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Serrulatic acid diastereomers identified from an antibacterial survey of *Eremophila*

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ABSTRACT

In an age of growing antimicrobial resistance, new antibacterial agents are desperately needed. A rapid antibacterial and phytochemical survey was designed to screen for antibacterial leads in plants. The survey was applied to over 90 Australian native plants from the genus *Eremophila*, revealing *Eremophila complanata* and *E. nivea* × *E. drummondii* as active against Gram positive bacteria. Thin layer chromatography with bioautography, flash chromatography and nuclear magnetic resonance led to the isolation and identification of two diastereomeric serrulatic acids. A single stereoisomer of 7,8,16-trihydroxyserrulat-19-oic acid has been previously described as its methyl ester. This paper describes the NMR of both serrulatic acids epimeric at C15 and their methyl esters, and demonstrates their Gram positive antibacterial activity. It is the first time that stereoisomers of this serrulatic acid have been found together in some *Eremophila* species. Further characterization of *E. complanata* additionally found an abundance of α-selinene and β-selinene. The study validates a rapid survey approach to finding antibacterial phytochemicals.

1. Introduction

The threat of antimicrobial resistance described by the World Health Organization [1] and US Centers for Disease Control and Prevention [2] has been highlighted by recent reports of bacteria resistant to our last lines of drugs [3,4]. Despite this, there have been few new drugs developed to combat resistant organisms [5]. Natural products from bacteria and fungi have provided our best defenses to date [6], and as plants similarly rely heavily on secondary metabolites to protect them from bacterial attack [7], it is possible that phytochemicals could lead to a new line of antibacterial agents.

We have focused our search for antibacterial leads on native Australian flora, including the genus *Eremophila* R.Br. (Scrophulariaceae). *Eremophila* are commonly known as Emu or Fuchsia Bushes and include 215 species growing as shrubs to small trees across the Australian mainland [8]. *Eremophila* have been reportedly utilized in the bush medicines of a number of Australian Aboriginal peoples, with patterns of use that suggest potential antibacterial qualities [9,10]. The antimicrobial potential of species in the genus has been and continues to be keenly studied [11–20].

2. Materials and methods

2.1. General

GC–MS characterization was undertaken on an Agilent GC System 7890A and inert MSD with triple-axis detector 7975C. TLCs were performed on Merck TLC silica gel $60F_{254}$ plates visualized under UV. NMR experiments were performed on a Bruker Ascend 500 MHz (125 MHz for ^{13}C) spectrometer in $CDCl_3$ and referenced to residual $CHCl_3$ (7.26 ppm for ^{1}H and 77.16 ppm for ^{13}C). Flash chromatography was undertaken with silica gel (Merck Kieselgel 60, 200–400 mesh). Rotary evaporation was at approximately 45 °C under reduced pressure. Disk diffusions were run on 6 mm blank disks with 10 μg gentamicin control disks supplied by Oxoid. Oxoid Mueller-Hinton agar, nutrient broth and bacteriological agar were made as per manufacturer instructions except where specified otherwise. Sigma-Aldrich tetracycline HCl was prepared from 1152 μg/ml tetracycline equivalent stock solution and iodonitrotetrazolium chloride from 2 mg/ml stock solution. All minimum inhibitory concentrations (MICs) were calculated in 96-well flat-based microtest plates and TLC bioautographies undertaken in 100 mm square petri dishes (Sarsdedt). HRMS were recorded using a Xevo TOF-MS in positive ESI V mode (Source temperature 80 °C, desolvation temperature 150 °C, Capillary 2.5 kV). IR spectra were recorded on a Perkin Elmer Spectrum Two. Specific rotations were measured on a

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2.2. Plant material and extraction

For rapid in-field extractions approximately 0.02 g of leaf material was collected from plants maintained in a private *Eremophila* garden in Inverell, north-western New South Wales, Australia and immediately placed in glass vials containing MeOH (~1 ml). Plant identification was confirmed by the garden's horticulturist. After overnight extraction, batches were directly injected into the GC–MS for characterization, or frozen until characterization. Larger quantities of aerial plant material for TLC bioautography and active compound isolation of *E. complanata* Chinnock (on *Myoporum montanum* root stock) and *E. nivea* × *E. drummondii* "Spring Affair" hybrid was collected from the same garden and vouchers lodged in the NCW Beadle Herbarium with the voucher numbers: *E. complanata*, NE 102411 and *E. nivea* × *E. drummondii* NE 102412. Samples were maintained frozen until extraction. All extractions carried out in the laboratory were undertaken at ambient temperature. Material from *E. complanata* (19.6 g) and *E. nivea* × *E. drummondii* (27.7 g) were coarsely chopped, immersed in sufficient MeOH to cover and sealed for 24 h at room temperature. Material was filtered using a combination of No. 1 Whatman filters and cotton wool. MeOH was removed using a rotary evaporator leaving crude extracts of *E. complanata* (3.42 g) and *E. nivea* × *E. drummondii* (3.98 g).

To further characterize *E. complanata*, and to review the serrulatic acid content of non-hybrid *E. drummondii* F.Muell. and *E. nivea* Chinnock, several tube stock specimens of each species were obtained from the Australian Arid Lands Botanic Garden (Port Augusta, SA, Australia). Representative specimens were vouchered at the BCW Beadle Herbarium (NE104754, NE105275 and NE105276, respectively), and plant material dried (5 days at 40 °C), coarsely ground and extracted with solvent. The *E. complanata* (42.6 g) material was extracted with DCM (425 ml) to obtain 2.0 g of crude extract. The non-hybridized *E. nivea* (3.93 g) and *E. drummondii* (8.49 g) materials were extracted with MeOH (80 ml and 85 ml, respectively) to obtain crude extracts (0.58 g and 1.68 g, respectively).

2.3. Phytochemical survey (GC–MS assisted dereplication)

The in-field MeOH extractions were characterized by GC–MS on an Agilent HP-5MS column (30 m × 250 μm × 0.25 μm; He; 50 °C for 0 min, 5 °C/min to 260 °C, hold 2 min). To limit solvent-related peaks, data was noted from a retention time of 3.5 min. Spectra were matched to the NIST11 library [21] and a small private *Eremophila* compound library maintained at the University of New England, Armidale. The top five peaks with over 5% area were assigned trivial names where commonly used and retention indices (RI) calculated and compared to the NIST Webbook [22]. An identification was rejected if the RI was not within five points from published values for non-isothermal and non-polar data. If the quality match of the spectra to the NIST11 library was under 80% the identification was rejected. Where no published RI was available, a compound was accepted if the quality of the spectra match was over 90%.

2.4. Semi-quantitative antibacterial survey

Disk diffusions were undertaken on in-field extraction material remaining from previous tests and after being concentrated by evaporating off the solvent and reconstituting in 30 μl of MeOH. To blank disks, 8 μl of the concentrated extract was added before being left to dry for > 30 min. The disk diffusion assay was adapted from the EUCAST method [23]: colonies of *Bacillus subtilis* ACM 2268 and *Escherichia coli* ATCC 25922 were selected and grown from the previous day and suspended in sterile saline to approximate a turbidity of 0.5 McFarland (~1–2 × 10^8 CFUs/ml). A swab was immersed in the suspension and spread across Mueller-Hinton agar plates. The disks were applied and the plates incubated aerobically for 16–20 h at 35 °C. MeOH control disks (8 μl) were added to preclude activity relating to the solvent. Zones of inhibition were read from the edge of the disk to bacterial growth and activity scored as inactive (≤ 1 mm) or active (+ for 2 mm, ++ for ≥ 3 mm).

2.5. TLC bioautography

The TLC bioautography method was adapted from Hamburger and Cordell [24]: TLC plates were cut to 90 × 90 mm and MeOH extract solutions loaded 10 mm from the base by spotting, alongside a terpinen-4-ol control in the form of 100% tea tree oil (Thursday Plantation). Plates were run in 100% ethyl acetate and the solvent evaporated. Several colonies of *B. subtilis* ACM 2268 were selected from a plate grown from the previous day and suspended in 0.9% saline to approximate a 0.5 McFarland turbidity standard. The bacteria were added to molten (< 40 °C) nutrient broth containing 0.4% bacteriological agar (after inoculation) achieving a bacterial density of ~1–2 × 10^7 CFUs/ml. The TLC plates were placed in square petri dishes, 10 ml of the inoculated molten agar was poured onto the plates, and the plates were left to incubate for 18–20 h aerobically at 35 °C. After incubation, the agar topped plates were sprayed with enough 2 mg/ml iodonitrotetrazolium chloride solution to soak the surface (~2.5 ml/plate) and incubated for a further 2 h. Subsequent clearings on the plate were interpreted as indicating inhibition. Silica from TLC plates ran under the same conditions as TLC bioautographies was excised at retention factors (R_fs) corresponding to clearing zones and soaked in MeOH, syringe filtered and characterized by GC–MS. The tea tree oil control clearing corresponded with terpinen-4-ol.

2.6. Isolation and characterization of serrulatic acids and their methyl esters

A portion of the *E. complanata* extract (735 mg) was fractionated by silica gel flash chromatography (94:5:1, DCM/MeOH/AcOH) and those fractions with components matching TLC bioautography clearing zones selected for NMR characterization (355 mg, **1a**, traces of **2a**).

A substantially purified fraction of the serrulatic acids could also be obtained from the crude extract of *E. complanata* by an acid/base partition. *E. complanata* extract (1.9 g) was dissolved in Et_2O (60 ml) and extracted with sat. $NaHCO_3$ (3 × 40 ml). The aqueous extracts were washed with Et_2O (40 ml) then acidified using 10 M HCl to pH 2.0 and extracted with DCM (3 × 50 ml). The combined organic extracts were concentrated under reduced pressure to give a brown wax (47 mg) consisting of mainly **1a** by ^{1}H NMR spectroscopy. Extracts from *E. complanata* tube stock and those grown at Inverell, both contained serrulatic acid **1a**.

Concentration of the ether partition afforded a residue (1.7 g) which was chromatographed using a gradient of ethyl acetate/hexanes (1:19 to 1:0) to give an orange wax (451 mg) which consisted of mainly α-selinene/β-selinene and an unidentified hydrocarbon.

A portion of the *E. nivea* × *E. drummondii* extract (1.26 g) was fractionated by silica gel flash chromatography (94:5:1, DCM/MeOH/AcOH) and those fractions with components matching TLC bioautography clearing zones selected for NMR characterization (339 mg, **2a**).

Acidic fractions were prepared from *Eremophila drummondii* extract (1.68 g) and *E. nivea* extract (0.58 g) as for *E. complanata* to give brown waxes (13 mg) and (11 mg) respectively.

2.6.1. rel-(1R,4S,11S,15R)-7,8,16-trihydroxyserrulat-19-oic acid (1a)

R_f 0.3 (94:5:1, DCM/MeOH/AcOH); $[\alpha]_D^{18}$ –83.0 (EtOH); IR (neat) ν_{max} 2927, 1657, 1481, 1463, 1375, 1288, 1219, 1186, 1114, 1001, 938, 907, 883, 797, 764, 732, 690 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 373.1987 $[M + Na]^+$ calculated for $C_{20}H_{30}O_5Na$, 373.1991.