Lanostane Triterpenoids from the Sri Lankan Basidiomycete *Ganoderma applanatum*

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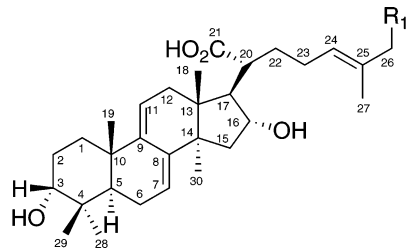
Two new lanostane-type triterpenoids, 3α,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid (**1**) and 3α,16α,26-trihydroxylanosta-7,9(11),24-trien-21-oic acid (**2**), along with three known lanostanoids, 16α-hydroxy-3-oxolanosta-7,9(11),24-trien-21-oic acid (**3**), 3α-carboxyacetoxy-24-methylen-23-oxolanost-8-en-26-oic acid (**4**), and 3α-carboxyacetoxy-24-methyl-23-oxolanost-8-en-26-oic acid (**5**), have been isolated from the EtOAc extract of the fruiting body of *Ganoderma applanatum*. The structures of **1**, **2**, and **3** were determined directly by the interpretation of spectroscopic data, while the structures of **4** and **5** were assigned by comparison of spectroscopic data against literature values.

The fruiting bodies of *Ganoderma* species have been widely used in traditional Chinese, Japanese, and Korean medicine to treat a variety of conditions.[1−3] These basidiomycetes are known to be prolific producers of lanostane-type triterpenoids, with over 100 such compounds having been recognized from the genus.[1] In a program directed at identifying biologically active secondary metabolites from Sri Lankan fungi we investigated *G. applanatum* (Fr.) Pat. [=*Elfvingia appalanata* (Pers.) Pat.] (Polyporaceae). The EtOAc extract of the fruiting bodies was moderately active against the P388 murine leukemia cell line. From this study we report the isolation and characterization of five lanostane triterpenoids, two of which are new (**1** and **2**), along with three known derivatives (**3**, **4**, and **5**), and their associated biological activities.

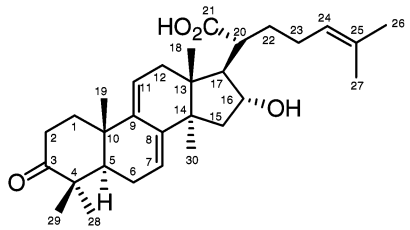
Following defatting of the crude EtOAc extract and initial chromatography on silica gel, two selected fractions were further purified by semipreparative reversed-phase HPLC to give compounds **1**−**3**. Two further compounds, **4** and **5**, were isolated from another of the silica gel fractions followed by chromatography on LH-20 (MeOH) with final purification by semipreparative reversed-phase HPLC.

The molecular formula of **1** was determined as $C_{30}H_{46}O_4$ by HRESIMS and was supported by observation of 30 resonances in the ^{13}C NMR spectrum. Between the ^{13}C and ^1H NMR spectra, seven tertiary methyl groups were established, two of them being vinylic (δ_H 0.61, 0.94, 0.95, 0.99, 1.09, 1.63, and 1.73 (3H, each s)). Seven methylenes and eight methines, including three sp^2 carbons (δ_H 5.19, 1H, t, J = 6.7 Hz; 5.40, 1H, d, J = 5.8 Hz, and 5.52, 1H, br s), and two oxymethines (δ_H 3.33, 1H, br s and 3.98, 1H, m) were also characterized. The remaining signals were accounted for by seven quaternary carbons; three of these were sp^2 alkene (δ_C 131.2, 142.0, and 146.0) and one was a carbonyl group (δ_C 177.2). The unsaturation required by the molecular formula of **1** could be satisfied in part from the above data, namely, the carbonyl resonance, indicative of a carboxylic acid (δ_C 177.2), and three olefinic bonds. This suggested that **1** was tetracyclic, a necessary assumption in order to account for the eight degrees of unsaturation required by the molecular formula. This preliminary analysis of the spectroscopic data suggested that **1** is most likely a lanostane-type triterpenoid, typical of many of the other metabolites previously isolated from the genus *Ganoderma*.

The structure of **1** was determined from detailed analysis of ^1H and ^{13}C NMR data including COSY, HSQC, DEPT, and ROESY experiments. The COSY spectrum revealed the connectivities from C-1 to C-3, C-5 to C-7, C-11 to C-12, and C-15 to C-17 and then



1 R_1 = H
2 R_1 = OH



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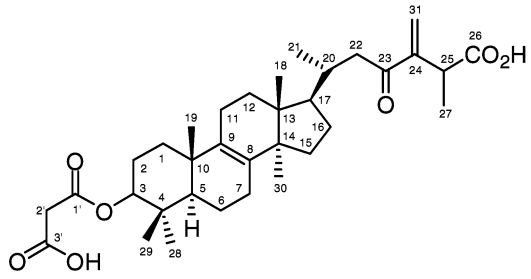

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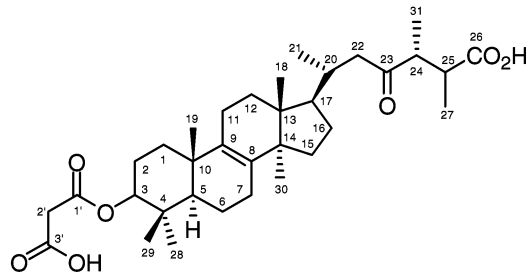

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Table 1. NMR Data for Compounds **1**−**3** in DMSO-d_6

	1				**2**				**3**			
C no.	δ_C [a]		δ_H [a] (J_{HH} Hz)	CIGAR	δ_C [a]		δ_H (J_{HH} Hz)	CIGAR	δ_C [a]		δ_H (J_{HH} Hz)	CIGAR
1	29.8	(CH_2)	β: 1.67 m	3, 5, 10, 19	29.7	(CH_2)	β: 1.67 m	5, 9, 10	36.1	(CH_2)	α: 1.70 ddd (4.5, 13.9, 18.2)	2, 3, 5, 9, 10, 19
			α: 1.82 m	9, 10			α: 1.81 m	2			β: 2.38 m	2, 3, 5, 10, 19
2	25.7	(CH_2)	α: 1.60 m	4	25.6	(CH_2)	α: 1.59 m	10	34.4	(CH_2)	α: 2.29 m	1, 3, 4, 10
			β: 1.90 m				β: 1.93 m				β: 2.90 ddd (6.1, 15.1, 20.5)	1, 3, 10
3	73.8	(CH)	3.33 br s		73.8	(CH)	3.33 br s	1	215.3	(C)		
4	37.2	(C)			37.0?	(C)			46.9	(C)		
5	42.8	(CH)	1.53 dd (5.3, 10.1)	6, 9, 10, 28, 29	42.8	(CH)	1.53 dd (5.9, 10.3)	6, 9, 10	50.5	(CH)	1.52 dd (3.8, 11.8)	4, 6, 7, 9, 10, 28, 29
6	22.6	(CH_2)	β: 2.04 m	4, 5, 8, 10	22.6	(CH_2)	β: 2.05 m	10	23.2	(CH_2)	α: 2.16 m	5, 7, 8, 9, 10, 11
			α: 2.06 m	7, 10			α: 2.04 m	10			β: 2.23 m	7, 8, 5, 10
7	120.6	(CH)	5.52 br s	5, 6, 9, 14	120.6	(CH)	5.53 br s	14, 5	120.1	(CH)	5.57 d (6.1)	5, 6, 9, 14
8	142.0	(C)			141.9	(C)			142.1	(C)		
9	146.0	(C)			145.9	(C)			144.2	(C)		
10	37.1	(C)			37.1	(C)			37.0	(C)		
11	115.1	(CH)	5.40 d (5.83)	8, 10, 13	115.1	(CH)	5.41 m	8, 10, 13	116.7	(CH)	5.52 d (5.6)	8, 10, 13
12	35.3	(CH_2)	β: 1.85 dd (6.6, 18.2)	9, 11, 13, 14, 18	35.3	(CH_2)	β: 1.86 dd (6.4, 17.8)	9, 11, 14, 13, 18	35.3	(CH_2)	α: 1.89 m	8, 9, 11, 13, 14, 18
			α: 2.18 br d (17.2)	9, 11, 13, 18			α: 2.18 m	18			β: 2.19 m	9, 11, 13, 18
13	43.9	(C)			43.9	(C)			43.8	(C)		
14	48.5	(C)			48.4	(C)			48.3	(C)		
15	43.4	(CH_2)	α: 1.45 br d (13.3)	13, 14, 16, 17, 30	43.3	(CH_2)	α: 1.44 br d (13.3)	13, 14, 16, 17, 30	43.3	(CH_2)	α: 1.45 br d (13.0)	13, 14, 16, 17, 30
			β: 2.10 dd (9.1, 12.9)	8, 14, 16, 30			β: 2.11 m	8, 14, 30			β: 2.13 m	8, 14, 16, 30
16	75.1	(CH)	3.98 m	14, 20	75.0	(CH)	3.99 m	14, 20	75.0	(CH)	β: 3.98 t (7.0)	14, 17, 20
17	56.2	(CH_2)	2.02 m	12, 13, 16, 18, 20, 21, 22	56.2	(CH)	2.03 m	12, 13, 16, 18, 20, 21, 22	56.2	(CH)	2.03 m	12, 13, 16, 18, 20, 21, 22
18	16.9	(CH_3)	0.61 s	12, 13, 14, 17	16.9	(CH_3)	0.62 s	12, 13, 14, 17	16.9	(CH_3)	0.65 s	12, 13, 14, 17
19	22.7	(CH_3)	0.99 s	1, 5, 9, 10	22.7	(CH_3)	1.00 s	1, 5, 9, 10	21.8	(CH_3)	1.21 s	5, 9, 10
20	46.9	(CH)	2.32 ddd (2.9, 11.3, 14.2)	13, 16, 21, 22, 23	46.9	(CH)	2.34 ddd (3.6, 12.1, 14.9)	17	46.9	(CH)	2.35 m	13, 16, 17, 21, 22, 23
21	177.2	(C)			177.2	(C)			177.2	(C)		
22	31.9	(CH_2)	a: 1.59 m	20	31.7	(CH_2)	a: 1.61 m	24	31.8	(CH_2)	a: 1.60 m	20, 21, 24
			b: 1.92 m	24, 25			b: 1.93 m				b: 1.91 m	21, 24, 25
23	26.0	(CH_2)	1.95 m		25.4	(CH_2)	1.98 m	24	26.0	(CH_2)	1.96 m	24, 25
24	124.3	(CH)	5.19 br t (6.72)	26, 27	123.1	(CH)	5.43 d (5.0)	26, 27	124.2	(CH)	5.20 t (7.2)	26, 27
25	131.2	(C)			135.7	(C)			131.1	(C)		
26	25.7	(CH_3)	1.63 s	24, 25, 27	66.5	(CH_2)	3.86 d (4.0)	24, 25, 27	25.6	(CH_3)	1.74 s	24, 25, 27
27	17.7	(CH_3)	1.73 s	24, 25, 26	13.6	(CH_3)	1.62 s	24, 25, 26	17.6	(CH_3)	1.64 s	24, 25, 26
28	28.7	(CH_3)	0.94 s	3, 29	28.7	(CH_3)	0.95 s	3, 4, 5, 29	25.4	(CH_3)	1.07 s	3, 4, 5, 29
29	22.8	(CH_3)	0.95 s	3, 4, 5, 28	22.8	(CH_3)	0.94 s	3, 4, 5, 28	22.1	(CH_3)	1.14 s	3, 4, 5, 28
30	26.2	(CH_3)	1.09 s	8, 13, 14	26.1	(CH_3)	1.10 s	8, 13, 14, 15	25.8	(CH_3)	1.08 s	8, 13, 14, 15
3(OH)			4.36 d (3.42)	2, 4			4.33 d (4.0)					
16(OH)			4.77 d (4.37)	16, 17			4.75 d (4.7)					

[a] Multiplicity determined from HSQC-DEPT experiments.

through C-20 and C-22 to C-24. The complete analysis of HSQC and CIGAR correlations (Table 1) confirmed the tetracyclic nature of **1**. This included the placement of the three angular methyl groups, Me-18, Me-19, and Me-30; the geminal dimethyl groups, Me-28 and Me-29, on C-4 in a 1,3 relationship to the hydroxyl-bearing carbon (C-3, δ_C 73.8); and the conjugated diene at $\Delta^{7,9(11)}$ (observed UV, λ_{max} of 243 nm, calc λ_{max} of 244 nm[4]). Placement of the carboxylic acid at C-20 (δ_C 46.9) was based on CIGAR correlations observed from δ_H 2.32 (H-20) and 2.02 (H-17) to the carbon at δ_C 177.2 (C-21). Likewise, placement of the hydroxyl at C-16 (δ_C 75.1) was determined from the CIGAR correlations observed from H-20 and H-17 to this carbon. The presence of the terminal gem-dimethyl groups, Me-26 and Me-27, were also established by the CIGAR correlations with each other and also with the protonated alkene carbon (δ_C 124.3, C-24). The appearance of the C-3 hydroxymethine proton at δ_H 3.33 as a broad singlet suggested an equatorial disposition for H-3. The appearance of H-3 is in keeping with previously reported results in this series.[5] The complete relative stereochemistry of **1** was determined by the analysis of ROESY correlations, which established the structure as 3α,16α-dihydroxylanosta-7,9(11),24-trien-21-oic acid. The ROESY correlation observed between H-16 (δ_H 3.98) and Me-18 (δ_H 0.61) established the α-orientation of the C-16 hydroxyl group, while the α-nature of the C-3 hydroxyl group was confirmed by ROESY correlations between H-3 (δ_H 3.33) and Me-29 (δ_H 0.95) and H-2β (δ_H 1.90), the latter two signals both correlating to Me-19 (δ_H 0.99). The ROESY correlations between Me-30 (δ_H 1.09) and H-17 (δ_H 2.02) indicated the α-orientation of H-17, while that between H-20 (δ_H 2.32) and Me-18 (δ_H 0.61) pointed to the α-orientation of the carboxylic group.[5] The C-3 epimer of **1** has previously been reported.[6]

Negative ion HRESIMS allowed assignment of the molecular formula of **2** as $C_{30}H_{46}O_5$ (*m*/*z* 485.3255 $[M - H]^-$). This molecular formula required one more oxygen atom than was found for **1**. The comparison of NMR data (Table 1) clearly showed that **1** and **2** are very closely related to each other, including stereochemistry, except in the vicinity of the three terminal carbon atoms of the side chain. The differences between the two compounds was explained by the placement of a hydroxyl group at C-26 (δ_C 66.5) in **2**, establishing the structure as 3α,16α,26-trihydroxylanosta-7,9-(11),24-trien-21-oic acid. The *E*-stereochemistry of the C-24 olefin was determined by observation of a ROESY correlation between H-24 (δ_H 5.43) and H-26 (δ_H 3.86).

The molecular formula of **3** was determined as $C_{30}H_{44}O_4$. Analysis of and comparison of the NMR data for **3** with the data for **1** clearly showed that the two compounds were similar in structure and stereochemistry, except that the secondary alcohol function at C-3 of **1** had been oxidized to a ketone function in **3** (δ_C 215.3). Thus, the structure of **3** was determined as 16α-hydroxy-3-oxolanosta-7,9(11),24-trien-21-oic acid. This work was presented at an international symposium in June of 2005.[7] Although ^{1}H and ^{13}C NMR data have been reported for this compound in the patent literature,[8] this was after the report at the symposium, and full spectroscopic data for this compound have not been reported elsewhere. For this reason physical data as well as assigned ^{1}H, ^{13}C NMR, CIGAR, and ROESY correlations for **3** have been included in the experimental and Supporting Information (ROESY data).

Two known lanostane triterpenoids, 3α-carboxyacetoxy-24-methylen-23-oxolanost-8-en-26-oic acid (**4**)[9] and 3α-carboxyacetoxy-24-methyl-23-oxolanost-8-en-26-oic acid (**5**),[9] were also isolated in this study. Compounds **4** and **5** were identified by comparison of spectroscopic (^{1}H NMR, ^{13}C NMR, 2D NMR) and MS data with those published in the literature.[9]

All compounds isolated in this study were evaluated for cytotoxicity against the P388 murine leukemia cell line.[10] While the initial crude extract displayed moderate activity (4.6 μg/mL), all isolated compounds, except **3**, were inactive (>125 μg/mL). 16α-Hydroxy-3-oxolanosta-7,9(11),24-trien-21-oic acid (**3**) was found to be only very weakly bioactive (111 μg/mL; 237 μM).

Experimental Section

General Experimental Procedures. Optical rotations were obtained using a Perkin-Elmer 341 polarimeter. UV spectra were recorded using a Dionex analytical HPLC system equipped with a UVD340U detector and connected to an Alltech ELSD 800. NMR experiments were recorded in DMSO-d_6 on a Varian INOVA 500 spectrometer at 23 °C, operating at 500 MHz for ^{1}H and 125 MHz for the ^{13}C nuclei, using the signals of the residual solvent protons and the solvent carbons as internal references (δ_H 2.60 and δ_C 39.6 ppm for DMSO-d_6). IR spectra were recorded on a Shimadzu FTIR-8201 PC spectrometer. HRESIMS were obtained on a Micromass LCT spectrometer using a probe voltage of 3200 V, an operating temperature of 150 °C, and a source temperature of 80 °C. Preparative HPLC was performed on a Shimadzu LC-4A instrument equipped with a SPD-2AS UV spectrophotometric detector (λ = 210 nm) using a C_{18} column (5 mL/min). Cytotoxicity against the P388 cell line was measured using a standard protocol.[10]

Fungal Material. The fruiting body of *Ganoderma applanatum* (450 g) was collected at Ingiriya, Sri Lanka, in December 2004. The fungus was identified by one of the authors (R.L.C.W.). A voucher specimen (SLF-F-002) is deposited in the herbarium of the Department of Plant Science, University of Colombo, Sri Lanka.

Extraction and Isolation. The freshly collected fruiting body of *G. applanatum* was cut into small pieces and extracted with EtOAc (2 × 500 mL of EtOAc). After concentration a portion of the crude extract (0.5 g) was suspended in aqueous MeOH (10%; 200 mL) and defatted using petroleum ether (2 × 200 mL). The polarity of the aqueous layer was increased to 40% H_2O, and the compounds of interest were extracted into $CHCl_3$ (2 × 200 mL). This extract (410 mg) was chromatographed on silica gel (50 g) with stepwise increase of the solvent polarity from petroleum ether/EtOAc (1:1) to EtOAc and finally EtOAc/MeOH (1:1) to yield fractions 1 (22 mg), 2 (50 mg), and 3 (96 mg). Fraction 1 was further purified by semipreparative reversed-phase HPLC (MeCN/H_2O, 70:30, Phenomenex Luna C_{18} (10 × 250 mm, 5 μm; 5 mL/min) to yield compounds **1** (3.4 mg) and **3** (5.4 mg). Semipreparative reversed-phase HPLC (MeCN/H_2O, 55:45; 5 mL/min) on fraction 2 led to compound **2** (2.5 mg). The known compounds, 3α-carboxyacetoxy-24-methylen-23-oxolanost-8-en-26-oic acid (**4**) (2.9 mg) and 3α-carboxyacetoxy-24-methyl-23-oxolanost-8-en-26-oic acid (**5**) (2.1 mg), were isolated from fraction 3 by size-exclusion chromatography on Sephadex LH-20 (MeOH) followed by semipreparative reversed-phase HPLC (MeCN/H_2O, 65:35).

3α,16α-Dihydroxylanosta-7,9(11),24-trien-21-oic acid (1): off-white powder; $[\alpha]^{20}_D$ +10 (*c* 0.1, DMSO); UV (MeCN/H_2O) λ_{max} 201, 237(sh), 244, 252(sh) nm; IR ν_{max} (diffuse reflectance KBr) 3330, 1710, 1685, 1448, 1375, 1263, 1064, 1028 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 469.3340 $[M - H]^-$ (calcd for $C_{30}H_{45}O_4$, 469.3318).

3α,16α,26-Trihydroxylanosta-7,9(11),24-trien-21-oic acid (2): off-white powder; $[\alpha]^{20}_D$ +28 (*c* 0.047, DMSO); UV (MeCN/H_2O) λ_{max} 201, 237(sh), 244, 252(sh) nm; IR ν_{max} (diffuse reflectance KBr) 3340, 1708, 1686, 1448, 1379, 1294, 1141, 1026 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 485.3255 $[M - H]^-$ (calcd for $C_{30}H_{45}O_5$, 485.3267).

16α-Hydroxy-3-oxolanosta-7,9(11),24-trien-21-oic acid (3): off-white powder; $[\alpha]^{20}_D$ +1.8 (*c* 0.39, DMSO); UV (MeCN/H_2O) λ_{max} 202, 237(sh), 243, 252(sh) nm; IR ν_{max} (diffuse reflectance KBr) 3338, 1718, 1685, 1452, 1375, 1253, 1113 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 467.3158 $[M - H]^-$ (calcd for $C_{30}H_{43}O_4$, 467.3161).

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Supporting Information Available: ^{1}H NMR spectra for compounds **1** and **2** as well as a table containing ROESY NMR correlations for compounds **1**−**3** are available free of charge from the Internet at http://pubs.acs.org.

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