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Author

Barnes, Emma C, Kavanagh, Angela M, Ramu, Soumya, Blaskovich, Mark A, Cooper, Matthew A, Davis, Rohan A

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Antibacterial serrulatane diterpenes from the Australian native plant *Eremophila microtheca*

Emma C. Barnes,[a] Angela M. Kavanagh,[b] Soumya Ramu,[b] Mark A. Blaskovich,[b] Matthew A. Cooper,[b] and Rohan A. Davis[a *]

[a]*Eskitis Institute, Griffith University, Nathan, QLD, 4111, Australia.*

[b]*Institute for Molecular Bioscience, University of Queensland, St Lucia, QLD, 4072, Australia.*

Abstract

Chemical investigations of the aerial parts of the Australian plant *Eremophila microtheca* resulted in the isolation of three new serrulatane diterpenoids, 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**1**), 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**2**), and 3,19-diacetoxy-8-hydroxyserrulat-14-ene (**3**) as well as the previously reported compounds verbascoside (**4**) and jaceosidin (**5**). Acetylation and methylation of the major serrulatane diterpenoid (**2**) afforded 3,8-diacetoxy-7-hydroxyserrulat-14-en-19-oic acid (**6**) and 3,7,8-trihydroxyserrulat-14-en-methyl-19-benzoate (**7**), respectively. The antibacterial activity of **1**-**7** was assessed against a panel of Gram-positive and Gram-negative bacterial isolates. All the serrulatane compounds exhibited moderate activity against *Streptococcus pyogenes* (ATCC 12344) with minimum inhibitory concentrations (MICs) ranging from 64—128 μg/mL. Serrulatane **1** demonstrated activity against all Gram-positive bacterial strains (MICs

* Corresponding author. Address: Eskitis Institute, Griffith University, Nathan, QLD, 4111, Australia. Tel.: +61 7 3735 6043; fax: +61 7 3735 6001. E-mail address: r.davis@griffith.edu.au (R.A. Davis).

64—128 μg/mL) except for *Enterococcus faecalis* and *Enterococcus faecium*. This is the first report of natural products from *Eremophila microtheca*.

Keywords

Eremophila microtheca; Myoporaceae; Antibacterial; Serrulatane; Diterpenoid

1. Introduction

While the Australian endemic genus *Eremophila* (family Myoporaceae) has been a source of > 100 structurally diverse compounds, mostly within the terpenoid structure class, a large number of the 215 species identified to date remain chemically under-investigated (Chinnock, 2007; Dictionary of Natural Products, 2012; Ghisalberti, 1994a; Ghisalberti, 1994b). *Eremophila* plants grow within the arid and semi-arid regions of Australia, and a number of these species are recorded as having been used by Australian Aboriginal people to treat a range of ailments such as colds, wounds, and scabies (Ghisalberti, 1994a; Latz, 1995; Low, 1990). A number of bioactivities have been reported for *Eremophila* extracts or isolated natural products, including antibacterial (Liu et al., 2006; Ndi et al., 2007a; Ndi et al., 2007b; Ndi et al., 2007c; Palombo and Semple, 2001, 2002; Smith et al., 2007; Wilkinson and Cavanagh, 2005), antiviral (Semple et al., 1998), anti-inflammatory (Liu et al., 2006), anti-malarial (Barnes et al., 2012), and cytotoxic activities (Beattie, 2009; Beattie et al., 2011). Furthermore, *Eremophila* extracts and/or natural products have been studied for their cardioactivity (Pennacchio et al., 1995; Pennacchio et al., 2005; Pennacchio et al., 1996, 1997) and have been investigated as potential treatments for

neurological disorders such as migraine (Grice et al., 2003; Rogers et al., 2002; Rogers et al., 2000, 2001).

Literature searches on the *Eremophila* samples contained within the Eskitis Institute's Nature Bank biota library (Nature Bank, 2011) identified a number of under-investigated species, including *Eremophila microtheca* (F. Muell. ex Benth.) (Dictionary of Natural Products, 2012; SciFinder, 2012). Only one reference could be found on *E. microtheca*, in which an extract of this species was included in a study of the antimicrobial activity of 72 *Eremophila* plants (Ndi et al., 2007a). No reports were found of natural products that had been isolated from *E. microtheca*, indicating that this plant had the potential to yield new and/or bioactive chemistry. Due to our continuing interest in the chemistry of *Eremophila* species (Barnes et al., 2011; Barnes et al., 2012) we thus prioritised *E. microtheca* for chemical investigation.

E. microtheca grows as an erect shrub up to 1.5 m tall with lilac flowers (Chinnock, 2007). The name *microtheca* comes from the Greek language, with '*micro*' meaning small, and '*theca*' case or container, which refers to the small nature of the fruit of this particular plant. This species is relatively rare in the wild, being found in just a few localities in Western Australia (Chinnock, 2007). However, this plant is becoming a common addition in many nurseries within Australia due to its attractive flowers and drought tolerance. This species also possesses a distinctive aroma that some find offensive. Chinnock reported that the odour of the foliage can be discerned from some distance, and that one large wild population of *E. microtheca* was discovered after the searchers followed the odour to the source (Chinnock, 2007).

Herein we report the isolation and structure elucidation of three new serrulatanes from *E. microtheca*. These natural products as well as two semi-synthetic derivatives were also examined for their anti-bacterial activity.

2. Results and discussion

The air-dried aerial parts of *E. microtheca* were extracted with sequential washes of CH_2Cl_2 and CH_3OH. Both extracts were combined and subsequently fractionated by semi-preparative HPLC (C_{18}-bonded silica, H_2O/CH_3OH). This afforded the new serrulatanes: 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**1**, 66 mg, 0.638% dry wt); 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**2**, 453 mg, 4.355% dry wt); and 3,19-diacetoxy-8-hydroxyserrulat-14-ene (**3**, 54 mg, 0.519 % dry wt) and the previously reported compound verbascoside (**4**, 44 mg, 0.418% dry wt) (Aligiannis et al., 2003; Andary et al., 1982). One semi-pure fraction from the HPLC work was further purified by size exclusion chromatography (Sephadex LH-20, CH_2Cl_2:CH_3OH) to afford the known flavonoid jaceosidin (**5**, 9 mg, 0.088% dry wt) (ApSimon et al., 1963; Nakasugi et al., 2000).

Compound **1** was isolated as an optically active brown gum and assigned a molecular formula of $C_{22}H_{30}O_6$ (eight degrees of unsaturation) on the basis of NMR (Tables 1 and 2) and HRESIMS data. The ^{1}H NMR spectrum of **1** indicated the presence of five methyl groups (δ_H 0.47, 1.20, 1.56, 1.65 and 2.00), one –OCHR– moiety (δ_H 5.19), and two olefinic protons (δ_H 5.10 and 6.96) and also contained several upfield signals that integrated for eight protons. The ^{13}C NMR and edited HSQC spectra of **1** indicated a total of 22 carbons (Table 2), including five methyls (δ_C 17.5, 18.8, 21.0, 21.5, 25.5), two carbonyls (δ_C 169.8 and 172.3), three methylenes (δ_C 25.4, 31.4, 37.4), six sp^2 quaternary carbons (δ_C 110.3, 126.6, 130.8, 134.7, 142.4, 147.7), two sp^2 methines (δ_C 119.8 and 124.3), and four sp^3 methines (δ_C 28.0, 31.2, 44.5, 69.6).

The extended spin system –(R)CH(CH_3)CH_2CH(OR)CH(R)CH(CH_3)CH_2CH_2CH=C(CH_3)$_2$ was readily established following interpretation of the COSY data for **1** and was supported by key HMBC correlations (Figure 1). For example, the methyl group at H-20 (δ_H 1.20) showed HMBC correlations to C-1 (δ_C 28.0), C-2 (δ_C 31.4), and C-9 (δ_C 134.7). HMBC correlations from δ_H 1.95 (H-13) to C-14 (δ_C 124.3) and C-15 (δ_C 130.8) and from δ_H 5.10 (H-14) to C-16 (δ_C 25.5) and C-17 (δ_C 17.5) confirmed that the side chain terminated with a di-methylated olefin moiety. Substructure searching of this spin system in conjunction with the taxonomic genus in DNP (Dictionary of Natural Products, 2012) indicated that **1** possessed a serrulatane skeleton.

The remainder of structure **1** was assembled as follows. An acetoxy group was attached to C-3 on the basis of strong HMBC correlations from both the downfield proton at δ_H 5.19 (H-3) and the methyl group at δ_H 2.00 (H-22) to the carbonyl at δ_C 169.8 (C-21) (Ndi et al., 2007c). H-4 showed HMBC correlations to carbons resonating at δ_C 119.8 (C-5), δ_C 134.7 (C-9), and δ_C 126.6 (C-10), indicating that it was adjacent to an aromatic system. H-1 (δ_H 3.26) also showed HMBC correlations to C-9 and C-10, allowing a cyclohexene (ring A) to be formed. The proton at δ_H 6.96 was attached to a carbon at δ_C 119.8 on the basis of HSQC data, and HMBC correlations from this sp^2 methine to C-4 and C-9 of ring A allowed it to be assigned to H-5.

At this point a subunit of $C_4O_4H_3$ remained to be elucidated. The four remaining carbons included one carbonyl (δ_C 172.3) and three aromatic quaternary (δ_C 110.3, 147.7 and 142.4) signals. The proton at H-5 (δ_H 6.96) possessed a HMBC correlation to the carbon at δ_C 147.7, placing it at C-7, while H-1 (δ_H 3.26) demonstrated a strong three-bond HMBC correlation to δ_C 142.4, positioning it at C-

8. This allowed the formation of the benzenoid system, ring B (Figure 1). The proton at H-5 also showed a HMBC correlation to the carbon at δ_C 172.3, hence a carbonyl group was placed at C-6 (δ_C 110.3). This left the equivalent of three -OH moieties to position within the structure, suggesting a carboxylic acid and two hydroxy groups at C-6, C-7 and C-8, respectively. While neither the phenolic or carboxylic acid protons were identified in the ^{1}H NMR spectrum of **1**, the NMR data supported the assigned substitution pattern of ring B after comparison with literature data (Forster et al., 1986). Furthermore, the IR spectrum of **1** showed strong absorptions at 3262, 1667, and 1731 cm^{-1}, which confirmed the presence of phenolic, aromatic carboxylic acid and ester moieties, respectively (Pretsch et al., 2009). Thus the planar structure of **1** was assigned.

Of note is the shielding effect seen for the secondary methyl group at H-18. It has been reported that when a secondary hydroxy group is located at C-3, the signal for H-18 is shifted significantly upfield (δ_H ~ 0.60 in $CDCl_3$, δ_H ~ 0.30 in DMSO-d_6) (Liu et al., 2006; Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993) compared to when the hydroxy group is not present (δ_H ~ 1.00 in both $CDCl_3$ and DMSO-d_6) (Liu et al., 2006; Ndi et al., 2007b; Ndi et al., 2007c; Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993). The acetoxy group at H-3 in **1** has a similar effect to a hydroxy moiety, with the chemical shift of H-18 being δ_H 0.47 in **1**.

The relative configuration of **1** was established through analysis of the ROESY and ^{1}H-^{1}H coupling constant data and with comparison to literature values. ROESY correlations between H-4/H-3, H-4/H-2β, H-3/H-2β, and H-3/H-20 placed these protons on the same face of ring A. The small ^{1}H-^{1}H coupling constants between H-4/H-3 ($J_{3,4}$ = 3.6 Hz) and H-3/H-2β ($J_{3,2\beta}$ = 4.2 Hz) supported the *cis* orientation of these protons. The relative configuration of the cyclohexene system

present in **1** is consistent with that reported for the majority of serrulatanes described in the literature. Only one serrulatane, compound **11**, isolated from *E. phyllopoda* has been found to have the opposite configuration at C-1 (Syah and Ghisalberti, 1997). The relative configuration of the related natural products **8** and **9** was established by X-ray crystallographic analysis (Croft et al., 1981; Croft et al., 1977), which allowed for the assignment of stereochemistry at C-11. The majority of previous studies on serrulatanes have assigned the C-11 relative configuration to be identical to that of **8** and **9**, on the basis of similar NMR spectroscopic data and biosynthetic grounds (Liu et al., 2006; Ndi et al., 2007b; Ndi et al., 2007c). Comparison of the NMR data of **1** with related serrulatanes showed a high degree of similarity for ^{1}H and ^{13}C chemical shifts about C-11, thus the relative configuration was determined to be the same as that of previously reported metabolites (Liu et al., 2006; Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993). Consequently the chemical structure of **1** was assigned as 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid.

Compound **2** was determined to have a molecular formula of $C_{20}H_{28}O_5$ based on the sodiated pseudomolecular ion at *m/z* 371.1844 in the (+)-HRESIMS (calcd. 371.1829), which equated to seven degrees of unsaturation. The molecular weight difference of 42 Da between **1** and **2** suggested that **2** contained a hydroxy rather than an acetoxy moiety. Comparison of the NMR data of compounds **1** and **2** (Tables 1 and 2) showed they had almost identical ^{1}H and ^{13}C chemical shifts for both rings and the alkene side chain. The only major differences were that **2** lacked the ^{1}H and ^{13}C signals of an acetoxy group, and that the H-3 and C-3 resonances of **2** (δ_H 4.05 and δ_C 64.9) resonated further upfield than that of **1** (δ_H 5.19 and δ_C 69.6). These data indicated that **2** was the de-acetyl derivative of **1**. Furthermore, the chemical shift of H-18 (δ_H 0.36) was in agreement with literature values reported when a hydroxy

group is located at C-3 in the pseudo-equatorial position (δ_H ~ 0.30 in DMSO-d_6) (Liu et al., 2006; Ndi et al., 2007b; Ndi et al., 2007c; Syah and Ghisalberti, 1997; Tippett and Massy-Westropp, 1993). While the phenolic protons were not observed in the ^{1}H NMR spectrum of **1**, a downfield hydroxy signal (δ_H 8.60) was detected for **2** and attached to C-8 based on HMBC correlations from this resonance to C-7, C-8, and C-9. The ROESY and ^{1}H-^{1}H coupling constant data for **2** were essentially identical to that of **1**, hence the chemical structure of **2** was assigned to 3,7,8-trihydroxyserrulat-14-en-19-oic acid.

The third new serrulatane to be identified, compound **3**, also displayed a very similar ^{1}H NMR spectrum to that of natural product **1** (Table 1). The only observed differences were that **3** possessed an additional aromatic proton resonance (δ_H 6.64), an extra methyl group (δ_H 2.03), and an –OCH_2– moiety (δ_H 4.92). The ^{13}C NMR spectrum of **3** (Table 2) was similar to that of **1** in that it contained two carbonyl signals (δ_C 170.1 and 169.8), however, it possessed an additional oxygenated carbon signal (δ_C 65.4) and lacked one of the phenolic carbons of **1**. The 2D NMR data of **3** indicated that it differed from **1** in the substitution of the benzene ring (ring B). It was determined that the hydroxy at C-7 in **1** had been replaced by a hydrogen (δ_H 6.64) in **3** as indicated by HMBC correlations from this proton to C-5 (δ_C 119.9) and C-9 (δ_C 127.4). H-5 and H-7 shared a HMBC correlation to δ_C 65.4 (C-19), which allowed the –OCH_2– moiety to be substituted at C-6. The methylene protons at H-19 (δ_H 4.92) showed a HMBC correlation to the carbonyl carbon at δ_C 170.1, as did the methyl group at δ_H 2.03, which established an acetoxy moiety at C-19.

The molecular formula obtained for **3** of $C_{24}H_{34}O_5$ (eight degrees of unsaturation) from the HRESIMS data was in agreement with the NMR data. After analyses of the ROESY spectrum and ^{1}H-^{1}H coupling constants the relative

configuration of **3** was found to be the same as that of **1** and **2**. The chemical structure of **3** was therefore determined to be 3,19-diacetoxy-8-hydroxyserrulat-14-ene. Natural product **3** is a positional isomer of serrulatane **10**, which was isolated from *E. neglecta* (Ndi et al., 2007c).

The previously reported natural products verbascoside (Aligiannis et al., 2003; Andary et al., 1982) and jaceosidin (ApSimon et al., 1963; Nakasugi et al., 2000) were assigned to compounds **4** and **5**, respectively, after MS and 1D/2D NMR spectroscopic data analyses and comparison with literature values. Verbascoside has reportedly been found in a number of *Eremophila* spp. (Dictionary of Natural Products, 2012). This is the first report of jaceosidin (**5**) having been isolated from an *Eremophila* sp..

Literature reports have identified a number of serrulatanes that demonstrate selective antimicrobial activity towards Gram-positive bacteria, and just one has been shown to inhibit a Gram-negative bacterial strain (Anakok et al., 2011; Liu et al., 2006; Ndi et al., 2007b; Ndi et al., 2007c; Smith et al., 2007). During the antibacterial screening of 72 *Eremophila* plants, Ndi et al. found that an extract of *E. microtheca* inhibited *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumonia* with MICs ranging from 31—125 μg/mL (Ndi et al., 2007a). On account of this data, we decided to screen our compounds against a panel of bacterial strains.

As serrulatane **2** had been isolated in such large quantities (453 mg), we decided that prior to antimicrobial screening a number of simple analogues would be generated in order to facilitate structure activity relationships for this particular diterpenoid class. In parallel, **2** was acetylated using Ac_2O and dry pyridine (Davis et al., 1999) and methylated using TMS-diazomethane in CH_3OH/CH_2Cl_2 (Garfunkle et al., 2009). Both reaction crudes were purified using semi-preparative HPLC (C_{18}-

bonded silica, CH_3OH/H_2O). The acetylation reaction afforded compound **6** (3,8-diacetoxy-7-hydroxyserrulat-14-en-19-oic acid, 16 mg, 53% yield), while the methylation procedure resulted in derivative **7** (3,7,8-trihydroxyserrulat-14-en-methyl-19-benzoate, 9 mg, 35% yield). The 1D/2D NMR spectroscopic and HRESIMS data obtained for each of these compounds confirmed their structures.

Compounds **1**-**7** were screened against a panel of nine Gram-positive and one Gram-negative bacterial strains (Table 3). The majority of the compounds **1**-**7** were found to be inactive at the highest concentration tested (128 μg/mL) against most bacteria, though all the serrulatane compounds were found to be moderately active (MICs 64—128 μg/mL) against *S. pyogenes*. Natural product **1** demonstrated activity at 128 μg/mL against all bacterial strains except for *Enterococcus faecalis* and *Enterococcus faecium*. Jaceosidin (**5**) had the greatest potency (MICs 16—32 μg/mL) against most *S. aureus* isolates, while verbascoside (**4**) was inactive against all bacterial strains.

Ndi et al. found that two serrulatanes (**12** and **13**) from *E. duttoni* possessed antibacterial activity against *S. aureus* (ATCC 29213, MIC 7.8—15.6 μg/mL), however, they were shown to be unstable upon separation. The acetylation of the hydroxy groups of these serrulatanes generated stable compounds that could be separated (**14** and **15**), however, this also resulted in a loss of antibacterial activity. This indicated that phenolic groups are required in order for these natural products to elicit an antibacterial effect, but such groups may also cause the instability of these compounds as diphenols can be easily oxidised to form quinones (Ndi et al., 2012). In our study, the acetylated compound **6** was active against *S. aureus* (ATCC 43300) and *S. pyogenes* (MICs 128 μg/mL and 64 μg/mL, respectively) while natural product **2** was only active against *S. pyogenes* (MIC 128 μg/mL). As only partial acetylation

was achieved for **6**, it may be that this has stabilised the compound while leaving one of the phenolic groups free for interaction with the target. Ndi et al. also found that serrulatane **10**, which is an isomer of **3**, was inactive (MIC > 250 μg/mL) against strains of *S. aureus*, *S. pyogenes*, and *S. pneumonia* (Ndi et al., 2007c), while in this investigation **3** was active against *S. pyogenes* (MIC 128 μg/mL).

3. Conclusions

Three new members of the serrulatane structure class and the known compounds verbascoside and jaceosidin were isolated from the aerial parts of *E. microtheca*. Acetylated and methylated derivatives of one of the new serrulatanes were generated and the antibacterial activity of all the natural products and semi-synthetic derivatives was assessed against a panel of Gram-positive and Gram-negative bacterial strains. Jaceosidin was shown to possess the most potent antibacterial activity with MIC values of 16—32 μg/mL against most *S. aureus* strains, including daptomycin, glycopeptide, and methicillin resistant strains. This is the first report of natural products from *E. microtheca*.

4. Experimental

4.1. General experimental procedures

Optical rotations were recorded on a Jasco P-1020 polarimeter. IR and UV spectra were recorded on a Bruker Tensor 27 spectrophotometer and a Jasco V-650 UV/Vis spectrophotometer, respectively. NMR spectra were recorded at 30 °C on either a Varian 500 MHz or 600 MHz Unity INOVA spectrometer. The latter

spectrometer was equipped with a triple resonance cold probe. The ^{1}H and ^{13}C chemical shifts were referenced to the solvent peaks for DMSO-d_6 at δ_H 2.49 and δ_C 39.5. LRESIMS were recorded on a Mariner Time-of-Flight spectrometer equipped with a Gilson 215 eight probe injector or a Waters ZQ mass spectrometer. HRESIMS were recorded on a Bruker Apex III 4.7 Tesla Fourier transform ion cyclotron resonance mass spectrometer or a Bruker micrOTOF-Q instrument with a Bruker ESI source. An Edwards Instrument company Bio-line orbital shaker was used for plant extractions. A Waters 600 pump equipped with a Waters 966 PDA detector and a Waters 717 Plus Autosampler connected to a Gilson FC204 fraction collector were used for semi-preparative separations. Alltech C_{18} bonded silica, 35-75 μm, 150 Å and Alltech stainless steel guard cartridges (10 × 30 mm) were used for pre-adsorption work. A ThermoElectron C_{18} Betasil 5 μm 143 Å (21.2 × 150 mm) column was used for semi-preparative HPLC separations. Size exclusion chromatography was undertaken using a LH-20 Sephadex column (35 × 310 mm). All solvents used for chromatography, $[\alpha]_D$, UV, and MS were Lab Scan HPLC grade, and the H_2O was Millipore Milli-Q PF filtered. All synthetic reagents were obtained from Sigma–Aldrich and used without further purification.

4.2. Plant material

E. microtheca was cultivated and identified by J. Glazebrook at Logan Village, Queensland, Australia and the aerial parts harvested in March 2011. A voucher specimen (RAD039) has been deposited at the Eskitis Institute, Griffith University, Brisbane, Australia.

4.3. Extraction and isolation

The air-dried aerial parts of *E. microtheca* (10.4 g) were poured into a conical flask (1 L) then sequentially extracted with CH_2Cl_2 (250 mL × 4) and CH_3OH (250 mL × 4) while being shaken at 150 rpm. All CH_2Cl_2 and CH_3OH extractions were combined and dried under reduced pressure to yield a brown gum (3.26 g). This crude extract was divided into ten equal portions, and each portion pre-adsorbed to C_{18}-bonded silica (~ 1 g), packed into a guard cartridge then attached to a semi-preparative C_{18}-bonded silica HPLC column. Isocratic conditions of H_2O/CH_3OH (9:1) were held for 10 min, followed by a linear gradient to CH_3OH over 40 min, then isocratic conditions of CH_3OH for 10 min, all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected for each run. From the ten HPLC separations, similar fractions were combined then analysed by (+)-LRESIMS and ^{1}H NMR spectroscopy. Fraction 30 contained verbascoside (**4**, 44 mg, 0.418% dry wt), fractions 41-42 contained semi-pure jaceosidin (**5**), fractions 46-47 afforded 3,7,8-trihydroxyserrulat-14-en-19-oic acid (**2**, 453 mg, 4.355% dry wt), fraction 51 was shown to contain 3-acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**1**, 66 mg, 0.638% dry wt), and fraction 52 yielded 3,19-diacetoxy-8-hydroxyserrulat-14-ene (**3**, 54 mg, 0.519 % dry wt). Fractions 41 and 42 were further purified on a LH-20 Sephadex size exclusion column using CH_2Cl_2/CH_3OH (1:1) as the eluent. Initially 100 mL was collected into a conical flask and discarded, then twenty-five fractions (25 × 10 mL) were collected. Fractions 9 and 10 afforded jaceosidin (**5**, 9 mg, 0.088% dry wt).

*4.3.1. 3-Acetoxy-7,8-dihydroxyserrulat-14-en-19-oic acid (**1**)*

Stable brown gum; $[\alpha]_D^{25}$ + 6.0 (*c* 0.033, CH_3OH); UV (CH_3OH) λ_{max} nm (log ε) 215 (4.28), 257 (3.75), 320 (3.27); IR ν_{max} (KBr) 3262 (br), 2961, 2928, 2874, 2565, 1731, 1667, 1618, 1481, 1434, 1371, 1291, 1245, 1026, 999 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6) see Tables 1 and 2; (-)-LRESIMS *m/z* (rel. int.) 389 (100) $[M - H]^-$; (+)-LRESIMS *m/z* (rel. int.) 413 (100) $[M + Na]^+$; (+)-HRESIMS *m/z* 413.1955 ($C_{22}H_{30}O_6Na$ $[M + Na]^+$ requires 413.1935).

*4.3.2. 3,7,8-Trihydroxyserrulat-14-en-19-oic acid (**2**)*

Stable brown gum; $[\alpha]_D^{25}$ + 9.1 (*c* 0.044, CH_3OH); UV (CH_3OH) λ_{max} nm (log ε) 216 (4.35), 225 (4.26), 259 (3.91), 325 (3.38); IR ν_{max} (KBr) 3267 (br), 2960, 2929, 2558, 1664, 1614, 1512, 1433, 1377, 1292, 1219, 1025, 998 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6) see Tables 1 and 2; (-)-LRESIMS *m/z* (rel. int.) 347 (100) $[M - H]^-$; (+)-HRESIMS *m/z* 371.1844 ($C_{20}H_{28}O_5Na$ $[M + Na]^+$ requires 371.1829).

*4.3.3. 3,19-Diacetoxy-8-hydroxyserrulat-14-ene (**3**)*

Stable brown gum; $[\alpha]_D^{25}$ + 5.5 (*c* 0.037, CH_3OH); UV (CH_3OH) λ_{max} nm (log ε) 207 (3.83), 227 (3.42), 260 (2.94), 287 (2.62), 324 (2.45); IR ν_{max} (KBr) 3262 (br), 2960, 2931, 2360, 2342, 1733, 1668, 1618, 1586, 1456, 1433, 1374, 1292, 1243, 1026, 1003 cm^{-1}; ^1H and ^{13}C NMR data (DMSO-d_6) see Tables 1 and 2; (-)-LRESIMS *m/z* (rel. int.) 401 (100) $[M - H]^-$; (+)-LRESIMS *m/z* (rel. int.) 425 (100) $[M + Na]^+$; (+)-HRESIMS *m/z* 425.2319 ($C_{24}H_{34}O_5Na$ $[M + Na]^+$ requires 425.2298).

4.3.4 Identification of known compounds

Compounds **4** and **5** were identified as the previously reported natural products verbascoside (Aligiannis et al., 2003; Andary et al., 1982) and jaceosidin

(ApSimon et al., 1963; Nakasugi et al., 2000) following 1D/2D NMR (^{1}H, ^{13}C, gCOSY, gHSQC, gHMBC, ROESY) and MS data analysis and comparison with literature values. The optical rotation value obtained for **4** ($[\alpha]_D^{27}$ – 68.0 [*c* 0.05, CH_3OH]) was in agreement with literature data (Aligiannis et al., 2003; Andary et al., 1982).

*4.4. Acetylation of **2***

Serrulatane **2** (23.9 mg, 0.0687 mmol) was dissolved in Ac_2O (1 mL) and dry pyridine (1 mL) and stirred at rt overnight (Davis et al., 1999). The solution was dried under N_2 and the resulting product pre-adsorbed to C_{18}-bonded silica (~ 1 g), packed into a guard cartridge, and subjected to semi-preparative HPLC using a C_{18}-bonded silica column. Isocratic conditions of H_2O/CH_3OH (9:1) were held for 10 min, followed by a linear gradient to CH_3OH over 40 min, then isocratic conditions of CH_3OH for 10 min all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected. 3,8-Diacetoxy-7-hydroxyserrulat-14-en-19-oic acid (**6**, 16 mg, 53%) eluted in fractions 40-43.

*4.4.1. 3,8-Diacetoxy-7-hydroxyserrulat-14-en-19-oic acid (**6**)*

Stable opaque gum; $[\alpha]_D^{26}$ + 17.2 (*c* 0.07, CH_3OH); UV (CH_3OH) λ_{max} nm (log ε) 211 (3.64), 246 (2.94), 309 (2.59); ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 0.44 (3H, d, *J* = 6.6 Hz, H-18), 1.14 (3H, d, *J* = 6.6 Hz, H-20), 1.23 (1H, ddt, *J* = 13.2, 6.6, 6.0 Hz, H-12), 1.39 (1H, ddt, *J* = 13.2, 7.2, 6.0 Hz, H-12), 1.56 (1H, m, H-2*β*), 1.57 (3H, s, H-17), 1.65 (3H, s, H-16), 1.93 (1H, m, H-11), 1.96 (2H, brd, *J* = 6.6 Hz, H-13), 2.00 (3H, s, H-22), 2.03 (1H, m, H-2*α*), 2.22 (3H, s, H-24), 2.94 (1H, d, brd, *J* = 3.0 Hz, H-

4), 3.01 (1H, qd, *J* = 6.6, 6.0 Hz, H-1), 5.11 (1H, t, *J* = 7.8 Hz, H-14), 5.18 (1H, ddd, *J* = 12.0, 4.8, 3.0 Hz, H-3), 7.24 (1H, s, H-5); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C 17.5 (C-17), 18.9 (C-18), 20.4 (C-24), 21.0 (C-22), 22.5 (C-20), 25.4 (C-13), 25.5 (C-16), 28.3 (C-1), 31.3 (C-2), 31.4 (C-11), 37.9 (C-12), 44.4 (C-4), 69.7 (C-3), 109.5 (C-6), 122.4 (C-10), 124.4 (C-14), 127.0 (C-5), 130.7 (C-15), 136.1 (C-9), 136.5 (C-8), 153.4 (C-7), 168.1 (C-23), 169.7 (C-21), 171.0 (C-19); (-)-LRESIMS *m/z* (rel. int.) 431 (100) $[M - H]^-$; (-)-HRESIMS *m/z* 431.2077 ($C_{24}H_{31}O_7$ $[M - H]^-$ requires 431.2075).

*4.5. Methylation of **2***

Serrulatane **2** (23.9 mg, 0.0687 mmol) was dissolved in CH_3OH:CH_2Cl_2 (1:1, 2 mL) before TMS-diazomethane (2.0 M in diethyl ether, 172 μL, 0.3435 mmol) was added dropwise (Garfunkle et al., 2009). The reaction was stirred for 20 min at rt then quenched with AcOH (50 μL). The solvent was removed under N_2 and the resulting product pre-adsorbed to C_{18}-bonded silica (~ 1 g), packed into a guard cartridge, and subjected to semi-preparative HPLC using a C_{18}-bonded silica column. Isocratic conditions of H_2O/CH_3OH (9:1) were held for 10 min, followed by a linear gradient to CH_3OH over 40 min, then isocratic conditions of CH_3OH for 10 min all at a flow rate of 9 mL/min. Sixty fractions (60 × 1 min) were collected. 3,7,8-Trihydroxyserrulat-14-en-methyl-19-benzoate (**7**, 9 mg, 35%) eluted in fractions 50-51.

*4.5.1. 3,7,8-Trihydroxyserrulat-14-en-methyl-19-benzoate (**7**)*

Stable brown gum; $[\alpha]_D^{25}$ + 3.2 (*c* 0.063, CH_3OH); UV (CH_3OH) λ_{max} nm (log ε) 217 (4.39), 227 (4.33), 262 (4.06), 328 (3.48); ^{1}H NMR (600 MHz, DMSO-d_6) δ_H 0.36 (3H, d, *J* = 7.2 Hz, H-18), 1.17 (3H, d, *J* = 6.6 Hz, H-20), 1.25 (1H, m, H-12), 1.45 (1H, brd, *J* = 12.6 Hz, H-2*β*), 1.46 (1H, m, H-12) 1.57 (3H, s, H-17), 1.65 (3H, s, H-16), 1.87 (1H, ddd, *J* = 12.6, 12.0, 6.0 Hz, H-2*α*), 1.97 (2H, ddd, *J* = 7.8, 7.2, 7.2 Hz, H-13), 2.11 (1H, brq, *J* = 7.2 Hz, H-11), 2.70 (1H, brd, *J* = 3.6 Hz, H-4), 3.20 (1H, qd, *J* = 6.6, 6.0 Hz, H-1), 3.87 (3H, s, H-21), 4.05 (1H, brdddd, *J* = 12.0, 4.2, 3.6, 3.0 Hz, H-3), 4.65 (1H, brd, *J* = 3.0 Hz, 3-OH), 5.13 (1H, t, *J* = 7.2 Hz, H-14), 6.95 (1H, s, H-5); ^{13}C NMR (125 MHz, DMSO-d_6) δ_C 17.5 (C-17), 18.8 (C-18), 21.8 (C-20), 25.5 (C-13), 25.7 (C-16), 28.5 (C-1), 29.9 (C-11), 34.5 (C-2), 38.5 (C-12), 48.1 (C-4), 52.3 (C-21), 64.8 (C-3), 109.6 (C-6), 119.5 (C-5), 124.7 (C-14), 128.9 (C-10), 130.2 (C-15), 135.8 (C-9), 142.6 (C-8), 146.5 (C-7), 170.0 (C-19); (+)-LRESIMS *m/z* (rel. int.) 363 (100) $[M + H]^+$; (-)-HRESIMS *m/z* 361.2018 ($C_{21}H_{29}O_5$ $[M - H]^-$ requires 361.2020).

4.6. Bacterial strains and control values

Compounds **1-7** were screened against one Gram-negative bacterial strain: *Escherichia coli* (ATCC 25922), and nine Gram-positive bacterial strains: *Enterococcus faecalis* (VanA clinical isolate), *Enterococcus faecium* (MDR Van A ATCC 51559), *Streptococcus pyogenes* (Group A ATCC 12344), *Streptococcus pneumoniae* (MDR ATCC 700677), *Staphylococcus aureus* (mMRSA clinical isolate), *Staphylococcus aureus* (MRSA ATCC 43300), *Staphylococcus aureus* (GISA NRS 17), *Staphylococcus aureus* (GISA NRS 1), and *Staphylococcus aureus* (MRSA DapRSA clinical isolate). The experiments were all performed in duplicate (n

= 2) with vancomycin, colistin, and daptomycin used as positive controls. Control MIC values for vancomycin were as follows: *E. faecalis* (> 64 μg/mL), *E. faecium* (> 64 μg/mL), *S. pyogenes* (0.5 μg/mL), *S. pneumoniae* (1 μg/mL), and *S. aureus* strains (1-4 μg/mL). The control MIC value for colistin was as follows: *E. coli* (≤ 0.03 μg/mL). Control MIC values for daptomycin were as follows: *E. coli* (> 64 μg/mL), *E. faecalis* (16 μg/mL), *E. faecium* (16 μg/mL), *S. pyogenes* (0.25 μg/mL), *S. pneumoniae* (4 μg/mL), and *S. aureus* strains (2-16 μg/mL). Positive growth control rows of bacteria and DMSO + bacteria as well as a negative control row of only media were included for every plate tested.

4.6.1. MIC assay

MICs were determined by a two-fold serial broth microdilution according to the recommendation of CLSI standards with an inoculum of 5×10^5 cfu/mL. The compounds along with standard antibiotics were serially diluted twofold across the wells of 96-well non-binding surface plates (NBS, Corning). Standards ranged from 64—0.03 μg/mL, and the compounds from 128—0.06 μg/mL with final volumes of 50 μL per well. Gram-positive and Gram-negative bacteria were cultured in Mueller Hinton broth (MHB) (Bacto laboratories, Cat. no. 211443) at 37 °C overnight. A sample of each culture was then diluted 40-fold in fresh MHB broth and incubated at 37 °C for 2-3 h. The resultant mid-log phase cultures were diluted to the final concentration of 5×10^5 cfu/mL, then 50 μL was added to each well of the compound containing 96-well plates. All the plates were covered and incubated at 37 °C for 24 h. MICs were the lowest concentration showing no visible growth.

4.6.2. MBC assay

Resazurin (30 μL, 0.01%) was added to each well of the 96-well plates after the MIC values were determined. The compounds were then incubated at 37 °C for a further 18 to 24 h. Wells with blue coloration indicated dead microorganisms, whereas wells with pink coloration indicated live microorganisms. The MBC value was determined by the lowest concentrations of the wells with blue coloration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://...

Figure Legends

Fig. 1. Chemical structures for the isolated natural products **1-5**, the semi-synthetic derivatives **6** and **7**, and related serrulatane natural products **8**-**15**

Fig. 2. Key COSY and HMBC correlations for **1**

Table Legends

Table 1. ^{1}H NMR data for serrulatanes **1**-**3**[a]

Table 2. ^{13}C NMR data for serrulatanes **1**-**3**[a]

Table 3. Antibacterial activity of compounds **1**-**7**

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