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Nor-*ent*-kaurane diterpenes and hydroxylactones from *Antennaria geyeri* and *Anaphalis margaritacea*

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Abstract

In a study of Pacific Northwest plants of the Asteraceae family, a nor-*ent*-kaurane diterpene and a known diterpene, both displaying antibacterial activities, were isolated together with scopoletin and sitosterol-3-*O*-β-glucopyranoside from the aerial parts of *Antennaria geyeri*. *Anaphalis margaritacea* afforded two hydroxylactones. The structures were established by one and two dimensional NMR techniques, IR and CIMS.

Keywords: *Antennaria geyeri*; *Anaphalis margaritacea*; Asteraceae; nor-*ent*-kaurane; Diterpenes; Hydroxylactones; Antibacterial

1. Introduction

As part of an ongoing study of the natural products chemistry of Pacific Northwest forest plants, *Antennaria geyeri* Gray and *Anaphalis margaritacea* (L.) B&H were investigated. Both plants belong to Gnaphalieae (Family Asteraceae), a small tribe of 22 genera and ≈320 species, which occur worldwide (Anderberg, 1994).

Antennaria geyeri is found in the open ponderosa pine forests of the semi-arid interior regions of the Pacific Northwest from eastern Washington State to California. Commonly called “pinewoods pussytoes”, this plant has no recorded description of medicinal use or chemical characterization. However, other members of this genus have been used by Native Americans to treat a variety of ailments from gastrointestinal disorders to dermatological problems. A common use of some members of this genus in both North America and Turkey has been as a cough and respiratory medicine (Mericli, 1983; Moerman, 1998). Flavonoids, ursolic acid and phenolic acids have been reported from other species of *Antennaria* (Borovkov and Belova, 1967; Mericli, 1983; Tira et al., 1969). Here we report a new nor-ent-kaurane diterpene, **1** and the known diterpene **2**, from *Antennaria geyeri* both with antibacterial activity. Scopoletin and sitosterol-3-*O*-β-glucopyranoside were also isolated.

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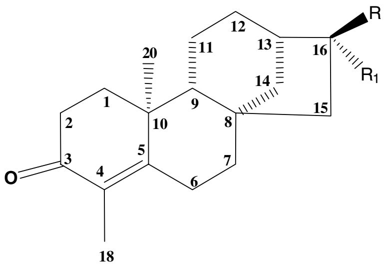
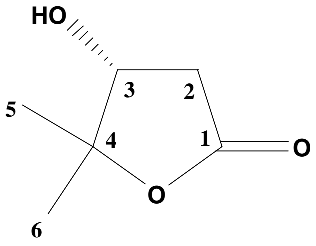


1 **R = H, R$_1$ = CH$_2$OH**
2 **R = CH$_2$OH, R$_1$ = OH**

In contrast to *Antennaria geyeri*, *Anaphalis margaritacea* (pearly everlasting) was widely used by many native American tribes across North America from coast to coast (Moerman, 1998). In common with *Antennaria geyeri*, many uses were for cough and respiratory problems as well as for colds and rheumatism. Previous chemical investigations of this plant revealed the presence of flavonoids and polyacetylenes (Bohlmann and Arndt, 1965; Wollenweber et al., 1993; Khattab, 1998). We report here two hydroxylactones, **3** and **4**, from *Anaphalis margaritacea*.



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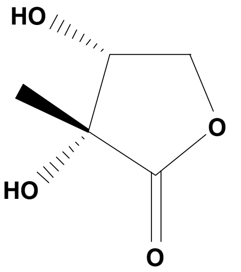

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Table 1
^{13}C-NMR and DEPT spectral data for compound **1** (125.7 MHz, CDCl$_3$, δ-values)

Carbon	1	
	Chemical shift	DEPT
C-1	36.8	CH$_2$
C-2	33.3	CH$_2$
C-3	199.1	C
C-4	128.2	C
C-5	164.4	C
C-6	27.0	CH$_2$
C-7	38.9	CH$_2$
C-8	44.5	C
C-9	54.2	CH
C-10	40.7	C
C-11	19.2	CH$_2$
C-12	31.1	CH$_2$
C-13	37.8	CH
C-14	36.4	CH$_2$
C-15	44.0	CH$_2$
C-16	43.3	CH
C-17	67.3	CH$_2$
C-18	11.0	CH$_3$
C-20	20.2	CH$_3$

2. Results and discussion

Fractionation of the CH$_2$Cl$_2$–MeOH (1:1) extract of the aerial parts of *Antennaria geyeri* on a silica gel column afforded a new nor-*ent*-kaurane diterpene, **1** and the known diterpene **2**. HRCIMS, ^{13}C-NMR and DEPT experiments suggested a molecular formula of C$_{19}$H$_{28}$O$_2$ for **1**, *m/z* 289.21665 (calc. 289.21675). The IR spectrum displayed absorption bands at 3420 cm^{-1} (free OH) and 1715 cm^{-1} (α,β-unsaturated carbonyl). The ^{13}C-NMR and DEPT spectra (Table 1) displayed 19 carbon signals as follows: two methyls, nine methylenes, three methines and five quaternary carbons. ^{1}H-NMR (CDCl$_3$) showed a methyl at δ 1.25 and an olefinic methyl at δ 1.78, both correlated in HMQC with carbons at δ 20.2 (C-20) and 11.0 (C-18), respectively. The olefinic methyl exhibited in HMBC experiments correlation with a carbonyl carbon at δ 199.1 and two quaternary carbons at δ 164.4 (C-5) and 128.2 (C-4). These data proved the presence of a CO–C(CH$_3$)=C pattern in the skeleton (Mahmoud et al., 2000). Furthermore, the tertiary methyl at δ 1.25 correlated with the carbon signals at δ 164.4 (C-5) and 54.2 (C-9). Those data established the A-ring of compound **1** and placed the carbonyl carbon at C-3 and the double bond at C4–C5. The broad doublet at δ 3.41 correlated with a carbon signal at δ 67.3 (t) in HMQC, indicating a hydroxylated methyl group. This primary alcohol signal exhibited a weak correlation with a multiplet signal at δ 2.03 (H-16) in the ^{1}H–^{1}H COSY spectrum. Additionally, it correlated with two carbons at δ 44.0 (t, C-15) and 43.3 (d, C-16) in the HMBC spectrum. Therefore, the primary alcohol group was placed at C-17. The chemical shift of the carbonyl carbon at δ 199.1 was in agreement with an α,β-unsaturated keto group (Mahmoud et al., 2000). Careful investigation of the ^{13}C, DEPT, HMQC and HMBC led to the assignment of all proton and carbon signals. The stereochemistry of the asymmetric carbons was deduced from NOESY experiments, H-17 exhibited correlations with H-13, H-14, and H-15 which suggested the α-orientation of hydroxymethyl at C-16 (Fig. 1). H-20 correlated with H-2α with H-13. Therefore, compound **1** was identified as 16β-H-17-hydroxy-3-oxo-19-nor-*ent*-kaur-4-ene.

The structure of **2** was determined from ^{1}H- and ^{13}C-NMR spectral comparison with those of **1**. The spectral data were identical with a compound reported from

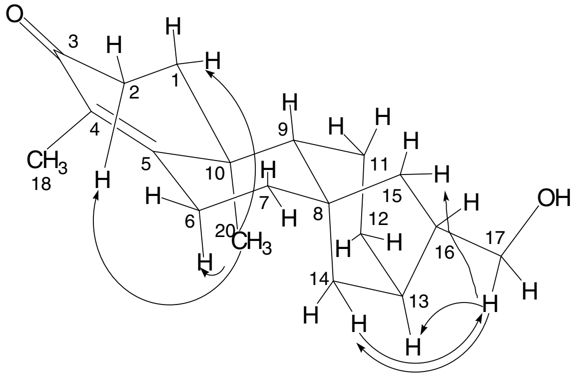

Fig. 1. Key NOESY correlations of compound **1**.

roots of *Exostema acuminatum* (Ito et al., 2000). 19-Norkaurene derivatives appear to be rare diterpenes (Bohlmann et al., 1980).

The methylene chloride–methanol extract (1:1) of the aerial parts of *Anaphalis margaritacea* afforded two hydroxylactones (**3**, **4**). Compound **3** was isolated as a colorless oil with $[\alpha]_D^{25} + 53$ ($CDCl_3$). Its molecular formula $C_6H_{10}O_3$ (confirmed by ^{13}C-NMR and DEPT analysis) was deduced from the CIMS. The ^{1}H-NMR spectra showed signals typical of two methyl groups: two singlets each 3H at δ 1.38 (H-5) and 1.44 (H-6) and their carbon signals at δ 21.0 (q) and 26.1 (q), respectively. The downfield proton signal which appeared as a doublet of doublets at δ 4.20 ($J = 3.5$, 6.5 Hz) correlated with an oxygenated carbon signal at δ 73.8 in the HMQC spectrum. The presence of downfield quaternary carbon signal at δ 87.6 in the ^{13}C-NMR suggested the presence of a second oxygenated carbon. The assignment of all proton signals and their connectivity to carbons was established from the results of the 2D ^{1}H–^{1}H COSY, HMQC and the long-range coupling HMBC experiments. Therefore, **3** was identified as (+)-(3*R*)-3-hydroxy-4,4-dimethyl-4-butyrolactone. Compound **3** was previously reported as a product of the degradation of coumarins (Ishii et al., 1992), previously unreported ^{13}C-NMR data are given in the Section 3. The structure of compound **4** was proven by NMR analysis which was identical with those previously reported for a compound obtained from *Hymenoxys* (Ahmed et al., 1995).

Antibacterial activities of **1** and **2** were evaluated against the test organisms, *Bacillus cereus*, *Pseudomonas aeruginosa* and *Escherichia coli*. From preliminary studies **1** had an estimated minimum inhibitory conc. (MIC) less than 1.5 mg/mL against *Staphylococcus aureus* and between 2.5 and 2 mg/mL against *B. cereus*, *P. aeruginosa* and *E. coli*. The MIC of **2** was less than 1.5 mg/mL against *S. aureus* and between 3 and 2 mg/mL against *B. cereus*, *P. aeruginosa* but showed no antibacterial effect against *E. coli*. However, compound **2** showed bacteriostatic activity on the basis of fluorescein diacetate assay. Generally, both compounds tended to act bacteriocidally at high concentration and bacteriostatically at low concentration, although no compound was bacteriocidal against all four bacteria at less than 1 mg/mL.

3. Experimental

3.1. General

^{1}H, ^{13}C-NMR and 2D spectra were recorded on JEOL 500 MHz, Lambda spectrometer with TMS as an internal standard. CI mass spectra were recorded on TSQ-70-Triple Stage Quadrupole mass spectrometer (70 eV). The IR spectra ($CHCl_3$) were recorded on a Perkin–Elmer FT-IR-spectrometer. Optical rotations were determined using a JASCO-20C automatic recording spectropolarimeter.

3.2. Plant material

Antennaria geyeri Gray was collected during the flowering stage in July 1998 along the Deschutes River, south of Bend, OR (Voucher #337579). *Anaphalis margaritacea* (L.) B&H was collected during flowering in August 1998, Benton County, OR, 6 miles southwest of Philomath (Voucher #337580). Voucher specimens are deposited at the University of Washington Herbarium in Seattle.

3.3. Extraction and isolation

The aerial parts of *Antennaria geyeria* (300 g) were extracted with CH_2Cl_2–MeOH (1:1, 2 L, 24 h), with the resulting extract evaporated under reduced pressure. The extract (4.5 g, 30%) was fractionated by silica gel column chromatography (CC) eluted with CH_2Cl_2–*n*-hexane in a step gradient by using different ratios. The first fraction (*n*-hexane–CH_2Cl_2 (75:25, 1 L, 1 g) was purified on a Sephadex LH-20 column (*n*-hexane–CH_2Cl_2–MeOH, 7:4:0.5) to give scopoletin (30 mg; Ballantyne et al., 1971). The second fraction (CH_2Cl_2–MeOH) (90:10, 1 L, 1 g) was purified on a Sephadex LH-20 column (*n*-hexane–CH_2Cl_2–MeOH, 7:4:1) to give sitosterol 3-*O*-β-glucopyranoside (20 mg; Iribarren and Pomilio, 1983). The third fraction (CH_2Cl_2–MeOH 85:15, 1 L, 2 g) also was purified on Sephadex LH-20 column. (*n*-hexane–CH_2Cl_2–MeOH, 7:4:1) to give a complex mixture of the two nor-*ent*-kaurane diterpenes, which were separated by TLC (*n*-hexane–diethylether 1:5, five runs), to give 16β-H-17-hydroxy-3-oxo-19-nor-*ent*-kaur-4-ene (10 mg) (**1**) and 16α,17-dihydroxy-3-oxo-19-nor-*ent*-kaur-4-ene (1.5 mg) (**2**).

The air-dried plant materials of *Anaphalis margaritacea* (500 g) were ground and extracted at room temperature with CH_2Cl_2–MeOH (1:1). The extracts were concentrated in vacuo to give a residue of 23 g, which was prefractionated by silica gel CC (6×120 cm) eluting with *n*-hexane (3 L) followed by a gradient of *n*-hexane–CH_2Cl_2 up to 100% CH_2Cl_2 and CH_2Cl_2–MeOH up to 15% MeOH (2 L of each solvent mixture). The 100% CH_2Cl_2 fraction was further submitted to a column (2×60 cm) of Sephadex LH-20 and eluted with *n*-hexane–CH_2Cl_2–MeOH (6:5:1) to afford (+)-(3*R*)-3-hydroxy-4,4-dimethyl-4-butyrolactone (6 mg) (**3**). The 5% MeOH–CH_2Cl_2 fraction was subjected to a silica gel CC (2×60 cm) eluted with gradient of *n*-hexane–CH_2Cl_2–MeOH to give two fractions. The first fraction (*n*-hexane–CH_2Cl_2–MeOH, 6:5:1) was further purified by CC (2×60) on Sephadex LH-20 eluted with *n*-hexane–CH_2Cl_2–MeOH (6:5:1) to give 2,3-dihydroxy-2-methylbutyrolactone **4** (4 mg).

16βH,17-hydroxy-3-oxo-19-nor-*ent*-kaur-4-ene (**1**): obtained as colorless oil; $[\alpha]_D^{25}: -72.0°$ ($CHCl_3$, $c = 0.47$); IR ($CHCl_3$) cm^{-1}; 3420, 2925, 1715, 1660, 1650, 1450; ^1H-NMR ($CDCl_3$, 500 MHz) δ: 3.41 (2H, br *d*, $J = 7.3$ Hz, H-17), 2.69 (1H, *ddd*, $J = 14.5, 3.0, 3.0$ Hz, H-6$_\beta$), 2.47 (1H, *ddd*, $J = 17.0, 15.0, 5.0$ Hz, H-2$_\alpha$), 2.35 (1H, *ddd*, $J = 17.0, 4.5, 2.0$ Hz, H-2$_\beta$), 2.23 (1H, *ddd*, $J = 14.5, 14.4, 6.5$ Hz, H-6$_\alpha$), 2.15 (1H, *m*, H-13$_\alpha$), 2.09 (1H, *ddd*, $J = 13.0, 5.0, 2.0$ Hz, H-1$_\alpha$), 2.03 (1H, *m*, H-16$_\beta$), 1.98 (1H, br *d*, $J = 12.0$ Hz, H-14$_\beta$), 1.78 (3H, *s*, H-18), 1.70 (2H, *m*, H-11), 1.61 (1H, *m*, H-12a), 1.56 (1H, *m*, H-15$_\beta$), 1.54 (1H, *m*, H-1$_\beta$), 1.54 (2H, *m*, H-7), 1.49 (1H, *m*, H-12b), 1.28 (1H, *m*, H-9), 1.25 (3H, *s*, H-20), 1.16 (1H, *m*, H-14$_\alpha$), 0.95 (1H, *dd*, $J = 13.5, 5.3$ Hz, H-15$_\alpha$); ^1H-NMR (pyridine-d_5, 500 MHz) δ : 3.64 (2H, *m*, H-17), 2.53 (1H, *ddd*, $J = 14.5, 3.0, 3.0$ Hz, H-6$_\beta$), 2.43 (1H, *ddd*, $J = 16.0, 14.5, 5.0$ Hz, H-2$_\alpha$), 2.39 (1H, *m*, H-13$_\alpha$), 2.35 (1H, *m*, H-2$_\beta$), 2.17 (1H, *m*, H-16$_\beta$), 2.05 (1H, *ddd*, $J = 14.5, 13.0, 6.0$ Hz, H-6$_\alpha$), 1.88 (1H, *m*, H-1$_\alpha$), 1.86 (3H, *s*, H-18), 1.84 (2H, *m*, H-14$_\beta$), 1.59 (1H, *m*, H-11$_\beta$), 1.53 (1H, *m*, H-15a), 1.45 (2H, *m*, H-12), 1.45 (1H, *m*, H-11$_\alpha$), 1.42 (2H, *m*, H-7), 1.36 (H, *ddd*, $J = 14.5, 14.0, 4.0$ Hz, H-1$_\beta$), 1.20 (1H, *m*, H-14$_\alpha$), 1.10 (1H, *m*, H-15b), 1.10 (1H, *m*, H-9), 1.08 (3H, *s*, H-20); CIMS $[M + H]^+$ *m/z* 289, $[M + H-H_2O]^+$ *m/z* 271; HRCIMS: *m/z* 289.21665 (calc. for $C_{19}H_{29}O_2$, 289.216755).

(+)-(3*R*)-3-hydroxy-4,4-dimethyl-4-butyrolactone. (**3**): colorless oil, $[\alpha]_D^{25} + 53$ ($CHCl_3, c = 0.34$); IR ($CHCl_3$) cm^{-1}; 3400, 1700, CIMS $[M + H-H_2O]^+$ *m/z* 113. ^1H-NMR ($CDCl_3$, 500 MHz) δ: 1.38 (3H, *s*, H-6), 1.44 (3H, *s*, H-7), 2.52 (1H, *dd*, J = 3.5, 18.0 Hz, H-3b), 2.91(1H, *dd*, J = 6.5, 18.0 Hz, H-3a), 4.20 (1H, *dd*, J = 3.5, 6.5 Hz, H-4); ^{13}C-NMR ($CDCl_3$, 125.7 MHz) δ: 21.0 (CH_3, C-6), 26.1 (CH_3, C-7), 38.2 (CH_2, C-3), 73.8 (CH, C-4), 87.6 (C, C-5), 174.2 (C, C-2).

3.4. Bioassay

The antibacterial activities of compounds **1** and **2** were evaluated using *S. aureus*, *B. cereus*, *P. aeruginosa* and *E. coli* in cup agar diffusion assay (United States Pharmacopoeia XXII, 1990). The media used were nutrient agar (Oxoid). The overnight culture of each organism was diluted in saline to contain 1×10^6 cell/mL. An aliquot of diluted culture (3 ml) was spread onto the surface of nutrient agar plate (40 ml each) and the excess bacterial suspension was withdrawn. The plates were incubated at 37 °C for 24 h.

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