AN ENT-KAURENE DERIVATIVE FROM AERIAL PARTS OF BACCHARIS RUFESCENS

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Abstract

From aerial parts of Baccharis rufescens Sprengel (Asteraceae:Astereae), the diterpene ent-3α,19-disuccinyloxy-kaur-16-ene (1) was isolated along with the triterpene oleanolic acid (4), and the flavonoids cirsimaritin (5,4'-dihidroxy-6,7-dimethoxyflavone) (5), and cirsiliol (5, 3',4'-trihidroxy-6,7-dimethoxyflavone) (6). Biological assays aimed to evaluate the incidence of compound 1 and its methyl ester derivative (2) on the food comsuption of adults of Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae), showed that compound 2 exhibited a significant antifeedant activity.

Resumen

De partes aéreas de Baccharis rufescens Sprengel (Asteraceae:Astereae) fue aislado el diterpeno ent-3α,19-disucciniloxi-kaur-16-eno (1) además del triterpeno ácido oleanólico (4) y los flavonoides cirsimaritina (5,4'-dihidroxi-6,7-dimetoxiflavona) (5) y cirsiliol (5, 3',4'-trihidroxi-6,7-dimetoxiflavona) (6). Ensayos biológicos dirigidos a evaluar la incidencia en el comportamiento alimentario del compuesto 1 y su metil éster (2) sobre adultos de Tribolium castaneum (Herbst) (Coleoptera: Tenebrionidae), permitieron asignar al compuesto 2 una significativa actividad antialimentaria.

Introduction

Baccharis constitutes the largest genus of the family Asteraceae with more than 400 species distributed in the American continent. Neo-clerodane, ent-labdane, and kaurane diterpenes have been reported to occur in this genus. The most common are the clerodane type diterpenes [1], while kaurane derivatives constitute minor findings. However, B. minutiflora Mart. yielded this kind of secondary metabolites as principal skeleton model [2].

In previous papers we have reported on the feeding-deterrent activities exhibited toward Tenebrio molitor (L) (Coleoptera:Tenebrionidae) larvae by some neo-clerodane diterpenoids isolated from this genus [3,4]. Computer-assisted conformational and electronic studies, as well as the analysis of the role that the hydrophobicity plays in the bioactivity have been carried out [5-7]. Recently, we have reported the anti-inflammatory

activities of some extracts from the aerial parts of B. rufescens [8]. Moreover, the essential oils constituents from this plant have been previously reported [9].

Several authors have studied the effects of natural products on Tribolium castaneum Herbst. (Coleoptera:Tenebrionidae), an important stored-product pest with a world wide distribution [10-11]. As part of a screening program for bioactive compounds against pest insects, we have studied the principal secondary metabolites presented in B. rufescens, as well as the antifeedant activity of the ent-kaurane **1**, and its methyl ester derivative (**2**).

Table 1: *1H nmr and 13C nmr chemical shifts (δ_C mult. in ppm, J in Hz) for Compounds 1-3 in $CDCl_3$. * Overlapped signals.[a]Determined at 75 MHz . [b]Determined at 50.23 MHz. [c]Determined at 400 MHz. [d]Determined at 200.13 MHz*

C	1[a]	2[b]	3[b]	H	1[c]	2[d]	3[d]
1	38.9 t	38.7 t	39.5 t	1	1.91* m	1.89* m	1.87* m
2	23.7 t	23.5 t	27.7 t	1′	1.03 m	0.85 m	0.95 m
3	80.2 d	80.5 d	80.8 d	2	1.71 m	1.71 m	1.71 m
4	41.3 s	41.3 s	41.2 s	2′			1.89 m
5	55.4 d	55.6 d	55.7 d	3	4.58 brt ($J_{w1/2}$=8)	4.59 dd (J= 9.2, 6)	3.40 br dd (J=11.2, 5, 1)
6	20.7 t	17.3 t	20.0 t	5	1.02 m	1.02 m	0.87 m
8	43.5 s	43.7 s	43.8 s	6	1.72 m	1.72 m	1.65 m
9	55.6 d	55.8 d	55.7 d	6′	1.49* m	1.49* m	1.66 m
10	38.7 s	39.3 s	38.6 s	7	1.57 m	1.55 m	1.55 m
11	18.2 d	18.3 d	18.3 d	7′	1.49* m	1.49 m	1.49 m
12	32.9 t	33.0 t	33.0 t	9	1.09 m	1.09 m	1.05 m
13	43.7 d	43.9 d	43.9 d	11	1.65* m	1.65* m	1.65 m
14	38.5 t	38.4 t	38.3 d	11′	1.55 m	1.55 m	1.55 m
15	48.5 t	48.6 t	48.8 t	12	1.67 * m	1.67 * m	1.70 m
16	155.2 s	155.4s	155.5s	12′	1.52 m	1.52 m	1.40 m
17	103.1 t	103.1 t	103.1t	13	2.70 br s	2.64 br s	2.64 m ($J_{W1/2}$ 3.5)
18	22.5 q	22.5 q	22.6 q	14	1.91* m	1.92* m	1.87* m
19	65.2 t	65.5 t	64.2 t	14′	1.14 m	1.13 m	1.10br m
20	17.5 q	17.3 q	18.1 q	15	2.05 br s	2.05 br s	2.07 br s
Succinic acid moiety				15′			
				17	4.79 br s	4.79 br s	4.79 br s
OCOR	174.3*s	172.6s	-	17′	4.73 br s	4.74 br s	4.72 br s
	172.2 s	172.2s	-	18	0.96 s	1.07 s	1.05 s
	171.8 s	171.7s	-	19	4.38 d (J= 12.0)	4.32 d (J= 12.0)	4.19 d (J= 12.0)
$-(CH_2)_2-$	29.3* t	29.3 t	-	19′	4.15 d (J= 12.0)	4.25 d (J= 12.0)	3.33 dd (J= 12.0, 1.0)
	29.1 t	29.1 t	-	20	1.02 s	0.98 s	0.98 s
	28.8 t	28.9 t	-	Succinic acid moiety			
		28.8 t	-	$-(CH_2)_2-$	2.64* br s	2.63* s, 2.66* s	-
OMe		51.8*q	-	$-OCH_3$	-	3.68 s, 3.72 s	-

Results and discussion

Compound **1** was obtained as yellow needles from acetone. The IR spectrum showed the presence of ester groups (1740 cm^{-1}), and an exociclic methylene group (1659, 887 cm^{-1}). The mass spectrum of **1** showed a [M^+] at m/z 504 and combustion elementary analysis gave a molecular formula $C_{28}H_{40}O_8$. EIMS fragment ions at m/z 386 (M^+-118), and m/z 268 (base peak, M^+-236) were suggestive of the presence of two succinyloxy groups in the molecule under study. This observation was supported by the

broad singlet signal centered at δ 2.64 integrating for eight protons in the 1H NMR spectrum, as well as by carbonyl signals at δ 171.8, 172.2, 174.3 (two carbons), and resonances at δ 28.8, 29.1, 29.3 (two carbons) in the ^{13}C NMR spectrum, which were in agreement with the aforementioned functionality.

All the ^{1}H NMR signals for compound **1** were assigned by comparing the NMR spectral data with those of known succinyl-kaurane derivatives [12] and analyzing the DEPT, ^{1}H-^{1}H COSY, HMQC, and HMBC high field experiments. A combination of ^{13}C NMR and DEPT spectra (Table 1) showed signals for two methyl groups, twelve sp^3 methylene carbons, four methines (one of them bearing oxygen), three sp^3 quaternary carbons, and an exocyclic methylene group.

Furthermore, the ^{1}H NMR spectrum exhibited signals assigned for two quaternary methyl groups as singlets at δ 0.96, and δ 1.02 (H-18 and H-20, respectively), one oxymethylene group at 4.38 (H-19, 1-H, d, J = 12 Hz) and 4.15 (H-19', 1-H, d, J = 12 Hz), and a exocyclic methylene group at δ 4.79 and 4.73 (H-17 and H-17', each 1-H, s, W1/2 = 3 Hz). Cross-peaks from the ^{1}H-^{1}H COSY spectrum showed the allylic coupling between the methylene group at δ 2.05 (H-15, 2H, br s), and the methinyl hydrogen H-13.

From these data the structure of compound **1** was assigned as an ent-kaur-16-ene diterpene, possessing two succinyloxy groups, one acyl group attached at C-19, and the other one on C-3. The location of the equatorial succinyloxy group on C-3 was established by a NOESY experiment, which showed significant cross-peaks between H-3ax and the equatorial methyl group (δ 0.96, H-18) on C-4. These experiments also exhibited a nOe effect between H-19 and H-20, H-17' with H-15, and H-17 with H-13 (Figure 1).

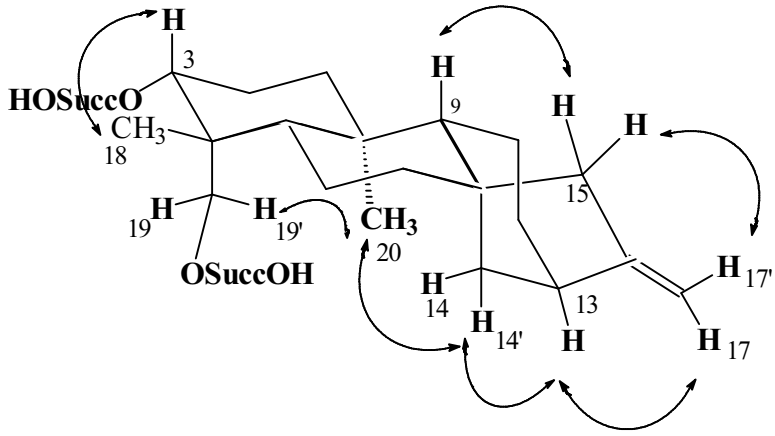


Figure 1: *Selected nOe effects observed in Compound 1.*

The HMBC correlation for compound **1** (Table 2) showed all the expected connectivity for the proposed kaurane skeleton. The compound **2** was prepared from **1** using diazomethane and its spectral data showed close resemblance to that of diterpene **1**. The ^{1}H NMR spectrum of **2** showed two methoxy groups signals as singlets at δ 3.72 and

δ 3.68, respectively, while the broad singlet at δ 2.64 (8 H) (compound **1**) due to the methylen protons of the two succinyloxy groups appeared in the dimethylester **2** as two singlets at δ 2.63 and δ 2.66. The ^{13}C NMR spectrum of **2** (Table 1) agrees with the proposed structure. Furthermore, saponification of **1** with NaH in CH_3OH followed by acid treatment, afforded the diol **3** whose physical constants and spectral data were identical to those for the known ent-kaur-16-ene [13]. In detail, the ^{1}H-^{13}C HETCOR spectra allowed us to the assignment of some of the non reported ^{1}H NMR signals for compound **3**. From the above mentioned data, the structure of **1** was established as ent-3α,19-disuccinyloxy-kaur-16-ene.

Table 2: *HMBC correlations for Compound 1 (Determined in Cl_3CD at 400 MHz)*

Proton(s)	Correlated carbons
3	2,18,19
7	5,8
12	16
14	15,16
15	8,16,17
17	16
18	5,19
19	18
19’	3,18
20	9

Table 3: *Nutritional and feeding deterrence indices of T. castaneum. RGR: relative growth rate. RCR: relative consumption rate. ECI: efficiency of conversion of ingested food. FDI: feeding deterrence index*

Compound	RGR (mg/mg/disk)	RCR (mg/mg/disk)	ECI (%)	FDI (%)
1	-0.001 ± 0.004	0.015 ± 0.009	-28.117 ± 3.978	12.76
2	-0.009 ± 0.016	0.008 ± 0.006	-658.300 ± 131.700	50.00

Insect nutritional experiments (Table 3) revealed that neither compound **1** nor **2** significantly reduced the insect growth rate (RGR), food consumption rate (RCR), and food use (ECI) of T. castaneum adults ($P>0.05$). This indicates that there is no effect on insect nutrition at the concentration here evaluated. However, when feeding deterrence indices (FDI) were calculated for compound **2**, a 50% antifeedant effect was founded.

Similar antifeedant effects on the subterranean termite Reticulitermes speratus Kolbe (Isoptera: Rhinotermitidae) [14], have been described after treated the feeding substrate with six ent-kauranes isolated from the fruits extract of Xylopia aethiopica (Dunal) A. Rich (Annonaceae). It has been reported the antifeedant activity presented by stevioside, rebaudioside A, and fifteen of their derivatives on Schizaphis graminum Rondani (Homoptera: Aphididae) [15]. The results here reported indicated that

compounds **1** and **2** have no effect on the nutritional indices using T. castaneum as insect model. Nevertheless, the antifeedant action presented by the derivative **2** agrees with other authors results [14-15]. Taking into account the limited number of assayed structures was not possible to define some structure-activity relationships.

Finally, from the phytochemical analysis here reported we can conclude that B. rufescens showed a diterpene pattern different to that exhibited by others species of the Baccharis genus from the central-western area of Argentina [4].

Experimental

General. Melting points were measured on a Leitz Wetzlar melting point apparatus and are reported uncorrected. Optical rotations were measured in acetone using a Perkin Elmer 341 polarimeter with a sodium lamp operating at 598 nm. Combustion analysis was carried out using an Eager 200 instrument (Instituto Universitario de Bioorgánica - Antonio González - La Laguna, Tenerife, Spain). NMR spectra were recorded at 400/75 MHz (1H/13C) (AMX - Bruker spectrometer) and 200.13/50.23 MHz ($^{1}H/^{13}C$) (Bruker AC-200 NMR spectrometer) using $CDCl_3$ as solvent and TMS as internal standard. Carbon multiplicities were determined by DEPT experiments. Connectivities were established by HMQC, HMBC and COSY spectral data. GC-MS were performed on a GCQ-Plus Finnigan-MAT apparatus, using a Restek-5ms (30 m length, 0.25 thick) column. TLC was carried out on Silica-gel F-254 (0.2 thick) with n-hexane/ AcOEt 1:1 as solvent. Detection of the products was made by spraying with a H_2SO_4:AcOH:H_2O (40:20:1) solution followed by heating. Column chromatography was performed on Lichoprep RP-18 (Merck, Darmstadt) and MeOH:H_2O 7:3 as eluent, VLC was performed over Silica gel (Kieselgel 60 H) using mixtures of n-hexane/AcOEt of increasing polarity as solvent system.

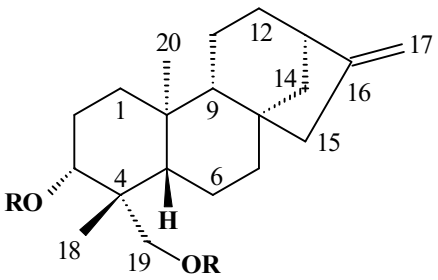
Plant material: Baccharis rufescens Sprengel var. rufescens was collected in Merlo, Dpto. Junín, San Luis, Argentina. A herbarium sample was deposited in the Herbario of the Universidad Nacional de San Luis (Luis A. Del Vitto, O. Giordano and E. Guerreiro voucher: N0 9102-(UNSL).

Extraction and isolation: Air dried aerial parts of B. rufescens (450g) were extracted with Me_2CO at room temperature for three times of two days each. After vacuum evaporation of the solvent, the residue (56g) was dissolved in a mixture of MeOH:H_2O (9:1), filtered and extracted with n-hexane in order to remove pigments and fatty materials. Water was added to the aqueous-alcohol fraction until the mixture became MeOH:H_2O 7:3 and this was then extracted with $CHCl_3$. The $CHCl_3$ extract was concentrated under vacuum and the resulting dark brown syrup (23g) was fractionated by flash-vacuum chromatography (VLC). The 50% AcOEt eluate was then purified using reverse phase Silica gel column (RP-18, and MeOH:H_2O 7:3 mixture as eluent). After crystallization from Me_2CO of the corresponding fractions, 90 mg of Compound **1** and 1200 mg of oleanolic acid were recovered.

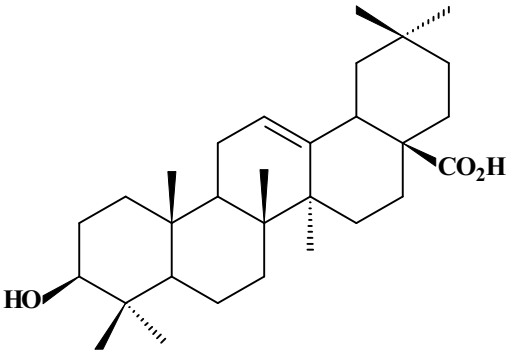
After purification of the more polar VLC-fraction (AcOEt) using a Sephadex LH-20 column (30 cm length, 0.25 thick), and MeOH as eluent, cirsimaritin (5,4'-dihidroxy,

6,7-dimethoxyflavone) (10 mg) and cirsiliol (3',4',5-trihidroxy-6,7-dimethoxy-flavone) (18 mg) were isolated.

Compound 1: ent-3α,19-disucciniloxy-kaur-16-ene: yellow crystals; mp: 202-203ºC, Elemental analysis: Found: C, 66.655; H, 7.926, (C28H40O8, Requires: C: 66.66, H: 7.93) $[\alpha]^{25}D$ –52.3º (c: 0.0367, acetone). IR (BrK) ν_{max} cm^{-1} 3437, 3070, 1739, 1659, 887. LR-EIMS 70 eV (%): 504$[M]^+$(7), 386 [M-succinic acid]$^+$ (18), 268 [M-2 succinic acid]$^+$ (Base peak, 100), 253 (80), 225 (65), 197 (37), 105 (30), 91 (35). ^1H and ^{13}C NMR spectral data: see Table 1.



1 R = SuccOH
2 R = SuccOMe
3 R = H



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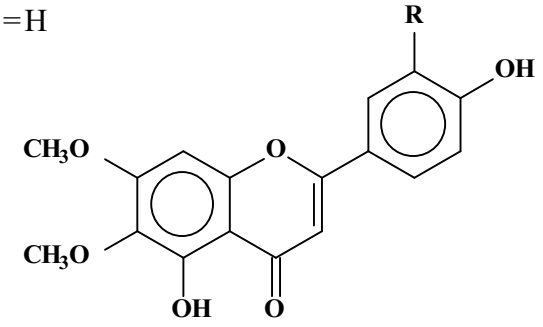

5 R = H
6 R = OH

Compound 2: Compound **1** (10 mg, 0.0198 mmol) was dissolved in Et2O (10 ml) and treated with diazomethane solution (Et2O). Purification of the crude by CC over silicagel (n-hexane-AcoEt , 7:3) afforded the methylester 2 (8.5 mg, 0.0159 mmol, 85 % yield) as yellow crystals; mp: 204-206 ºC. $[\alpha]^{20}D$ –56.2º (c: 0.0143, acetone), IR (BrK) ν_{max}, cm^{-1} 1738, 1725, 1692, 887. LR-EIMS 70 eV (%): 532 $[M]^+$(1), 400 [M-methylsuccinate]$^+$ (15), 268 [M-2 methylsuccinate]$^+$ (Base peak, 100). ^1H and ^{13}C NMR spectral data: see Table 1.

Compound 3: (ent-3α, 19-dihydroxy-kaur-16-ene): 10 mg of compound 1 (0.0198 mmol) were dissolved in MeOH (10 ml) and 10 mg of NaH (0.41 mmol, 60 % paraffin oil) were added in small portions over 5 min. After 2 hours with stirring at 20 oC the usual work-up furnished a mixture which was purified by CC over silicagel (n-hexane-AcoEt :1:1) and 7 mg of 3 (0.0230 mmol, 70 % yield) were recovered as yellow needles; mp: 187-189 ºC, $[\alpha]^{20}D$ – 63.5º (c 0.0125 acetone), IR (BrK) ν_{max}, cm^{-1} 3450, 3400, 1655, 885. LR-EIMS 70 eV (%): 304 $[M]^+$ (1), 286 $[M - H_2O]^+$ (25), 271 (27), 255 (35), 243 (40), 227 (35), 185 (33), 159 (55), 145 (60), 133 (40), 131 (78), 121 (38), 119 (56), 107 (50), 105 (75), 91 (100).

^1H and ^{13}C NMR spectral data: see Table 1.

Flour disk bioassay: Nutritional studies were done using adults of T. castaneum randomly selected. Flour disks were prepared using 200 μL of a stirred suspension of wheat flour in water (20g in 50mL) [11]. The disks were left in a chamber at 25 ± 1°C, 65% relative humidity to dry, and to equilibrate for one day (75 ± 8 mg/disk). Solutions of each compound (40 mg/mL, acetone), were prepared. Aliquots of 5 μL were spread evenly on each flour disks (200 μg/disk of the assayed compound). Controls were treated using 5 μL of acetone. The solvent was allowed to evaporate for 24 h, and two disks of the same treatment were placed in each plastic vial (diameter 3 cm, height 2 cm). The disks were weighed, and a ten group-weighed of unsexed adults of T. castaneum were added to each vial. Five replicates were set up for each compound and control, and each experiment was repeated three times. After 5 days, the flour disks and live insects were weighed again, and survival of the insects was recorded. Nutritional indices [11], were calculated as previously described: Relative Growth Rate (RGR) = (A–B)/(5B), where A is the weight (mg) of live insects on the fifth day divided by the number of live insects on the fifth day, B is the original weight (mg) of insects divided by the original number of insects. Relative Consumption Rate (RCR) = D/(5B), where D is biomass ingested (mg)/number of live insects on the fifth day. Efficiency of Conversion of Ingested food (ECI) (%) = (RGR/RCR) x 100. Feeding Deterrence Index (FDI) (%) = [(C-T)/C] X 100, where C is the consumption of control disks (mg) and T is the consumption (mg) of treated disks.

Statistical Analysis. Data of flour disk bioassay were treated by ANOVA test.

Acknowledgements

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