Oleanane Triterpenes from *Junellia tridens*

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Received May 5, 2000

Three novel triterpenes, 3,4-*seco*-olean-12-ene-3,28-dioic acid (**4**), 3α-hydroxyolean-11-en-28,13*β*-olide (**5**), and 3α-hydroxyoleane-11:13(18)-dien-28-oic acid (**6**), were isolated from the aerial parts of the Argentinean shrub, *Junellia tridens*. Another five compounds—oleanolic (**1**), oleanonic (**2**), and epioleanolic acids (**3**), all biosynthetically related to the three new oleananes, and epibetulinic acid (**7**) and sitosterol (**8**)—were also isolated. Structures were elucidated primarily by 1D and 2D NMR and mass spectrometry, and all protons and carbons of the three novel compounds were fully assigned by NMR. We report the minimum inhibitory concentrations of these compounds against *Mycobacterium tuberculosis* and conclude that they are responsible for antitubercular activity originally observed in the crude plant extract. LC-MS data is provided on the occurrence of triterpenes **1**−**6** in six other species of *Junellia*.

As part of an International Cooperative Biodiversity Group (ICBG) program exploring the arid land plants of Latin America for new biologically active agents, we have investigated the chemistry of *Junellia tridens* (Lag.) Mold. (Verbenaceae). The genus *Junellia* includes 45 species endemic to the Andes Mountain and Patagonian regions of South America.[1] *J. tridens* is a spiny, low growing shrub, which was collected near Rio Gallegos in the Santa Cruz Province of Southern Argentina. The finding of antitubercular activity in the CH_2Cl_2/MeOH extract of the aboveground plant parts motivated the use of bioactivity-guided fractionation to identify antimycobacterial compounds. Tuberculosis (TB) remains the world's leading cause of mortality from an infectious agent, and the discovery of new antitubercular drugs has taken on increased importance in recent years, as multidrug-resistant strains have emerged and the incidence of TB worldwide has been rising.[2]

Based on preliminary bioactivity data of hexane, EtOAc, and CH_2Cl_2/MeOH extracts of the plant, we targeted the EtOAc-soluble portion of the CH_2Cl_2/MeOH extract of *J. tridens* for bioactivity-guided fractionation and chemical identification of active compounds. Several iterations of chromatographic separation and bioassay of the resultant fractions led to the isolation of a series of related triterpene acids possessing moderate antimycobaterial activity. Three of the active compounds (**4**, **5**, and **6**) have not, to our knowledge, been reported in the literature. Proof of the novel structures is provided primarily by 2D NMR, HSQC, and HMBC experiments in which all protons and carbons were fully assigned (Table 1).

Results and Discussion

The EtOAc-soluble portion of the CH_2Cl_2/MeOH extract of *J. tridens* was fractionated by flash silica chromatography to generate 36 fractions. Each of these was tested for activity against *M. tuberculosis* $H_{37}Rv$ in the BACTEC 460 radiorespirometric assay. The results of this screening indicated that a set of fractions eluting with 20% EtOAc in hexane contained enhanced activity compared to the crude plant extracts. To find the source of the activity, each active fraction was subjected to a combination of flash chromatography and HPLC. In total, eight crystalline compounds were isolated and characterized. Compounds **1**−**7** exhibited spectroscopic characteristics typical of pentacyclic triterpene acids, including 30 peaks in the ^{13}C NMR spectra, and seven methyl groups in the ^1H NMR and DEPT analysis.

Negative ion HRFABMS of **4** showed an ion consistent with a molecular formula of $C_{30}H_{47}O_4$. Electron ionization gave a fragmentation pattern typical of Δ^{12} oleanane-type triterpenes where cleavage of the C-8−C-14 and the C-9−C-11 bonds yielded a major fragment ion at *m*/*z* 248 corresponding to the D- and E-ring portion of an oleanane triterpene bearing one carboxylic acid.[3] Other peaks at *m*/*z* 233 and 203 corresponded to demethylation and decarboxylation of this fragment, respectively. NMR showed a single olefinic proton at δ_H 5.47 (H-12) and two olefinic carbons at δ_C 123.2 (C-12) and δ_C 145.1 (C-13), indicating a pentacyclic Δ^{12}-triterpene. The four oxygens in the molecular formula could be accounted for by two carbonyl signals at δ_C 177.1 and 180.6, corresponding to two carboxylic acid groups. The carbonyl carbon of the most downfield acid could be assigned to position 28 by its three-bond HMBC coupling with H-18, itself positioned by HMBC couplings with C-12, -13, -14, -16, -17, and -28. In contrast, the acid at δ_C 177.1 could only be located at C-3 because of its HMBC couplings with protons at positions 1 and 2. These positions for the carboxylic acids are also consistent with mass spectral fragmentation patterns. The upfield portion of the ^1H NMR showed that two methyl groups at δ_H 0.93 and 0.75 were split into doublets, while the remaining five were singlets. The doublets originated from an isopropyl group in which the methyl groups 23 and 24 were coupling with H-4. Additional evidence for an isopropyl moiety was supplied by a three-bond HMBC coupling between each methyl group doublet and the carbon of the adjacent geminal methyl group. Methyl groups 23 and 24 also gave HMBC correlations with C-5. The combination of an acid at C-3 and an isopropyl group at C-5 led us to conclude that we had isolated the novel 3,4-*seco*-olean-12-

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10.1021/np0002233 CCC: $19.00
Published on Web 10/27/2000

Table 1. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and HMBC Data for Compounds **4**−**6** in Pyridine-d_5[a]

position	**4**			**5**			**6**		
	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC	δ_C	δ_H	HMBC
1	34.4 t	1.94 m	3	34.0 t	1.76 m	3, 10	33.1 t	1.87 m	
		1.90 m			1.59 m	2, 3, 5, 10		1.72 m[b]	
2	29.9 t	2.51 m	3	26.7 t	2.03 m		26.0 t	2.09 t (12.8)	
		2.49 m			1.78 m	3, 4, 10		1.85 m	
3	177.1 s			75.6 d	3.61 br s	1, 5	75.0 d	3.65 t (2.5)	1, 4, 5, 23, 24
4	25.9 d	1.92 m		38.6 s			37.7 s		
5	48.4 d	1.15 d (11.5)		49.1 d	1.69 dd		48.5 d	1.82 m	
6	19.0 t	1.33 m[b]		18.4 t	1.48 m		18.3 t	1.56 m	
		1.30 m[b]			1.43 m			1.44 m	
7	32.7 t	1.42 t		31.9 t	1.36 m		32.6 t	1.35 m[c]	9
		1.31 m[b]			1.12 m			1.40 m	
8	40.0 s			42.2 s[b]			40.9 s		
9	38.9 d	1.98 t		54.0 d	2.14 s	10, 11, 12, 14, 25, 26	54.4 d	2.27 m[d]	11,12
10	40.6 s			37.4 s			37.0 s		
11	24.6 t	2.06 m		136.8 d	6.16 d (10.3)	13	126.9 d	5.84 d (10.5)	8, 9, 13
		1.99 m						6.67 dd (10.6, 2.9)	
12	123.2 d	5.47 t (3.5)	14, 11, 9	127.8 d	5.53 dd (10.2, 3.0)	13	125.6 d		9, 13, 14, 18
13	145.1 s			90.1 s			136.2 s		
14	43.2 s			42.6 s[b]			42.0 s		
15	28.8 t	2.14 t		26.1 t	1.72 m		25.1 t	2.01 t (13.7)	8, 17, 14, 27
		1.21 m			1.06 m			1.06 m	
16	24.3 t	2.10 m		22.1 t	2.07 m	17, 28	35.9 t	2.62 d (13.4)	14, 17, 18, 28
		1.95 m			1.25 m			1.50 m	17, 28
17	47.2 s			44.7 s			48.3 s		
18	42.6 d	3.28 dd (9.9, 4.2)	12, 13, 14, 16, 17, 28	51.2 d	2.16 d (2.8)	13, 16	132.7 s		
19	46.9 t	1.77 t		37.7 t	1.75 m[b]	20, 30	40.7 t	2.73 d (14.1)	13, 17, 19, 20, 30, 18
		1.26 m			1.22 m			2.16 d (14.3)	13, 14, 17, 18, 20, 30
20	31.5 s			31.9 s			32.3 s		
21	34.8 t	1.44 t		34.9 t	1.30 m		37.1 t	1.34 m[c]	17
		1.20 m			1.11 m			1.71 m[b]	17
22	33.7 t	2.04 m		28.3 t	1.66 m		33.0 t	2.26 m[d]	14, 17, 28,18
		1.82 m			1.75 m[b]			1.79 m	17, 28
23	25.4 q	0.93 d (6.7)	24, 5, 4	29.6 q	1.18 s	3, 4, 5, 24	28.9 q	1.22 s	3, 4, 5, 24
24	19.6 q	0.75 d (6.7)	23, 5, 4	22.5 q	0.86 s[c]	3, 5, 23	21.9 q	0.89 s	3, 4, 5, 23
25	20.0 q	0.84 s	5, 10, 9, 1	18.6 q	0.90 s	1, 5, 9, 10	17.9 q	1.00 s	1, 5, 9, 10
26	18.0 q	1.02 s	14, 8, 9, 7	20.0 q	1.22 s	7, 8, 9, 14	16.8 q	1.11 s	7, 8, 9, 14
27	26.4 q	1.24 s	14, 8, 15	18.6 q	0.91 s	8, 13, 14, 15	19.7 q	0.99 s	8, 13, 14
28	180.6 s			180.0 s			178.5 s		
29	33.8 q	0.96 s	30, 19, 20, 21	33.6 q	0.86 sc	19, 20, 21, 30	32.0 q	0.92 s	19, 20, 30
30	24.2 q	1.00 s	19, 20, 29, 21	23.9 q	0.74 s	19, 20, 21, 29	24.1 q	0.88 s	19, 20, 29

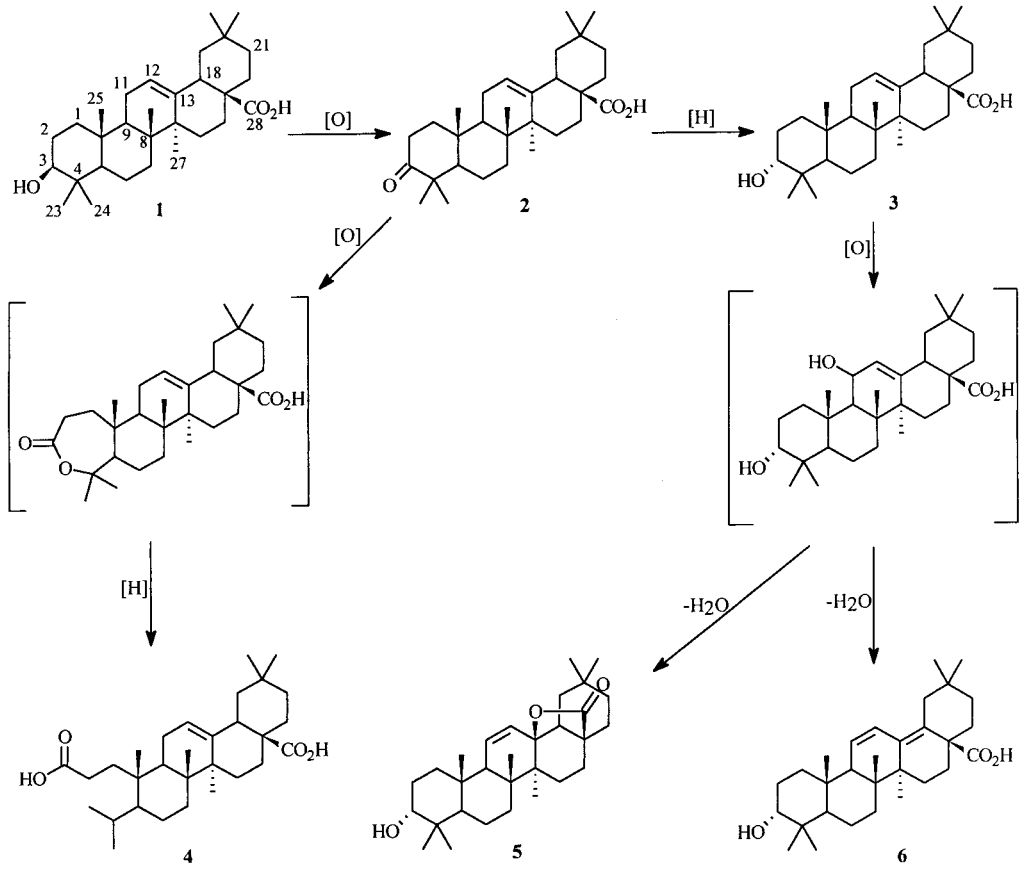
[a] Assignments based on ^{1}H, ^{13}C, DEPT, HSQC, and HMBC experiments. Coupling constants (Hz) are in parentheses. [b−d] Entries marked with the same symbol are interchangeable within each column.

ene-3,28-dioic acid (**4**), a pentacyclic triterpene in which the A-ring appears to have opened by lactone hydrolysis and reduction at position 4 as shown in Scheme 1.

The molecular formula of **5** was confirmed as $C_{30}H_{47}O_3$ by positive-ion HRFABMS. HSQC data indicated an olefin in which the proton at δ_H 5.53 was attached to the carbon at δ_C 127.8 and the proton at δ_H 6.16 was attached to the carbon at δ_C 136.8. Comparison with published NMR data[4] indicated that these values were typical for a pentacyclic Δ^{11}-triterpene. Moreover, HMBC correlations between each of these olefinic protons and the unusually far downfield carbon at position 13 (δ_C 90.1) indicated not only an 11,12 double bond, but also a 28,13-lactone. HMBC coupling of C-13 with H-18 and the protons of methyl group 27 provided further evidence for a 28,13-lactone. The stereochemistry of the alcohol in the 3-position was determined by analysis of the ^{1}H NMR coupling constant of H-3, where a very narrow triplet (δ_H 3.61, J = 3 Hz) indicated an equatorial proton and hence an α-orientation for the alcohol. In contrast, the splitting pattern of H-3 in the ^{1}H NMR of the known 3β-hydroxy epimer appears as a double doublet (δ_H 3.23, J = 10, 6). Further evidence for a 3α-hydroxyoleanane is given by an upfield shift of C-3 (δ_C 75.6) as compared with C-3 of the 3β-hydroxy conformer (δ_C 78.9).[4] Based on these data, we conclude that **5** is the novel lactone, 3α-hydroxyolean-11-en-28,13β-olide.

HREIMS showed that **6** had a molecular formula of $C_{30}H_{46}O_3$. The presence of four olefinic carbons (δ_C 125.6, 126.9, 132.7, and 136.2) in the ^{13}C NMR, two downfield proton doublets in the ^{1}H NMR, and UV quenching on TLC plates suggested a conjugated diene. HMBC coupling between the olefinic doublet at δ_H 5.84 and C-8, -9, and -13 and the coupling of the double doublet at δ_H 6.67 with C-9, -13, -14, and -18 led us to conclude that we had isolated an 11,13(18)-diene. Other outstanding features of the ^{13}C NMR were a carbonyl at δ_C 178.5, indicating a carboxylic acid, and a methine at δ_C 75.0, indicating a 3-hydroxy group. For reasons similar to those given for lactone **5**, namely, a narrow triplet in the ^{1}H NMR (δ_H 3.61, J = 3 Hz), it was concluded that the 3-hydroxy substituent was present in an axial or α-configuration. The carboxylic acid could be assigned to C-28 based on its HMBC coupling with the four methylene protons in positions 22 and 16, as well as by comparison with the NMR data of compound **4** and other 28-oleanane acids. These data led us to conclude that we had isolated 3α-hydroxyoleane-11:13(18)-dien-28-oic

Scheme 1. Proposed Biosynthetic Pathway for the Production of Oleanane Triterpenes in *J. tridens*



acid (**6**). This diene differs only in the stereochemistry at C-3 from 3β-hydroxyoleane-11:13(18)-dien-28-oic acid recently isolated from callus tissue cultures of *Paeonia* species.[5] In addition, diene **6** has been reported as part of a glycoside;[6] however, the aglycon was not isolated, and no proton NMR evidence has been provided as proof of an α-orientation of the 3-OH.

Comparison of ^{1}H and ^{13}C NMR and MS data with the literature indicated that compounds **1**, **2**, and **3** were oleanolic,[7] oleanonic,[8] and epioleanolic acids,[9] respectively. Compounds **7** and **8** were identified as epibetulinic acid and sitosterol by ^{1}H NMR and by TLC comparison with authentic samples.

All of the triterpenes isolated from *J. tridens* appear to be derived biosynthetically from oleanolic acid (**1**), the most abundant triterpene in the plant. Oxidation at the 3-position leads to oleanonic acid (**2**), which in turn can be reduced at C-3 to give 3-epioleanolic acid (**3**).[10] By a mechanism analogous to that proposed by Ikuta et al.,[5] compounds **5** and **6** may be derived from the epi-compound by dehydration of an intermediate allylic alcohol. A proposed pathway is shown in Scheme 1.

The minimum inhibitory concentrations (MIC) of compounds **1**−**7** against *M. tuberculosis* were determined in the BACTEC 460 radiorespirometric assay. Although all of the triterpenes tested exhibited some activity, 3-epioleanolic acid (**3**) and oleanonic acid (**2**) were the most potent, with MIC values of 16 μg/mL, still an order of magnitude less potent than the first-line antitubercular drug, rifampin (Table 2). Based on the results of these bioassays, we conclude that the antitubercular activity of *J. tridens* resides with its oleanane triterpenes. This observation is consistent with the previous finding of our laboratory and others that low polarity pentacyclic triterpenes with a hydroxy or keto group in the A or B rings and an acid group in the E ring possess moderate antitubercular activity.[11,12] In addition, the lipophilicity of these triterpenes is likely to allow them to rapidly penetrate the lipid-rich mycobacterial cell wall.

Table 2. MIC Values of Compounds **1**−**7** against *M. tuberculosis* $H_{37}Rv$

compound	MIC (μg/mL)
oleanolic acid (**1**)	50
oleanonic acid (**2**)	16
3-epioleanolic acid (**3**)	16
3,4-*seco*-olean-12-ene-3,28-dioic acid (**4**)	128
3α-hydroxyolean-11-en-28,13β-olide (**5**)	64
3α-hydroxyoleane-11:13(18)-dien-28-oic acid (**6**)	64
epibetulinic acid (**7**)	50
rifampin[a]	0.16

[a] First-line antitubercular drug for comparison.[13]

Normal-phase LC−MS was used to generate chemical profiles of six other *Junellia* species from Argentina. By comparing the profile of the EtOAc extract of each species to a set of purified standards obtained from the original *J. tridens* extract, we were able to determine the identities and relative quantities of triterpenes present in the extracts. The results of the LC−MS study, shown in Table 3, indicated large amounts of both oleanolic (**1**) and epioleanolic acids (**3**) and lesser amounts of several other triterpenes in *J. spissa*, *J. tetragonocalyx*, *J. aspera*, *J. seriphioides*, and *J. tridens*. In contrast, *J. mulinoides* and *J. ligustrina* contained no detectable levels of triterpenes **1**−**6**. Compound **4**, though originally isolated from *J. tridens*, was apparently present in low enough concentration as to be undetectable. Although this study has demonstrated that LC−MS analysis of triterpenes can

Table 3. Distribution of Compounds **1**−**6** in Several *Junellia* Species[a]

species	1	2	3	4	5	6
J. aspera (Gillies & Hook) Mold.	+	−	+	−	+	+
J. ligustrina (Lag.) Mold.	−	−	−	−	−	−
J. mulinoides (Speg.) Mold.	−	−	−	−	−	−
J. seriphioides (Gillies & Hook) Mold.	++	+	++	−	+	+
J. spissa (Sandw.) Mold.	++	+	++	−	+	+
J. tetragonocalyx (Tronc.) Mold.	++	+	++	−	+	+
J. tridens (Lag.) Mold.	++	+	++	−	+	+

[a] LC−MS evidence for large quantities, ++; small quantities, +; not present, −.

provide a tool for chemotaxonomic comparisons within the genus *Junellia*, the reason these compounds are present in some *Junellia* species and absent in others is a topic for future research.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO P1020 polarimeter. NMR spectra were recorded in pyridine-d_5 on a Bruker Avance DRX-500 NMR spectrometer with residual pyridine as reference. HR-FABMS with *m*-NBA as matrix and HREIMS were obtained on a JEOL HX 110 mass spectrometer. Melting points were determined on a Fisher-Johns melting point apparatus and are uncorrected. Flash chromatography was performed using Si gel 40 (32−63 μ, Scientific Adsorbents Incorporated, Atlanta Georgia). HPLC was carried out using a Varian 9002 pump, a Varian Star 9040 refractive index detector, and an Alltech Econosil 10-μ silica 10 × 250 mm column. Visualization of all compounds on Si gel TLC was accomplished by spraying with a solution of 0.5% anisaldehyde, 10% glacial acetic acid, and 5% concentrated sulfuric acid in methanol followed by gentle heating.

Plant Material. Aerial parts of *J. tridens* were collected and identified by Renee H. Fortunato in January 1995, in the Departamento Guer Aike, Santa Cruz Province, Argentina (51° 37′ S; 69° 36′ W). A voucher specimen (RF 4912) has been deposited at the herbarium of the Instituto Nacional de Tecnología Agropecuaria (INTA), Castelar, 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 Separation. The dried, ground biomass (722 g) from the aerial parts of *J. tridens* was extracted three times at room temperature with a 1:1 mixture of CH_2Cl_2 and MeOH. After evaporation of the combined extracts under reduced pressure, a brown residue (250 g) was obtained. This material was extracted three times with EtOAc to give 83 g of insoluble, yellow powder and 165 g of a soluble, brown residue. The soluble portion was adsorbed to 300 g of Si gel (32−63 μ) and loaded onto a flash column packed with a 76 × 450 mm bed of the same Si gel. The extract was eluted with solvent of gradually increasing polarity using a gradient of 100% hexane to 100% EtOAc. After pooling, 36 discrete fractions were obtained. Compound **1**, though present in several fractions, was isolated from fraction 23 (1.5 g). Compounds **2** and **3** were obtained from HPLC separation of fraction 22 (1.4 g). Fraction 27 (3.8 g) generated an additional 28 subfractions when it was subjected to further flash silica chromatography using an isocratic mobile phase of 12% EtOAc in hexane. Normal-phase HPLC purification of the twelfth subfraction (41 mg) with 15% EtOAc in hexane yielded the pure compounds **4** and **5**. Recrystallization of the seventh subfraction (64 mg) from hexane led to the isolation of compound **6**.

LC−MS. Approximately 0.5 g of a CH_2Cl_2/MeOH extract from each of the species investigated was triturated with 10 mL of EtOAc. The resultant slurry was filtered, and the filtrate was passed through a 4-cm plug of Si gel. An additional 10 mL of EtOAc was passed through the plug, and the combined eluent was evaporated to one-tenth its original volume and passed through a 0.45-μ filter. This procedure was repeated separately for each plant species in the study. LC−MS of individual extracts as well as the standards was performed on a Finnegan TSQ 7000 mass spectrometer in APCI positive mode with a Si gel column (Alltech Econosil 5 μ, 4.6 × 250 mm). The HPLC used an isocratic mobile phase of 15% EtOAc in hexane with a flow rate of 1 mL/min. Under these conditions, compounds **1**−**6** and **8** had the following retention times and ions: **1**, 20.9 min, *m/z* 439 $[M + H - H_2O]^+$; **2**, 8.67 min, *m/z* 455 $[M + H]^+$, 437 $[M + H - H_2O]^+$; **3**, 10.5 min, *m/z* 439 $[M + H - H_2O]^+$; **4**, 12.5 min, *m/z* 473 $[M + H]^+$, 455 $[M + H - H_2O]^+$; **5**, 18.6 min, *m/z* 455 $[M + H]^+$, 437 $[M + H - H_2O]^+$; **6**, 12.4 min, *m/z* 437 $[M + H - H_2O]^+$; **8**, 15.7 min, *m/z* 397 $[M + H - H_2O]^+$. The occurrence of these compounds in several species of *Junellia* is reported in Table 3.

3,4-*seco*-Olean-12-ene-3,28-dioic acid (4): isolated by HPLC as an off-white powder; mp >250 °C (dec); $[\alpha]^{25}_D$ +54.4° (*c* 0.006, $CHCl_3$); ^{1}H NMR, ^{13}C NMR, and HMBC, see Table 1; HRFABMS *m/z* 471.3487 $[M - H]^-$ (calcd for $C_{30}H_{47}O_4$, 471.3474); EIMS *m/z* 248 (76), 233 (10), 203 (72), 43 (100).

3α-Hydroxyolean-11-en-28,13*β*-olide (5): obtained by HPLC as a white powder; mp 240−244 °C; $[\alpha]^{25}_D$ +45.5° (*c* 0.055, acetone); ^{1}H NMR, ^{13}C NMR, and HMBC, see Table 1; HRFABMS *m/z* 455.3516 $[M + H]^+$ (calcd for $C_{30}H_{47}O_3$, 455.3525); EIMS *m/z* 454 $[M]^+$ (63), 410 (100), 408 (54).

3α-Hydroxyoleane-11:13(18)-dien-28-oic acid (6): obtained as colorless crystals (hexane/EtOAc); mp 258−261 °C; $[\alpha]^{25}_D$ −151.6° (*c* 0.23, $CHCl_3$); ^{1}H NMR, ^{13}C NMR, and HMBC, see Table 1; HREIMS *m/z* 454.3465 (calcd for $C_{30}H_{46}O_3$, 454.3447).

Determination of Antitubercular Activity. The crude fractions and pure compounds were tested against the pathogen *M. tuberculosis* H_{37}Rv (ATCC 27294) using the BACTEC 460 radiorespirometric assay as previously described.[12,13] The crude fractions were tested at a concentration of 100 μg/mL in DMSO and a percent of inhibition, defined as 1 − (growth index of test sample/growth index of control) × 100, was calculated. The pure compounds were dissolved in DMSO and assayed in a series of 2-fold dilutions to determine MIC values. The MIC was defined as the lowest concentration of compound that inhibited 99% of the growth of the organisms.

Acknowledgment. The authors thank Dr. Arpad Somogyi for the acquisition of HRMS, Ing. Ag. Renee H. Fortunato for plant collections and taxonomic identification, and the Southwest Environmental Health Sciences Core (SWEHSC) for LC−MS experiments. This work was supported by the ICBG "Bioactive Agents from Dryland Biodiversity of Latin America" Grant 2 U01 TW 00316 from the National Institutes of Health (NIH), the National Science Foundation (NSF), and the U.S. Department of Agriculture (USDA) to B.N.T. The contents of this study are solely the responsibility of the authors and do not necessarily represent the official views of the NIH, NSF, or USDA.

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NP0002233