Trypanocidal Withanolides and Withanolide Glycosides from *Dunalia brachyacantha*

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Two new withanolide glycosides, (20*R*,22*R*)-*O*-(3)-α-L-rhamnopyranosyl(1→4)-*β*-D-glucopyranosyl-1α,12*β*-diacetoxy-20-hydroxywitha-5,24-dienolide (**3**) and (20*R*,22*R*)-*O*-(3)-*β*-D-xylopyranosyl(1→3)-[*β*-D-xylopyranosyl(1→4)]-*β*-D-glucopyranosyl-1α-acetoxy-12*β*,20-dihydroxywitha-5,24-dienolide (**4**), were isolated from the leaves and root of *Dunalia brachyacantha*. Their aglycones, (20*R*,22*R*)-1α,12*β*-diacetoxy-3*β*,20-dihydroxywitha-5,24-dienolide (or 1α,12*β*-diacetyldunawithagenine) and (20*R*,22*R*)-1α-acetoxy-3*β*,12*β*,-20-trihydroxywitha-5,24-dienolide (or 1α-acetyl-12*β*-hydroxydunawithagenine), are novel. The known 18-acetoxywithanolide D (**1**) and 18-acetoxy-5,6-deoxy-5-withenolide D (**2**) were also isolated from the leaves. These last two compounds were shown to be responsible for the trypanocidal, leishmanicidal, and bactericidal activities manifested by the crude ethanolic extract. The structures were deduced from spectroscopic data and on the basis of chemical evidence.

The withanolides are steroidal lactones that are mainly distributed in the different genera of the family Solanaceae. The first withanolide glycosides, named dunawithanines, have been isolated from *Dunalia australis* (Griseb.) (syn. *Iochroma australe* and *Acnistus australis*).[1] In the course of screening extracts from Bolivian plants against *Trypanozoma cruzi*, *Leishmania* spp., *Bacillus subtilis*, and *Staphylococcus aureus*, *Dunalia brachyacantha* (Griseb.) Sleumer was found to be active. *D. brachyacantha* is a shrub known for its medicinal uses to alleviate stomach ache among the Raqaypampeños, an ethnic group settled in the Mizque province of Cochabamba in Bolivia. In a preliminary pharmacological study, the leaf extract of this species displayed moderate antimalarial activity in vivo and good activity in vitro.[2] Withaferin A has been previously reported from this species,[3] and the leaves and flowers of specimens native to Argentina have yielded several new witha-2,24-dienolides and 18,20-hemiacetal-type derivatives.[4] The bioassay-guided purification of the leaf extract led to the isolation and identification of two known acetoxywithanolides (**1** and **2**), which displayed antiparasitic and antimicrobial activity. We have also isolated two new inactive withanolide glycosides named dunawithanines G (**3**) and H (**4**) from leaves and root, respectively. The structures of these four compounds were established by spectroscopic methods, mainly NMR and MS techniques.

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Results and Discussion

The dried leaves of *D. brachyacantha* were extracted with aqueous ethanol, and the crude extract was submitted to in vitro bioassays against Gram-positive bacteria, *Leishmania* and *Trypanosoma cruzi* strains. The last stages in the purification of this active extract were monitored by means of in vitro test procedures on promastigote forms of *Leishmania* spp. and epimastigote forms of *Trypanosoma cruzi*, as previously described.[5] The aqueous residue obtained after evaporation of the alcohol was partitioned against methylene chloride, in which the highest activity was found. The organic layer was treated with a mixture of 10% aqueous methanol and petroleum ether. The lower aqueous layer, which retained the highest activity, was fractionated by chromatography on LH-20 Sephadex gel. Two known bioactive compounds, 18-acetoxywithanolide D (**1**) and 18-acetoxy-5,6-desoxy-5-withenolide D (**2**), were isolated and characterized by spectroscopic methods.[6] Although inactive, the Sephadex fractions, including compounds of higher molecular weight, were purified by vacuum liquid chromatography (VLC) on silica gel and yielded dunawithanine G (**3**) as the major component of the leaf crude extract.

The dried root of the same collection of *D. brachyacantha* was extracted with aqueous alcohol to yield an inactive crude extract. After separation by chromatography on silica gel, this extract afforded a second new withanolide glycoside, dunawithanine H (**4**).

Compounds **1** and **2** show IR absorptions due to the presence of an α,*β*-unsaturated six-membered lactone (1709 and 1705 cm^{-1}) and an α,*β*-unsaturated ketone (1694 and 1689 cm^{-1}). The ^1H NMR spectra (Table 1) displayed signals with chemical shifts and splitting patterns usually described for the steroidal structure of withanolide D.[7,8]

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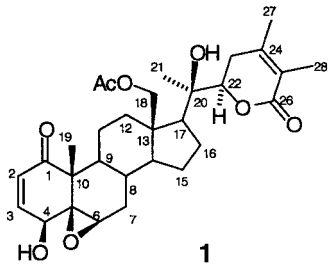
Table 1. ^{1}H NMR Data for Withanolides **1**−**4** and Peracetylated Derivatives **3a** and **4a**[a]

H	1	2	3	3a	4	4a
1e			4.85 t (2.3)	4.95 t (2.3)	5.01 brt ($W_{1/2}$=8)	4.91 brs
2e	6.20 d (10)	5.95 d (10.3)	2.14 dd (16. 2)		2.18 m	2.12 m
2a			1.75 dd (15. 3)		1.84 brt (13)	1.72 brt (12)
3a	6.94 dd (10, 5.8)	6.79 dd (10.5, 4.5)	3.87 m	3.80 m	3.89 m	3.71 m
4e	3.77 d (5.8)	4.63 d (4.5)	2.48 dd (14, 5.5)		2.53 dd (11, 4)	2.36 m
4a			2.27 t (13.3)		2.34 t (11)	2.18 m
6	3.25 brs ($W_{1/2}$=5)	5.92 d (4.6)	5.45 brdd (3.4, 1.4)	5.52 brd (3.4, 1.4)	5.54 brd (2.8)	5.48 brm ($W_{1/2}$=10)
7e	2.18 dt (15.2, 3)	2.12 dd (13, 4)	2.00 dd (13.3, 3.3)		2.01 brd (14)	2.03 m
7a	1.25 brt (13)		2.27 t (13.3)		2.35 t (12)	
8a	1.66 m		1.48 m		1.43 m	
9a			1.52 m		1.44 m	1.46 m
11e			1.55 dd (13.3, 3.3)		1.57 dm (11)	1.57 m
11a			1.30 t (13.3)		1.40 t (12)	1.38 m
12a			4.57 dd (10.3, 4)	4.63 dd (10.3, 4)	3.79 dd (11.2, 2)	4.61 dd (10, 5)
14a			1.05 m		1.08 m	
17a			1.75 m		1.88 m	1.74 m
18	4.15 d (11.6)	4.21 d (11.8)	0.91 s	0.98 s	0.81 s	0.96 s
18'	4.11 d (11.5)	4.16 d (11.6)				
19	1.40 s	1.44 s	1.03 s	1.06 s	1.09 s	1:03 s
21	1.38 s	1.42 s	1.18 s	1.26 s	1.20 s	1.24 s
22a	4.25 dd (13.3, 3.6)	4.25 dd (13.3, 3.6)	4.36 dd (13.3, 3.5)	4.31 dd (13.3, 3.5)	4.68 dd (13.2, 3)	4.32 dd (13, 3.4)
23e	2.08 brd (16)	2.11 dd (16, 2)	2.09 dd (16.6, 4)		2.22 dd (14, 3)	2.02 m
23a	2.40 brt (16)	2.40 brt (16)	2.45 t (16.6)		2.63 brt (14)	2.39 t (15)
27	1.89 s	1.89 s	1.81 s	1.88 s	1.87 brs	1.87 s
28	1.95 s	1.96 s	1.91 s	1.93 s	1.97 brs	1.92 s
C_1OAc			2.02 s		2.05 s	
C_{12}OAc			1.91 s			
C_{18}OAc	2.05 s	2.06 s				

H	3	3a	4	4a
glucose				
1	4.34 d (7.6)	4.57 d (7)	4.39 d (7.8)	4.38 d (8)
2	3.21 t (8.3)	4.80 dd (8.7, 7)	3.40 t (7.5)	4.85 t (8.2)
3	3.44 m	5.18 t (9)	3.80 m	3.87 brt (8.6)
4	3.51 m	3.83 t (9.3)	3.80 m	3.69 t (8.9)
5	3.28 m	3.58 m	3.34 m	3.49 ddd (9, 5, 2)
6	3.62 dd (11.9, 4.9)	4.27 dd (11.7, 3.3)	3.80 dd (14.0, 4.0)	4.02 dd (12.1, 5)
6'	3.73 dd (11.9, 1.7)	4.41 dd (11.7, 2.7)	3.88 brd (14)	4.38 dd (12, 2)
rhamnose			**xylose**	
1	4.81 d (1.6)	4.80 d (1.6)	4.56 d (6.5)	4.70 d (7.2)
2	3.82 dd (3.2, 1.9)	5.03 m	3.39 m	4.86 dd (9.1, 7.2)
3	3.60 dd (9.3, 3.6)	5.18 dd (9.3, 3.3)	3.34 m	5.12 t (9.1)
4	3.39 t (9.5)	5.06 t (9.3)	3.52 m	4.99 ddd (9.1, 7, 5)
5	3.83 qd (9.4, 6.6)	3.83 m	3.25 dd (12.1, 10)	3.42 dd (12.3, 7.1)
6	1.26 d (6.1)	1.16 d (6)		
5'			3.98 dd (11.5, 4.5)	4.28 dd (12.2, 4.9)
			xylose'	
1			4.84 m ($W_{1/2}$=8)	4.59 d (6.2)
2			3.44 m	4.92 dd (8.8. 6)
3			3.44 m	5.13 t (8.5)
4			3.56 m	4.97 td (9, 4)
5			3.34 dd (11.5, 12)	3.33 dd (12, 8.7)
5'			4.06 dd (12.1, 5.1)	4.21 dd (11.7, 5.3)

[a] **1**, **2**, **3a**, and **4a** in $CDCl_3$, **3** in $CDCl_3$−CD_3OD, 9:1, **4** in CD_3OD; coupling constants (*J*) in Hz.



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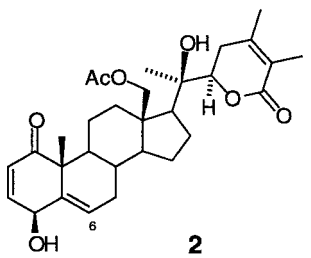

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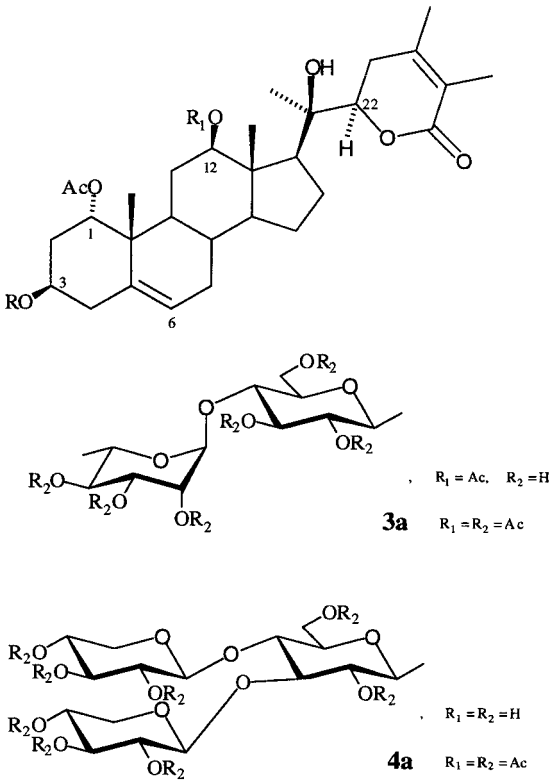
The ^{13}C NMR spectra were in agreement with those previously described for the 18-acetoxywithanolide D (**1**) and 18-acetoxy-5,6-deoxy-5-withenolide D (**2**) (Table 2).[6] Confirmation of these structures and full proton and carbon assignments (Tables 1 and 2) were made possible by analyses of COSY H−H, HMQC, and HMBC experiments. The *β*-OH orientation at C-4 was confirmed by a cross-peak observed between H-4α and H-6 in the ROESY experiment, as previously described for the (17*S*,20*R*,22*R*,24*S*,25*R*)-4*β*-acetyloxy-5*β*,6*β*:18,20-diepoxy-18-hydroxy-1-oxowitha-2-enolide.[4] A ROE cross-peak between $-CH_2$-18 and CH_3-21 allowed establishment of the 17*β*-orientation of the side chain. Observation of a characteristic positive Cotton effect at 240 nm in the CD spectra of **1** and **2** confirmed the 22-*R* configuration in these withanolides.[9]

Highly polar compounds **3** and **4** exhibited NMR signals for di- and triosidic glycosylated withanolides, respectively (Tables 1 and 2), but with the same aglycone, 1α-acetoxy-3*β*,12*β*,20-trihydroxywitha-5,24-dienolide, which can be

Table 2. ^{13}C NMR Data for Withanolides **1**−**4** and Peracetylated Derivative **4a**[a]

C	1	2	3	4	4a	C	3	4	4a
1	202.3	203.5	75.4	75.0	74.4				
2	132.1	129.0	33.8	33.2	33.6		glucose		
3	142.0	142.9	73.8	73.4	74.5	1	101.1	100.6	99.3
4	69.8	69.4	37.9	37.4	38.1	2	73.6	73.5	73.2
5	63.8	139.0	137.0	136.5	136.9	3	75.6	72.0	76.6
6	62.5	130.5	124.6	124.2	124.1	4	79.1	75.4	74.6
7	31.5	31.1	31.4	30.8	31.2	5	75.2	77.1	73.2
8	29.6	32.5	30.3	30.2	30.0	6	61.1	60.5	62.2
9	44.2	42.9	41.0	41.2	40.8				
10	48.0	49.3	40.5	40.3	40.2		rhamnose	xylose	
11	22.1	22.8	27.0	28.2	29.6	1	101.9	101.9	100.2
12	34.9	35.1	81.0	79.7	80.2	2	71.1	72.3	70.8
13	45.2	45.4	47.2	47.6	47.2	3	71.2	75.3	72.1
14	56.2	56.4	56.1	54.9	56.0	4	72.6	69.1	69.4
15	23.6	23.7	23.4	24.1	23.3	5	69.7	65.3	62.4
16	21.8	21.7	23.3	22.8	23.2	6	17.4		
17	54.9	54.9	55.2	61.4	55.1			xylose′	
18	61.7	61.9	10.1	8.3	9.9	1		102.1	100.2
19	17.6	22.8	19.3	19.0	19.2	2		71.8	71.3
20	74.7	74.8	74.9	73.9	74.8	3		74.9	71.7
21	21.2	21.2	21.0	23.8	21.5	4		69.0	69.5
22	80.0	80.9	81.2	78.4	80.6	5		64.8	62.7
23	31.3	31.6	31.5	32.6	31.2				
24	149.0	148.5	150.5	150.3	148.6				
25	122.0	122.1	121.7	121.2	122.0				
26	166.0	165.8	167.3	167.3	165.8				
27	12.3	12.5	12.3	11.9	12.4				
28	20.5	20.6	20.6	20.2	nd				
C_1OAc			171.1	170.5	170.2				
C_1OAc			21.0	20.7	nd				
$C_{12}OAc$			170.6		170.0				
$C_{12}OAc$			21.7		21.6				
$C_{18}OAc$	171.0	171.1							
$C_{18}OAc$	20.4	21.2							

[a] **1**, **2**, and **4a** in $CDCl_3$, **3** in $CDCl_3$−CD_3OD, 9:1; nd: not determined.



also named 1α-acetyl-12β-hydroxydunawithagenine[10] or 12β-hydroxyphysalolactone B.[1,11] In compound **3**, this genin is acetylated at position 12, thus corresponding to the new 12β-acetoxyphysalolactone B.[12] The positive FABMS spectrum of **3** showed an ion $[M + Li]^+$ at *m*/*z* 873, analyzed for $C_{44}H_{66}O_{17}Li$. The loss of the sugar moiety yielded the ion at *m*/*z* 563, corresponding to the lithiated aglycone, thus analyzed for 556 amu ($C_{32}H_{44}O_8$). The ^1H NMR spectrum of **3** displays signals for an ethylenic proton (δ 5.45, brdd, J = 3.4, 1.4 Hz, H-6), a CHOH group (δ 3.87, m, H-3), the H-22 signal of the lactone (δ 4.36, dd, J = 13.3, 3.5 Hz) characteristic for the corresponding protons in the series, and two CHOAc groups (δ 4.85, t, J = 2.3 Hz, H-1 and δ 4.57, dd, J = 10.3, 4 Hz, H-12). Compound **4** exhibits NMR spectra similar to those of **3**, with the major differences due to the absence of an acetate group at C-12. In **4**, H-12 resonates at δ 3.79 (Δ = −0.78 ppm) and C-12 resonates at δ 79.7 (Δ = −1.3 ppm). Also in **4**, only carbonyl signals are observed for the δ-lactone (δ 167.3) and the C-1 acetate (δ 170.5) (Tables 1 and 2). The flat structure of the aglycone as well as full proton and carbon assignments were established by analyses of the 2D NMR experiments (COSY, HMQC, and HMBC) (Table 3). Proton H-12 is in the α-axial position based on its large diaxial coupling constant value (J = 10.3 Hz). The β-equatorial orientation of H-1 was suggested by the small coupling constants with the two H-2 protons. This was confirmed by the observation of an Overhauser effect between H-1, CH_3-19, and H-11eq (δ 1.55) (Table 3). Methine H-3 (δ 3.87) was deduced to be in an α-axial orientation on the basis of comparison of its chemical shift with those reported for similar derivatives.[1,11,12] The close chemical shift values for C-18 and C-21 precluded observation of clear-cut ROEs between these methylic groups and, hence, made it difficult to ascertain configuration of the orientation of the side chain at C-17 in **3** and **4**. The establishment of the configuration of C-17 in withanolides was the subject of an article by Gottlieb, but its deductions were based on the comparison of α and β 17-hydroxylated compounds.[13] In our case, we consider

Table 3. HMBC and ROESY Correlations for Dunawithanine G (**3**)[a]

H	HMBC correlation	ROESY correlation
1	C-5, C-10, (Me*C*=O)OC_1	H-2e, H-2a, H-11e, H-19
2e		H-1, H-2a
2a		H-19, H-4a, H-2e
3		H-4a, H-4e, H-1 glc
4e	C-2, C-3, C-5, C-6, C-10	H-3, H-4a, H-6
4a		H-2a, H-19
6	C-4, C-8, C-10	H-4e, H-7e
7		H-6
11e		H-11a, H-1
11a		H-11e
12	C-13, C-17, C-18, (Me*C*=O)OC_{12}	
16a		H-21
18	C-12, C-13, C-14, C-17	
19	C-1, C-5, C-9, C-10	H-1, H-2a, H-4a
21	C-17, C-20, C-22	H-16a
22	C-26	
23e		H-23a
27	C-24, C-25, C-26	
28	C-23, C-24, C-25	
C_1O*Ac*	(Me*C*=O)OC_1	
C_{12}O*Ac*	(Me*C*=O)OC_{12}	
glucose		
1	C-3, C-2 glc, C-5 glc	H-3, H-2 glc, H-3 glc, H-5 glc
2	C-1 glc	H-4 glc, H-1 glc
3		H-1 glc, H-1 rha
4	C-1 rha	H-1 rha, H-2 glc
5		H-1 glc
6		H-6′ glc
6′		H-6 glc
rhamnose		
1	C-2 rha, C-3 rha, C-5 rha	H-3 glc, H-4 glc, H-2 rha
2		H-1 rha, H-4 rha
3		H-4 rha, H-5 rha
4	C-5 rha, C-6 rha	H-2 rha, H-3 rha, H-6 rha
5	C-4 rha	H-3 rha, H-6 rha
6		H-4 rha, H-5 rha

[a] a = axial, e = equatorial.

that the low variation of chemical shifts for C-14 and C-15 is an argument in favor of the β-orientation of the side chain at C-17 within the series of compounds **1**−**4**. The configuration at C-20 in **3** and **4** was proposed to be 20*R* on the basis of the chemical shifts of H-21 in **3**, **4**, and previously reported 20-hydroxycholesterol derivatives.[4,7,14] The 22*R* configuration was defined by the positive Cotton effect at 250 nm (**3**) and 248 nm (**4**).[9]

Thus, the genin of **3** is the (20*R*,22*R*)-1α,12β-diacetoxy-3β,20-dihydroxywitha-5,24-dienolide, and that of **4** is (20*R*,22*R*)-1α-acetoxy-3β,12β,20-trihydroxywitha-5,24-dienolide, previously reported as the aglycone of dunawithanine F.[10] Identification of these aglycones as derivatives of dunawithagenine[11] permitted adoption of the stereochemical configuration defined by crystallographic methods for the natural dunawithagenine.[15]

The ^{1}H NMR spectrum of glycoside **3** shows two anomeric signals at δ 4.34 (d, J = 7.6 Hz) and δ 4.81 (d, J = 1.6 Hz) and a methyl doublet at δ 1.26 (J = 6.1 Hz), which correlates in the COSY spectrum to an osidic proton at δ 3.83 (qd, J = 9.4, 6.6 Hz), thus suggesting the presence of a 6-desoxyhexose. The *J*-modulated ^{13}C NMR spectrum of **3** shows 10 carbons between δ 61 and 79, nine oxymethines, and one oxymethylene, along with a methyl signal at δ 17.4, confirming the presence of one hexose and one 6-desoxyhexose in the osidic part of **3**. The determination of the nature of these two sugars was done by analyses of COSY and HOHAHA experiments. The sugar identity was confirmed by comparison with the spectra of the peracetylated derivative **3a** (Tables 1 and 2). One terminal α-L-rhamnose and a monosubstituted β-D-glucose constitute the chain, which is linked to position 3 of the aglycone, as demonstrated by the observation of a ROE between H-1 of the glucose and H-3 of the genin (Table 3). Complete assignments of the osidic carbons of **3** and **3a** were possible from analyses of the HMQC correlations. The sugar sequence was established as [rhamnose(1→4)glucose] since in the HMBC spectrum of **3** H-1 of rhamnose was ^3J correlated with C-4 of the glucose (Table 3) and H-4 of the glucose was not deshielded by acetylation (δ 3.83, t, J = 9.3 Hz). Hence, compound **3** is (20*R*,22*R*)-*O*-(3)-α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl-1α,12β-diacetoxy-20-hydroxywitha-5,24-dienolide.

The LSIMS (with NBA/LiI) spectrum of **4** showed two ions at *m/z* 965 ([M + Na]$^+$ analyzed for $C_{46}H_{70}O_{20}Na$) and at *m/z* 949 ([M + Li]$^+$; $C_{46}H_{70}O_{20}Li$). In the ^{1}H NMR spectrum of **4**, three signals for anomeric protons of three sugar residues were detected at δ 4.39 (d, J = 7.0 Hz, glc), 4.56 (d, J = 6.5 Hz, xyl), and 4.84 (m, $W_{1/2}$ = 8.0 Hz, xyl′). The signals correlated in the HMQC experiment to three anomeric carbons at δ 100.6 (glc), 101.9 (xyl), and 102.1 (xyl′). In the *J*-modulated carbon spectrum, three oxymethylenic carbons appear from δ 60.5 to 65.3, supporting the presence of one hexose and two pentoses, which were identified as one β-D-glucose and two β-D-xyloses by COSY and HOHAHA experiments (Table 1). The complete NMR assignments of glycoside **4** were made possible by analyses of the 2D NMR spectra of the acetylated derivative **4a**. The observation of signals shifted to high frequencies for nonanomeric protons of xyloses in **4a** proved that these units are terminal (Table 2). Similarly, the observation of the displacement of H-3 (δ 3.87 in **4a** compared to δ 3.80 in **4**) and of H-4 (δ 3.69 instead of δ 3.80) of the diacetylglucose demonstrated that the sugar sequence is β-D-xylopyranosyl(1→3)-[β-D-xylopyranosyl(1→4)]-β-D-glucopyranose. Thus, withanolide **4** is (20*R*,22*R*)-*O*-(3)-β-D-xylopyranosyl(1→3)-[β-D-xylopyranosyl(1→4)]-β-D-glucopyranosyl-1α-acetoxy-12β,20-dihydroxywitha-5,24-dienolide.

The analyses of tandem mass spectra (MS/MS) of these glycosides and of their acetylated derivatives support the NMR results and the proposed structures for **3** and **4**. In the lithiated positive FAB spectrum of **3**, the loss of the terminal rhamnose is evidenced by ions at *m/z* 726 [M + Li − rhamnose]$^+$ and at *m/z* 720 [M + H − rhamnose]$^+$. The detection of ions at *m/z* 593 and 755 is produced by two cleavages in the osidic cycle to form the $^{1,5}X_{glc}$ and $^{1,5}X_{rha}$ ions, respectively, and thus confirm the glucose-rhamnose sugar moiety. The retro-Diels−Alder fragmentation of cycle B in the aglycone part and the loss of its side chain produced ions at *m/z* 369 ($C_{21}H_{30}O_5Li$) and 169 ($C_9H_{13}O_3$). The MS/MS fragmentation of the [M + Li]$^+$ ion of compound **3a** (*m/z* 1125, $C_{56}H_{78}O_{23}Li$) yields ions at *m/z* 881 ($^{1,5}X_{rha}$) and 593 ($^{1,5}X_{glc}$), confirming the osidic sequence as terminal rhamnose-glucose. Another major ion was detected at *m/z* 777 ([M + Li − $C_{12}H_{17}O_8$ − OAc]$^+$), which can be attributed to the loss of the terminal rhamnose and acetic acid conjointly. Bond cleavages of the osidic linkage at C-3 produced ions at *m/z* 563 ([aglycone + Li]$^+$), 547 ([aglycone + Li − O]$^+$), and 585 ([disaccharide + Li]$^+$), and the loss of two acetic acid units from *m/z* 547 produced the ion at *m/z* 427.

The MS/MS spectrum of **4** ([M + Na]$^+$ at *m/z* 965, $C_{46}H_{70}O_{20}Na$) showed a major ion at *m/z* 905 ([M + Na − AcOH]$^+$). The other fragments involving the aglycone were detected at *m/z* 479 ([aglycone + Na − AcOH]$^+$) and 461 ([aglycone + Na − AcOH − H_2O]$^+$), confirming the formula for the aglycone as $C_{30}H_{44}O_7$. The ion at *m/z* 869

Table 4. Antiparasitic and Antibacterial Activities of 18-Acetoxywithanolide D (**1**) and 18-Acetoxy-5,6-deoxy-5-withenolide D (**2**)

		strains[a]			
compd	conc[b]	*Bs*	*Sa*	*Ec*[e]	*Sf*[e]
1	1.0	+	+		
	0.5	+	+		
	0.25	+	+		
	0.125	+	+		
	0.0625	+	−		
	0.0312	−	−		
2	1.0	+	+	−	−
	0.5	+	+	−	−
	0.25	+	+	−	−
	0.125	+	+	−	−
	0.0625	+	−	−	−
	0.0312	−	−	−	−

compd	conc[c]	*Tc*[d]	*Lb*[d]	*La*[d]	*Ld*[d]
1	50	++	+++	+++	+++
	25	0	+++	+++	+++
	10	0	++	+	+
2	50	+++	+++	+++	+++
	25	+++	+++	+++	+++
	10	+	+++	+++	+++
	1	0	++	+	+
	0.1	0	0	0	0
pentamidine	10	+++	+++	+++	+++

[a] *Bs* = *Bacillus subtilis*, *Sa* = *Staphylococcus aureus*, *Ec* = *Escherichia coli*, *Sf* = *Shigella flexneri*, *La* = *Leishmania amazonensis*; *Lb* = *Leishmania braziliensis*; *Ld* = *Leishmania donovani*; *Tc* = *Trypanosoma cruzi*. [b] In mg/mL. [c] In μg/mL. [d] 0: number of epimastigotes or promastigotes identical to control; +: 75% epimastigotes or promastigotes, with few degenerative forms; ++: 50% epimastigotes or promastigotes, with few degenerative forms; +++: total lysis of parasites. [e] Not tested for **1**.

($[M + Na]^+ - C_6H_8O$) results from a retro-Diels−Alder fragmentation of the δ-lactone ring. The spectrum exhibited a major product ion at *m*/*z* 781 resulting from the loss of the δ-lactone ring (125 uma) and an acetate residue.[16] The fragmentation pattern of the osidic part of compounds **4** and **4a** is characterized by the loss of the two terminal pentoses at *m*/*z* 667 (**4**) and 777 (**4a**) and the presence of $^{1,5}X_{xyl}$-type ions at *m*/*z* 861 and 1097, respectively. Similarly, the $^{1,5}X$-type cleavage in the glucosidic cycle yielded product ions at *m*/*z* 567 (**4**) and 593 (**4a**).

With the present report of the isolation of 18-acetoxywithanolides in *D. brachyacantha*, three Solanaceae species, including *Acnistus arborescens*[17] and *Iochroma fuchsioides*,[6] are now known to contain acetoxywithanolides. The in vitro activities of the known compounds **1** and **2** (Table 4) suggest that these two compounds are responsible for the observed antiprotozoal activity of the crude extract. This constitutes the first report of antileishmanial and antitrypanosomial (Chagas' disease) activities for steroidal lactones. The only reported bioactive withasteroids structurally close to **1** and **2** are 20-deoxywithanolide D, which is active against Gram-positive bacteria, withanolide D, which is cytotoxic and immunosuppressive, and withaferin A, which exhibits antimicrobial, cytotoxic, and immunostimulating activities.[18]

Experimental Section

General Experimental Procedures. UV spectra were recorded on a Hitachi Perkin-Elmer 200 and a PV 8720 Philips spectrometer. IR spectra were measured on a Beckman AccuLab 4 spectrometer. ^1H and ^{13}C NMR spectra were run with a Bruker AC-300 NMR spectrometer at 300 and 75 MHz, respectively, using $CDCl_3$ or CD_3OD or $CDCl_3-CD_3OD$ (9:1) as solvent. Two-dimensional NMR experiments were performed using standard Bruker microprograms. The EIMS, FABMS, and LSIMS were measured with a Autospec VG or a Zabspect-T spectrometer. CD spectra were taken in CH_3OH with a Micrograph Jobin Yvon spectrograph. Optical rotations were determined with a Perkin-Elmer 241 polarimeter.

Plant Material. *Dunalia brachyacantha* (Griseb.) Sleumer. in Hooks was collected at the Cota-Cota valley (3300 m above sea level) in La Paz, Bolivia, in August 1996. The plant was kindly authenticated by Dr. G. Bourdy, and a voucher specimen under GB 1770 was deposited at the National Herbarium of the San Andrés University in La Paz.

Parasites. The following strains were used: *Leishmania amazonensis* IFLA/BR/67/PH8, *Leishmania braziliensis* MHOM/BR/75/M 2903, and *Leishmania donovani* MHOM/IN/83/HS-70, for determination of leishmanicidal activity, and *Trypanosoma cruzi* strain Tulahuen for evaluation of trypanocidal activity. The strains were obtained from IBBA (La Paz, Bolivia), a WHO reference laboratory, and their identifications were confirmed by isoenzyme analysis.

Microorganisms. *Escherichia coli* ATCC-8739, *Shigella flexneri* ATCC-120222, *Staphylococcus aureus* ATCC-25923/6538, and *Bacillus subtilis* ATCC-6633 were used and obtained from the National Institute of Hygiene (Santiago, Chile).

Extraction and Isolation. Dried and powdered leaves (114 g) were percolated with EtOH−H_2O (70:30) for 21 days, renewing solvent (1.5 L) weekly. The hydroethanolic solution was evaporated and pooled. The extract was dissolved in H_2O and partitioned with CH_2Cl_2 (500 mL). The CH_2Cl_2 extract was partitioned between MeOH−H_2O (90:10) and petroleum ether, and the aqueous methanolic extract (4.78 g) showed the total lysis of *Leishmania* spp. parasites (TL*L*P at 10 μg/mL). The extract was chromatographed on Sephadex LH-20 using MeOH (975 mL) as solvent. Among the collected active fractions, fraction 3 (TL*L*P; 1.9 g) was purified by chromatography on a silica gel 60 H under VLC system, using CH_2Cl_2−MeOH mixtures of increasing polarity as eluent [100:0 (230 mL); 99:1 (300 mL); 98:2 (300 mL); 97:3 (300 mL); 96:4 (300 mL); 95:5 (300 mL); 90:10 (300 mL); 80:20 (300 mL); 0:100 (150 mL)]. Fractions 12 and 13 [CH_2Cl_2−MeOH (80:20)] afforded compound **3** (1018 mg, 0.89% of dried leaves).

Fractions 4 and 5 (1.07 g) from the Sephadex CC exhibited a TL*L*P activity and were chromatographed on a silica gel 60 (43 g) liquid CC to give 48 fractions eluted with mixtures of $CHCl_3$−MeOH [100:0 (86 mL); 99:1 (258 mL); 98:2 (176 mL); 97:3 (135 mL); 96:4 (135 mL); 94:6 (180 mL); 92:8 (180 mL); 88:12 (180 mL); 85:15 (135 mL); 80:20 (180 mL); 75:25 (0.5 L); 0:100 (0.5 L)]. Fraction 9 [$CHCl_3$−MeOH (97:3); 42.3 mg] and fraction 10 [$CHCl_3$−MeOH (97:3); 23.1 mg] were purified by preparative TLC on silica gel (500 μm) eluted with EtOAc−hexane (90:10 or 70:30). Pure compounds **1** [3.9 mg, 0.0034%; R_f 0.36 in EtOAc−hexane (90:10)] and **2** [15 mg, 0.013%; R_f 0.51 in EtOAc−hexane (90:10)] were obtained.

Dried powdered root (1409 g) was macerated for a week in 3 L of EtOH−H_2O (70:30). The hydroethanolic solution was evaporated and the aqueous layer lyophilized. The dried extract (2 g) was chromatographed over a silica gel 60 column (80 g) eluted with mixtures of $CHCl_3$−MeOH−H_2O [100:0:0 (3.3 L); 99:1:0 (1.4 L); 80:20:0 (2.4 L); 70:30:2 (8 L); 60:40:7 (0.4 L)]. Fractions 54−61 [$CHCl_3$−MeOH−H_2O (80:20:0)] yielded pure compound **4** (208 mg, 0.015% of dried root).

Acetylation of Glycosides. 3 (10 mg) was suspended in CH_2Cl_2 (1.5 mL) and stirred at room temperature for 50 h with Ac_2O (0.1 mL) and 4-(dimethylamino)pyridine (20 mg). CH_2Cl_2 was added to the reacting mixture, and the resulting mixture was rinsed with a $CuSO_4$ aqueous solution and dried with Na_2SO_4 to yield **3a** (9.2 mg). A similar procedure was applied to 30 mg of **4** (4.5 mL of CH_2Cl_2, 0.3 mL of Ac_2O, and 60 mg of 4-(dimethylamino)pyridine) to yield a mixture from which derivative **4a** was purified (28 mg).

Leishmanicidal Activity. In vitro test procedure on promastigote culture of *Leishmania* spp: compounds were aseptically dissolved in liquid medium and DMSO (final concentration of DMSO less than 0.1%) to obtain final concentrations of 50, 25, 10, 1, and 0.1 μg/mL. The solution was filtered

through a Millipore membrane (0.22 μm) and placed in Titertek 96 microcells (Flow Laboratories). All assays were done in triplicate. Each cell was cultured with 50 000 parasites/mL at 27 °C. The activity of the compounds was evaluated after 72 h by optical observation on a drop of culture with an inverted phase microscope, by comparison with control cells without extracts and with pentamidine-containing cells.

Trypanocidal Activity. In vitro procedure on the epimastigote form of *T. cruzi*: *T. cruzi* epimastigotes were cultured in LIT (liver infusion tryptose) medium supplemented with 10% fetal calf serum at 28 °C with an inoculum of 10^6 cells/mL. Samples (4 mg) were aseptically dissolved in 50 μL of DMSO and liquid medium to obtain final concentrations of 50, 20, 10, 1, and 0.1 μg/mL. All assays were carried out in triplicate. Final DMSO concentration was less than 0.5%. Parasites were counted after 48 h of contact with the samples in a haemocytometer, and the activity of the test substances was assessed by comparison with controls without extract and with pentamidine-containing cells.

Antibacterial Activity. The culture of microorganisms was performed in tubes with 3 mL of tryptic soy broth medium (30 g/L) at 37 °C for 18 h. Compounds were dissolved in a mixture of DMSO and water (1:1) to obtain final concentrations of 30 and 10 μg/mL. Petri dishes were prepared with 20 mL of tryptic soy broth medium (40 g/L) inoculated with 0.1 mL of test organisms (1 500 000 bacteria/mL). In all test plates, holes (Ø = 8 mm) were made and filled with 0.1 mL of solution of compound. Plates were incubated at 37 °C for 18 h. Diameters of inhibition were measured.

18-Acetoxywithanolide D (1): $[\alpha]^{25}_D$ +36° (*c* 0.27, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 242 (4.66) nm; IR ($CHCl_3$) ν_{max} 3443, 1732, 1709, 1680, 1250, 1132 cm^{-1}; CD $[\theta]_{240}$ +15300 (*c* 0.0012; $CHCl_3$); EIMS *m/z* 403 (4), 385 (5), 369 (14), 343 (100), 327 (12), 325 (16), 313 (6), 300 (7), 283 (8), 257 (7), 239 (11), 215 (34), 197 (9), 181 (12), 169 (58), 159 (16), 149 (75), 126 (62), 125 (59), 124 (97); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.

18-Acetoxy-5,6-deoxy-5-withenolide D (2): $[\alpha]^{25}_D$ +54° (*c* 0.94, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 242 (4.52) nm; IR ($CHCl_3$) ν_{max} 3437, 1738, 1705, 1694, 1254, 1128 cm^{-1}; CD $[\theta]_{240}$ +14 900 (*c* 0.0012; $CHCl_3$); EIMS *m/z* 512 (10), 494 (27), 452 (5), 434 (15), 387 (7), 385 (10), 369 (7), 327 (82), 311 (37), 309 (21), 291 (8), 282 (11), 265 (19), 251 (12), 237 (15), 223 (19), 211 (16), 195 (14), 185 (20), 169 (73), 159 (24), 150 (7), 145 (31), 133 (28), 126 (96), 125 (100); ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.

Dunawithanine G (3): mp 135−137°; $[\alpha]^{25}_D$ −2.5° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 206 (4.96) nm; IR ($CHCl_3$) ν_{max} 3391, 1734, 1714, 1692, 1248, 1130 cm^{-1}; CD $[\theta]_{227}$ −16 400, $[\theta]_{250}$ +14 800 (*c* 0.0012; MeOH); FABMS (positive, Gly+LiCl) *m/z* 873 $[M + Li]^+$, 755, 753, 593, 563, 547,427, 369, 251, 175, 169; FABMS (positive, NBA+LiCl) *m/z* 726 $[M + H - rhamnose]^+$, 720 $[M + Li - rhamnose]^+$, 558, 466, 304, 228, 169, 133; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.

Peracetylated dunawithanine G (3a): LSIMS (positive, NBA+LiI) *m/z* 1125 $[M + Li]^+$, 1065, 972, 895, 777, 661, 525, 446, 397, 355, 313, 244, 202, 160; FABMS/MS (positive) *m/z* 1125 $[M + Li]^+$, 1066, 1007, 941, 881, 835, 777, 691, 657, 593, 585, 563, 547, 487, 427, 251, 175, 111; ^{1}H NMR, see Table 1.

Dunawithanine H (4): mp 119−121°; $[\alpha]^{25}_D$ −5.3° (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (5.19) nm; IR ($CHCl_3$) ν_{max} 3401, 1712, 1658, 1256, 1120 cm^{-1}; CD $[\theta]_{222}$ −17 100, $[\theta]_{248}$ +5700 (*c* 0.0012, MeOH); LSIMS (positive, NBA+LiI) *m/z* 965 $[M + Na]^+$, 949 $[M + Li]^+$, 815, 736, 661, 617, 534, 455, 355, 319, 261, 207, 136; FABMS/MS (positive) *m/z* 965 $[M + Na]^+$, 907, 905, 875, 861, 781, 755, 667, 567, 537, 479, 467, 461, 317, 173; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.

Peracetylated dunawithanine H (4a): LSIMS (positive, NBA+LiI) *m/z* 1327 $[M + Li]^+$, 1217, 1015, 972, 883, 751, 661, 547, 446, 397, 313, 202, 160; FABMS/MS (positive) *m/z* 1327 $[M + Li]^+$, 1267, 1209, 1167, 1097, 1051, 979, 787, 777, 668, 593, 547, 511, 427, 390, 251, 175, 97; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2.

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