.

Fungal Isolation: Sea foam was collected on the 23rd of May 2015 at New River beach, New Brunswick, Canada (N45° 8′ 16.644″, W66° 32′ 19.325″) on an incoming tide and immediately placed into sterile, 50 mL conical centrifuge tubes (Falcon™). Approximately 1 mL of sea foam was transferred onto yeast-malt agar (2 g/L yeast extract, 10 g/L malt extract, 10 g/L glucose, 20 g/L agarose, and 0.05 g/L chloramphenicol) prepared with artificial seawater (distilled water, 18 g/L Instant Ocean sea salt®) and incubated at ambient room temperature (20 – 22 °C). Fungal growth was subcultured onto fresh yeast-malt seawater agar until pure cultures were

obtained. A total of 16 unique fungal isolates were obtained and screened for antibiotic activity, with the extract of SC1-079G exhibiting significant inhibitory activity of bacterial growth *in vitro*.

Identification: SC1-079G was identified (by JAJ) to be a *Penicillium* sp. fungus through examination of fruiting bodies using microscopy.[24] Attempts to identify fungal isolate SC1-079G through DNA sequencing were unsuccessful as DNA from the internal transcribed spacer (ITS) region could not be amplified after repeated unsuccessful attempts to isolate genomic DNA using published procedures.[25] On 2% malt extract agar, the isolate grew as a flat, filamentous, green colony with a dull surface and filiform margin. Light microscopy (40× magnification) revealed that the isolate possessed septate hyphae and branching conidiophores with circular spores characteristic of *Penicillium* spp. (Supporting information Figure S1).

Fermentation and isolation: SC1-079G was fermented in 2.0% malt extract broth at room temperature with shaking (150 rpm) for 2 weeks (10L; 100 × 100 mL batches in 250 mL Erlenmeyer flasks stoppered with foam baffles). Fermentation cultures were sonicated for 30 seconds, the fungal material was removed by filtration and the spent broth was extracted with ethyl acetate (EtOAc, 3 × 3 L). The organic fractions were combined and concentrated in vacuo, and the resulting extract (993 mg) was loaded onto C_{18} silica (16 g) and eluted sequentially with water (H_2O) (100 mL), methanol (MeOH) (100 mL), and EtOAc (100 mL). The MeOH fraction (563 mg) was subjected to C_{18} flash chromatography [stepwise gradient from 100% H_2O to 100% acetonitrile (CH_3CN) in 10% increments] to give 11 fractions. Fraction six (1:1 H_2O/CH_3CN, 26 mg) was subjected to further isocratic reversed-phase HPLC (3:2 H_2O/CH_3CN) to give **1** (5 mg).

2,4,6,9-tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (1). $[\alpha]_D^{22}$: 0 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ε) 216 (3.44), 258 (3.30), 268 (3.22), 344 (3.07), 370 (2.99)

nm; ^{1}H and ^{13}C NMR data, see Table 1 and Supporting information Figures S2-S7; HRMS *m/z* 365.0996 $[M + Na]^+$ (calcd for $C_{19}H_{18}O_6Na^+$, 365.0996).

ECD Computational Methods: An exhaustive molecular mechanics conformer search of **1** was performed through systematic (Spartan 20, Wavefunction Inc., Irvine, CA, USA), Monte Carlo (Spartan 20), and GMMX (PCModel 10, Serena Software, Bloomington, IN, USA) searches using the MMFF94 force field. These methods identified 291, 104, and 79 conformers respectively that were consolidated into a set of 297 unique conformers. The geometries of the unique conformers were optimized by ab initio calculation at the HF/3-21G level (Spartan 20), and the conformer set was reduced to 67 by eliminating duplicates and conformers with energies >40kJ/mol above the lowest energy conformer. The conformer set was further reduced by sequential DFT optimization and frequency calculation (ORCA 5.01, Max Planck Institute fuer Kohlenforschung, Mülheim an der Ruhr, Germany) at the HF-3c (67 conformers), BLYP-D3BJ/def2-SVPD (67 conformers), and B3LYP-D3BJ/def2-TZVPD (60 conformers) levels with duplicate and high energy conformers (*i.e.* relative Gibbs free energies >40kJ/mol) being removed at each step.[26,27] Electronic transition and rotational strength calculations were performed by TDDFT at the CAM-B3LYP-D3BJ/def2-TZVPD//B3LYP-D3BJ/def2-TZVPD level on all conformers that accounted for more than 0.1% of the Boltzmann distribution of the Gibbs free energies of the 60 B3LYP-D3BJ/def2-TZVPD conformers (i.e. 35 conformers with DG < 12.9kJ/mol). None of the conformers analysed by DFT displayed imaginary frequencies indicative of saddle-points demonstrating that they were all true minima of the potential energy surface. The conductor-like polarizable continuum (CPCM) implicit solvation model was used in all ab initio and DFT calculations to account for the effect of solvation in methanol. The calculated ECD spectrum was constructed using the Gibbs free energy Bolzmann-weighted average of the

oscillator and rotational strength values obtained for each of the conformers' excited states using SpecDis v1.71.[28] The bandwidth and UV shift used to generate the calculated ECD spectrum (0.29 eV and -25 nm respectively) were obtained by comparison of the experimental and calculated UV spectra using the SpecDis v1.71 cross-section algorithm.[28]

Biological Assays: Antimycobacterial assays against *Mycobacterium tuberculosis* H37Ra (ATCC 25177) and *Mycobacterium smegmatis* (ATCC 700084) and antibacterial and antifungal assays against *Escherichia coli* (25922), *Pseudomonas aeruginosa* (10145) *Enterococcus faecium* (ATCC 35667), *Staphylococcus aureus* (ATCC 29213), methicillin resistant *S. aureus* (ATCC 33591), *Candida albicans* (ATCC 14053), and *Saccharomyces cerevisiae* (ATCC 9763) were performed as previously described.[19,29]

Results and Discussion

The molecular formula of **1** was determined as $C_{19}H_{18}O_6$ based on high resolution electrospray ionization mass spectrometry (HRESIMS) and NMR data (Table 1), implying 11 degrees of unsaturation. Analysis of the ^{1}H, ^{13}C, and HSQC data indicated the presence of three methyl groups, one methylene carbon, one tertiary alcohol, 12 olefinic/aromatic carbons (two of which were protonated and three of which were attached to oxygen), two ketone carbonyls, and four exchangeable protons. The planar structure of **1** was elucidated by analysis of its 2D NMR data, and two fragments were identified: ^{1}H-^{1}H COSY correlations [H-1' – H-2', H-2' – H-4' (long range), H-2' – H-5' (long range)] and HMBC correlations (H-4' to C-2'/C-3' and H-5' to C-2'/C-3') suggested the presence of a prenyl group; while HMBC correlations (H-8 to C-6/C-6a/C-7/C-9/C-9a and H-5 to C-3/C-3a/C-4/C-6) suggested a tricyclic phenalenedione skeleton (Figure 2). This was further supported by HMBC correlations from 9-OH to C-1/C-7/C-8/C-9/C-9a, 6-OH to C-

6/C-6a, 4-OH to C-3/C-3a/C-4/C-5, and H-10 to C-6a/C-7/C-8. HMBC correlations from H-1′ to C-1/C-2/C-3 established that the prenyl group was bonded to C-2 and completed the planar structure of **1**. The C-4' methyl group was determined to be *cis* in relation to H-2' through NOESY correlations between H-2' and H-4'. 2,4,6,9-Tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (**1**) was determined to be a racemic mixture based on the absence of an optical rotation and only noise level features observed in the electron circular dichroism spectrum, contrary to the theoretical spectra (Figure 3) and the spectra of similar molecules.[30–33]

The antimicrobial activity of **1** was assessed against a panel of two Gram-negative [*E. coli* (25922) and *P. aeruginosa* (10145)], three Gram-positive [*E. faecium* (ATCC 35667), *S. aureus* (ATCC 29213), and methicillin resistant (MR) *S. aureus* (ATCC 33591)], two fungi [*C. albicans* (ATCC 14053) and *S. cerevisiae* (ATCC 9763)], and two mycobacteria [*M. tuberculosis* H37Ra (ATCC 25177) and *M. smegmatis* (ATCC 700084)]. Compound **1** was found to have selective weak activity against *S. aureus* [IC_{50} 38.6 (33.2 – 44.7) μM, MIC 146 μM], MR *S. aureus* [IC_{50} 50.1 (44.9 – 55.9) μM, MIC 292 μM], and *M. tuberculosis* [IC_{50} 45.8 (42.3 – 49.7) μM, MIC 73.0 μM], while being inactive against the remaining organisms (>292 μM). Similar phenalenones have been found to be immunomodulators,[34] DNA polymerase inhibitors,[35] cytotoxic,[36] antifungal,[37] anti-HIV,[31] and antibacterial.[38] This is the first report of a phenalenone derivative possessing inhibitory activity against *M. tuberculosis*; previous phenalenones have been tested against *M. phlei*, *M. smegmatis*, and *M. tuberculosis* but were found to be inactive.[36,39]

Although phenalenone derivatives have previously been isolated as optically inactive racemic mixtures, it has been shown that these are artefacts of the employed isolation protocol and have come about from spontaneous addition of solvent to an unstable triketone biosynthetic intermediate.[34,37,40] As our isolation procedure involved no opportunity for prenylation of a

biosynthetic precursor, we propose that **1** is the first naturally occurring, racemic phenalenedione being produced by mixed biogenesis either nonenzymatically or by an enzyme with relaxed specificity.

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Author contributions

SLC isolated the fungus SC1-079G. NJM and JAJ identified SC1-079G and confirmed its taxonomy. KMG isolated compound **1**. NJM elucidated the structure of compound **1**. NJM and CAG performed the in silico calculations. N.J.M. evaluated the bioactivity of compound **1**. All authors have given approval to the final version of the manuscript.

Competing interests

The authors declare there are no competing interests.

Data Availability

The data generated or analyzed during this study are provided in full within the published article and its supplementary materials. NMR data for compound **1** has been deposited to the Natural Products Magnetic Resonance Database (NP- MRD; www.np-mrd.org) and has been assigned the NP-MRD ID: NP0331782.

Supporting Information

HRMS and NMR spectra for compound **1** (PDF).

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Table 1. ^{1}H (700 MHz) and ^{13}C (175 MHz) NMR of **1** in DMSO-d_6.

position	δ_C, type	δ_H, mult (*J* in hz)
1	202.6, C	
2	135.7, C	
3	200.0, C	
3a	101.8, C	
3a[1]	137.2, C	
4	166.9, C	
5	99.1, CH	6.37, s
6	166.2, C	
6a	112.3, C	
7	149.2, C	
8	117.8, CH	6.83, s
9	164.0, C	
9a	106.1, C	
10	25.4, CH_3	2.80, s
1'	40.7, CH_2	2.50, m
2'	116.0, CH	4.91, t (7.7, 1.5)
3'	81.9, C	
4'	25.5, CH_3	1.44, s
5'	17.2, CH_3	1.27, s
OH-2		5.92, bs
OH-4		13.59, s
OH-6		12.00, s
OH-9		13.06, s

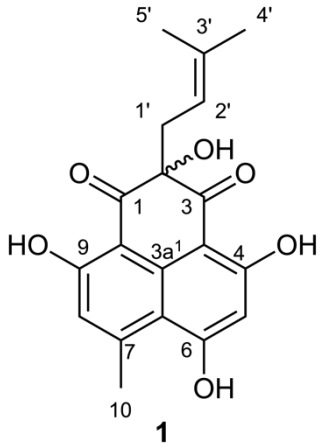

Figure 1. 2,4,6,9-Tetrahydroxy-7-methyl-2-prenyl-1*H*-phenalene-1,3(2*H*)-dione (**1**).

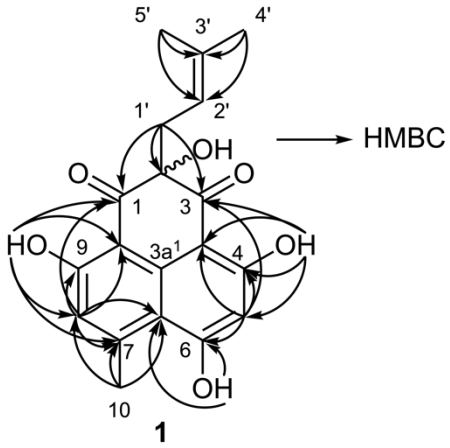

Figure 2. Key HMBC correlations of **1**.

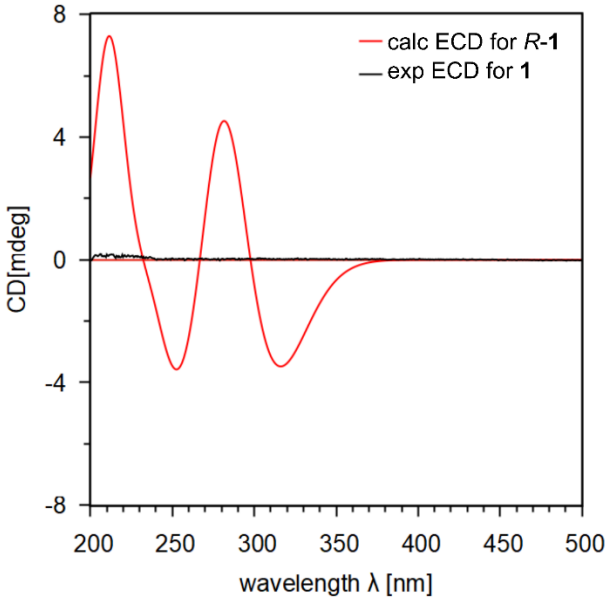

Figure 3. Experimental and TDDFT simulated ECD spectra for **1**.

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