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Pseudocnoside A, a new cytotoxic and antiproliferative triterpene glycoside from the sea cucumber *Pseudocnus dubiosus leoninus*

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A new triterpene glycoside, pseudocnoside A (**1**), was isolated from the sea cucumber *Pseudocnus dubiosus leoninus*. The structure of the new compound was established on the basis of extensive NMR spectroscopic analysis (^{1}H and ^{13}C NMR, ^{1}H,^{1}H-COSY, HMBC, HSQC, TOCSY and NOESY), HR-ESI-MS data and chemical transformations. In addition, the cytotoxicity and antiproliferative activities of **1** and structurally related triterpene glycosides isolated from the sea cucumbers *Psolus patagonicus* and *Hemioedema spectabilis* were evaluated against cancer cell lines A-549 and HeLa.

Keywords: *Pseudocnus dubiosus leoninus*; sulphated triterpene glycosides; cytotoxic; antiproliferative

1. Introduction

Sea cucumbers (class Holothuroidea) have been shown to contain a variety of triterpene glycosides based on a 'holostanol' skeleton ((3β,5α,20*S*)-3,20-dihydroxylanostano-18,20-lactone) and a sugar chain of two to six monosaccharide units linked to the C-3 of the aglycon (Maier, 2008). These substances are produced in the skin and in the Cuvier's tubules of sea cucumbers and are ejected when the animals are disturbed. This behaviour may be associated with a defensive function due to the ability of holothurins to form complexes with cholesterol and other Δ^5-sterols from cell membranes. This membranolytic activity probably determines the wide spectrum of their biological effects, including antifungal, cytotoxic, antiviral, haemolytic and immunomodulatory properties (Kalinin, Aminin, Avilov, Silchenko, & Stonik, 2008). Recently, we have reported on the antifungal activities of patagonicosides A–C, three sulphated triterpene glycosides from the sea cucumber *Psolus patagonicus* (Careaga, Munian, & Maier, 2011).

As a continuation of our search for bioactive metabolites from cold-water echinoderms of the South Atlantic (Chludil & Maier, 2005; Chludil, Seldes, & Maier, 2002; Maier et al., 2001; Murray, Muniain, Seldes, & Maier, 2001), we have investigated the glycoside fraction of the sea cucumber *Pseudocnus dubiosus leoninus* (Semper, 1867) (Dendrochirotida, Cucumariidae). This small holothurian is frequently found from the patagonian intertidal rocks to 200 m in the

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South Atlantic. No chemical investigation of *P. dubiosus leoninus* has been reported as yet. Here, we describe the isolation and structure elucidation of a new sulphated triterpene glycoside, pseudocnoside A (**1**) (Figure 1), from the EtOH extract of *P. dubiosus leoninus*. Compound **1** and structurally related triterpene glycosides isolated from the sea cucumbers *P. patagonicus* (Careaga et al., 2011) and *Hemioedema spectabilis* (Chludil et al., 2002) were evaluated for their cytotoxicity and antiproliferative activities on two cancer cell lines (A-549 and HeLa) in order to establish structure–activity correlations.

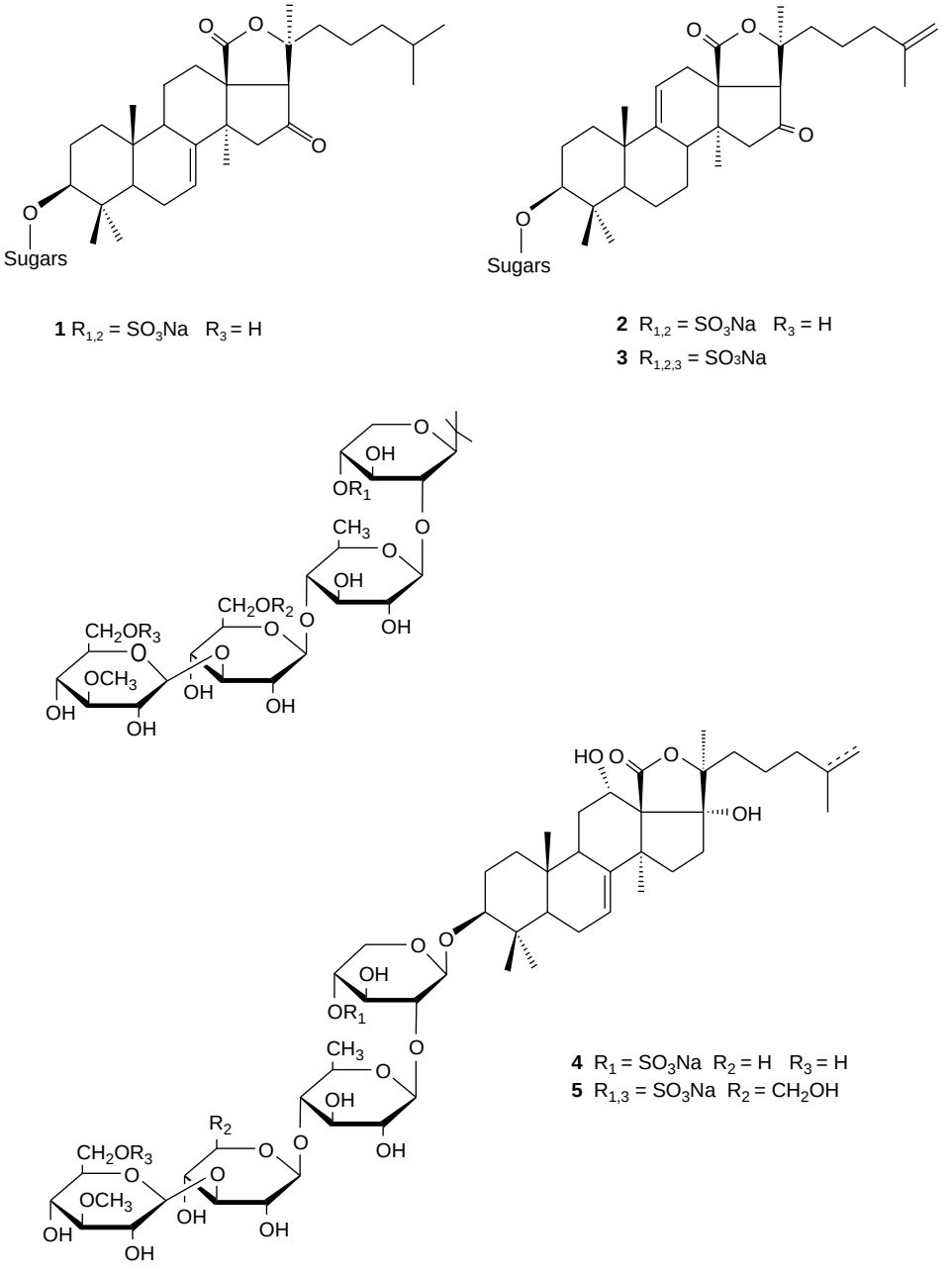

Figure 1. Pseudocnoside A (**1**), hemoiedemosides A (**2**) and B (**3**), patagonicosides B (**4**) and C (**5**).

2. Results and discussion

Purification of the MeOH extract of *P. dubiosus leoninus* by vacuum dry column chromatography on C_{18} reversed-phase (RP) silica gel and finally by RP-high performance liquid chromatography (HPLC) on a Bondclone C_{18} yielded a new triterpene glycoside, pseudocnoside A (**1**), as the major component.

An examination of the ^{1}H and ^{13}C NMR spectra of pseudocnoside A (**1**) indicated the presence of a triterpenoid aglycon with one olefinic bond, a keto group and one lactone C═O group bonded to an oligosaccharide chain composed of four sugar units. The structure of the aglycon of **1** was determined on the basis of spectroscopic data and by its comparison with those of triterpene glycosides isolated from the sea cucumber *Cucumaria japonica* (Chludil, Murray, Seldes, & Maier, 2003). The aglycon of **1** belongs to the holostane type [based on the signals of the 18(20)-lactone at δ(C) 179.7 (C(18)) and 84.4 (C(20)) in the ^{13}C NMR spectrum] and contains a C(7)═C(8) bond (δ(C) 122.7 (C(7)) and 145.1 (C(8)); δ(H) 5.76 (H-C(7))) and a keto group at C-16 (δ(C) 213.6 (C(16)). The presence of a keto group at C(16) was evidenced by the signals of C(15) and C(17) in the ^{13}C NMR spectrum at δ(C) 52.8 and 64.5, respectively, together with signals at δ(H) 2.57, 2.74 (H-C(15)) and 2.90 (H-C(17)) in the ^{1}H NMR spectrum. The ^{1}H NMR spectrum also showed seven methyl groups, five characteristics of a holostane skeleton at δ 1.25 (s, H-32), 1.14 (s, H-30), 1.34 (s, H-31), 1.32 (s, H-19) and 1.50 (s, H-21), and two of terminal methyl groups at δ 0.90 (s, H-27) and 0.92 (s, H-26) in the side chain. $^{1}H,^{1}H$-COSY, HSQC and HMBC spectra allowed the assignment of all the proton and carbon resonances of the aglycon. The relative stereochemistry of all chiral centres of the aglycon was established with the aid of a NOESY experiment (Figure 2). The above data indicated that the structure of the aglycon moiety was 16-keto-holost-7-en-3β-ol, the same as that of cucumarioside A_2-3, isolated from the sea cucumber *C. japonica* (Drozdova et al., 1993).

The carbohydrate chain of **1** consisted of four monosaccharide residues as deduced from the ^{13}C NMR spectrum, which showed the signals of four anomeric C-atoms at 105.6–106.1 ppm, correlated by the HSQC spectrum with the corresponding signals of anomeric H-atoms at 4.78 (d, $J = 7.2$ Hz), 4.88 (d, $J = 8.1$ Hz), 5.03 (d, $J = 7.8$ Hz) and 5.41 (d, $J = 7.8$ Hz). The coupling constants of the anomeric H-atoms were indicative of β-configuration of the glycosidic bonds. The presence of 3-*O*-methylglucose, glucose, quinovose and xylose in the ratio 1:1:1:1 was established by acid hydrolysis with aqueous 2 N trifluoroacetic acid followed by GC analysis of the corresponding peracetylated alditols (Maier et al., 2001). All H- and C-atom chemical shifts of the oligosaccharide chain of **1** could be assigned by $^{1}H,^{1}H$-COSY, HSQC, HMBC, TOCSY and NOESY experiments. The four carbohydrate units belong to the D-series, as determined by

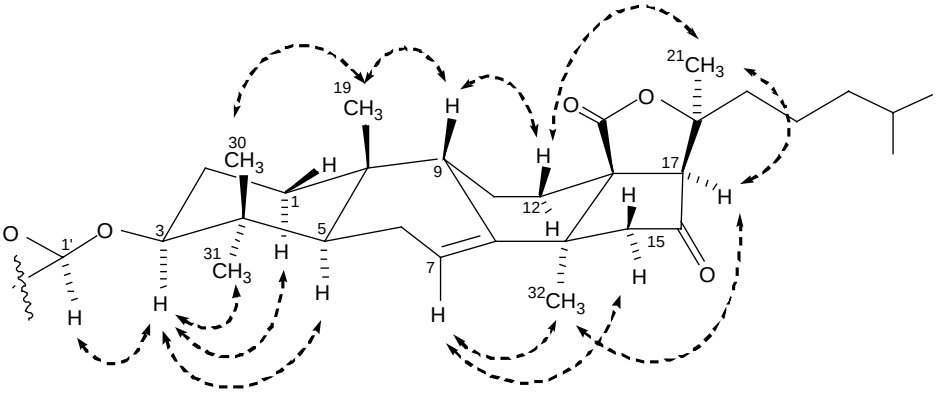

Figure 2. NOESY correlations of the aglycon of pseudocnoside A (**1**).

the GC/MS analysis of the mixture of 1-{(acetyl)[(*S*)-2-hydroxypropyl]amino}-1-deoxyalditol acetate derivatives following the procedure described in Maier et al. (2001).

The HR-ESI-MS (negative-ion mode) of pseudocnoside A (**1**) exhibited a *pseudo*-molecular ion peak at *m/z* 1267.4288 ([M − Na]$^-$; calcd 1267.4488). The MS/MS spectrum of this ion indicated the peaks for fragment ions at *m/z* 1147.5565 ([M − $NaSO_4$−H−Na]$^-$) and 827.3620 ([M − 3-*O*-Me-Glc-*O*-Glc-OSO_3Na]$^-$), confirming the presence of two sulphated groups in the carbohydrate chain and the presence of a terminal unit of a sulphated 3-*O*-methylglucose. This and NMR spectroscopic data allowed the determination of the molecular formula as $C_{54}H_{84}O_{28}S_2Na_2$.

The interglycosidic linkages were deduced from NOESY (Figure 3) and HMBC correlations, where cross-peaks between H-C(1′) of the xylose residue and H-C(3) of the aglycon, H-C(1″) of the quinovose and H-C(2′) of the xylose residue, H-C(1‴) of the glucose and H-C(4″) of the quinovose unit, and H-C (1⁗) of 3-*O*-methylglucose and H-C(3‴) of the glucose unit, respectively, were observed. The carbohydrate chain of **1** is a linear tetrasaccharide consisting of D-xylose (sulphated at C-4), D-quinovose, D-glucose (sulphated at C-6) and a terminal 3-*O*-methyl-D-glucose. The oligosaccharide chain of **1** is identical to that of patagonicoside A (Murray et al., 2001), hemoiedemoside A (Chludil et al., 2002) and cucumechinosides A and C (Miyamoto, Togawa, Higuchi, Komori, & Sasaki, 1990).

On the basis of all the above data, the structure of pseudocnoside A (**1**) was established as 3-*O*-[3-*O*-methyl-β-D-glucopyranosyl-(1 → 3)-6-*O*-sodiumsulphonato-β-D-glucopyranosyl-(1 → 4)-β-D-quinovopyranosyl-(1 → 2)-4-*O*-sodiumsulphonato-β-D-xylopyranosyl]-holost-7-en-16-one (Figure 1).

Studies on the structure–activity relationship for sea cucumber glycosides revealed that their biological activities depend on both the aglycon and the carbohydrate chain structures (Kalinin et al., 2008). Several triterpene glycosides containing a linear tetrasaccharide chain have shown cytotoxic activity on tumour cell growth *in vitro* (Hang, Xu, Yi, Gong, & Jiao, 2010; Silchenko et al., 2008; Sun et al., 2007). Usually, the literature deals mainly with sea cucumber triterpene glycosides exhibiting cytotoxic activity in tumour cells rather than antiproliferative effects. Recently, we have shown that patagonicoside A, a disulphated triterpene glycoside isolated from the sea cucumber *P. patagonicus* and its desulphated analogue showed antiproliferative activity in three tumour cell lines (Hep3B, MDA-MB231 and A549) and displayed lower levels of cytotoxicity than most triterpene glycosides already reported (Careaga, Bueno, Muniain, Alché, & Maier, 2009).

These results enabled us to test compound **1** for *in vitro* cytotoxicity and antiproliferative activity on two human tumour cell lines (A549 and HeLa) following the procedure described in Careaga et al. (2009) and compare these activities with those of four structurally related triterpene glycosides, hemoiedemosides A (**2**) and B (**3**) and patagonicosides B (**4**) and C (**5**)

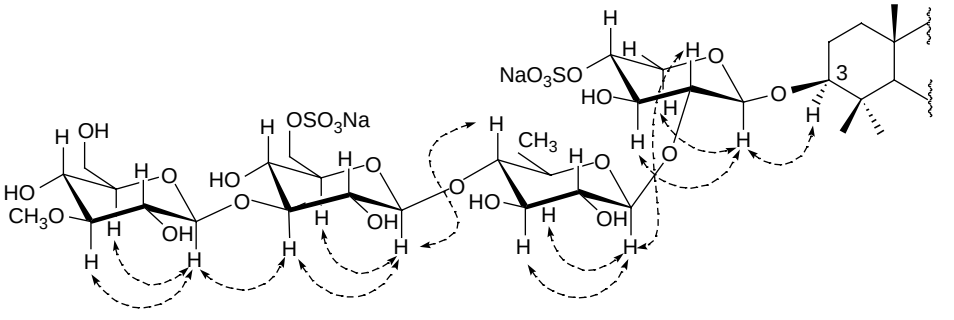

Figure 3. NOESY correlations of the oligosaccharide chain of pseudocnoside A (**1**).

Table 1. *In vitro* cytotoxic and antiproliferative activities of compounds **1**–**5** against human tumour cells A549 and HeLa.

	A549		HeLa	
Cell line	CC_{50} (μM)	IC_{50} (μM)	CC_{50} (μM)	IC_{50} (μM)
1	39.17	14.53	74.41	57.36
2	29.71	7.43	21.96	9.95
3	5.96	3.16	2.80	2.15
4	8.87	9.73	8.58	7.94
5	4.04	5.56	3.39	3.57

(Figure 1) isolated from *H. spectabilis* (Chludil et al., 2002) and *P. patagonicus* (Careaga et al., 2011), respectively. The IC_{50} and CC_{50} values were determined on the basis of the observed cell viability after 24 h of drug exposure (Table 1).

The antiproliferative activity of **1** in A549 cells ($IC_{50} = 14.53$ μM) was considerably higher than in HeLa cells ($IC_{50} = 57.36$ μM). Frequently, different tumour cell lines exhibit a differential sensitivity to the cytotoxic effects of sea cucumber glycosides (Jin et al., 2009). The differences in the antiproliferative activity of glycoside **1** may be ascribed to a higher sensitivity of A549 tumour cells when treated with compound **1**.

Hemoiedemosides A (**2**) and B (**3**) differ only in the degree of sulphation of the oligosaccharide chain. Trisulphated glycoside **3** contains an additional sulphate group at C-6 of the terminal 3-*O*-methylglucose unit. Glycosides **2** and **3** exhibited stronger antiproliferative activity than compound **1** with similar IC_{50} values in A549 and HeLa cell lines (Table 1). Besides, the higher cytotoxicity of glycoside **3** on both cell lines may be related to the presence of a third sulphate group in the carbohydrate chain. Similar results were observed for okhotosides B_1, B_2 and B_3. Monosulphated okhotoside B_1 showed lower cytotoxicity than their disulphated analogues B_2 and B_3 in HeLa cell line (Silchenko et al., 2008).

Patagonicosides B (**4**) and C (**5**) exhibited similar antiproliferative and cytotoxic activities in both cell lines with IC_{50} values comparable to those for hemoiedemoside B (**3**) (Table 1). It is important to point out that compound **2** exhibited a low cytotoxicity not only for A549 and HeLa cells but also for Vero cells, with a CC_{50} value of 30.02 μM. The fact that compound **2** displayed a low level of cytotoxicity for tumoural and normal cells, together with its remarkable antiproliferative effect, suggests that **2** is a more suitable molecule than other sea cucumber triterpene glycosides to impede tumour cell growth *in vitro*.

3. Experimental

3.1. *General*

1-{(Acetyl)[(*S*)-2-hydroxypropyl]amino}-1-deoxyalditol acetate derivatives of monosaccharides were analysed using a Shimadzu GCMS-QP5050 chromatograph equipped with a capillary column (Ultra II Hewlett Packard, 50 m × 0.20 mm). Preparative HPLC: SP liquid chromatograph equipped with a Spectra Series P100 solvent delivery system, a Rheodyne manual injector and a refractive index detector using a Bondclone 10μ column (30 cm × 7.8 mm i.d.). The samples were eluted with CH_3OH/H_2O (55:45) with a flow of 2 ml/min. Thin layer chromatography (TLC): pre-coated Si gel F254 (*n*-BuOH/HOAc/H_2O 12:3:5) and C_{18} RP plates (55:45% MeOH/H_2O); detection by spraying with *p*-anisaldehyde (5% EtOH). Optical rotations: Perkin-Elmer 343 polarimeter. ^1H and ^{13}C NMR spectra: in C_5D_5N/D_2O (4:1) on a Bruker AM 500 spectrometer. HR-ESI-MS (negative-ion mode): Bruker Daltonics micrOTOF-QII mass spectrometer (Bruker Daltonics, Billerica, MA, USA).

3.2. *Animal material*

Specimens of *P. dubiosus leoninus* were collected by dredge from beds of the scallop *Zygochlamys patagonica* during May 2007 and October 2009 at a depth of 100–120 m in the South Atlantic Ocean. Individuals of 20–40 mm were removed manually from each shell scallop and immediately frozen. The organisms were identified by Dr C. Muniain. A voucher specimen is deposited at the Museo Argentino de Ciencias Naturales 'Bernardino Rivadavia', Buenos Aires, Argentina (MACN No. 39439).

3.3. *Extraction and purification procedures*

The sea cucumbers (39 g), frozen prior to storage, were homogenised in EtOH (1.5 l) and centrifuged. The EtOH was evaporated and the residue was partitioned between MeOH/H_2O 9:1 (300 ml) and cyclohexane (100 ml). The glassy material obtained after evaporation of the MeOH extract was subjected to vacuum dry column chromatography on Davisil C_{18} RP (35–70 μm) using H_2O, H_2O/MeOH mixtures with increasing amounts of MeOH and finally MeOH as eluents. Triterpene glycosides were eluted with 60% and 70% MeOH. These fractions were combined and submitted to RP-HPLC to give the pure glycoside **1** (3 mg; t_R 47.5 min) as a white amorphous powder. $[\alpha]_D^{20}$ −39.1 (c 0.18, pyridine).

^{1}H NMR (500 MHz, C_5D_5/D_2O (4:1)): δ 1.50 (2H, m, H-1); 1.99, 2.19 (2H, m, H-2); 3.34 (1H, m, H-3); 1.11 (1H, m, H-5); 2.11 (2H, m, H-6); 5.76 (1H, m, H-7); 3.79 (1H, m, H-9); 1.71, 1.95 (2H, m, H-11); 2.14β, 2.34α (2H, m, H-12); 2.57 (1H, m, H-15); 2.74 (1H, d, J = 15.8 Hz, H-15); 2.90 (1H, s, H-17); 1.32 (3H, s, CH_3-19); 1.50 (3H, s, CH_3-21); 1.72, 1.87 (2H, m, H-22); 1.50 (2H, m, H-23); 1.22 (2H, m, H-24); 1.57 (1H, m, H-25); 0.92 (3H, s, CH_3-26); 0.90 (3H, s, CH_3-27); 1.14, (3H, s, CH_3-30); 1.34 (3H, s, CH_3-31); 1.25 (3H, s, CH_3-32); 4.78 (d, J = 7.2 Hz, C-1′-H); 4.08 (m, C-2′-H); 4.07 (t, J = 8.9 Hz, C-3′-H); 5.25 (m, C-4′-H); 3.77 (m, 4.70 m, C-5′-H); 5.03 (d, J = 7.8 Hz, C1″-H); 3.97 (m, C2″-H); 3.97 (m, C3″-H); 3.52 (t, J = 9.1 Hz, C4″-H); 3.68 (m, C5″-H); 1.62 (d, J = 6.1 Hz, C1″-3H); 4.88 (d, J = 8.1 Hz, C1‴-H); 3.81 (m, C2‴-H); 4.36 (m, C3‴-H); 3.88 (m, C4‴-H); 4.30 (m, C5‴-H); 4.87, 5.29 (m, C6‴-2H); 5.41 (d, J = 7.8 Hz, C1⁗-H); 3.92 (m, C2⁗-H); 3.78 (m, C3⁗-H); 4.22 (m, C4⁗-H); 4.04 (m, C5⁗-H); 4.35 (m, C6⁗-H); 4.54 (brd, J = 10.5 Hz, C6⁗-H); 3.93 (s OCH_3).

^{13}C NMR (500 MHz, C_5D_5/D_2O (4:1)): δ (C-1) 36.7; (C-2) 27.9; (C-3) 89.7; (C-4) 40.3; (C-5) 49.2; (C-6) 24.1; (C-7) 122.7; (C-8) 145.1; (C-9) 47.7; (C-10) 36.4; (C-11) 23.2; (C-12) 30.4; (C-13) 57.7; (C-14) 46.5; (C-15) 52.8; (C-16) 213.6; (C-17) 64.5; (C-18) 179.7; (C-19) 23.5; (C-20) 84.4; (C-21) 26.9; (C-22) 39.9; (C-23) 23.7; (C-24) 40.1; (C-25) 28.4; (C-26) 23.6; (C-27) 23.4; (C-30) 18.1; (C-31) 29.4; (C-32) 32.9; (C-1′) 105.7; (C-2′) 83.8; (C-3′) 75.6; (C-4′) 76.6; (C-5′) 65.0; 64.55; (C-1″) 105.6; (C-2″) 76.7; (C-3″) 74.4; (C-4″) 89.1; (C-5″) 72.3; (C-6″) 18.8; (C-1‴) 106.0; (C-2‴) 72.3; (C-3‴) 87.8; (C-4‴) 71.2; (C-5‴) 75.8; (C-6‴) 68.6; (C-1⁗) 106.1; (C-2⁗) 74.9; (C-3⁗) 88.7; (C-4⁗) 71.1; (C-5⁗) 79.1; (C-6⁗) 62.6; OCH_3 61.5.

(−) HR-ESI-MS, m/z 1267.4288 $[M - Na]^-$, $C_{54}H_{84}O_{28}S_2Na$ (1267.4488 calcd); (−) HR-ESI-MS/MS of the ion $[M - Na]^-$ at m/z 1147.5565 $[M - NaSO_4 - Na - H]$ and 827.3620 $[M - 3\text{-}O\text{-Me-Glc-}O\text{-Glc-OSO}_3Na]^-$.

3.4. *Cell culture and reagents*

Human lung carcinoma A549 cells were grown in Eagle's minimal essential medium supplemented with 10% inactivated fetal bovine serum (FBS) (MEM 10%). HeLa cells were maintained in Dulbecco's modified Eagle's minimum essential medium and Ham's F-12 medium containing 10% FBS (DMEM 10%). Vero cells, a cell line initiated from the kidney of a normal adult African green monkey, were grown in Eagle's minimal essential medium

supplemented with 5% inactivated FBS (MEM 5%). Cell cultures were maintained in a 4% CO_2 humidified atmosphere at 37°C.

3.5. *Antiproliferative assay*

We seeded 2.4×10^6 cells in 96-well plates together with different concentrations of triterpene glycosides 1–5 in duplicate, and incubated at 37°C for 24 h in a 4% CO_2 atmosphere. Then, cells were fixed with 10% formaldehyde for 15 min at room temperature, washed once with distilled water and stained with 0.05% crystal violet in 10% ethanol over 30 min. Afterwards, cells were washed once and eluted with a solution of 50% ethanol and 0.1% acetic acid in water. The absorbance of each well was measured on a Eurogenetics MPR-A 4i microplate reader using a test wavelength of 590 nm.

3.6. *Cytotoxicity assay*

Cell viability was determined as previously reported (Careaga et al., 2009). Briefly, cell viability in the presence of the compound was determined using the cleavage of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by the mitochondrial enzyme succinate dehydrogenase to give a blue product (formazan). The absorbance of each well was measured on a Eurogenetics MPR-A 4i microplate reader using a test wavelength of 570 nm and a reference wavelength of 630 nm by duplicate.

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