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Antiproliferative Oleanane Saponins from *Dizygotheca elegantissima*

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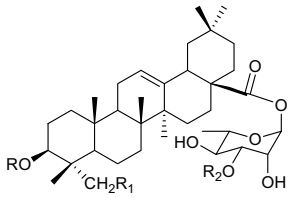
Four new oleanane saponins (**1**-**4**), together with three known saponins, were isolated from the aerial parts of *Dizygotheca elegantissima* R. Vig. & Guillaumin. Their structures were elucidated by 1D and 2D NMR experiments including 1D TOCSY, DQF-COSY, HSQC, and HMBC spectroscopy, as well as ESIMS analysis. The antiproliferative activity of all isolated compounds was evaluated.

Keywords: *Dizygotheca elegantissima*, Araliaceae, Triterpenes, Glycosides, NMR.

In our ongoing search for new bioactive compounds from plants belonging to the family Araliaceae growing at the Botanical Garden of Palermo [1-3], we have performed a phytochemical study of the aerial parts of *Dizygotheca elegantissima* R. Vig. & Guillaumin [4]. Plants of the family Araliaceae have been used in Asian countries as folk remedies for treatment of pain, rheumatic arthritis, fracture, sprains, and lumbago [5,6]. Four new oleanane saponins (**1**-**4**) were isolated from *D. elegantissima*, along with three known saponins. Taking into account the fact that some triterpene saponins have been found to possess cytotoxic activity against various tumor cell lines [2,7], the antiproliferative activity of **1**-**7** was evaluated [8].



	R	R_1	R_2
1	Glc-(1→2)-Glc	H	H
2	Glc-(1→2)-Glc	H	Ac
3	Glc-(1→6)-Glc-(1→2)-Xyl	H	H
4	Glc-(1→6)-Glc-(1→2)-Xyl	OH	H

Compound **1** was assigned the molecular formula $C_{48}H_{78}O_{17}$, as determined by ^{13}C, ^{13}C DEPT NMR, and positive HRESIMS (*m/z* 927.5311 $[M + H]^+$). The ESIMS of **1** showed a $[M + H]^+$ ion at *m/z* 927 and prominent fragments at *m/z* 781 $[M - 146 + H]^+$ and 765 $[M - 162 + H]^+$, due to the loss of one deoxyhexose and one hexose units, respectively, and *m/z* 457 $[M – (162 + 162 + 146) + H]^+$, due to the loss of one deoxyhexose and two hexose units. A peak at *m/z* 457 was attributed to the aglycone moiety. Data from the ^{13}C NMR spectrum (see Experimental Section and Table 1) suggested a triterpenoid glycoside structure. The ^1H NMR spectrum of the aglycone portion of **1** showed signals for seven tertiary methyl groups (δ 0.81, 0.86, 0.95, 0.97, 1.05, 1.10, 1.24), a typical signal of H-3ax at δ 3.22 (dd, *J*=12.0, 4.5 Hz), and a characteristic olefinic proton at δ 5.33 (t, *J*=3.5 Hz). The ^{13}C NMR spectrum showed for the aglycone moiety 30 signals that could be correlated unambiguously to the corresponding proton chemical shifts from the HSQC experiment, leading to the identification of the aglycone as oleanolic acid [3].

The sugar portion of **1** exhibited, in the ^1H NMR spectrum (Table 2), three anomeric proton signal resonances (δ 5.90, d, *J* = 1.8 Hz; 4.50, d, *J* = 7.8 Hz; 4.35, d, *J* = 8.0 Hz) and one methyl doublet (δ 1.28, d, *J* = 6.5 Hz). The 1D TOCSY and 2D NMR experiments indicated that two β-glucopyranose, and one α-rhamnopyranose moieties were present (Tables 1 and 2). The configurations of the sugar units were assigned after hydrolysis of **1** with 1N 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 [7]. The absence of any ^{13}C NMR glycosidation shift for the α-L-rhamnopyranosyl, and one of the β-D-glucopyranosyl moieties indicated that these sugars were terminal units. Glycosidation shift was observed for C-2_{glc} (δ 83.5). The chemical shifts of H-1_{rha} (δ 5.90) and C-1_{rha} (δ 94.0) indicated that the α-L-rhamnopyranosyl unit was involved in an ester linkage with the C-28 carboxylic group. Thus, compound **1** was identified as 3β-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-olean-12-en-28-*O*-[α-L-rhamnopyranosyl] ester.

Compound **2** was assigned the molecular formula $C_{50}H_{80}O_{18}$, as shown by its HRESIMS data (*m/z* 969.5415 $[M + H]^+$), in combination with the ^{13}C NMR spectrum. The ^{13}C and ^{13}C DEPT spectra (Table 1) showed 50 signals, of which 30 were assigned to the aglycone, 18 to the sugar, and two to an acetyl group. 1D TOCSY, DQFCOSY, and HSQC NMR experiments showed the presence of two β-D-glucopyranosyl and one α-L-rhamnopyranosyl unit (Tables 1 and 2). The HSQC spectrum also showed glycosylation shifts for C-2 (δ 84.0) of one glucopyranosyl unit and

Table 1: ^{13}C NMR data for glycosyl moieties of compounds **1** - **3** (CD_3OD, 600 MHz)[a].

	1	**2**	**3**
position	δ_C	δ_C	δ_C
Rha-1	94.0	94.3	
2	71.0	69.5	
3	73.2	74.6	
4	72.2	71.0	
5	72.0	72.4	
6	18.3	18.0	
Xyl 1			105.0
2			81.6
3			77.8
4			70.4
5			66.0
6			
GlcI 1	104.5	104.7	103.9
2	83.5	84.0	74.7
3	77.0	76.8	77.8
4	71.0	71.0	70.9
5	77.8	78.0	76.7
6	62.3	62.5	69.4
GlcII 1	103.7	103.6	103.3
2	74.9	75.0	75.0
3	77.8	77.8	77.9
4	71.2	71.3	71.4
5	78.0	78.0	78.0
6	62.6	62.6	62.4
$\underline{CH_3}CO$		21.0	
$\underline{CH_3CO}$		172.8	

[a] chemical shifts in ppm; assignments confirmed by COSY, TOCSY, HSQC, and HMBC experiments.

Table 2: ^1H NMR data for glycosyl moieties of compounds **1** - **3** (CD_3OD, 600 MHz)[a].

	1	**2**	**3**
position	δ_H	δ_H	δ_H
Rha-1	5.90 d (1.8)	5.88 d (1.5)	5.88 d (1.8)
2	3.78 dd (2.5, 1.8)	3.90 dd (3.0, 1.5)	3.75 dd (2.5, 1.8)
3	3.48 dd (9.0, 2.5)	5.05 dd (9.0, 3.0)	3.46 dd (9.0, 2.5)
4	3.71 t (9.o)	3.72 t (9.0)	3.70 t (9.0)
5	3.60 m	3.80 m	3.60 m
6	1.28 d (6.5)	1.30 d (6.0,)	1.27 d (6.5)
Xyl 1			4.40 d (7.5)
2			3.56 dd (9.0, 7.5)
3			3.57 t (9.0)
4			3.62 m
5a			3.90 dd (11.0, 2.5)
5b			3.30 dd (11.0, 5.0)
GlcI 1	4.50 d (7.8)	4.48 d (7.8)	4.56 d (7.8)
2	3.54 dd (9.5, 7.8)	3.56 dd (9.5, 7.8)	3.46 dd (9.5, 7.8)
3	3.63 t (9.5)	3.64 t (9.5)	3.42 t (9.5)
4	3.60 t (9.5)	3.62 t (9.5)	3.36 t (9.5)
5	3.41 m	3.43 m	3.39 m
6a	3.64 dd (12.0, 5.0)	3.68 dd (12.0, 5.0)	3.85 dd (12.0, 5.0)
6b	3.89 dd (12.0, 3.0)	3.92 dd (12.0, 3.0)	4.18 dd (12.0, 3.0)
GlcII 1	4.35 d (8.0)	4.32 d (8.0)	4.36 d (8.0)
2	3.40 dd (9.5, 8.0)	3.38 dd (9.0, 8.0)	3.32 dd (9.0, 8.0)
3	3.37 t (9.5)	3.35 t (9.0)	3.35 t (9.0)
4	3.39 t (9.5)	3.39 t (9.0)	3.38 t (9.0)
5	3.47 m	3.42 m	3.40 m
6a	3.69 dd (12.0, 5.0)	3.66 dd (12.0, 5.0)	3.66 dd (12.0, 5.0)
6b	3.88 dd (12.0, 3.0)	3.88 dd (12.0, 2.5)	3.88 dd (12.0,2.5)
$\underline{CH_3}CO$		2.02 (s)	

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

acylation shifts for H-3 (δ 5.05) and C-3 (δ 74.6) of the rhamnopyranosyl unit. The acyl moiety was identified as an acetyl group from the NMR data [9]. An unambiguous determination of the sequence and linkage sites was obtained from HMBC correlations. The configurations of the sugar units were assigned as for compound **1**. In this way, those of **2** were determined to be D-glucose and L-rhamnose in the ratio 2:1. On the basis of this evidence, **2** was established as the new compound 3β-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-olean-12-en-28-*O*-[(3-*O*-acetyl)-α-L-rhamnopyranosyl] ester.

Compound **3** was obtained as an amorphous powder with the molecular formula $C_{53}H_{86}O_{21}$, as deduced by HRESIMS (*m/z* 1059.5746 $[M + H]^+$) and confirmed by ^{13}C NMR and ^{13}C DEPT data. The MS/MS spectrum of compound **3** showed prominent fragments at *m/z* 735 $[M - (162 + 162) + H)]^+$, and 589 $[M - (162 + 162 + 146) + H)]^+$.

The spectroscopic data of the aglycone moiety of **3** were identical to those of **1**. Analysis of sugar chains NMR data (Tables 1 and 2) of compound **3** and comparison with those of **1,** revealed **3** to differ from **1** only in the sugar chain at C-3. The sugar portion at C-3 of **3** exhibited three anomeric proton resonances (δ 4.56, d, *J* = 7.8 Hz; 4.40, d, *J* = 7.5 Hz; 4.36, d, *J* = 8.0 Hz) in the ^1H NMR spectrum (Table 2). The 1D TOCSY and 2D NMR experiments indicated that two β-glucopyranose, and one β-xylopyranose moieties were present (Tables 1 and 2). The absence of any ^{13}C NMR glycosidation shift for one of the β-glucopyranosyl moieties indicated that this sugar was a terminal unit. Glycosidation shifts were observed for C-6_{glc} (δ 69.0), and C-2_{xyl} (δ 81.6) (Table 1). Direct evidence for the sugar sequence and linkage site to the aglycone was derived from the HMBC experiment that showed unequivocal correlations between resonances at δ 4.40 and δ 91.8 (H-1_{xyl}—C-3), indicating that xylose was linked to C-3 of the aglycone. Thus, compound **3** was identified as 3β-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl]-olean-12-en-28-*O*-α-L-rhamnopyranosyl ester.

Comparison of the NMR spectral data of compound **4** (molecular formula $C_{53}H_{86}O_{22}$) with those of **3** showed these compounds to be identical in the sugar portion, but different in their aglycones. The ^{13}C NMR spectra showed, for the aglycone moiety, signals that could be unambiguously correlated to the corresponding proton chemical shifts from the HSQC experiment, leading to the identification of the aglycone as hederagenin [10]. Thus, **4** was determined to be 3β-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl]-hederagenin-28-O-[α-L-rhamnopyranosyl] ester.

The three known triterpene glycosides were identified by detailed NMR and MS analyses and comparison with literature data as 3β-*O*-[β-D-galactopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-α-L-arabino-pyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside [11]; 3-*O*-β-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-*α*-L-rhamnopyranosyl-(1→2)-α-L-rhamnopyranosyl oleanolic acid 28-*O*-α-L-arabinopyranosyl-(1→2)-*α*-L-rhamnopyranoside [11], and 3β-*O*-*α*-L-arabinopyranosyl-olean-12-en-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester [12].

In the framework of a research project aimed at searching for cytotoxic triterpene saponins from medicinal plants, all compounds isolated from *D. elegantissima* were assayed against human uterine cervical adenocarcinoma (HeLa), human lymphocyte cells T (Jurkat), and human breast adenocarcinoma (MCF7) cell lines. All compounds showed an IC_{50} higher than 50 μM [13,14].

Experimental

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. UV spectra were recorded on a Perkin-Elmer-Lambda spectrophotometer. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All the 2D NMR spectra were acquired in CD_3OD or $CDCl_3$ 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. Standard pulse sequences and phase cycling were used for DQF-COSY, TOCSY, HSQC, and HMBC experiments. NMR data were processed on a Silicon Graphics Indigo2 Workstation using UXNMR software. HRESIMS were acquired in positive ion mode on a Q-TOF premier spectrometer equipped with an electrospray ion source (Waters-Milford, MA, USA). Column chromatography was performed over Sephadex LH-20. HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Shimadzu RID-10A refractive index detector and Shimadzu injector on a C_{18} μ-Bondapak column (30 cm x 7.8 mm, 10 μm Waters, 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 x 25 m).

Plant material: The aerial parts of *D. elegantissima* (Veitch ex Mast.) R. Vig. & Guillaumin were collected in Palermo, Italy, during May 2007 and were identified by Prof. Giuseppe Venturella of the Dipartimento di Scienze Botaniche, University of Palermo, Italy, where a voucher specimen is deposited.

Extraction and isolation: The dried powdered *D. elegantissima* aerial parts (300 g) were defatted with *n*-hexane, and then extracted with MeOH to give 13.4 g of extract. The MeOH extract was partitioned between *n*-BuOH and AcOEt, to afford *n*-BuOH soluble (4 g) and AcOEt portions (5.4 g). A portion of the *n*-BuOH residue (2.5 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) or $CHCl_3$-MeOH-H_2O (40:9:1), and grouped to obtain 8 fractions (1-8). Fraction 2 (245 mg) was chromatographed using RP-HPLC (MeOH-H_2O (7:3)) to yield 3β-*O*-[β-D-galactopyranosyl-(1→3)-β-D-glucopyranosyl-(1→2)-α-L-arabino-pyranosyl oleanolic acid 28-*O*-α-L-rhamnopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (8 mg), and 3-*O*-β-D-galactopyranosyl-(1→2)-α-L-arabinopyranosyl-(1→3)-α-L-rhamno-pyranosyl-(1→2)-α-L-rhamnopyranosyl oleanolic acid 28-*O*-α-L-arabinopyranosyl-(1→2)-α-L-rhamnopyranoside (2.8 mg). Fraction 3 (203 mg) was chromatographed using RP-HPLC with MeOH-H_2O (7:3) to yield compounds **3** (8 mg), **4** (11 mg), and **1** (7 mg). Fractions 4 (120 mg) and 5 (30 mg) were subjected to RP-HPLC with MeOH-H_2O (7:3) to yield compounds **1** (6.5 mg) and **2** (3 mg) from fraction 4, and **1** (4 mg), and 3β-*O*-α-L-arabinopyranosyl-olean-12-en-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl] ester (2 mg), from fraction 5.

3β-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-olean-12-en-28-*O*-[*α*-L-rhamnopyranosyl] ester (1)

Yield: 0.012%.

$[\alpha]_D$: +37.4 (*c* 1.00, MeOH).

^{1}H NMR data of the aglycone (600 MHz, CD_3OD): 0.81 (3H, s, Me-26), 0.86 (3H, s, Me-24), 0.95 (3H, s, Me-29), 0.97 (3H, s, Me-25), 1.05 (3H, s, Me-30), 1.10 (3H, s, Me-23), 1.24 (3H, s, Me-27), 2.96 (1H, dd, *J* = 12.0, 5.0 Hz, H-18), 3.22 (1H, dd, *J* = 12.0, 4.5 Hz, H-3), 5.35 (1H, t, *J* = 3.5 Hz, H-12).

^{13}C NMR data of the aglycone (150 MHz, CD_3OD) δ: 16.0 (C-25), 16.5 (C-24), 18.0 (C-26), 19.5 (C-6), 23.5 (C-16), 23.8 (C-30), 24.6 (C-11), 26.2 (C-27), 26.6 (C-2), 28.0 (C-23), 28.2 (C- 15), 30.8 (C-20), 33.4 (C-29), 33.7 (C-7), 34.0 (C-22), 34.5 (C-21), 37.7 (C-10), 39.0 (C-4), 39.9 (C-1), 40.3 (C-8), 42.3 (C-14), 42.8 (C-18), 46.7 (C-19), 47.0 (C-17), 48.8 (C-9), 57.0 (C-5), 90.8 (C-3), 124.3 (C-12), 144.2 (C-13), 177.8 (C-28).

^{1}H and ^{13}C NMR of the sugar moiety, see Tables 1 and 2.

ESIMS *m/z* 927 $[M + H]^+$, 765 $[M - 162 + H]^+$, 781 $[M – 146 + H]^+$, 619 $[M – (162 + 146) + H]^+$, 457 $[M – (162 + 162 + 146) + H]^+$.

HRESIMS *m/z* 927.5311 $[M + H]^+$ (calcd for $C_{48}H_{78}O_{17}$, 927.5318).

3β-*O*-[β-D-glucopyranosyl-(1→2)-β-D-glucopyranosyl]-olean-12-en-28-*O*-[(3-*O*-acetyl)-α-L-rhamnopyranosyl] ester (2)

Yield: 0.002%.

$[\alpha]_D$: +35.2 (*c* 1.00, MeOH).

NMR data of the aglycone see compound **1**.

^{1}H NMR and ^{13}C NMR sugar moiety: Tables 1 and 2.

ESIMS: *m/z* 969 $[M + H]^+$, 781 $[M – (146 – 42) + H]^+$, 645 $[M – (162 + 162) + H]^+$.

HRESIMS *m/z* 969.5415 $[M + H]^+$ (calcd for $C_{50}H_{80}O_{18}$, 969.5424).

3β-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl]-olean-12-en-28-*O*-α-L-rhamnopyranosyl ester (3)

Yield: 0.005%.

$[\alpha]_D$: +40 (*c* 1.00, MeOH)

NMR data of the aglycone see compound **1.**

^{1}H NMR and ^{13}C NMR of sugar moiety: Tables 1 and 2.

ESIMS: *m/z* 1059 $[M + H]^+$, 735 $[M - (162 + 162) + H]^+$, 589 $[M - (162 + 162 + 146) + H]^+$.

HRESIMS *m/z* 1059.5746 $[M + H]^+$ (calcd for $C_{53}H_{86}O_{21}$, 1059.5741).

3β-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl-(1→2)-β-D-xylopyranosyl]-hederagenin-28-O-[α-L-rhamnopyranosyl] ester (4)

Yield: 0.007%.

$[\alpha]_D$: +39.4 (*c* 1.00, MeOH).

^{1}H NMR data of the aglycone (600 MHz, CD_3OD): 0.75 (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), 2.27 (1H, d, *J* = 12.8, Hz, H-18), 3.62 (1H, dd, *J* = 12.0, 4.5 Hz, H-3); 3.32 (1H, d, *J* = 12.4 Hz, H-23a), 3.63(1H, d, *J* =12.4 Hz, H-23b), 5.28 (1H, t, *J* = 3.2 Hz, H-12).

^{13}C NMR data of the aglycone (150 MHz, CD_3OD) δ: 13.0 (C-24), 18.0 (C-26), 18.2 (C-6), 18.5 (C-25), 24.0 (C-11), 24.5 (C-30), 24.7(C-16), 25.5 (C-2), 26.0 (C-27), 28.0 (C-15), 31.3 (C-20), 32.5 (C-7), 33. 5 (C-29), 33.5 (C-22), 34.0 (C-21), 37.9 (C-10), 39.5 (C-1), 42.5 (C-18), 43.0 (C-4), 41.0 (C-8), 47.0 (C-19), 47.6 (C-17), 48.0 (C-5), 43.2 (C-14), 48.2 (C-9), 48.5 (C-5), 87.1 (C-3), 64.4 (C-23), 124.0 (C-12), 144.3 (C-13), 178.3 (C-28).

^{1}H and ^{13}C NMR of the sugar moiety, see compound **3**.

ESIMS: *m/z* 1075 $[M + H]^+$, 751 $[M - (162 + 162) + H]^+$, 605 $[M - (162 + 162 + 146) + H]^+$.

HRESIMS *m/z* 1075.5733 $[M + H]^+$ (calcd for $C_{53}H_{86}O_{22}$, 1075.5740).

Cell culture and cytotoxic assay: HeLa (human cervical carcinoma), Jurkat (human lymphocyte cells T), and human breast adenocarcinoma (MCF7) cell lines were obtained from American Type Cell Culture (ATCC) (Rockville, MD, USA). Cells were maintained in DMEM and/or RPMI 1640, supplemented with 10% FBS, 100 mg/L streptomycin, and 100 IU/mL penicillin (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2. To ensure logarithmic growth, cells were subcultured every 2 days. Stock solutions (1 mg/ml) of all compounds were prepared in DMSO and stored at 4°C. In all experiments, the final concentration of DMSO did not exceed 0.3%, v/v. Cells were seeded in 96-well plates (1 x 10^4/well). One day after seeding, cells were exposed to either increasing concentrations of each compound or vehicle only and incubated for the established times. The number of HeLa, Jurkat

and MCF7 viable cells was quantified by MTT assay [8,13] and CellTiter-Blue1 Cell Viability assay (Promega). Appropriate controls were included in preliminary experiments to exclude any interferences of each compound with the assays. The IC_{50} values were obtained from dose-response curves.

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