

Triterpenoid Saponins from *Lippia alba* (Mill.) N. E. Brown

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Das folhas de *Lippia alba* foram isoladas duas saponinas. As estruturas destas saponinas foram estabelecidas empregando métodos espectroscópicos, principalmente RMN mono e bi-dimensional e espectrometria de massas. Estes novos compostos foram caracterizados como ácido 3-*O*-β-D-glucopiranosil-28-*O*-(α-L-rhamnopyranosil-(1→3)-β-D-xilopiranosil-(1→4)-α-L-rhamnopyranosil-(1→2)-α-L-arabinopyranosil)-16α,23-di-hidróxi-olean-12-en-28-óico, designada Lippiasaponina I (**2**) e como ácido 3-*O*-β-D-glucopiranosil-28-*O*-(α-L-rhamnopyranosil-(1→3)-β-D-xilopiranosil-(1→4)-α-L-rhamnopyranosil-(1→3)-α-L-arabinopyranosil)-16α,23-di-hidróxi-olean-12-en-28-óico, designada Lippiasaponina II (**3**).

Two saponins were isolated from the leaves of *Lippia alba*. Their structures were established using one- and two-dimensional NMR spectroscopy and mass spectrometry. These new compounds were elucidated as 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→2)-α-L-arabinopyranosyl)-16α,23-dihydroxy-olean-12-en-28-oic acid, named as Lippiasaponin I (**2**) and as 3-*O*-β-D-glucopyranosyl-28-*O*-(α-L-rhamnopyranosyl-(1→3)-β-D-xylopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→3)-α-L-arabinopyranosyl)-16α,23-dihydroxy-olean-12-en-28-oic acid, named Lippiasaponin II (**3**).

Keywords: *Lippia alba*, verbenaceae, saponins

Introduction

Lippia alba (Mill.) N. E. Brown (Verbenaceae) is a shrub widely distributed throughout South America and it is popularly known as 'cidreira' or 'false melissa', designation derived from other medicinal plants (*Melissa officinalis* L. and *Cymbopogon citratus* (DC) Stapf.) also used in the popular medicine in cases of respiratory distress. Traditionally the tea from its leaves is largely utilized in popular medicine from all Brazilian regions as a tranquilizer and also in gastrointestinal and respiratory disorders. From the pharmacological point of view, antifungal activity was reported for hydroalcoholic extracts.^{1,2} Inhibition of HSV-1 (strain 29R/acyclovir resistant) was reported for the *n*-butanol fraction, and antipoliiovirus activity for the ethyl acetate fraction.³ In addition, sedative and myorelaxant

effects *in vivo* were reported for the hydroalcoholic extracts⁴ and antiulcerogenic activity was described for the leaves' infusion.⁵

Most chemical studies of *L. alba* are related to the essential oil composition and at least three chemotypes have been proposed based on the volatile chemical composition of its leaves.⁶ For the aerial parts, the presence of flavonoids,⁷ iridoid and phenylethanoid glycosides⁸ was reported. For a detailed review see Pascual *et al.*⁹ and references therein quoted.

In the present work, we report the isolation and structural characterization of two new saponins. To the best of our knowledge, the presence of saponins in *Lippia alba* has not been previously reported. The complete hydrogen and carbon assignments of the new compounds was accomplished using 2D NMR experiments including ¹H, ¹H-COSY, RCT, ¹³C, DEPT 90 and 135, HSQC, HMQC and HMBC.

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Results and Discussion

Solvent partition and chromatographic procedures allowed the isolation of the main triterpenoid saponins from the aerial parts of *L. alba*: Lippiasaponin I (**2**) and Lippiasaponin II (**3**). Basic hydrolysis of a mixture of **2** and **3** afforded only one prosapogenin (**1**).

Compound 1

The ^{13}C NMR spectrum of the prosapogenin (**1**) showed 36 signals, whereas the DEPT spectrum revealed 6 methyls, 11 methylenes, 11 methines and 8 quaternary carbon atoms. Six carbons could clearly be assigned to the sugar moiety, identified as β -glucopyranose by the NMR signals of the anomeric position (δ ^{13}C 105.8; δ ^1H 4.39).

The ^1H NMR and ^{13}C NMR spectrum of the prosapogenin **1** in CD_3OD displayed characteristic signals of a triterpene aglycone derived from oleanolic acid, showing a triplet at δ 5.29, which correlated in the HSQC spectrum with an olefinic carbon doublet at δ 123.4, assigned to C-12. Six three-hydrogen singlets at δ 0.70, δ 0.98, δ 0.79, δ 1.38, δ 0.87 and δ 0.96 could be assigned to the C-24, C-25, C-26, C-27, C-29 and C-30 methyl groups respectively. A signal at δ 181.3 in the ^{13}C NMR spectrum was assigned to the carboxylic acid at C-28.¹⁰

Three oxygenated carbons were observed at δ 83.5 (CH), δ 64.9 (CH_2) and δ 75.3 (CH). The former was assigned as glycosidated C-3, confirmed by the HMBC correlation between the anomeric hydrogen at δ 4.39 and the carbon at δ 83.5. The oxygenated carbon at δ 65.0 (CH_2), in principle could be assigned to C-23 or C-24.¹⁰

This hydroxymethylene group was assigned to C-23 considering the ^{13}C NMR chemical shifts of the methyl groups. It is reported that a hydroxymethylene group at the 4α -position (C-23) provokes a shielding for the 4β -methyl group (C-24) to *ca.* δ 11-13.¹¹ The shielded methyl group (δ 13.4, s) was assignable to the 4β -methyl group (C-24), thus, this hydroxyl was linked at C-23 (δ 65.0).

The remaining oxygenated carbon at δ 75.3 (CH) was linked to the hydrogen at δ 4.45 (m). The COSY spectrum showed that this hydrogen was coupled to hydrogens at δ 1.35 and δ 1.85, assigned to C-15 by HMBC correlations, thus unambiguously locating this hydroxyl group at C-16. The 16α -configuration was evident from the small J values of H-16 (broad multiplet at δ 4.45) in the ^1H NMR spectrum, characteristic of an equatorial hydrogen.

Therefore, the structure of **1** was elucidated as 3- O - β -D-glucopyranosyl- 16α -23-dihydroxy-olean-12-en-28-oic acid. Total carbon and hydrogen assignments are shown on Table 1.

Compound 2

The FAB MS (positive ion mode) of compound **2** displayed a quasi-molecular ion peak at m/z 1229 [$\text{M} + \text{Na}^+$] suggesting a molecular formula of $\text{C}_{58}\text{H}_{94}\text{O}_{26}$. In addition, a fragment ion at m/z 1050 indicated the loss of a hexose moiety.

The ^{13}C NMR spectrum of **2** showed 58 signals, whereas the DEPT spectrum revealed 8 methyls, 13 methylenes, 29 methines and 8 quaternary carbon atoms. The comparison of the ^1H and ^{13}C data (Table 1) indicated the same partial structure of compound **1**. The major differences were observed at C-28 (δ 175.6) and the presence of five anomeric carbons instead of one. The shielding of C-28 was attributed to glycosylation of the carboxyl group.¹⁰

The ^{13}C NMR signals of the four additional anomeric carbons were located at δ 94.0, δ 101.4, δ 102.5 and δ 106.5 and correlated to anomeric hydrogens at δ 5.60, δ 5.03, δ 5.13 and δ 4.53 respectively in the HSQC experiments (Table 1).

HMBC, COSY, RCT, and 2D J -Resolved spectra were used for the complete assignment of the resonances of each monosaccharide moiety, starting from the anomeric hydrogens.

The analysis of these spectra revealed the presence of a β -D-xylose ($J_{\text{H-1, H-2}} = 7.7\text{Hz}$), an α -L-arabinose in a predominant $^1\text{C}_4$ conformation ($^3J_{\text{H-1, H-2}} = 3.8\text{Hz}$)¹² and two α -rhamnoses ($J_{\text{H-1, H-2}} = 1.6\text{Hz}$ and $J_{\text{H-1, H-2}} = 1.4\text{Hz}$) identified through the observation of COSY correlations between two methyl doublets at δ 1.23 and δ 1.29 and glycosidic hydrogens at δ 3.99 and 3.68, respectively. Sequencing of the glycosidic chains was achieved by analysis of HMBC experiments. For the tetraglycosidic chain linked to the carboxylic C-28 of the aglycone, the HMBC showed cross-peaks between H-1 (δ 5.13) of a terminal rhamnose and C-3 (δ 84.1) of xylose, between H-1 of xylose (δ 4.53) and C-4 (δ 83.4) of an inner rhamnose, between H-1 (δ 5.03) of an inner rhamnose and C-2 (75.8) of arabinose and between H-1 (δ 5.60) of arabinose and C-28 (δ 175.6). This last correlation confirmed the linkage between C-2 of arabinose and C-1 of inner rhamnose. The assignment of D or L configurations was confirmed by GC (Gas Chromatography) of the corresponding acetylated 1-deoxy-1-(2-hydroxypropylamino) alditols, prepared from the monosaccharides obtained by acid hydrolysis of compound **2**, and co-injection with authentic standards.¹³ Therefore, compound **2** was identified as 3- O - β -D-glucopyranosyl-28- O -(α -L-rhamnopyranosyl-(1 \rightarrow 3))- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 2)- α -L-arabinopyranosyl)- 16α ,23-dihydroxy-olean-12-en-28-oic

Table 1. ^{13}C NMR and ^1H NMR data of Prosapogenin, Lippiasaponin I and Lippiasaponin II (in CD_3OD)

Attribution	DEPT	Prosapogenin (1)		Lippiasaponin I (2)		Lippiasaponin II (3)	
		δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)	δ_{C}	δ_{H} (J in Hz)
Aglicone							
1	CH_2	39.6	0.98 m 1.62 m	39.6	1.61 m 1.65 m	39.6	0.96 m 1.62 m
2	CH_2	26.4	1.74 m 1.95 m	26.3	1.90 m 1.90 m	26.4	1.75 m 1.93 m
3	CH	83.5	3.63 m*	83.6	3.64 m*	83.8	3.66 dd (5.4, 12.0)
4	C	43.9	-	43.5	-	43.9	-
5	CH	48.3	1.23 m	48.4	1.23 d (6.2)	48.5	1.20 d (4.2)
6	CH_2	18.9	1.37 m 1.48 m	19.0	1.37 m 1.47 m	19.2	1.37 m 1.47 m
7	CH_2	33.8	1.28 m 1.64 m	33.7	1.37 m 1.61 m	33.7	1.48 m 1.58 m
8	C	40.6	-	40.7	-	40.8	-
9	CH	48.2	1.69 m	48.1	1.67 m	48.1	1.67 m
10	C	37.7	-	37.6	-	37.7	-
11	CH_2	24.5	1.90 m 1.90 m	24.5	1.90 m 1.90 m	24.5	1.90 m 1.90 m
12	CH	123.4	5.29 br t	123.7	5.34 t (3.4)	123.5	5.32 t (3.5)
13	C	145.2	-	145.0	-	144.8	-
14	C	42.8	-	42.3	-	42.8	-
15	CH_2	36.2	1.35 m 1.85 m	36.4	1.15 m 1.39 m	36.6	1.47 m 1.67 m
16	CH	75.3	4.45 m	74.6	4.48 br t	74.5	4.46 s
17	C	49.6	-	50.0	-	50.3	-
18	CH	42.1	3.00 dd (4.0, 12.0)	42.1	3.02 dd (3.9, 12.0)	42.2	2.96 dd (4.0, 12.0)
19	CH_2	47.7	1.02 m 2.28 t (12.0)	47.6	1.04 dd (12.0, 3.9) 2.27 t (12.0)	47.9	1.05 dd (4.0, 12.0) 2.28 t (12.0)
20	C	31.4	-	32.0	-	31.3	-
21	CH_2	36.6	1.14 m 1.93 m	36.4	1.43 dd (3.9, 12.0) 1.75 m	36.6	1.16 m 1.93 m
22	CH_2	32.7	1.76 m 1.90 m	31.9	1.90 m 1.90 m	32.1	1.89 m 1.92 m
23	CH_2	65.0	3.27 m 3.62 m	65.2	3.36 d (12.0) 3.62 d (12.0)	65.4	3.30 m 3.64 d (12.0)
24	CH_3	13.4	0.70 s	13.5	0.72 s	13.6	0.74 s
25	CH_3	16.6	0.98 s	16.6	0.99 s	16.7	0.99 s
26	CH_3	17.8	0.79 s	18.0	0.77 s	17.8	0.77 s
27	CH_3	27.3	1.38 s	27.3	1.37 s	27.2	1.35 s
28	C	181.3	--	175.6	-	177.1	-
29	CH_3	33.4	0.87 m	33.3	0.87 s	33.3	0.87 s
30	CH_3	24.9	0.96 s	25.1	0.96 s	24.9	0.94 s
3-O-sugar							
Gly							
1	CH	105.8	4.39 d (7.7)	105.7	4.40 d (7.7)	105.7	4.39 d (7.7)
2	CH	75.7	3.16 t (8.2)	75.6	3.17 t (8.0)	75.7	3.16 t (8.8)
3	CH	78.4	3.34 m*	78.3	3.35 t (8.0)	78.3	3.33 t (8.8)
4	CH	71.6	3.27 m*	71.6	3.29 m	71.6	3.28 t (8.8)
5	CH	77.8	3.27 m*	77.7	3.27 m	77.8	3.26 m
6	CH_2	62.8	3.67 m* 3.83 dd (3.2, 10.0)	62.7	3.70 m 3.81 dd (3.5, 12.0)	62.7	3.68 dd (5.4, 12.0) 3.82 m

Table 1. continuatin

28-O-sugars					
Ara					
1	CH	94.0	5.60 d (3.8)	95.5	5.43 d (5.2)
2	CH	75.8	3.77 t (3.8)	77.1	3.54 t (9.5)
3	CH	71.2	3.86 m	76.6	3.55 m
4	CH	67.0	3.82 dd (3.5, 9.0)	70.9	3.52 m
5	CH ₂	63.8	3.50 m*	66.7	3.28 d (12.0)
			3.89 dd (9.0, 12.0)		3.91 m
Rha I					
1	CH	101.4	5.03 d (1.4)	101.3	5.28 d (1.6)
2	CH	72.1	3.85 m	71.9	3.91 dd (1.6, 3.4)
3	CH	72.4	3.84 m	72.4	3.81 dd (3.4, 9.0)
4	CH	83.4	3.55 t (9.0)	84.3	3.52 t (9.0)
5	CH	69.0	3.68 m	69.0	3.70 m
6	CH ₃	18.1	1.29 d (6.2)	18.4	1.31 d (6.2)
Xyl					
1	CH	106.5	4.53 d (7.7)	107.0	4.48 d (7.7)
2	CH	76.2	3.34 t (8.6)	76.4	3.37 t (8.8)
3	CH	84.1	3.45 t (8.6)	84.3	3.45 t (8.8)
4	CH	69.9	3.53 m	69.9	3.52 m
5	CH ₂	67.1	3.18 t (8.3)	67.2	3.21 dd (9.0, 12.0)
			3.82 m		3.86 dd (5.5, 12.0)
Rha II					
1	CH	102.5	5.13 d (1.6)	102.6	5.13 d (1.6)
2	CH	72.3	3.94 dd (1.6, 3.5)	72.3	3.95 dd (1.6, 3.4)
3	CH	72.2	3.71 dd (3.5, 9.2)	72.2	3.71 dd (3.4, 8.8)
4	CH	74.0	3.38 t (9.2)	74.0	3.37 t (9.5)
5	CH	70.0	3.99 dd (9.2, 7.0)	70.0	3.99 dd (5.4, 9.5)
6	CH ₃	17.9	1.23 d (7.0)	17.9	1.23 d (6.2)

* overlapping; br = broad.

acid, and named Lippiasaponin I. These sugar assignments are in accordance with literature data.^{14,15}

Compound 3

As in the case of compound **2**, the ¹³C NMR spectrum of **3** showed 58 signals, whereas the DEPT spectrum revealed 8 methyls, 13 methylenes, 29 methines and 8 quaternary carbon atoms (Table 1).

A comparative analysis of ¹H and ¹³C NMR, and COSY spectra of **3**, showed great similarities in the chemical shift of both compounds, but some differences in the resonances of the arabinose unit linked at C-28. All ¹³C signals of this arabinose were deshielding shifted (Table 1). Besides, the *J* of H-1 Ara (δ 5.43) changed notably from 3.8 Hz (**2**) to 5.2 Hz (**3**).

The analysis of the COSY, RCT and 2D J-Resolved spectra of compound **3** revealed as in compound **2**, the

presence of a β -D-xylose ($J_{H-1, H-2} = 7.7$ Hz), a β -D-glucose ($J_{H-1, H-2} = 7.7$ Hz) and two α -rhamnosides ($J_{H-1, H-2} = 1.6$ Hz and $J_{H-1, H-2} = 1.6$ Hz). The main difference between **2** and **3** was observed in the α -L-arabinose unit, which in **3** was in a predominant ⁴C₁ conformation ($^3J_{H-1, H-2} = 5.2$ Hz).¹²

For the tetraglycosidic chain linked to the carboxylic C-28 of the aglycone, the HMBC showed cross-peaks between H-1 (δ 5.13) of a terminal rhamnose and C-3 (δ 84.3) of xylose, between H-1 of xylose (δ 4.48) and C-4 (δ 84.3) of an inner rhamnose, between H-1 (δ 5.28) of an inner rhamnose and C-3 (76.6) of arabinose and between H-1 (δ 5.43) of arabinose and C-28 (δ 177.1). These data indicated the linkage between C-3 of arabinose and C-1 of the inner rhamnose, pointing to the main structural difference between Lippiasaponins I (**2**) and II (**3**). A value for the $^3J_{H-1, H-2}$ coupling of 3.8 Hz (Ara-H1) was observed in Lippiasaponin I. This data indicated a ¹C₄ arabinose conformation.¹² On the other hand, we observed

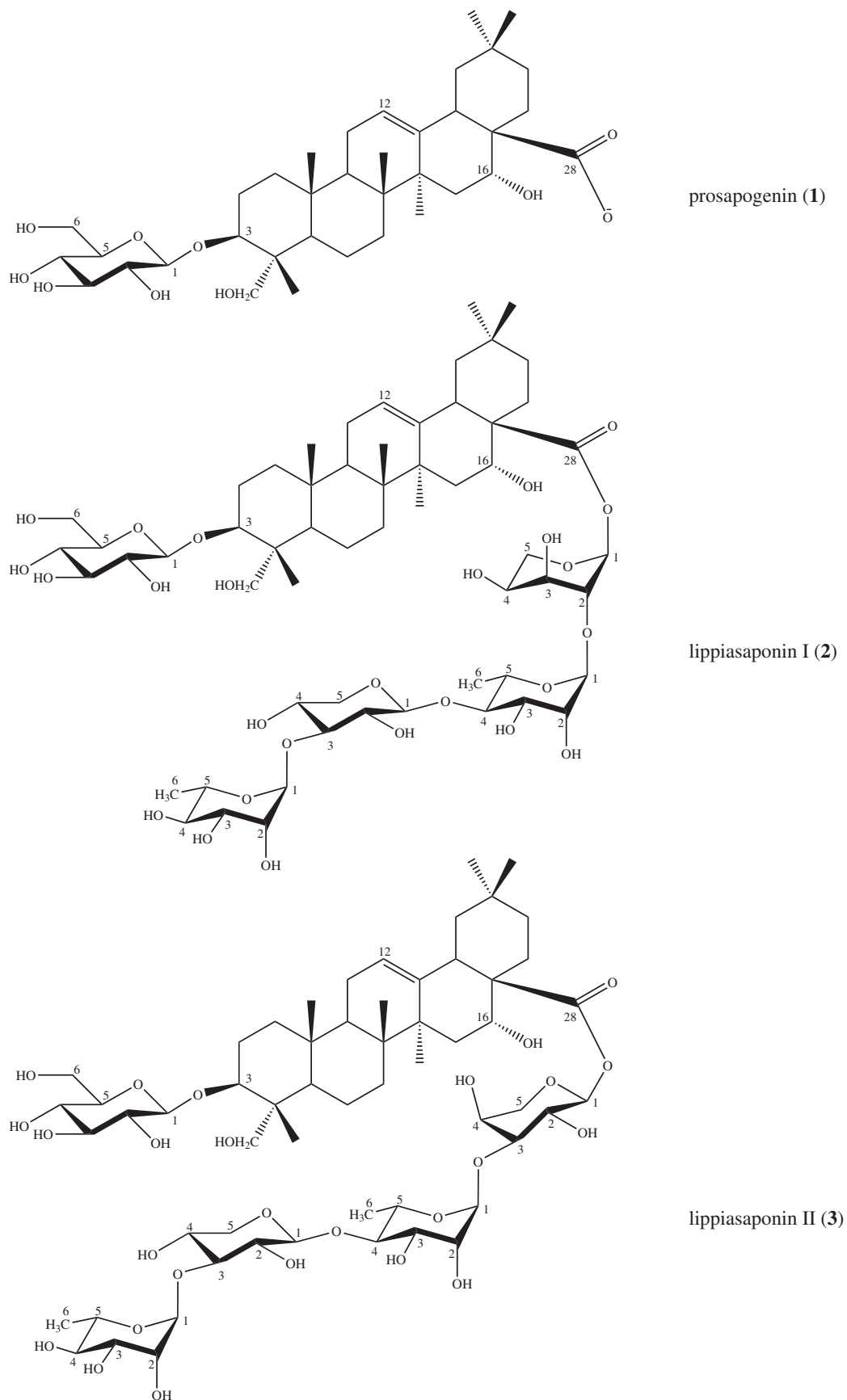


Figure 1. Structures of the prosapogenin (1), lippiasaponin I (2) and lippiasaponin II (3).

in Lippiasaponin II a value of 5.2 Hz (Ara-H1) which suggests an α -L-arabinopyranoside in 4C_1 conformation.¹² The problem of the configuration and conformation of arabinopyranoses in esters of hindered triterpene carboxylic acids has been discussed at length using 1H and ${}^{13}C$ NMR arguments.^{12,16} Particularly puzzling is the fact that in α -L-arabinosyl ester the ${}^3J_{H-1-H-2}$ couplings vary from 2.8 to 6.2 Hz depending on the equilibrium between 1C_4 and 4C_1 conformations.

The α -L-arabinopyranoside absolute stereochemistry was determined by comparison with methyl α -L-arabinopyranoside.¹⁷ The C-1 of arabinopyranose in esters of triterpene carboxylic acids differs from C-1 of methyl α -L-arabinopyranoside, because the priority order changes with the atomic number of atoms, therefore, the α -L-arabinopyranoside is *S/R/S/S*. In the molecular model of compound **2** we observed that the dihedral angle between Ara-H1 and Ara-H2 is approximately 80-90° on α -L-arabinopyranoside chair 1C_4 conformation, which explains the small *J*, in accordance with Karplus curve. The 1C_4 conformation on Lippiasaponin I (**2**) can be explained by *O*-Rha substitution at C-2 in Ara, which increases the population of 1C_4 conformation.¹² Additional proof was obtained through the observation of a *W* coupling between H-3 (δ 3.86) of arabinose and H-5 (δ 3.50).

In the molecular model of α -L-arabinopyranoside chair 4C_1 conformation the dihedral angle between Ara-H1 and Ara-H2 is approximately 180°, which points to a high value for the coupling constant. The predominance of 4C_1 conformation on Lippiasaponin II (**3**) can be explained by *O*-Rha substitution at C-3 in Ara, since the C-2 is hindered by the proximity of the *O*-substituent at position 1.

Therefore, compound **3** was identified as 3-*O*- β -D-glucopyranosyl-28-*O*-(α -L-rhamnopyranosyl-(1 \rightarrow 3))- β -D-xylopyranosyl-(1 \rightarrow 4)- α -L-rhamnopyranosyl-(1 \rightarrow 3)- α -L-arabinopyranosyl)-16 α ,23-dihydroxy-olean-12-en-28-oic acid, and named Lippiasaponin II. To the best of our knowledge, this is the first report of the natural occurrence of **2** and **3**.

Experimental

General procedures

NMR experiments were performed on a Bruker AM-500 (500 MHz) spectrometer. NMR data were reported as δ values, and referenced to the residual signal of the solvent (CD_3OD). The MS experiments were performed on a UltratOF-Q, Bruker Daltonics, Billerica, MA spectrometer (positive ion mode). Silica gel 60 (0.063-0.200 mm, Merck) and silica gel 60 (0.040-0.063 mm,

Merck) were used for column chromatography. TLC was performed on precoated silica gel 60 F₂₅₄ plates (Merck). HPLC separations were performed on a Shimadzu (FRC-10A) liquid chromatographer equipped with a UV-Vis detector using a preparative ODS column (column A, Shim-pack 20 x 250 mm, Shimadzu; detector: 254 nm; flow rate: 5 mL min⁻¹).

Plant material

Aerial parts of *Lippia alba* were collected in Cacupé, Florianópolis, Brazil, in April 2003. A voucher specimen (FLOR-31267) is deposited at the herbarium ICN (Federal University of Santa Catarina, Brazil).

Extraction and isolation

Material was dried and the leaves were separated from the stems and flowers. Leaves (840 g) were ground to powder and macerated two times with 96% ethanol. The alcohol extract (89.2 g) was concentrated, suspended in water, and then partitioned successively with petroleum ether (boiling range 30-60 °C), CH_2Cl_2 , EtOAc and *n*-BuOH (150 mL, 6 times with each solvent). The *n*-BuOH-soluble fraction (2.8 g) was applied to a silica gel 60 column (0.063-0.200 mm) eluting with the organic phase of the mixture EtOAc:MeOH: isopropanol:H₂O:HOAc (7:1:2:5:0.2) to give five sub-fractions (1-5). Fraction 5 (265 mg) was further chromatographed over silica gel 60 (0.040-0.063 mm), eluting with the mixture EtOH:EtOAc:H₂O (120:60:5) to yield two sub-fractions (5.1-5.2). Fraction 5.1 (218 mg) was separated by preparative HPLC (column A) using $CH_3CN:H_2O$, 70:30 (v:v) as eluant to yield **2** (35 mg) and **3** (32 mg).

Acid hydrolysis of Lippiasaponin I (**2**)

Compound **2** (2 mg) was hydrolyzed in 2 mol L⁻¹ TFA at 120 °C for 1 h 30 min. The hydrolyzed monosaccharides were derivatized to the acetylated 1-deoxy-1-(2-hydroxypropylamino) alditols following published procedures¹² and analysed by GC using a Hewlett-Packard Ultra-2 column (50m x 0.2 mm, thickness of liquid phase 0.11 μ m) and identified by coinjection with authentic standards prepared in a similar way.

Prosapogenin (**1**)

White powder, 1H NMR and ${}^{13}C$ NMR spectral data: see Table 1.

Lippiasaponin I (2)

White powder, $[\alpha]_D^{25} = -30.43$ (C = 0.013, MeOH); FAB MS $m/z = 1229$ (100%); 1050 (10%); ^1H NMR and ^{13}C NMR spectral data: see Table 1.

Lippiasaponin II (3)

White powder; ^1H NMR and ^{13}C NMR spectral data: Table 1.

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