Elatumic Acid: A New Ursolic Acid Congener from *Omphalocarpum elatum* Miers (Sapotaceae)

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A new triterpene diastereomer, **1**, of the previously reported 3β,6β,19α-trihydroxy-urs-12-en-28-oic acid-24-carboxylic acid methyl ester was obtained from the stem bark of *Omphalocarpum elatum* Miers (Sapotaceae) along with α-amyrin acetate (**2**), spinasterol (**3**), spinasterol 3-*O*-β-D-glucopyranoside (**4**), and tormentic acid (**5**). The structures of the isolates were established on the basis of NMR and mass spectrometric data and by comparison with those previously reported in the literature. Compound **1** showed weak antibacterial activity against *E. aerogenes* ATCC13048 and EA3, *K. pneumoniae* ATCC29916, and *P. aeruginosa*; it also displayed moderate cytotoxicity against CCRF-CEM, CEM/ADR5000, and MDA-MB231 cells.

Key words: Omphalocarpum elatum, Triterpene, Cytotoxicity, Antimicrobial

Introduction

Omphalocarpum elatum Miers is a woody tree with about 30 m in height and about 80 cm in diameter. The leaves are 12 to 25 cm in length and 4 to 8 cm wide (Louppe *et al*., 2008). The species is widespread in tropical Africa, especially in Sierra Leone, Ivory Coast, Ghana, Gabon, and Cameroon (Louppe *et al*., 2008). During our investigations of new bioactive compounds with triterpenic skeletons in the Sapotaceae, *O. elatum*, another species of this plant family was subjected to phytochemical studies. We recently reported four interesting compounds with triterpenic structures, including donellanic acids A – C and omphalocarpoidone from the Sapotaceae (Djoumessi *et al.*, 2012; Fru *et al.*, 2013). Although a part of the metabolites that we previously identified did not exhibit pronounced bio-activities, many studies performed on triterpenes described some of them as cytotoxic (Ud Din *et al.*, 2013), and others as anti-inflammatory (Checker *et al*., 2012; Srivastava *et al*., 2013) or antimicrobial metabolites (Mokoka *et al*., 2013). As a continuation of our investigations, a new type of ursolic acid was identified and tested for its cytotoxic and antibacterial activities.

Results and Discussion

Compound **1** was obtained as a colourless powder. Its molecular formula $C_{31}H_{48}O_7$, consistent with

Table I. NMR data of compound **1** (CD_3OD, 600 MHz) and the reported diastereomer (Sun *et al.*, 2012).

Position	**1**		Diastereomer		Position	**1**		Diastereomer	
	^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C		^{1}H (*J* in Hz)	^{13}C	^{1}H (*J* in Hz)	^{13}C
1	1.29 (1H, m), 1.45 (1H, m)	35.4	1.63 (2H, m)	42.00	17	–	48.8	–	49.26
2	1.54 (1H, m), 2.02 (1H, m)	26.0	1.60 (2H, m)	27.74	18	2.54 (1H, br s)	53.3	2.52 (1H, s)	55.36
3	3.70 (1H, pseudo-t, 2.9)	75.6	3.90 (1H, dd, 3.6, 12)	77.40	19	–	73.6	–	73.82
4	–	53.3	–	56.42	20	1.38 (1H, m)	43.1	0.94 (1H)	43.30
5	2.07 (1H, m)	45.9	1.50 (1H, m)	54.15	21	1.26 (1H, m), 1.75 (1H, m)	27.3	1.73 (2H, m)	27.49
6	4.37 (1H, m)	71.4	3.84 (1H, m)	72.06	22	1.65 (1H, m), 1.75 (1H, m)	39.1	1.73 (2H, m)	39.18
7	1.49 (1H, m), 1.94 (1H, m)	41.4	1.46 (1H, m), 1.69 (1H, m)	42.03	23	–	178.9	1.48 (3H, s)	12.88
8	–	40.8	–	40.93	24	1.55 (3H, s)	20.0	–	180.49
9	2.01 (1H, br s)	48.8	1.78 (1H, m)	49.40	25	1.40 (3H, s)	17.3	1.33 (3H, s)	17.63
10	–	37.5	–	37.42	26	1.13 (3H, s)	18.8	1.07 (3H, s)	18.65
11	2.01 (1H, m) 2.07 (1H, m)	24.5	2.05 (2H, m)	24.80	27	1.41 (3H, s)	25.1	1.32 (3H, s)	25.03
12	5.35 (1H, t, 3.7)	129.7	5.32 (1H, t, 3.6)	129.72	28	–	182.5	–	182.52
13	–	139.5	–	139.51	29	1.22 (3H, s)	27.0	1.20 (3H, s)	27.27
14	–	43.3	–	43.28	30	0.96 (3H, d, 6.7)	16.6	0.93 (3H, s)	16.77
15	1.02 (1H, m), 1.91 (1H, m)	29.6	0.98 (1H, m), 1.86 (1H, ddd, 4.8, 13.2)	29.82	MeO	3.68 (3H, s)	52.0	3.69 (3H, s)	52.59
16	1.54 (1H, m), 2.59 (1H, dt, 4.6, 13.2)	26.7	1.51 (1H, m), 2.55 (1H, ddd, 4.8, 13.2)	26.82	–	–	–	–	–

eight double bond equivalents, was deduced from the pseudo-molecular ion at m/z 555.3295 (calcd. for $[M+Na]^+$, 555.3292) in the HR-ESI mass spectrum. It responded positively to the Liebermann-Burchard test indicative of triterpenes (Kenny, 1952). The IR spectrum showed absorption bands of OH groups (3500 cm^{-1}) and C=O groups (1694 cm^{-1}). The NMR spectrum (Table I) revealed the presence of seven CH_3, eight CH_2, seven CH, and nine quaternary carbon atoms. The CH_3 groups included a methoxy group at δ 3.68/52.0 ppm, while among the CH groups, two were oxygenated (δ 3.70/75.6 ppm and 4.37/71.4 ppm) and one a part of a C=C double bond (δ 5.35/129.7 ppm). Furthermore, the quaternary carbon atoms observed included an olefinic carbon atom (δ_C 139.5 ppm), an oxygenated sp^3 carbon atom (δ_C 73.6 ppm), and two carboxylic functions one of which being esterified (δ_C 178.9 and 182.5 ppm). The signals of the trisubstituted double bond observed in the NMR spectrum (Table I) at δ 5.35/129.7 ppm and δ_C 139.5 ppm suggested an ursolic acid scaffold for compound **1** (Lee *et al.*, 2011). This assumption was supported by the multiplicity of one of the CH_3 groups appearing in the ^{1}H NMR spectrum as a doublet (δ_H 0.96 ppm, $J = 6.7$ Hz) and showing HMBC correlations (Fig. 1) with a CH_2 group at δ 1.26, 1.75/27.3 ppm, a downfield quaternary carbinol at δ_C 73.6 ppm, and a CH group at δ 1.38/43.1 ppm which are typical chemical shifts observed for 19α-hydroxy-urs-12-en-28-oic acid congeners of **1** such as the tormentic or pomolic acids (Lee *et al.*, 2010; Saimaru

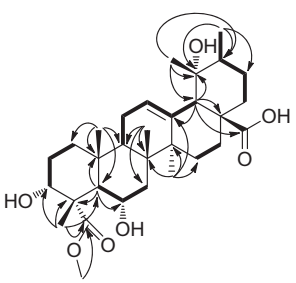

Fig. 1. COSY (bold) and HMBC (arrow) correlations of compound **1**.

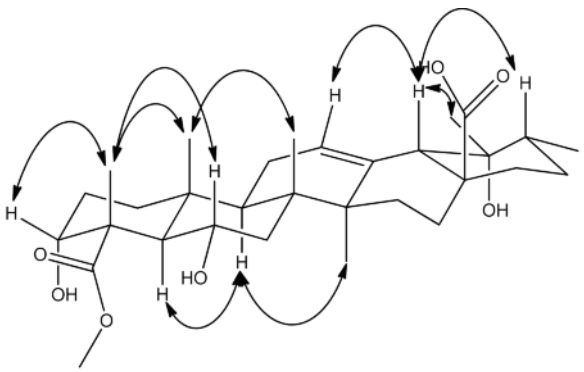

Fig. 2. NOESY correlations of compound **1**.

et al., 2007). The COSY spectrum (Fig. 1) of **1** exhibited correlations of H-1 (δ_H 1.29 and 1.45 ppm) to H-2 (δ_H 1.54 and 2.02 ppm) and H-3 (δ_H 3.70 ppm), while H-5 (δ_H 2.07 ppm) correlated with H-6 (δ_H 4.37 ppm) and H-7 (δ_H 1.49 and 1.94 ppm). The aforementioned information along with HMBC correlations of the CH_3 group at δ_H 1.13 ppm (H-26) and C-8 (δ_C 40.8 ppm), C-7 (δ_C 41.4 ppm), C-14 (δ_C 43.3 ppm), and C-9 (δ_C 48.8 ppm) as well as HMBC correlations of H-25 (δ_H 1.40 ppm) and C-1 (δ_C 35.4 ppm), C-10 (δ_C 37.5 ppm), C-5 (δ_C 45.9 ppm), and C-9 (δ_C 48.8 ppm) allowed the deduction of the fused rings A and B contained in the urs-12-ene skeleton. Moreover, H-9 (δ_H 2.01 ppm) revealed COSY correlations with H-11 (δ_H 2.01 and 2.07 ppm) and H-12 (δ_H 5.35 ppm), while this latter showed similar interactions with a proton at δ_H 2.54 ppm (H-18). Likewise, protons of CH_2-15 at δ_H 1.02 and 1.91 ppm correlated with those at δ_H 1.54 and 2.59 ppm (H-16). The foregoing data in conjunction with HMBC correlations displayed between H-27 (δ_H 1.41 ppm) and C-15 (δ_C 29.6 ppm), C-8 (δ_C 40.8 ppm), C-14 (δ_C 43.3 ppm), and C-13 (δ_C 139.5 ppm) as well as correlations of H-18 (δ_H 2.54 ppm) and C-16 (δ_C 26.7 ppm), C-17 (δ_C 48.8 ppm), and C-13 (δ_C 139.5 ppm) led to the establishment of the fused rings C and D. The complete assignment of compound **1** led us to a related, previously reported triterpene isolated from *Uncaria macrophylla* and identified as 3β,6β,19α-trihydroxy-urs-12-en-28-oic acid-24-carboxylic acid methyl ester (Sun *et al.*, 2012). Nevertheless, the optical rotation values of both compounds [−5.6 (*c* 0.12, MeOH) for compound **1** and +4.9 (MeOH) for the reported compound] indicated that they have either an enantiomeric or a diastereomeric relationship. The former possibility could be excluded based on differences in the NMR spectra. Thus, the OH group at C-3 of compound **1** was axially oriented since H-3 (δ_H 3.70 ppm) showed NOESY correlations (Fig. 2) with H-24 (δ_H 1.55 ppm), which in turn correlated with H-6 (δ_H 4.37 ppm), and H-25 (δ_H 1.40 ppm). Accordingly, the structure of **1** was iden-

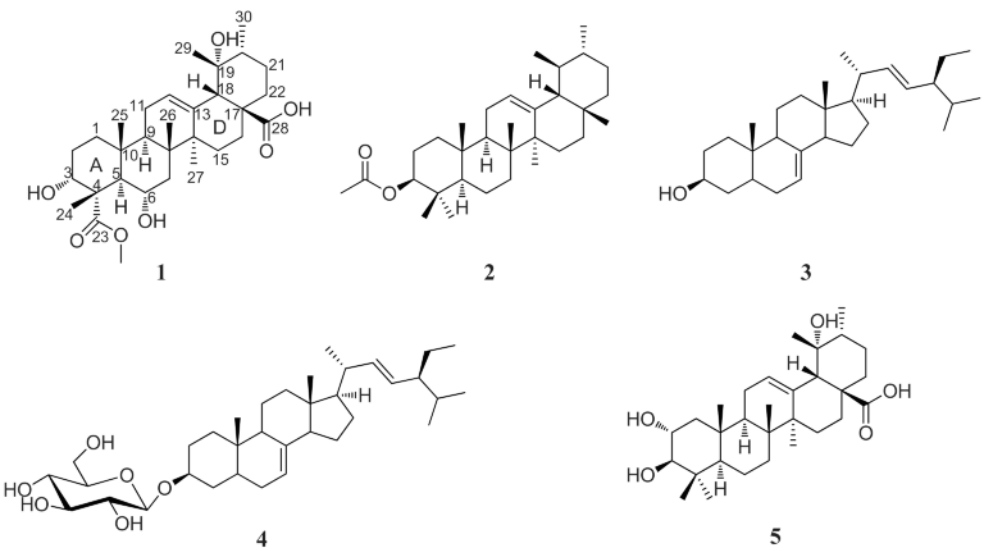

Fig. 3. Chemical structures of the isolated compounds elatumic acid (**1**), α-amyrin acetate (**2**), spinasterol (**3**), spinasterol 3-*O*-β-D-glucopyranoside (**4**), and tormentic acid (**5**).

Table II. MIC values of compound **1** and tetracycline against the tested bacterial strains.

Bacterial strain		MIC [μg/mL]	
		1	Tetracycline
E. coli	ATCC8739	–	64
	AG100	–	128
	AG102	–	8
E. aerogenes	ATCC13048	512	32
	EA3	512	< 4
	EA27	–	128
	CM64	–	< 4
K. pneumoniae	ATCC29916	256	< 4
	KP55	–	16
	KP63	–	< 4
P. aeruginosa	PA01	512	< 4

Table III. IC_{50} values of compound **1** and doxorubicin towards cancer cell lines.

Cell line	IC_{50} [μM]	
	1	Doxorubicin
CCRF-CEM	16.60 ± 2.17	0.20 ± 0.06
CEM/ADR5000	67.91 ± 4.78	195.12 ± 14.30
MDA-MB231	46.73 ± 4.07	1.10 ± 0.28
HCT116(p53 + /+)	> 72.03	1.41 ± 0.29
U87MG	> 72.03	1.06 ± 0.15
HepG2	> 72.03	3.83 ± 0.94

tified as 3α,6α,19α-trihydroxy-urs-12-en-28-oic acid-23-carboxylic acid methyl ester (Fig. 3). The trivial name elatumic acid was assigned.

The structures of the known compounds (Fig. 3) α-amyrin acetate (**2**) (Ali, 2013), spinasterol (**3**) (Kojima *et al.*, 1990), spinasterol 3-*O*-β-D-glucopyranoside (**4**) (Kojima *et al.*, 1990), and tormentic acid (**5**) (Lee *et al.*, 2010) were determined based on their NMR data in conjunction with those reported in the literature.

The results of the antibacterial assays indicated that compound **1** has weak antibacterial activity against *E. aerogenes* ATCC13048 and EA3, *K. pneumoniae* ATCC29916, and *P. aeruginosa* PA01 with MIC values above 100 μg/mL (Kuete, 2010), whilst no activity against other tested microorganisms was observed (Table II). In the cytotoxicity assay (Table III), compound **1** also displayed moderate activities with IC_{50} values above 10.0 μM against CCRF-CEM (16.60 μM), CEM/ADR5000 (67.91 μM), and MDA-MB231 (46.73 μM) cells. More than 50% growth proliferation was recorded with HCT116(p53 + /+), U87MG, and HepG2 cell lines at up to 72.03 μM. The activity of **1** was, however, better than that of doxorubicin towards the resistant CEM/ADR5000 cell line.

Conclusion

A new pentacyclic triterpenoid was identified during phytochemical studies of *Omphalocarpum elatum* Miers along with four known metabolites. The new compound was investigated for its antimicrobial and cytotoxic activities. It showed a weak antimicrobial activity against four microbial strains and was inactive against the other seven. Although compound **1** was more potent than doxorubicin against the CEM/ADR5000 cancer cell line, it gave moderate to no cytotoxicity in the other cell lines. Its reported diastereomer did not show any cytotoxic activity against the HepG2 cancer cell line.

Experimental

Instrumentation

Column chromatography (CC) and thin-layer chromatography (TLC) were performed on silica gel 60 Å (40 – 63 μm) and $60F_{254}$ (Merck, Darmstadt, Germany), respectively. ^{1}H, ^{13}C, and 2D-NMR spectra were recorded on a Bruker AVANCE III-600 MHz spectrometer (Bruker, Karlsruhe, Germany) equipped with a 5-mm inverse TCI cryoprobe using standard pulse sequences. Melting points were recorded on a Krüss optronic apparatus (Hamburg, Germany). The IR spectra were recorded on a Bruker Tensor 27 IR spectrometer equipped with a diamond ATR unit. HR-ESI-MS was carried out with a Q-ToF ULTIMA-III quadrupole TOF mass spectrometer (Waters, Eschborn, Germany). Optical rotation was measured on a Perkin Elmer model 241 polarimeter (Offenbach, Germany) at 546 and 578 nm and was extrapolated to 589 nm using Drude's equation.

Plant material

The stem bark of *O. elatum* Miers was collected in April 2013 from the Dja reserve in the eastern region of Cameroon. The plant was identified by the staff of the national herbarium of Cameroon in Yaoundé, where a voucher specimen was conserved under the specimen No. 6209 SRFCAM.

Extraction and isolation

The air-dried and powdered stem bark (2.3 kg) was macerated in a mixture of CH_2Cl_2/MeOH (1:1) (10 L) for 48 h. The solvent was evaporated at 40 °C *in vacuo* to afford 90 g of a dark red residue which was

further extracted by a liquid-solid process yielding a $CHCl_3$ fraction (42 g) and a residue (46 g). The $CHCl_3$ fraction was subjected to repeated silica gel CC using *n*-hexane (Hex) and Hex/ethyl acetate (EA) in gradient conditions. One hundred and ninty-five sub-fractions were collected from which α-amyrin acetate (**2**) [Hex/EA (95:5); 30 mg] and spinasterol (**3**) [Hex/EA (90:10); 150 mg] were obtained. Sub-fractions 169 – 180 obtained from the mixture Hex/EA (3:7) were further chromatographed using a $CHCl_3$/MeOH gradient. Fifty sub-fractions were collected from which tormentic acid (**5**) [$CHCl_3$/MeOH (97:3); 5 mg], compound **1** [$CHCl_3$/MeOH (95:5); 15 mg], and spinasterol 3-*O*-β-D-glucopyranoside (**4**) [$CHCl_3$/MeOH (92.5:7.5); 10 mg] were isolated.

Elatumic acid (3α,6α,19α-trihydroxy-urs-12-en-28-oic acid-23-carboxylic acid methyl ester) (**1**): Colourless powder. – M.p. 229.3 – 230.2 °C. – $[\alpha]_D^{20}$ −5.6 (*c* 0.12, MeOH). – IR (ATR): $\nu = 3500, 2929, 1694, 1450, 1378, 1266\ cm^{-1}$. – ^1H and ^{13}C NMR: see Table I. – HR-ESI-MS: $m/z = 555.3295$ $[M + Na]^+$ (calcd. for $C_{31}H_{48}O_7Na$, 555.3292). – ESI-MS: $m/z = 555.3$ $[M + Na]^+$.

Antibacterial assay

The studied microorganisms included reference (ATCC) and multidrug-resistant strains of *Pseudomonas aeruginosa* (PA01), *Klebsiella pneumoniae* (ATCC29916, KP55, KP63), *Escherichia coli* (ATCC8739, AG100, AG102), and *Enterobacter aerogenes* (ATCC13048, EA3, EA27, CM64) obtained from the American Type Culture Collection. They were maintained on agar slants at 4 °C and subcultured on fresh appropriate agar plates 24 h prior to any antimicrobial test. Nutrient agar and Sabouraud glucose agar were used for the activation of bacteria and fungi, respectively. Mueller Hinton broth (MHB) was used for the determinations of the minimal inhibitory concentration (MIC) and minimum microbiocidal concentration (MMC). Mueller Hinton agar (MHA) was also used for the determination of the MMC on these species (Kuete *et al.*, 2011). Tetracycline (Sigma-Aldrich, St. Quentin Fallavier, France) was used as reference antibiotic (RA) against bacteria. *p*-Iodonitrotetrazolium chloride (INT; Sigma-Aldrich) was used as microbial growth indicator (Mativandlela *et al*., 2006). The MIC determination on bacteria was conducted using the rapid INT colorimetric assay according to described methods with some modifications. Briefly, the test sample was first dissolved in 10% (v/v) DMSO/MHB to give a final concentration of 512 μg/mL and serially diluted twofold to obtain concentration ranges. One hundred μL of each concentration were added to a well of a 96-well microplate containing 95 μL of MHB and 5 μL of inoculum standardized at $1.5 \cdot 10^6$ CFU/mL by adjusting the optical density to 0.1 at 600 nm using a Shimadzu UV-120-01 spectrophotometer (Hayward, CA, USA) (Tereschuk *et al.*, 1997). The final content of DMSO in the well was less than 3% [3% (v/v) DMSO does not alter the growth of the test organisms]. The negative control well cotained 195 μL MHB and 5 μL standard inoculum (Zgoda and Porter, 2001). The plate was covered with a sterile plate sealer, then agitated to mix the contents of the wells using a plate shaker, and incubated at 37 °C for 24 h. The assay was repeated three times in triplicate. The MIC values of samples were detected following addition 40 μL of 0.2 mg/mL INT and incubation at 37 °C for 30 min (Mativandlela *et al*., 2006). Viable microorganisms reduced the yellow dye to a pink colour. MIC is defined as the lowest sample concentration that prevents this change and exhibits complete inhibition of bacterial growth (Kuete *et al*., 2008).

Cytotoxicity assay

The resazurin reduction assay (O'Brien *et al*., 2000) was performed to assess the cytotoxicity of compound **1** and doxorubicin towards various sensitive and resistant cancer cell lines, including the leukemia CCRF-CEM and CEM/ADR5000, breast MDA-MB231, colon HCT116, glioblastoma U87MG, and hepatocarcinoma HepG2 cell lines. The assay is based on the reduction of the indicator dye, resazurin (Sigma-Aldrich, Schnelldorf, Germany), to the highly fluorescent resorufin by viable cells. Non-viable cells rapidly lose their metabolic capacity to reduce resazurin and, thus, do not produce fluorescent signals anymore. Briefly, adherent cells were detached by treatment with 0.25% trypsin/EDTA (Invitrogen, Darmstadt, Germany), and an aliquot of $1 \cdot 10^4$ cells was placed in each well of a 96-well cell culture plate (Thermo Scientific, Langenselbold, Germany) in a total volume of 200 μL. Cells were allowed to attach overnight and then were treated with different concentrations of compounds. For suspension cells, aliquots of $2 \cdot 10^4$ cells per well were seeded in 96-well plates in a total volume of 100 μL. The studied compound was immedi-

ately added in varying concentrations in an additional 100 μL of culture medium to obtain a total volume of 200 μL/well. After 72 h, resazurin [20 μL, 0.01% (w/v)] in distilled water was added to each well, and the plates were incubated at 37 °C for 4 h. Fluorescence was measured on an Infinite M2000 ProTM plate reader (Tecan, Crailsheim, Germany) using an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Every assay was done at least twice with six replicates each. The viability was evaluated based on a comparison with untreated cells. IC_{50} values represent the compound concentrations required to inhibit 50% of cell proliferation and were calculated from a calibration curve by linear regression using Microsoft Excel.

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