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Article

Triterpenic Acids from *Eugenia moraviana*

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Um novo triterpeno caracterizado como ácido 6α-hidroxibetulínico foi isolado das folhas e caule da planta, vulgarmente conhecida no Brasil como Cambuí, *Eugenia moraviana* (Myrtaceae), juntamente com três outras substâncias conhecidas, identificadas como ácido platânico, ácido betulínico e β-sitosterol. Através da análise de espectros incluindo NOE e experimentos de RMN em duas dimensões foi realizada a atribuição inequívoca dos deslocamentos químicos de ^{1}H e de ^{13}C do ácido 6α-hidroxibetulínico(ácido-3β,6α-diidróxi-20(29)-lupen-28-óico) e do ácido platânico.

A novel triterpene, characterized as 6α-hydroxybetulinic acid, was isolated from the leaves and stems of *Eugenia moraviana* (Myrtaceae), known in Brazil as Cambuí, together with three known compounds, platanic acid, betulinic acid and β-sitosterol. Unequivocal ^{1}H and ^{13}C assignments of 6α-hydroxybetulinic acid (3β,6α-dihydroxy-20(29)-lupen-28-oic acid) and platanic acid were undertaken by spectral analysis including NOE and 2 D NMR experiments.

Keywords: *Eugenia moraviana*, 6α-hydroxybetulinic acid, platanic acid, betulinic acid

Introduction

Eugenia moraviana Berg. (Myrtaceae) is a tree that occurs in South America, mainly in Brazil, Argentina and Paraguai[1]. The leaves of some *Eugenia* species are used in folk medicine for several therapeutics finalities[2,3]. Compounds such as flavonoids, triterpenes, tannins and especially essential oils constituted of monoterpenes and sesquiterpenes have already been isolated from the genus *Eugenia*[4-6]. However, no reports were found on the *Eugenia moraviana* constituents. Our phytochemical studies on the leaves and stems of *E. moraviana* resulted in the isolation of a new lupane-type triterpenoid, which was characterized as 6α-hydroxybetulinic acid (**1**) together with three known compounds identified as platanic acid (**2**), betulinic acid and β-sitosterol[7]. Compound **2** and betulinic acid have been attracting much attention in natural products chemistry because they present biological activities, *e.g.* anti-HIV[8], antitumor promoter[9], antimalarial and anti-inflammatory activities[10,11]. Previous assignments of ^{13}C signals [8,12,13] led to divergence between the C-18 and C-19 chemical shifts of compounds **1** and **2** and also between the C-15 and C-21 chemical shifts of **2**. In the present study we used techniques like COSY, HETCOR, HETCORLR and NOE difference, acquired at 300 MHz (^{1}H), to report an unequivocal and complete ^{1}H and ^{13}C assignment of compounds **1** and **2**. Peng and cols [14] reported the betulinic acid resonance assignment through a combination of high resolution NMR spectroscopy and a computer-assisted structure elucidation expert system. The authors run ^{13}C/DEPT, DQF COSY, HMBC, HMQC and NOESY spectra, acquired with a 720 MHz (^{1}H) spectrometer.

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Results and Discussion

Structural elucidation and NMR signal assignments of 6α-hydroxybetulinic acid **(1)**

Compound **1**, mp 285-288 °C; was isolated as white crystals. Its EIMS spectrum showed a molecular peak $[M]^{+\bullet}$ at m/z 472 corresponding to the formula $C_{30}H_{48}O_4$ and other peaks at m/z 248, 203, 187 and 175, which are characteristic for a pentacyclic triterpene skeleton of the lupane series[15]. The IR spectrum showed absorptions due to hydroxyl (3454 cm^{-1}), carboxyl (1685 cm^{-1}) and exo-methylene (1639 and 879 cm^{-1}) groups. The complete and unequivocal ^{1}H and ^{13}C chemical shifts assignments of **1** were assisted by DEPT, COSY (^{1}H x ^{1}H), HETCOR (^{13}C x ^{1}H) and HETCORLR (^{13}C x ^{1}H) spectra (Table 1). The ^{13}C NMR spectral data of **1** were compared with those from 6β-hydroxybetulinic acid[16]. According to our results the C-17 was erroneously

assigned and the C-15/C-21 and C-18/C-19 were interchanged. The antiperiplanar coupling ($J_{6,7a}$ and $J_{6,5a}$) of 10.4 Hz and the synclinal coupling ($J_{6,7e}$) of 3.8 Hz for H-6 proton at δ 4.35, indicated that the hydroxyl group at C-6 is α-orientation. The NOE difference NMR experiment was also performed to confirm the hydroxyl group orientation at C-6. Irradiation of the H-6 signal at δ 4.35 produced an enhancement in the methyl protons resonances at δ 1.45 (H-24, 5.0%), 0.96 (H-25, 6.5%) and 1.19 (H-26, 5.9%), which reveals a coaxial relationship between H-6, H-24, H-25 and H-26 thus confirming the equatorial position of the hydroxyl group at C-6. According to the observed NOE enhancements the structure of compound **1** should be as shown in Figure 1.

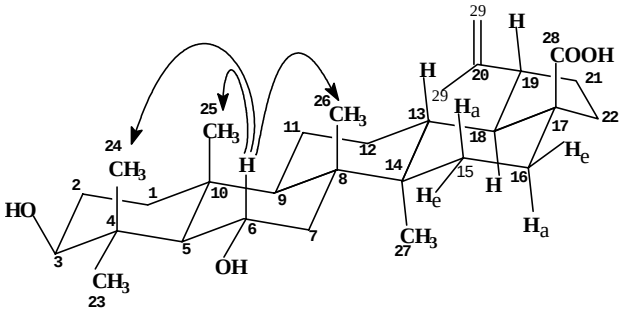

Figure 1. Schematic representation of selected NOE difference spectroscopy of **1**.

Assignments of C-15, C-18, C-19 and C-21 in the platanic acid **(2)**

To unequivocally assign carbons 15, 18, 19 and 21 in compound **2** the NOE difference spectra and a HETCOR spectrum were obtained. Initially the proton at δ 3.71 was irradiated and a NOE enhancement was observed at δ 2.24 (H-29). Irradiation of the signal at δ 2.40 provided NOE enhancements at δ 1.61(Ha-16) and 1.10 (H-27).

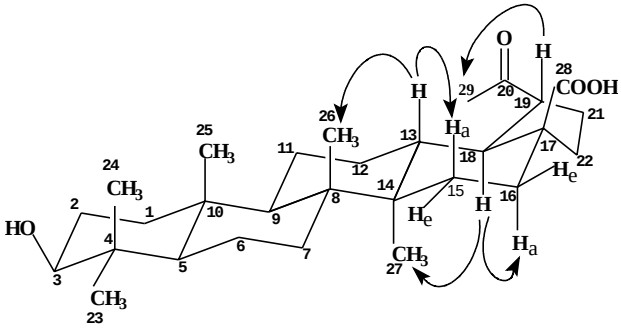

Figure 2. Schematic representation of selected NOE difference spectroscopy of **2**.

Table 1. $^{13}C^{a}$ (75.5MHz) and 2D NMR data of 6α-hydroxybetulinic acid (**1**) in pyridine-d_5

C	δ $^{13}C^{b}$	δ ^{13}C (DEPT)	HETCOR ($^{13}Cx^{1}H$) δ ^{1}H	COSY ($^{1}Hx^{1}H$) (^{2}J, ^{3}J and ^{4}J)	HETCORLR (^{2}J and ^{3}J)
1	38.7 (CH_2)	39.3 (CH_2)	1.10; 1.70	1.70; 1.10; 1.90	0.96
2	27.3 (CH_2)	28.2 (CH_2)	1.90	1.10	
3	78.9 (CH)	78.7 (CH)	3.60	2.01	
4	38.9 (C)	40.4 (C)	-		1.45; 2.01 (^{2}J)
5	57.1 (CH)	61.4 (CH)	1.23	4.35	0.96; 1.45; 1.70; 2.01
6	69.6 (CH)	67.9 (CH)	4.35	1.23; 1.99	
7	42.6 (CH_2)	47.5 (CH_2)	1.99	4.35	1.19
8	40.7 (C)	42.3 (C)	-		1.19 (^{2}J)
9	50.4 (CH)	50.7 (CH)	1.51		0.96; 1.19
10	37.2 (C)	39.6 (C)	-		
11	21.2 (CH_2)	21.2 (CH_2)	1.19; 1.48	1.48; 1.19; 1.98	
12	25.6 (CH_2)	26.2 (CH_2)	1.27; 1.98	1.19	
13	38.5 (CH)	38.3 (CH)	2.75		1.11
14	42.3 (C)	43.0 (C)	-		2.61; 1.19
15	30.6 (CH_2)	30.4 (CH_2)	1.31; 1.93		1.11
16	29.3 (CH_2)	32.9 (CH_2)	1.57; 2.61	2.61; 1.57	
17	48.0 (C)	56.7 (C)	-		1.31
18	48.0 (CH)	49.8 (CH)	1.76	3.55	2.61
19	48.8 (CH)	47.8 (CH)	3.55	1.76	
20	150.7 (C)	151.5 (C)	-		1.80 (^{2}J)
21	30.0 (CH_2)	31.2 (CH_2)	1.53; 2.23		
22	34.0 (CH_2)	37.7 (CH_2)	1.59; 2.27	2.27; 1.59	
23	28.0 (CH_3)	32.1 (CH_3)	2.01	3.60; 1.45	1.45
24	16.2 (CH_3)	16.6 (CH_3)	1.45	2.01	2.01; 1.23
25	16.8 (CH_3)	17.6 (CH_3)	0.96		
26	18.7 (CH_3)	18.0 (CH_3)	1.19		1.99
27	14.9 (CH_3)	14.9 (CH_3)	1.11		1.93
28	181.1 (C)	179.2 (C)	-		1.59; 1.76
29	109.8 (CH_2)	110.2 (CH_2)	4.79; 4.96	4.96; 4.79; 1.80	1.80
30	19.4 (CH_3)	19.5 (CH_3)	1.80	4.79; 4.96	4.79; 4.96

[a]Chemical shifts on δ scale (ppm) from TMS. [b]6β-hydroxybetulinic acid in $CDCl_3$ and 50MHz[16].

Interpretation of the observed NOE enhancements suggests a structure for **2** as shown in Figure 2 that shows all protons mentioned above in 1,3-diaxial interaction.

After the interpretation of the NOE enhancements we were able to identify carbons 18 and 19 in the HETCOR spectrum. The proton at δ 2.40 (H-18) correlates with the carbon at δ 49.9, and the proton at δ 3.71 (H-19) correlates the carbon at δ 52.1. To assign carbons C-15 and C-21, the proton at δ 2.53 (H-13) was irradiated and the NOE enhancement was observed on the protons at δ 1.57 (Ha-15) and 1.03 (H-26). Those protons are on 1,3-diaxial positions with respect to H-13 (see Figure 2). The HETCOR spectrum indicated the correlation between the proton at δ 1.57 (H-15) and the carbon at δ 28.8. The carbon at δ 30.3, that showed no correlation, must be C-21.

The complete assignments for the ^{1}H and ^{13}C NMR signals, as well the ^{1}H x ^{1}H and ^{13}C x ^{1}H correlations of **2** are reported in Table 2.

Table 2. ^{13}C[a](75.5MHz) and 2D NMR data of platanic acid (**2**) in pyridine-d_5

C	δ ^{13}C (DEPT)	HETCOR (^{13}Cx^{1}H) δ ^{1}H	COSY(^{1}Hx^{1}H) (^2J, ^3J and ^4J)
1	39.3 (CH_2)	0.94; 1.58	1.58; 0.94; 1.86
2	28.4 (CH_2)	1.86	0.94; 1.58; 3.46
3	78.2 (CH)	3.46	1.86
4	39.6 (C)	—	—
5	55.9 (CH)	0.79	1.39
6	18.8 (CH_2)	1.55	—
7	34.8 (CH_2)	1.39	0.79
8	41.1 (C)	—	—
9	50.9 (CH)	1.35	—
10	37.8 (C)	—	—
11	21.2 (CH_2)	1.39	—
12	27.8 (CH_2)	1.97	2.53
13	37.6 (CH)	2.53	1.97; 2.30
14	42.7 (C)	—	—
15	28.8 (CH_2)	1.57; 2.30	—
16	32.4 (CH_2)	1.61; 2.62	2.62; 1.61
17	56.6 (C)	—	—
18	49.9 (CH)	2.40	3.71
19	52.1 (CH)	3.71	2.40
20	212.0 (C)	—	—
21	30.3 (CH_2)	1.28; 1.83	1.83; 1.28
22	37.5 (CH_2)	1.63; 2.23	2.23; 1.63
23	28.7 (CH_3)	1.23	—
24	16.4 (CH_3)	0.82	—
25	16.4 (CH_3)	1.02	—
26	16.4 (CH_3)	1.03	—
27	14.9 (CH_3)	1.10	—
28	179.1 (C)	—	—
29	29.7 (CH_3)	2.24	—

[a]Chemical shifts on δ scale (ppm) from TMS.

Experimental

General

Mps: uncorr. IR: KBr pellet; EIMS: 70 eV; ^{1}H (300 MHz) and ^{13}C (75,5 MHz) NMR spectra were obtained in pyridine-d_5 with TMS as internal reference; CC: silica gel 60 (70-230 mesh) and silica gel 60 (230-400 mesh); TLC: silica gel F254 (0.25 mm in thickness).

Plant material

Leaves and stems of the plant were collected in September 1996, at the edge of Paraná river, Porto Rico city, State of Paraná, Brazil and identified by Dr. Graziela Maciel Barroso, Jardim Botânico do Rio de Janeiro and Dr. Lucia Elena Soares e Silva, Universidade Federal de Brasilia. A voucher specimen (HUM 2163) was kept at the herbarium of the Biological Department of the Universidade Estadual de Maringá.

Isolation

Air-dried and powdered leaves and stems (920 g) of *E. moraviana* Berg., were extracted with 95% aq. EtOH (18 L) at room temp. The combined EtOH extracts were concd in vacuum and after lyophilization yielded 84g of crude ethanolic extract. The crude ethanolic extract was partitioned with n-hexane (900 mL), chloroform (500 mL), ethyl acetate (500 mL) and n-butanol(500 mL), yielding 11.7g (32%), 27.3g (32.5%), 8.2g (9.8%) and 20.4g (24.3%) respectively. Part of the chloroformic fraction (6.0 g) was subjected to CC on silica gel (101 g) and eluted with different ratios of n-hexane and EtOAc. The appropriate frs (monitored by TLC analysis) were combined resulting in 11 frs. Frs 6-9, eluted with n-hexane-EtOAc (4:1, 2:3) afforded ppts, which were purified by recristalization on MeOH to give betulinic acid (603 mg –10%). Fr. 10 (763.4mg) was subjected to CC on silica gel, eluted with n-hexane, n-hexane-$CHCl_3$ (1:1), $CHCl_3$, $CHCl_3$-EtOAc (1:1), EtOAc, EtOAc-MeOH (1:1) and MeOH. The frs $CHCl_3$-EtOAc (1:1) afforded ppts, which by recristalization on $CHCl_3$-MeOH gave 6α-hydroxybetulinic acid (**1**) (30 mg-3.9%). The combined frs n-hexane and n-hexane-$CHCl_3$ (1:1) (571mg) were subjected to repeated CC on silica gel, eluted with n-hexane-$CHCl_3$ mixts, and flash CC on silica gel, eluted with $CHCl_3$-MeOH mixts of increasing polarity to give platanic acid (**2**) (25 mg-4.3%). The n-hexane fraction (4.4 g) was refractionated successively by CC on silica gel, eluted with n-hexane-EtOAc mixts of increasing polarity which afforded β-sitosterol (20.5mg – 0.5%) and betulinic acid (180mg – 4.0%).

6α-hydroxybetulinic acid (**1**)*:* White crystals, mp 285-288°. IR ν_{max} /cm^{-1}: 3454, 1685, 1639, 1038 and 879 (KBr). EIMS m/z (rel. int.): 472 $[M]^+_\bullet$ (0.5), 454 (2.9), 436 (3.7), 248 (10.9), 220 (3.9), 219 (5.6), 207 (11.3), 203 (14.9), 189 (25.8), 187 (66.9), 175 (17.5) and 43 (100). ^{1}H NMR (300 MHz, pyridine-d_5): δ 0.96 (3H, s, H-25), 1.11

(3H, s, H-27), 1.19 (3H, s, H-26), 1.45 (3H, s, H-24), 1.80 (3H, s, H-30), 2.01 (3H, s, H-23), 3.60 (1H, m, H-3), 4.35 (1H, dt, *J* 10.4 and 3.8 Hz, H-6), 4.79 (1H, brs, H-29), and 4.96 (1H, d, *J* 2,3 Hz, H-29).

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