Sesquiterpene lactones from *Elephantopus scaber*

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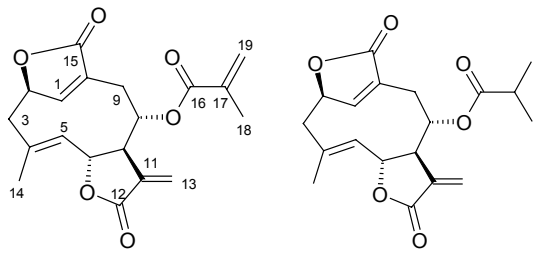
The ethanolic and acetone extracts of the whole plant of *Elephantopus scaber* were found to contain ethyl hexadecanoate, ethyl-9,12-octadecadienoate, ethyl-(*Z*)-9-octadecenoate, ethyl octadecanoate, lupeol, stigmasterol, stigmasterol glucoside, deoxyelephantopin (**1**) and two new germacranolide sesquiterpene lactones named 17,19-dihydrodeoxyelephantopin (**2**) and *iso*-17,19-dihydrodeoxyelephantopin (**3**) whose stereostructures were determined by spectroscopic methods, comparison with reported data and single-crystal X-ray analysis.

Key Words: *Elephantopus scaber*, Sesquiterpene lactones, Deoxyelephantopin, 17,19-Dihydrodeoxyelephantopin, Iso-17,19-dihydrodeoxyelephantopin.

Introduction

The plant *Elephantopus scaber* L. (Compositae) is known as "Tawmonlar" in Myanmar traditional medicine. It is used as an antiviral and antimalarial remedy and applied for the treatment of hepatitis [1]. It contains unique sesquiterpene lactones like deoxy-elephantopin and related compounds, which have been largely studied by several groups due to the anticancer and antitumor activities of these compounds [2-9]. We have now isolated two new sesquiterpene lactones, 17,19-dihydrodeoxy-elephantopin (**2**) and *iso*-17,19-dihydro-deoxyelephantopin (**3**) together with the known deoxyelephantopin (**1**). We wish to report here the characterization of the new compounds and the determination of the structure of **2,** using X-ray crystallographic analysis.



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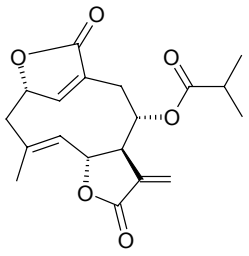

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Results and Discussion

A combination of column chromatography on silica gel, PTLC and preparative HPLC of the ethanol and acetone extracts of *E. scaber* gave two new sesquiterpene lactones, **2** and **3** along with the known sesquiterpene lactone deoxyelephantopin

(**1**), previously isolated from *E. scaber* [4,10-14]. Deoxyelephantopin (**1**) ($[\alpha]_D^{20}$ = -63°, c = 0.02, $CHCl_3$) was obtained by preparative HPLC as colourless needles. The molecular formula $C_{19}H_{20}O_6$ was deduced from the HRESI MS data. The presence of the bands at 1762 and 1655 cm^{-1} in the IR spectrum and the ^1H NMR data indicated **1** to contain an α-methylene-γ-lactone moiety and a methacrylate side chain which was established by the mass spectrum with the fragment at *m/z* 258 [M-$C_4H_6O_2$]$^+$. The ^{13}C NMR spectrum (Table 2) confirmed all of the above assignments and confirmed the structure **1**.

Table 1: ^1H NMR (300 MHz, $CDCl_3$) data of compounds **1-3** [*J* values (Hz) in parentheses]

H	1	2	3
1	7.08 br s	7.05 br s	7.14 br s
2	5.46 td (1.8, 3.9)	5.45 td (1.8, 3.9)	5.37 d (4.8)
3_a	2.69 ddd (1.2, 2.1,13.4)	2.69 ddd (0.6, 1.8, 13.2)	2.38 dd (5.1, 14.7)
3_b	2.85 dd (4.5, 13.8)	2.85 dd (4.5, 13.5)	2.93 br dd (3.6, 12.0)
5	4.77 br d (10.5)	4.77 br d (10.5)	5.10 dq (1.5, 10.5)[a]
6	5.13 dd (8.1, 10.5)	5.11 dd (7.8, 10.2)	5.12 dd (9, 10.5)[a]
7	2.94 dt (3.6, 7.5)	2.91 dd (3.6, 7.8)	3.13 dt (3.3, 6.6)
8	4.65 ddd (2.1, 3.6, 11.4)	4.53 ddd (2.1, 3.6, 11.4)	4.44 ddd (3.9, 7.5, 12.3)
9_a	2.78 d (12.3)	2.73 t (12)	2.68 dd (3.9, 12.6)
9_b	3.02 ddd (1.7, 3.0, 12.6)	2.97 br d (12.9)	3.00 d (12.6)
13_a	5.65 br d (3.3)	5.67 dd (0.6, 3.3)	5.70 br d (3.3)
13_b	6.23 br d (3.9)	6.30 dd (0.6, 3.9)	6.28 br d (3.9)
14	1.85 d (1.5)	1.84 d (1.5)	1.78 d (0.9)
17	-	2.53 sp (6.9)	2.54 sp (7.2)
18	1.93 dd (1.2, 1.4)	1.21 d (6.9)	1.21 d (6.9)
19	5.66 d (1.5)	1.14 d (6.9)	1.14 d (6.9)
19	6.14 t (1.2)		

[a]coupling constants were measured in acetone.

Compound **2** ($[\alpha]_D^{20}$ = -95°, c = 0.02, $CHCl_3$) was obtained as colourless needles. Its molecular formula was deduced to be $C_{19}H_{22}O_6$ on the basis of NMR and HRESI MS measurements. The ^1H NMR spectrum of compound **2** was very similar to that of **1**, here too the presence of an α-methylene-γ-lactone moiety was revealed by the IR bands at 1761 and 1659 cm^{-1} and by ^1H NMR signals at δ = 5.67 (dd, *J* = 0.6, 3.3 Hz, 1H, H-13_a) and 6.30 (dd, *J* = 0.6, 3.9 Hz, 1H, H-13_b). The IR band at 1748 cm^{-1} and the ^1H NMR signals at δ = 7.05 (br s, 1H, H-1) and 5.45 (td, *J* = 1.8, 3.9 Hz, 1H, H-2), and additionally the carbon signal at δ = 172.4 (C-15) indicated the presence of an α,ß-unsaturated lactone moiety as in **1**. Careful comparison of the ^1H, ^{13}C NMR, HMBC and HMQC spectra showed that **1** and **2** possess the same sesquiterpene skeleton. The only difference was found in the side chain where the protons of the *exo* methylene and methyl groups of the methacrylate ester in **1** were replaced by signals at δ = 2.53 (sp, *J* = 6.9 Hz, 1H, H-17), δ = 1.21 (d, *J* = 6.9 Hz, 3H, H-18) and δ = 1.14 (d, *J* = 6.9 Hz, 3H, H-19) due to the presence of an isopropyl group. The ^{13}C NMR spectrum of **2** (see Table 2)

Table 2 : ^{13}C NMR spectral assignments of **1-3** (75.5 MHz, $CDCl_3$)

C	1	2	3	C	1	2	3
1	153.5	153.2	149.3	11	134.0	133.9	133.9
2	81.4	81.4	79.5	12	169.3	169.3	174.3
3	41.2	41.4	40.1	13	123.6	123.6	123.3
4	135.5	135.9	135.3	14	20.0	20. 1	21.6
5	133.6	133.7	125.3	15	172.5	172.4	176.5
6	78.0	78.0	78.8	16	166.4	176.2	169.4
7	52.2	52.2	49.7	17	135.9	33.8	33.8
8	71.5	70.9	73.3	18	18.2	19. 1	19.2
9	33.4	33.5	30. 1	19	126.6	18.5	18.5
10	128.3	128.5	131.5				

showed the presence of 19 carbon atoms, among them three carbonyl groups, which appeared at δ = 176.2, 172.4 and 169.3. Two carbonyl groups were attributed to the lactone carbonyl at C-12 and C-15 whereas the remaining carbonyl group (δ = 176.2) was assigned to belong to the side chain. The downfield shift of this ester carbonyl as well as the absence of the sp^2 carbon signal at δ = 135.9 confirmed the presence of the isopropyl ester side chain, which was also explained the base peak at *m/z* 71 in the EI mass spectrum.

Unequivocal proof of structure of **2** was derived from a single-crystal X-ray analysis. A perspective view of the solid-state conformation is shown in Fig. 1. Details of the analysis are given in the experimental part.

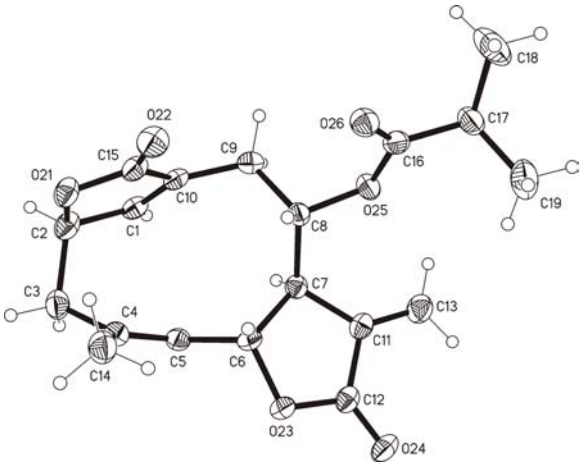

Fig. 1: Crystal structure of 17,19-dihydrodeoxyelephantopin (**2**) showing 50 % thermal ellipsoids. The bonds between carbon and hydrogen are shown as thin lines. Both C18 and C19 are saturated carbon atoms in contrast to the previous structure **1** where C19 was an unsaturated car bon.

Iso-17,19-dihydrodeoxyelephantopin (**3**) ($[\alpha]_D^{20}$ = +43°, c = 0.02, $CHCl_3$) was obtained as colourless crystals and the molecular weight and formula were determined to be the same as in **2** ($C_{19}H_{22}O_6$). Comparison of the ^1H and ^{13}C NMR spectra of **3** and **2** indicated as major difference the downfield shift of protons H-5 and H-7 which appeared in **3** at $\delta = 5.10$ and 3.13, respectively, and the upfield shift of C-5 at $\delta = 125.3$. It is evident that the carboxyl group of the α,β–unsaturated lactone in **3** is in close proximity to both H-5 and H-7 protons, therefore a paramagnetic shielding was observed in **3**. These results lead to the conclusion that **3** differs from **2** in the configuration at C-2. All ^1H and ^{13}C NMR data of **2** and **3** are in agreement with well-defined literature values for **1** and its stereoisomer *iso*-deoxyelephantopin [12-14].

Biological activities

Deoxyelephantopin (**1**) is known to have a high anti-tumour activity [2-9]. Compounds **2** and **3** were investigated therefore for anticancer activity *in vitro* in a panel of 34 human tumour cell lines, with deoxyelephantopin (**1**) for comparison. The screening comprised cell lines derived from bladder, central nervous system, colon, gastric, head & neck, lung, mammary, ovarian, pancreatic, prostate, renal and uterus cancers, as well as cell lines established from melanomas and pleuramesothelioma. As shown in Table 3, the new substances **2** and **3** exhibited a mean IC_{70}-value of 4.0 µg/ml and 4.3 µg/ml, respectively, compared to a mean IC_{70}-value of 1.1 µg/ml of **1**. However, with regard to the tumour selectivity, some differences were found between the compounds. Whereas the melanoma derived cell line MEXF 394NL was sensitive to all three compounds, **1** effected pronounced activity in the mammary cancer cell line MAXF 401NL, **2** was highly effective in the renal cancer cell line RXF 944L, and **3** showed marked activity to the large cell lung cancer LXFL 529L.

The antibiotic activity of **1-3** was negligible. In the agar diffusion test, inhibition zones of 11 mm diameter were observed at 100 µg/platelet with *Staphylococcus aureus*, and no activity was observed against *Bacillus subtilis* and *Candida albicans* at this concentration.

Experimental Section

General

Melting points are uncorrected; optical rotations: Perkin Elmer model 241 polarimeter. ^1H (300 and 600 MHz), ^{13}C NMR (75.5 and 125.7 MHz) NMR spectra were measured on a Bruker AMX 300 and on a Varian Inova 600 (599.740 MHz) spectrometer. ESI mass spectra were recorded on a LCQ Finngan Mass Spectrometer, HR-ESI mass spectra were recorded on APEX IV, 7T, FT-ICR MS Bruker Daltonik.

Table 3: *In vitro* antitumor activities of **1-3** in 4 selected human tumour cell lines

compound	mean IC_{70}	tumour selectivity	
	[µg/ml]	selective */total	most sensitive cell lines**
1	1.1	4/34	MEXF 394NL, MAXF 401NL
2	4.0	3/34	MEXF 394NL, RXF 944L
3	4.3	3/34	MEXF 394NL, LXFL 529L

* cell lines with individual IC_{70} < 1/2 (mean IC_{70}) / total cell lines

** individual IC_{70} < 1/3 (mean IC_{70})

GC-MS were measured on a TRACE GC-MS ThermoFinnigan mass spectrometer. Preparative HPLC was performed on a Kromasil column (100 C18, 7 µm, 250 × 20 mm i.d., flow rate 8 ml/min, detection at 210 nm) using CH_3CN-H_2O (40 : 60) as the mobile phase. HPLC-MS were run on a LCQ Finnigan and Flux Instruments Rheos 4000 was used as pump and Linear UVIS-205 was used as detector. IR spectra were recorded on a Perkin-Elmer 1600 Series FT-IR spectrometer as KBr pellets. Chromatography was carried out on silica gel (230-400 mesh). Thin layer chromatography (TLC) were performed on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). R_f values were measured on Polygram SIL G/UV_{254} (Macherey-Nagel & Co.). The X-ray structure analysis was performed at 100 K using a Bruker rotating anode X-ray source operating at CuK_α radiation (wavelength 1.5418 Å), equipped with osmic focussing mirrors, Bruker platform goniometer and a Bruker SMART 6000 CCD detector.

Human tumour cell lines and cell proliferation assay

A modified propidium iodide assay was used to assess the effects of the compounds on the growth of the human tumour cell lines. Cells were treated concentration-dependent with the test compounds and surviving cells were stained with a fluorescence dye. Details of the test procedure have been described elsewhere [15]. Twenty-two cell lines of the Oncotest cell line collection were used. These cell lines were established from human tumour xenografts growing on nude mice [16,17]. The other cell lines were kindly provided by the US National Cancer Institute (Bethesda, MD) or were supplied by the ATCC (Rockville, MD).

Plant material

Elephantopus scaber L. (Compositae) was collected in Bago, Myanmar, in March 2002 and a voucher specimen, voucher no. Y. H. V. 1004 has been deposited in the Herbarium of the Department of Botany, Yangon University.

Extraction and isolation

The air-dried whole plant materials (250 g) were extracted each with ethanol (6 × 1 l) and acetone (6 × 1 l) at room temperature for 3 days. The extracts were concentrated under reduced pressure at 40 °C. The ethanol extract (7 g) was chromatographed on a silica gel column and eluted successively with cyclohexane followed by a cyclohexane/EtOAc gradient and EtOAc/MeOH (9:1). The eluates were monitored by TLC and grouped into 5 fractions. The GC of the fraction 1 delivered four fatty acid esters (ethyl hexadecanoate, ethyl-9,12-octadecadienoate, ethyl-(*Z*)-9-octadecenoate, ethyl octadecanoate). 1.06 g of lupeol was obtained from fraction 2, fraction 3 delivered stigmasterol (0.1 g). The fraction 4 was washed with MeOH and purified by PTLC ($CHCl_3$: MeOH, 98:2) to afford 88 mg of a mixture of **1** and **2** (same R_f = 0.32 on TLC). HPLC-MS showed two molecular peaks at *m/z* 344 and 346. The mixture was separated using preparative HPLC (CH_3CN/H_2O 30:70) to give **1** (15 mg, R_t = 36.53 min) and **2** (5 mg, R_t = 39.02 min). The purification of fraction 5 gave stigmasterol glucoside (0.75 g). TLC of the acetone extract (7 g) exhibited a similar spot like **2**. Silica gel column chromatography using the same solvent system delivered the fraction of interest (0.176 g) as a mixture of **1** and **3** which was further purified by PTLC and

preparative HPLC (CH_3CN/H_2O, 40:60) to afford **1** (24 mg) and **3** (4 mg).

*Deoxyelephantopin, 2-Methyl-acrylic acid 10-Methyl-5-methylene-6,14-dioxo-7,13-dioxa-tricyclo[10.2.1.0*4,8*]pentadeca-1(15),9-dien-3-yl ester* (**1**)

Colourless needles from CH_2Cl_2/cyclohexane, m.p. 198-200 °C; $[\alpha]_D^{20}$ = -63° (c = 0.02, $CHCl_3$). – IR (KBr): ν = 1745, 1715, 1632, 1156 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75.5 MHz, $CDCl_3$) see Table 1 and 2. – (+)-ESI MS: *m/z* (%) = 367.1152 ($[M+Na]^+$); EI MS (70 eV): *m/z* (%) = 344 (1), 258 (8), 69 (100), 71 (35); (+)-ESI HRMS = 345.1259 $[M+H]^+$ (calcd. 345.13381 for $C_{19}H_{21}O_6$).

*3.6. 17,19-Dihydrodeoxyelephantopin, Isobutyric acid 10-methyl-5-methylene-6,14-dioxo-7,13-dioxa-tricyclo-[10.2.1.0*4,8*]pentadeca-1(15),9-dien-3-yl ester* (**2**)

Colourless needles from CH_2Cl_2/cyclohexane, m.p. 208-210 °C. – $[\alpha]_D^{20}$ = -95° ($CHCl_3$, c = 0.02). – IR (KBr): ν = 1760, 1748, 1732, 1648, 1157 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75.5 MHz, $CDCl_3$) see Table 1 and 2. – (+)-ESI MS: *m/z* (%) = 369 ($[M+Na]^+$, 6) 347 ($[M+H]^+$, 3); EI MS (70 eV): *m/z* (%) = 346 (4.8), 258 (16.8), 71 (85.2), 43 (100); (+)-ESI HRMS = 347.1416 $[M + H]^+$ (calcd. 347.14946 for $C_{19}H_{23}O_6$).

*Iso-17,19-dihydrodeoxyelephantopin, isobutyric acid 10-methyl-5-methylene-6,14-dioxo-7,13-dioxa-tricyclo[10.2.1.0*4,8*]pentadeca-1(15),9-dien-3-yl ester* (**3**)

Colourless crystals from CH_2Cl_2/cyclohexane, m.p. 183-185 °C. – $[\alpha]_D^{20}$ = + 43° (c = 0.02, $CHCl_3$). – IR (KBr): ν = 1756, 1745, 1730, 1651, 1152 cm^{-1}. – ^1H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75.5 MHz, $CDCl_3$) see Table 1 and 2. – (+)-ESI MS *m/z* (%) = 369 ($[M+Na]^+$, 6) 347 ($[M+H]^+$, 3); EI-MS (70 eV): *m/z* (%) = 346 (8), 258 (21), 71 (100), 43 (94); (+)-ESI HRMS =347.1416 ($[M+H]^+$) (calcd. 347.14946 for $C_{19}H_{23}O_6$).

Table 4: Crystal data and Structure refinement for 17,19-dihydrodeoxyelephantopin (**2**).

Empirical formula	$C_{19}H_{22}O_6$
Formula weight	346.37
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 6.3837(3) Å α = 90°
	b = 10.4589(6) Å β = 90°
	c = 25.8504(15) Å γ = 90°
Volume	1725.94(16) $Å^3$
Z	4
Density (calculated)	1.333 Mg/m^3
Absorption coefficient	0.822 mm^{-1}
F(000)	736
θ range for data collection	3.42 to 59.15°
Reflections collected	14551
Independent reflections	2473 [R(int) = 0.0338]
Completeness to θ = 59.15°	98.9 %
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2473 / 0 / 226
Goodness-of-fit on F^2	1.073
Final R indices [I>2sigma(I)]	R1 = 0.0254, wR2 = 0.0825
R indices (all data)	R1 = 0.0267, wR2 = 0.0851
Largest diff. peak and hole	0.153 and -0.142 $e.Å^{-3}$

R indices defined as: $R_1(\%) = \Sigma\,||F_o|-|F_c||/\Sigma|F_o|$, $wR_2 = [\Sigma w(F_o^2-F_c^2/\Sigma w(F_o^2)^2]^{1/2}$

Crystal structure determination of **2**

17,19-Dihydrodeoxyelephantopin (**2**) was crystallised by slowly evaporating the solvent from a cyclohexane/dichloromethane solution of 2 mg/ml **2** at room temperature. Colourless thin needles of about 0.3 × 0.2 × 0.1 mm^3 size appeared within about 2 days.

The crystal was shock frozen in perfluoro polyether oil in a loop and a dataset was collected at 100K. Ten 180° φ scans were collected. The integration of the data was done with the program SAINT [18]. All data were corrected *semi*empirically for systemic errors such as absorption using SADABS [19]. The program XPREP [20] was used to determine the space group and to prepare the files for structure determination. The structure was solved by direct methods using SHELXS [21] and refined against F^2 by least squares [22]. All non-

hydrogen atoms were refined anisotropically. The hydrogen atoms were placed in geometrically ideal position and refined with a riding model, in which the methyl groups can rotate on its local axis and the refinement statistics are given in Table 4. The crystallographic data of structure **2**, discussed in this publication, have been deposited at the Cambridge crystallographic data centre (CCDC) under the CCDC number 242439.

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