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Anti-mycobacterial diynes from the Canadian medicinal plant *Aralia nudicaulis*

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ABSTRACT

Ethnopharmacological relevance: Aralia nudicaulis, or wild sarsaparilla, is used as a traditional medicinal plant for the treatment of various illnesses by many of the Canadian First Nations. Iroquois and Algonquin First Nations of Eastern Canada use a tea prepared from dried *Aralia nudicaulis* rhizome as a cough medicine and for the treatment of tuberculosis. Previous investigations of aqueous extracts of *Aralia nudicaulis* rhizomes have shown it to possess antimycobacterial activity.
Aim of the study: To isolate and identify antimycobacterial constituents from *Aralia nudicaulis* rhizomes.
Materials and methods: Methanolic extracts of *Aralia nudicaulis* rhizomes were subjected to bioassay guided fractionation using the microplate resazurin assay (MRA) to assess inhibitory activity against *Mycobacterium tuberculosis* strain H37Ra. The antimycobacterial constituents were identified by NMR, MS and polarimetry.
Results: Two C17 polyacetylenes with significant antimycobacterial activity were isolated from the *Aralia nudicaulis* rhizome extract. The polyacetylenes were identified as (3*R*)-falcarinol and (3*R*, 9*R*, 10*S*)-panaxydol. Falcarinol and panaxydol displayed MICs of 25.6 μM and 36.0 μM and IC_{50}s of 15.3 μM and 23.5 μM against *Mycobacterium tuberculosis* H37Ra.
Conclusions: Falcarinol and panaxydol were identified as the principal constituents responsible for the antimycobacterial activity of *Aralia nudicaulis* rhizomes validating an ethnopharmacological use of this plant by the Canadian First Nations.

1. Introduction

The medicinal ethnobotanical knowledge of the Canadian First Nations peoples has been shown to be a valid resource for identifying bioactive plants with studies demonstrating a high degree of correlation between traditional medicinal uses and observed biological activity (McCutcheon et al., 1992, 1994, 1995, 1997; Jones et al., 2000). The Algonquin and Iroquois First Nations of the North Eastern Woodlands (*i.e.* presently Eastern Canada and the North Eastern United States) have an established history of using plants for medicinal purposes (Moerman, 1998). Whilst *Aralia nudicaulis* (wild sarsaparilla) has many documented medicinal uses by First Nations communities across the whole of Canada (MacKinnon et al., 2009), infusions of *Aralia nudicaulis* rhizomes were commonly used by Algonquin peoples as cough medicines (Erichsen-Brown, 1989) and by Iroquois peoples for the treatment of tuberculosis (Herrick, 1994). The ethnobotanical uses of this plant in conjunction with the results of a recent study that indicated that aqueous extracts of *Aralia nudicaulis* rhizomes exhibited significant antimycobacterial activity (Webster et al., 2010) prompted a detailed investigation of the antituberculosis properties this plant.

The objective of the current research was the bioassay guided fractionation of *Aralia nudicaulis* rhizome extracts to isolate and identify the antimycobacterial constituents of this traditionally used medicinal plant.

2. Materials and methods

2.1. General experimental procedures

All solvents for extraction and isolation were ACS certified or HPLC grade. NMR spectra were recorded on Varian Unity 300 and 400 instruments at 300 MHz and 400 MHz for ^{1}H and 75 and 100 MHz for ^{13}C using standard 1D and 2D pulse programmes. HRESIMS data were recorded on Thermo LTQ Exactive Orbitrap

Abbreviations: $[\alpha]_D$, specific rotation measured at 589 nm and the temperature indicated; bs, broad singlet; *c*, concentration in g/100 mL; d, doublet; DMSO, dimethyl sulfoxide; ED_{50}, median effective dose; EtOAc, ethyl acetate; HPLC, high performance liquid chromatography; HRESIMS, high resolution electrospray ionization mass spectrometry; IC_{50}, median inhibitory concentration; IR, infra-red; LC–MS, liquid chromatography–mass spectrometry; m, multiplet; MeOH, methanol; MIC, minimum inhibitory concentration; MRA, microplate resazurin assay; *n*BuOH, 1-butanol; NMR, nuclear magnetic resonance; q, quartet; s, singlet; t, triplet.

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LC–MS. Optical rotations were determined on an Optical Activity Ltd. AA-10 polarimeter. Silicycle SiliaFlash F60 silica gel (40–63 μm, 60 Å) was used for flash chromatography and semi-preparative HPLC was performed using a Waters 510 pump, a Phenomenex Luna silica column (10 μm, 100 Å, 250 × 10 mm) and a Waters R401 refractive index detector at a flow rate of 4 mL/min. Antimycobacterial testing was performed using modified Middlebrook 7H9 broth base (BBL™ MGIT™, Becton Dickinson, Mississauga, Ontario) in non-tissue culture treated, low-binding, black 96-well microtitre plates sealed with polyester films (50 μm). Fluorometric readings (in relative fluorescence units, RFU) were recorded using a Molecular Devices Gemini EM dual-scanning microplate spectrofluorometer with a 530 nm excitation filter and a 590 nm emission filter operating in top-scan mode and are the mean values of 30 reads per well.

2.2. Plant material and extraction

Rhizomes of *Aralia nudicaulis* (wet weight: 432 g) were collected by hand in May 2010 from an unmanaged woodlot on the University of New Brunswick campus, Saint John, New Brunswick, Canada (45°18′22.7″N; 66°05′35.9″W). Loose soil and other debris were immediately removed from the rhizomes by rinsing with deionized water, the rhizomes were then freeze dried (dry weight: 35.0 g) and stored at −20 °C. Plants were identified by Dr Stephen Clayden of the New Brunswick Museum and a voucher specimen has been deposited in the New Brunswick Museum Herbarium (Number: NBM VP-37477). Freeze dried rhizome (35.0 g) was ground into a fine powder and exhaustively extracted with MeOH (250 mL) for 8 h using a Soxhlet extractor. The resulting solution was concentrated *in vaccuo* to give a crude methanolic extract (5.39 g).

2.3. Mycobacterial strains and growth conditions

Mycobacterium tuberculosis strain H37Ra (ATCC 25177) was grown in Mycobacteria Growth Indicator Tubes (MGIT™) containing modified Middlebrook 7H9 broth (7 mL), BBL™ MGIT™ PANTA™ (polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin) antibiotic mixture (280 μg) in BBL™ MGIT™ oleic acid–albumin–dextrose–catalase enrichment (800 μL) and Tween 80 (4 μL). Cultures were incubated (37 °C; 5% CO_2) in a humid environment for one week before being diluted to a turbidity equivalent to a McFarland 1.0 standard (10^7 CFU) using 0.05% Tween 80 in modified Middlebrook 7H9 broth. The resulting mycobacterial suspensions (1.5 mL) were cryogenically preserved for up to four weeks (−20 °C), and thawed and diluted to a concentration of 2.0×10^6 cells/mL with modified Middlebrook 7H9 broth (1:5) immediately prior to use in bioassays.

2.4. Microplate resazurin assay (MRA)

Stock solutions of rifampin (100 and 20 μg/mL) and test fractions (10 mg/mL) were prepared with sterile-filtered DMSO and stored at 4 °C. Antibiotic solutions were used within one month of preparation and fraction solutions were used within one week. Immediately prior to use, stock solutions (20 μL) were diluted with modified Middlebrook 7H9 broth (980 μL) and the resulting test solutions (100 μL) transferred to non-peripheral wells of a 96-well microtitre plate and inoculated with suspensions of *Mycobacterium tuberculosis* (100 μL). To reduce evaporation from the plates, sterile water (200 μL) was added to perimeter wells. In addition to the rifampin positive controls, negative controls [2% DMSO in modified Middlebrook 7H9 broth (100 μL) inoculated with suspensions of *Mycobacterium tuberculosis* (100 μL)] and blanks [1% DMSO in modified Middlebrook 7H9 broth (200 μL), and test solutions (100 μL) with modified Middlebrook 7H9 broth (100 μL)] were included in each plate. All controls and fractions were tested in triplicate. Plates were incubated (37 °C; 5% CO_2) for 6 days in a humid environment before a 1:1 mixture of an aqueous solution of resazurin (0.25 mg/mL) and 10% Tween 80 (50 μL) was added to all wells. Plates were then incubated for a further 24 h, sealed with an adhesive polyester film, and mycobacterial growth was assessed fluorometrically at 37 °C.

Fluorescence values were corrected for any background fluorescence of the media and test fractions by subtracting the fluorescence readings of the appropriate blanks from the mean fluorescence readings of the control and test wells. The percentage inhibition of mycobacterial growth was then defined as 1 − (mean test or positive control well fluorescence/mean negative control well fluorescence) × 100 (Collins and Franzblau, 1997). Fractions that caused more than 50% inhibition were considered to have significant activity against *Mycobacterium tuberculosis*.

2.5. Extraction, isolation and identification

The fractionation of *Aralia nudicaulis* was guided by the MRA assessment of inhibitory activity against *Mycobacterium tuberculosis* H37Ra. The crude extract was initially fractionated by a modified Kupchan solvent–solvent partition protocol to give five fractions as follows: The organic extract (5.39 g) was taken up in 9:1 MeOH/H_2O (200 mL) and extracted with hexane (3 × 100 mL) before being diluted with H_2O (100 mL) and extracted with CH_2Cl_2 (3 × 100 mL). The aqueous fraction was then concentrated, taken up in H_2O (200 mL) and extracted with EtOAc (3 × 100 mL) and *n*BuOH (3 × 100 mL). The five partition fractions were concentrated *in vaccuo*, the hexane (739 mg) and CH_2Cl_2 (708 mg) fractions were combined and subjected to flash chromatography (50 g silica) using a stepwise gradient of hexane to EtOAc (10% increments of EtOAc, 350 mL per eluent) to afford eleven fractions. Fractions 3 (436 mg) and 4 (249 mg) exhibited significant antimycobacterial activity and were further purified by normal phase HPLC with fractions being collected as chromatographically homogenous peaks detected by differential refractometry. Fraction 3 (421 mg) was eluted with 19:1 hexane/EtOAc and fraction 4 (241 mg) with 9:1 hexane/EtOAc to give falcarinol (**1**; 112 mg) and panaxydol (**2**; 108 mg) respectively.

2.6. Spectroscopic and spectrometric data

(3*R*)-Falcarinol (**1**). Colorless oil; $[\alpha]^{25}_D = -54.1^\circ$ (*c* 1.85, $CHCl_3$); IR (thin film) υ_{max} 3368, 2926, 2855, 2256, 984, 930 cm^{-1}; ^1H NMR (benzene-d_6, 300 MHz) δ 5.69 (1H, ddd, *J* = 17.0, 10.1, 5.2 Hz, H-2), 5.34 (1H, m, H-10), 5.32 (1H, m, H-9), 5.24 (1H, dt, *J* = 17.0, 1.2 Hz, H-1a), 4.88 (1H, dt, *J* = 10.1, 1.2 Hz, H-1b), 4.55 (1H, m, H-3), 2.73 (2H, m, H_2-8), 1.80 (2H, m, H_2-11), 1.45 (1H, bs, 3-OH), 1.27 (2H, m, H_2-16), 1.21 (2H, m, H_2-15), 1.18 (6H, m, H_2-12, H_2-13, H_2-14), 0.90 (3H, t, *J* = 6.9 Hz, H_3-17); ^{13}C NMR (benzene-d_6, 100 MHz) δ 136.9 (d, C-2), 133.0 (d, C-10), 122.6 (d, C-9), 116.1 (t, C-1), 80.2 (s, C-7), 75.7 (s, C-4), 71.6 (s, C-5), 65.3 (s, C-6), 63.5 (d, C-3), 32.2 (t, C-16), 29.6 (t, C-12), 29.5 (t, C-13), 29.5 (t, C-14), 27.4 (t, C-11), 23.1 (t, C-16), 17.8 (t, C-8), 14.4 (q, C-17); ESIMS *m*/*z* 243.2 (62), 235.2 (100), 203.2 (91), 181.1 (42), 149.0 (58) 147.1 (57) 117.1 (44), 105.1 (50); HRESIMS *m*/*z* 245.1898 [M+H$^+$] (calculated for $C_{17}H_{25}O$, 245.1905).

(3*R*, 9*R*, 10*S*)-Panaxydol (**2**). Colorless oil; $[\alpha]^{25}_D = -176.3^\circ$ (*c* 1.56, $CHCl_3$); IR (thin film) υ_{max} 3399, 2929, 2856, 2257, 986, 931 cm^{-1}; ^1H NMR (benzene-d_6, 300 MHz) δ 5.70 (1H, ddd, *J* = 17.0, 10.2, 5.2 Hz, H-2), 5.26 (1H, ddd, *J* = 17.0, 1.6, 1.2 Hz, H-1a), 4.90 (1H, *I* = 10.2, 1.6, 1.2 Hz, H-1b), 4.57 (1H, m, H-3), 2.83 (1H, m,H-9), 2.59 (1H, m, H-10), 2.30 (1H, ddd, *J* = 17.7, 5.5, 0.9 Hz, H-8a), 2.00 (1H, ddd, *J* = 17.7, 7.0, 1.0 Hz, H-8b), 1.62 (1H, bs, 3-OH), 1.26 (2H, m, H_2-16), 1.24 (4H, m, H_2-11, H_2-12), 1.20 (2H, m, H_2-15), 1.19 (4H, m, H_2-13, H_2-14), 0.91 (3H, t, *J* = 6.9 Hz, H_3-17); ^{13}C NMR (benzene-d_6, 100 MHz) δ 136.8 (d, C-2), 116.2 (t, C-1), 77.3 (s, C-4), 76.3 (s,