Antiproliferative Oleanane Saponins from *Meryta denhamii*

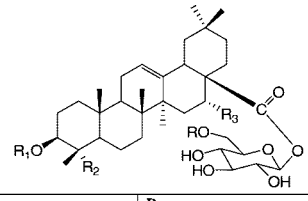
Giuseppina Cioffi,[†] Fabrizio Dal Piaz,[†] Antonio Vassallo,[†] Fabio Venturella,[‡] Paolo De Caprariis,[†] Francesco De Simone,[†] and Nunziatina De Tommasi*[,†]

Dipartimento di Scienze Farmaceutiche, Università di Salerno, Via Ponte Don Melillo, 84084 Fisciano (SA), Italy, and Dipartimento di Scienze Farmacologiche, Università di Palermo, Via Forlanini 1, 90123 Palermo, Italy

Received January 22, 2008

Eight new oleanane saponins (**1**−**8**) together with four know saponins (**9**−**12**) were isolated from the aerial parts of *Meryta denhamii*. Their structures were elucidated by 1D and 2D NMR experiments including 1D TOCSY, DQF-COSY, ROESY, HSQC, and HMBC spectroscopy, as well as ESIMS analysis. The antiproliferative activity of all compounds was evaluated using three murine and human cancer cell lines: J774.A1, HEK-293, and WEHI-164.

In our ongoing search for new bioactive compounds from Araliaceae plants growing at the Botanical Garden of Palermo,[1,2] we have performed a phytochemical study of the aerial parts of *Meryta denhamii* Seem (Araliaceae), a plant never before investigated. Plants of family Araliaceae have been used as folk remedies for the treatment of pain, rheumatic arthritis, fracture, sprains, and lumbago in Asian countries.[3] Eight new oleanane saponins (**1**−**8**) were isolated from *M. denhamii*, along with four known saponins. Since triterpenoid saponins have been reported to possess cytotoxic activity,[2,4] the antiproliferative activity of **1**−**8** was evaluated in the cell lines J774.A1, HEK-293, and WEHI-164.



	R	R_1	R_2	R_3
1	Rha-(1—>4)-Glc	HSO_3	CH_2OH	H
2	Glc	HSO_3	CH_2OH	H
3	Rha-(1—>4)-Glc_I	Glc_{IV}-(1—>4)-Glc_{III}-(1—>3)-Ara	CH_3	H
4	Rha-(1—>4)-Glc_I	Glc_{III}-(1—>3)-Ara	CH_3	H
5	Rha-(1—>4)-Glc_I	Glc_{III}-(1—>2)-Glucur	CH_3	H
6	H	Glc_{III}-(1—>2)-Glucur	CH_3	OH
7	Rha-(1—>4)-Glc_{II}-(1—>3)-Glc_I	Glc_{IV}-(1—>4)-Glc_{III}-(1—>3)-Ara	CH_3	OH
8	Rha-(1—>4)-Glc_{II}-(1—>3)-Glc_I	Glc_{IV}-(1—>4)-Glc_{III}-(1—>3)-Ara	CH_3	H
9	Rha-(1—>4)-Glc_I	HSO_3	CH_3	H
10	Glc	HSO_3	CH_3	H
11	Rha-(1—>4)-Glc_I	Ara	CH_3	H
12	H	Glc-(1—>3)-Ara	CH_3	H

Results and Discussion

The *n*-BuOH-soluble portion of a MeOH extract of the aerial parts of *M. denhamii* was subjected to Sephadex LH-20 column chromatography, followed by RP-HPLC, to afford eight new oleanane saponins (**1**−**8**) and four known saponins (**9**−**12**).

The negative-ion HRESIMS spectrum of **1** showed a single peak at *m*/*z* 1021.4652 ascribable to the molecular formula $C_{48}H_{78}O_{21}S$, suggesting the presence of a sulfate group in the molecule. The high-resolution daughter ion spectrum of this ion showed a largely predominant fragment at *m*/*z* 551.3048 [M − (162 + 162 + 146) − H]$^-$ consistent with the loss of an esterified sugar chain and indicating that the glycosidic moiety was composed of two hexose and one deoxyhexose unit. A minor peak at *m*/*z* 453.3372 [M − (162 + 162 + 146) − 98 − H]$^-$, due to elimination of H_2SO_4 from the negatively charged aglycone, confirmed the presence of a sulfate group in **1**.[5]

The ^{1}H NMR spectrum of the aglycone moiety of compound **1** showed signals corresponding to six tertiary methyls at δ 0.73, 0.83, 0.94, 0.96, 1.00, and 1.18 and a typical H3-ax at δ 3.96 (1H, dd, J = 12.4, 4.5 Hz) due to the presence of a substitution at C-3. The downfield shift observed for H-3 was indicative of substitution on the OH group.[6] Further features were a signal at δ 5.28 (1H, t, J = 3.5 Hz), ascribable to an olefinic proton, and signals at δ 3.31 (1H, d, J = 12.4 Hz) and 3.61 (1H, d, J = 12.4 Hz) ascribable to protons of a primary alcoholic function. The ^{13}C NMR chemical shifts of all protonated carbons, assigned unambiguously by the HSQC spectrum, suggested a hederagenin derivative (Table 1).[6] The sulfate group was placed at C-3 on the basis of the downfield shifts of the H-3 (δ 3.96) and C-3 (δ 82.8) signals, consistent with the presence of a sulfate group.[7,8] The sugar portion of **1** exhibited three anomeric proton resonances (δ 5.37, d, J = 7.5 Hz; 4.89, d, J = 1.6 Hz; 4.47, d, J = 7.6 Hz) and one methyl doublet (δ 1.28, d, J = 6.2 Hz), suggesting the occurrence of one deoxyhexose unit. The oligosaccharide structure was deduced using 1D TOCSY and 2D NMR experiments, which indicated that two β-glucopyranose and one α-rhamnopyranose were present (Tables 2 and 3). The configurations of the sugar units were assigned after hydrolysis of **1** with 1 N HCl. The hydrolysate was trimethylsilated, and GC retention times of each sugar were compared with those of authentic sugar samples prepared in the same manner. Glycosidation shifts were observed for C-$6_{glc\text{-}C28}$ (δ 68.7) and C-4_{glcI} (δ 79.3). Chemical shift of H-1_{glc} (δ 5.37) and C-1_{glc} (δ 95.5) indicated that this sugar unit was involved in an ester linkage with the C-28 carboxylic group.[9] On the basis of these data, compound **1** was identified as the 3-sulfate ester of 3β,23-dihydroxyolean-12-en-28-oic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

The molecular formula of **2** was established unequivocally to be $C_{42}H_{68}O_{17}S$ on the basis of the HRESIMS negative-ion spectrum, showing an ion peak at *m*/*z* 875.4108. The high-resolution product ion spectrum of this species generated the same fragments detected in the MS/MS spectrum of **1** (*m*/*z* 551.3045 and 453.3374), suggesting that **1** and **2** had the same aglycone, but that the esterified sugar chain of **2** consisted of only two hexose.

The NMR data (^{1}H, ^{13}C, 1D TOCSY, DQF-COSY, HSQC, HMBC) of **2** in comparison to those of **1** revealed that compound **2** differed from **1** only in the absence of the terminal α-L-rhamnopyranosyl unit. The configuration of the sugar unit was assigned after hydrolysis of **2** with 1 N HCl. Thus, **2** was identified

* To whom correspondence should be addressed. Tel: +39-089-969754. Fax: +39-089-969602. E-mail: detommasi@unisa.it.
[†] Università di Salerno.
[‡] Università di Palermo.

10.1021/np8000464 CCC: $40.75
Published on Web 04/29/2008

Table 1. ^{13}C NMR Data of the Aglycon Moieties of Compounds **1**, **3**, and **6** (CD_3OD, 150 MHz)[a]

position	**1** δ_C	**3** δ_C	**6** δ_C
1	39.5	39.9	39.8
2	25.0	26.6	26.6
3	82.8	91.0	91.0
4	43.0	39.0	39.0
5	48.5	57.0	57.2
6	18.2	19.5	18.5
7	32.5	33.7	34.2
8	41.0	40.3	40.6
9	48.2	48.8	48.2
10	37.9	37.7	37.8
11	24.0	24.6	23.8
12	124.0	124.3	123.7
13	144.3	144.2	144.0
14	43.2	42.3	42.0
15	28.0	28.2	36.6
16	24.7	23.5	74.6
17	47.6	47.0	50.0
18	42.5	42.8	41.9
19	47.0	46.7	47.7
20	31.3	30.8	30.8
21	34.0	34.5	33.0
22	33.5	34.0	36.0
23	65.2	28.0	29.3
24	64.4	16.5	17.8
25	18.5	16.0	16.6
26	18.0	18.0	16.0
27	26.0	26.2	27.7
28	178.3	178.2	177.6
29	33.5	33.4	33.0
30	24.5	23.8	24.5

[a] Chemical shifts are given in δ (ppm); assignments were confirmed by HSQC and HMBC experiments.

as the 3-sulfate ester of 3β,23-dihydroxyolean-12-en-28-oic acid 28-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside.

Compound **3** was assigned a molecular formula of $C_{65}H_{106}O_{31}$, as determined by positive-ion HRESIMS (*m/z* 1405.6588 [M + Na]$^+$). Tandem mass spectra obtained by collision-induced dissociation (CID) of ions at *m/z* 1405.7 showed fragments at *m/z* 1259.6 [M − 146 + Na]$^+$ and 1097.5 [M − (146 + 162) + Na]$^+$ and a predominant peak at *m/z* 935.5 [M − (146 + 162 + 162) + Na]$^+$, due to the loss of one deoxyhexose and two hexose moieties, respectively. An intense signal at *m/z* 493.1 was also observed, consistent with a sodiated three-sugar chain [(146 + 162 + 162) + Na]$^+$. These results suggested that **3** had an esterified sugar chain composed of a deoxyhexose and two hexose units. Fragment ions detected at *m/z* 773.4 [M − (146 + 162 + 162) − 162 + Na]$^+$ and 611.3 [M − (146 + 162 + 162) − (162 + 162) + Na]$^+$ demonstrated that a second glycoside chain was also present in **3**.

The ^{13}C NMR spectrum of **3** displayed 65 carbon resonances, of which 30 were assigned to the aglycone and 35 to the sugar moiety. The ^{13}C NMR spectrum (Table 2 and Experimental Section) suggested a triterpenoid glycoside structure. The ^{13}C NMR spectrum of the aglycone portion exhibited resonances assigned to seven tertiary methyl, two sp^2-hybridized, one hydroxymethine (δ 91.0), and one carboxylic (δ 178.2) carbons (Table 1). The combined NMR data indicated that the aglycone of **3** was oleanolic acid.[10] In compound **3**, C-28 appeared at δ 178.2 in the ^{13}C NMR spectrum and H-18 appeared at δ 2.92 (dd, J = 12.0 and 4.0 Hz) in the ^1H NMR spectrum, indicating that the carboxyl group was glycosilated. Attachment of another glycoside chain at C-3 was suggested by the significant downfield shift observed for this carbon in **3** relative to the corresponding signal in oleanolic acid derivatives.[10] Assignments of all NMR signals of the aglycone portion were ascertained from a combination of 1D TOCSY, DQF-COSY, and HSQC experiments.

The sugar portion of **3** exhibited six anomeric proton resonances (δ 5.36, d, J = 7.5 Hz; 4.90, d, J = 1.6 Hz; 4.68, d, J = 7.6 Hz; 4.60, d, J = 7.5 Hz, 4.48, d, J = 7.6 Hz; 4.36, d, J = 5.5 Hz) and one methyl doublet (δ 1.30, d, J = 6.2 Hz) in the ^1H NMR spectrum (Table 3). The 1D TOCSY and 2D NMR experiments indicated that four β-glucopyranose, one α-arabinopyranose, and one α-rhamnopyranose groups were present (Tables 2 and 3). The configurations of the sugar units were assigned after hydrolysis of **3** with 1 N HCl.

Table 2. ^{13}C NMR Data for Glycosyl Moieties of Compounds **1**−**7** (CD_3OD, 600 MHz)[a]

position	**1** δ_C	**2** δ_C	**3** δ_C	**4** δ_C	**5** δ_C	**6** δ_C	**7** δ_C
GlcC28 1	95.5	95.5	95.7	95.7	95.5	95.3	95.5
2	76.0	73.4	75.6	75.5	75.9	75.0	75.4
3	77.6	77.7	77.8	77.6	77.6	77.8	77.8
4	70.6	70.1	71.0	70.8	70.5	71.0	71.0
5	77.0	77.6	76.9	76.7	77.0	78.1	77.0
6	68.7	69.0	69.0	68.6	69.0	62.3	68.9
GlcI 1	104.1	104.3	103.9	104.0	104.0		104.2
2	74.9	72.3	75.0	74.9	75.0		75.0
3	77.7	78.3	77.0	77.8	77.5		86.3
4	79.3	71.2	79.3	79.3	78.8		70.3
5	77.8	77.9	77.2	77.0	76.9		77.6
6	62.0	62.2	62.0	62.0	62.1		62.0
Rha 1	102.8		102.5	102.6	102.3		102.4
2	72.0		72.2	72.3	72.4		72.2
3	71.7		71.9	72.0	71.9		72.0
4	73.4		73.5	73.7	73.7		73.6
5	70.2		70.3	70.5	70.5		70.0
6	18.0		18.1	18.2	18.3		18.2
GlcII 1							103.1
2							75.2
3							77.3
4							79.0
5							77.0
6							61.9
Ara 1			107.4	107.4			107.0
2			71.8	71.8			71.6
3			83.0	82.6			82.8
4			69.0	69.0			69.0
5			66.0	66.0			66.0
GlcIII 1			103.7	104.9	104.4	104.2	104.0
2			76.0	75.8	75.0	74.9	76.0
3			77.0	78.0	77.5	77.8	76.8
4			79.5	70.5	72.0	71.0	79.3
5			77.0	78.1	77.8	78.0	77.1
6			61.5	62.0	62.6	62.4	61.8
GlcIV1			104.4				104.3
2			75.0				75.5
3			77.5				78.0
4			72.0				71.1
5			77.8				77.8
6			62.6				62.6
Glucur 1					105.1	105.1	
2					82.0	82.0	
3					76.5	76.5	
4					72.0	71.7	
5					77.0	76.8	
6					176.0	175.6	

[a] Chemical shifts are given in δ (ppm); assignments were confirmed by HSQC and HMBC experiments.

The absence of any ^{13}C NMR glycosidation shift for the α-L-rhamnopyranosyl and one of the β-glucopyranosyl moieties indicated that these sugars were terminal units. Glycosidation shifts were observed for C-6$_{glc\text{-}C28}$ (δ 69.0), C-4$_{glcI}$ (δ 79.3), C-3$_{ara}$ (δ 83.0), and C-4$_{glcIII}$ (δ 79.5) (Table 2). The chemical shifts of H-1$_{glc}$ (δ 5.36) and C-1$_{glc}$ (δ 95.7) indicated that this sugar unit was involved in an ester linkage with the C-28 carboxylic group.

Direct evidence for the sugar sequence and their linkage sites to the aglycone was derived from the HMBC experiment, which showed unequivocal correlations between resonances at δ 4.36 and δ 91.0 (H-1$_{ara}$−C-3), indicating that arabinose was linked to C-3 of the aglycone; a cross-peak between δ 4.60 and δ 83.0 (H-1$_{glcIII}$−C-3$_{ara}$) indicated that glucose III was the second unit,

Table 3. ^{1}H NMR Data for Glycosyl Moieties of Compounds **1**−**7** (CD_3OD, 600 MHz)[a]

position	**1** δ_H	**2** δ_H	**3** δ_H	**4** δ_H	**5** δ_H	**6** δ_H	**7** δ_H
GlcC28 1	5.37 d (7.5)	5.39	5.36 d (7.5)	5.41	5.37 d (7.5)	5.36	5.37 d (7.5)
2	3.38 dd (9.5, 7.5)	3.32	3.35 dd (9.5, 7.5)	3.38	3.38 dd (9.5, 7.5)	3.32	3.40 dd (9.5, 7.5)
3	3.46 t (9.5)	3.44	3.41 t (9.5)	3.47	3.46 t (9.5)	3.64	3.45 t (9.5)
4	3.42 t (9.5)	3.47	3.46 t (9.5)	3.45	3.48 t (9.5)	3.71	3.48 t (9.5)
5	3.58 m	3.60	3.58 m	3.58	3.60 m	3.40	3.59 m
6a	3.85 dd (12.0, 5.0)	3.86	3.84 dd (12.0, 5.0)	3.83	3.85 dd (12.0, 5.0)	3.68	3.89 dd (12.0, 5.0)
6b	4.12 dd (12.0, 3.0)	4.13	4.10 dd (12.0, 3.0)	4.15	4.12 dd (12.0, 3.0)	3.84	4.10 dd (12.0, 3.0)
GlcI 1	4.47 d (7.6)	4.50	4.48 d (7.6)	4.47	4.47 d (7.6)		4.49 d (7.6)
2	3.21 dd (9.5, 7.6)	3.27	3.18 dd (9.5, 7.6)	3.21	3.21 dd (9.5, 7.6)		3.43 dd (9.5, 7.6)
3	3.53 t (9.5)	3.39	3.50 t (9.5)	3.53	3.53 t (9.5)		3.64 t (9.5)
4	3.62 t (9.5)	3.30	3.65 t (9.5)	3.62	3.64 t (9.5)		3.60 t (9.5)
5	3.30 m	3.42	3.32 m	3.38	3.32 m		3.30 m
6a	3.73 dd (12.0, 5.0)	3.68	3.70 dd (12.0, 5.0)	3.73	3.73 dd (12.0, 5.0)		3.73 dd (12.0, 5.0)
6b	3.84 dd (12.0, 3.0)	3.88	3.81 dd (12.0, 3.0)	3.84	3.84 dd (12.0, 3.0)		3.80 dd (12.0, 3.0)
Rha 1	4.89 d (1.6)		4.90 d (1.6)	4.89	4.89 d (1.6)		4.90 d (1.6)
2	3.88 dd (3.0, 1.6)		3.86 dd (3.0, 1.6)	3.89	3.90 dd (3.0, 1.6)		3.82 dd (3.0, 1.6)
3	3.70 dd (9.0, 3.0)		3.69 dd (9.0, 3.0)	3.70	3.70 dd (9.0, 3.0)		3.73 dd (9.0, 3.0)
4	3.48 t (9.0)		3.49 t (9.0)	3.48	3.48 t (9.0)		3.48 t (9.0)
5	4.03 m		4.05 m	4.03	4.03 m		4.05 m
6	1.28 d (6.2)		1.30 d (6.2)	1.28	1.28 d (6.2)		1.30 d (6.2)
GlcII 1							4.52 d (7.6)
2							3.23 dd (9.5, 7.6)
3							3.50 t (9.5)
4							3.60 t (9.5)
5							3.36 m
6							3.70 dd (12.0, 5.0)
							3.81 dd (12.0, 3.0)
Ara 1			4.36 d (5.5)	4.38			4.34 d (6.0)
2			3.74 dd (5.5, 9.4)	3.77			3.71 dd (5.5, 9.4)
3			3.78 dd (1.5, 3.0)	3.80			3.77 dd (1.5, 3.0)
4			4.10 m	4.09			4.05 m
5a			3.94 dd (12.0, 3.5)	3.96			3.91 dd (12.0, 3.5)
5b			3.63 dd (12.0, 2.0)	3.65			3.62 dd (12.0, 2.0)
GlcIII 1			4.60 d (7.5)	4.60	4.62 d (7.5)	4.60	4.62 d (7.5)
2			3.32 dd (9.5, 7.5)	3.45	3.25 dd (9.5, 7.5)	3.27	3.32 dd (9.5, 7.5)
3			3.60 t (9.5)	3.50	3.38 t (9.5)	3.40	3.57 t (9.5)
4			3.64 t (9.5)	3.42	3.30 t (9.5)	3.29	3.67 t (9.5)
5			3.38 m	3.48	3.42 m	3.42	3.41 m
6			3.73 dd (12.0, 5.0)	3.70	3.70 dd (12.0, 5.0)	3.72	3.75 dd (12.0, 5.0)
			3.84 dd (12.0, 3.0)	3.90	3.87 dd (12.0, 3.0)	3.88	3.88 dd (12.0, 3.0)
GlcIV1			4.68 d (7.6)				4.70 d (7.5)
2			3.30 dd (9.5, 7.6)				3.27 dd (9.5, 7.6)
3			3.34 t (9.5)				3.30 t (9.5)
4			3.32 t (9.5)				3.33 t (9.5)
5			3.40 m				3.38 m
6			3.68 dd (12.0, 5.0)				3.70 dd (12.0, 5.0)
			3.83 dd (12.0, 3.0)				3.82 dd (12.0, 3.0)
Glucur 1					4.47 d (7.5)	4.47	
2					3.66 dd (9.5, 7.5)	3.65	
3					3.55 t (9.5)	3.55	
4					3.50 t (9.5)	3.52	
5					3.59 m	3.58	
6							

[a] *J* values are in parentheses and reported in Hz; chemical shifts are given in δ (ppm); assignments were confirmed by DQF-COSY, 1D TOCSY, HSQC, and HMBC experiments.

and a cross-peak between δ 4.68 and δ 79.5 (H-1_{glcIV}−C-4_{glcIII}) indicated that glucose IV was the terminal unit of the trisaccharide chain at C-3. Similarly the sequence of the trisaccharide chain at C-28 was indicated by the cross-peaks between C-6_{glc} (δ 69.0) and H-1_{glcI} (δ 4.48) and between C-4_{glcI} (δ 79.3) and H-1_{rha} (δ 4.90). Thus, compound **3** was identified as 3β-*O*-[β-D-glucopyranosyl-(1→4)-β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl] olean-12-en-28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester.

Compound **4** was obtained as an amorphous powder with the molecular formula $C_{59}H_{96}O_{26}$ as deduced from the HRESIMS spectrum and confirmed by ^{13}C NMR and ^{13}C DEPT data. The MS/MS spectrum of compound **4** showed a prominent fragment at *m/z* 773.4 $[M - (146 + 162 + 162) + Na)]^+$. The spectroscopic data of the aglycone moiety of **4** were identical to those of **3**. The proton coupling network within each sugar residue was established, using a combination of 1D TOCSY, DQF-COSY, and HSQC experiments. Once again, direct evidence for the sugar sequence and the linkage sites was derived from the HSQC and HMBC data. Comparison of NMR data of the sugar moieties (Tables 2 and 3) of **4** with those of **3** indicated that **4** differed from **3** only by the absence of a terminal glucopyranosyl moiety on the C-3 sugar chain. The structures of the sugar units were determined as reported for compound **3**. Thus, compound **4** was defined as 3β-*O*-[β-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl] olean-12-en-28-*O*-[α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester.

Compound **5** was obtained as an amorphous powder ($C_{60}H_{96}O_{28}$) as deduced by HRESIMS and confirmed by ^{13}C and ^{13}C DEPT NMR data. The proton coupling network within each sugar residue was established, using a combination of 1D TOCSY, DQF-COSY, and HSQC experiments. Direct evidence for the sugar sequence

and the linkage sites was derived from the HSQC and HMBC data. Comparison of NMR data of the sugar moiety (Tables 2 and 3) of **5** with those of **4** indicated that **5** differed from **4** only by the presence of one glucuronopyranosyl unit instead of one arabinopyranosyl moiety on the C-3 sugar chain. The configuration of the sugar units was determined as reported for compound **1**. Thus, compound **5** was defined as 3*β*-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl] olean-12-en-28-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester.

Compound **6** (molecular formula $C_{48}H_{76}O_{20}$) showed a quasi-molecular ion peak at *m*/*z* 971.4842 $[M - H]^-$ in the negative HRESIMS. The ^{13}C and ^{13}C DEPT spectra showed 48 resonances, of which 30 were assigned to the aglycone and 18 to the sugar portion. The ^{13}C NMR spectra showed, for the aglycone moiety, signals that could be correlated unambiguously to the corresponding proton chemical shifts from the HSQC experiment, leading to the identification of the aglycone as echinocystic acid (Table 1).[11] Analysis of the NMR data (Tables 2 and 3) of compound **6** and comparison with those of **5** revealed **6** to differ from **5** only in the sugar chain at C-28. The structure of the sugar chain at C-28 was deduced using 1D TOCSY and COSY experiments, leading to the identification of one glucopyranosyl moiety. Thus, compound **6** was defined as 3*β*-*O*-[*β*-D-glucopyranosyl-(1→2)-*β*-D-glucuronopyranosyl]echinocystic acid 28-*O*-*β*-D-glucopyranosyl ester.

Compound **7** had the molecular formula $C_{71}H_{116}O_{37}$, as determined by ^{13}C and ^{13}C-DEPT NMR data and the $[M + Na]^+$ peak at *m*/*z* 1583.8 in the ESIMS spectrum. The ^{13}C and ^{13}C DEPT NMR spectra showed 71 signals, of which 30 were assigned to a triterpenoid moiety and 41 to the saccharide portion. The spectroscopic analysis revealed the presence of a carboxylic group located at C-28 and was in agreement with echinocystic acid as the aglycone of compound **7**.[11] Seven anomeric protons were observed in the ^1H NMR spectrum of **7** at *δ* 4.34 (d, *J* = 6.0 Hz), 4.49 (d, *J* = 7.6 Hz), 4.52 (d, *J* = 7.6 Hz), 4.62 (d, *J* = 7.5 Hz), 4.70 (d, *J* = 7.5 Hz), 4.90 (d, *J* = 1.6 Hz), and 5.37 (d, *J* = 7.5 Hz). The chemical shifts of all the individual protons of the seven sugar units were ascertained from a combination of 1D TOCSY and DQF-COSY spectral analysis, and the ^{13}C NMR chemical shifts (Table 2) of their attached carbons were assigned unambiguously from the HSQC spectrum. In this way, the sugar units were identified as one α-arabinopyranoside, one α-rhamnopyranoside, and five *β*-glucopyranoside units.[12] In the HSQC experiment, glycosidation shifts were observed for C-6_{glc} (*δ* 68.9), C-3_{glcI} (*δ* 86.3), C-4_{glcII} (*δ* 79.0), C-3_{ara} (*δ* 82.8), and C-4_{glcIII} (*δ* 79.3). The absence of any glycosidation shifts for one glycopyranosyl unit and one rhamnopyranoside suggested that these sugars were terminal units. The anomeric carbon signal at *δ* 95.5 (C-1_{glc}) and the carbonyl signal at *δ* 177.6 revealed the presence of an ester glycosidic linkage between C-28 and one glucopyranosyl unit.[9] Direct evidence for the sugar chain linked at C-3 was derived from the results of the HMBC experiment, which showed unequivocal correlation peaks between H-1_{ara}−C-3, H-1_{glcIII}−C-3_{ara}, and H-1_{glcIV}−C-4_{glcIII}. Similarly, the sequence of the tetrasaccharide chain at C-28 was indicated by the cross-peaks between H-1_{rha}−C-4_{glcII}, H-1_{glcII}−C-3_{glcI}, and H-1_{glcI}−C-6_{glc}. The configuration of the sugar units was determined as reported for compound **1**. Thus, compound **7** was established as 3*β*-*O*-[*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl]echinocystic acid 28-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester.

Compound **8** ($C_{71}H_{116}O_{36}$) was identified as a further triterpene derivative possessing saccharide chains at C-3 and C-28. Comparison of NMR spectroscopic data of compound **8** (Experimental Section) with those of compound **7** showed these to be identical in the sugar portion but different in the aglycone. The spectroscopic data for the aglycone moiety of **8** were identical to those of compound **3**. Therefore, the structure 3*β*-*O*-[*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-α-L-arabinopyranosyl] olean-12-en-28-*O*-[α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranosyl] ester was assigned to **8**.

Table 4. In Vitro Antiproliferative Activity of Compounds **1**−**12**[a]

	cell line (IC_{50} μM)		
compound	J774.A1[b]	HEK-293[c]	WEHI-164[d]
1	nd[e]	nd[e]	nd[e]
2	nd[e]	nd[e]	nd[e]
3	3.0 ± 0.001	nd[e]	nd[e]
4	3.6 ± 0.007	nd	7 ± 0.03
5	6.9 ± 0.012	nd[e]	nd[e]
6	0.19 ± 0.001	0.23 ± 0.012	0.34 ± 0.036
7	0.32 ± 0.007	1.3 ± 0.021	1.7 ± 0.023
8	9.0 ± 0.032	nd[e]	nd[e]
9	nd[e]	nd[e]	nd[e]
10	nd[e]	nd[e]	nd[e]
11	2.5 ± 0.022	nd[e]	3.4 ± 0.03
12	3.9 ± 0.022	nd[e]	4.6 ± 0.03
6-MP[f]	0.003 ± 0.005	0.007 ± 0.004	0.015 ± 0.006

[a] The IC_{50} value is the concentration of compound that affords a 50% reduction in cell growth (after a 3-day incubation). [b] J774.A1 = murine monocyte/macrophage cell lines. [c] HEK-293 = human epithelial kidney cell lines. [d] WEHI-164 = murine fibrosarcoma cell lines. [e] nd = not detectable. [f] 6-MP = 6-mercaptopurine.

The four known triterpene glycosides were identified as the 3-sulfate ester of 3*β*-hydroxyolean-12-en-28-oic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (**9**),[13] 3-sulfate ester of 3*β*-hydroxyolean-12-en-28-oic acid 28-*O*-[*β*-D-glucopyranosyl-(1→6)]-*β*-D-glucopyranoside (**10**),[14] 3*β*-*O*-α-L-arabinopyranosylolean-12-en-28-*O*-α-L-rhamnopyranosyl-(1→4)-*β*-D-glucopyranosyl-(1→6)-*β*-D-glucopyranoside (**11**),[15] and 3*β*-*O*-α-L-arabinopyranosyl-(1→2)-*β*-D-glucopyranosylolean-12-en-28-*O*-*β*-D-glucopyranoside (**12**),[16] by detailed NMR and MS analyses and comparison with literature data.

The antiproliferative activity of compounds **1**−**12** was evaluated against the J774.A1, WEHI-164, and HEK-293 cell lines (Table 4). Sulfate derivatives **1**, **2**, **9**, and **10** were completely inactive. Compounds **6** and **7**, having echinocystic acid as the aglycone, were the most active constituents, while compounds **3**−**5**, **8**, **11**, and **12**, having oleanolic acid as the aglycone, were low in activity. Absence of an α-OH function at C-16 of the oleanane skeleton reduced the antiproliferative activity, as was observed for similar derivatives tested previously.[12]

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 241 polarimeter equipped with a sodium lamp (589 nm) and a 1 dm microcell. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. 2D NMR spectra were acquired in CD_3OD in the phase-sensitive mode with the transmitter set at the solvent resonance and TPPI (time proportional phase increment) used to achieve frequency discrimination in the ω_1 dimension. The standard pulse sequence and phase cycling were used for DQF-COSY, TOCSY, HSQC, HMBC, and ROESY experiments. The NMR data were processed on a Silicon Graphics Indigo2 Workstation using UXNMR software. ESIMS and MS/MS spectra (positive and negative mode) were obtained from an LCQ Advantage ThermoFinnigan spectrometer, equipped with Xcalibur software. HRESIMS and HRESIMS/MS spectra were acquired in positive- and negative-ion mode using a Q-TOF Premier spectrometer equipped with a nanospray source. Column chromatography was performed on Sephadex LH-20. HPLC separations were conducted using a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and Shimadzu injector on a C_{18} *μ*-Bondapak column (30 cm × 7.8 mm, flow rate 2.0 mL min^{-1}). GC analyses were performed using a Dani GC 1000 instrument on a L-CP-Chirasil-Val column (0.32 mm × 25 m).

Plant Material. The aerial parts of *M. denhamii* were collected in Palermo, Italy, during April 2002 and were identified by Prof. Giuseppe

Venturella of the Dipartimento di Scienze Botaniche, University of Palermo, Italy, where a voucher specimen (PAL 70577) is deposited.

Extraction and Isolation. The dried, powdered *M. denhamii* plant material (400 g) was defatted with *n*-hexane and then extracted with MeOH (3 × 2 L) to give 22.5 g of extract. The MeOH extract was dissolved in H_2O, and this was partitioned between *n*-BuOH and AcOEt, to afford an *n*-BuOH-soluble portion (9 g) and AcOEt portion (5 g). A portion of the *n*-BuOH residue (4.0 g) was separated on a Sephadex LH-20 column, using MeOH as eluent. Fractions were collected, analyzed by TLC (silica 60 F_{254} gel-coated glass sheets with *n*-BuOH−HOAc−H_2O (60:15:25) and $CHCl_3$−MeOH−H_2O (40:9:1)), and grouped to obtain eight fractions (1−8).

Fraction 2 (120 mg) was purified by preliminary SPE followed by RP-HPLC with MeOH−H_2O (3:2) to afford compounds **7** (8.0 mg) and **8** (14.0 mg). Fraction 3 (173 mg) was chromatographed using RP-HPLC (MeOH−H_2O (3:2)) to yield compounds **3** (6.5 mg), **4** (22 mg), and **11** (10 mg). Fractions 4 (80 mg) and 5 (30 mg) were subjected to RP-HPLC with MeOH−H_2O (1:1) to yield compounds **5** (4.5 mg) and **6** (12 mg) from fraction 4, and **12** (22 mg) from fraction 5, respectively. Fraction 6 (250 mg) was purified by preliminary SPE followed by RP-HPLC with MeOH−H_2O (1:1) to afford compounds **1** (30.0 mg), **2** (9.0 mg), **9** (8.0 mg), and **10** (8.0 mg).

Compound 1: amorphous powder; $[\alpha]_D^{25}$ +1.12 (*c* 0.1, MeOH); ^{1}H NMR data of the aglycone (CD_3OD, 600 MHz) δ 0.73 (3H, s, Me-23), 0.83 (3H, s, Me-25), 0.94 (3H, s, Me-29), 0.96 (3H, s, Me-30), 1.00 (3H, s, Me-26), 1.18 (3H, s, Me-27), 3.96 (1H, dd, J = 12.4, 4.5 Hz, H-3); 3.31 (1H, d, J = 12.4 Hz, H-23a), 3.61(1H, d, J = 12.4 Hz, H-23b), 5.28 (1H, t, J = 3.5 Hz, H-12); ^{13}C NMR data of the aglycone, see Table 1; ^{1}H and ^{13}C NMR of the sugar moiety, see Tables 2 and 3; HRESIMS *m/z* 1021.4652 $[M - H]^-$ (calcd for $C_{48}H_{77}O_{21}S$: 1021.4669); HRESIMS/MS (parent ion 1021.4652) *m/z* 551.3048 [M − (146 + 162 + 162) − H]$^-$ (calcd for $C_{30}H_{47}O_7S$, 551.3043), 453.3372 [M − (146 + 162 + 162) − 98 − H]$^-$ (calcd for $C_{30}H_{45}O_3$, 453.3369).

Compound 2: amorphous powder; $[\alpha]_D^{25}$ +15.2 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR data of the aglycone were superimposable on those of **1**; ^{1}H and ^{13}C NMR of the sugar moiety, see Tables 2 and 3; HRESIMS *m/z* 875.4108 $[M - H]^-$ (calcd for $C_{42}H_{67}O_{17}S$, 875.4090); HRESIMS/MS (parent ion 875.4108) *m/z* 551.3045 [M − (162 + 162) − H]$^-$ (calcd for $C_{30}H_{47}O_7S$, 551.3043), 453.3374 [M − (162 + 162) − 98 − H]$^-$ (calcd for $C_{30}H_{45}O_3$, 453.3369).

Compound 3: amorphous powder; $[\alpha]_D^{25}$ +39.9 (*c* 0.1, MeOH); $_1$H NMR data of the aglycone (see ref 17); ^{13}C NMR data of the aglycone, see Table 1; ^{1}H and ^{13}C NMR of the sugar moieties, see Tables 2 and 3; HRESIMS *m/z* 1405.6588 $[M + Na]^+$ (calcd for $C_{65}H_{106}O_{31}Na$, 1405.6616); MS/MS (parent ion 1405.7) *m/z* 1259.6 [M − 146 + Na]$^+$, 1097.5 [M − (146 + 162) + Na]$^+$, 935.5 [M − (146 + 162 + 162) + Na]$^+$, 773.4 [M − (146 + 162 + 162) − 162 + Na]$^+$, 611.3 [M − (1462 + 162 + 162) − (162 + 162) + Na]$^+$, 493.1 [(146 + 162 + 162) + Na]$^+$.

Compound 4: amorphous powder; $[\alpha]_D^{25}$ 42.5 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR of the aglycone were superimposable on those of **3**; ^{1}H and ^{13}C NMR of the sugar moieties, see Tables 2 and 3; HRESIMS *m/z* 1243.6039 $[M + Na]^+$ (calcd for $C_{59}H_{96}O_{26}Na$, 1243.6088); MS/MS (parent ion 1243.6) *m/z* 1097.4 [M − 146 + Na)]$^+$, 935.4 [M − (146 + 162) + Na)]$^+$, 773.4 [M − (146 + 162 + 162) + Na)]$^+$.

Compound 5: amorphous powder; $[\alpha]_D^{25}$ 23.6 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR of the aglycone were superimposable on those of **3**; ^{1}H and ^{13}C NMR of the sugar moieties, see Tables 2 and 3; HRESIMS *m/z* 1263.6018 $[M - H]^-$ (calcd for $C_{60}H_{95}O_{28}$, 1263.6008); MS/MS (parent ion 1263.6) *m/z* 793.5 [M − (146 + 162 + 162) − H]$^-$.

Compound 6: amorphous powder; $[\alpha]_D^{25}$ 40.3 (*c* 0.1, MeOH); ^{1}H NMR data of the aglycone, see ref 6; ^{13}C NMR data of the aglycone, see Table 1; ^{1}H and ^{13}C NMR of the sugar moieties, see Tables 2 and 3; HRESIMS *m/z* 971.4842 $[M - H]^-$ (calcd for $C_{48}H_{75}O_{20}$, 971.4852); MS/MS (parent ion 971.5) *m/z* 809.4 [M − 162 − H]$^-$.

Compound 7: amorphous powder; $[\alpha]_D^{25}$ +36.0 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR of the aglycone, see ref 6; ^{1}H and ^{13}C NMR of the sugar moieties, see Tables 2 and 3; ESIMS *m/z* 1583.8 $[M + Na]^+$ (calcd for $C_{71}H_{116}O_{37}Na$, 1583.7182).

Compound 8: amorphous powder; $[\alpha]_D^{25}$ 45.1 (*c* 0.1, MeOH); ^{1}H and ^{13}C NMR of the aglycone were superimposable with those of compound **3**; ^{1}H and ^{13}C NMR of the sugar moieties were superimposable with those of compound **7**; ESIMS *m/z* 1567.8 $[M + Na]^+$ (calcd for $C_{71}H_{116}O_{36}Na$, 1567.7182).

Acid Hydrolysis of Compounds 1−8. A solution of each compound (2.0 mg) in 1 N HCl (1 mL) was stirred at 80 °C in a stoppered vial for 4 h. After cooling, the solution was evaporated under a stream of N_2. Each residue was dissolved in 1-(trimethylsilyl)imidazole and pyridine (0.2 mL), and the solution was stirred at 60 °C for 5 min. After drying the solution, the residue was partitioned between H_2O and $CHCl_3$. The $CHCl_3$ layer was analyzed by GC using a L-CP-Chirasil-Val column (0.32 mm × 25 m). Temperatures of the injector and detector were 200 °C for both. A temperature gradient system was used for the oven, starting at 100 °C for 1 min and increasing up to 180 °C at a rate of 5 °C/min. Peaks of the hydrolysate were detected by comparison with retention times of authentic samples of D-glucuronic acid, D-glucose, L-arabinose, and L-rhamnose (Sigma Aldrich) after treatment with 1-(trimethylsilyl)imidazole in pyridine.

Antiproliferative Assay. The J774.A1 murine monocyte/macrophage, WEHI-164 murine fibrosarcoma, and HEK-293 human epithelial kidney cells were grown as reported previously.[18] All reagents for cell culture were from Hy-Clone (Euroclone, Paignton Devon, U.K.); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2*H*-tetrazolium bromide] and 6-mercaptopurine (6-MP) were from Sigma Chemicals (Milan, Italy). The J774.Al, WEHI-164, and HEK-293 (3.4×10^4 cells) were plated on 96-well microtiter plates and allowed to adhere at 37 °C in 5% CO_2 and 95% air for 2 h. The medium was then replaced with 50 μL of fresh medium, a 75 μL aliquot of 1:4 serial dilution of each test compound was added, and then the cells were incubated for 72 h. In some experiments, serial dilutions of 6-MP were added. The cell viability was assessed through an MTT conversion assay.[18–20] The optical density (OD) of each well was measured with a microplate spectrophotometer (Titertek Multiskan MCC/340) equipped with a 620 nm filter. The viability of each cell line in response to treatment with tested compounds and 6-MP was calculated as % dead cells = 100 − (OD treated/OD control) × 100. Table 4 shows the results expressed as IC_{50} values (μM), the concentration that inhibited cell growth by 50% as compared to the control.

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NP8000464