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ent-Pimarane type diterpenes from *Gnaphalium gaudichaudianum*

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Abstract

Fractionation of the methanol extract of *Gnaphalium gaudichaudianum* DC afforded one new and six known *ent*-pimarane diterpenes together with velutin, squalene and stigmasterol. The structure of the new compound was established on the basis of extensive 1D and 2D NMR spectroscopic data interpretation. Two of the isolated compounds exhibited moderate toxicity in the *Artemia salina* toxicity test.

Keywords: *Gnaphalium gaudichaudianum*; Asteraceae; Diterpenes; *ent*-Pimar-15-ene-3α,8α-diol; *Artemia salina* toxicity test

1. Introduction

The genus *Gnaphalium* comprises 50 species, of which 22 grow in Argentina. One of them, *Gnaphalium gaudichaudianum* DC, is an annual herb 40–60 cm high that grows in arid soils and is native to southeastern Brazil, Uruguay and north and central Argentina (Zuloaga and Morrone, 1999). Different parts of the plant are employed in folk medicine as an expectorant and emmenagogue (Bestien, 1983).

Phytochemical investigations of the genus revealed the presence of flavonoids and diterpenes as major constituents (Maruyama et al., 1974; Escarria et al., 1977; Torrenegra et al., 1980, 1992; Bohlmann and Ziesche, 1980; García et al., 1982; Guerreiro et al. 1982); although acetylenes and carotenoids have also been isolated (Bohlmann and Ziesche, 1980). Previous studies of *G. gaudichaudianum* reported the presence of flavonoids and kaurenic acid derivatives (García et al., 1982; Guerreiro et al., 1982).

The present study was undertaken to identify the constituents of the active fractions of this plant showing toxic activity toward larvae of the crustacean *Artemia salina* (brine shrimp). This assay has long been utilized as a simple, rapid and reliable method to detect antitumor or cytotoxic activity in plant extracts (McLaughlin, 1991). From the active fractions, we have isolated stigmasterol, squalene, the flavone velutin (4′,5-dihydroxy-3′,7-dimethoxyflavone) (Das et al., 1970), the new pimarane type diterpene *ent*-pimar-15-ene-3α,8α-diol (**1**) and six additional known ones: *ent*-pimara-8(14),15-dien-19-oic acid (**2**) (Wenkert and Buckwalter, 1972), *ent*-pimara-8(14),15-dien-3α-ol (**3**) (Ansell et al., 1993), *ent*-pimara-8(14),15-dien-19-ol (**4**) (Wenkert and Buckwalter, 1972), *ent*-pimara-8(14),15-dien-18-oic acid (**5**) (Matsuo et al., 1976), *ent*-pimar-15-ene-8α,19-diol (**6**) (Wenkert and Buckwalter, 1972) and *ent*-pimara-8(14),15-dien-3α,19-diol (**7**) (García et al., 1985). The structure and stereochemistry of compound **1** was determined by a combination of spectroscopic techniques and molecular modeling.

2. Results and discussion

Compound **1** was isolated as a white solid with a molecular composition of $C_{20}H_{34}O_2$ as inferred from HR-EIMS. The IR spectrum showed absorption bands for hydroxyl groups (ν_{max} 3414 cm^{-1}) and a mono-

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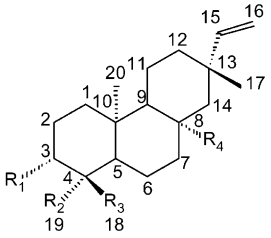
Compound	R_1	R_2	R_3	R_4	
1	OH	CH_3	CH_3	OH	
2	H	COOH	CH_3	-	Δ 8-14
3	OH	CH_3	CH_3	-	Δ 8-14
4	H	CH_2OH	CH_3	-	Δ 8-14
5	H	CH_3	COOH	-	Δ 8-14
6	H	CH_2OH	CH_3	OH	
7	OH	CH_2OH	CH_3	-	Δ 8-14



substituted double bond (ν_{max} 3086, 1637, 919 cm^{-1}). The combined analysis of the ^{13}C NMR and DEPT spectra revealed the presence of 20 carbon signals assigned to four methyls, seven methylenes, two methines and three quaternary carbons; one quaternary and one tertiary carbinol carbons and two olefinic carbons. The earlier evidence and the index of hydrogen deficiency suggested that compound **1** was a tricyclic diterpene with a double bond and two hydroxyl groups. The ^1H NMR spectrum showed signals of four tertiary methyl groups at δ 0.81, 0.91, 0.93 and 0.99; one carbinol proton at δ 3.21 (*dd*, $J = 11.1$, 5.2 Hz, H-3β) and three signals of an ABX system corresponding to the three vinyl protons from the monosubstituted double bond at δ 5.09 (*dd*, $J = 11.0$, 1.2 Hz, H-16A), δ 5.14 (*dd*, $J = 17.9$, 1.2 Hz, H-16B) and δ 5.98 (*dd*, $J = 17.9$, 11.0 Hz, H-15), confirming a pimar-15-ene derivative. From a detailed analysis of the COSY-90 and COSY-45 data we identified five spin systems, H-1/H-2/H-3; H-5/H-6/H-7; H-9/H-11/H-12; H-14; H-15/H-16 (Table 1). The only methylene proton cross-peak not tilted in the COSY-45 was the one corresponding to the pair H-14α/H-14β, clearly indicating that those two protons form an isolated AB spin system, as a consequence of the substitution of C-8 by the OH group (Cavanagh et al., 1996). Connections between the spin systems were established by a NOESY experiment (Table 1). The presence of NOE cross-peaks between H-1α and H-20, H-3β and H-18, H-14α and H-15, and the equatorial methyl group H-17 with H-12α, H-12β, H-14α and H-14β as expected, clearly established the relative stereochemistry of the chiral centers as shown in the formula. Full and unambiguous ^1H and ^{13}C NMR assignments were performed by HSQC and HMBC experiments (Table 1).

Previous phytochemical works on various species of the *Gnaphalium* genus yielded labdane and kaurene type diterpenes. This is the first report of pimarane derivatives in the genus. This fact is in agreement with the biosynthetic pathway for diterpenes where pimaranes are intermediates between labdane and kaurane skeletons.

Compounds **1**, **3**, **4**, **6**, **7**, squalene and velutin were inactive in the brine shrimp toxicity test while the diterpenes **2** and **5** showed moderate activity ($LC_{50} = 27$ and 32 μg/ml, respectively).

Table 1
^1H, ^{13}C NMR and NOESY data of compound **1**[a]

Position	δ_C	δ_H	NOESY[b]
1α	37.8 *t*	1.71 *dt* (13.1, 3.5)	2α, 20
1β		0.98	2β, 5β
2α	27.2 *t*	1.61 *m*	1α, 20
2β		1.61 *m*	1β, 3β, 5β
3β	79.1 *d*	3.21 *dd* (11.1, 5.2)	2β, 5β, 18
4	38.9 *s*		
5β	55.6 *d*	0.82	1β, 2β, 3β, 6β, 9β
6α	17.8 *t*	1.63 *qd* (13.4, 3.7)	7α
6β		1.49	5β, 7β
7α	42.0 *t*	1.78 *dt* (13.4, 3.2)	6α
7β		1.22	6β
8	72.3 *s*		
9β	56.2 *d*	0.85	5β, 11β, 14β
10	37.0 *s*		
11α	17.4 *t*	1.47 *qd* (13.4, 3.1)	12α, 16B, 20
11β		1.47 *m*	9β, 12β
12α	36.1 *t*	2.01 *dq* (13.7, 3.1)	11α, 16B, 17
12β		1.21 *dd* (13.7, 4.4)	11β, 17
13	36.5 *s*		
14α	53.4 *t*	1.68 *dd* (14.0, 3.1)	15, 16A, 16B, 17
14β		1.23	9β, 17
15	147.5 *d*	5.98 *dd* (17.9, 11.0)	14α, 16A, 16B, 17
16A	112.0 *t*	5.09 *dd* (11.0, 1.2)	14α, 15
16B		5.14 *dd* (17.9, 1.2)	11α, 12α, 14α, 15, 17
17	28.3 *q*	0.91 *s*	12α, 12β, 14α, 14β, 15, 16B
18	32.4 *q*	0.99 *s*	3β
19	15.5 *q*	0.81 *s*	20
20	15.5 *q*	0.93 *s*	1α, 2α, 11α, 19

[a] Recorded in $CDCl_3$, chemical shifts are reported as δ values (ppm) from TMS at 600 MHz for ^1H and 50.32 MHz for ^{13}C. *J* values in parentheses are given in Hz.
[b] Key NOESY cross peaks.

3. Experimental

3.1. General experimental procedures

The IR spectra were recorded on a Nicolet Avatar 360 FT-IR. The optical rotation values were obtained on a Jasco P-1010 polarimeter. The NMR spectra of compound **1**, including ^1H NMR, COSY-45, COSY-90,

NOESY, HSQC and HMBC, were recorded on a Brüker Advance DRX-600 at 600 MHz while the ^{1}H and ^{13}C NMR spectra of compounds **2**–**8** were recorded on a Brüker AC-200 NMR spectrometer at 200.13 and 50.32 MHz, respectively, using $CDCl_3$ as the solvent and tetramethylsilane (TMS) as internal standard. HRMS spectrum were obtained with a Varian Mat CH-5 mass spectrometer using electron impact. Chromatographic separations were achieved by vacuum liquid chromatography (VLC) and column chromatography (CC) using silica gel 60 (40–63 μm, Merck) or octadecyl-functionalized silica gel (Aldrich) or by centrifugal chromatography in a Chromatotron Model 7924 T using silica gel 60 PF_{254} (Merck 7749) plates (2 mm thick, total silica length 7 cm). Preparative TLC was performed on silica gel 60 G F254, 16×5 cm (L×H) plates, 0.2 mm thick, 15 mg maximum sample loading. Analytical TLC was performed on precoated silica gel 60 F254 plates (Merck) and detection was achieved by spraying with sulfuric acid in EtOH, followed by heating. All solvents were distilled before use.

3.2. Plant material

Gnaphalium gaudichaudianum DC was collected in Córdoba, Argentina, in February 1999 and was identified by Dr. Luis Ariza Espinar. A voucher specimen has been deposited at the Museo Botánico, Universidad Nacional de Córdoba, Argentina, under No. CORD 741.

3.3. Extraction and isolation

Dried and powdered whole plants of *G. gaudichaudianum* (279 g) were extracted exhaustively with CH_2Cl_2. The extract was concentrated at reduced pressure at 40 °C. The residue (9.9 g) was suspended in MeOH–H_2O (4:1) and extracted with hexane. The aqueous-MeOH solution was evaporated under vacuum and the aqueous residue was partitioned between Et_2O, CH_2Cl_2 and EtOAc, successively. The hexane and Et_2O extracts showed toxicity in the brine shrimp assay with $LC_{50} = 31$ and 19 μg/ml, respectively. The hexane extract (7.4 g) was chromatographed over reversed phase silica gel eluting sequentially with MeOH–H_2O, 7:3 and 9:1; MeOH and EtOAc. A total of six fractions (fraction size 300 ml) were collected and combined on the basis of their TLC profiles. Fraction 2 (488.2 mg) was rechromatographed over silica gel (40 g) with a step gradient of hexane–acetone (49:1; 24:1; 47:3; 23:2; 22:3); a total of 101 fractions were collected (fraction size 20 ml). Subfraction 24–27 (23.0 mg) was purified by preparative TLC, using toluene–Et_2O, 4:1, affording **2** (8.4 mg) and **3** (3.7 mg). Subfraction 49–60 (70.1 mg) was subjected to CC over silica gel (3 g) eluting with CH_2Cl_2 to afford compounds **1** (3.4 mg) and **5** (2.1 mg). Subfractions 32 and 80 consisted of pure compounds **4** (5.9 mg) and **5** (3.2 mg), respectively. Subfraction 62–77 (103.6 mg) was further fractionated through a silica gel column (4.8 g) using hexane–EtOAc, 4:1. Preparative TLC of subfraction 5 from the subfraction 62–77 (16.1 mg) (hexane–EtOAc, 4:1), allowed the isolation of compound **6** (9.3 mg). Fraction 3 (122.0 mg) was subjected to a silica gel column eluting with a gradient of hexane–EtOAc (49:1; 47:3; 93:7 and 9:1). Subfraction 40 gave stigmasterol (13.3 mg), identified by comparison with an authentic sample; subfraction 48 was purified by preparative TLC with CH_2Cl_2–EtOAc, 9:1, yielding compound **1** (8.6 mg); and subfraction 77–81 was purified in the same manner to afford compound **7** (4.0 mg). Fraction 4 (344.1 mg), purified by CC, yielded squalene (3.1 mg) and stigmasterol (10.0 mg). The spectral properties of squalene were identical to those previously described in the literature (Pouchart and Behnke, 1993).

The ether extract (2.5 g) was subjected to VLC over Si gel eluting sequentially with CH_2Cl_2, CH_2Cl_2–MeOH (99:1; 49:1; 24:1; 47:3 and 9:1) and pure MeOH (12 fractions, 250 ml each). The fractions were combined, based on the analysis of their TLC profiles, into 5 new fractions: 1–3, 4–6, 7–8, 9 and 10–12. Fraction 4–6 (83.7 mg) was subjected to a CC using silica gel (4.2 g) and eluting with hexane–EtOAc, 9:1, to obtain 9 subfractions. Subfraction 3 (46.8 mg) was purified by centrifugal chromatography, benzene–Et_2O, 9:1, yielding compounds **2** (6.0 mg) and **4** (2.7 mg). Fraction 7–8 (939.9 mg) was fractionated over a silica gel column (34 g) with hexane and hexane–acetone (9:1 and 4:1). Further purification of subfraction 35 yielded velutin (**8**) (20.8 mg) and subfractions 44/45 afforded diterpene **1** (29.8 mg). Fraction 9 (201.7 mg) after repetitive CC separations gave compound **2** (5.0 mg).

The compounds **2**–**8** were identified by comparison of their physical and spectroscopic data with those reported in the literature (García et al., 1985; Matsuo et al., 1976; Wenkert and Buckwalter, 1972; Ansell et al., 1993; Das et al., 1970).

3.4. *ent*-Pimar-15-ene-3α,8α-diol (*1*)

White solid; mp 137.5–138.5 °C; $[\alpha]_D^{25}$ −17.5° ($CHCl_3$, *c* 1.36), IR ν_{max}^{AgCl} cm^{-1}: 3414, 3086, 2936, 2869, 1637, 919; ^{1}H NMR and ^{13}C NMR, see Table 1; HRMS *m*/*z* $[M]^+$ 306.2551 (calc. for $C_{20}H_{34}O_2$, 306.2559).

3.5. Brine shrimp toxicity test (BSTT)

The BSTT was performed according to standard protocols (Mongelli et al., 1999). The LC_{50} values were determined in μg/ml, using the Finney probit analysis computer program (McLaughlin, 1991).

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