Mycobacterium tuberculosis Growth Inhibition by Constituents of *Sapium haematospermum*

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Four novel compounds consisting of two new pimaranes, lecheronol A (**1**) and lecheronol B (**2**), an acylated cycloartane, 3-*O*-*β*-lauroyl-cycloart-(23*E*)-en-25-ol (**10**), and a highly oxygenated novel chalconoid, α,*β*,3,4,5,2′,4′,6′-octahydroxydihydrochalcone (**12**), were isolated along with seven known triterpene derivatives and three flavonol glucosides from *Mycobacterium tuberculosis* growth-inhibiting fractions of the CH_2Cl_2/MeOH (1:1) extract of the aerial parts of *Sapium haematospermum*. Compounds **1**, **3** (3α-hydroxyolean-12-ene), **8** [3α-hydroxylup-20(29)-en], and **9** (cycloartanol) were found most active, with MIC values of 4, 12.2, 13.4, and 8 *μ*g/mL, respectively. Cytotoxicity tests in Vero cells for compounds **1**, **3**, **8**, and **9** gave IC_{50} values of 104.8, 127.2, 127.2, and 102.4 *μ*g/mL, respectively.

Sapium haematospermum Müll (Euphorbiaceae) is a plant native to drier regions of South America usually found growing as a small tree up to 5 m tall. In the locality where the plant was collected in northern Argentina, it is commonly known as "lecheryn" and is traditionally used in the treatment of teeth ailments. Some species of the genus *Sapium*, especially Chinese tallow (*Sapium sebiferum*), have been the focus of investigations owing to the potential of sapium fat as a substitute for cocoa butter.[1] Phytochemical investigations on other species in the genus have so far led to the identification of an anti-inflammatory alkaloid,[2] antihypertensive phenolic compounds,[3] toxic phorbol esters,[4] toxic cucurbitacins,[5] and hydrolyzable tannins and flavonoids.[6]

The CH_2Cl_2/MeOH (1:1) extract of the aerial parts of *S. haematospermum* was included in an initial screening carried out as part of an effort at discovery of bioactive agents from dryland biodiversity of Latin America under the International Cooperative Biodiversity Group (ICBG) program. In the present study, this extract was found active in inhibiting the growth of *Mycobacterium tuberculosis* and, thus, merited further chemical investigation. Below we describe compounds obtained from bioactive fractions of the extract and evaluation of their inhibitory effect on the growth of *M. tuberculosis*.

Results and Discussion

On discovery that the CH_2Cl_2/MeOH (1:1) extract of *S. haematospermum* inhibited growth of *M. tuberculosis* with a MIC value of 25 *μ*g/mL, bioassay-directed fractionation of the extract was undertaken as described in the Experimental Section. This resulted in the identification of 3α-hydroxyolean-12-ene (**3**),[7a] 3*β*-hydroxyolean-12-ene (**4**),[7b] 3α-hydroxyurs-12-ene (**5**),[7c] 2α,3*β*,23-trihydroxyolean-12-en-28-oic acid (**6**),[7d] 3*β*-hydroxylup-20(29)-ene (**7**),[7e] 3α-hydroxylup-20(29)-ene (**8**),[7f] cycloartanol (**9**),[8] stigmast-5-en-3-*O*-*β*-D-glucopyranoside (**11**),[9] kaempferol-3-*O*-*β*-D-glucopyranoside (**13**),[10] and quercetin-3-*O*-*β*-D-glucopyranoside (**14**)[10] as known compounds from bioactive fractions. Compounds **1**, **2**, **10**, and **12** were identified as novel on the basis of analysis of spectroscopic data.Compound **1** was isolated as a white powder from one of the bioactive fractions after extensive chromatographic purification. Its molecular composition, $C_{20}H_{30}O_3$, was then inferred from HRFABMS. The IR spectrum for this compound indicated the presence of a ketone and hydroxyl groups through characteristic absorptions. The combined analysis of its ^{13}C and DEPT NMR spectra then revealed the presence of 20 carbons assigned to four methyls, four methylenes, two methines, two quaternary carbons, two tertiary carbinols, one carbonyl, and four olefinic carbons. These, together with the molecular composition, suggested that **1** possessed 6 degrees of unsaturation. The presence of two *π*-bonds and a ketone function further indicated that the compound was a tricyclic diterpene. Olefinic methine and quaternary carbon signals observed in the ^{13}C NMR spectrum at *δ* 128.6 (C-14) and 137.6 (C-8), respectively, confirmed the presence of a trisubstituted double bond, while those at *δ* 144.5 (C-15) and 115.5 (C-16) in the ^{13}C NMR and at *δ* 5.94 (H-15), 4.90 (H-16a), and 5.03 (H-16b) in the ^1H NMR spectra made the presence of an exocyclic vinyl group readily apparent. All these indicated that compound **1** possessed a pimara-8(14),15-diene skeleton. Also identifiable in the ^1H and ^{13}C NMR spectra were, however, two signals at *δ* 3.99 (br s, H-1) and 3.29 (dd, J = 12.2 and 2.2 Hz, H-12), compatible with two hydroxy-bearing methines, and a signal ascribable to a ketone group at *δ* 211.1 (C-3). On the basis of extensive selective 1D-TOCSY experiments, the spin systems H_1-H_2, $H_5-H_6-H_7$, $H_9-H_{11}-H_{12}$, and $H_{14}-H_{15}-H_{16}$ were identified. Connections between the identified spin systems were then established through analysis of the HMBC spectrum as shown in Table 1. In addition, $^3J_{C,H}$ correlations between one of the methyls in the *gem*-dimethyl group ($C_{18}-C_4-C_{19}$, also identified using the HMBC spectrum) and both C_3 and C_5, between H_{16} and C_{13}, between H_{15} and C_{17}, and between H_{14} and both C_{17} and C_7 enabled the specific location of groups that were

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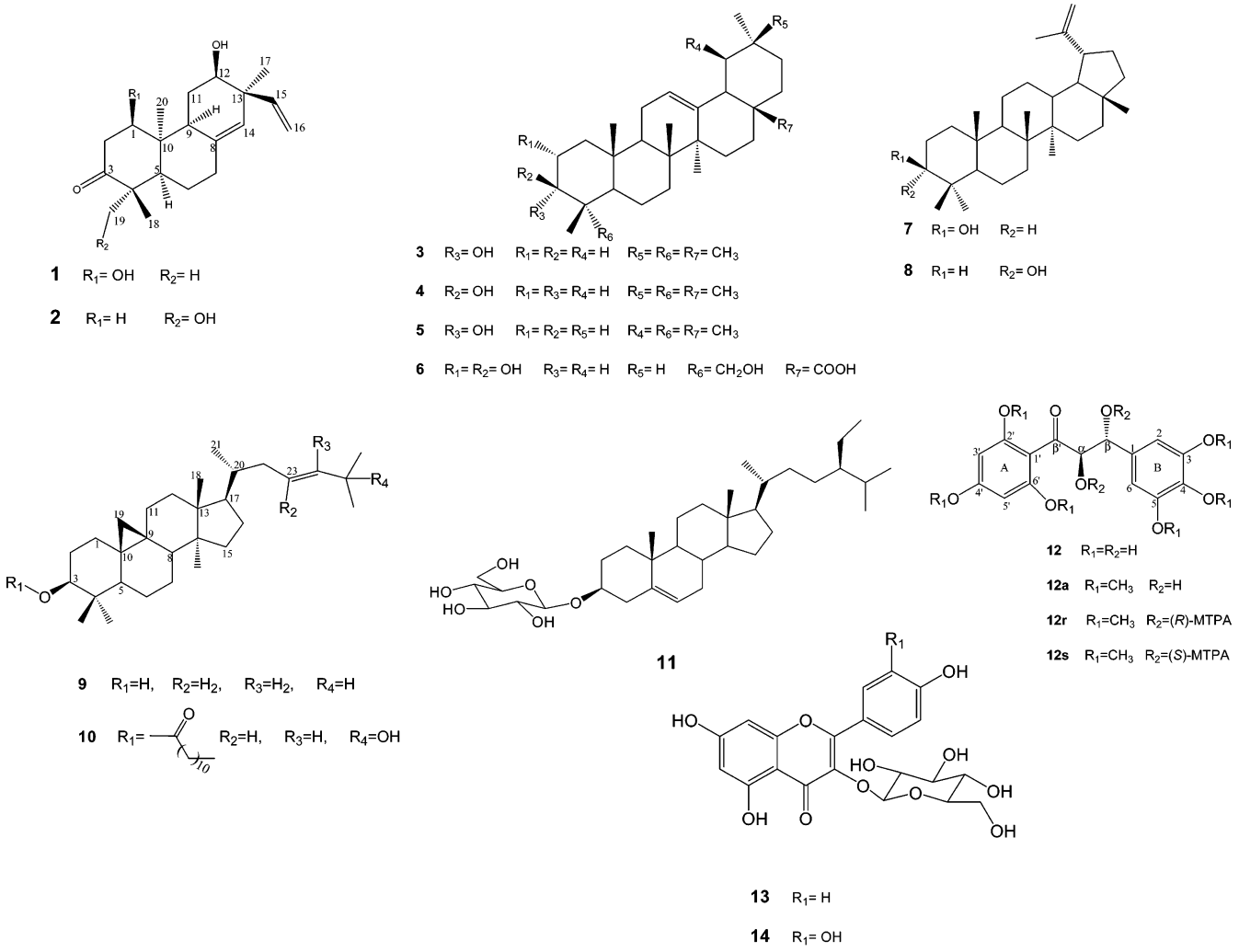
Chart 1



not readily identifiable as part of spin systems already identified from the 1D-TOCSY experiments. A β-equatorial orientation for the vinyl group at C-13 was determined on the basis of the characteristic ^{13}C chemical shifts for C-15, C-16, and C-17 and multiplicities observed for H-15, H-16, and H-17 and a comparison of these with those of related compounds.[11−13] The observation of NOE effects at H_5, $H_{2\alpha}$, H_{19}, and H_{20} on irradiation of H_1, at H_9, $H_{11\alpha}$, and H_{17} on irradiation of H_{12}, and at H_1, H_{20}, and H_9 on irradiation of H_5 in selective 1D-NOE experiments then clearly established the relative stereochemistry of the chiral centers and also revealed an A/B *cis* (5α,20α) ring fusion.[13,14] The structure of compound **1** was, therefore, established as 1β,-12β-dihydroxy-(5α,9α,20α)-13-*epi*-pimara-8(14),15-dien-3-one and has been named lecheronol A.

The HRFABMS of compound **2** gave the same molecular composition as with **1**, $C_{20}H_{30}O_3$. However, a quick comparison of its ^1H NMR spectrum with that of compound **1** revealed the absence of the methine signal seen in **1** at around 4 ppm and the appearance, in **2** instead, of a pair of doublets at δ 3.67 (d, J = 10.8 Hz, 1H) and 4.21 (d, J = 10.8 Hz, 1H). Both these protons correlated with a ^{13}C signal at δ 63.2 in the HSQC NMR spectrum that was identified as a methylene signal from both the HSQC and DEPT-135 NMR spectra. These facts confirmed a geminal relationship between these two protons and helped identify the correlating ^{13}C signal as an oxymethylene, while also suggesting hydroxyl substitution at one of the methyl groups. This conclusion is in agreement with the observation of just three methyl resonances at δ 21.8, 25.0, and 25.6 in the ^{13}C and at δ 1.10, 1.10, and 1.14 in the ^1H NMR spectra in **2** compared to four methyl resonances in **1**. The HMBC spectrum further indicated that this oxymethylene group is attached at the C_4 position in either the β- (C_{18}) or α-position (C_{19}). A decision in favor of C_{19} followed from observation of NOE effects at H_5 and H_{20} on irradiation of both H_{19a} and H_{19b} in a selective 1D-NOE experiment. A signal at δ 3.45 (dd, J = 12.0, 3.5 Hz) in the ^1H NMR spectrum indicated equatorial orientation of the hydroxyl group at C_{12} in **2**. On the other hand, chemical shifts for C_{13}, C_{15}, C_{16}, and C_{17} along with multiplicities of their protons and NOE data also allowed the determination of the configuration at the C_{13} position. These and comparison of the NMR data with those of **1** enabled the characterization of **2**, named here lecheronol B, as 12β,19-dihydroxy-(5α,9α,20α)-13-*epi*-pimara-8(14),15-dien-3-one.

Compound **10** showed a quasimolecular ion in its HR-FABMS, consistent with the formula $C_{42}H_{72}O_3$. The IR spectrum, on the other hand, showed the presence of hydroxy (3498 cm^{-1}) and ester (1715 cm^{-1}) functionalities. Its ^{13}C NMR spectrum showed signals for several methylenes in the range between δ 29.8 and 32.3. These together with a methyl signal at δ 0.85 (t, J = 6.9 Hz, 3H) and several overlapping methylenes between δ 1.21 and 1.31 in the ^1H NMR spectrum and a signal at δ 176.2 in the ^{13}C NMR spectrum confirmed the presence of an acylated fatty acid. The remaining signals in the ^{13}C spectrum, as given in Table 2, were characteristic of a cycloartane type

Table 1. ^{13}C and ^{1}H NMR Spectral Data for **1** (DMSO-d_6) and **2** (CD_3OD)

	1					**2**				
pos.	^{1}H mult. (J = Hz)	^{13}C	TOCSY	HMBC	NOE	^{1}H (J = Hz)	^{13}C	TOCSY	HMBC	NOE
1eq	3.99 br s	82.4	H-2	C-2, C-3, C-20	H-2_{ax}, H-5, H-19, H-20	1.55 m	37.6	H-2	C-2, C-3, C-9	
1ax						2.00 ddd (14.9, 7.3, 2.3)			C-5, C-20	
2ax	2.49 d (12.6)	51.8	H-1	C-1, C-3, C-4		2.27 dt (14.9, 2.3)	34.6	H-1	C-1, C-4	H-1_{eq}, H-5, H-19, H-20
2eq	2.06 d (12.6)			C-3, C-4		2.75 td (14.9, 7.3)			C-1, C-10	H-1_{ax}, H-6, H-18
3		211.1					218.0			
4		44.5					51.5			
5ax	1.77 dd (12.2, 1.3)	52.7	H-6, H-7	C-3, C-4, C-6, C-20	H-1, H-2_{ax}, H-6_{ax}, H-9, H-19, H-20	1.55 ov[a]	55.2	H-6, H-7	C-1, C-9, C-18 C-19, C-20	H-1, H-2_{ax}, H-6_{ax}, H-9, H-19, H-20
6ax	1.68 m	22.6	H-5, H-7	C-8, C-10		1.64 ov	23.1	H-5, H-7	C-4, C-8	
6eq	1.40 m			C-4, C-5		1.64 ov				
7ax	2.36 dd (14.5, 2.2)	34.7	H-5, H-6	C-5, C-8, C-9		2.46 td (15.8, 4.0)	34.7	H-5, H-6	C-5, C-8, C-9, C-14	
7eq	2.08 td (14.5, 5.7)			C-8		2.13 m			C-8, C-14	
8		137.6					137.5			
9ax	2.19 dd (11.8, 6.5)	51.8	H-11, H-12	C-5, C-7, C-12, C-14	H-2_{ax}, H-11_{eq}, H-12	2.03 dd (11.8, 6.0)	51.5	H-11, H-12	C-5, C-8, C-10, C-14	H-2_{ax}, H-11_{eq}, H-12
10		45.6					37.9			
11ax	1.26 ddd (12.6, 12.2, 1.8)	28.8	H-9, H-12	C-9, C-10, C-12		1.53 ddd (12.6, 11.8, 1.8)	28.2	H-9, H-12	C-9, C-12, C-13	
11eq	1.42 ddd (12.6, 6.5, 2.2)			C-8, C-12		1.67 ddd (12.6, 6.0, 3.5)			C-8, C-9, C-12	
12ax	3.29 dd (12.2, 2.2)	75.0	H-9, H-11	C-15	H-9, H-11_{eq}, H-17	3.45 dd (12.0, 3.5)	75.8	H-9, H-11	C-9, C-14, C-15	H-9, H-11_{eq}, H-17
13		44.6					44.3			
14	5.08 s	128.6		C-9, C-7, C-12, C-17	H-15, H-16a, H-18, H-20	5.12 s	128.1		C-7, C-9, C-15, C-17	H-15, H-16a H-18, H-20
15	5.94 dd (17.2, 10.9)	144.5	H-16	C-14, C-17	H-16a, H-16b	5.99 dd (17.7, 10.7)	142.9	H-16	C-12, C-16, C-17	H-16a, H-16b
16a	4.90 dd (17.5, 1.4)	115.5	H-15, H-16b	C-13, C-15	H-15, H-16b, H-18	5.07 dd (17.7, 1.9)	115.0	H-15, H-16b	C-13, C-15	H-15, H-16b, H-18
16b	5.03 dd (10.9, 1.4)		H-15, H-16a	C-13, C-15		5.14 dd (10.7, 1.9)		H-15, H-16a	C-13, C-15	
17ax	1.05 s	27.1		C-12, C-13, C-14, C-15	H-12, H-14	1.14 s	25.6			H-12, H-14
18eq	0.63 s	15.8		C-3, C-4, C-5, C-19		1.10 s	25.0			
19a	0.62 s	17.8		C-3, C-4, C-5, C-18		3.67 d (10.8)	63.2	H-19b	C-3, C-4, C-5, C-18	H-1_{eq}, H-2_{ax}, H-5, H-19
19b						4.21 d (10.8)		H-19a		
20ax	1.09 s	29.5		C-1, C-5, C-9, C-10	H-1, H-5, H-6_{ax}, H-19	1.10s	21.8			H-1_{eq}, H-5, H-6_{ax}, H-19

[a] ov=overlapped.

triterpene. Signals for seven methyl groups, consisting of one secondary methyl (δ_H 0.96, d, J = 6.4 Hz, 3H, H-21), two *gem*-dimethyl groups (C_{28}−C_4−C_{29} and C_{26}−C_{25}−C_{27}, as identified by the HMBC spectrum), and two tertiary methyl singlets at δ 1.33 and 1.32, in addition to two upfield shifted doublets that appeared as an AX system (δ 0.54 and 0.30 both d, J = 3.4 Hz, H-19) assignable to a cyclopropyl methylene group, enabled the identification of the triterpene as a cycloartane type.[15] Observation of signals at δ 3.52 (dd, J = 11.5, 4.2 Hz, H-3) in the ^{1}H NMR spectrum and at δ 124.7 (C_{23}) and 141.8 (C_{24}) and 69.9 (C_{25}) corresponding to a Δ^{23} unsaturation and hydroxylation at C_{25}, respectively, confirmed the identity of the triterpene portion as cycloart-(23*Z*)-ene-3β,25-diol.[16] This together with the molecular formula obtained in the HRFABMS then enabled the identification of the acylated fatty acid as lauric acid. HMBC correlation from the H_3 to the carbonyl carbon confirmed attachment of the lauroyl group at C_3. All the foregoing finally led to the structure of compound **10** as 3-*O*-lauroylcycloart-(23*Z*)-ene-3β,25-diol.

$C_{15}H_{14}O_9$ was arrived at as the molecular formula for compound **12** on the basis of its positive and negative ion HRFABMS, ^{13}C, and DEPT NMR spectra. Its IR spectrum showed bands for hydroxyl groups (3577, 3492, 3271 cm^{-1}),

Table 2. ^{13}C and ^{1}H NMR Spectral Data for **10** (pyridine-d_5)

position	^{13}C	^{1}H (J in Hz)
1	32.3	1.57 m, 1.24 m
2	30.1	1.75 m, 1.60 m
3	78.1	3.52 dd (11.5, 4.2)
4	41.3	
5	47.6	1.33 m
6	21.6	1.59 m, 0.79 m
7	26.5	1.79 m, 1.24
8	48.3	1.50 m
9	20.2	
10	26.8	
11	26.8	1.98 m, 1.14 m
12	32.6	1.57 m (2H)
13	45.7	
14	49.2	
15	35.1	1.31 m (2H)
16	28.5	1.35 m, 1.12 m
17	52.4	1.64 m
18	18.6	1.00 s (3H)
19	29.9	0.54 d (3.4), 0.30 d (3.4)
20	37.0	1.50 m
21	18.8	0.96 d (6.4)
22	39.7	2.29 m, 1.87 m
23	124.7	5.95 ddd (16.5, 8.3, 5.4)
24	141.8	5.94 d (16.5)
25	69.9	
26	26.4	1.33 s (3H)
27	26.4	1.32 s (3H)
28	25.8	1.08 s (3H)
29	15.0	1.10 s (3H)
30	19.7	0.90 s (3H)
1′	176.2	
2′	35.1	2.52 t (7.7, 2H)
3′	25.8	1.79 quintet (7.7)
4′−11′	29.8−32.3	1.21−131 overlapped
12′	14.4	0.85 t (6.9)

Table 3. ^{13}C and ^{1}H NMR Spectral Data for Compound **12** (DMSO-d_6)

position	^{13}C	^{1}H (J in Hz)	HMBC
α	72.5	4.45 d (5.0)	C-β, C-1′, C-β′
β	84.1	4.92 d (5.0)	C-α, C-1, C-2, C-6, C-β′
1	128.0		
2	107.8	6.41 s	C-β, C-3, C-4, C-5
3	146.6		
4	134.3		
5	146.6		
6	107.8	6.41 s	C-β, C-3, C-4, C-5
β′	198.4		
1′	101.3		
2′	163.8		
3′	96.4	5.89 s	C-1′, C-3′, C-4′, C-6′
4′	167.9		
5′	96.4	5.89 s	C-1′, C-2′, C-6′
6′	163.8		

hydrogen-bonded carbonyl (1626 cm^{-1}), and aromatic rings (1460 cm^{-1}). The ^{1}H NMR (Table 3) revealed two aromatic singlets at δ 5.89 and 6.41 both integrating for two protons and two coupling aliphatic methine protons at δ 4.45 (d, J = 5.0 Hz) and 4.92 (d, J = 5.0 Hz), whose chemical shifts and coupling patterns indicated the presence of a −CO−CH(OH)−CH(OH)− subunit. These data and the UV absorption maxima at 333, 312, 287, 227, and 203 nm were indicative of a dihydrochalcone skeleton.[17] In agreement, the ^{13}C and DEPT-135 NMR spectra revealed the presence of a hydrogen-bonded carbonyl, 12 aromatic carbons (eight quaternary and two methines each representing two carbons), and two aliphatic carbinols. The two-proton singlet at δ 6.41 showed long-range correlations with carbon resonances at δ 146.6 (C_3 and C_5), 134.3 (C_4), and 128.0 (C_1) in the HMBC spectrum, thus identifying members of the first aromatic ring (ring-B). Comparison of these chemical shifts with those in myricetin also confirmed the structure of the ring as being an aromatic ring bearing hydroxy substitutions at positions 3, 4, and 5.[10] Similarly, the aromatic proton singlet at δ 5.89 showed correlations with the carbons at δ 167.9 ($C_{4'}$), 163.8 ($C_{2'}$ and $C_{6'}$), and 101.3 ($C_{1'}$), confirming that the remaining aromatic carbons constituted the second ring (ring-A). Here again the obtained values were in agreement with those in α,β,2,4,6′-pentahydroxydihydrochalcone and confirmed hydroxy substitutions at positions 2′, 4′, and 6′.[18] Though attempts at crystallization of **12** were unsuccessful to enable crystal structure determination, configurations at the two stereocenters (C_α and C_β) in the aliphatic chain were inferred as *erythro* from intramolecular hydrogen bondings in the IR spectrum (3577 cm^{-1}, free ArOH; 3496 cm^{-1}, OH···OH; 3271 cm^{-1}, ArOH···O═C) and the coupling constant of 5.0 Hz between H_α and H_β observed in the ^{1}H NMR spectrum.[19] The absolute configuration at these stereocenters was confirmed via preparation of (*R*)- and (*S*)-MTPA esters (Mosher's esters designated **12r** and **12s**, respectively) of the 3,4,5,2′,4′,6′-hexamethyl ether derivative **12a**. Comparison of the ^{1}H NMR spectrum of **12r** and **12s** showed a negative $\Delta\delta^{RS}$ value for protons in the B-ring owing to higher shielding effect of the aryl group in **12r** (Figure 1) and a positive $\Delta\delta^{RS}$ value for H_α and A-ring protons because of higher shielding in **12s** (Table 4, Figure 1). However, with the *anti*-α,β-diol orientation inferred from lack of any NOE effects at either H_α or H_β at selective excitation of either one, instead of the expected negative $\Delta\delta^{RS}$ value for H_β, no change in chemical shift was observed.[20] The foregoing confirmed an *R*-configuration at C_α and suggested an *S*-configuration at C_β. Confirmation for the configuration at C_β was obtained by considering chemical shift differences ($\Delta\delta = \delta_{u,OMe} - \delta_{l,OMe}$) between the methoxy group in (*R*)- and (*S*)-MTPA esterified at C_α and C_β when the absolute configuration of the MTPA moiety matches that of the dialkanol (*R*,*R* or *S*,*S* = *l*, like) and in which either of the configurations are inverted (*R*,*S* or *S*,*R* = *u*, unlike) as shown in Figure 1. This $\Delta\delta$ value for compound **12** was found to range from +0.03 to +0.18, as shown in Table 4. With the C_α configuration already determined, identification of the configuration as *R* was made possible on the basis of comparison of the positive $\Delta\delta^{ul}$ value with data available for MTPA derivatives of dialkanols.[21] These finally enabled the identification of compound **12** as α(*R*),β-(*S*),3,4,5,2′,4′,6′-octahydroxydihydrochalcone.

Compounds **1**−**14** were tested for their inhibitory activity toward the growth of *M. tuberculosis*. The MIC values for these compounds are as shown in Table 5. The most active fraction (F1) was found to possess an MIC value of 4 μg/mL. However, its major constituents, compounds **3** and **8**, were found active, with MIC values of 12.2 and 13.4 μg/mL. In compounds with either the oleanene or lupene skeleton, it appears that a 3α-hydroxy substitution augurs well for activity. On the other hand, hydroxy substitution at C_{19} instead of C_1, as is inferred from the comparison of activity of compounds **1** and **2**, may lead to loss of activity. The findings that acylation of the 3-hydroxyl group as in **10** causes abrogation of activity parallel those for 24,25-epoxycycloartan-3-ol and its 3-acetate derivative.[22] In addition, hydroxylation of the 3′ position in compounds **13** and **14** appears to increase activity. In the Vero cell cytotoxicity assay, the most active compounds, **1**, **3**, **8**, and **9**, showed IC_{50} values of 104.8, 127.2, 127.2, and 102.4 μg/mL, respectively. Selectivity indices of 26.2, 10.4, 10.4, and 12.8, respectively, for these compounds suggest lack of appreciable toxicity against mammalian cells.

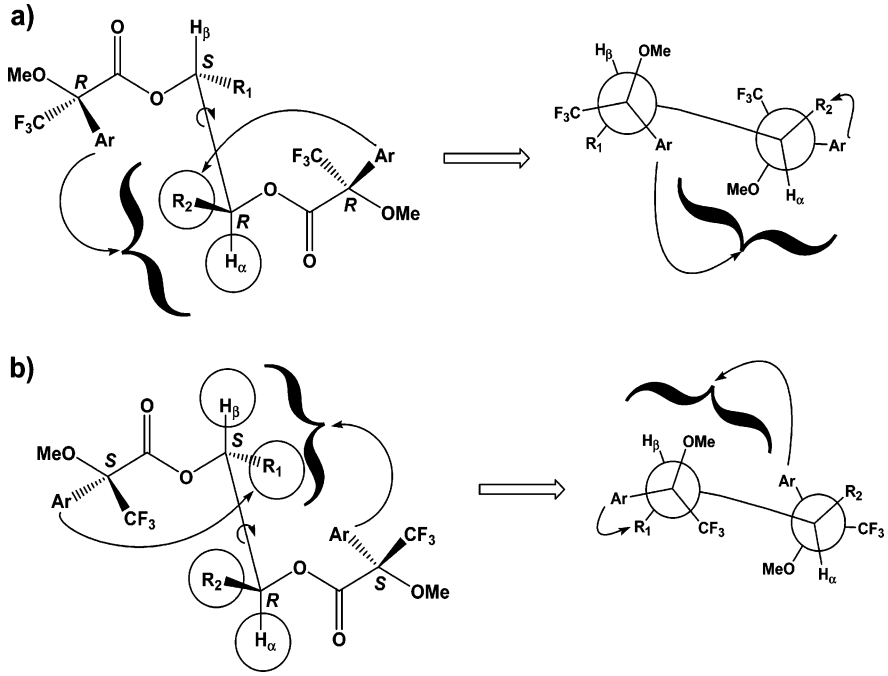


Figure 1. (a) Compound **12r** (*u* at C_β and *l* at C_α). (b) Compound **12s** (*l* at C_β and *u* at C_α). Groups shielded by aryl rings are shown by arrows.

Table 4. ^{1}H NMR Spectral Data for (*R*)- and (*S*)-MTPA Esters of **12a** (pyridine-d_5)[a]

position	**12r**	**12s**	$\Delta\delta^{R-S}$	$\Delta\delta(u-l)$
α	5.83	5.82	+0.01	
β	6.11	6.11	0.00	
2	6.51	6.64	−0.13	
6	6.51	6.64	−0.13	
3′	5.93	5.92	+0.01	
5′	5.93	5.92	+0.01	
OMe (MTPA at C_α)	3.33[a]	3.42[b]		+0.03−0.18
OMe (MTPA at C_β)	3.51[a]	3.45[b]		+0.03−0.18

[a] Values carrying the same superscript may be interchangeable.

Experimental Section

General Experimental Conditions. IR (as a film on a diamond cell) was measured on a Thermo Nicolet Avatar 360 FT-IR spectrometer. Optical rotations were acquired using a Perkin-Elmer 241 polarimeter. A JEOL HX110A mass spectrometer was used in recording HRFAB mass spectra. NMR spectra (^{1}H, selective 1D-NOE, selective 1D-TOCSY, ^{13}C, DEPT-135, DEPT-90, HSQC, HMBC, DQF-COSY) were recorded using either a Bruker DRX-500 or DRX-600 spectrometer in $CDCl_3$ (compounds **3**, **4**, **5**, **7**, and **8**), DMSO-d_6 (compounds **1**, **11**, **13**, and **14**), CD_3OD (compounds **2** and **6**), or pyridine-d_5 (compounds **9**, **10**, and **11**). Chemical shifts were expressed in ppm (*δ*) using partially deuterated solvent chemical shifts as reference. The mixing times used in recording selective 1D-NOE and selective 1D-TOCSY spectra were 350 and 60.9 ms, respectively. In fraction purification and compound isolation, a Sephadex LH-20 LPLC system composed of a Büchi 688 pump and a 47.5 × 3.5 cm column, an Analtech centrifugal TLC system composed of an RHSY solvent pump and 8 mm Si-gel rotors, and a Varian Star semiprep HPLC system equipped with a model 230 pump and a model 310 variable-wavelength detector were used.

Plant Material. The plant material was collected in October 2000 in Argentina (27°18′ S; 58°37′ W) by Renée H. Fortunato. A voucher specimen (RHF 6057) has been deposited at the Instituto Nacional de Tecnologia Agropecuaria (INTA), Buenos Aires, Argentina. Intellectual Property Rights Agreements for plant collections and collaborative research have been fully executed between the University of Arizona and INTA.

Extraction and Isolation. The aerial parts of the plant (850 g) were powdered and extracted with 5 L of CH_2Cl_2/MeOH, 1:1, followed by 5 L of MeOH, giving a combined 80 g of viscous mass. This extract was then fractionated on silica gel CC using 0−100% EtOAc in hexanes, yielding 32 fractions. Of these, fractions F1, F2, F5, F7, F9, F11, F12, and F14 were found to cause ≥90% growth inhibition of *Mycobacterium tuberculosis* and were, as a result, selected for further work. Direct HPLC purification of F1 using a Dynamax-100Å C-18 column (20 × 300 mm, 8 *μ*, 15 mL/min, Varian) with MeOH/H_2O (85:100 to 100:0 in 10 min to 100:0 13 min) yielded compounds **3** (13.7 mg), **5** (12.7 mg), and **8** (34.5 mg) with t_R = 18.6, 20.5, and 21.6 min, respectively. Centrifugation and HPLC of the resultant supernatant from F2 yielded compounds **4** (4.1 mg) and **7** (12.6 mg) with t_R = 12.6 and 11.4 min, respectively, using a C-18 column (Reliasil, 10 × 250 mm, 10 *μ*, Column Engineering, at 5.4 mL/min) and MeOH/0.15% formic acid in H_2O, 95:5 to 100:0 in 5 min and at 100:0 for 10 min. Next, F5 was purified using CH_2Cl_2 in a centrifugal TLC system, and compound **10** (5.5 mg) was isolated from the purified fraction with HPLC (MeOH/H_2O, 70:30 to 100:0 in 20 min, t_R = 13.0 min). Fraction F7 gave compound **2** (9.5 mg) after centrifugal TLC purification with CH_2Cl_2/MeOH, 99:1. Compound **9** (5.1 mg) was obtained after centrifugal TLC purification (CH_2Cl_2/MeOH, 98:2) and HPLC (MeOH/H_2O, 80:20 to 100:0 in 14 min to 100:0 in 6 min, t_R = 14.8 min) of fraction F9. On the other hand, compound **1** (11.2 mg) was isolated from F11 after Sephadex filtration (CH_2Cl_2/MeOH, 3:7) and HPLC purification (MeCN/H_2O, 40:60 to 80:20 in 20 min, t_R = 12.4 min). To obtain compound **6** (3.0 mg), fraction F12 was purified using centrifugal TLC (hexane/*i*-PrOH/MeOH, 6:1:1). Finally, compounds **11**−**14** were isolated from F14 after centrifugation of the fraction, which gave a precipitate (**11**, 50.2 mg), followed by Sephadex filtration (MeOH), which gave compound **12** (81.9 mg) and a mixture of compounds **13** and **14**. These compounds were then separated using HPLC (MeCN/H_2O, 10:90 to 45:55 in 20 min) to yield **13** (6.3 mg) and **14** (6.5 mg) with t_R = 13.3 and 13.9 min, respectively.

MIC versus *M. tuberculosis*. Activity of the crude extract, fractions, and isolated compounds was determined against *M. tuberculosis* H_{37}Rv (ATCC 27294) in the microplate Alamar Blue assay as previously described.[23] Percentage inhibition for crude extracts and fractions was defined as 1 − (test well fluorescence units/mean fluorescence units of triplicate wells containing only bacteria) × 100. The MIC was defined as the

Table 5. *M. tuberculosis* Growth Inhibition by Compounds **1**−**14** (MIC values in μg/mL)

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	rifampin[a]
MIC	4	128	12.2	64	>128	>128	>128	13.4	8	>128	128	>128	>128	94	0.06

[a] Control.

lowest drug concentration that effected an inhibition of ≥90% relative to untreated cultures.

Cytotoxicity Assay. Cytotoxicity against Vero cells (ATCC CCL-81) in the CellTiter 96 aqueous nonradioactive cell proliferation assay was determined as previously described.[24] The IC_{50} is defined as the reciprocal dilution resulting in 50% inhibition of the Vero cells. Maximum cytotoxicity (100%) was determined by lysing the cells with sodium dodecyl sulfate (Sigma Chemical Co., St. Louis, MO). Selectivity indices were then calculated by dividing the IC_{50} value with the MIC value.

Methylation of 12. Compound **12** (8 mg) was dissolved in 1.0 mL of dry DMSO, an excess of fresh CH_2N_2 (in Et_2O) was added, and the reaction was allowed to progress overnight at room temperature. The 3,4,5,2′,4′,6′-hexamethyl ether derivative, **12a** (5.3 mg), was then purified using preparative TLC.

Preparation of (*R*)- and (*S*)-MTPA Ester Derivatives of 12a. 12a was divided into two portions (each 2.6 mg) and treated with (*S*)-(+)α- and (*R*)-(−)α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μL) in anhydrous pyridine (2.0 mL) overnight at room temperature to afford the (*R*)- and (*S*)-MTPA ester derivatives, **12r** and **12s**, respectively. The reaction mixture in each case was purified over a small Si gel (2 g) column using CH_2Cl_2/MeOH (99:1). For ^{1}H NMR data (C_5D_5N) of **12r**, see Table 4; **12s**, see Table 4.

Lecheronol A (1): 1β,12β-dihydroxy-(5α,9α,20α)-13-*epi*-pimara-8(14),15-dien-3-one; $[\alpha]_D^{25}$ +8° (*c* 0.03, DMSO); white powder; HRFAB$^+$ $[M + H]^+$ 319.2273 (monoisotopic calc 319.2265), $C_{20}H_{30}O_3$; IR ν_{max} (cm^{-1}) 3391, 2932, 2872, 1714, 1387, 1039; ^{13}C NMR (125 MHz, DMSO-d_6) and ^{1}H NMR (500 MHz), see Table 1; ^{1}H NMR (500 MHz, $CDCl_3$) 5.74 (dd, J = 17.1, 10.9 Hz, H-15), 5.21 (dd, J = 10.9, 1.1 Hz, H-16b), 5.08 (dd, J = 17.1, 1.1 Hz, H-16a), 5.06 (s, H-14), 4.08 (br s, H-1), 3.64 (m, H-12), 1.11 (s, H-20), 1.05 (s, H-17), 1.02 (H-18 or H-19), 0.99 (H-18 or H-19); ^{13}C NMR (125 MHz, $CDCl_3$) 213.3 (C-3), 146.1 (C-15), 136.4 (C-8), 128.3 (C-14), 113.9 (C-16), 82.6 (C-1), 73.1 (C-12), 52.6 (C-5), 51.6 (C-9), 47.2 (C-4), 44.3 (C-10), 43.0 (C-13), 42.9 (C-2), 34.4 (C-7), 27.3 (C-20), 26.4 (C-11), 26.3 (C-18), 22.3 (C-6), 20.9 (C-19), 19.5 (C-17).

Lecheronol B (2): 12β,19-dihydroxy-(5α,9α,20α)-13-*epi*-pimara-8(14),15-dien-3-one; $[\alpha]_D^{25}$ +3° (*c* 0.05, MeOH); white powder; HRFAB$^+$ *m*/*z* 341.2076 $[M + Na]^+$ (monoisotopic, calc 341.2085), $C_{20}H_{30}O_3$; IR ν_{max} (cm^{-1}) 3362, 2941, 2845, 1701, 1302, 1062; ^{13}C NMR (125 MHz, CD_3OD) and ^{1}H NMR (500 MHz), see Table 1; ^{1}H NMR (500 MHz, $CDCl_3$) 5.74 (dd, J = 17.2, 10.9 Hz, H-15), 5.21 (dd, J = 10.9, 1.3 Hz, H-16b), 5.08 (dd, J = 17.2, 1.3 Hz, H-16a), 5.05 (s, H-14), 3.66 (m, H-12), 3.78 (d, J = 10.9 Hz, H-19a), 3.47 (d, J = 10.9 Hz, H-19b), 1.11 (s, H-20), 1.18 (s, H-18), 1.04 (s, H-17); ^{13}C NMR (125 MHz, $CDCl_3$) 217.2 (C-3), 146.2 (C-15), 136.2 (C-8), 128.1 (C-14), 113.5 (C-16), 73.2 (C-12), 65.8 (C-19), 55.2 (C-5), 51.4 (C-9), 50.9 (C-4), 43.1 (C-13), 37.9 (C-1), 36.4 (C-10), 35.6 (C-7), 34.1 (C-2), 26.1 (C-11), 24.3 (C-18), 22.2 (C-20), 22.1 (C-6), 19.3 (C-17).

3-*O*-β-Lauroylcycloart-(23*E*)-ene-25-ol (10): white powder; $[\alpha]_D^{25}$ +22.7° (*c* 0.1, C_5H_5N); HRFAB$^+$ $[M + H]^+$ *m*/z 625.5533 (monoisotopic calc 625.5541), $C_{42}H_{72}O_3$; IR ν_{max} (cm^{-1}) 3498, 2915, 2852, 1715, 1468, 1023; ^{13}C NMR (125 MHz, pyridine-d_5) and ^{1}H NMR (500 MHz), see Table 1.

α,β,3,4,5,2′,4′,6′-Octahydroxydihydrochalcone (12): orange powder; $[\alpha]_D^{25}$ −10.4° (*c* 0.01, MeOH); HRFAB$^+$ $[M + H]^+$ *m*/*z* 339.0716 (monoisotopic calc 339.0711), $C_{15}H_{14}O_9$; UV (MeOH) λ_{max} (log ϵ) 333 (2.9), 312 (2.4), 287 (3.4), 227 (1.8), 203 (3.3) nm; IR ν_{max} (cm^{-1}) 3577, 3492, 3271, 1626, 1460, 1160, 1021; ^{13}C NMR (125 MHz, DMSO-d_6) and ^{1}H NMR (500 MHz), see Table 1.

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