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ScienceDirect
Phytochemistry 68 (2007) 2684–2690
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Antimicrobial compounds from *Eremophila serrulata*

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Received 23 April 2007; received in revised form 28 May 2007
Available online 12 July 2007

Abstract

We report a search for antimicrobial compounds in the Australian plant *Eremophila serrulata*. Bioassay directed fractionation of a diethyl ether extract prepared from the leaves of *E. serrulata* led to the isolation of two compounds, an *o*-naphthoquinone, 9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-*bc*]pyran-7,8-dione (**2**), and a serrulatane diterpenoid, 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**3**). Two other known serrulatane-type diterpenoids, 8,20-dihydroxyserrulat-14-en-19-oic acid (**4**) and 8,20-diacetoxyserrulat-14-en-19-oic acid (**5**) were also isolated. None of these compounds had previously been tested for antimicrobial activity. Compounds **2**–**5** showed antimicrobial activity against *Staphylococcus aureus* (ATCC 29213) with minimum inhibitory concentrations (MICs) ranging from 15.6 to 250 μg/mL. Compound **2** was the most active with an MIC of 15.6 μg/mL and a minimum bactericidal concentration (MBC) of 125 μg/mL. This compound also showed antimicrobial activity against other Gram-positive bacteria including *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. No activity was observed for this compound against all Gram-negative bacteria tested.

Keywords: *Eremophila serrulata*; Myoporaceae; Antimicrobial activity; Serrulatane diterpenoids; Naphthoquinone

1. Introduction

The arid-land plant genus *Eremophila* (Myoporaceae), native only to Australia, includes over 200 different species (Chinnock, 2007). A small proportion of *Eremophila* species have recorded medicinal uses in traditional Aboriginal cultures for symptoms possibly indicative of bacterial infection such as skin sores and sore throat (Barr et al., 1993; Ghisalberti, 1994).

Previously, preparations from a small number of recorded medicinal *Eremophila* species including *E. duttonii* and *E. alternifolia* have been shown to have antibacterial activity against Gram-positive bacteria (Palombo and Semple, 2001; Shah et al., 2004). Recently, in a study focussing on *Eremophila* species producing large quantities of leaf resin, we have shown that extracts of a number of other *Eremophila* species not known to have been used in traditional medicine, including *Eremophila serrulata* (Cunn. ex A.DC.) Druce have antimicrobial activity against Gram-positive organisms including clinical isolates of multi-drug-resistant *Staphylococcus aureus* (MRSA) (Ndi et al., 2007). The resin from a number of *Eremophila* species, extractable with organic solvents such as diethyl ether and acetone (Ghisalberti, 1994, has been the subject of previous chemical investigation with a large variety of novel diterpenoids isolated (Ghisalberti, 1995). However, very little is known about the compounds responsible for the antimicrobial activity of these plants.

E. serrulata is a shrub growing 1–2.5 m tall. It is widely distributed in inland South Australia, Western Australia, the Northern Territory and New South Wales. It has hairy leaves, often obscured by the resin covering the leaf surface (Jessop and Toelken, 1986). A previous chemical investigation of this plant (Croft et al., 1977) led to the isolation of 8,16-dihydroxyserrulat-14-en-19-oic acid which is a typical example of the most common class of diterpenoids found

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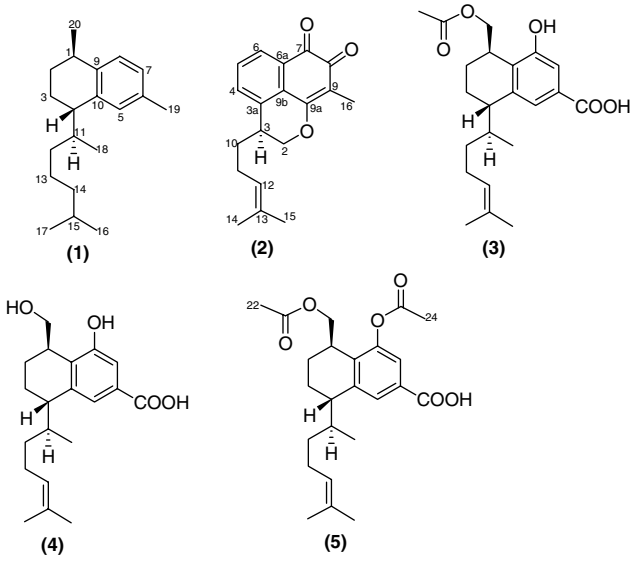
0031-9422/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2007.05.039

in *Eremophila* species – the serrulatanes (Croft et al., 1977; Forster et al., 1986). The general structure of the serrulatanes (**1**) is shown below. Another study (Ghisalberti, 1992), led to the isolation of a dimethoxy derivative of a tricyclic diterpene phenolic acid which contains the 3-*epi*-pseudopterosin skeleton.

In this paper, we report the isolation (from the leaves of *E. serrulata*), structural elucidation, and antimicrobial activity of two new compounds: a naphthoquinone, 9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-*bc*]pyran-7, 8-dione (**2**), and a serrulatane-type diterpenoid, 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**3**). Also for the first time we report the antimicrobial activity of the known 8,20-dihydroxyserrulat-14-en-19-oic acid (**4**) and 8,20-diacetoxyserrulat-14-en-19-oic acid (**5**).



2. Results and discussion

The diethyl ether extract of *E. serrulata* exhibited antimicrobial activity against *S. aureus* ATCC 29213 with a minimum inhibitory concentration of (MIC) of 125 μg/mL. The $NaHCO_3$ soluble, NaOH soluble and neutral CH_2Cl_2 fractions obtained by partitioning the crude diethyl ether extract were also examined for antimicrobial activity. Both the NaOH soluble and the neutral fractions were found to be the most active with each having an MIC of 125 μg/mL.

Bioassay guided fractionation of the NaOH soluble and the neutral CH_2Cl_2 fractions led to the isolation of two new compounds, 9-methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-*bc*]pyran-7,8-dione (**2**) and a serrulatane diterpenoid, 20-acetoxy-8-hydroxyserrulat-14-en-19-oic acid (**3**) together with two known (Forster et al., 1986) serrulatane-type diterpenoids, 8,20-dihydroxyserrulat-14-en-19-oic acid (**4**) and 8,20-diacetoxyserrulat-14-en-19-oic acid (**5**). The numbering system for compound 2 follows that for biflorin in Fonseca et al. (2003).

All these compounds were active against *S. aureus* ATCC 29213 with the most active being compound **2**, which had an MIC of 15.6 μg/mL. The structures of these compounds were elucidated using one- and two-dimensional NMR, IR and UV/visible spectroscopy and mass spectrometry. Compounds **4** and **5** were identified by comparing their NMR and mass spectral data with reported values (Forster et al., 1986).

Compound **2** was isolated as an amorphous orange solid. The molecular formula was determined to be $C_{19}H_{20}O_3$ from the molecular ion peak at *m/z* 296.1412 (Calcd. 296.1413) in the high-resolution EI mass spectrum (HREIMS), indicating 10 degrees of unsaturation.

The UV spectrum in $CHCl_3$ showed absorptions at λ_{max} 268, 344, and 452 nm characteristic of a typical *o*-naphthoquinone (Thomson, 1971) and the IR spectrum showed peaks at 1706 and 1640 cm^{-1} in agreement with the presence of two conjugated carbonyl groups. This was further supported by the presence of the $[M+2]^+$ ion peak in the EI mass spectra which is characteristic of an *ortho*-naphthoquinone but not a *para*-naphthoquinone (Oliver and Rashman, 1971).

The ^{13}C and the APT NMR spectra indicated a total of 19 carbons (Table 1) with three being methyls (δ_C 8.2, 18.3, and 26.3), three methylenes with one directly attached to an oxygen atom (δ_C 26.9, 34.6, and 71.9), 4 sp^2 methines (δ_C 125.2, 129.0, 132.5, and 136.7), 1 sp^3 methine (δ_C 37.9), 6 quaternary sp^2 carbons (δ_C 117.6, 127.7, 131.4, 134.1, 140.4, and 165.4) and two carbonyls 181.5, and 181.7. In total, the 10 sp^2 carbons (five double bond functionalities) and the two carbonyls accounted for seven degrees of unsaturation. This suggested that the compound contained three rings.

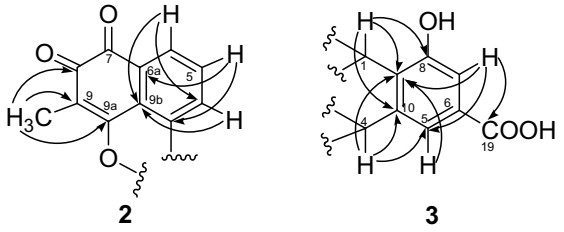
Amongst the signals in the ^{1}H NMR spectrum were those for three methyl singlets (δ_H 1.61, 1.68, and 1.91), a tri-substituted vinyl group with the olefinic proton resonating at δ_H 5.14 (*t*, $J = 6.0$ Hz), one benzylic proton (δ_H 2.99, *m*), two oxy-methylene protons δH 4.62, *dd*, $J = 11.1$, 2.1 Hz; δH 4.40, *dd*, $J = 11.1$, 3.3 Hz , and three aromatic protons (δ_H 7.51, *m*; 7.56, *m*; and δ_H 7.89, *d*, $J = 6.6$ Hz).

In the COSY spectrum, correlations were observed between the aromatic protons at δ_H 7.89 and 7.56 ppm indicating that they were adjacent to each other and also between the aromatic protons at δ_H 7.56 and 7.51 ppm. The benzylic proton showed couplings with the oxy-methylene protons and the protons of a methylene group at δ_H 1.78 and 1.69 in the COSY spectrum. This benzylic proton, exhibited HMBC correlations with the quaternary aromatic carbons at δ_C 127.7 and 140.4 as well as with the aromatic sp^2 methine carbon at δ_C 136.7 (δ_H 7.51, *m*) while the oxy-methylene protons showed HMBC correlation with the methylene carbon at δ_C 34.6, the quaternary aromatic carbon at δ_C 140.4 and the quaternary sp^2 carbon at δ_C 165.4 ppm thus indicating the presence of a pyran ring fused to the aromatic ring. HMBC correlations of the other

Table 1
NMR data for compounds **2** and **3**

C/H	**2**		C/H	**3**	
	$\delta^{13}C$	$\delta^{1}H$ (*J* in Hz)		$\delta^{13}C$	$\delta^{1}H$ (*J* in Hz)
2	71.9	4.62, *dd* (11.1, 2.1)4.40, *dd* (11.1, 3.3)	1	34.0	3.42, *m*
3	37.9	2.99, *m*	2	23.1	1.84, *m*
3a	140.4	–	3	21.0	1.83, 1.73, *m*
4	136.7	7.51, *m*	4	44.2	2.66, *m*
5	132.5	7.56, *m*	5	123.6	7.39, *br s*
6	129.0	7.89, *d* (6.6)	6	130.8	–
6a	131.4	–	7	114.2	7.25, *d* (1.8)
7	181.7	–	8	156.9	–
8	181.5	–	9	130.4	–
9	117.6	–	10	144.2	–
9a	165.4	–	11	39.6	1.94, *m*
9b	127.7	–	12	35.0	1.26, 1.09, *m*
10	34.6	1.78, 1.69, *m*	13	27.7	1.98, 1.84, *m*
11	26.9	2.12, *m*	14	126.1	4.95, *t br* (7.0)
12	125.2	5.14, *t* (6.0)	15	132.8	–
13	134.1	–	16	26.3	1.52, *br s*
14	18.3	1.61, *br s*	17	18.2	1.62, *br s*
15	26.3	1.68, *br s*	18	19.6	0.98, *d* (6.6)
16	8.2	1.91, *s*	19	171.0	–
			20	66.9	4.32, *dd* (10.2, 4.0) 4.06, *t br* (10.2)
			21	173.7	–
			22	21.3	2.03, *s*

Spectra obtained at 600 MHz in CD_3OD.



2 **3**

Fig. 1. HMBC correlations of the aromatic moiety of **2** and **3**.

aromatic protons are shown in Fig. 1. The methylene protons at δ_H 2.12 ppm which coupled with the olefinic proton (δ_H 5.14) and the methylene protons at δ_H 1.78 and 1.69 in the COSY spectrum, showed HMBC correlation with the benzylic carbon at δ_C 37.9 (δ_H 2.99, *m*). This indicated that the tri-substituted vinylic group was part of a side chain attached to the benzylic carbon through the methylene carbon at δ_C 34.6. COSY correlation between the protons of this methylene carbon and the benzylic proton further supported the attachment of the side chain at this location. Cross peaks of the olefinic methine proton (δ_H 5.14, t, $J = 6.0$ Hz) with a methylene carbon at δ_C 34.6, a quaternary carbon at δ_C 134.1, and two methyl carbons at δ_C 18.3 and δ_C 26.3 were observed in the HMBC spectrum.

Other HMBC correlations observed were the correlations from the allylic methyl protons (δ_H 1.91) to the quaternary sp^2 carbons δ_C 117.6, δ_C 165.4, and the carbonyl at δ_C 181.5. The second carbonyl (δ_C 181.7) was placed adjacent to the one at δ_C 181.5 based on evidence from the mass and UV spectra, which suggested the compound is an *o*-naphthoquinone. The presence of an aromatic benzene ring, a pyran ring, four sp^2 carbons and two carbonyls accounted for nine degrees of unsaturation. Hence, with no additional carbon signal, it was concluded that the carbonyl at δ_C 181.7 was directly linked to the aromatic ring thus accounting for the remaining one degree of unsaturation. Based on these spectral studies, structure **2** was established for this new compound.

Compound **2** is structurally similar to biflorin, a diterpene quinone which was first isolated from *Capraria biflora* L. (Scrophulariaceae) (D'Albuquerque et al., 1962; Comin et al., 1963). An unpublished report of the isolation of biflorin from *Eremophila latrobei* has been cited in the literature (Forster et al., 1986). Biflorin was the first *o*-naphtho[1,8-*bc*]pyran quinoid to be found in nature and it was shown to have antimicrobial properties against Gram-positive organisms (D'Albuquerque et al., 1962). Compound **2** differs from biflorin in that it lacks the double bond between C-2 and C-3 and the aromatic methyl group attached to C-6. Compound **2** is the first example in nature of an *o*-naphtho[1,8-*bc*]pyran quinoid with this 19 carbon skeleton. This compound also shows structural similarities to the mansonones which are sesquiterpenoid quinones with potent activity against MRSA (Shin et al., 2000, 2004; Suh et al., 2006). Compound **2** has a similar bicarbocyclic skeleton as compound **3**. This suggests that they might be biogenetically related and that compound **2** might have come from precursors of serrulatane diterpenoids. The 19 carbon skeleton suggests that compound **2** is probably derived from a nor-serrulatane diterpenoid. If this is

the case then it is possible that the relative stereochemistry of H-3 in compound **2** might be the same as that of H-11 in the serrulatane diterpenoids. However, this still needs to be proven.

Compound **3** was isolated as a pale yellow oil. The molecular formula was determined to be $C_{22}H_{30}O_5$ from the molecular ion peak at *m/z* 374.2091 (Calcd. 374.2093) in the high resolution EI mass spectrum (HREIMS). This implied that the compound had eight degrees of unsaturation. The IR spectrum showed the presence of hydroxyl (3366 cm^{-1}), and carbonyl (1717 and 1691 cm^{-1}) functional groups.

The ^{13} C and the APT NMR spectra indicated a total of 22 carbons, four of which were methyls (δ_C 18.2, 19.6, 21.3 and 26.3), 5 methylenes with one directly attached to an oxygen atom (δ_C 21.0, 23.1, 27.7, 35.0 and 66.9), 3 sp^2 methines (δ_C 114.2, 123.6, and 126.1), 3 sp^3 methines (δ_C 34.0, 39.6, and 44.2), 5 quaternary sp^2 carbons (δ_C 130.4, 130.8, 132.8, 144.2, and 156.9) and two carbonyls (171.0 and 173.7). In total, the 8 sp^2 carbons (four double bond functionalities) and the two carbonyls accounted for six degrees of unsaturation. This suggested that the compound contained two rings.

The ^{1}H NMR spectrum showed signals for one acetyl methyl (δ_H 2.03, *s*), two methyl singlets (δ_H 1.52 and 1.62), a methyl doublet (δ_H 0.98, $J = 6.6$ Hz), an oxy-methylene group (δ_H 4.32, *dd*, $J = 4.0$ and 10.2 Hz; δ_H 4.06, *br t*, $J = 10.2$ Hz), two aromatic protons (δ_H 7.39, *br s* and 7.25, *d*, $J = 1.8$ Hz) suggesting the presence of an aromatic ring, a tri-substituted vinyl group with the olefinic proton resonating at 4.95 ppm (*t br*, $J = 7.0$ Hz), and two benzylic protons (δ_H 2.66, *m*; 3.42, *m*). The benzylic proton shift at 3.42 ppm suggested the presence of a *peri*-hydroxyl group (Ghisalberti, 1995). The presence of the hydroxyl group was further supported by the appearance of the band at 3366 cm^{-1} in the IR spectrum.

In the COSY spectrum, the benzylic proton at δH 3.42 (δ_C 34.0) which showed vicinal coupling with the oxy-methylene protons δ_H 4.06 (1H, *t br*, $J = 10.2$ Hz; δ_C 66.9) and δ_H 4.32 (1H, *dd*, $J = 10.2$, 4.0 Hz; δ_C 66.9) also coupled to the methylene protons at δ_H 1.84, (*m*; δ_C 23.1). The protons of another methylene group (δ_H 1.83, *m*; 1.73, *m*; δ_C 21.0) exhibited coupling with the methylene protons at δ_H 1.84 and the benzylic proton at δ_H 2.66 (δ_C 44.2) thereby forming a six-membered carbocyclic ring. This indicated a bicyclic structure for the compound with the carbocyclic ring fused to an aromatic ring accounting for the remaining two degrees of unsaturation. The corresponding carbons bearing the different protons were identified from the HMQC spectrum.

HMBC correlations between the two aromatic protons (δH 7.39, *br s* and 7.25, *d*, $J = 1.8$ Hz) with the other sp^2 aromatic carbons and the carbonyl carbon at δ_C 171.0 (Fig. 1) indicated that they were located on either side of a quaternary carbon (δ_C 130.8) with a carbonyl substituent (δ_C 171.0). Also in the HMBC spectrum, the benzylic methine proton at δ_H 2.66 showed cross peaks with the methylene carbons at δ_C 23.1, δ_C 21.0, and δ_C 35.0; the quaternary carbons at δ_C 130.4 and δ_C 144.2; the aromatic sp^2 methine at δ_C 123.6 and the sp^3 methine group at δ_C 39.6, while the other benzylic protons at δ_H 3.42 showed cross peaks with the oxy-methylene carbon at δ_C 66.9, the methylene carbon at δ_C 21.0 and the quaternary sp^2 carbons at δ_C 144.2 and 156.9, thus locating the aromatic ring. HMBC correlations of the aromatic moiety are shown in Fig. 1. The ^{13}C NMR shift for the sp^2 carbon at δ_C 156.9 which also showed HMBC correlations with the aromatic protons at δ_H 7.25 was consistent with that of a phenolic carbon thus placing the phenolic hydroxyl group *peri*- to the benzylic proton at δ_H 3.42. HMBC interaction between the oxy-methylene protons and the acetyl carbonyl carbon at δ_C 173.7 which also correlated with the acetyl methyl protons at δ_H 2.03 indicated that an acetoxy group was linked directly to the methylene carbon at δ_C 66.9. As there were no other signals in the ^{1}H and ^{13}C NMR spectra for an alkoxy group, the carbonyl carbon which showed HMBC cross peaks with the aromatic protons was considered to be the carbonyl of a carboxylic acid group. This is supported by the presence in the IR spectrum of the band at 1691 cm^{-1}.

The other signals in the ^{1}H and ^{13}C NMR spectra that also included a signal for an alkyl methine (δ_H 1.94, *m*, δ_C 39.6) and two methylenes (δ_H 1.26, *m*, 1.09, *m*, δ_C 35.0 and δ_H 1.98, *m*, 1.84, *m*, δ_C 27.7) were assigned as a side chain connected to the bicyclic moiety. The COSY spectrum showed couplings of the olefinic proton (δ_H 4.95, *t br*, $J = 7.0$ Hz; δ_C 126.1) to the methylene protons at δ_H 1.98, 1.84 (δ_C 27.7). These methylene protons coupled with the protons of the other methylene group at δ_H 1.26 and 1.09 ppm (δ_C 35.0), which in turn showed coupling with the alkyl methine proton. Interactions between the alkyl methine and the methyl protons at δ_H 0.98 (δ_C 19.6) were also observed in the COSY spectrum. The above couplings were also confirmed in the HMBC spectrum. HMBC correlations were also observed between the olefinic protons and the methyl carbons at 26.3 and 18.2 ppm. HMBC interactions of the alkyl methine proton with the methylene carbon at 21.0 ppm (δ_H 1.83, *m*, 1.73, *m*) and the quaternary aromatic carbon at δ_C 144.2 together with the coupling observed in the COSY spectrum between this alkyl methine proton and the benzylic proton at δ_H 2.66 (δ_C 44.2) indicated that the alkyl methine group (and hence the side chain) was attached to the benzylic carbon. From the above spectral data, structure **3** was established for this new compound. The relative stereochemistry of this compound was assumed to be the same as that of compound **4**, a serrulatane diterpenoid whose relative stereochemistry had been shown to be the same as that of other serrulatane diterpenoids isolated from different *Eremophila* species (Forster et al., 1986). The structure of compound **4** is shown above. This assumption about the stereochemistry was made on the basis of the structural similarity existing between the two compounds. The serrulatanes represent the most common

Table 2
Antimicrobial activity of compounds **2**–**5** against *S. aureus* ATCC 29213

Compound	Antimicrobial activity MIC (μg/mL)
2	15.6
3	125
4	250
5	250

Table 3
Antimicrobial activity of compound **2** against six different microorganisms

Microorganisms	Antimicrobial activity	
	MIC (μg/mL)	MBC (μg/mL)
Staphylococcus aureus ATCC 29213	15.6	125
Staphylococcus aureus ATCC 25923	7.8	15.6
Streptococcus pneumoniae ATCC 49619	7.8	7.8
Streptococcus pyogenes ATCC 10389	7.8	15.6
Escherichia coli ATCC 25922	NA	NA
Salmonella typhimurium ATCC 13311	NA	NA
Pseudomonas aeruginosa ATCC 27853	NA	NA

NA means not active at maximum concentration tested (250 μg/mL).

class of diterpenes encountered so far in the genus *Eremophila*.

Compounds **2**–**5** showed antimicrobial activity against *Staphylococcus aureus* (ATCC 29213) with minimum inhibitory concentrations (MICs) ranging from 15.6 to 250 μg/mL (Table 2). Compound **2** was the most active with an MIC of 15.6 μg/mL and a minimum bactericidal concentration (MBC) of 125 μg/mL. This compound further showed antimicrobial activity against other Gram-positive bacteria including *Staphylococcus aureus* (ATCC 25923), *Streptococcus pyogenes*, and *Streptococcus pneumoniae*. The MICs and the MBCs ranged from 7.8 to 15.6 μg/mL and 7.8 to 125 μg/mL, respectively (see Table 3). No activity was observed for this compound against all Gram-negative bacteria tested. This, together with the recent isolation of two new antimicrobial serrulatane diterpenoids (serrulatic acids) from *Eremophila sturtii* (Liu et al., 2006), confirm previous findings that *Eremophila* extracts are active against Gram-positive organisms (Palombo and Semple, 2001; Shah et al., 2004; Ndi et al., 2007).

The results of this study together with an unpublished report of previous isolation of biflorin from *Eremophila latrobei* (Forster et al., 1986) and the recent isolation of two new antimicrobial serrulatane diterpenoids from *Eremophila sturtii* (Liu et al., 2006) suggest that at least two different classes of compounds (*o*-naphthoquinones and serrulatanes) are responsible for the antimicrobial activity in extracts of *Eremophila* species.

3. Experimental

3.1. General experimental procedures

Merck silica gel 60, Sephadex LH-20 (Sigma, St Louis, MO), were used for column chromatography. High performance liquid chromatography (HPLC) separations were performed on Shimadzu LC-6A system with Activon Goldpak C-18 semi-preparative (25 × 1 cm) HPLC column. 1D and 2D NMR spectra were acquired on a Varian INOVA 600 MHz spectrometer. The chemical shifts of signals in the NMR spectra are referenced to residual solvents. IR spectra were measured on a FT-IR-8400 S Shimadzu spectrometer. UV spectra were recorded on a Shimadzu UV-1700 Pharma Spec spectrophotometer. Optical rotations were measured on an Atago AP100 polarimeter. High and low resolution mass spectra were obtained on a Kratos Concept ISQ magnetic sector mass spectrometer.

3.2. Plant materials

Leaves of *E. serrulata* were collected in March 2005 in northern South Australia, 135.2 km north of Glendambo (South Australian Government Department of Environment and Heritage collection permit number K24937). A voucher specimen (AD191475) was deposited at the State Herbarium of South Australia, Adelaide. Species identification was confirmed by Dr. R. Chinnock, *Eremophila* taxonomist, State Herbarium of South Australia.

3.3. Antimicrobial assays

3.3.1. Bacterial strains and media

Staphylococcus aureus ATCC 29213 obtained from stock cultures preserved at −70 °C at the Sansom Institute, University of South Australia was used as the test microorganism in the bioassay guided fractionation process. *Staphylococcus aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 10389, *Streptococcus pneumoniae* ATCC 49619, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922, from the same collection were used to test the most active compound isolated. All bacteria were grown on blood agar plates (Colombia agar – CM331, Oxoid, Basingstoke, England) supplemented with 5% v/v sheep blood) at 37 °C. The *Streptococcus* species were incubated at 37 °C in the presence of 5% carbon dioxide (CO_2). Brain Heart Infusion broth (CM 225, Oxoid) was used for the experiments to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for the *Streptococcus* species while cation adjusted MH II broth (Becton Dickinson, France) was used for the *Staphylococcus* species and the Gram-negative organisms tested.

3.3.2. Broth micro dilution assay for determination of MIC and MBC

Broth micro dilution was used to determine the MIC of extract, fractions and pure compounds against *Staphylococcus aureus* ATCC 29213. Duplicate twofold serial dilutions of test sample (100 μL/well) were prepared in sterile round-bottom 96-well plates (Sarstedt, Technology

Park, South Australia), in the appropriate broth containing 2% DMSO. Bacterial cell suspension (100 μL) corresponding to 1×10^6 CFU/mL was added in all wells except those in columns 10, 11 and 12 of the plate which served as saline, test sample and media sterility controls, respectively. Controls for bacterial growth without test sample were also included on each plate. The final concentration of bacteria in the assay was 5×10^5 CFU/mL and that of DMSO was 1% v/v. Plates were then placed on a shaker for 10 min and incubated at 37 °C overnight. After incubation, the MIC was determined as the lowest concentration at which no growth was observed in the duplicate wells. Vancomycin and gentamicin (Sigma, St Louis, MO), were used as positive controls for the Gram-positive and Gram-negative organisms, respectively. Following the determination of the MIC, the MBC was determined by transferring a 10 μL aliquot from each of the wells at the concentration corresponding to the MIC and those concentrations above into 190 μL of appropriate broth in a sterile 96-well plate. The plates were incubated under the same conditions as in the MIC experiment with the *Streptococcus* species incubated in the presence of 5% CO_2. The presence or absence of bacterial growth was determined by visual inspection. The MBC was considered to be the lowest concentration of the compound at which no growth occurred.

3.4. Extraction and isolation

The fresh leaves of *E. serrulata* (500 g) were soaked in diethyl ether (Analytical grade, Merck, Australia) overnight in a closed container to extract leaf resins. The solvent was drained and evaporated to dryness in vacuo at 40 °C to produce 40 g of a dark greenish residue which was re-dissolved in CH_2Cl_2 and washed sequentially with 8% w/v aq. $NaHCO_3$ and 5% w/v aq. NaOH solution as described previously (Forster et al., 1986). The aqueous basic fractions were acidified with 10% H_2SO_4, re-extracted with CH_2Cl_2 and filtered through activated charcoal. The different CH_2Cl_2 portions were dried using anhydrous Na_2SO_4, filtered and evaporated to yield $NaHCO_3$-soluble (MIC = 1000 μg/mL), NaOH-soluble (MIC = 125 μg/mL) and neutral CH_2Cl_2 (MIC = 125 μg/mL) fractions, respectively.

The NaOH soluble fraction (11 g) was subjected to vacuum liquid chromatography eluting with CH_2Cl_2 and increasing amounts of ethyl acetate. Eighteen fractions were collected and grouped based on their TLC profile into four major fractions (F1–F4).

Fraction F1 eluted from the main column with CH_2Cl_2 formed a powder when dissolved in chloroform. This was filtered to afford a yellow powder (MIC = 500 μg/mL) and a filtrate (MIC = 125 μg/mL). The filtrate (225 mg) was passed through a Sephadex column eluting with CH_2Cl_2/MeOH 3:1 to afford 32 fractions, which were grouped into three major fractions (F1-1, F1-2 and F1-3). Fraction F1-1 (MIC = 15.6 μg/mL) was further separated isocratically by RP-HPLC using 75% MeOH/water with 0.1% formic acid as eluent and a flow rate of 2 mL/min. Seventy-two fractions were collected and grouped into eight different fractions (F1-1-1 to F1-1-8). Fraction F1-1-4 afforded 10 mg of pure compound **2** (MIC = 15.6 μg/mL) as an amorphous orange solid.

Fraction F3 (500 mg, MIC = 250 μg/mL) eluted from the vacuum liquid chromatography column with CH_2Cl_2/MeOH 1:1 was passed through a reverse phase HPLC column using 65% MeOH/water with 0.1% formic acid as eluent and a flow rate of 2 mL/min. Seventy fractions were collected and grouped into three main fractions (F3-1 to F3-3). F3-3 afforded 20 mg of 8,20-dihydroxyserrulat-14-en-19-oic acid (**4**) (MIC = 250 μg/mL) as a pale yellowish oil.

Treatment of 2.5 g of the neutral fraction (MIC = 125 μg/mL) by vacuum liquid chromatography eluting with CH_2Cl_2 and increasing amounts of ethyl acetate afforded 22 fractions which were grouped into six major fractions (ESAF1–6) based on their TLC profile.

Fraction ESAF4 which came out from the main column with CH_2Cl_2/EtOAc 1:1 was further separated by RP-HPLC using 75% MeOH/water with 0.1% formic acid as eluent and a flow rate of 2 mL/min. Fifty fractions were collected and grouped into four different fractions (ESAF4-1 to ESAF4–4). ESAF4–2 (MIC = 125 μg/mL) was passed through a Sephadex column eluting with CH_2Cl_2/MeOH 3:1 to afford 35 fractions, which were grouped into three fractions (ESAF4 -2-1 to ESAF4-2-3). ESAF4-2-3 afforded 25 mg of compound **3** (MIC = 125 μg/mL) as a pale yellowish oil. Similarly, fraction ESAF4-4 afforded 30 mg 8,20-diacetoxyserrulat-14-en-19-oic acid (**5**) (MIC = 250 μg/mL) as a pale yellowish oil after it was passed through a Sephadex column and eluted with CH_2Cl_2/MeOH 3:1.

The R_f values for compounds **2**, **3**, **4**, and **5** on normal phase TLC (Merck, Silica gel 60, F_{254}, Darmstadt, Germany) when methylene chloride:methanol (9:1) was used as the mobile phase were 0.750, 0.388, 0.350, and 0.488, respectively. These spots were also present in the original diethyl ether extract before the partitioning with the bases.

3.4.1. 9-Methyl-3-(4-methyl-3-pentenyl)-2,3-dihydronaphtho[1,8-bc]-pyran-7,8-dione (*2*)

Orange amorphous solid; $[\alpha]_D^{24} + 93°$ (MeOH; *c* 0.150); $\lambda_{max}^{CHCl_3}$ nm (log ε): 268 (4.3), 344 (3.0), 452 (3.3); ν_{max} ($CHCl_3$) cm^{-1}: 1706, 1640, 1615, 1575; ^1H and ^{13}C NMR data are shown in Table 1; HREIMS *m/z*: 296.1412 $[M]^+$, LREIMS *m/z*: 298 $[M+2]^+$, 268, 225.

3.4.2. 20-Acetoxy-8-hydroxyserrulat-14-en-19-oic acid (*3*)

Pale yellow oil; $[\alpha]_D^{25} - 63°$ (MeOH; *c* 0.268); λ_{max}^{MeOH} nm (log ε): 249 (3.9), 302 (3.4); ν_{max} (CH_2Cl_2) cm^{-1}: 3366, 2961, 2930, 1717, 1691, 1609, 1578; ^1H and ^{13}C NMR data are shown in Table 1; HREIMS *m/z*: 374.2091 $[M]^+$, LREIMS *m/z*: 374 $[M]^+$, 314, 243, 230, 204, 159,131, 109, 69.

Acknowledgements

This research was supported by a University of South Australia Collaborative Research Grant. We thank Dr. Bob Chinnock (State Herbarium, Adelaide Botanic Gardens) for plant identification, Martin O'Leary (State Herbarium, Adelaide Botanic Gardens) for assistance with plant collection and field identification, Mr. Marshall Hughes (Central Science Laboratory, University of Tasmania) for performing the HREIMS and LREIMS, and Mr. Phil Clements (School of Chemistry & Physics, University of Adelaide) for performing the NMR experiments on the compounds.

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