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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Triterpenoids from the stem bark of *Vitellaria paradoxa* (Sapotaceae) and derived esters exhibit cytotoxicity against a breast cancer cell line

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Abstract A study of the chemical constituents of the stem bark of *Vitellaria paradoxa* (Sapotaceae) has resulted in the isolation and characterization of a new ursane-type triterpenoid, 2β,3β,19α-trihydroxyurs-12-en-28-oic acid (**1**), together with seven known compounds: betulinic acid (**2**), 1α,2β,3β,19α-tretrahydroxyurs-12-en-28-oic acid (**3**), *β*-sitosterol (**7**), sigmasterol (**8**), (-)-epicatechin (**9**), (+)-catechin (**10**) and quercetin (**11**). The structure of the novel, ursane-type acid **1** was elucidated on the basis of detailed spectroscopic analysis including IR, HRMS (ESI), 1D and 2D NMR and a comparison to previously described, related natural products. Preliminary cytotoxicity assays against the MDA-MB-231 breast cancer cell line indicated that betulinic acid **2** and its corresponding methyl ester **5** were the most active compounds tested with IC_{50} values of 19.9 µM (17.2–23.1 µM, 95% CI) and 32.9 µM (24.9–43.4 µM, 95% CI), respectively. Esterification of acids **1–3** afforded the corresponding methyl esters **4–6** for additional structure-activity relationship (SAR) analysis. In general, the activity against the MDA-MB-231 breast cancer cell line increased upon esterification of the triterpenoids screened.

Keywords *plant-derived natural products* · ursane family · isolation · structure elucidation · Apoptosis/necrosis assay

Introduction

Cancer refers to any of a group of more than 100 distinct diseases that are characterized by the uncontrolled proliferation of abnormal cells in the body. The genotypic and phenotypic diversity between tumors originating from distinct organs and through distinct mutations necessitate the continued development of novel cytotoxic molecules as well as the characterization of natural products with cytotoxic properties. Despite a diminished focus on natural products recently, compounds originally isolated from natural sources make a significant contribution to the landscape of pharmaceutical agents (Harvey et al. 2015, Gennari et al. 2007) approved by the US Food and Drug Administration (FDA). Furthermore, the World Health Organization estimates that 25% of pharmaceutical drugs are derived from plants known for efficacy among practitioners of traditional medicine (WHO 2003).

In recent years, the use of natural products for drug discovery has declined in favor of combinational methods

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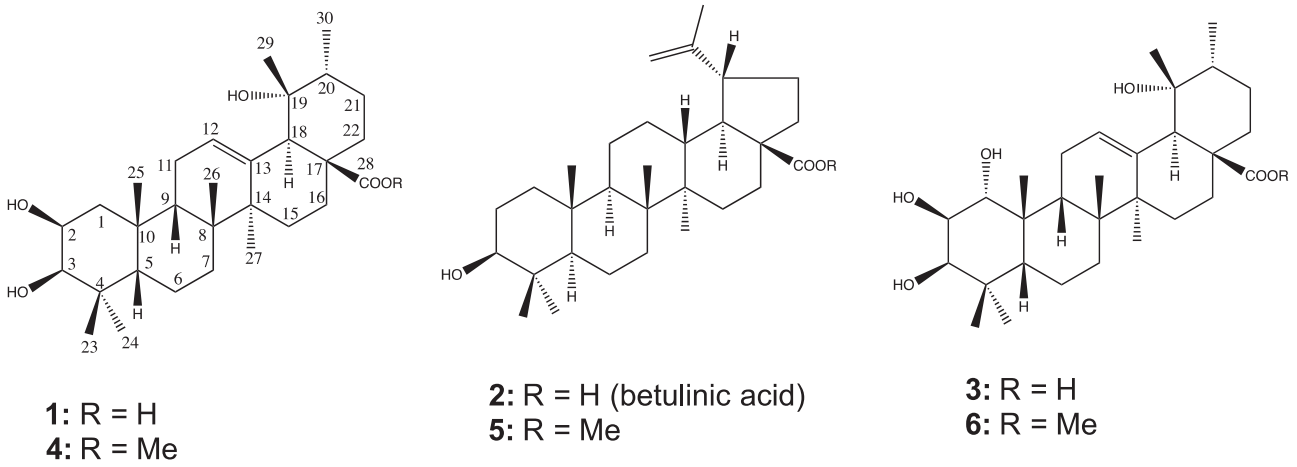

Fig. 1 Triterpenoid acids from *V. paradoxa* and their methyl ester derivatives

which can rapidly generate large libraries of potential lead compounds. In their perspective, Paterson and Anderson (Paterson and Anderson 2005) suggest that it may be time to revisit the prevailing dogma and consider ways in which natural products may continue to inspire the development of new drugs. One approach that we have applied in this report is to use bioactive natural products as a starting point for the design and synthesis of targeted therapeutic agents with specific structural modifications.

The diversity of flora in West Africa represents a promising source for novel anti-cancer agents. *Vitellaria paradoxa* C. F. Gaertn (Sheanut tree) has long been used in traditional medicine for the treatment of various ailments, including inflammation, fever, skin irritations, dermatitis, sunburn, rheumatism, diarrhea, stomach ache and ulcers (Orwa et al. 2009, Moore 2008, Foyet et al. 2015). Moreover, according to an ethno-botanical survey, the bark of the trunk and stems of *V. paradoxa* has been used historically for the treatment of cancer (Jiofack et al. 2010). Following this survey, a preliminary antiproliferation screen of the methanol extract from *V. paradoxa* produced very low GI_{50} values (concentration of the extract causing 50% growth inhibition of the cells) compared with four cancer cell lines (NCI-H460, MCF7, PC3 and HeLa) using a sulforhodamine B (SRB) assay (Tagne et al. 2014). The results from these preliminary studies compelled us to run more comprehensive experiments to isolate and characterize the bioactive compounds responsible for the observed cytotoxic effects.

Herein, we report the isolation and structure elucidation of a new ursane-type triterpenoid, 2β,3β,19α-trihydroxyurs-12-en-28-oic acid (**1**) from the air-dried stem bark of *V. paradoxa* along with seven known compounds. Triterpenoids **1–3** together with their corresponding derived methyl esters **4–6**, were evaluated for cytotoxic activity against the MDA-MB-231 breast cancer cell line.

Results and discussion

Isolation and characterization

A total of 3.2 kg of powdered stem bark of *V. paradoxa* was extracted by sonication, successively with hexanes, ethyl acetate, methanol and water. The MeOH extract was subjected to column chromatography eluting successively with hexanes, hexanes-EtOAc, EtOAc, EtOAc-MeOH and MeOH. Three triterpenoids (**1–3**, Fig. 1) were obtained together with five non-triterpenoids (**7–11**, Fig. 3). Esterification of acids **1–3** afforded their corresponding methyl esters **4–6** (Fig. 1).

Acid **1** was obtained as a white powder from silica gel column chromatography eluting with 55% EtOAc/hexanes. It is soluble in MeOH, stains on TLC with iodine or H_2SO_4 and has a melting point of 260–262 °C. The molecular formula was determined to be $C_{30}H_{48}O_5$ on the basis of HRMS (ESI), (*m/z* 489.1668 $[M+H]^+$) indicating seven degrees of unsaturation. The IR spectrum of acid **1** confirmed the presence of a carboxylic acid group (carbonyl: 1687 cm^{-1}; broad O–H stretch; 2900–3400). Acid **1** displayed an optical rotation of $[\alpha]_D^{26} + 25.5^o$ (*c* 0.22, THF).

The ^1H NMR spectrum (600 MHz, MeOH-d_4) of compound **1** exhibited signals indicative of seven methyl groups at δ_H 1.37 (3H, s), 1.22 (3H, s), 1.04 (3H, s), 1.01 (3H, s), 0.96 (3H, d, 12 Hz), 0.89 (3H, s), and 0.81(3H, s), twenty-four methylene and methine groups, two of which are attached to oxygen atoms at δ_H 3.97 (1H, dt, 6.0, 12.0 Hz) and 3.35(1H, d, 6.0 Hz), and one olefinic proton at δ_H 5.32 (1H, t, 6.0 Hz) (Table 1). The ^{13}C NMR spectrum (150 MHz, MeOH-d_4) exhibited 30 carbon signals that were assigned by a DEPT experiment as seven methyl (δ_C 27.6, 25.7, 23.5, 21.1, 16.1, 15.5, 15.2), eight methylene (δ_C 44.1, 37.6, 32.7, 28.2, 26.0, 25.2, 23.3, 17.9), seven methine,

Table 1 ^{1}H and ^{13}C NMR Data (600 and 150 MHz) of compounds **1–6** (δ_H, $CDCl_3$, MeOH-d_4, *J* in Hz)

C#	1		2		3		4		5		6	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	1.55(1H, m)	41.1	0.88(1H, m)	38.7	3.45(1H, d,	81.4	1.55(1H, m)	41.1	0.88(1H, m)	38.7	3.48 (1H, d, 8.0)	79.8
	1.35(1H, m)		1.55(1H, m)		12.0)		1.32(1H, m)		1.65(1H, m)			
2	3.95 (1H, td, 6.0, 12.0Hz)	65.8	1.75 (2H, m)	27.6	3.63(1H, dd, 3.5, 12.0)	71.7	3.97 (1H, dt, 6.0, 12.0)	65.7	1.75 (2H, m)	27.4	3.68 (1H, dd, 4.0, 12)	70.4
3	3.35(1H, d, 6.0Hz)	78.7	2.98(1H, m)	77.2	3.39(1H, d, 3.5)	80.9	3.35(1H, d, 6.0)	78.7	3.13(1H, dd, 4.0, 12.0)	79.0	3.42(1H, d, 4.0)	79.3
4		38.1		39.0		42.5		38.1		38.7		41.1
5	1.23(1H, m)	48.7	0.65(1H, m)	55.4	1.34(1H, br, d)	49.1	1.20(1H, m)	48.1	0.65(1H, m)	55.4	1.35(1H, m)	48.2
6	1.45(1H, m)	17.9	1.45(1H, m)	18.4	1.78(1H, m)	19.6	1.53(1H, m)	17.9	1.45(1H, m)	18.3	1.78 (1H, m)	18.0
	1.40 (1H, m)		1.40 (1H, m)		0.94(1H, br)		1.48 (1H, m)		1.50 (1H, m)		0.94 (1H, m)	
7	1.65(1H, m)	32.7	1.40(2H, m)	34.4	1.58(1H, m)	34.3	1.62(1H, m)	32.6	1.40(2H, m)	34.3	1.58(1H, m)	32.6
	1.35(1H,m)				1.29(1H, m)		1.32(1H,m)				1.29(1H, m)	
8		38.9		40.7		41.8		41.3		40.7		40.4
9	1.85(1H, m)	46.8	1.33(1H, m)	50.4	2.00(1H, m)	49.4	1.89(1H, m)	46.8	1.25(1H, m)	50.6	2.05(1H, m)	48.4
10		38.0		37.2		44.4		39.9		37.2		42.9
11	2.00(2H, m)	23.3	1.42(1H, m)	20.9	2.55(1H, m)	28.4	2.00(2H, m)	23.4	1.45(1H, m)	20.9	2.67(1H, m)	26.9
			1.21(1H, m)		2.17(1H, dd, 4, 12)				1.25(1H, m)		2.22(1H, dd, 4, 12)	
12	5.32(1H, t, 6.0Hz)	128.0	1.00(1H, m)	25.6	5.29(1H, m)	130.8	5.32(1H, m)	128.1	1.00(1H, m)	25.5	5.32 (1H, t, 4)	129.4
			1.89(1H, m)						1.75(1H, m)			
13		138.7	2.26(1H, td, 4.0, 12.0)	38.1		138.9		138.6	2.13(1H, m)	38.3		137.4
14		41.3		42.5		38.9		41.7		42.4		37.4
15	1.00(1H, m)	28.2	1.26(1H, m)	30.6	1.00 (1H, m)	29.8	1.00(1H, m)	28.1	1.35(1H, m)	30.6	1.00 (1H, m)	28.2
	1.77(1H, m)		1.85(1H, m)		1.77(1H, m)		1.77(1H, m)		1.81(1H, m)		1.77(1H, m)	
16	1.52 (1H, m)	25.9	1.55 (1H, m)	32.2	1.51(1H, m)	26.8	1.52 (1H, m)	25.8	1.45 (1H, m)	32.2	1.51(1H, m)	25.2
	2.62 (1H, td, 6.0, 12 Hz)		2.19 (1H, m)		2.5(1H, m)		2.67 (1H, td, 6.0, 12)		2.18 (1H, m)		2.61(1H, m)	
17		47.9		55.9		49.0		48.1		56.6		48.1
18	2.52(1H, s)	53.7	1.55(1H, m)	49.0	2.48(1H, s)	55.1	2.54(1H, s)	53.7	1.55(1H, m)	49.5	2.53(1H, s)	53.6
19		72.2	3.00 (1H, m)	47.1		73.6		72.1	2.95 (1H, m)	47.0		72.1
20	1.35(1H, m)	41.7		150.8	1.34(1H, m)	43.2	1.32(1H, m)	41.7		150.1	1.34(1H, m)	41.6
21	1.35(2H, m)	25.2	1.22(1H, m)	29.7	1.51(1H, m)	27.5	1.65(1H, m)	25.2	1.20(1H, m)	29.7	1.51(1H, m)	25.8
			1.45(1H, m)		1.48(1H, m)		1.25(1H, s)		1.40(1H, m)		1.48(1H, m)	
22	1.65(1H, m)	37.6	1.45(1H, m)	36.8	1.72(1H, m)	39.2	1.65(1H, m)	37.4	1.45(1H, m)	37.0	1.72(1H, m)	37.5
	1.75(1H, m)		1.82(1H, m)		1.62 (1H, m)		1.75(1H, m)		1.86(1HH, m)		1.62 (1H, m)	
23	1.40(3H, s)	23.5	0.94(3H, s)	28.6	1.17(3H, s)	29.3	1.37(3H, s)	23.5	0.89(3H, s)	28.0	1.22(3H, s)	27.7
24	0.89 (3H, s)	21.1	0.77 (3H, s)	16.2	0.86(3H, s)	22.5	0.89 (3H, s)	21.0	0.75 (3H, s)	15.4	0.90(3H, s)	21.0
25	1.02(3H, s)	15.5	0.65(3H, s)	16.3	1.00 (3H, s)	13.1	1.01(3H, s)	15.4	0.68(3H, s)	16.0	1.03 (3H, s)	11.5
26	0.81(3H, s)	16.1	0.87(3H, s)	16.4	0.77(3H, s)	17.8	0.71(3H, s)	16.0	0.89(3H, s)	16.1	0.72(3H, s)	16.2
27	1.21(3H, s)	25.7	0.88(3H, s)	14.9	1.33(3H, s)	25.1	1.21(3H, s)	25.6	0.84(3H, s)	14.7	1.37(3H, s)	23.6
28		180.8		177.7		182.4		179.1		176.7		179.1
29	1.01(3H, s)	27.6	4.70(1H, d, 4.0Hz)	110.1	0.97(3H, s)	27.2	1.01(3H, s)	27.8	4.69(1H, d, 4.0)	109.4	0.99(3H, s)	25.6
			4.57(1H, d, 4.0 Hz)						4.53(1H, d, 4.0)			
30	0.96 (3H, d, 12)	15.2	1.65 (3H, s)	19.4	0.92(3H, d, 8.0)	16.8	0.96 (3H, d, 12.0)	15.1	1.61 (3H, s)	19.4	0.95(3H, d, 8.0)	15.1
31							3.60(3H, s)	50.7	3.60(3H, s)	51.3	3.60(3H, s)	50.7

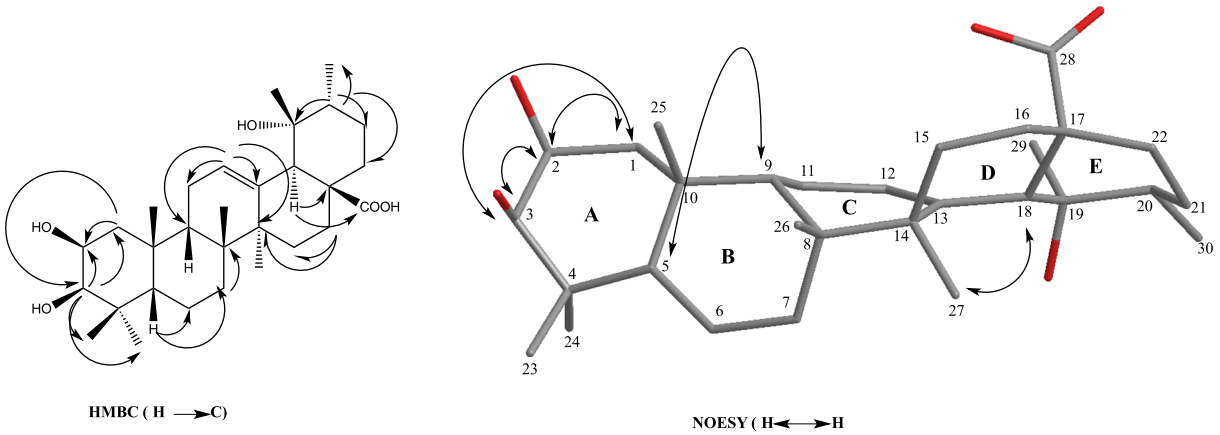

Fig. 2 Main HMBC and NOESY correlations of compound **1**

including two –CH(OH) (δ_C 78.7, 65.8), four –CH (δ_C 53.7, 48.0, 46.8, 41.7) and three sp^2-hybridized carbons (δ_C 180.8 (C=O), 138.7 (C=C), 127.9 (=CH), one tertiary alcohol (72.2 (C–OH)) and five quaternary carbons (δ_C 47.9, 41.3, 38.9, 38.1, 38.0) (Table 1). The above NMR data are characteristic of the triterpenoid class of natural products (Peng et al. 1998, Liu et al. 2016).

The presence of a methyl doublet assigned to proton H-20 (δ 0.96, d, 3H, $J = 12$ Hz) and a quaternary carbon atom at δ 72.2, attributed to C-19, are signals typical of an ursane-type triterpenoid (Eyong et al. 2015, Liu et al. 2016). The ^{1}H and ^{13}C NMR data (Table 1) were assigned using ^{1}H–^{1}H COZY, HSQC, HSQC–TOCSY, HMBC and NOESY spectra (Supporting Information). In the ^{1}H–^{1}H COZY, correlations were observed between H-2 (δ 3.95, td, $J = 6.0$, 12.0 Hz) and H-3 (δ 3.35, d, $J = 6.0$ Hz) with a coupling constant $^3J_{\text{H-2,H-3}} = 6.0$ Hz suggesting a cis coupling (Minch 1994). Also observed were correlations between H-2, H-3, H-1a (δ 1.55, m) and H-1b (δ 1.35, m) from the ring A nucleus. The ring A correlations were observed in the HSQC–TOCSY and TOCSY experiments. Additional ^{1}H–^{1}H-COZY correlations were identified between H-5 (δ 1.23, m), H–6a (1.45, m), H-6b (1.40, m), H-7a (1.65, m) and H-7b (1.35, m) in ring B, H-12 (δ 5.32, t, 6.0 Hz), H-11 (δ 2.00, m, 2H) and H-9 (δ 1.85, m) for ring C, H-16a (δ 2.62 td, 6.0, 12.0 Hz), H-16b (δ 1.52, m), H-15a (δ 1.77, m) and H-15b (δ1.00, m) for ring D, and correlations between Me-30 (δ 0.96, d, 12), H-20 (1.35, m), H-21 (1.35, m), H-22a (1.75, m) and H-22b (1.65, m) for ring E. The HSQC–TOCSY correlations of H-12 (δ 5.32) with C-11 (δ 23.3), C-9 (46.8) and C-12 (128.0), H-2 (δ 3.97) with C-1(δ 41.1), C-2(65.8) and C-3(78.7), H-16$_\beta$(δ 2.62) with C-16(δ 25.9) and C-15(δ 28.2) support our assignment. Typical in the HMBC spectrum are correlations of H-12(δ 5.32) with C-11(δ 23.3), C-14(41.3), C-9(46.8), and C-18 (53.7) supporting H-12 connectivity in ^2J or ^3J to these carbon atoms (Fig. 2). The HMBC correlations between H-16$_\beta$(δ 2.62) with C-15(δ 28.2), C-17(47.91), C-18(53.7) and C-28 (180.8) support the ring D connectivity. Moreover, the NOESY correlations between H-18 (δ 2.52) with H-27 (1.21), H-2 (3.95) with H-3 (3.35) and H-5 (1.23) with H-9 (1.85) confirmed the relative configuration of C-2, C-3, C-5, C-9 and C-18 in Fig. 2. The coupling constant between H-2, H-3 ($^3J_{\text{H-2,H-3}} = 6.0$ Hz) indicated a syn configuration of the two hydrogens and thus a syn diol as previously observed in similar triterpenoids (Eyong et al. 2015). Therefore, compound **1** was determined to be 2β,3β,19α-trihydroxyurs-12-en-28-oic acid.

Compound **2** was obtained as a white powder from a silica gel column eluting with 25 → 27.5% EtOAc/hexanes. It is soluble in methanol, stains on TLC in iodine or H_2SO_4, has a melting point of 282–285 °C, and gave LRMS ($APCI^+$) Calcd. for $C_{30}H_{47}O_3$ $(M–H)^+$ 455.4, found *m/z*: 455.3. These properties along with the IR and NMR data (supplemental information) matched those previously reported for betulinic acid (Peng et al. 1998) (Fig. 1).

Compound **3** was obtained as a white powder from a silica gel column eluting with 70 → 80% EtOAc/hexanes. It is soluble in methanol, stains on TLC in iodine or H_2SO_4, has a melting point of 262–264 °C and gave LRMS($APCI^+$) Calcd. for $C_{30}H_{47}O_6$ $(M–H)^+$ 503.4, found *m/z*: 503.3. These properties along with the IR and NMR data (SI) matched those previously reported to the known 1α,2β,3β,19α-tretrahydroxyurs-12-en-28-oic acid (Eyong et al. 2015) (Fig. 1).

To obtain derivatives for structure activity relationship (SAR) studies, esterification was carried out on compounds **1–3** using diazomethane generated *in situ* from 1-methyl-3-nitro-1-nitrosoguanidine (MNG) (McKay 1948) to afford methyl esters **4–6** (Fig. 1).

After esterification of compound **1** with diazomethane, compound **4** was purified by column chromatography on silica gel using 50% EtOAc/hexanes (75% yield) and isolated as a white powder. It is soluble in MeOH, stains on

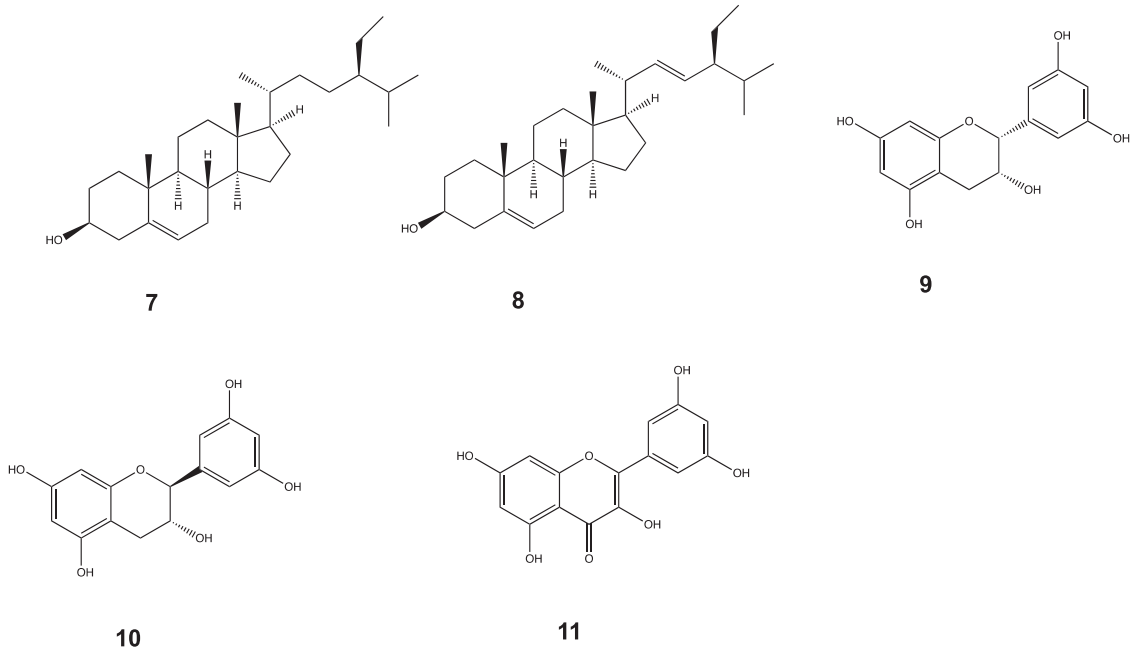

Fig. 3 Non-triterpenoids from the methanol extract of *V. paradoxa*

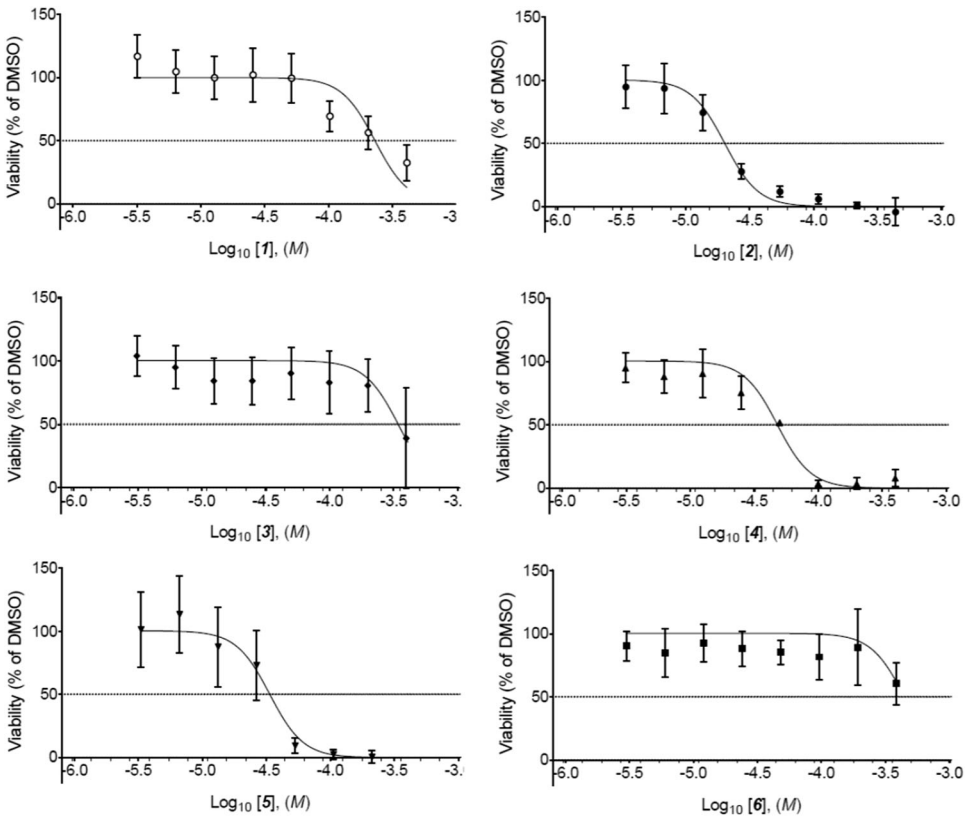

Fig. 4 Triterpenoids **2**, **4** and **5** display cytotoxic activity against MDA-MB-231 breast cancer cells. MDA-MB-231 cells were plated at a density of 2000 cells per well and treated with the indicated compounds for 3 days followed by cell viability assay. Error bars indicate the standard deviation of three independent measurements. The curves indicate the best fit of a non-linear curve fitting the equation, $Y = 100/(1 + 10^{X-\log\ IC50})$

TLC in iodine or H_2SO_4 and had a melting point of 103–105 °C. The IR spectrum showed λ_{max}: 3438, 2928, 2873, 1708, 1456, 1378 cm^{-1} and LRMS(APCI$^+$) Calcd. for $C_{31}H_{51}O_5$ $(M + H)^+$ 503.4, found *m/z*: 503.18. The ^1H NMR (600 MHz) and ^{13}C NMR spectrum of compound **4** in MeOH-d_4 was similar to compound **1** except for the

Table 2 IC_{50} values calculated from Fig. 4 for the indicated compounds upon a 3 day exposure to MDA-MB-231 breast cancer cells

Compounds	1	2	3	4	5	6
IC_{50} (μM)	230	19.9	63.5	46.7	32.9	430
95% Confidence intervals (μM)	179–295	17.2–23.1	51.5–78.3	39.8–54.9	24.9–43.4	328–564

appearance of a new methyl singlet at δ 3.60 (3H, s) in the ^{1}H NMR and at δ 50.7 in the ^{13}C NMR (Table 1).

After esterification of acid **2** with diazomethane, methyl ester **5** was purified by column chromatography on silica gel using 20% EtOAc/hexanes (95% yield) and isolated as a white powder. It is soluble in CH_2Cl_2, stains on TLC with iodine or H_2SO_4 and melts at 205–207 °C. An IR spectrum showed signals at 3437, 2927, 1698, 1452, 1378 cm^{-1} and a low resolution LRMS(APCI$^+$) was obtained: Calcd. for $C_{31}H_{51}O_3$ $(M+H)^+$ 471.4, found *m/z*: 471.20. The ^{1}H NMR (600 MHz) and ^{13}C NMR spectra of methyl ester **5** in $CDCl_3$ were similar to those of (3β)-3-hydroxy-lup-20(29)-en-28-oic acid (**2**) except for the appearance of a new methyl singlet at δ 3.60 (3H, s) for the ^{1}H NMR and at δ 51.3 for the ^{13}C NMR (Table 1).

After esterification of compound **3** with diazomethane, compound **6** was purified by column chromatography on silica gel using 65% EtOAc/hexanes (75% yield) and isolated as a white powder. It is soluble in MeOH, stains on TLC with iodine or H_2SO_4 and exhibits a m.p. 155–157 °C. The IR spectrum showed signals at 3435, 2929, 2871, 1700, 1454, 1378 cm^{-1} and a low resolution MS(APCI$^+$) was obtained: Calcd. for $C_{31}H_{51}O_6$ $(M+H)^+$ 519.4, found *m/z*: 519.16. The ^{1}H NMR (600 MHz) and ^{13}C NMR spectra of ester **6** in MeOH-d_4 were similar to those of acid **3** except for the appearance of the new methyl peak at δ 3.60 (3H, s) in the ^{1}H NMR and at δ 50.7 in the ^{13}C NMR (Table 1).

In addition to the triterpenoids, five non-triterpenes were obtained and include β-sitosterol (**7**), sigmasterol (**8**), (−)-epicatechin (**9**), (+)-catechin (**10**), and quercetin (**11**) (See Fig. 3) (Zhang et al. 2014).

Cytotoxic activity

We next sought to determine the cytotoxic activity of each of these compounds in the context of breast cancer. For this, we chose the triple-negative MDA-MB-231 breast cancer cell line, commonly used for *in vitro* assays (Lacroix and Leclercq 2004). The compounds screened exhibited differential activity, with compounds **2, 4** and **5** showing the highest cytotoxic activity against MDA-MB-231 cells (Fig. 4 and Table 2). To determine whether the compounds induced programmed cell death or necrosis, we next performed staining with annexin V and propidium iodide (PI). Necrosis was not observed with compounds **2** and **5** at the approximate IC_{50} doses, however, the proportion of cells positive for annexin V increased (38 and 45%, respectively, relative to 20% in vehicle-treated cells) indicating induction of apoptosis (Fig. 5).

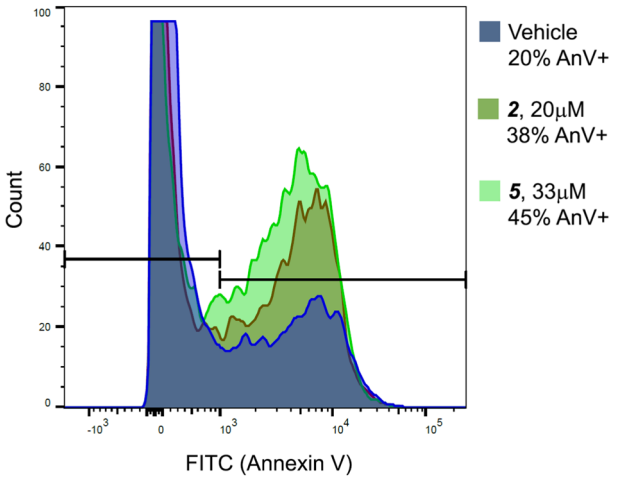

Fig. 5 Induction of apoptosis by triterpenoids **2**, and **5**. Cells treated with IC_{50} doses of the indicated compounds for 24 h were stained with annexin V and propidium iodide (PI). Flow cytometry was performed and the proportion of cells positive for annexin V is indicated. Note that the y-axis is shortened to better indicate the differences in the annexin V-positive populations. The proportion of cells positive for annexin V is indicated in the legend

Discussion

In the present investigation, the methanolic extract of the stem bark of *V. paradoxa* afforded eight compounds belonging to three main classes of natural products: triterpenoids (**1–3**), steroids (**7–8**) and flavonoids (**9–11**). Of the triterpenoids isolated, 2β,3β,19α-trihydroxyurs-12-en-28-oic acid (**1**) is reported for the first time. Esterification of the triterpenoids was carried out for SAR studies and a general trend was determined. The methyl esters **4–6** were more active than the isolated carboxylic acids **1–3** against the MDA-MB-231 breast cancer cell line. This trend could be due to greater cell permeability of the more lipophilic ester derivatives. The carboxylic acid natural products carry a negative charge at physiological pH and this could hinder passive diffusion across cell membranes. Observed IC_{50} values of these compounds are near the range of clinically useful chemotherapeutic agents such as cisplatin (IC_{50} = 6.7 μM for MDA MB 231 cells, *data not shown*). Clinically, breast cancer can be divided into distinct subtypes that have

prognostic and therapeutic implications. Most breast cancer patients have a subtype defined by the expression of the estrogen receptor (ER), progesterone receptor (PR), or amplification of HER-2/Neu (Chave et al. 2010, Foulkes et al. 2010). These markers allow classification of breast cancer tumors as hormone receptor positive or HER-2/Neu amplified. However, a fourth type of breast cancer referred to as triple-negative breast cancer (TNBC) does not express ER, PR, or have HER-2/Neu amplification. The name of this cancer type, triple-negative is based on the lack of these three molecular biomarkers. Given the lack of validated molecular targets and the poor outcome of patients with TNBC, there is a clear need for a greater understanding of TNBC and for the development of novel therapies to treat this specific cancer type.

The transport of drugs across membranes involves one or more of the following processes: (1) passive diffusion, (2) filtration, (3) bulk flow, (4) active transport, (5) facilitated transport, (6) ion-pair transport, (7) endocytosis, and (8) exocytosis. Passive drug absorption depends on a number of physicochemical factors, the three most important of which are lipophilicity, solubility and charge (Alavijeh et al. 2005). The formation of methylesters from the corresponding carboxylic acids has a considerable impact on the net charge of these compounds since esters are neutral and acids are negatively charged at physiological pH. We also observed a considerable decrease in the melting points of the esters which corresponds with decreased polarity and intramolecular interactions (e.g. hydrogen bonding). These results suggest that esterification of the natural products may have improved their passive cell membrane permeability. Lipophilic substances that do not possess a charge tend to more readily diffuse passively across the hydrophobic cellular membranes. These substances are more hydrophobic with a tendency to be driven into cellular membranes from an aqueous environment.

Conclusion

Phytochemical studies on the methanol extract of the stem bark of *V. paradoxa* afforded a new ursane triterpenoid **1**, together with two known ursane (**3**) and lupeol-type (**2**) triterpenoids. Methylation of compounds **1–3** afforded their methyl esters **4–6**. In a general trend, the methyl esters were more active than the isolated natural triterpenoids **1–3** against the MDA-MB-231 breast cancer cell line. Further derivatization of these triterpenoids could lead to a better understanding of structure-activity relationships for this class of natural products. Additional screening against a broader range of cancer cell lines may reveal additional utility of these compounds as drug leads and may fuel investigations into their putative cellular receptors and a molecular level understanding of their mode of action.

Experimental

Plant material

The stem bark of *V. paradoxa* was harvested in Garoua-Cameroon (July, 2012) and identified by Mr. Bamba Jean Paul (plant taxonomist) of the National School of Fauna, Garoua, where voucher specimens were deposited (HEFGN 6276). The stem bark was collected, cut into small pieces, dried at ambient temperature (~25 °C) and powdered.

Extraction and isolation

A total of 3.2 kg of powdered stem bark was extracted by sonication, successively with hexane, ethyl acetate, methanol and water. A portion (400 g) of the MeOH extract was then subjected to silica gel F60, 40–63 µm column chromatography using a hexanes, hexanes-ethyl acetate gradient, ethyl acetate, ethyl acetate-methanol gradient and finally methanol as eluent. Eight natural products: 2β,3β,19α-trihydroxyurs-12-en-28-oic acid (**1**, 60 mg), betulinic acid (**2**, 700 mg), 1α,2β,3β,19α-tretrahydroxyurs-12-en-28-oic acid (**3**, 200 mg), *β*-sitosterol (**7**, 100 mg), sigmasterol (**8**, 80 mg), (-)-epicatechin (**9**, 45 mg), (+)-catechin (**10**, 75 mg), and quercetin (**11**, 20 mg) were isolated.

*2β,3β,19α-trihydroxyurs-12-en-28-oic acid (**1**)*

White powder (MeOH) obtained from Hexane-EtOAc 55%; mp 260–262 °C; $[\alpha]_{25D}$ + 25.5 (c 0.22, THF); IR ν_{max} 2953, 1702, 1449 cm^{-1}. ^{1}H NMR (MeOH-d_4, 600 MHz,): $\delta = 5.32$ (1H, t, 6.0 Hz, H-12), 3.95 (1H, td, 6.0, 12.0 Hz, H-2), 3.35 (1H, d, 6.0 Hz, H-3), 2.62 (1H, td, 6.0, $J = 12$ Hz, H-16a), 2.52(1H, s, H-18), 2.00(2H, m, H-11), 1.85(1H, m, H-9), 1.77(1H, m, H-15a), 1.75(1H, m, H-22a), 1.65(1H, m, H-22b), 1.65(1H, m, H-7a), 1.55(1H, m, H-1b), 1.52 (1H, m, H-16b), 1.45(1H, m, H-6a), 1.40 (1H, m, H-6b), 1.40(3H, s, H-23), 1.35(1H, m, H-1a), 1.35(1H, m, H-7b), 1.35(1H, m, H-20), 1.35(2H, m, H-21), 1.23(1H, m, H-5), 1.21(3H, s, H-27), 1.02(3H, s, H-25), 1.01(3H, s, H-29), 1.00(1H, m, H-15b), 0.96 (3H, d, $J = 12.0$ Hz, H-30), 0.89 (3H, s, H-24), 0.81(3H, s, H-26) and ^{13}C NMR (MeOH-d_4, 150 MHz,): δ = 180.8(C-28), 138.7(C-13), 128.0 (C-12), 78.7(C-3), 72.2 (C-19), 65.8(C-2), 53.7 (C-18), 48.7(C-5), 47.9 (C-17), 46.8 (C-9), 41.7 (C-20), 41.1(C-1), 41.3 (C-14), 38.9 (C-8), 38.1 (C-4), 38.0 (C-10), 37.6 (C-22), 32.7(C-7), 28.2 (C-15), 27.6 (C-29), 25.9(C-16), 25.7(C-27), 25.2 (C-21), 23.5(C-23), 23.3 (C-11), 21.1(C-24), 17.9(C-6), 16.1(C-26), 15.5

(C-25), 15.2(C-30), LRMS(APCI$^+$) *m/z*: Calcd. for $C_{30}H_{47}O_5$ $(M–H)^+$; 487.4. Found 487.3.

*(3β)-3-Hydroxy-lup-20(29)-en-28-oic acid or Betulinic acid (**2**)*

White powder (MeOH) obtained from Hexane-AcOEt 25–27.5%. mp 282–285 °C. ^{1}H NMR (MeOH-d_4, 600 MHz,): $\delta = 4.70$(1H, d, 4.0 Hz, H-29a), 4.57(1H, d, 4.0 Hz, H-29b), 3.00 (1H, m, H-19), 2.98(1H, m, H-3), 2.26(1H, td, $J = 4.0$ 12.0 Hz, H-13), 2.19 (1H, m, H-16a), 1.89(1H, m, H-12a), 1.85(1H, m, H-15a), 1.82(1H, m, H-22a), 1.75 (2H, m, H-2), 1.65 (3H, s, H-30), 1.55(1H, m, H-18), 1.55 (1H, m, H-16b), 1.55(1H, m, H-1a), 1.45(1H, m, H-6a), 1.45(1H, m, H-22b), 1.45(1H, m, H-21a), 1.42(1H, m, H-11a), 1.40 (1H, m, H-6b), 1.40(2H, m, H-7), 1.33(1H, m, H-9), 1.22 (1H, m, H-21b), 1.21(1H, m, H-11b), 1.00(1H, m, H-12b), 0.94(3H, s, H-23), 0.88(1H, m, H-1b), 0.88(3H, s, H-27), 0.87(3H, s, H-26), 0.65(3H, s, H-25), 0.65(1H, m, H-5) and ^{13}C NMR (MeOH-d_4, 150 MHz,): $\delta = 177.7$(C-28), 150.8 (C-20), 110.1(C-29), 77.2(C-3), 55.9(C-17), 55.4(C5), 50.4 (C-9), 49.0(C-18), 47.1(C-19), 42.5(C-14), 40.7(C-8), 39.0 (C-4), 38.7 (C-1), 38.1(C-13), 37.2(C-10), 36.8(C-22), 34.4 (C-7), 32.2(C-16), 30.6(C-15), 29.7(C-21), 28.6(C-23), 27.6 (C-2), 25.6(C-12), 20.9(C-11), 19.4(C-30), 18.4(C-6), 16.4 (C-26), 16.3(C-25), 16.2(C-24), 14.9(C-27) LRMS(APCI$^+$) *m/z*: Calcd. for $C_{30}H_{47}O_3$ $(M–H)^+$; 455.4. Found 455.33

*1α,2β,3β,19α-tretrahydroxyurs-12-en-28-oic acid (**3**)*

White powder (MeOH) obtained from Hexane-AcOEt 70%. mp 262 °C. ^{1}H NMR (MeOH-d_4, 600 MHz,): $\delta = 5.29$(1H, m, H-12), 3.63(1H, dd, $J = 3.5$, 12.0 Hz, H-2), 3.45(1H, d, $J = 12.0$ Hz, H-1), 3.39(1H, d, $J = 3.5$ Hz, H-3), 2.55(1H, m, H-11a), 2.50(1H, m, H-16a), 2.48(1H, s, H-18), 2.17 (1H, dd, $J = 4$, 12 Hz, H-11b), 2.00(1H, m, H-9), 1.78(1H, m, H-6a), 1.77(1H, m, H-15a), 1.72(1H, m, H-22b), 1.62 (1H, m, H-22a), 1.58(1H, m, H-7a), 1.51(1H, m, H-21a), 1.51(1H, m, H-16b), 1.48(1H, m, H-21b), 1.34(1H, m, H-20), 1.34(1H, br, H-5), 1.33(3H, s, H-27), 1.29(1H, m, H-7b), 1.17(3H, s, H-23), 1.00 (1H, m, H-15b), 1.00 (3H, s, H-25), 0.97(3H, s, H-29), 0.94(1H, br, H-6b), 0.92(3H, d, 8.0, H-30), 0.86(3H, s, H-24), 0.77(3H, s, H-26) and ^{13}C NMR (MeOH-d_4, 150 MHz,): $\delta = 182.4$(C-28), 138.9(C-13), 130.8(C-12), 81.4(C-1), 80.9(C-3), 73.6(C-19), 71.7(C-2), 55.1(C-18), 49.4(C-9), 49.1(C-5), 49.0(C-17), 44.4(C-10), 43.2(C-20), 42.5(C-4), 41.8(C-8), 39.2(C-22), 38.9(C-14), 34.3(C-7), 29.8(C-15), 29.3(C-23), 28.4(C-11), 27.5(C-21), 27.2(C-29), 26.8(C-16), 25.1(C-27), 22.5(C-24), 19.6(C-6), 17.8(C-26), 16.8(C-30), 13.1(C-25); LRMS(APCI$^+$) *m/z*: Calcd. for $C_{30}H_{47}O_6$ $(M–H)^+$; 503.4. Found 503.30

*2β,3β,19α-trihydroxyurs-12-en-28-oic methyl ester (**4**)*

White powder (MeOH) obtained from hexane-AcOEt 50%. mp 103–105 °C. ^{1}H NMR (MeOH-d_4, 600 MHz,): $\delta = 5.32$ (1H, m, H-12), 3.97 (1H, dt, $J = 6.0$, 12.0 Hz, H-2), 3.60 (3H, s, H-31), 3.35(1H, d, $J = 6.0$ Hz, H-3), 2.67 (1H, td, $J = 6.0$, 12 Hz, H-16a), 2.54(1H, s, H-18), 2.00(2H, m, H-11), 1.89(1H, m, H-9), 1.77(1H, m, H-15a), 1.75(1H, m, H-22a), 1.65(1H, m, H-22b), 1.65(1H, m, H-21a), 1.62(1H, m, H-7a), 1.55(1H, m, H-1a), 1.53(1H, m, H-6a), 1.52 (1H, m, H-16a), 1.48 (1H, m, H-6b), 1.37(3H, s, H-23), 1.32(1H, m, H-20), 1.32(1H, m, H-7b), 1.32(1H, m, H-1b), 1.25(1H, s, H21b), 1.21(3H, s, H-27), 1.20(1H, m, H-5), 1.01(3H, s, H-29), 1.01(3H, s, H-25), 1.00(1H, m, H-15b), 0.96 (3H, d, $J = 12.0$ Hz, H-30), 0.89 (3H, s, H-24), 0.71(3H, s, H-26), and ^{13}C NMR (MeOH-d_4, 150 MHz,): $\delta = 179.1$(C-28), 138.6(C-13), 128.1(C-12), 78.7(C-3), 72.1(C-19), 65.7(C-2), 53.7(C-18), 50.7(C-31), 48.1(C-17), 48.1(C-5), 46.8(C-9), 41.7(C-20), 41.7(C-14), 41.3(C-8), 41.1(C-1), 39.9(C-10), 38.1(C-4), 37.4(C-22), 32.6(C-7), 28.1(C-15), 27.8(C-29), 23.5(C-23), 25.8(C-16), 25.6(C-27), 25.2(C-21), 23.4 (C-11), 21.0(C-24), 17.9(C-6), 16.0(C-26), 15.4(C-25), 15.1 (C-30); LRMS(APCI$^+$) *m/z*: Calcd. for $C_{31}H_{51}O_5$ $(M + H)^+$; 503.4. Found 503.18.

*(3β)-3-Hydroxy-lup-20(29)-en-28-oic acid methyl ester or Betulinic acid methyl ester (**5**)*

White powder (CH_2Cl_2) obtained from Hexane-AcOEt 20%, mp 205–207 °C. ^{1}H NMR ($CDCl_3$, 600 MHz,): $\delta =$ 4.69(1H, d, $J = 4.0$ Hz, H-29a), 4.53(1H, d, $J = 4.0$ Hz, H-29b), 3.60(3H, s, H-31), 3.13(1H, dd, $J = 4.0$, 12.0 Hz, H-3), 2.95 (1H, m, H-19), 2.18 (1H, m, H-16a), 2.13(1H, m, H-13), 1.86(1H, m, H-22a), 1.81(1H, m, H-15b), 1.75 (2H, m, H-2), 1.75(1H, m, H-12a), 1.65(1H, m, H-1a), 1.61 (3H, s, H-30), 1.55(1H, m, H-18), 1.50 (1H, m, H-6a), 1.45 (1H, m, H-16b), 1.45(1H, m, H-6b), 1.45(1H, m, H-11a), 1.45 (1H, m, H-22b), 1.40(1H, m, H-21a), 1.40(2H, m, H-7), 1.35(1H, m, H-15a), 1.25(1H, m, H-11b),1.25(1H, m, H-9), 1.20(1H, m, H-21b), 1.00(1H, m, H-12b), 0.89(3H, s, H-26), 0.89(3H, s, H-23), 0.88(1H, m, H-1b), 0.84(3H, s, H-27), 0.75 (3H, s, H-24), 0.68(3H, s, H-25), 0.65(1H, m, H-5) and ^{13}C NMR (MeOH-d_4, 150 MHz,): $\delta = 176.7$(28), 150.1(20), 109.4(C-29), 79.0(C-3), 56.6(C-17), 55.4(C-5), 51.3(C-31), 50.6(C-9), 49.5(C-18), 47.0(C[19), 42.4(C-14), 40.7(C-8), 38.7(C-4), 38.7 (C-1), 38.3(C-13), 37.2(C-10), 37.0(C-22), 34.3(C-7), 32.2(C-16), 30.6(C-15), 29.7(C-21), 28.0(C-23), 27.4(C-2), 25.5(C-12), 20.9(C-11), 19.4(C-30), 18.3(C-6), 16.1(C-26), 16.0(C-25), 15.4(C-24), 14.7(C-27); LRMS(APCI$^+$) *m/z*: Calcd. for $C_{31}H_{51}O_3$ $(M + H)^+$; 471.4. Found 471.20.

*1α,2β,3β,19α-tretrahydroxyurs-12-en-28-oic acid methyl ester (**6**)*

White powder (MeOH) obtained from Hexane-AcOEt 65%, mp 155–157 °C. ^{1}H NMR (MeOH-d_4, 600 MHz,): $\delta = 5.32$ (1H, t, $J = 4$ Hz, H-12), 3.68 (1H, dd, $J = 4.0$, 12 Hz, H-2), 3.60 (3H, s, H-31), 3.48 (1H, d, $J = 8.0$ Hz, H-1), 3.42(1H, d, $J = 4.0$ Hz, H-3), 2.67(1H, m, H-11a), 2.61(1H, m, H-16a), 2.53(1H, s, H-17), 2.22(1H, dd, $J = 4$, 12 Hz, H-11b), 2.05(1H, m, H-9), 1.78 (1H, m, H-6a), 1.77(1H, m, H-15a), 1.72(1H, m, H-22a), 1.62 (1H, m, H-22b), 1.58(1H, m, H-7a), 1.51(1H, m, H-21a), 1.51(1H, m, H-16b), 1.48(1H, m, H-21b), 1.37(3H, s, H-27), 1.35(1H, m, H-5), 1.34(1H, m, H-20), 1.29(1H, m, H-7b), 1.22(3H, s, H-23), 1.03 (3H, s, H-25), 1.00 (1H, m, H-15b), 0.99(3H, s, H-29), 0.95(3H, d, $J = 8.0$ Hz, H-30), 0.94 (1H, m, H-6b), 0.90(3H, s, H-24), 0.72(3H, s, H-26), and ^{13}C NMR (MeOH-d_4, 150 MHz,): δ = 179.1(C-28), 137.4(C-13), 129.4(C-12), 79.8(C-1), 79.3 (C-3), 72.1(C-19), 70.4(C-2), 50.7(C-31), 53.6(C-18), 48.4 (C-9), 48.2(C-5), 48.1(C-17), 42.9(C-10), 41.6(C-20), 41.1 (C-4), 40.4(C-8), 37.5(C-22), 37.4(C-14), 32.6(C-7), 28.2 (C-15), 27.7(C-23), 26.9(C-11), 25.8(C-21), 25.6(C-29), 25.2(C-16), 23.6(C-27), 21.0(C-24), 18.0(C-6), 16.2(C-26), 15.1(C-30), 11.5(C-25); LRMS(APCI$^+$) *m/z*: Calcd. for $C_{31}H_{51}O_6$ $(M + H)^+$; 519.4. Found 519.16.

Analytical measurements

High resolution mass spectrometry (HRMS) was carried out using an Accela liquid chromatograph coupled to an Orbitrap Q-Exactive Focus mass spectrometer. Compounds were injected (5 µL) into the LC system consisting of a 15 cm × 2.1 mm (5 µm) Eclipse Plus-C8 column at a constant flow rate of 350 µL/min). A binary mobile phase gradient containing 0.1 % (v/v) formic acid in water (A) and acetonitrile (B) was applied as follows: 97% A for 5 min, to 98% B in 30 min, held for 5 min, back to 97% A in 1 min, and equilibrated for 5 min at 97% A. Analytes were introduced into the mass spectrometer using positive electrospray ionization (+ESI). Full-scan accurate mass spectra (*m/z* range: 50–750) of eluting compounds were obtained at high resolution (70,000 FWHM) on the Orbitrap mass analyzer using internal calibration (accuracy of measurements < 1 ppm) and processed using Xcalibur 4.0.27.10 software. Electrospray source conditions were as follows: sheath and auxiliary gas flow 49 and 12 arbitrary units (a.u.), respectively; electrospray voltage 3.5 kV; capillary temperature 300 °C; and S-Lens RF level 50 V.

Optical rotations were recorded on a polarimeter in MeOH at 26 °C. *Fourier* Transform Infrared (FTIR) spectra were recorded as thin films. 1D (^{1}H, ^{13}C and DEPT) and 2D (^{1}H–^{1}H COZY, HSQC, HSQC-TOCSY, HMBC, TOCSY, NOESY and ROESY) NMR spectra were recorded at 600 MHZ spectrometer equipped with a cryoprobe. The chemical shifts are quoted relative to TMS, and the coupling constants are provided in Hz. The chromatographic silica gel (450–550 mesh) purchased from SiliCycle, F60, 40–63 µm 60 Å was used.

Chemicals

1-Methyl-3-nitro-1-nitrosoguanidine (wetted with ca. 50% water) a product of TGI, Lot. JBCSI-TA was used to generate diazomethane (McKay 1948). Potassium hydroxide and diethyl ether were purchased from Sigma-Chemical Co. (St Louis, MO, USA). MTS reagent (CellTiter 96 AQ Assay) was purchased from TCI (Mfr No: M0527).

Biological assays

MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (Corning), supplemented with 10% fetal bovine serum (Gibco) and antibiotics (Lonza). For the determination of cytotoxicity, cells were plated in 96-well plates (Nest Scientific) at a density of 2000 cells per well in 100 µl of media per well. On the following day, the media was aspirated, and 100 µl of media in which a serial dilution of DMSO-dissolved compound (or DMSO as a control) was added to wells in triplicate. 3 days later, the relative numbers of metabolically active cells was determined by addition of the CellTiter-Glo reagent (Promega) and measurement of chemiluminesence according to the manufacturer's protocol using a Fluoroskan Ascent FL (Thermo Scientific). To calculate the percent viability, signal from background wells (media only) was subtracted from each well and then the remaining chemiluminesence value was normalized to signal from wells with the equivalent amounts of DMSO. Apoptosis induction was measured using the Annexin V / Propidium Iodide staining kit (BD Biosciences) per manufacturer's instructions. Assays were performed in at least triplicate.

Statistical analysis

Graphing was done using GraphPad Prism 6 (GraphPad Software). IC_{50} values were calculated by fitting a non-linear curve using the "log-inhibitor vs normalized response" function given by the equation $Y = 100/(1 + 10^{X\text{-log IC50}})$.

Methylation

Methylation of carboxylic acids **1–3** with diazomethane. To a stirred slurry of 50% aq. KOH (10 mL) in Et_2O (5.0 mL) at 0 °C, 1-methyl-3-nitro-1-nitroso guaidine (11.25 mg, 0.08 mmol) was added slowly. The resulting yellow colored organic layer containing diazomethane was separated, dried

over KOH pellets and added to acids **1–3** (0.04 mmol) in Et_2O (2 mL). The reaction mixture was stirred for 2 h at 0 °C to rt, excess CH_2N_2 was quenched with acetic acid and the solvent was removed under reduced pressure. Column chromatographic purification of the crude products over silica gel using hexanes-EtOAc gradient systems afforded methyl esters **4–6** (75–95% yield).

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