Triterpenoids from Brazilian *Ilex* Species and Their in Vitro Antitrypanosomal Activity

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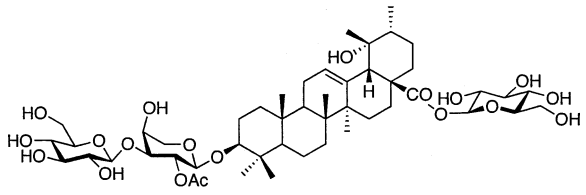
From the leaves of *Ilex affinis* and *Ilex buxifolia*, two adulterant species of "erva maté" (*Ilex paraguariensis*), three new triterpenoid glycosides were isolated. Affinoside 1 (3*β*-*O*-{*β*-D-glucopyranosyl-(1→3)-[2-*O*-acetyl-(1→2]}-α-L-arabinopyranosyl pomolic acid 28-*O*-*β*-D-glucopyranosyl ester, **1**) was isolated from *I. affinis*, while buxifolioside I (28-*O*-*β*-D-glucopyranosyl ester of (20*S*)-3α,19α-dihydroxyurs-12-ene-23,28-dioic acid, **7**) and buxifolioside II (28-*O*-*β*-D-glucopyranosyl ester of (20*S*)-3*β*,19α-dihydroxyurs-12-en-24,28-dioic acid, **8**) were isolated from *I. buxifolia*. Along with these new compounds, ilexoside II (**2**), ursolic acid (**3**), 28-nor-ursolic acid (**4**), 3*β*-*O*-acetylursolic acid (**5**), and uvaol (**6**) were also isolated. The observed results confirm the structural specificity of the *I. paraguariensis* triterpenoids and reinforce a previous proposal to detect maté adulteration by triterpenoid analysis. In addition, the in vitro antitrypanosomal activity of some *Ilex* triterpenoids is also reported.

For centuries, the dried leaves of *Ilex paraguariensis* St. Hil. (Aquifoliaceae) have been used in the east-central part of South America by the Guarani Indians to prepare maté, a stimulating beverage. Maté is still frequently used for this purpose nowadays in Brazil, and the annual consumption is estimated to be around 500 000 tons.[1] Because of the emigration of Brazilians to Western Europe and Eastern Asia, exports of maté are also increasing on a regular basis.[2] However, maté adulteration by incorporation of the leaves of other South American *Ilex* species has been reported.[3] As a result, not only does the taste of the beverage become modified[4] but some of the adulterant species may contain secondary metabolites with unknown physiological and pharmacological activities.[5] In consequence, there is a need to design analytical methods to allow the rapid determination of maté authenticity. Some years ago, taking advantage of the *I. paraguariensis* saponin profile[6−10] we proposed to use *Ilex* saponins as chemical markers.[11] For this purpose we initiated a program aimed at systematically studying the saponin content of all South American *Ilex* species.[4,6−9,11−19] Continuing this study, in the present work, we report the isolation and structural elucidation of triterpenes and triterpenoid glycosides from the leaves of two other *Ilex* species, namely, *I. affinis* Gardner and *I. buxifolia* Gardner, which are known as "congonha-de-Goias" and "congonha-do-campo", respectively. These two species are distributed in Central Brazil, East Bolivia, and Northeast Paraguay.[20,21]

Results and Discussion

Three new triterpenoid glycosides were isolated during this study. A new compound isolated from the leaves of *I. affinis* was named affinoside 1 (**1**), and two new compounds isolated from *I. buxifolia* were named buxifolioside I (**7**) and buxifolioside II (**8**). Together with these derivatives, ilexoside II (**2**), ursolic acid (**3**), 28-nor-usolic acid (**4**), 3*β*-*O*-acetylursolic acid (**5**), and uvaol (**6**) were also isolated. The structure of the new compounds was elucidated using chemical and spectroscopic methods. The in vitro antitrypanosomal activity of five compounds isolated during this study and of nine compounds previously isolated from other *Ilex* was examined.



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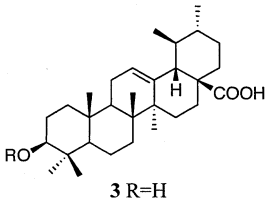
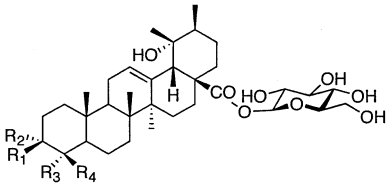


3 R=H
5 R= $COCH_3$



7 R_1= H, R_2 = OH, R_3 = COOH, R_4 = CH_3
8 R_1= OH, R_2 = H, R_3 = CH_3, R_4 = COOH

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Table 1. ^{1}H NMR Data for Sugar Portions of Compounds **1**, **7a**, and **8a** in C_5D_5N[a]

	1		**7a**		**8a**
position	δ_H (J in Hz)	position	δ_H (J in Hz)	position	δ_H (J in Hz)
	Ara		Glc		Glc
1′	4.68 d (7.8)	1′	6.31 d (8.6)	1′	6.28 d (8.3)
2′	6.02 dd (7.8, 10.2)	2′	5.74 dd (8.6, 9.8)	2′	5.73 dd (8.3, 9.4)
3′	4.21 dd (3.9, 10.2)	3′	5.99 t (9.8)	3′	5.97 t (9.4)
4′	4.46 m	4′	5.60 t (9.8)	4′	5.59 t (9.4)
5′	3.67 m	5′	4.39 ddd (2.1, 4.5, 9.8)	5′	4.38 ddd (2.0, 4.2, 9.4)
	4.14 m	6′	4.30 dd (2.1, 12.4)	6′	4.30 dd, (2.0, 12.5)
CH_3CO	2.24 s		4.58 dd, (4.5, 12.4)	4.57 dd, (4.4, 12.5)	
	GlcI				
1″	5.06 d (7.7)				
2″	3.85 dd (7.7, 9.0)				
3″	4.20 t (9.0)				
4″	4.17 t (9.0)				
5″	4.00 m				
6″	4.37 dd (4.6, 12.0)				
	4.56 br. d (12.0)				
	GlcII				
1‴	6.30 d (8.2)				
2‴	4.24 dd (8.2, 8.8)				
3‴	4.31 t (8.8)				
4‴	4.38 t (8.8)				
5‴	4.06, m				
6‴	4.40 dd (3.0, 11.8)				
	4.48 d (11.8)				

[a] Assignments were confirmed by COSY, 1D-TOCSY, HSQC, and HMBC experiments.

The ethanolic extract of the leaves of *I. affinis* afforded, after chromatographic procedures, three compounds. The major component was compound **2**, which was identical to ilexoside II, a saponin previously isolated from *I. crenata*[22] and also from the leaves of *Randia formosa*, from which it was named randiasaponin III.[23] Compounds **1** and **2** were peracetylated to form the known derivative **2a**.[22] Compound **3** was rapidly identified as ursolic acid after comparison of its NMR spectral data with literature.[7,8,16]

The HRFABMS of compound **1** (ion peak observed at *m*/*z* 970.5138) indicated the molecular formula $C_{49}H_{78}O_{19}$, representing a 42 amu increase from compound **2**. Acid hydrolysis of **1** afforded two sugars, identified as glucose and arabinose by co-TLC. The ^{1}H NMR spectrum of **1** (Table 1) displayed three anomeric proton signals (δ 4.68, d, J = 7.8 Hz; 5.06, d, J = 7.7 Hz and 6.30, d, J = 8.2 Hz). The ^{13}C NMR spectrum of **1** (Table 2) displayed 17 sugar signals between δ 60 and 107, allowing the identification of the sugar portion of **1** as two glucose residues and one arabinose unit. The ^3J H-1/H-2 value of each sugar residue gave evidence of their antiperiplanar orientation, reflecting the α-configuration of the L-arabinose moiety and the β-configuration of the two D-glucose residues. The ^{13}C NMR spectrum of **1** was very similar to that of **2**. Accordingly, compound **1** could be assigned as a pomolic acid derivative, with the presence of an acetyl group within the structure of **1** (^{13}C NMR δ CH_3 21.6, δ CO 170.0) being the sole difference from **2**. The ROESY spectrum of **1** displayed correlations between each axial anomeric proton and the coaxial H-3 and H-5 sugar protons. A HMBC correlation between the deshielded anomeric signal at δ 6.30 (glc-H1) and the carbonyl signal at δ 177.1 (C-28) confirmed the presence of a 28-*O*-β-D-glucopyranosyl ester also suggested from the shielded glc-1-anomeric carbon (δ 96.0). The HMBC experiment displayed the expected ara-H-1 (δ 4.68)/aglycon C-3 (δ 89.3) and glc-H-1 (δ 5.06)/ara-C3 (δ 81.4) correlations. To fully attribute each proton signal and to determine their coupling pattern with a high resolution (Table 1), a 1D SELTOCSY spectrum was obtained from each anomeric proton. This experiment allowed the unambiguous identification of the deshielded sugar signal at δ 6.02 as ara-H-2. Such a chemical shift for this proton, together with its correlation on the HMBC spectrum with the carbonyl at δ 170.0, allowed the definitive localization of the acetyl group at the position C-2 of the L-arabinose moiety. Hence, the structure of **1** was identified as 3β-*O*-{β-D-glucopyranosyl-(1→3)-[2-*O*-acetyl-(1→2]}-α-L-arabinopyranosyl pomolic acid 28-*O*-β-D-glucopyranosyl ester. This new compound has been named affinoside l.

Table 2. ^{13}C NMR Data of the Sugar Portions of Compounds **1**, **7a**, and **8a** in C_5D_5N[a]

	1		**7a**		**8a**
position	δ_C	position	δ_C	position	δ_C
	Ara		Glc		GlcI
1′	105.0	1′	92.5	1′	92.5
2′	72.4	2′	71.0	2′	71.0
3′	81.4	3′	73.6	3′	73.5
4′	69.7	4′	69.0	4′	69.0
5′	67.3	5′	73.1	5′	73.1
CH_3CO	21.6	6′	62.3	6′	62.3
	170.0				
	GlcI				
1″	106.7				
2″	74.8				
3″	78.6				
4″	71.7				
5″	78.7				
6″	63.0				
	GlcII				
1‴	96.0				
2‴	74.3				
3‴	79.1				
4‴	71.4				
5‴	79.5				
6‴	62.5				

[a] Assignments were confirmed by HSQC and HMBC experiments.

The ethanol extract of the leaves of *I. buxifolia* was suspended in water and extracted first with chloroform and then with *n*-butanol. Evaporation of the chloroform gave a dark green viscous material. After repeated column chromatographic purification, this afforded compounds **3** (50 mg), **4** (3.6 mg), **5** (48 mg), and **6** (98 mg). These compounds were identified after spectral data comparison with 28-nor-

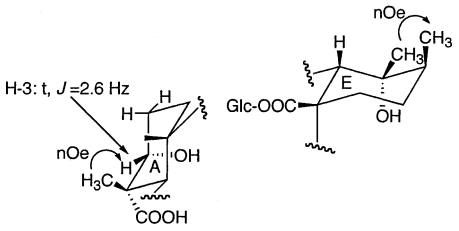

Figure 1. Selected ^{1}H NMR data observed for the A and E rings of **7a**.

ursolic acid (a sapogenin previously isolated from the fruits of *Symphoricarpus albus*[24]), 3β-acetylursolic acid (a sapogenin previously isolated from *Tripterygium hypoglaucum*[25]), and uvaol (a derivative previously isolated from *I. aquifolium*[26] and *I. latifolia*[27]), respectively.

The butanol fraction obtained after extraction of the aqueous suspension of the ethanol extract of *I. buxifolia* leaves afforded saponins **7** and **8**. After acid hydrolysis of **7** and **8**, glucose was the only sugar derivative that could be identified (co-TLC analysis). Peracetylation of **7** and **8** yielded **7a** and **8a**, respectively. The mass spectrum of **7a** and **8a** displayed an ion peak at *m/z* 897 $[M + Na]^+$, indicating their isomeric nature. The ^{1}H and ^{13}C NMR spectrum of **7a** and **8a** revealed characteristic signals for an ursolic-type aglycon[11] esterified by a glucose residue (**7a**: δ glc-H1 6.31, δ glc-C1 92.5; **8a**: δ glc-H1 6.28, δ glc-C1 92.5). For each compound, the location of the glucose moiety was determined to be at position C-28 of the aglycon after observation of a double correlation, in the HMBC spectrum, between the aglycon carbonyl atom at δ 176.4 (for **7a** and **8a**) and, on one hand, the glc-H-1 and, on the other hand, the aglycon H-18 (δ 3.04). Consequently, this also ascertained that the second carboxylic function (**7a**: δ 179.4; **8a**: δ 177.0) was not substituted. Compounds **7a** and **8a** were also shown to both belong to the 20*S* series from the characteristic E ring ^{13}C NMR chemical shift.[16] Indeed, in both cases, the chemical shift of C-18, assigned unambiguously using a combination of HMQC and HMBC experiments, at δ 47.3 and 47.2, respectively, versus δ 54.6 in the case of **1**, helped fix the proposed C-20 stereochemistry, which was fully consistent with the observation of a correlation in the ROESY spectrum between CH_3-29 (δ 1.37) and CH_3-30 (δ 0.96) (Figure 1). The discrepancies between the ^{13}C NMR spectrum of **7a** and **8a** mainly concerned the aglycon C-3−C-5 sequence. Of main importance was the chemical shift of C-5 (**7a**: δ 51.3; **8a**: δ 57.0). The γ-effect-shielded resonance of C-5 in the case of **7a** helped assign the *S*-configuration of C-4,[28] so therefore the remaining carboxyl group was at the C-23 position. The ^{1}H NMR spectrum of **7a** displayed also an unusual feature: the aglycon H-3 signal appeared as a triplet ($J = 2.6$ Hz). This suggested an equatorial orientation for this proton that was also confirmed by observation of a ROESY correlation between H-3 and CH_3-24 (Figure 1). On this basis, **7a** was identified as the peracetylated derivative of the 28-*O*-β-D-glucopyranosyl ester of (20*S*)-3α,19α-dihydroxyurs-12-ene-23,28-dioic acid and **8a** as the peracetylated derivative of the 28-*O*-β-D-glucopyranosyl ester of (20*S*)-3β,19α-dihydroxyurs-12-ene-24,28-dioic acid. Compound **8** is hence the stereoisomer at the 20-position of ilexsaponin A1 previously identified from *I. pubescens*.[29]

Interestingly, aglycons with a OH-3α configuration have not been isolated before from the genus *Ilex*.[30] In addition, the data reported in this work help substantiate our previously proposed method of the detection of *I. paraguariensis* adulteration by analysis of maté crude triterpenoid glycoside content. No *Ilex* species presenting a saponin profile similar to that of *I. paraguariensis* has been found so far.

Table 3. Antitrypanosomal Activity [IC_{50} (μM)] of Selected *Ilex* Triterpenoids[a,b]

compound	*T. brucei*	*T. cruzi*
ursolic acid (**3**)	4	4
3β-*O*-acetylursolic acid (**5**)	17	>32
brevicuspisaponin 2	16	>32
E6	4	10
sumarin	0.08	
nifurtimox		0.39

[a] For protocols used, see Experimental Section. [b] Compounds E7, E8, brevicuspisaponins 1 and 2, ilexoside II (**2**) and its peracetylated counterpart (**2a**), matesaponin 1, matesaponins 3 and 4, pedunculoside, 3β-*O*-acetylursolic acid, and uvaol (**6**) exhibited IC_{50} >32 μM against both *T. brucei* and *T. cruzi*.

Since several triterpenoids possess antiparasitic activity, compounds **2**, **2a**, **3**, **5**, **6**, and matesaponin 1,[6] matesaponins 3 and 4,[7] 3-*O*-[β-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl]oleanolic acid (E6), oleanolic acid 3-*O*-[α-L-arabinopyranosyl-(1−2)-β-D-galactopyranosyl]-28-β-D-glucopyranosyl ester (E7), oleanolic acid 3-*O*-[β-D-glucopyranosyl-(1−2)-β-D-galactopyranosyl]-28-β-D-glucopyranosyl ester (E8),[14] pedunculoside,[13,15,17] and brevicuspisaponins 1 and 2[16] were examined for their in vitro antitrypanosomal activity (Table 3).[31,32] Derivative **3** and E6, which both have a free carboxyl group at position C-28, were found to be the most active compounds, suggesting that this kind of triterpenoid could be of interest in the design of a new class of antitrypanosomal derivatives.

Experimental Section

General Experimental Procedures. Melting points were obtained with a Köfler melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 241 polarimeter. IR spectra were recorded with a Perkin-Elmer 881 spectrophotometer. ^{1}H (500 MHz) and ^{13}C (125.7 MHz) NMR experiments were recorded on a Bruker AVANCE AMX 500 spectrometer equipped with a 5 mm multinuclear inverse probehead with *z*-shielded gradient. 1D-TOCSY selective excitation spectra were acquired using waveform generator-based GAUSS-shaped pulses, with mixing times ranging from 100 to 150 ms; tetramethylsilane was used as internal reference (δ 0). EIMS data were obtained on a MS50 spectrometer. Positive-ion FABMS were registered using a glycerol or poly(ethylene glycol) matrix and performed on a VG ZAB HS spectrometer. Column chromatography was performed over silica gel, and HPLC separations were carried out on a Bruker LC-21 system equipped with UV detection (λ 220 nm) and LiChroprep C_{18} 40−63 μm or CROM Nucleosid C_{18}, 5 μm columns. TLC was performed on silica gel F_{254} (Merck) plates and visualized using the vanilin-sulfuric acid reagent and heating.

Plant Material. Leaves of *I. affinis* and *I. buxifolia* were collected in Espírito Santo, Brazil, and Marliéria, Brazil, respectively. A herbarium specimen of each (*I. affinis*: MS 8242; *I. buxifolia*: MS 8287) are on deposit in the Herbarium of the Botany Department of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

Extraction and Isolation. Fresh leaves of *I. affinis* (190 g) were crushed and extracted with 2 L of EtOH at room temperature (3 × 7 days). The ethanolic extract was evaporated to dryness under reduced pressure to afford a green residue (18.3 g). A portion of this residue (8.0 g) was repeatedly chromatographed on a normal-phase silica gel column (40−60 μm) using $CHCl_3$−EtOH−H_2O (60:40:5) as eluent, giving enriched fractions containing **1**−**3** (85 mg, 1.4 g, 146 mg, respectively). HPLC purification of **1** and **3**, using MeOH−H_2O (2:1) as eluent, afforded 5.3 and 17.6 mg, respectively. Acetylation of an aliquot of **2** (20 mg) afforded **2a** (23

mg). Final RP-HPLC purification of **2** and **2a** was performed using a flow rate of 2 mL/min and as eluent CH_3CN-H_2O (40:60) for **2** (t_R 25 min) and 70:30 for **2a** (t_R 18 min). TLC analysis of **1**−**3** was achieved using as eluent $CHCl_3$−MeOH (96:4) for **2a** and **3** and $CHCl_3$−EtOH−H_2O (50:40:5) for **1** and **2**. After hydrolysis the sugar component was eluted by TLC analysis using EtOAc−MeOH−HOAc−H_2O (13:3:4:3).

The air-dried leaves of *I. buxifolia* (490 g) were ground and extracted with 4 L of EtOH at room temperature (3 × 7 days). The ethanolic extract was evaporated to dryness under reduced pressure to afford a green residue (45.3 g). Water was added to this residue, and the solution was extracted successively with chloroform and *n*-butanol. The chloroform fraction (13.2 g) afforded compounds **3** (50 mg), **4** (3.6 mg), **5** (48 mg), and **6** (98 mg). After repeated column chromatography [silica gel 40−60 μm using $CHCl_3$−EtOH−H_2O (60:40:5) as eluent], the *n*-butanol fraction (7.2 g) yielded glycosides **7** (18 mg) and **8** (55 mg). Acetylation of aliquots of **7** (18 mg) and **8** (55 mg), followed by column chromatography purification (silica gel 40−60 μm using a gradient from 2 to 5% of MeOH in $CHCl_3$), gave **7a** (8 mg) and **8a** (21 mg).

Affinoside 1 [3*β*-*O*-{*β*-D-glucopyranosyl-(1→3)-[2-*O*-acetyl-(1→2)]}-α-L-arabinopyranosyl pomolic acid 28-*O*-*β*-D-glucopyranosyl ester] (1): amorphous white powder; $[\alpha]_{589}^{20}$ +3° and $[\alpha]_{578}^{20}$ +15° (*c* 0.2, MeOH); IR (KBr) ν_{max} 3422, 2930, 1735, 1637, 1375, 1256, 1075 cm^{-1}; ^1H NMR and ^{13}C NMR, aglycon moiety data similar to those of the aglycon of **2**;[22,23] ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), sugar moiety, see Tables 1 and 2; FABMS *m/z* 993.4 $[M + Na]^+$; HRFABMS *m/z* $[M + H]^+$ calcd for $C_{49}H_{78}O_{19}$ 970.5138, found 970.5137.

Peracetylated 28-*O*-*β*-D-glucopyranosyl ester of (20*S*)-3α,19α-dihydroxyurs-12-en-23,28-dioic acid (7a): amorphous white powder; $[\alpha]_{589}^{20}$ +13° and $[\alpha]_{578}^{20}$ +20° (*c* 0.2, MeOH); IR (KBr) ν_{max} 3446, 2938, 1740, 1456, 1372, 1236, 1063 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz), aglycon moiety, δ 5.92 (1H, t, J = 2.6 Hz, H-3), 3.04 (1H, s, H-18), 1.76 (1H, m, H-5), 1.67 (3H, s, Me-27), 1.50 (3H, s, Me-24), 1.37 (3H, s, Me-29), 1.22 (3H, s, Me-25), 1.06 (3H, s, Me-26), 0.96 (3H, d, J = 6.9 Hz, Me-30); ^{13}C NMR (C_5D_5N, 125 MHz), aglycon moiety, δ 35.1 (C-1), 24.6 (C-2), 74.5 (C-3), 47.3 (C-4), 51.3 (C-5), 20.7 (C-6), 31.4 (C-7), 40.4 (C-8), 47.1 (C-9), 38.2 (C-10), 24.2 (C-11), 128.2 (C-12), 138.3 (C-13), 42.4 (C-14), 29.3 (C-15), 26.8 (C-16), 48.6 (C-17), 47.3 (C-18), 73.3 (C-19), 42.9 (C-20), 24.8 (C-21), 31.7 (C-22), 179.4 (C-23), 24.4 (C-24), 13.6 (C-25), 17.5 (C-26), 24.0 (C-27), 176.4 (C-28), 29.6 (C-29), 16.0 (C-30); ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), sugar moiety, see Tables 1 and 2; FABMS *m/z* 897 $[M + Na]^+$; HRFABMS *m/z* $[M + Na]^+$ calcd for $C_{36}H_{56}NaO_4$ 687.3719, found 687.3720.

Peracetylated 28-*O*-*β*-D-glucopyranosylester of (20*S*)-3*β*,19α-dihydroxyurs-12-en-24,28-dioic acid (8a): amorphous white powder; $[\alpha]_{589}^{20}$ +20° and $[\alpha]_{578}^{20}$ +20° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3432, 2968, 1757, 1452, 1369, 1227, 1035 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz), aglycon moiety, δ 4.90 (1H, dd, J = 12.0, 4.5 Hz, H-3), 3.02 (1H, s, H-18), 1.71 (3H, s, Me-27), 1.52 (3H, s, Me-23), 1.37 (3H, s, Me-29), 1.24 (3H, s, Me-25), 1.23 (1H, m, H-5), 0.97 (3H, s, Me-26), 0.96 (3H, d, J = 7.1 Hz, Me-30); ^{13}C NMR (C_5D_5N, 125 MHz), aglycon moiety, δ 39.2 (C-1), 24.9 (C-2), 80.1 (C-3), 48.3 (C-4), 57.0 (C-5), 20.8 (C-6), 34.0 (C-7), 40.2 (C-8), 47.4 (C-9), 38.1 (C-10), 24.2 (C-11), 128.0 (C-12), 138.4 (C-13), 42.3 (C-14), 29.2 (C-15), 26.8 (C-16), 48.6 (C-17), 47.2 (C-18), 73.3 (C-19), 42.9 (C-20), 24.7 (C-21), 31.7 (C-22), 24.3 (C-23), 177.0 (C-24), 13.9 (C-25), 17.3 (C-26), 24.1 (C-27), 176.4 (C-28), 29.6 (C-29), 16.0 (C-30); ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), sugar moiety, see Tables 1 and 2; ^1H NMR and ^{13}C NMR see Table 2; FABMS *m/z* 897 $[M + Na]^+$; HRFABMS *m/z* 687.3719 $[M + Na]^+$ (calcd for $C_{36}H_{56}NaO_4$, 687.3720).

Saponin Acetylation and Hydrolysis. These reactions were performed as previously described.[6]

Bioassays. The in vitro antitrypanosomal tests were performed according to the WHO procedure, using a common microdilution method in a 96-multiwell microtiter integrated system.[31,32] The antitrypanosomal activity was evaluated against *T. brucei* (strain S427) and *T. cruzi* (strain Tulahuen CL2).

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