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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.

Keywords: *Eremophila*, antibacterial, serrulatic acid, Australia, survey, phytochemistry

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 60 F_{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 Rudolph Research Analytical Autopol I.

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 x 250 µm x 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 minutes. 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 x 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 ≥ 3mm).

2.5. TLC Bioautography

The TLC bioautography method was adapted from Hamburger and Cordell [24]: TLC plates were cut to 90 x 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 x 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.

***rel*-(1*R*,4*S*,11*S*,15*R*)-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.

***rel*-(1*R*,4*S*,11*S*,15*S*)-7,8,16-Trihydroxyserrulat-19-oic acid (2a)**. R_f 0.3 (94:5:1, DCM/MeOH/AcOH); $[\alpha]_D^{18}$ –74.1 (EtOH); IR (neat) ν_{max} 2927, 2870, 1656, 1480, 1463, 1375, 1286, 1218, 1185, 1114, 1000, 937, 907, 882, 797, 764, 732, 690, 650 cm^{-1}; ^{1}H NMR and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 373.1998 $[M + Na]^+$ calculated for $C_{20}H_{30}O_5Na$, 373.1991.

***rel*-(1*R*,4*S*,11*S*,15*R*)-Methyl-7,8,16-trihydroxyserrulat-19-oate (1b)**. A solution of anhydrous methanolic HCl was prepared by dissolving acetyl chloride (100 mg) in MeOH (5 mL) and stirring for 10 minutes. A solution of acid **1a** (50 mg, 0.13 mmol) was added and the mixture maintained for 96 h at ambient temperature before being heated with stirring at 60°C under nitrogen for 17 h. The volatiles were removed under reduced pressure then residual HCl removed by adding toluene and again evaporating under reduced pressure. Purification of the residue by flash chromatography (19:1 DCM/MeOH) afforded a waxy solid (2 mg, 4%); R_f 0.5 (94:5:1, DCM/MeOH/AcOH); ^{1}H NMR and ^{13}C NMR data, see Table 3; HRESIMS *m/z* 387.2143 $[M + Na]^+$ calculated for $C_{21}H_{32}O_5Na$, 387.2147.

***rel*-(1*R*,4*S*,11*S*,15*S*)-Methyl-7,8,16-trihydroxyserrulat-19-oate (2b)**. Treatment of **2a** (50 mg) as per the synthesis of **1b** with the modification that the mixture was heated to 60 °C for 72 hours afforded **2b** (5 mg, 10%); R_f 0.5 (94:5:1, DCM/MeOH/AcOH); IR (neat) ν_{max} 2929, 1673, 1440, 1303, 1223, 1031, 947, 792 cm^{-1}; HRESIMS *m/z* 387.2147 $[M + Na]^+$ calculated for $C_{21}H_{32}O_5Na$, 387.2147.

2.7. Minimum Inhibitory Concentrations

The MIC assay was adapted from the Clinical and Laboratory Standards Institute [25] but included the 'sloppy agar' adaption for poorly soluble compounds [26] and the addition of a tetrazolium salt to assist interpretation [13]. Agar was prepared by adding 0.15% w/v bacteriological agar to nutrient broth. Active fractions were reconstituted in DMSO and dispersed into the sloppy agar with a starting concentration (after inoculation) of 2 mg/ml (tetracycline 64 µg/ml), and serially diluted such that each subsequent row was half the concentration of the preceding well. Bacteria (*B. subtilis* ACM 2268, *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC29213 and *Staphylococcus epidermidis* ACM 3978) were subcultured from a Mueller-Hinton plate grown from the previous day and suspended in 0.9% saline to approximate a 0.5 McFarland turbidity standard. The suspension was diluted so the final bacterial concentration in each well was ~5 x 10^5 CFUs/ml. Plates were incubated aerobically for 16-20 h at 35°C after which 40 µl of 0.2 mg/ml iodonitrotetrazolium chloride was added to each well. Plates were re-incubated for a further 2-3 h. Colored wells indicated metabolism of the iodonitrotetrazolium chloride while clear wells were interpreted as indicating inhibition. Fractions were run in triplicate within each plate and the experiment repeated. The triplicates were averaged and the repeated experiments reported as a range. DMSO control wells indicated no inhibition resulted from the use of this solvent at the concentrations employed (maximum 2% v/v).

3. Results and discussion

3.1. Rapid antibacterial survey and GC-MS assisted dereplication

The high throughput semi-quantitative antibacterial survey of MeOH leaf extracts from 91 species, subspecies, hybrids and varieties of *Eremophila* revealed those plants with antibacterial promise in the disk diffusion screen against the rapid growing Gram-positive organism, *B. subtilis* (Table 1; Supplementary Table S.1). Most noteworthy were *E. complanata* and *E. nivea* × *E. drummondii.* A screen by Ndi, Semple, Griesser and Barton [15] previously identified an *E. complanata* extract as having antibacterial qualities but did not identify the active compounds. This is the first time the hybrid *E. nivea* × *E. drummondii* has been reviewed for antimicrobial activity. There was no noteworthy activity of any of the plants tested against the Gram-negative *Escherichia coli* (zones of inhibition < 1 mm). GC-MS-assisted dereplication identified known antimicrobial compounds such as α-pinene, β-pinene, cinnamic acid, farnesol, caryophyllene, germacrene D and p-cymene in several plants. However, the two notably active specimens included peaks which could not be matched by the NIST11 library and so a potential cause for the antimicrobial activity could not be identified.

Table 1

Antibacterial extracts as indicated by a *B. subtilis* disk diffusion assay with GC-MS dereplication

Species	Inhibition	Compounds (% peak)
E. aureivisca	+	U_{1220}(24); U_{1313}(7); Cinnamic acid(7); $U_{1495\text{-}6}$(17); U_{1843}(17)
E. calorhabdos	+	α-Pinene(9); $U_{1022\text{-}7}$(13); $U_{1832\text{-}3}$(13); 9,12,15-Octadecatrien-1-ol, (Z,Z,Z)-(13); U_{2113}(10)
E. complanata	++	$U_{1418\text{-}23}$(23); Selinene(18)*; $U_{2628\text{-}31}$(45)
E. decipiens subsp. *decipiens*	+	α-Pinene(15); β-Pinene(9); $U_{1495\text{-}6}$(19); $U_{2636\text{-}40}$(11); U_{2662}(10)
E. gilesii x *E. latrobei*	+	α-Pinene(15); U_{988}(9); Germacrene D(7); $U_{1495\text{-}6}$(30); U_{2351}(7)
E. lehmanniana	+	Caryophyllene(10); cis-β-Farnesene(8); Elixene(11); $U_{2599\text{-}601}$(36); U_{2776}(9)
E. lucida	+	$U_{1711\text{-}4}$(12); Farnesol(11); trans,trans-Farnesal(33); Octadecanal(11)
E. microtheca	+	p-Cymene(15); $U_{2599\text{-}601}$(72)
E. nivea × *E. drummondii*	++	Viridiflorene(8); $U_{2636\text{-}40}$(87)
E. scaberula	+	Caryophyllene(6); Bicyclogermacrene(81)
E. subfloccosa subsp. *glandulosa*	+	α-Pinene(14); Caryophyllene(11); Germacrene D(5); $U_{1495\text{-}6}$(49)
E. virens	+	Calamenene(10); U_{1804}(87)
Gentamicin (10 µg)	++	

Semi-quantitative evaluation of inhibition measured as a clearing from disk edge to *B. subtilis* growth: + = 2 mm, ++ = ≥ 3 mm; 8 µl extract-MeOH solution per disk; U, Unidentified peak(s), retention indices in subscript. See Supplementary Table S.1 for the complete survey. *The original GC-MS data identified this compound as γ-selinene, however NMR analysis on *E. complanata* revealed a mix of α- and β- selinenes.

3.2. Isolation of antimicrobial constituents

Owing to their activity, further material was collected and extracted from the same sources of *E. complanata* and *E. nivea* × *E. drummondii*. Thin layer chromatography (TLC) and TLC bioautography of the extracts against *B. subtilis* suggested the active compounds in both to be polar acids. GC-MS analysis

of the corresponding MeOH extracted TLC silica was unable to identify the corresponding compounds, suggesting poorly volatile compounds.

Fractions from *E. complanata* and *E. nivea* × *E. drummondii* extracts were isolated which corresponded to the highly active regions of the TLC bioautographies and back-tested for activity using an MIC assay. Consistent with the initial screening, activity was greatest against Gram positive organisms, including *S. aureus*, *S. epidermidis* and *B. subtilis* (Table 2). Both fractions had poor activity against Gram-negative *E. coli* and *P. aeruginosa* (> 500 µg/ml).

Table 2

MICs of active fractions (µg/ml)

Species	*S. aureus*	*S. epidermidis*	*B. subtilis*	*P. aeruginosa*	*E. coli*
E. complanata (**1a**, traces **2a**)	250-333	63-125	125-208	>500	>500
E. nivea × *E. drummondii* (**2a**)	250-333	83-125	125-333	>500	>500
Tetracycline	0.13-0.25	32-64	0.063	8-16	1-8

3.3. Characterization of antimicrobial compounds

The active fractions of *E. complanata* and *E. nivea* × *E. drummondii* revealed unique stereoisomers of 7,8,16-trihydroxyserrulat-19-oic acid differing at the distal stereocenter. The relationship in **1a** and **2a** between the ring stereochemistry and the C15 stereocenter was not clear and so the C15 center has been arbitrarily assigned *vide infra* (Fig. 1). Compound **1a** was isolated from *E. complanata* and found to be accompanied by traces of **2a**. Purification of the extract of *E. nivea* × *E. drummondii* afforded **2a** and there was no evidence of the second diastereomer **1a**.

The acid **2a** was first isolated from *E. drummondii* by Croft et al. [27] and the relative configuration around the ring and the distal C15 stereocenter assigned on the basis of an X-ray crystal structure of the methyl ester **2b** reported by Hall et al. [28]. In the original publication, only a few of the prominent peaks in the 60 MHz ^{1}H NMR of **2b** were identified and the paper predates the routine use of ^{13}C NMR. The single stereoisomer reported and used in the study was isolated by crystallization following chromatography, however the presence of a second diastereomer in the extracts obtained by Croft et. al

cannot be ruled out. More recently, the diastereomer **2a** has been reported to have inhibitory activity against the enzymes PTP1B and α-glucosidase, associated with type 2 diabetes, however, the authors of that study did not report any data for the diastereomer used [29].

In an attempt to identify the two diastereomers isolated from *E. complanata* and *E. nivea* × *E. drummondii*, an authentic sample of serrulatic acid **2a** was sought from *E. drummondii*. The acidic fractions from *E. drummondii* (and *E. nivea*) were thus prepared from commercially grown tube stock and analyzed by NMR. Despite Croft et al. only reporting one diastereomer in *E. drummondii*, the extracts in the current study showed that both **2a** and **1a** were present in near equal amounts (see Supplementary Fig. S.1), complicating the interpretation of data presented in previous works [18, 27, 28]. *Eremophila nivea* was found to contain only **2a**.

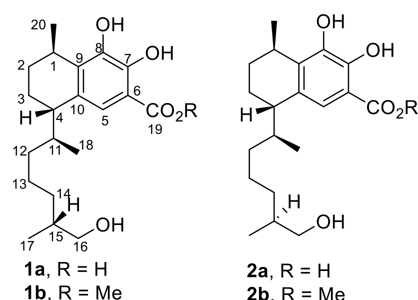

Fig. 1. Structure and stereochemistry of serrulatic acids **1** and **2**.

As these serrulatic acids are not well described in the literature, a discussion of the NMR for **1a** isolated from *E. complanata* is warranted and assignments are made in Table 3 with 2D correlations shown in Fig. 2. The ^{1}H NMR exhibited a single aromatic resonance at δ 7.27 ppm, a diastereotopic pair at δ 3.47 and 3.40 ppm and three methyl doublets at δ 1.22, 0.93 and 0.87 ppm. The CH_3 doublet at δ 1.22 ppm showed a COSY crosspeak with the benzylic C1-H at δ 3.24 ppm which also coupled to the C2 diastereotopic pair at δ 1.91 and 1.48 ppm. The C11 methine at δ 1.87 ppm was assigned on the basis of a coupling to the methyl group at δ 0.93 ppm and C4 resonance at δ 2.55 ppm. A HMBC correlation was seen between benzylic C4-H at δ 2.55 ppm and the aromatic resonance at δ 120.9 ppm which established the location of the aromatic proton. A HMBC correlation was seen between the resonance at δ 7.27 ppm and the carbonyl at δ 173.7 ppm confirming the serrulatic acid structure.

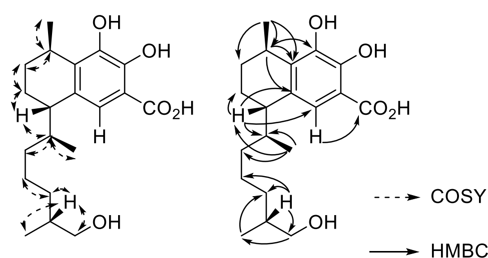

Fig. 2. COSY and HMBC correlations observed for **1a**.

The serrulatic acid isolated from *E. nivea* × *E. drummondii* **2a** had the same connectivity as **1a** and the chemical shifts for the fused-ring resonances differed by less than 0.1 ppm in the ^{13}C NMR spectra of **1a** and **2a**. The largest differences were in the ^{1}H and `^{13}C chemical shifts assigned as C12, C13 and C14 linking the ring and the distal stereocenter (see Supplementary Fig. S.2). The ^{1}H NMR chemical shifts were displaced between 0.1-0.4 ppm and the ^{13}C NMR chemical shifts had a maximum difference of 0.51 ppm. On the basis of the similarities of the ring-resonances in both diastereomers, the stereochemistry at C1 and C4 were assigned as being the same. Inversion of the stereocenter at C11 adjacent to the ring is possible, however, the C11 resonances in both spectra had very similar chemical shifts in the ^{1}H NMR and differed by only 0.4 ppm in the ^{13}C NMR. Additionally, there was no difference in the chemical shift of the adjacent C4-H resonances between diastereomers. On the basis of these chemical shifts, the two diastereomers were assigned as being epimeric at C15. Although there is a crystal structure for methyl ester **2b**, the lack of spectroscopic data to correlate with this diastereomer, the similarity of the NMR spectra between diastereomers, and the presence of both diastereomers in the *E. drummondii* extract means that the relative configuration at C15 in each diastereomer cannot be confidently assigned.

Table 3

Comparison of characterized and published NMR chemical shift values.

	1a		**2a**			**1b**		**2b**		**Lit** [27]
Pos.	^{1}H	^{13}C	^{1}H	^{13}C	$\Delta\delta\ ^{13}C_{1a-2a}$	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	3.24 (ddq, 6.8, 6.8, 3.0)	27.8	3.25 (ddq, 7.0, 7.0, 3.4)	27.8	0.0	3.24 (m)	27.6	3.23 (ddq, 7.0, 7.0, 2.6)	27.6	3.20 (m)
2	1.91 (m) 1.48 (m)	27.2	1.93 (dddd, 12.6, 12.6, 6.3, 3.1) 1.48 (m)	27.2	+0.09	1.93 (13.3, 13.3, 6.0, 3.3) 1.49 (m)	26.9	1.93 (dddd, 13.3, 13.3, 6.2, 3.3) 1.48 (m)	26.9	
3	1.83 (m) 1.66	19.7	1.84 (m) 1.66 (m)	19.8	-0.06	1.85 (m) 1.66 (m)	19.6	1.84 (ddd, 13.3, 5.6, 2.8) 1.71 (dddd, 13.3, 5.0, 2.8, 2.8)	19.7	
4	2.55 (m)	42.0	2.57 (br ddd, 5.2, 5.2, 4.4)	42.0	0.0	2.54 (ddd, 5.5, 5.5, 3.3)	42.0	2.55 (ddd, 5.6, 5.6, 3.4)	42.0	2.50 (m)
5	7.27 (br s)	120.9	7.27 (br s)	120.9	-0.06	7.19 (s)	120.2	7.18 (s)	120.2	7.12 (s)
6		108.6		108.7	0.03		109.3		109.7	
7		145.8		145.9	0.0		145.5		145.5	
8		141.9		141.9	0.0		141.8		141.8	
9		137.3		137.3	0.0		136.3		136.3	
10		131.4		131.4	0.0		131.1		131.1	
11	1.87 (m)	37.6	1.86 (m)	38.0	-0.39	1.81 (m)	38.2	1.81 (m)	38.3	
12	1.30 (m) 1.21 (m)	32.8	1.24 (m) 1.05 (m)	33.33	-0.51	1.23 (m) 1.07 (m)	33.4	1.26 (m) 1.05 (m)	33.54	
13	1.31 (m) 1.20 (m)	24.7	1.42 (m) 1.07 (m)	25.1	-0.40	1.33 (m) 1.17 (m)	25.0	1.41 (m) 1.07 (m)	25.1	
14	1.05 (m, 2H)	32.8	1.34 (m) 1.00 (m)	33.29	-0.45	1.04 (m, 2H)	33.2	1.33 (m) 1.01 (m)	33.37	
15	1.54 (m)	35.3	1.56 (ddddq, 7.0, 7.0, 7.0, 7.0, 7.0)	35.6	-0.28	1.55 (m)	35.6	1.55 (m)	35.7	
16	3.47 (dd, 10.5, 5.8 Hz) 3.40 (dd 10.5, 6.5 Hz)	68.5	3.49 (dd, 10.6, 5.8) 3.41(dd, 10.6, 6.6)	68.3	0.19	3.46 (dd, 10.8, 5.9)	68.4	3.46 (br dd, 9.9, 6.3) 3.38 (br dd, 9.9, 6.7)	68.3	3.38 m
17	0.87 (d, 6.7)	16.3	0.87 (d, 6.7)	16.6	-0.25	0.87 (d, 6.7)	16.4	0.88 (d, 6.9)	16.6	0.85 (d, 7)
18	0.93 (d, 6.9)	18.6	0.96 (d, 7.0)	18.7	-0.14	0.92 (d, 6.8)	18.7	0.93 (d, 7.0)	18.7	0.90 (d, 7)
19		173.7		173.8	-0.09		170.9		170.9	
20	1.22 (d, 7.0)	20.7	1.22	20.7	0.11	1.21 (d, 7.0)	20.7	1.22 (d, 7.0)	20.7	1.20 (d, 7)
Ester						3.93 (s, 3H)	52.2	3.93 (s, 3H)	52.3	3.90 (s)
OH	4.50 (br s) 10.39 (br s)		4.31 (br s) 10.5 (br s)			5.67 (br s) 10.50 (br s)		5.70 (br s) 10.55 (br s)		

^{13}C (125 MHz; $CDCl_3$) and ^{1}H NMR (500 MHz; $CDCl_3$) chemical shift data in ppm (multiplicity, *J*); multiplet chemical shifts are given as the center of the peak from the 2D COSY NMR; I, indeterminable (under another peak). Literature values from [27] obtained at 60 MHz ($CDCl_3$).

As the phytochemistry of *E. complanata* does not appear to be well described in the literature, additional plant material was obtained as tube stock and an extract was further characterized. Fractions of the Et_2O partition were found to contain abundant α-selinene (**3**) and β-selinene (**4**) by comparing ^{13}C NMR data with published literature [30] (Supplementary Table S.2).

4. Conclusion

Both diastereomers of 7,8,16-trihydroxyserrulat-19-oic acid demonstrated activity against Gram positive bacteria which indicates that the relative stereochemistry at C15 in these molecules is unimportant. Their activity reaffirms the potential of *Eremophila* and validates the status of the genus in Australian Aboriginal medicines. The presence of multiple serrulatic stereoisomers within the same genus highlight the care needed in identifying such compounds in *Eremophila*. More generally, the rapid antibacterial screening platform with dereplication of known compounds by GC-MS has demonstrated potential in identifying antimicrobial compounds. Limitations of the method should be noted, namely that variations in leaf samples collected in the field mean extraction efficiencies remain unknown. Such a limitation will tend to select for highly active compounds extracted in lower abundance or moderately active compounds extracted in higher abundance. Additionally, it must be noted that GC-MS is limited to identifying only the more volatile known compounds in an extract. More than 19,000 vascular species of plants have been described in Australia alone [31]. The trade-off of reduced accuracy at the early screening stage for increased speed is therefore necessary if it is to be applied to other genera.

Acknowledgments: Plant material was provided by Tim Kolaczyk and his private *Eremophila* garden. The authors wish to dedicate this paper to the memory of Tim who passed away during the project.

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Supplementary Materials: See supplementary materials for the full table of *Eremophila* species surveyed, NMR data and NMR spectra.

Supplementary Materials

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

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Table S.1

Eremophila specimens reviewed with semi-quantitative *B. subtilis* disk diffusion assay

Species	Inhibition	
	B. subtilis	*E. coli*
E. abietina ssp. *ciliata*	-	-
E. accrescens	-	-
E. adenotricha	-	-
E. alternifolia x *E. bignoniflora*	-	-
E. alternifolia x *E. maculata*	-	-
E. alternifolia x *E. purpurascens*	-	-
E. arachnoides ssp. *tenera*	-	-
E. arbuscular	-	-
E. aureivisca	+	-
E. barbata	-	-
E. brevifolia	-	-
E. caerulea ssp. *caerulea*	-	-
E. calorhabdos	+	-
E. calorhabdos x *E. splendens*	-	-
E. christophorii	-	-
E. clarkei	-	-
E. clavata	-	-
E. compacta	-	-
E. complanata	++	-
E. debilis	-	-
E. decipiens ssp. *decipiens*	+	-
E. decipiens ssp. *linearifolia*	-	-
E. delisseri	-	-
E. dempsteri	-	-
E. densifolia ssp. *densifolia*	-	-
E. divaricata ssp. *callewatta*	-	-
E. divaricata ssp. *divaricata*	-	-
E. divaricata x *E. polyclada*	-	-
E. forrestii	-	-
E. georgei	-	-
E. gibbifolia	-	-
E. gibsonii	-	-
E. gilesii x *E. latrobei*	+	-
E. glabra ssp. *carnosa*	-	-
E. glabra x *E. biserrata*	-	-
E. glabra x *E. veneta*	-	-
E. hillii	-	-
E. hygrophana	-	-
E. interstans ssp. *interstans*	-	-
E. ionantha	-	-
E. jucunda	-	-
E. laanii	-	-
E. lachnocalyx	-	-
E. lehmanniana	+	-
E. longifolia x *E. scoparia*	-	-
E. lucida	+	-
E. macdonnellii (Green leaf form)	-	-
E. macdonnellii (Simpson Desert form)	-	-
E. mackinlayi	-	-
E. maculata ssp. *brevifolia*	-	-
E. maculata x *E. duttonii*	-	-
E. maculata x *E. viscida*	-	-
E. microtheca	+	-
E. muellerana	-	-
E. nivea	-	-
E. nivea × *E. densifolia*	-	-
E. nivea × *E. drummondii*	++	-
E. oldfieldii ssp. *angustifolia*	-	-
E. oldfieldii x *E. oppositifolia*	-	-
E. ovata	-	-
E. pantonii	-	-
E. parvifolia	-	-
E. polyclada	-	-
E. psilocalyx	-	-
E. psilocalyx x *E. dempsteri*	-	-
E. pterocarpa ssp. *pterocarpa*	-	-
E. racemosa	-	-
E. racemosa x *E. maculata*	-	-
E. resinosa	-	-
E. rugosa	-	-
E. saligna	-	-
E. santalina	-	-
E. sargentii	-	-
E. scaberula	+	-
E. scoparia	-	-
E. serpens	-	-
E. splendens	-	-
E. splendens x *E. maculata*	-	-
E. stenophylla	-	-
E. subfloccosa ssp. *glandulosa*	+	-
E. subfloccosa ssp. *lanata*	-	-
E. subfloccosa ssp. *subfloccosa*	-	-
E. subteretifolia	-	-
E. sulcata	-	-
E. tetraptera	-	-
E. tietkensii	-	-
E. veneta	-	-
E. vernicosa	-	-
E. veronica	-	-
E. virens	+	-
E. weldii	-	-
Methanol (8 µl)	-	-
Gentamycin (10 µg)	++	++

Semi-quantitative evaluation of bacterial inhibition measured as a clearing from disk edge to bacterial growth: - = ≤ 1 mm, + = 2 mm, ++ = ≥ 3 mm; 8 µl extract-MeOH solution per disk.

Table S.2

^{13}C NMR data from *E. complanata* (Et_2O partition, fractions 4-5) and comparison with published values for α-selinene **(3)** and β-selinene **(4)**.

Peak (ppm)	α-selinene (ppm)*	β-selinene (ppm)*
151.0	151.0	151.0
150.8		150.9
135.2	135.1	
121.1	120.9	
108.47		
108.45	108.2	
108.3		108.1
105.6		105.3
56.2		
52.2		
50.1		49.9
47.0	46.8	
46.9	46.7	
46.7		
46.0		45.8
45.5		
42.1		41.9
41.4		41.2
40.4	40.2	
38.1	37.9	
37.3		
37.1		36.9
36.1		35.9
33.7		
32.5	32.3	
32.1		
29.8		
29.6		29.5
29.5		
29.1	28.9	
28.6		
27.1		
26.97 / 27.0	26.8	26.8
26.5		
23.6		23.5
23.1	22.9	
22.9		
21.3	21.2	
21.18		
21.17		21.0
21.0	20.9	
19.5		
18.9		
16.5		16.3
15.8	15.6	
14.3		

* H.J. Williams, I. Sattler, G. Moyna, A. Ian Scott, A.A. Bell, S. Bradleigh Vinson, Diversity in cyclic sesquiterpene production by Gossypium hirsutum, Phytochemistry 40(6) (1995) 1633-1636; The fraction appears to contain a 3rd compound which could not be identified; experimental and published data in $CDCl_3$.

Fig. S.1

^{13}C NMR spectra comparing the presence of **1a** and **2a** in the plants under investigation.

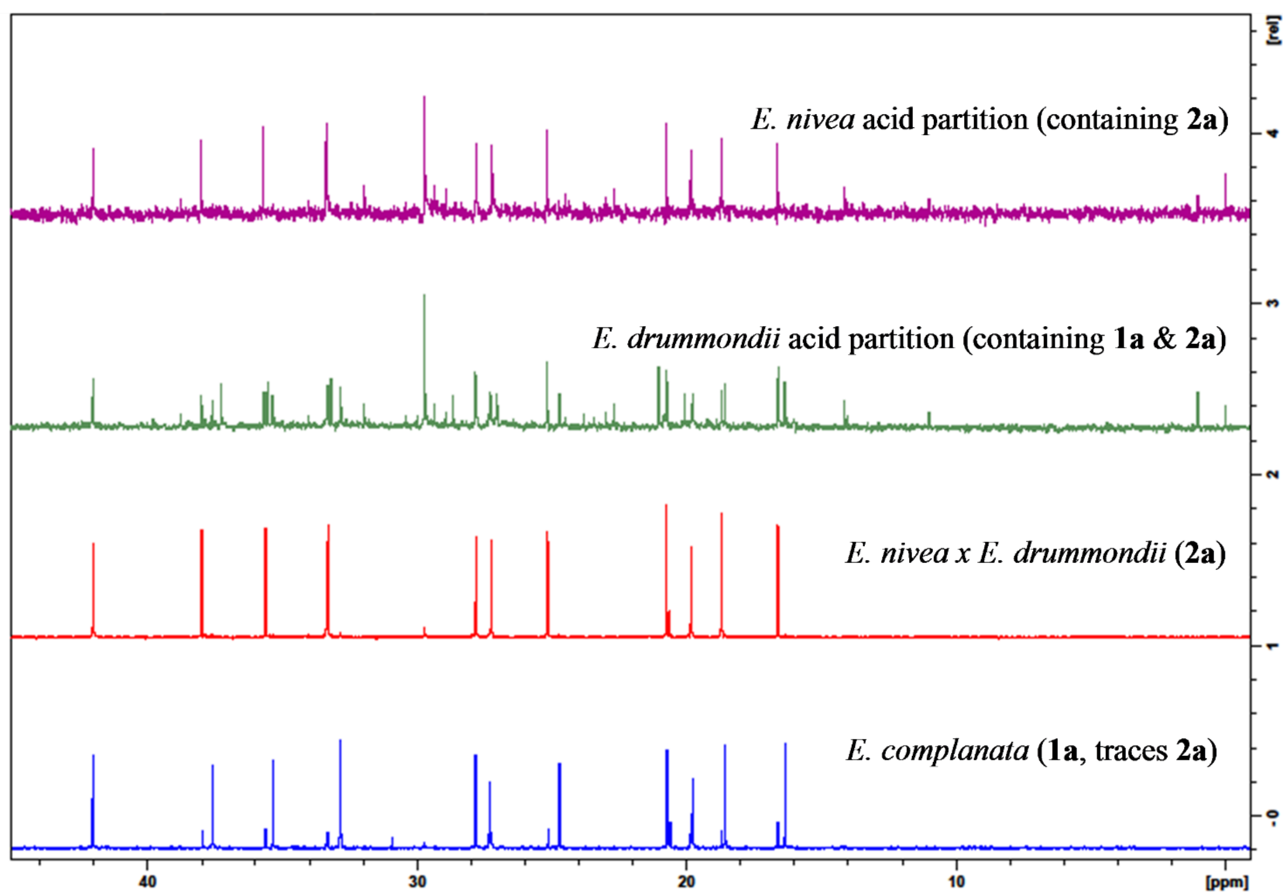


Fig. S.2

^{1}H NMR spectra for **1a** and **2a** focusing on the aliphatic region.

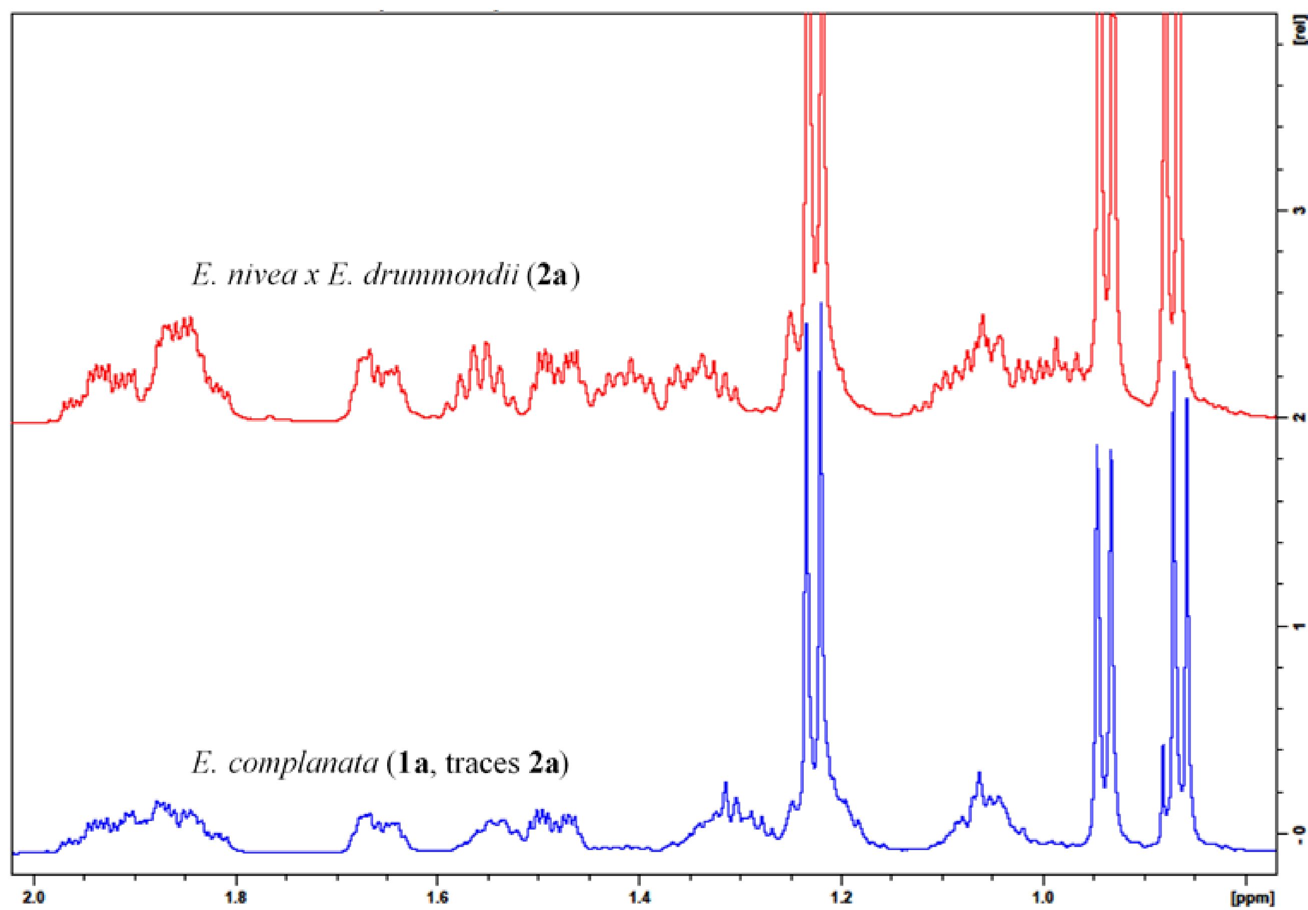


Fig. S.3

^{1}H NMR spectrum for **1a**

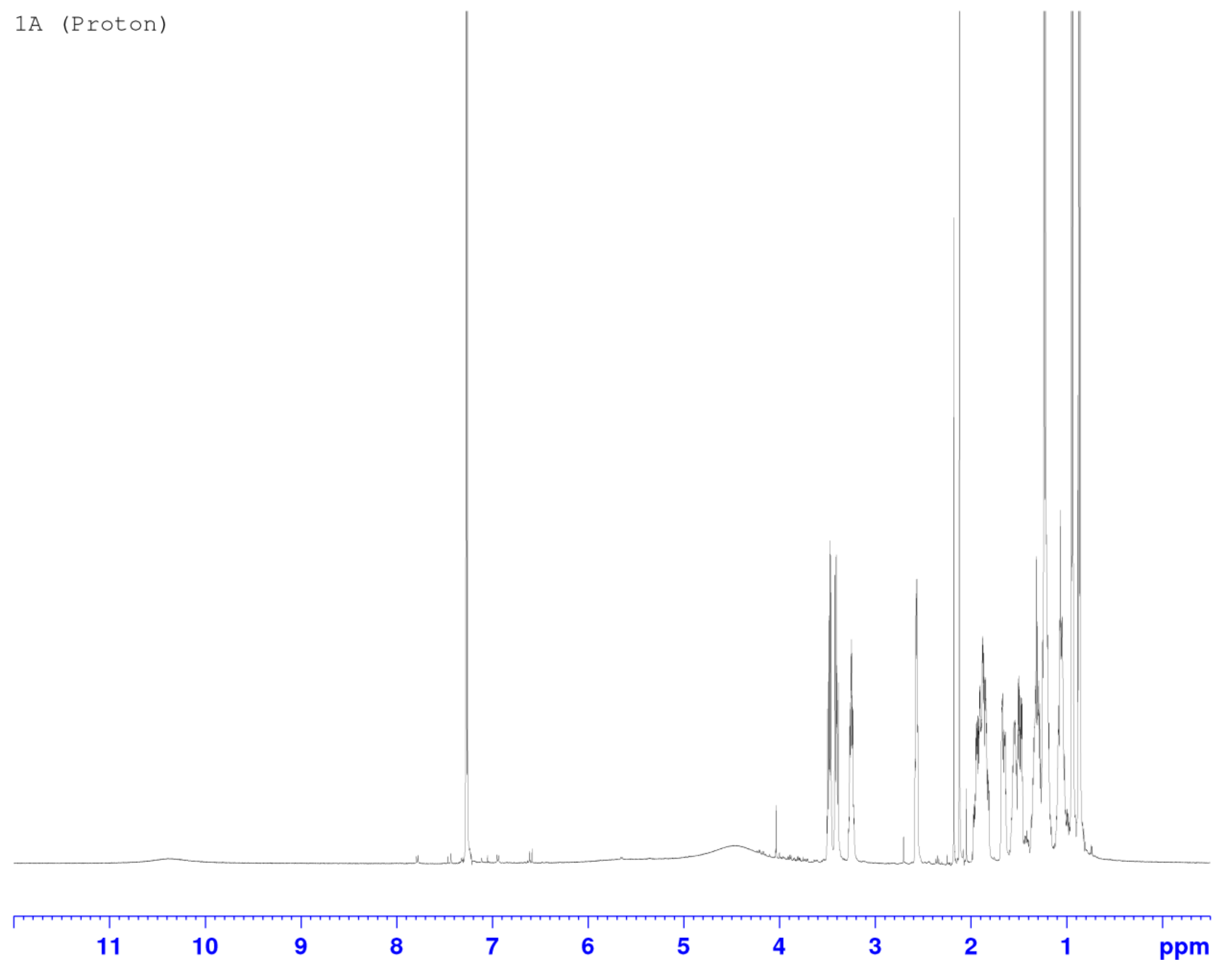
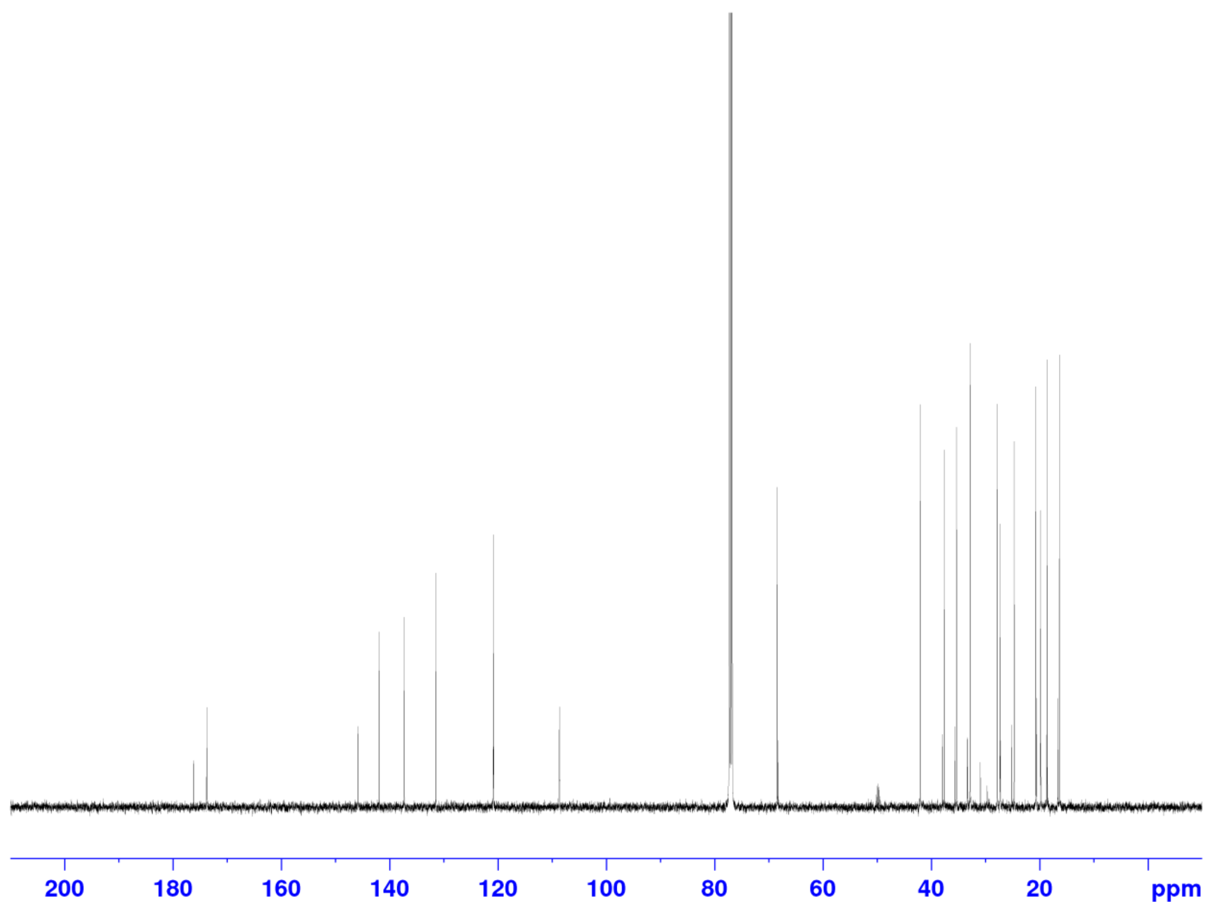


Fig. S.4

^{13}C NMR spectrum for **1a**

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DE                    6.50 usec
TE                   298.0 K
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TD0                      1
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NUC1                   13C
P1                   10.00 usec
PLW1           78.00000000 W
SFO2           500.1620006 MHz
NUC2                    1H
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PLW13           0.17989001 W

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Fig. S.5

^{1}H NMR spectrum for **2a**

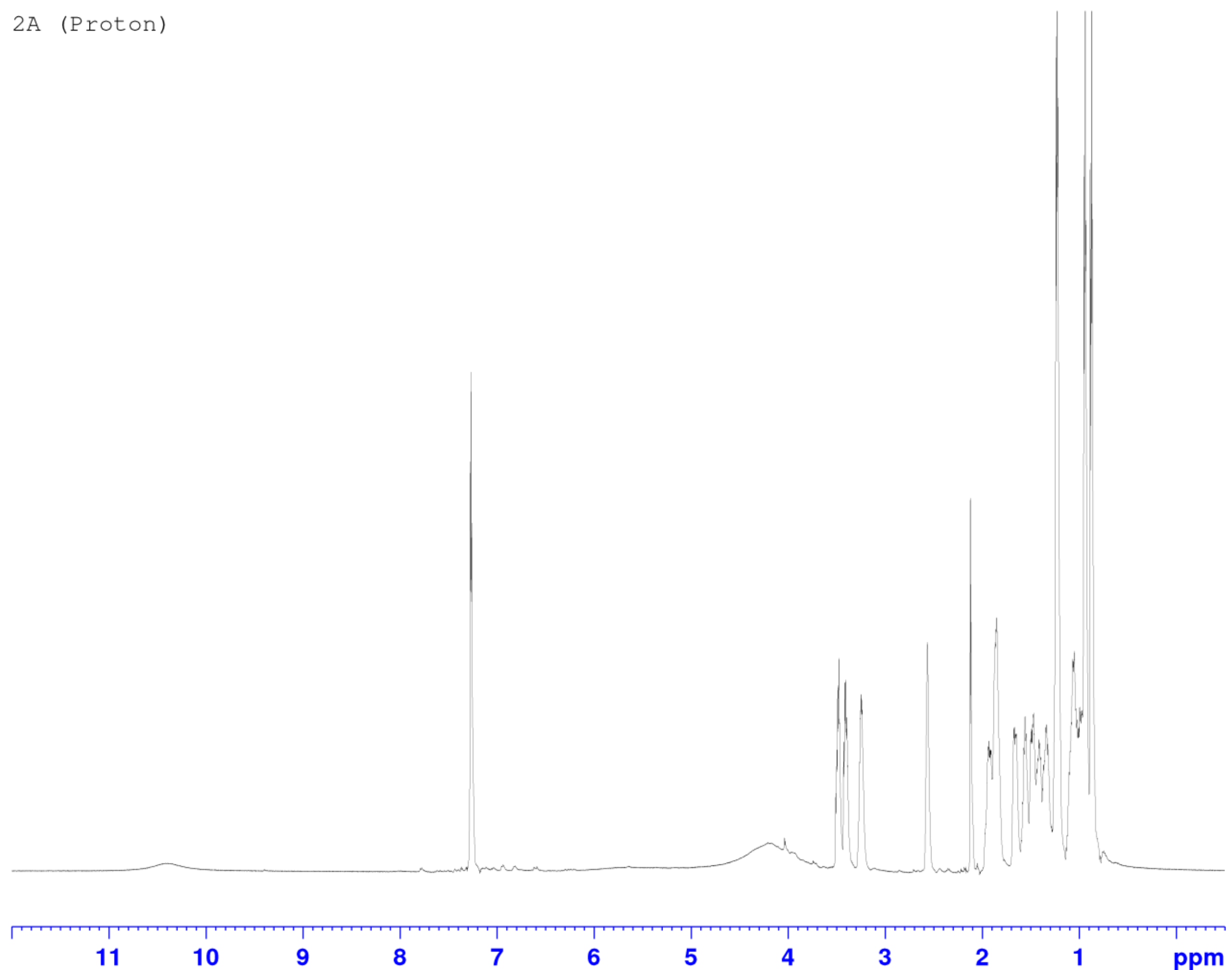
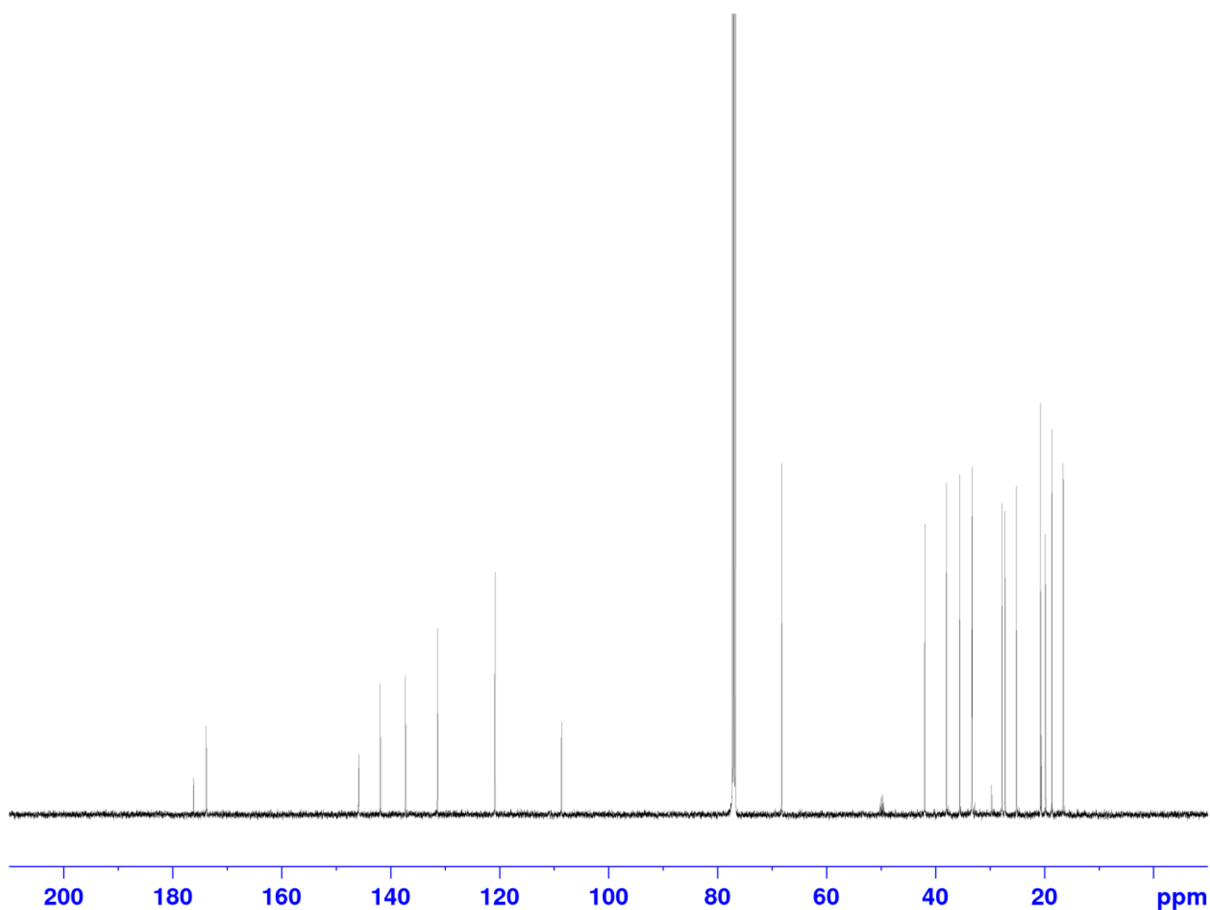


Fig. S.6

^{13}C NMR spectrum for **2a**



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FIDRES 0.908261 Hz
AQ 1.1010048 sec
RG 191.36
DW 16.800 usec
DE 6.50 usec
TE 298.0 K
D1 2.00000000 sec
D11 0.03000000 sec
TD0 1
SFO1 125.7779086 MHz
NUC1 13C
P1 10.00 usec
PLW1 78.00000000 W
SFO2 500.1620006 MHz
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PLW13 0.17989001 W

F2 - Processing parameters
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SF 125.7653351 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.40

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