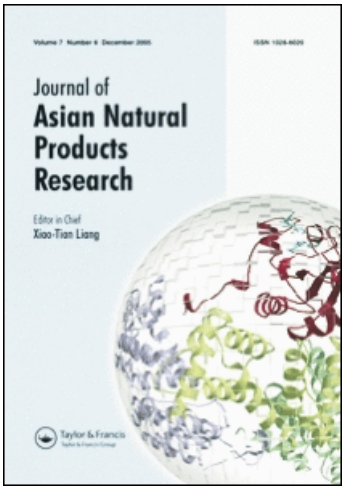

Journal of Asian Natural Products Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713454007

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To cite this Article Ma, CHUN-HUI , Fan, MING-SONG , Lin, LI-PING , Tang, WEI-DONG , Lou, LI-GUANG , Ding, JIAN and Huang, CHENG-GANG(2008) 'Cytotoxic triterpenoid saponins from *Vaccaria segetalis*', Journal of Asian Natural Products Research, 10: 2, 177 — 184
To link to this Article: DOI: 10.1080/10286020701394381
URL: http://dx.doi.org/10.1080/10286020701394381

Journal of Asian Natural Products Research, Vol. 10, No. 2, February 2008, 177–184

Cytotoxic triterpenoid saponins from *Vaccaria segetalis*

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(Received 8 October 2006; revised 27 February 2007; in final form 21 March 2007)

By the guidance of bioassay, one new cytotoxic triterpenoid saponin, 3-*O*-[β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranosyl] quillaic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-fucopyranosyl-(1 → 4)]-β-D-fucopyranoside (**1**), and five known cytotoxic triterpenoid saponins, vaccaroside E (**2**), vaccaroside G (**3**), vaccaroside B (**4**), segetoside H (**5**) and segetoside I (**6**), were isolated from *Vaccaria segetalis*. Their structures were established on the basis of ESI-MS, IR, extensive NMR (^{1}H NMR, ^{13}C NMR, TOCSY, ^{1}H–^{1}H COSY, DEPT, HMQC, HMBC and ROESY) analyses, chemical degradation, and by comparing with previously reported data. Compounds **1**–**6** showed moderate cytotoxic activities against LNcap, P-388 and A-549 cell lines with IC_{50} values in the range 0.1–12.9 μM.

Keywords: *Vaccaria segetalis*; *Vaccaria pyramidata*; Triterpenoid saponin; Quillaic acid; Vaccaroside I; Cytotoxic activity

1. Introduction

The plant *Vaccaria segetalis* (Neck.) Garcke (syn. *V. pyramidata* Medik) is an annual herb widely distributed in Asia, Europe and other parts of the world [1]. The seed of the plant, popularly known as Wang–Bu–Liu–Xing, has a prominent role in traditional Chinese medicine. Its main uses include the promotion of diuresis and milk secretion, activation of blood circulation and the relief of carbuncle [2]. This plant is known to be rich in cyclopeptides [3–6] and triterpenoid glycosides [7–11]. In the present study, by the guidance of bioassay, one new cytotoxic triterpenoid saponin, 3-*O*-[β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranosyl] quillaic acid 28-*O*-β-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-fucopyranosyl-(1 → 4)]-β-D-fucopyranoside (**1**), and five known cytotoxic triterpenoid saponins, vaccaroside E (**2**) [8], vaccaroside G (**3**) [8], vaccaroside B (**4**) [9], segetoside H (**5**) [12] and segetoside I (**6**) [12] were isolated from *V. segetalis*. The isolation and structural elucidation of the new compound and the cytotoxic activities of compounds **1**–**6** against LNcap, P-388 and A-549 cell lines are reported.

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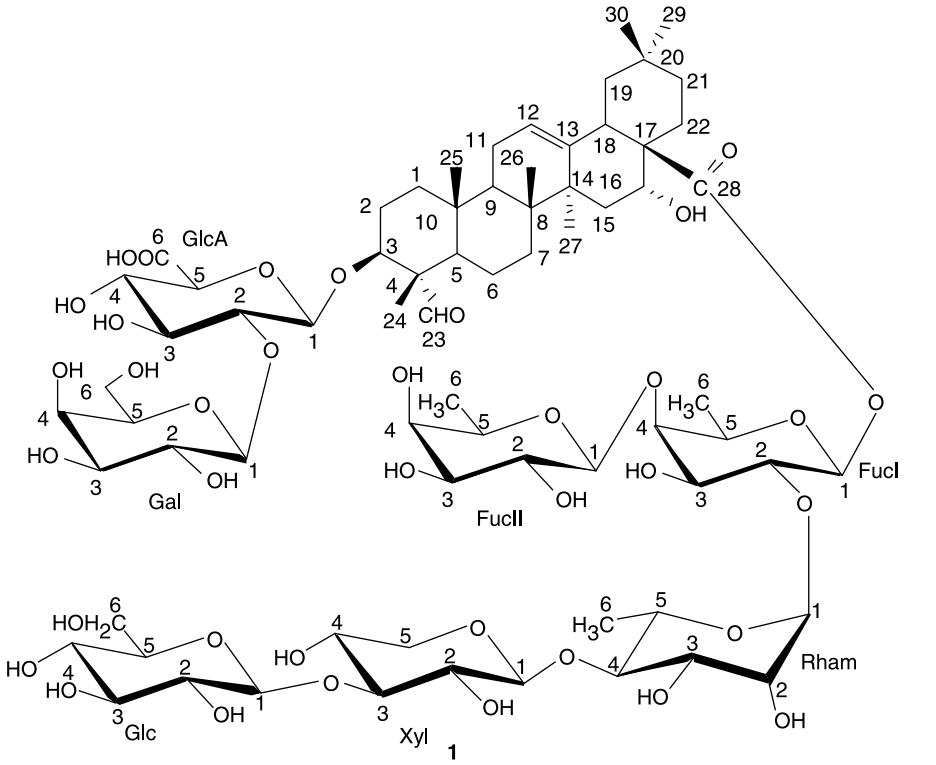
Journal of Asian Natural Products Research
ISSN 1028-6020 print/ISSN 1477-2213 online © 2008 Taylor & Francis
http://www.tandf.co.uk/journals
DOI: 10.1080/10286020701394381

2. Results and discussion

Vaccaroside I (**1**), an amorphous solid, its molecular formula $C_{71}H_{112}O_{37}$ was determined from the ion peak at *m/z* 1579.6778 $[M + Na]^+$ in the HRESI-MS spectrum. Its spectral features and physicochemical properties suggested **1** to be a triterpenoid saponin (figure 1). Of the 71 carbons, 30 were assigned to the aglycone part, 41 to the oligosaccharide moiety (table 1). The IR spectrum showed absorptions at 3407 cm^{-1} (OH) and 1731 cm^{-1} (ester carbonyl). The six sp^3 hybrid carbons at δ 10.9, 15.8, 17.3, 24.6, 27.2, 32.9, and two sp^2



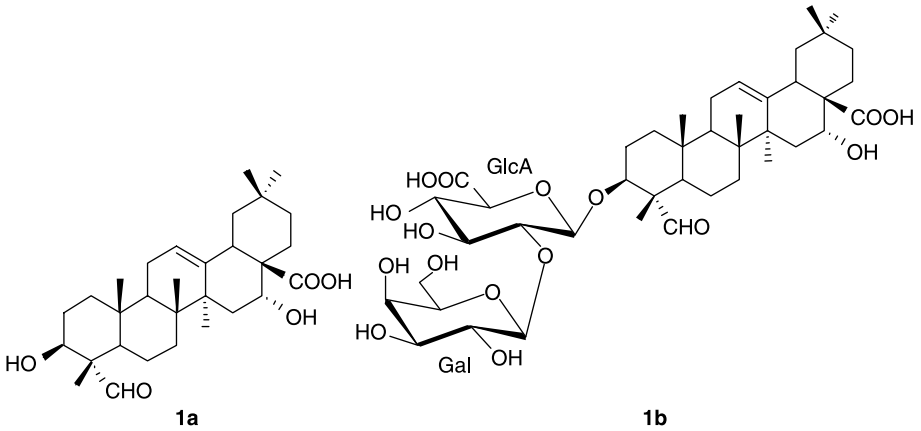


Figure 1. Structure of compounds **1**, **1a** and **1b**.

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectral data of compound **1** (C_5D_5N, *J* in Hz)[†].

Position	δ_c	*Position*	δ_c	δ_H	*Position*	δ_c	δ_H	*Position*	δ_c	δ_H
1	38.3 t	21	36.1 t		3	75.1 d	4.19 (1H, m)	2	71.9 d	4.82 (1H, m)
2	25.1 t	22	32.0 t		4	70.2 d	4.68 (1H, m)	3	72.5 d	4.71 (1H, m)
3	83.6 d	23	209.5 d		5	78.5 d	4.14 (1H, m)	4	83.7 d	4.44 (1H, m)
4	55.3 s	24	10.9 q		6	62.0 t	4.55 (1H, m)	5	68.4 d	4.52 (1H, m)
							4.60 (1H, m)			
5	48.5 d	25	15.8 q		FucI			6	18.8 q	1.69 (3H, d, 6.3)
6	20.7 t	26	17.3 q		1	94.7 d	6.02 (1H, d, 8.1)	Xyl		
7	32.9 t	27	27.2 q		2	74.4 d	4.65 (1H, m)	1	106.3 d	5.25 (1H, d, 7.2)
8	40.4 s	28	176.2 s		3	76.9 d	4.23 (1H, m)	2	76.0 d	4.08 (1H, m)
9	47.1 d	29	32.9 q		4	84.1 d	4.05 (1H, m)	3	88.2 d	4.12 (1H, m)
10	36.4 s	30	24.6 q		5	71.7 d	4.00 (1H, m)	4	69.4 t	4.11 (1H, m)
11	23.9 t	GluA			6	17.5 q	1.64 (3H, d, 6.5)	5	66.8 t	4.23 (1H, m)
										3.51 (1H, m)
12	122.3 d	1	103.5 d	4.95 (1H, d, 8.0)	FucII			Glu		
13	144.6 s	2	81.9 d	4.29 (1H, m)	1	106.8 d	5.05 (1H, d, 7.7)	1	105.7 d	5.29 (1H, d, 8.0)
14	42.3 s	3	77.8 d	4.37 (1H, m)	2	75.5 d	4.08 (1H, m)	2	75.0 d	4.19 (1H, m)
15	36.4 t	4	71.5 d	4.23 (1H, m)	3	73.1 d	4.59 (1H, m)	3	78.4 d	4.28 (1H, m)
16	74.1 d	5	74.9 d	4.61 (1H, m)	4	76.7 d	4.14 (1H, m)	4	70.3 d	4.63 (1H, m)
17	49.4 s	6	172.6 s		5	73.4 d	3.72 (1H, m)	5	78.6 d	4.04 (1H, m)
18	41.7 d	Gal			6	18.6 q	1.63 (3H, d, 5.9)	6	62.3 t	4.54 (1H, m)
										4.61 (1H, m)
19	47.6 t	1	106.1 d	5.31 (1H, d, 8.1)	Rham					
20	30.9 s	2	74.8 d	4.59 (1H, m)	1	101.4 d	6.40 s			

[†] The assignments were based on H–H COSY, HSQC and HMBC experiments.

hybrid carbons at δ 122.3 (*d*) and 144.6 (*s*) coupled with the information from ^{1}H NMR (six methyl proton singlets and a broad vinyl proton singlet at δ 5.62) indicated that the aglycone possessed an olean-12-ene skeleton. After an extensive 2D-NMR study, the aglycone was identified as quillaic acid (**1a**), a common aglycone of the triterpenoid glycosides [13,14] (figure 1). The chemical shifts of C-3 (δ 84.2) and C-28 (δ 176.2) indicated that **1** was a bisdesmosidic glycoside. The ^{1}H NMR and ^{13}C NMR spectra of **1** displayed seven sugar anomeric protons (δ 4.95, d, $J = 8.0$ Hz; 5.31, d, $J = 8.1$ Hz; 5.05, d, $J = 7.7$ Hz; 6.02, d, $J = 8.1$ Hz; 5.29, d, $J = 8.0$ Hz; 6.40, s; 5.25, d, $J = 7.2$ Hz) and carbons (δ 103.5, 106.1, 106.8, 94.7, 105.7, 101.4, 106.3), respectively (table 1). Alkaline hydrolysis of **1** furnished a prosapogenin (**1b**), identified as quillaic acid 3-*O*-β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranoside from its spectral data (figure 1). Acid hydrolysis afforded quillaic acid and the monosaccharide components, identified as fucose, galactose, xylose, rhamnose, glucose (2:1:1:1:1) based on GLC analysis. The other monosaccharide was identified as glucuronic acid on TLC by comparing with an authentic sample.

The assignment of the oligosaccharide chain at C-28 was determined by a combination of TOCSY, COSY, ROESY, DEPT, HMBC, and HSQC experiments. Starting from the anomeric protons of each sugar unit, all the hydrogens within each spin system were delineated using TOCSY, COSY and ROESY spectra. On the basis of the assigned protons, the ^{13}C NMR resonances of each sugar unit were identified by HSQC and further confirmed by HMBC. All the monosaccharides were in the pyranose forms as determined from their ^{13}C NMR spectral data. The β-anomeric configuration for the fucose, galactose, glucuronic acid and xylose were based on their $^{3}J_{H1,H2}$ coupling constants (7–8 Hz). The α anomeric configuration for the rhamnose was judged by their C_5 data (δ 68.4). The absolute configurations of these sugars were chosen in keeping with those mostly encountered among other plant glycosides.

The linkage of the sugar units at the side chain was established from the following HMBC correlations: H-1 of the glucose with C-4 of xylose; H-1 of the xylose with C-4 of rhamnose; H-1 of the rhamnose with C-2 of fucose I; H-1 of the fucose II with C-4 of fucose I, while the attachment of the saccharide chain to C-28 of the aglycone was based on the correlation between H-1 of fucose I and the C-28 of the aglycone (figure 2). The same conclusion with regard to the sugar sequence was also drawn from the ROESY experiment (figure 2). The linkage was also supported from the fragmentation patterns observed in the ESI-MS/MS experiment. MS/MS analysis of the molecular ion at *m/z* 1555 $[M - H]^-$ gave a daughter ion at *m/z* 1409 $[(M - H) - 146]^-$ due to the loss of the terminal fucose, at *m/z* 1393 $[(M - H) - 162]^-$ by the loss of one of the terminal haxanoses (galactose or glucose), at *m/z* 1217 $[(M - H) - 162 - 176]^-$ with the loss of the gal–glcA fragment, and at *m/z* 823 $[(M - H) - 162 - 132 - 146 - 147 - 145]^-$ due to the loss of the pentasaccharide chain linked to C-28. Further loss of the terminal galactose at C-3 afforded another ion at *m/z* 643 $[(M - H) - 162 - 132 - 146 - 147 - 145 - 163 - OH]^-$ confirming the attachment of glucuronic acid to C-3 of the aglycone (figure 2). MS/MS analysis of the molecular ion at *m/z* 1579 $[M + Na]^+$ gave a daughter ion at *m/z* 1241 $[(M + Na) - 163 - 175]^+$ by the loss of the gal–glcA fragment, and at *m/z* 801 $[(M + Na) - 163 - 175 - 162 - 132 - 146]^+$ due to the loss of the gal–glcA fragment at C-3 and glu–xyl–rham fragment at C-28. Thus, the structure of **1** is established as 3-*O*-[β-D-galactopyranosyl-(1 → 2)-β-D-glucuronopyranosyl] quillaic acid 28-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-fucopyranosyl-(1 → 4)]-β-D-fucopyranoside, named as vaccaroside I.

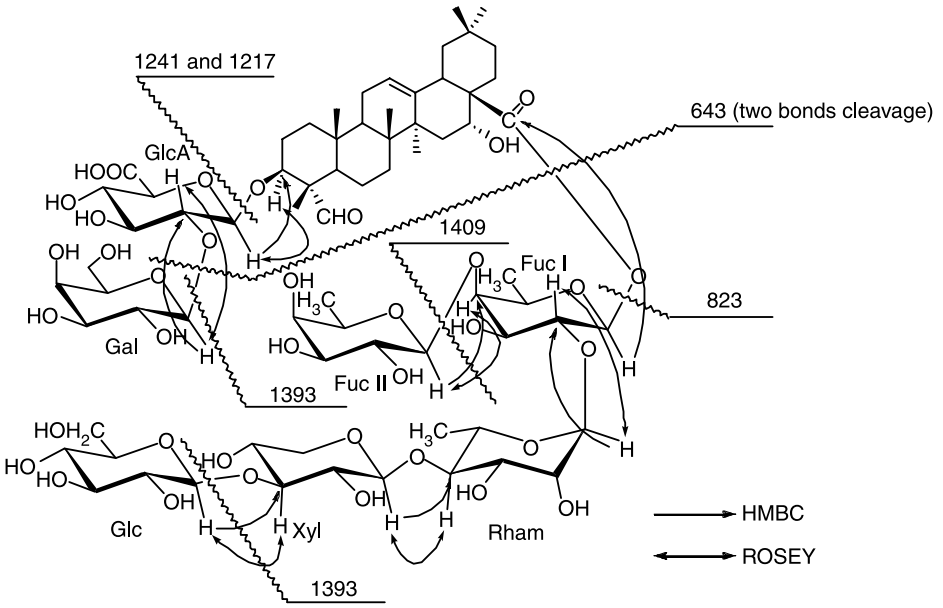


Figure 2. Key HMBC and NOESY correlations and ESI-MS/MS fragmentations for **1**.

The six isolated compounds (**1**–**6**) were evaluated for their cytotoxic activities against LNcap, A-549, and P-388 cell lines. Topotecan and decotaxel were used as positive controls. These compounds exhibited moderate cytotoxicity as shown in table 2.

3. Experimental

3.1 General experimental procedures

IR spectra were recorded on a Nicolet Manga-750 FT-IR spectrophotometer (KBr disc). Optical rotation was obtained on a JASCO DEP 370 polarimeter. Mass spectra were measured on a Finnigan LCQ-DECA instrument (ESI-MS) and a Bruker Daltonics APEXIII 7.0 TESLA FTMS spectrometer (HRESI-MS). NMR spectra were run on a Bruker AM-400 instrument with TMS as internal standard. Silica gel (160–200 and 200–300 mesh, Qingdao Haiyang Chemical Co. Ltd.), Lobar LiChroprep Rp-18 (40–60 μm; Merck), Sephadex LH-20 (Amersham, Biosciences) and MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Industries Ltd) were used for column chromatography. Precoated plates (silica gel GF_{254}, 0–40 μ), activated at 110°C for 2 h, were used for TLC analysis. The visualisation of TLC

Table 2. The cytotoxic activities (IC_{50} value, μM) of compounds **1**–**6**.[†‡]

Cell lines	***1***	***2***	***3***	***4***	***5***	***6***	*Topotecan*	*Decotaxel*
LNcap	3.6	3.4	2.5	4.2	12.9	1.2	0.053	
A-549	1.0	3.0	11.0	1.0	7.2	0.4		<0.01
P-388	0.8	9.4	0.7	3.7	1.6	0.1		<0.01

[†] LNcap, human prostate cancer cell line; A-549, human lung cancer cell line; P-388, mouse leukaemia cell line.
[‡] Topotecan and decotaxel as positive control.

was realised by spraying 20% H_2SO_4/EtOH reagent. All solvents were distilled prior to use. Petroleum ether: 60–90°C. Semi-preparative HPLC was performed using an ODS column (Inertsil ODS-3 column, 250 × 10 mm, UV detector, 210 nm). GLC was carried out with a Shimadzu GC-14B apparatus, equipped with a 5% OV225/AW-DMCS-Chromosorb W (80–100 mesh) column (2.5 m × 3 mm), as well as a hydrogen-flame ionisation detector; column temperature was set at 160°C; N_2 was used as carrier gas; flow rate was 30 ml/min.

3.2 Plant material

The seeds of *Vaccaria segetalis* were purchased from Shanghai Yanghetang Traditional Chinese Medicine Co. Ltd, Shanghai, China, in July 2005 and identified by Professor Jingui Shen (Shanghai Institute of Materia Medica, CAS). A voucher specimen (VS-072005) has been deposited at the herbarium of Shanghai Institute of Materia Medica, CAS.

3.3 Extraction and isolation

The seeds of *V. segetalis* (5 kg) were extracted with 95% EtOH. After evaporation of ethanol *in vacuo*, the residue (410 g) was suspended in water and then extracted successively with petroleum ether, CH_2Cl_2, EtOAc and n-BuOH. The n-BuOH fraction showing cytotoxic activities was subjected to silica gel column chromatography with a $CHCl_3$/MeOH solvent system (5:1, 2.5:1, 1:1 and 0:1) to afford subfractions A, B, C, and D, respectively. The cytotoxic subfraction D was chromatographed by semi-preparative HPLC (Inertsil ODS-3 column, 250 × 10 mm, 35–38% CH_3CN/0.06% TFA in H_2O, 3.5 ml/min, UV detector, 210 nm) to afford **1** (40 mg), **2** (10 mg), **3** (12 mg), **4** (9 mg), **5** (14 mg), and **6** (15 mg), respectively.

3.3.1 Vaccaroside I (1). Amorphous powder. $[\alpha]_D^{20}$ −16.0 (*c* 0.668, MeOH/H_2O = 1/1); IR ν_{max} (cm^{-1}): 3407, 2931, 1731, 1677, 1384, 1205, 1137, 1072; ^1H NMR (C_5D_5N, 400 MHz): δ 0.86, 1.03, 1.06, 1.13, 1.47 and 1.85 (each 3H, s, H-25, H-29, H-30, H-26, H-24 and H-27), 3.42 (1H, dd, J = 13.2, 4.3 Hz, H-18), 4.13 (1H, m, H-3), 5.22 (1H, brt, H-16), 5.61 (1H, brs, H-12), 9.92 (1H, s, H-23); other NMR spectral data, see table 1; ESI-MS (positive mode) *m/z*: 1579 $[M + Na]^+$ and 1557 $[M + H]^+$, ESI-MS (negative mode) *m/z*: 1555 $[M - H]^-$; HRESI-MS (positive mode) *m/z*: 1579.6778 $[M + Na]^+$ (calcd for $C_{71}H_{112}O_{37}$, 1579.6798).

The known compounds were identified as vaccaroside E (**2**), vaccaroside G (**3**), vaccaroside B (**4**), segetoside H (**5**), segetoside I (**6**), quillaic acid (**1a**) and prosapogenin (**1b**) by comparing their spectral data with those reported in the literature [8,9,12].

3.4 Acid hydrolysis of compound 1

Compound **1** (10 mg) was heated in 1 ml 1 M HCl (dioxane-H_2O, 1:1) at 80°C for 2 h in a water bath. After dioxane was removed, the solution was extracted with EtOAc (1 ml × 3). The extraction was washed with H_2O and then combined to give quillaic acid **1a** (4 mg). The monosaccharide portion was neutralised by passing through an exchange resin (Amberlite MB-3) column, and concentrated (dried overnight), then treated with 1-(trimethylsilyl)

imidazole at room temperature for 2 h. After the excess reagent was decomposed with water, the reaction product was extracted with hexane (1 ml × 2). The TMSi derivatives of the monosaccharides were identified to be fucose, galactose, rhamnose, xylose, glucose (2:1:1:1:1) by co-GLC analyses with standard monosaccharides. The glucuronic acid was detected by co-TLC analysis with an authentic sample (R_f: 0.15, solvent: MeCOEt/iso-PrOH/Me_2CO/H_2O, 20:10:7:6).

3.5 Alkaline hydrolysis of compound 1

Compound **1** (20 mg) in 2 ml 1 M KOH was heated at 80°C for 2 h. After cooling down, the reaction mixture was neutralised with 1 M HCl and then extracted with n-BuOH (1 ml × 3). The organic layers were combined and then evaporated to dryness *in vacuo*. The residue was subjected to HPLC purification (Inertsil ODS-3 column, 1.0 × 25 cm, 35–38% CH_3CN/0.06% TFA in H_2O, 3.0 ml/min, UV detector, 210 nm) and afforded prosapogenin **1b** (8 mg).

3.5.1 Compound 1b. Amorphous powder. IR ν_{max} (cm^{-1}): 3427, 2927, 1679, 1077; ^1H NMR (C_5D_5N, 400 MHz): δ 0.83, 0.98, 1.06, 1.18, 1.43 and 1.83 (each 3H, s, H-25, H-29, H-30, H-26, H-24, and H-27), 2.83 (1H, t, $J = 13.2$ Hz), 3.57 (1H, dd, $J = 13.2$, 4.1 Hz, H-18), 4.10 (1H, dd, $J = 12.0$, 4.3 Hz, H-3), 5.24 (1H, brt, H-16), 5.62 (1H, brs, H-12), 9.92 (1H, s, H-23), 4.96 (1H, d, $J = 7.9$ Hz, GluA-1), 4.23 (1H, m, GluA-2), 4.35 (1H, m, GluA-3), 4.22 (1H, m, GluA-4), 4.60 (1H, m, GluA-5), 5.27 (1H, d, $J = 7.4$ Hz, Gal-1), 4.58 (1H, m, Gal-2), 4.17 (1H, m, Gal-3), 4.65 (1H, m, Gal-4), 4.13 (1H, m, Gal-5), 4.56 and 4.60 (2H, m, Gal-6); ^{13}C NMR (C_5D_5N, 100 MHz): δ 38.2 (C-1), 24.9 (C-2), 83.6 (C-3), 55.1 (C-4), 48.4 (C-5), 20.5 (C-6), 32.9 (C-7), 40.3 (C-8), 47.3 (C-9), 36.4 (C-10), 23.9 (C-11), 122.3 (C-12), 145.0 (C-13), 42.2 (C-14), 36.3 (C-15), 74.8 (C-16), 49.0 (C-17), 41.6 (C-18), 47.2 (C-19), 31.2 (C-20), 36.2 (C-21), 32.9 (C-22), 209.4 (C-23), 10.9 (C-24), 15.7 (C-25), 17.4 (C-26), 27.4 (C-27), 179.9 (C-28), 33.1 (C-29), 24.8 (C-30), 103.5 (GluA-1), 82.3 (GluA-2), 77.7 (GluA-3), 71.7 (GluA-4), 75.0 (GluA-5), 172.0 (GluA-6), 106.1 (Gal-1), 75.3 (Gal-2), 75.5 (Gal-3), 70.2 (Gal-4), 78.6 (Gal-5), 62.0 (Gal-6); ESI-MS (positive mode) *m/z*: 847 $[M + Na]^+$.

3.6 Acid hydrolysis of compound 1b

The methods of acid hydrolysis of compound **1b** and the identification of its sugars were the same as those described in section 3.4. By this procedure, galactose and glucuronic acid were detected in the acid hydrolysis products of compound **1b**.

3.7 Cytotoxicity assay

Two human cancer cell lines, prostate cancer cell line LNcap and lung cancer cell line A549, mouse leukaemia cell line P388, obtained from American Type Culture Collection (Rockville, MD), were used for the cytotoxicity assay. Cells were maintained in RPMI-1640 medium supplemented with 10% (V/V) foetal bovine serum, 2 mmol/L glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin (GIBCO, Grand Island, NY, USA) in a highly humidified atmosphere of 95% air with 5% CO_2 at 37°C. The cytotoxicity of the compounds

against LNcap and A-549 was analyzed by the sulforhodamine B (SRB, Sigma) assay [15]. Briefly, the cells were seeded at 6000 cells/well in 96-well plates (Falcon.CA), and allowed to attach overnight. The cells were treated in triplicate with grade concentrations of compounds at 37°C for 72 h. Then, they were fixed with 10% trichloroacetic acid and incubated for 60 min at 4°C. Then the plates were washed and dried. SRB solution (0.4% W/V in 1% acetic acid) was added and the culture was incubated for an additional 15 min. After the plates were washed and dried, bound stain was solubilised with Tris buffer, and the optical densities were read on the plate reader (model VERSA Max, Molecular Devices) at 515 nm. The cytotoxicity of compounds against mouse leukaemia cell line P388 cells was measured by Methyl-Thiazol-Tetrazolium (MTT, Sigma) assay [16]. 8.0×10^3 cells in 100 L culture medium per well were seeded into 96-well plates, then exposed to gradient concentrations of compounds for additional 72 h. At the end of culture, 20 L of MTT (5 mg/ml) was added to each well and the plate was placed at 37°C for 4 h; then 100 L 'triplex solution' (10% SDS/5% isobutanol/12 mM HCl) was added and the cells were incubated at 37°C overnight. The optical density was detected at 570 nm using a microplate spectrophotometer. The growth inhibitory rate of treated cells was calculated by following formula: $[1 - (A_{515\text{treated}}/A_{515\text{control}})] \times 100\%$. The results were also expressed as IC_{50} (the compound concentration required for 50% growth inhibition of tumour cells), which was calculated by the Logit method.

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