

Triterpenic Acids from *Eugenia moraviana*

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Um novo triterpeno caracterizado como ácido 6 α -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 ^1H e de ^{13}C do ácido 6 α -hidroxibetulínico (ácido-3 β ,6 α -diidróxi-20(29)-lupen-28-óico) e do ácido platânico.

A novel triterpene, characterized as 6 α -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 ^1H and ^{13}C assignments of 6 α -hydroxybetulinic acid (3 β ,6 α -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*, 6 α -hydroxybetulinic acid, platanic acid, betulinic acid

Introduction

Eugenia moraviana Berg. (Myrtaceae) is a tree that occurs in South America, mainly in Brazil, Argentina and Paraguai¹. 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 6 α -hydroxybetulinic acid (**1**) together with three known compounds identified as platanic acid (**2**), betulinic acid and β -sitosterol⁷. Compound **2** and betulinic acid have been attracting much attention in natural products chemistry because they present biological activities, e.g. anti-HIV⁸, antitumor promoter⁹, 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 (^1H), to report an unequivocal and complete ^1H and ^{13}C assignment of compounds **1** and **2**. Peng and cols¹⁴ 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 $^{13}\text{C}/\text{DEPT}$, DQF COSY, HMBC, HMQC and NOESY spectra, acquired with a 720 MHz (^1H) spectrometer.

Results and Discussion

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

Compound **1**, mp 285-288 °C; was isolated as white crystals. Its EIMS spectrum showed a molecular peak $[\text{M}]^{+\bullet}$ at m/z 472 corresponding to the formula $\text{C}_{30}\text{H}_{48}\text{O}_4$ and other peaks at m/z 248, 203, 187 and 175, which are characteristic for a pentacyclic triterpene skeleton of the lupane series¹⁵. 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 ^1H and ^{13}C chemical shifts assignments of **1** were assisted by DEPT, COSY ($^1\text{H} \times ^1\text{H}$), HETCOR ($^{13}\text{C} \times ^1\text{H}$) and HETCORLR ($^{13}\text{C} \times ^1\text{H}$) spectra (Table 1). The ^{13}C NMR spectral data of **1** were compared with those from 6 β -hydroxybetulinic acid¹⁶. According to our results the C-17 was erroneously

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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.

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).

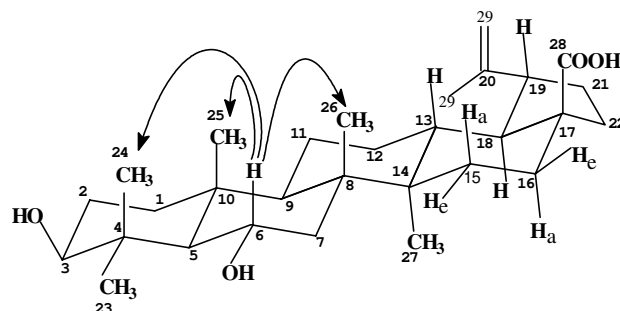


Figure 1. Schematic representation of selected NOE difference spectroscopy of **1**.

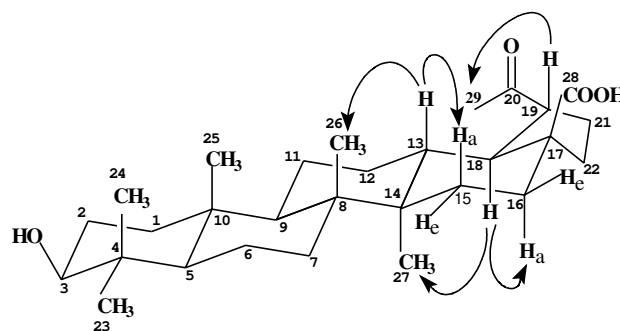


Figure 2. Schematic representation of selected NOE difference spectroscopy of **2**.

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

C	$\delta^{13}\text{C}^b$	$\delta^{13}\text{C}$ (DEPT)	HETCOR ($^{13}\text{C}_x^1\text{H}$) $\delta^1\text{H}$	COSY ($^1\text{H}_x^1\text{H}$) (2J , 3J and 4J)	HETCORLR (2J and 3J)
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 (2J)
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 (2J)
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 (2J)
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

^aChemical shifts on δ scale (ppm) from TMS. ^b 6β -hydroxybetulinic acid in CDCl_3 and 50MHz¹⁶.

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 (H-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 ^1H and ^{13}C NMR signals, as well the $^1\text{H} \times ^1\text{H}$ and $^{13}\text{C} \times ^1\text{H}$ correlations of **2** are reported in Table 2.

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

C	$\delta^{13}\text{C}$ (DEPT)	HETCOR ($^{13}\text{C} \times ^1\text{H}$) $\delta^1\text{H}$	COSY($^1\text{H} \times ^1\text{H}$) (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	—

^aChemical shifts on δ scale (ppm) from TMS.

Experimental

General

Mps: uncorr. IR: KBr pellet; EIMS: 70 eV; ^1H (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 Paran river, Porto Rico city, State of Paran, Brazil and identified by Dr. Graziela Maciel Barroso, Jardim Botnico 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 Maring.

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 recrystallization 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-CHCl₃ (1:1), CHCl₃, CHCl₃-EtOAc (1:1), EtOAc, EtOAc-MeOH (1:1) and MeOH. The frs CHCl₃-EtOAc (1:1) afforded ppts, which by recrystallization on CHCl₃-MeOH gave 6 α -hydroxybetulinic acid (**1**) (30 mg-3.9%). The combined frs n-hexane and n-hexane-CHCl₃ (1:1) (571mg) were subjected to repeated CC on silica gel, eluted with n-hexane-CHCl₃ mixts, and flash CC on silica gel, eluted with CHCl₃-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%).

6 α -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]⁺ (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). ^1H 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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