Cytotoxic Triterpenoid Saponins from the Roots of *Cephalaria gigantea*

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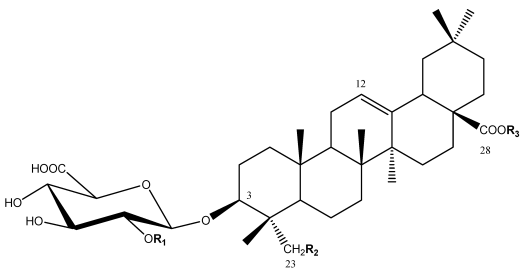
Three new oleanane-type saponins, giganteosides L (1), M (2) and N (3) along with eight known ones were isolated from the roots of *Cephalaria gigantea*. Their structures were established as 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-oleanolic acid, 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-hederagenin, 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-hederagenin, respectively, by means of spectroscopic methods (1D and 2D NMR, HR-ESI-MS). Cytotoxic activity of monodesmosides was investigated *in vitro* using three cancer cell lines, namely, human non pigmented melanoma MEL-5 and human leukemia HL-60. Giganteosides D (4) and E (5) showed antiproliferative effect on human cell lines with IC_{50} values in the range 3.15—7.5 μM.

Key words *Cephalaria gigantea*; Dipsacaceae; oleanane-type saponin; giganteoside; cytotoxic activity

The roots of *Cephalaria gigantea* (LEDEB.) BOBROV (Dipsacaceae), an endemic plant of Caucasus growing in Georgia,[1)] contain a large amount of triterpene saponins.[2—4)] The roots are well-known in traditional medicine as sedative and anti-inflammatory remedies.[1)] Previous works reported the identification of six triterpene glycosides.[5,6)] Antifungal and antiprotozoal activities of giganteosides D and E, major monodesmosides, have been evaluated.[7)] The present paper describes isolation and structure elucidation of three new triterpene saponins, named giganteosides L (**1**), M (**2**) and N (**3**) from the roots of *Cephalaria gigantea*. Their structures were established on the basis of 2D-NMR experiments (gs-COSY, gs-HMQC, gs-HMBC and gs-HSQC-TOCSY) and mass spectrometry (MALDI-TOF, ESI-HR-MS). Saponins containing glucuronic acid in the sugar chain are reported for the first time in *Cephalaria* species. In addition, eight known triterpene glycosides, giganteosides D (**4**),[5)] E (**5**),[5)] G (**6**),[6)] H (**7**),[6)] I (**8**),[8)] K (**9**),[8)] J (**10**)[9,10)] and J_1 (**11**)[9,10)] were isolated from this plant.

Dried root of *C. gigantea* was extracted with boiling 80% MeOH and after filtration the solution was evaporated. The solid residue was dissolved in methanol and precipitated in acetone. A combination of low pressure liquid chromatography and repeated silica gel column chromatography allowed the purification of eleven glycosides (**1**—**11**) including three new ones. The structures of the new saponins (**1**—**3**) were established on the basis of acid hydrolysis, NMR (Tables 1, 2) and MS data.

Giganteoside L (**1**) was assigned the molecular formula $C_{54}H_{86}O_{24}$ by high-resolution electrospray ionisation mass spectrometry (HR-ESI-MS) ($[M+Na]^+$ quasi-molecular ion at *m/z* 1141.5443; $C_{54}H_{86}NaO_{24}$ requires *m/z* 1141.5407). Six *tert*-methyls [^{1}H-NMR: δ 0.82 (s, H_3-24), 0.85 (s, H_3-26), 0.91 (s, H_3-29), 0.95 (s, H_3-30), 1.00 (s, H_3-23), 1.16 (s, H_3-25) and 1.29 (s, H_3-27); ^{13}C-NMR: δ 16.2 (C-25), 17.2 (C-24), 17.8 (C-26), 24.1 (C-30), 26.3 (C-27), 28.6 (C-23) and 33.5 (C-29), and a trisubstituted double bond [^{1}H-NMR: δ 5.24 (br s, H-12); ^{13}C-NMR: δ 123.9 (C-12) and 144.9 (C-13)] were attribuable to an olean-12-ene skeleton, in which one hydroxy group was assigned to C-3, together with one carboxylic function at C-17 [^{13}C-NMR: δ 178.1 (C-28)]. Therefore, the aglycone was determined to be oleanolic acid. Upon acid hydrolysis, compound **1** yielded glucuronic acid



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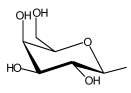
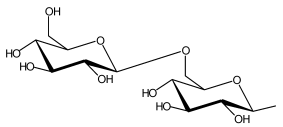
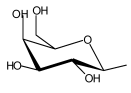
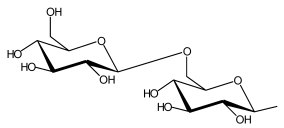
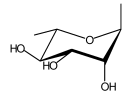
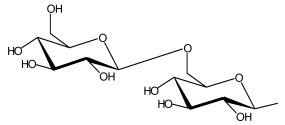
1		H	
2		OH	
3		OH	

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Table 1. ^{1}H-NMR Spectral Data for the Sugar Moieties of Saponins **1**—**3** in CD_3OD

	1	**2**	**3**
C-3-*O*-sugar			
GlcA			
1	4.45, d, *J*=7.8 Hz	4.55, d, *J*=7.7 Hz	4.55, d, *J*=7.7 Hz
2	3.55	3.55	3.42
3	3.57	3.58	3.41
4	3.38	3.38	3.40
5	3.50	3.50	3.55
Gal			
1	4.53, d, *J*=7.5 Hz	4.58, d, *J*=7.5 Hz	
2	3.56	3.56	
3	3.46	3.44	
4	3.85	3.81	
5	3.45	3.43	
6	3.70	3.66	
Rham			
1			5.34, br s
2			3.96
3			3.76
4			3.36
5			3.93
6			1.21
C-28-*O*-sugar			
Glc I			
1	5.34, d, *J*=7.9 Hz	5.34, d, *J*=7.9 Hz	5.34, d, *J*=7.9 Hz
2	3.33	3.32	3.32
3	3.53	3.54	3.50
4	3.41	3.41	3.41
5	3.52	3.52	3.50
6	4.11	4.13	4.14
	3.76	3.75	3.76
Glc II			
1	4.33, d, *J*=7.5 Hz	4.33, d, *J*=7.5 Hz	4.34, d, *J*=7.5 Hz
2	3.19	3.18	3.20
3	3.58	3.59	3.57
4	3.28	3.26	3.28
5	3.25	3.25	3.25
6	3.84	3.79	3.82
	3.60	3.58	3.56

GlcA=β-D-glucuronopyranosyl; Gal=β-D-galactopyranosyl; Rha=α-L-rhamnopyranosyl; Glc=β-D-glucopyranosyl.

Table 2. ^{13}C-NMR Spectral Data for Saponins **1**—**3** in CD_3OD

	1	**2**	**3**
Aglycone			
3	90.9	83.8	81.7
12	123.9	123.8	123.8
13	144.9	144.9	144.9
23	28.6	64.7	64.7
24	17.2	14.0	13.9
25	16.2	16.6	16.6
26	17.8	17.9	17.9
27	26.3	26.3	26.4
28	178.1	178.1	178.1
29	33.5	33.5	33.5
30	24.1	24.1	24.1
C-3-*O*-sugar			
GlcA			
1	105.4	104.3	104.3
2	83.1	82.1	78.8
3	78.0	78.0	78.0
4	73.8	73.7	74.1
5	76.8	76.1	76.6
6	177.0	177.0	177.0
Gal			
1	106.2	105.7	
2	74.1	74.1	
3	74.7	74.7	
4	69.8	69.9	
5	76.8	76.8	
6	61.7	62.0	
Rham			
1			101.8
2			71.9
3			72.1
4			74.0
5			70.0
6			18.0
C-28-*O*-sugar			
Glc I			
1	95.8	95.8	95.8
2	73.8	73.7	73.8
3	78.2	78.2	77.8
4	71.0	70.9	71.0
5	77.8	77.9	77.8
6	69.5	69.5	69.5
Glc II			
1	104.5	104.6	104.6
2	75.1	75.2	75.1
3	79.2	79.2	79.1
4	71.5	71.6	71.5
5	78.0	78.0	78.0
6	62.7	62.8	62.7

GlcA=β-D-glucuronopyranosyl; Gal=β-D-galactopyranosyl; Rha=α-L-rhamnopyranosyl; Glc=β-D-glucopyranosyl.

(GlcA), galactose (Gal) and glucose (Glc) as the sugar components identified on TLC by comparison with authentic samples. In the ^{13}C-NMR spectrum four anomeric carbons at δ 106.2, 105.4, 104.5 and 95.8 (Table 2) indicated the presence of four sugars in **1**. The resonances of C-3 at δ 90.9 and C-28 at δ 178.1 for **1** were characteristic of a bisdesmoside in agreement with the results of alkaline hydrolysis.

Interglycosidic and sugar-aglycone linkages as well as signal and structural assignments of the sugars were deduced on the basis of the following arguments. First of all the gs-HMQC[11] spectral analysis displayed the connectivities between proton and carbone anomeric atoms.Then, gs-HSQC-TOCSY[12] experiment showed for each sugar residue the intra-correlated peaks between anomeric proton and sugar carbons. Thus, subsequent examination of the HSQC-TOCSY diagram indicated the occurrence of two glucose, one galactose and one glucuronic acid units (Table 1). Finally, the sequence and linkage site of the sugar moieties were established using long-range correlation peaks observed in the gs-HMBC[13] diagram. The HMBC spectrum showed correlations between C-3 (δ 90.9) and GlcA H-1 (δ 4.45), and between Gal H-1 (δ 4.53) and GlcA C-2 (δ 83.1). The sequence of the sugar chain at C-28 was identified from the HMBC correlation between GlcI C-6 (δ 69.5) and GlcII H-1 (δ 4.33). A cross peak correlating GlcI H-1 (δ 5.34) and aglycone C-28 (δ 178.1) implying an ester linkage between the disaccharide chain and oleanolic acid moiety. The complementary data from the gs-COSY[14] spectrum was used to obtain a full assignment of the proton resonances (Table 1). Moreover, the anomeric configurations for individual monosaccharides were determined to be β-form based on the characteristic the $J_{1,2}$ coupling constants and the evidence of the ^{13}C-NMR data.[15,16] Thus, the structure of saponin **1** was elucidated as 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-

Table 3. Concentrations Inducing 50% Cellular Viability (IC_{50}) after 72 h Treatment in Cancer Cell Lines in the Presence of Monodesmosides

	IC_{50} (μM)	
	MEL-5	HL-60
Giganteoside D	7.50	3.15
Giganteoside E	7.50	6.80
Camptothecin	0.40	0.05

IC_{50} from MTT (MEL-5) or WST1 assay (HL-60).

glucopyranosyl]-oleanolic acid.

The molecular formula of giganteoside M (**2**) was deduced to be $C_{54}H_{86}O_{25}$ from HR-ESI-MS (measure: 1157.5342, calculated for $C_{54}H_{86}NaO_{25}$, 1157.5356). Comparison of the NMR data (^{1}H, ^{13}C, COSY, HMQC, HMBC) of **2** and those of **1**, indicated that the structures of the two saponins were identical except the replacement of oleanolic acid in **1** by hederagenin in **2**. Thus the structure of **2** was determined as 3-*O*-[β-D-galactopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-hederagenin.

The molecular formula of giganteoside N (**3**) was established as $C_{54}H_{86}O_{24}$ by HR-ESI-MS (*m/z* 1141.5388 [M+Na]$^+$, calculated for $C_{54}H_{86}NaO_{24}$, 1141.5407). ^{13}C-NMR shifts of C-3 and C-28 indicated that compound **3** was a bisdesmoside. Its spectral features indicated that **3** possessed the same aglycone as that of **2**, but differed in terms of the sugar structures. The ^{1}H-NMR spectrum of **3** showed four anomeric proton signals at δ 5.34, 5.34, 4.53 and 4.34. The HSQC-TOCSY correlations were indicative of two β-D-glucose, one β-D-glucuronic acid and one α-L-rhamnose units. The HMBC correlations indicating the same disaccharide chain that **2** linked to carboxylic group at C-28. The HMBC spectrum showed correlations between aglycone C-3 (δ 81.77) and GlcA H-1 (δ 4.53), GlcA C-2 (δ 78.85) and Rha H-1 (δ 5.34). Comparison of the NMR data (^{1}H, ^{13}C, COSY, HMQC, HMBC) of **3** and those of **2**, indicated that the structures of the two saponins were identical except the replacement of galactose moiety in **2** by rhamnose moiety in **3**. It can be concluded that the structure of **3** was 3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucuronopyranosyl]-28-*O*-[β-D-glucopyranosyl-(1→6)-β-D-glucopyranosyl]-hederagenin.

Bidesmoside saponins are not active *in vitro*. Monodesmosides were obtained from bidesmosides by alkaline hydrolysis and evaluated for their cytotoxicity against MEL-5 and HL-60 cell lines by using the metabolic MTT or WST1 cell viability assay, campthotecin was used as positive control.[17]

Among the tested compounds **4** and **5** exhibited interesting antiproliferative effect at lower concentrations 3.15 μM≤ IC_{50}≤7.5 μM (Table 3).

The leukemia HL-60 line is more sensitive to these monodesmosides with a dose-dependent effect (Fig. 1). The cytotoxic activity may be related to the rhamnose residue of the sugar chain attached at C_3 of the aglycone as it has been reported previously for hederacolchiside A.[18,19]

Experimental

General ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker DRX-500 spectrometer in CD_3OD solutions. TMS was used as an internal standard in ^{1}H and ^{13}C measurements. Standard Bruker pulse sequences were used for two-dimensional experiments (gs-COSY, gs-HMQC, gs-HMBC and gs-HSQC-TOCSY). High-Resolution Mass Spectra (HR-MS) were obtained on an Applied Biosystems MALDI-TOF Voyager Spec. The experiments were performed on a Jeol JMS-700 (Jeol LTD, Akishima, Tokyo, Japan) double focusing mass spectrometer, equipped with an electrospray ionization (ESI) source operating under positive ion mode. Samples diluted in H_2O/CH_3OH (50/50) were introduced into the ESI interface *via* a syringe pump (PHD 2000 infusion, Harvard Apparatus, Holliston, MA, U.S.A.) at a 30 μl min^{-1} flow rate. A 5-kV acceleration voltage was applied and the elemental composition of ions was checked at a typical resolving power of 8000 (10% valley) using a mixture of PEGs as internal standard. Melting points were determined on a BUCHI Melting Point B-540 apparatus. Optical rotations $[\alpha]_D^{25}$ were measured on a Perkin-Elmer model 341 Orot Polarimeter. TLC analysis of saponins and sugars were performed on precoated silica gel plates (Kiesegel 60F254, Merck) using the following solvent systems: $CHCl_3$–MeOH–H_2O (26 : 14 : 3) [system 1]; *n*-BuOH–HOAc–H_2O (4 : 1 : 5) [system 2]; $CHCl_3$–MeOH (25 : 1) [system 3]; CH_2Cl_2–MeOH–H_2O (50 : 25 : 5) [system 4]; spots were detected by spraying the plates with phosphoric acid naphtoresorcinol for sugars and H_2SO_4 for saponins followed by heating at 110 °C.

Material The roots of *Cephalaria gigantea* was collected in Tskhinvali region of Georgia (October, 2001) and dried in the shade. A voucher specimen is kept in the department of Pharmacobotany, Institute of Pharmacochemistry, Tbilisi, Georgia (roots No. 87243).

Extraction and Isolation Dried and powdered roots of *C. gigantea* (500 g) were extracted with boiling 80% MeOH (4 l). The extract was concentrated under vacuum. After evaporation of solvent the dry extract (141 g) was disolved in MeOH (500 ml) and precipitated in acetone (3 l). The precipitate was filtered and dried to obtain a crude saponins fraction (100 g). Twenty grams of crude saponins were subjected to low pressure liquid chromatography (LPLC) (ChromatoSPAC Prep 100) Lichroprep C-18, 15—25 μm, 50×4 cm and eluted with MeOH–H_2O (10 to 80% of MeOH) to afford 5 fractions. Fraction 4, containing the most polar triterpene saponins, was subjected to column chromatography on silica gel (0.04—0.063 mm, Merck) and eluted with $CHCl_3$–MeOH–H_2O (26 : 14 : 3) to give **1** (120 mg), **2** (45 mg), **3** (90 mg), **4** (80 mg), **5** (35 mg), **6** (150 mg), **7** (120 mg), **8** (35 mg), **9** (40 mg), **10** (20 mg), **11** (25 mg).

Acid Hydrolysis of 1, 2 and 3 The saponin (3 mg) was heated with aqueous 10% HCl (3 ml) in a sealed tube at 100 °C for 4 h. The sapogenin was extracted with Et_2O and then the aqueous layer was neutralized with *N*,*N*-dioctylmethylamine (10% in $CHCl_3$) and dried. The sapogenin and sugars were identified by TLC analysis with authentic samples in system 3 (*Rf* 0.30, 0.68) and 4 (*Rf* 0.32, 0.49, 0.57, 0.83) respectively.

Alkaline Hydrolysis of 1, 2 and 3 The saponin (4 mg) was heated in 5% aqueous KOH (4 ml) in a sealed tube at 100 °C for 90 min. After neutralization with 10% HCl (pH 5) the prosapogenin was extracted with *n*-BuOH. TLC analysis were performed using systems 1 and 2.

Giganteoside L (**1**): A white amorphous powder. $[\alpha]_D^{25}$ −5.9° (*c*=0.2, MeOH); mp 243 °C; HR-ESI-MS *m/z*: 1141.5443 [M+Na]$^+$ (Calcd for $C_{54}H_{86}NaO_{24}$, 1141.5407). ^{1}H-NMR data for sugar part, see Table 1. ^{13}C-NMR data for aglycone and sugar parts see Table 2.

Giganteoside M (**2**): A white amorphous powder. $[\alpha]_D^{25}$ +4.8° (*c*=0.87, MeOH); mp 221 °C; HR-ESI-MS *m/z*: 1157.5342 [M+Na]$^+$ (Calcd for $C_{54}H_{86}NaO_{25}$, 1157.5356). ^{1}H-NMR data for sugar part, see Table 1. ^{13}C-NMR data for aglycone and sugar parts see Table 2.

Giganteoside N (**3**): A white amorphous powder. $[\alpha]_D^{25}$ −23.0° (*c*=0.3, MeOH); mp 236 °C; HR-ESI-MS *m/z*: 1141.5388 [M+Na]$^+$ (Calcd for $C_{54}H_{86}NaO_{24}$, 1141.5407). ^{1}H-NMR data for sugar part, see Table 1. ^{13}C-NMR data for aglycone and sugar parts see Table 2.

Cellular Viability (MTT or WST1 Test) Human non pigmented melanoma MEL-5 cells (gift of Dr. G. Degiovanni, University of Liége) were cultured in medium DME supplemented with 5% heat inactivated (1 h at 57 °C) FBS. Human leukemia HL-60 cells (American tissue culture collection) were cultured in T25 containing 8 ml of RPMI-1640 supplemented with 10% heat inactivated (1 h at 57 °C) FBS, 1% L-glutamine, 1% non essential amino-acids, 1% sodium pyruvate (stock solutions from BioWhittaker) and 100 U/ml penicillin–100 μg/ml streptomycin. Adherent cells were cultured at a density 5000 (MEL-5) cells/200 μl/well in NUNC 90-well microplates. After 24 h, the cells were exposed to various concentrations of the tested drugs for 72 h. Corresponding controls with analogous concentrations of DMSO were carried out in pararell. After treatment, the medium was removed and the MTT test was realized. Ten microliters of a stock solution of 3 mg/ml MTT (Sigma) in PBS was added with 90 μl of DMEM during 40 min in the dark and in the incubator. The culture medium was removed and 100 μl DMSO was added at room temperature and mixed to dissolve the

dark blue crystals. Plate were analyzed on a Labsystems Multiskan MS (type 352) reader at 570 and 620 nm. For non adherent HL-60 line, 50000 cells were seeded in 200 μl directly with the substances and after 72 h, the WST1 colorimetric assay (from Boerhinger) was applied. Twenty microliters WST-1 was added directly to the RPMI medium containing the cells, at the end of he treatment. After 30 min in the incubator, plates were shaken and absorbance was recorded at two wavelenghts (450, 620 nm), against a background control as blank (medium plus WST-1 without cells. 8 wells were used per each condition and two experiments were performed. Means±S.E.M. were calculated and IC_{50} determination was achieved in a regression analysis of the results a different concentrations of each drug. The statistical analysis with the paired Student *t* test was performed (*p* values lower than 0.05 were considered as significant). The results of the cellular assays are expressed as percentages (controls taken as 100%).

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