13- *EPI*-NEOCLERODANES FROM *BACCHARIS MARGINALIS*

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ABSTRACT

In addition to the known diterpenes, desoxyarticulin and dihydrotucumanoic acid, a new neoclerodane diterpene was isolated from *Baccharis marginalis*. The structures of the compounds were elucidated by spectroscopic evidence.

KeyWords: *Baccharis marginalis*, *ent*-clerodanes, dihydrotucumanoic acid, 13-*epi*- dihydrotucumanoic acid.

INTRODUCTION

In a previous paper[1], the characterization of desoxyarticulin **1** and dihydrotucumanoic acid **2** from the CH_2Cl_2 extract of *Baccharis marginalis* was described.

Further examination of the minor constituents from the column chromatography of the CH_2Cl_2 extract of *B. marginalis* resulted in the isolation of an additional diterpenoid, 13-epi-dihydrotucumanoic acid **3**.

EXPERIMENTAL

General:

M.p. uncorr. Melting points were measured (in triplicate) on a Stuart-Scientific SMP3 apparatus and are uncorrected. EIMS and HREIMS spectra were taken on a Micromass Autospect spectrometer. ^{1}H-, ^{13}C- (DEPT 135 and DEPT 90), sel. 1D ^{1}H NOESY, sel. 1D ^{1}H TOCSY, 2D HSQC and 2D HMBC spectra were recorded in $CDCl_3$ solutions and are referenced to the residual peaks of $CHCl_3$ at δ 7.26 ppm and δ 77.0 ppm for ^{1}H- and ^{13}C-, respectively, on a Bruker Avance 400 Digital NMR spectrometer, operating at 400.1MHz for ^{1}H and 100.6 MHz for ^{13}C. Chemical shifts are reported in δ ppm and coupling constants (*J*) are given in Hz. Silica gel (Merck 200-300 mesh) was used for C.C. and silica gel plates HF-254 for TLC. TLC spots were detected by heating after spraying with 25% H_2SO_4 in H_2O.

Extraction and Isolation of Diterpenoids

Dried and finely powdered aerial parts of *B. marginalis* (3000 g) collected in Fray Jorge National Park (IV Region), Chile in December 2004, were extracted with dicloromethane at room temperature for 72 hrs, affording 500 g of a syrup. Part of this crude material (200g) was chromatographed on a silica gel column (800g), and eluted with mixtures of petrol and EtOAc of increasing polarity. Fractions of 100 mL were taken and combined upon TLC, to produce eight fractions. The fraction 6 (3.7 g) was acetylated with Ac_2O and pyridine in the usual manner, obtaining 4.3 g of acetilated extract. One part of this extract (2.00g) was further separated and purified by silica gel chromatography to give compounds **4**-**7**. The other part was treated with CH_2N_2 in ether to yield compounds **8** and **9**.

Acetylcholinesterase Inhibition Assay.

The assay for measuring acetylcholinesterase (AChE) activity was carried out according to Gutierrez et al[2]. Briefly, some 50 µL of AChE solution (0.25 µ/mL) in phosphate buffer (8 mM K_2HPO_4, 2.3 mM NaH_2PO_4, 150 mM NaCl, 0.05% Tween 20, pH 7.6) and 50 µL of the sample dissolved in the same buffer were added to the wells. The plates were incubated for 30 min at room temperature before the addition of 100 µL of the substrate solution [40 mM Na_2HPO_4, 0.2 mM 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), 0.24 mM acetylthiocholine iodide (ACTI) in distilled water, pH 7.5]. The absorbance was read in a Multiskan Ascent Instrument microplate reader at 405 nm after 3 min. Enzyme activity was calculated as a percentage compared to a control using buffer and enzyme solution only. Compounds were tested at 100 mg/mL the results of this inhibition are shown in Table 2. When the enzyme inhibition was >50% at this concentration, further dilutions were undertaken and the corresponding IC_{50} values were determined. The IC_{50} values were calculated from five individual determinations (Table 3).

2,3-diacetoxy-dihydrotucumanoic acid* (4):** IR $\eta^{(KBr)}_{máx}$ cm^{-1}: 3600-3400, 3000-2850, 1780-1700, 1480-1000. ^{1}H NMR (400 MHz, $CDCl_3$): ***δ 0.74 (3H, *s*, H-20), 0.79 (3H, *d, J* = 5.4 Hz, H-17), 0.95 (3H, *d, J* = 6.6 Hz, H-16), 1.06 (3H, *s*, H-19), 1.13 (3H, *s*, H-18), 1.97 (3H, *s*, OAc), 2.11 (3H, *s*, OAc), 2.14 (1H, *dd, J* = 14.7, 8.4 Hz, H-14), 2.37 (1H, *dd, J* = 14.7, 5.9 Hz, H-14'), 5.07 (1H, *d, J* = 3.4 Hz, H-3), 5.21 (1H, *m*, H-2). ^{13}C NMR ($CDCl_3$): See Table 1.

2,3-diacetoxy-13-epi-dihydrotucumanoic acid* (5):** IR $\eta^{(KBr)}_{máx}$ cm^{-1}: 3600-3000, 2110, 2260, 1600, 1040-990, 820, 760. ^{1}H NMR (400 MHz, $CDCl_3$): ***δ 0.73 (3H, *s*, H-20), 0.78 (3H, *d, J* = 4.2 Hz, H-17), 0.99 (3H, *d, J* = 6.4 Hz, H-16), 1.06 (3H, *s*, H-19), 1.12 (3H, *s*, H-18), 1.99 (3H, *s*, OAc), 2.11 (3H, *s*, OAc), 2.27 (1H, *dd, J* =14.5, 5.3 Hz, H-14), 5.06 (1H, *d, J* = 3.3 Hz, H-3), 5.25 (1H, *m*, H-2). ^{13}C NMR ($CDCl_3$): See Table 1.

2-acetoxy-dihydrotucumanoic acid* (6):** IR $\eta^{(KBr)}_{máx}$cm^{-1}: 3600-3200, 2980-2900, 1720, 1700, 1620, 1440-1000. ^{1}H NMR (400 MHz,$CDCl_3$): ***δ 0.72 (3H, *s*, H-20), 0.78 (3H, *d, J* = 5.1 Hz, H-17), 0.96 (3H, *d, J* = 6.6 Hz, H-16), 1.10 (3H, *s*, H-19), 1.28 (3H, *s*, H-18), 2.09 (3H, *s*, OAc), 2.13 (1H, *dd, J* = 14.8, *8*.2 Hz, H-14), 2.38 (1H, *dd, J* = 14.8, 5.9 Hz, H-14'), 3.69 (1H, *d, J*= 3.3 Hz, H-3), 5.15 (1H, *m*, H-2). ^{13}C NMR ($CDCl_3$): See Table 1.

***2-acetoxy-13-epi-dihydrotucumanoic acid* (7)**: IR $\eta^{(KBr)}_{máx}$ cm^{-1}: 3500-3420, 2980-2900, 1680, 1700, 1460-840, 700-460. ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.71 (3H, *s*, H-20), 0.77 (3H, *d, J* = 5.4 Hz, H-17), 0.96 (3H, *d, J* = 6.5 Hz, H-16), 1.08 (3H, *s,* H-19), 1.24 (3H, *s*, H-18), 2.08 (3H, *s,* OAc), 2.30 (1H, *dd, J* = 14.7, 5.4 Hz, H-14), 3.68 (1H, *d, J*= 3.3 Hz, H-3), 5.15 (1H, *m*, H-2).

***2-acetoxy-methyl-dihydrotucumanoate* (8)**: IR $\eta^{(KBr)}_{máx.}$: 3600-3400, 3020-2840, 1720, 1440-800 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 0.70 (3H, *s*, H-20), 0.76 (3H, *d*, *J* = 5.7 Hz, H-17), 0.94 (3H, *d*, *J* = 6.6 Hz, H-16), 1.08 (3H, *s*, H-19), 1.26 (3H, *s*, H-18), 2.07 (3H, *s*, OAc), 2.13 (1H, *dd*, *J* = 14.8, 8.3 Hz, H-14), 2.31 (1H, *dd*, *J* = 14.8, 5.9 Hz, H-14'), 3.64 (3H, *s*, OMe), 3.68 (1H, *d, J* = 3.3 Hz, H-3), 5.14 (1H, *m*, H-2). ^{13}C NMR ($CDCl_3$): See Table 1.

***2-acetoxy-3-hydroxy-methyl-13-epi-dihydrotucumanoate* (9)**: IR $\eta^{(KBr)}_{máx}$ cm-1.: 3540-3460, 3000-2895, 1700, 1740, 1450-860 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$): *δ* 0.71 (3H, *s*, H-20), 0.77 (3H, *d, J* = 4.2 Hz, H-17), 0.93 (3H, *d, J* = 6.4 Hz, H-16), 1.04 (3H, *s*, H-19), 1.10 (3H, *s*, H-18), 1.95 (3H, *s*, OAc), 2.08 (3H, *s*, OAc), 2.31 (1H, *dd, J* = 14.8, 5.4 Hz, H_2-14), 3.63 (3H, *s*, OMe), 5.06 (1H, *d, J* = 3.4 Hz, H-3), 5.21 (1H, *m*, H-2).

^{13}C NMR ($CDCl_3$): See Table 1

Table 1. ^{13}CNMR spectral data of compounds **2, 4-6, 8, 9** ($CDCl_3$, TMS, δ in ppm).

C	2	4	5	6	8	9
1	26.2	26.5	27.8	26.5	22.1	22.7
2	68.4	70.6	69.4	73.6	73.6	70.6
3	77.9	75.9	75.8	77.2	77.2	75.9
4	76.2	76.6	76.0	76.8	76.7	76.6
5	40.5	41.1	39.0	40.9	40.9	41.1
6	29.0	29.3	30.8	29.4	29.3	29.3
7	25.3	22.7	22.0	22.0	26.5	26.5
8	35.7	35.9	35.9	36.0	36.0	35.9
9	37.9	38.6	38.6	38.8	38.8	38.6
10	37.9	38.8	38.8	38.6	38.6	38.8
11	35.6	35.7	35.7	35.6	35.6	35.7
12	31.4	31.5	31.4	32.1	32.0	31.5
13	30.5	30.9	30.8	30.9	30.9	30.9
14	41.3	41.4	41.0	41.4	41.4	41.4
15	174.3	173.7	171.4	173.9	173.8	171.7
16	18.1	18.5	18.0	18.5	18.5	18.5
17	15.9	15.9	15.6	15.9	15.9	15.9
18	21.1	20.4	20.4	21.3	21.3	20.4
19	19.6	21.0	19.6	19.9	19.9	21.0
20	16.7	16.9	16.3	17.2	17.1	16.9
$OCO\underline{C}H_3$		21.2	21.7	21.8	21.8	21.2
$O\underline{C}OCH_3$		170.2	170.3	169.8	169.9	170.2
$O\underline{C}OCH_3$		170.1	169.8			
$OCO\underline{C}H_3$		21.5	21.5			
$COO\underline{C}H_3$					51.3	51.3

In **Table 2** we can see that compounds **6** and **7** have an inhibitory activity, where we can calculated the IC_{50} for each, (**Table 3**) assuming that the stock concentration for each compound is of 2.0 mg/mL.

Table 2: Results of the Acetylcholinesterase Inhibition Assay (100 mg/mL).

Compound	C_1	C_2	C_3	C_4	C_5
4	31.1	18.9	15.2	3.0	3.0
5	9.4	17.1	12.7	17.7	33.1
6	100.0	4.9	-6.1	-8.5	-3.0
7	78.5	65.2	65.7	68.5	67.4

Table 3: Inhibitory activity of compounds **6** and **7.**

Compound	IC_{50} (mg/mL)
6	**0.369**
7	**0.031**
Galantamine	**1.1 x 10^{-3} (3 x 10^{-3}mM)**

RESULTS AND DISCUSSION

The CH_2Cl_2 extract of the aerial parts of *Baccharis marginalis* was subjected to column chromatography on silica gel, using increasing proportions of ethyl acetate in petrol as solvent, to afford some diterpene enriched fractions. Repeated chromatography of the petrol-ethylacetate fraction (10:1), led to the purification of compounds **1** and **2**, whereas the next fraction (9:1) led to the isolation of a mixture of **2** and **3**. This mixture was treated with acetic anhydride in pyridine affording 4 compounds **4** - **7**.

The ^{1}H NMR spectrum of compound **4** showed signals for two secondary and three tertiary methyl groups of a clerodane skeleton between δ 0.71 and 1.28 ppm, signals at δ 2.11 and 1.97 corresponding to the acetate groups, and two signals at 5.07 and 5.21 ppm, corresponding to the protons geminal to two acetate groups respectively. In addition, the ^{1}H NMR spectrum of **4** showed two one-proton double doublets at δ 2.14 (J= 14.7, 8.4 Hz) and 2.37 (J= 14.7, 5.9 Hz), which were assigned to the C-14 methylene protons. Comparison of the spectroscopic data of compound **4** with those of **2,** showed only minor differences for the skeleton proton signal, in particular, the signals for H-2 and H-3 were shifted downfield from 3.94 and 3.50 to 5.21 and 5.07 ppm respectively, indicating that **4** must be the diacetyl derivative of **2**. The ^{13}CNMR spectrum of **4** confirmed all the above results and defined the proposed structure as ***2,3-diacetoxy-dihydrotucumanoic acid***.

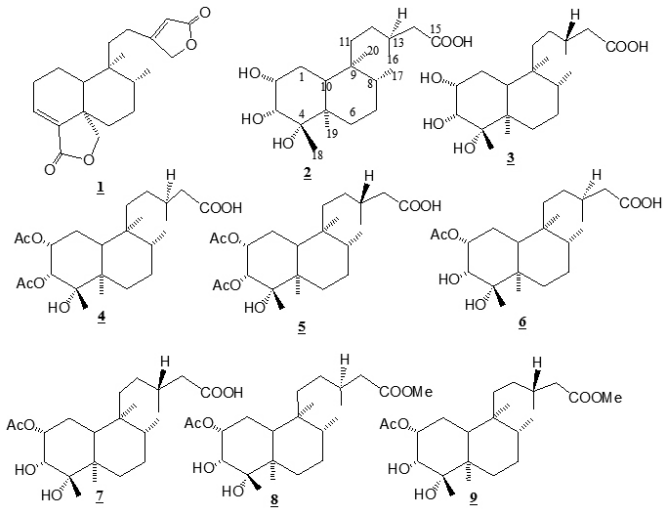

Fig. 1. Compounds *1-9* obtained from *B. marginalis.*

The ^{1}H and ^{13}C NMR spectra of compound **5** are very similar to the spectra corresponding to compound **4** with exception for the signals for the side chain, C-14 methylene protons, which in the case of **5**, appeared as a doublet at 2.27 ppm (J=5.3 Hz), ($\delta_{C\text{-}14}$ 41.0). C-15 was shifted upfield 2.3 ppm from **4** to **5.** This fact indicated that there is a difference in the configuration of C-13. So the proposed structure for compound **5** is ***2,3-diacetoxy-13-epi-dihydrotucumanoic acid.***

Compound **6** and **7** were characterized as their methyl ester derivatives **8** and **9** respectively. The ^{1}H NMR spectra of compounds **6** and **7** indicated that they were monoacetylated in C-2 (see Experimental) and as in the case of **4** and **5** they were epimers in C-13. The signals for H-14 are different in both compounds, in the case of compound **6** , H-14 appeared as a pair of double-doublet at δ 2.13 (J=15, 8.2 Hz) and 2.38 (J=15, 5.9 Hz), but in the case of compound **7**, H-14 appeared as a double-doublet at 2.30 ppm (J=14.7, 5.4 Hz). The ^{13}CNMR spectra of **8** and **9** confirmed all the above results and defined the proposed structures for **6** and **7** as ***2-acetoxy-dihydrotucumanoic acid*** and ***2-acetoxy-13-epi-dihydrotucumanoic acid*** respectively.

The absolute configuration of compounds **4** - **7** were not established, but it is very probably that correspond to that shown in the formulae, as also shown by related neoclerodane of known configuration isolated so far from *Baccharis* species[3,4]

The accumulation of 13-epimeric diterpenoids in members of the genus *Baccharis* could be of systematic value.

Compounds **4** - **7** were tested for inhibition of the acetylcholinesterase (AChE) activity (Table **2**). All the compounds obtained showed moderate inhibitory activity toward AChE, the better activity enzyme inhibitors were the compounds **6** and **7** with IC_{50} values of 0.369 and 0.031mg/mL respectively. The compound most active was **7** in comparison with the reference compound; the alkaloid galanthamine tested in parallel in the same assays was more potent (IC_{50} 1.1 x 10^{-3} mg/mL (3 x 10^{-3} mM)

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