Antimicrobial Compounds from the Australian Desert Plant *Eremophila neglecta*

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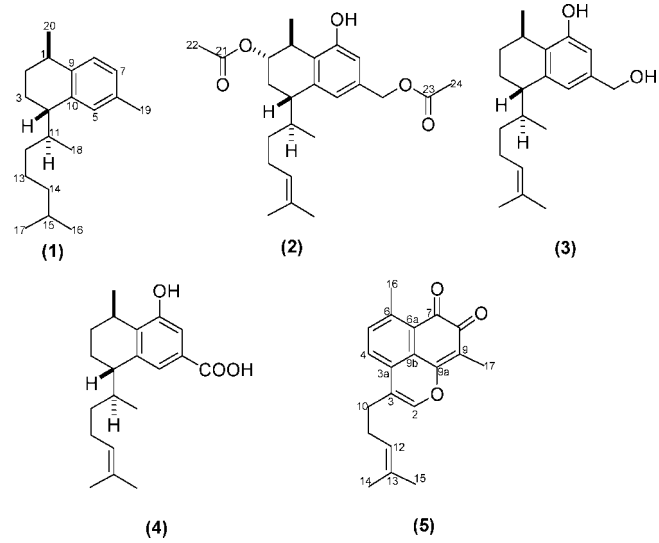
A crude extract from the Australian desert plant *Eremophila neglecta* has recently been shown to possess antibacterial activity in a survey of candidate plants that may bear novel antimicrobial compounds. Bioassay-directed fractionation of the Et_2O extract of *E. neglecta* using a broth microdilution assay led to the isolation of three new serrulatane-type diterpenoids, 2,19-diacetoxy-8-hydroxyserrulat-14-ene (**2**), 8,19-dihydroxyserrulat-14-ene (**3**), and 8-hydroxyserrulat-14-en-19-oic acid (**4**), and a known *o*-naphthoquinone commonly referred to as biflorin (**5**). The structures of **2**–**5** were determined using 1D and 2D NMR, FTIR, and high-resolution mass spectrometry. Compounds **3**–**5** showed antimicrobial activity against Gram-positive bacteria including *Staphylococcus aureus, Streptococcus pyogenes*, and *S. pneumoniae*. The minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs) ranged from 6.5 to 101.6 μM and 12.7 to 202.9 μM, respectively. No activity was observed for these compounds against Gram-negative bacteria.

The plant genus *Eremophila* (Myoporaceae) is known to produce different classes of secondary metabolites. These include terpenes, flavonoids, sterols, lignans, and fatty acids.[1] Of these different classes, the terpenes are the most studied, with several different monoterpenes, sesquiterpenes, and diterpenes isolated and identified.[2] Nine different classes of diterpenoids have been identified in the genus *Eremophila*, with the most common being the serrulatanes, represented by the bicarbocyclic structure **1**.[1] Most of these diterpenoids are unique to the genus *Eremophila*. Although preparations from some *Eremophila* species have been shown to have antimicrobial activity principally against Gram-positive organisms,[3–5] very limited work has been done to identify the actual compounds responsible for this activity. So far only two of the *Eremophila* species traditionally used by Australian Aboriginal people have been investigated for their antimicrobial constituents.[6,7] Bioactivity-guided fractionation of *E. sturtii* yielded 3,8-dihyroxyserrulatic acid and serrulatic acid as the active constituents, while serrulat-14-en-7,8,20-triol and serrulat-14-en-3,7,8,20-tetraol have recently been identified as the principal active constituents in *E. duttonii*. In a study focusing on *Eremophila* species producing large quantities of leaf resin, we have shown that 33 out of 72 *Eremophila* species tested had antimicrobial activity against Gram-positive organisms.[8] The most active of these, including *E. neglecta*, showed antimicrobial activity against clinical isolates of multiresistant methicillin-resistant *Staphylococcus aureus* (mMRSA).[8] None of these extracts had been investigated previously to identify the antimicrobial compounds.

E. neglecta J. Black is a shrub that grows to about 1–2.5 m tall. It is widely distributed in inland South Australia and the Northern Territory of Australia. It has hairy branches and orange flowers, and the leaves are highly resinous.[9] Infusions of the leaves of *E. neglecta* have been taken by Australian Aboriginal people for their general well-being.[10] No previous phytochemical study on *E. neglecta* has been reported. In this paper we report the isolation, structural elucidation, and antimicrobial activity of three new serrulatane-type diterpenoids, 2,19-diacetoxy-8-hydroxyserrulat-14-ene (**2**), 8,19-dihydroxyserrulat-14-ene (**3**), and 8-hydroxyserrulat-14-en-19-oic acid (**4**), and the known *o*-naphthoquinone (**5**) from the leaves of *E. neglecta.*



Results and Discussion

The Et_2O extract of *E. neglecta* exhibited antimicrobial activity against *S. aureus* ATCC 29213 with a minimum inhibitory concentration (MIC) of 62.5 μg/mL. The *n*-hexane-, CH_2Cl_2-, and MeOH-soluble fractions obtained by partitioning the crude Et_2O extract were also examined for antimicrobial activity. Both the *n*-hexane- and the CH_2Cl_2-soluble fractions were found to be the most active, each having an MIC of 62.5 μg/mL.

Bioassay-guided fractionation of a portion of the CH_2Cl_2 fraction led to the isolation of three new compounds, 2,19-diacetoxy-8-hydroxyserrulat-14-ene (**2**), 8,19-dihydroxyserrulat-14-ene (**3**), and 8-hydroxyserrulat-14-en-19-oic acid (**4**) together with biflorin, a known naphthoquinone that was first isolated from *Capraria bifloria* L. (Scrophulariaceae).[11,12] Other authors have cited an unpublished report of the isolation of biflorin from *Eremophila latrobei*.[13] 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.[11] Biflorin as well as compounds **3** and **4** were active against *S. aureus* ATCC 29213, while compound **2** was not active at the maximum concentration tested (621.5 μM).

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Table 1. NMR Spectroscopic Data (600 MHz) for Compounds **2–4**

	2[a]		**3**[b]		**4**[b]	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	36.2, CH	3.15 dq (6.6, 5.0)	26.8, CH	3.09 d quint (6.6, 3.0)	27.3, CH	3.18 d quint (7.0, 2.7)
2	78.8, CH	4.89 dt (8.7, 5.0)	27.3, CH_2	a 1.88 m ; b 1.48 ddt (13.0, 5.0, 3.0)	27.0, CH_2	a 1.95 m; b 1.52 m
3	28.6, CH_2	a 2.13 dt (13.0, 5.0); b 1.45 dt (13.0, 8.7)	19.4, CH_2	a 1.86 m; b 1.69 ddt (13.5, 5.0, 3.0)	19.2, CH_2	a 1.89 m; b 1.73 m
4	42.9, CH	2.72 dt (8.7, 5.0)	42.5, CH	2.58 dt (5.6, 3.0)	42.5, CH	2.65 dt (5.7, 2.8)
5	119.0, CH	6.76 br s	120.3, CH	6.70 br d (1.4)	124.1, CH	7.54 br d (1.4)
6	135.8, qC		137.9, qC		126.4, qC	
7	113.9, CH	6.63 br d (1.6)	111.1, CH	6.64 br d (1.4)	113.2, CH	7.29 br d (1.4)
8	157.2, qC		153.6, qC		153.2, qC	
9	129.5, qC		129.1, qC		136.5, qC	
10	141.9, qC		141.5, qC		141.7, qC	
11	35.3, CH	2.27 m	38.0, CH	1.86 m	38.0, CH	1.89 m
12	33.1, CH_2	a 1.19 m; b 0.91 dddd (13.5, 10.6, 8.5, 5.1)	33.5, CH_2	a 1.27 dddd (13.0, 10.0, 7.0, 3.0); b 1.09 dddd (13.0, 10.0, 9.4, 5.0)	33.4, CH_2	a 1.25 dddd (13.0, 9.9, 7.1, 3.0); b 1.09 dddd (13.0, 10.0, 9.4, 5.0)
13	27.8, CH_2	a 2.03 m; b 1.82 m	26.2, CH_2	a 1.97 m;b 1.79 m	26.2, CH_2	a 1.96 m; b 1.79 m
14	126.3, CH	5.07 t (6.6)	124.9, CH	4.96 t (7.0)	124.6, CH	4.96, t (7.0)
15	132.9, qC		131.2, qC		131.4, qC	
16	26.4, CH_3	1.67 br s	25.7, CH_3	1.63 br s	25.6, CH_3	1.62 br s
17	18.2, CH_3	1.57 br s	17.6, CH_3	1.53 br s	17.6, CH_3	1.524 br s
18	19.1, CH_3	1.05 d (6.6)	18.7, CH_3	0.94 d (6.6)	18.7, CH_3	0.96 d (6.6)
19	68.1, CH_2	4.97 br s	65.4, CH_2	4.56 br s	172.1, qC	-
20	20.2, CH_3	1.22 d (6.6)	21.0, CH_3	1.18, d (6.6)	20.8, CH_3	1.20 d (7.0)
21	173.4, qC					
22	21.8, CH_3	2.00 s				
23	173.1, qC					
24	21.4, CH_3	2.05, s				

[a] CD_3OD was used as solvent. [b] $CDCl_3$ was used as solvent.

The structures of compounds **2–4** were elucidated using 1D and 2D NMR spectroscopy, FT-IR, and mass spectrometry. Biflorin was identified by comparing its NMR and MS data with reported values.[14]

As a result of the close structural relationship of compounds **2–4**, the detailed structural determination of compound **3** will first be considered. Compound **3** was isolated as a pale yellow, amorphous solid. The molecular formula was determined to be $C_{20}H_{30}O_2$ from the molecular ion peak at *m/z* 302.2248 (calcd 302.2246) in the high-resolution EI mass spectrum (HREIMS). This implied that the compound had six degrees of unsaturation. The IR spectrum displayed absorption bands for hydroxy (3593 cm^{-1}) and C=C (1618 cm^{-1}) functional groups.

The ^{13}C and the APT NMR spectra (Table 1) indicated a total of 20 carbons, four of which were methyls (δ_C 17.6, 18.7, 21.0, and 25.7), five methylenes with one directly attached to an oxygen atom (δ_C 19.4, 26.2, 27.3, 33.5, and 65.4), three sp^3 methines (δ_C 26.8, 38.0, and 42.5), three sp^2 methines (δ_C 111.1, 120.3, and 124.9), and five quaternary sp^2 carbons (δ_C 129.1, 131.2, 137.9, 141.5, and 153.6). All these resonances accounted for a partial molecular formula of $C_{20}H_{28}O$. The remaining two hydrogen atoms and one oxygen suggested the presence of hydroxy groups, as evidenced by the IR absorption band at 3593 cm^{-1}. The eight sp^2 carbons (four double-bond functionalities) accounted for four degrees of unsaturation. This suggested that the compound contained two rings.

The ^1H NMR spectrum (Table 1) showed resonances for two methyl singlets (δ_H 1.63, br s; 1.53, br s), two methyl doublets (δ_H 1.18, d, J = 6.6 Hz; 0.94, d, J = 6.6 Hz), an oxymethylene group (δ_H 4.56, br s), a trisubstituted vinyl group with the olefinic proton resonating at 4.96 ppm (t, J = 7.0 Hz), two aromatic protons that appeared as broad singlets but resolved into broad doublets upon Lorentzian/Gaussian resolution enhancement[15] (δ_H 6.70, br d, J = 1.4 Hz; 6.64, br d, J = 1.4 Hz), and two benzylic protons (δ_H 3.09, d quint, J = 6.6, 3.0 Hz; 2.58, dt, J = 5.6, 3.0 Hz). The benzylic proton shift at 3.09 ppm suggested the presence of a *peri*-hydroxy group.[2] The presence of the aromatic and benzylic protons indicated that the compound contained a benzene ring.

In the COSY spectrum, the benzylic proton at δ_H 3.09 (δ_C 26.8), which showed vicinal coupling with a methyl doublet at δ_H 1.18 (δ_C 21.0), also coupled to the methylene protons at δ_H 1.88, m; 1.48, ddt, J = 13.0, 5.0, 3.0 Hz (δ_C 27.3). These methylene protons were connected to the benzylic proton at δ_H 2.58 (δ_C 42.5) by the methylene protons at δ_H 1.86, m; 1.69, ddt, J = 13.5, 5.0, 3.0 Hz (δ_C 19.4), thereby forming a six-membered carbocyclic ring. This supported a bicyclic structure for the compound with the carbocyclic ring fused to an aromatic ring, thus accounting for the remaining two degrees of unsaturation.

HMBC correlations between the two aromatic protons (δ_H 6.70, br d; 6.64, br d, each J = 1.4 Hz) with the other sp^2 aromatic carbons and the oxymethylene carbon (Figure 1) indicated that they were located on either side of a quaternary carbon (δ_C 137.9) with an oxymethylene substituent (δ_H 4.56, br s, δ_C 65.4). In addition, the aromatic proton at δ_H 6.64 showed a correlation with a quaternary aromatic carbon at δ_C 153.6 in the HMBC spectrum. The chemical shift of this carbon was consistent with that of a phenolic carbon. A HMBC correlation was also observed between this carbon and the benzylic proton at δ_H 3.09, placing the phenolic hydroxy group *peri*- to the benzylic proton.

The remaining resonances in the ^1H and ^{13}C NMR spectra, which included a resonance for an alkyl methine (δ_H 1.86, m, δ_C 38.0) and two methylenes (δ_H 1.27, dddd, J = 13.0, 10.0, 7.0, 3.0 Hz; 1.09, dddd, J = 13.0, 10.0, 9.4, 5.0 Hz; δ_C 33.5 and δ_H 1.97, 1.79, m; δ_C 26.2), were assigned as a side chain connected to the bicyclic moiety. The COSY spectrum showed couplings of the alkyl methine proton to the methyl protons at δ_H 0.94 and the methylene protons at 1.27 and 1.09 ppm. The methylene protons at δ_H 1.97 and 1.79 (δ_C 26.2) connected the olefinic proton (δ_H 4.96, t, J = 7.0 Hz) to the methylene protons at 1.27 and 1.09 ppm. The above couplings were also confirmed in the HMBC spectrum. HMBC correlations were also observed between the olefinic protons and the methyl carbons at 25.7 and 17.6 ppm. HMBC correlations of the alkyl methine proton with the methylene carbon at 19.4 ppm (δ_H 1.86, m, 1.69, ddt, J = 13.5, 5.0, 3.0 Hz) and the quaternary sp^2 carbon at δ_C 141.5 suggested that the methine group (and hence the side chain) was directly attached to the benzylic carbon at δ_C 42.5 ppm

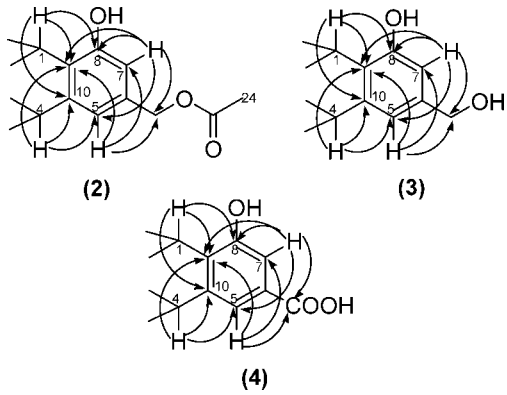

Figure 1. HMBC correlations of the aromatic moiety of compounds **2**–**4**.

(δ_H 2.58, dt, J = 5.6, 3.0 Hz). This was confirmed by the presence of a significant ion at *m/z* 191 in the mass spectrum arising from the loss of the side chain (C_8H_{15}) from the molecular ion (*m/z* 302). This cleavage, referred to as the C-4/C-11 cleavage,[13] is very common in the mass spectra of serrulatane diterpenoids. From the above data the structure of this new compound was established to be **3**, and it was named 8,19-dihydroxyserrulat-14-ene.

It has been noted previously that it is difficult to assign the relative configuration in the serrulatane series on the basis of NMR data alone.[16] The structure, absolute configuration, and the numbering system of the serrulatane skeleton **1** have been defined[17,18] using X-ray crystallographic analysis. To date, all the different serrulatane diterpenoids that have been isolated from *Eremophila* species were shown to have the same configurational characteristics around C-1, C-4, and C-11. Consequently the relative configuration of these carbons for compound **3** was assumed to be the same as in structure **1**.

Compound **2** was isolated as a pale yellow oil. The molecular formula was determined to be $C_{24}H_{34}O_5$ from the molecular ion peak at *m/z* 402.2402 (calcd 402.2406) in the high-resolution EI mass spectrum (HREIMS). This indicated the presence of four additional carbons and three additional oxygens when compared to compound **3** and two additional degrees of unsaturation. The IR spectrum showed the presence of hydroxy (3582 cm^{-1}) and carbonyl (1732 cm^{-1}) functional groups.

Analysis of the NMR spectroscopic data of compound **2** revealed that this compound was a serrulatane diterpenoid that was similar to compound **3**. Its side chain was attached to the same position on the bicyclic ring as in **3**, and the oxymethylene carbon at δ_C 68.1 (δ_H 4.97, br s) showed HMBC correlations with two aromatic protons at δ_H 6.76, br s, and 6.63, br d, J = 1.6 Hz. Similarly to **3**, the aromatic proton at δ_H 6.63 showed an HMBC correlation to the phenolic quaternary carbon at δ_C 157.2. An HMBC correlation between this carbon and the benzylic proton at δ_H 3.15 placed the phenolic hydroxy group *peri* to this benzylic proton. The benzylic proton at δ_H 3.15 appeared as a broadened quintet in the ^{1}H NMR spectrum, but it resolved into a doublet of quartets upon Lorentzian/Gaussian resolution enhancement[15] (dq, J = 6.6, 5.0 Hz). The substitution pattern on the aromatic ring for this compound was similar to that of **3** (Figure 1). The major differences were the presence of resonances in the carbon NMR spectrum for two additional carbonyl carbons (δ_C 173.4 and 173.1) accounting for the additional two degrees of unsaturation. Extra resonances for two *O*-acetyl methyl singlets at δ_H 2.00, s, δ_C 21.8 and δ_H 2.05, s, δ_C 21.4 were also observed in the ^{1}H and ^{13}C NMR spectra. The APT NMR spectrum for this compound indicated that it contained four sp^3 methylene carbons and four sp^3 methine carbons, i.e., one methylene carbon less and one methine carbon more when compared to **3**. The presence of an oxymethine carbon at δ_C 78.8 (δ_H 4.89, dt, J = 8.7, 5.0 Hz) in addition to the oxymethylene carbon at δ_C 68.1 (δ_H 4.97, br s) in this compound suggested that a proton of one of the methylene groups in **3** had been substituted by an oxygen-containing group. An HMBC correlation between the oxymethine proton at δ_H 4.89 and the carbonyl carbon at δ_C 173.4 as well as between this carbonyl carbon and the *O*-acetyl methyl protons at δ_H 2.00 confirmed that the oxygen-containing group was in fact an acetoxy group. The coupling in the COSY spectrum between the oxymethine proton at δ_H 4.89, dt, J = 8.7, 5.0 Hz (δ_C 78.8) with the benzylic proton at δ_H 3.15 dq, J = 6.6, 5.0 Hz (δ_C 36.2) and also with the methylene protons at δ_H 2.13, dt, J = 13.0, 5.0 Hz and 1.45, dt, J = 13.0, 8.7 Hz (δ_C 28.6) located the acetoxy group at position 2 (Table 1). The appearance of the benzylic proton at δ_H 3.15 as a doublet of quartets in the ^{1}H NMR spectrum further confirms the attachment of the acetoxy group to C-2. The oxymethylene protons at δ_H 4.97, which showed HMBC correlations with the aromatic methine carbons at δ_C 119.0 and 113.9 (carbons connected to the aromatic protons), also showed an HMBC correlation with the carbonyl carbon at δ_C 173.1. In addition this carbonyl carbon showed an HMBC correlation with the *O*-acetyl methyl protons at δ_H 2.05. This indicated that compound **2** possessed a second acetoxy group, which was attached to the methylene group at C-19. Hence structure **2** was established for this new compound.

The configurational assignments at C-1, C-4, and C-11 were assumed to be the same as that of previous serrulatane diterpenoids isolated from other plant species in the genus *Eremophila*. The relative configuration at C-2 was determined by 2D ROESY NMR experiments. In the ROESY spectrum strong correlations were observed between H-2 and H-20, H-2 and H-4, H-20 and H-4, and also H-4 and H-18. This indicates that H-2 is cofacial with H-20, H-4, and H-18.

Compound **4** was isolated as a white powder. The molecular formula was determined to be $C_{20}H_{28}O_3$ from the molecular ion peak at *m/z* 316.2035 (calcd 316.2038) in the HREIMS. This implied that the compound had seven degrees of unsaturation. The IR spectrum displayed absorption bands for hydroxy (3582 cm^{-1}), carbonyl (1726 cm^{-1}), and C=C (1688 cm^{-1}) functional groups.

The ^{1}H and ^{13}C NMR chemical shift assignments for this compound were very similar to those of the corresponding resonances for compound **3** and revealed the same structural features present in **3**. The only major difference was the absence of the resonance of the oxymethylene group at δ_C 65.4 (δ_H 4.56, br s) present in compound **3**. This resonance was replaced by a resonance for one carbonyl carbon (δ_C 172.1). The carbonyl carbon was considered to be the carbonyl of a carboxylic acid group, as there was an ion peak at *m/z* 271 in the mass spectrum, indicating the loss of a carboxylic acid group from the molecular ion (*m/z* 316). The presence of the carboxylic acid group accounted for the extra degree of unsaturation and the one additional oxygen atom present in this compound when compared to **3**. HMBC correlations between the *m*-substituted aromatic protons (δ_H 7.54, br d, J = 1.4 Hz; δ_H 7.29, br d, J = 1.4 Hz) and the carbonyl carbon of the carboxylic acid group (Figure 1) revealed that the carboxylic acid group was attached at the same position as the oxymethylene group in **3**. This new compound was named 8-hydroxyserrulat-14-en-19-oic acid, and its configuration was assumed to be the same as that for **3**.

Compounds **3**–**5** showed antimicrobial activity against *Staphylococcus aureus* (ATCC 29213) with MIC and MBC values ranging from 25.3 to 49.3 μM and 25.8 to 202.9 μM, respectively (Table 2). Compound **2** was not active at the maximum concentration tested (621.5 μM). Compounds **3**–**5** further showed antimicrobial activity against other Gram-positive bacteria including *S. aureus* (ATCC 25923), *Streptococcus pyogenes*, and *S. pneumoniae*. The MIC and MBC values are shown in Table 2. No activity was observed for these compounds against all Gram-negative bacteria tested. This is in line with previous findings, which indicated that *Eremophila* extracts are active only against Gram-positive organisms.[3,5] The

Table 2. Antimicrobial Activity of Compounds (**3**–**5**) against Different Microorganisms[a]

microorganism	antimicrobial activity, MIC (MBC), μM		
	3	**4**	**5**
Staphylococcus aureus ATCC 29213	25.8 (25.8)	49.3 (49.3)	25.3 (202.9)
Staphylococcus aureus ATCC 25923	25.8 (25.8)	49.3 (99.0)	25.3 (50.6)
Streptococcus pneumoniae ATCC 49619	12.9 (12.9)	24.7 (49.3)	6.5 (12.7)
Streptococcus pyogenes ATCC 10389	12.9 (12.9)	24.7 (99.0)	101.6 (202.9)
Escherichia coli ATCC 25922	NA	NA	NA
Salmonella typhimurium ATCC 13311	NA	NA	NA
Pseudomonas aeruginosa ATCC 27853	NA	NA	NA

[a] NA means not active at maximum concentration tested. Vancomycin had an MIC value of 0.7 μM against *Staphylococcus aureus* ATCC 29213. Gentamicin had an MIC value of 1.0 μM against *E. coli* ATCC 25922.

previous isolation of biflorin from *Eremophila latrobei*[13] (unpublished report) and the recent isolation of two new antimicrobial serrulatane diterpenoids (serrulatic acids) from *E. sturtii*[6] together with the results presented above suggest that at least two different classes of compounds (*o*-naphthoquinones and serrulatanes) are responsible for the antimicrobial activity in the *Eremophila* species. The recent identification of serrulata-14-en-7,8,20-triol and serrulat-14-en-3,7,8,20-tetraol as the active antimicrobial constituents in *E. duttonii*[7] and the strong antimicrobial activity of compound **3** indicate that serrulatanes other than serrulatic acids can also have antimicrobial activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on an Atago AP100 polarimeter. UV spectra were recorded on a Shimadzu UV-1700 Pharma Spec spectrophotometer. IR spectra were measured on an FT-IR-8400 S Shimadzu spectrometer. 1D and 2D NMR spectra were acquired on a Varian INOVA 600 MHz spectrometer. Chemical shifts are referenced to residual solvent resonances. High- and low-resolution mass spectra were obtained on a Kratos Concept ISQ magnetic sector mass spectrometer. Merck Si gel 60 (70–230 mesh ASTM) and Sephadex LH-20 (Sigma, St. Louis, MO) were used for column chromatography. HPLC experiments were performed on a Shimadzu LC-6A system with Activon Goldpak C-18 reversed-phase and normal-phase (SiO_2) semipreparative (25 × 1 cm) HPLC columns.

Plant Material. Leaves of *E. neglecta* were collected in March 2005 in northern South Australia, 119 km north of Marla (South Australian Government Department of Environment and Heritage collection permit number K24937). A voucher specimen (AD191474) 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.

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. *S. aureus* ATCC 25923, *Streptococcus pyogenes* ATCC 10389, *S. pneumoniae* ATCC 49619, *Salmonella typhimurium* ATCC 13311, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922, from the same collection, were used to further test the active compounds. 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.

Antimicrobial Activity. Broth microdilution was used to determine the MIC of extracts, fractions, and pure compounds against *Staphylococcus aureus* ATCC 29213. Duplicate 2-fold serial dilutions of the 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. A 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.

Extraction and Isolation. The fresh leaves of *E. neglecta* (1 kg) were soaked in Et_2O (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 80 g of a dark greenish residue. A portion (40 g) of this residue was redissolved in 70% MeOH and washed sequentially with *n*-hexane and CH_2Cl_2 to afford the *n*-hexane, CH_2Cl_2, and aqueous MeOH portions, respectively. The *n*-hexane and the CH_2Cl_2 portions were dried individually using anhydrous Na_2SO_4, filtered, and evaporated *in vacuo* using a rotary evaporator to yield the *n*-hexane (5.1 g; MIC = 62.5 μg/mL) and CH_2Cl_2 (24 g; MIC = 62.5 μg/mL) fractions, respectively. The aqueous MeOH portion was also evaporated to dryness to afford the MeOH fraction (1.5 g; MIC = 250 μg/mL).

A portion of the CH_2Cl_2 fraction (10 g) was subjected to vacuum liquid chromatography eluting with *n*-hexane and increasing amounts of EtOAc. Thirty fractions were collected and grouped on the basis of their TLC profiles into seven major fractions (F1 to F7). Fractions F2 and F3 were the most active fractions (with each having an MIC of 7.8 μg/mL) followed by fraction F4 (MIC = 31.3 μg/mL).

Fraction F2 (360 mg) was passed through a Sephadex LH-20 column eluting with CH_2Cl_2/MeOH (3:1) to afford 40 fractions, which were grouped into three major fractions (F2-1, F2-2, and F2-3). Similarly, fraction F3 was also separated using a Sephadex LH-20 column and grouped into three major fractions (F3-1, F3-2, and F3-3). On the basis of TLC analysis, F2-1 and F3-1 were grouped together and called fraction A (MIC > 250 μg/mL). In the same way, F2-2 and F3-2 as well as F2-3 and F3-3 were grouped to afford fraction B (MIC = 15.6 μg/mL) and fraction C (MIC = 7.8 μg/mL), respectively. Fraction B (100 mg) was separated isocratically by RP-HPLC using 75% MeOH/H_2O with 0.1% HCO_2H as eluent and a flow rate of 2 mL/min. Seventy fractions were collected and grouped into five different fractions (B1 to B5). Fraction B3 (MIC = 7.8 μg/mL) was further separated using normal-phase HPLC with *n*-hexane/EtOAc (8:2) plus 0.1% HCO_2H as eluent and a flow rate of 2 mL/min. Sixty-five fractions were collected. Fractions 17 and 18 afforded compound **2** (10 mg) as a pale yellow oil, which did not show any antimicrobial activity. Fractions 22–65 (2 mg; MIC = 7.8 μg/mL) were a mixture. Fraction B4 was treated in a similar manner, and 40 fractions were collected. Fractions 32–35 afforded the *o*-naphthoquinone **5** (2 mg; MIC = 25.3 μM). Fraction C (200 mg; MIC = 7.8 μg/mL) was separated using normal-phase HPLC with *n*-hexane/EtOAc (8:2) plus 0.1% HCO_2H as eluent and a flow rate of 2 mL/min. Seventy fractions were collected and grouped into five main fractions (C1 to C5). Fraction C3 (MIC = 7.8 μg/mL) was passed through a Sephadex LH-20 column eluted with CH_2Cl_2/MeOH (3:1) to afford compound **3** (20 mg; MIC = 25.8 μM) as a pale yellow, amorphous solid.

Separation of fraction F4 (1.3 g; MIC = 31.3 μg/mL) with RP-HPLC using 75% MeOH/H_2O with 0.1% HCO_2H as eluent (flow rate of 2 mL/min) and grouping the fractions obtained based on their TLC

profile afforded seven major fractions (F4-1 to F4-7). Fraction F4-6 (150 mg; MIC = 15.6 μg/mL) was further passed through a normal-phase HPLC column with *n*-hexane/EtOAc (8:2) plus 0.1% HCO_2H as eluent and a flow rate of 2 mL/min. Seventy fractions were collected and grouped into four major fractions. Fractions 13–17 afforded compound **4** (50 mg; MIC = 49.3 μM) as a white powder.

2,19-Diacetoxy-8-hydroxyserrulat-14-ene (2): pale yellow oil; $[\alpha]^{25}_D$ −27.5 (*c* 0.182, MeOH); UV (MeOH) λ_{max} (log ε) 280 (sh), 285 (3.29) nm; IR (CH_2Cl_2) ν_{max} 3582, 2962, 2928, 1732, 1688, 1585, 1433, 1047, 1024 cm^{-1}; ^1H and ^{13}C NMR data are shown in Table 1; LREIMS *m/z* 402 $[M]^+$, 342 (28), 282 (26), 257 (41), 233 (69), 171 (100), 157 (27), 109 (90), 69 (50); HREIMS *m/z* 402.2402 (calcd for $C_{24}H_{34}O_5$, 402.2406).

8,19-Dihydroxyserrulat-14-ene (3): pale yellow, amorphous solid; $[\alpha]^{25}_D$ −64.8 (*c* 0.216, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.32), 284 (3.27) nm; IR (CH_2Cl_2) ν_{max} 3593, 2961, 2930, 2872, 1618, 1580, 1456 cm^{-1}; ^1H and ^{13}C NMR data are shown in Table 1; LREIMS *m/z* 302 $[M]^+$ (31), 271 (14), 218 (43), 201 (14), 191 (100), 173 (38), 161 (94), 69 (43); HREIMS *m/z* 302.2248 (calcd for $C_{20}H_{30}O_2$, 302.2246).

8-Hydroxyserrulat-14-en-19-oic acid (4): white powder; $[\alpha]^{25}_D$ −81.7 (*c* 0.943, MeOH); UV (MeOH) λ_{max} (log ε) 254 (3.93), 303 (3.47) nm; IR (CH_2Cl_2) ν_{max} 3582, 2964, 2930, 2872, 1726, 1688, 1578, 1454 cm^{-1}; ^1H and ^{13}C NMR data are shown in Table 1; LREIMS *m/z* 316 $[M]^+$ (23), 271 (9), 245 (12), 232 (100), 217 (11), 205 (94), 187 (34), 161 (66), 145 (24), 69 (95); HREIMS *m/z* 316.2035 (calcd for $C_{20}H_{28}O_3$, 316.2038).

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