Novel Cytostatic Lanostanoid Triterpenes from *Ganoderma australe*

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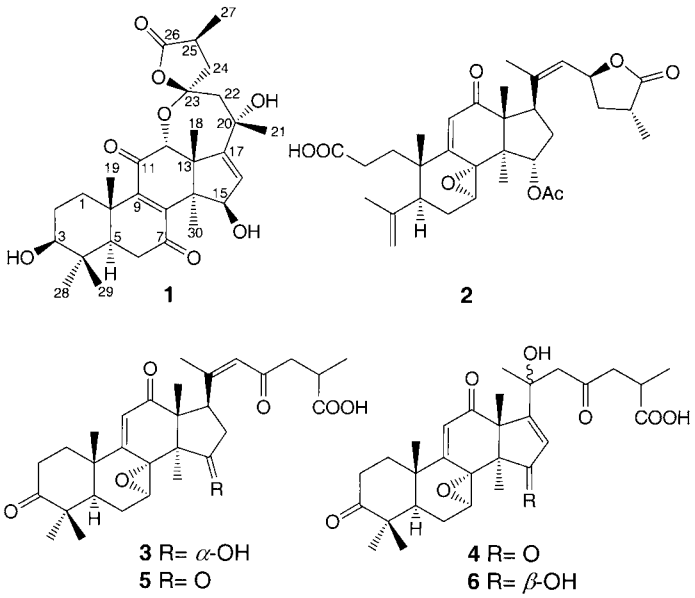
Two new compounds **1** and **2**, together with the known sterols ergosterol, 5,6-dehydroergosterol, and ergosterol peroxide, and four polyoxygenated lanostanoid triterpenes named applanoxidic acid A (**3**), C (**4**), F (**5**), and G (**6**) were isolated from the fungus *Ganoderma australe*. The structures of the new compounds were elucidated by means of spectroscopic techniques, while the known triterpenoids **3**–**6** were identified by comparing their spectral data with those reported in the literature. Compounds **1**–**6** inhibited the viability and growth of the HL-60 cell line.

1. Introduction. – Colombia is a privileged country from the point of view of biodiversity [1]. Fungi are represented by a large number of species, several of which have been used traditionally by the indigenous inhabitants and country farmers in ritual ceremonies and for medicinal purposes, as well as being a food source. However, fungi are underexploited there in comparison with other cultures in which they have been widely applied from time immemorial in folk medicine [2]. As part of our ongoing investigation into constituents of *Ganoderma australe* [3], we now describe the isolation and structure elucidation of two new compounds, austrolactone (**1**) and australic acid (**2**).

The structures of the new compounds **1** and **2** were elucidated by NMR-spectroscopic techniques (^{1}H- and ^{13}C-NMR, COSY, ROESY, HSQC, and HMBC) and mass spectrometry.

In our cytotoxicity assay, compounds **1**–**6** specifically inhibited the viability and growth of the HL-60 cell line, although australic acid (**2**; $IC_{50} = 94 \pm 6$ μM) and applanoxidic acid A (**3**; $IC_{50} = 132 \pm 22$ μM) were the most-active compounds. The mechanism by which these compounds display their cytotoxic effects is, at least in part, through activation of the apoptotic cell-death pathway as demonstrated by morphological and biochemical analyses.

2. Results and Discussion. – *G. australe* (dry weight 0.700 kg) was collected in the region of Nuquí, Departamento del Choco, Colombia. Freshly collected plant material was immersed in EtOH at room temperature for several days. The extract was then



decanted and evaporated, and the residue was extracted with $CHCl_3$. Repeated column chromatography and prep. TLC gave the known sterols ergosterol, 5,6-dehydroergosterol, and ergosterol peroxide, and six polyoxygenated lanostanoid triterpenes **1**–**6**.

Austrolactone **(1)**, obtained as an amorphous solid, showed in the EI-MS a molecular ion at *m/z* 528, while the HR-EI-MS displayed the M^+ at *m/z* 528.2767 corresponding to the molecular formula $C_{30}H_{40}O_8$ (calc. 528.2723). The IR spectrum exhibits absorption bands due to OH (3444 cm^{-1}), γ-lactone (1770 cm^{-1}), and α,β-unsaturated C=O (1696 cm^{-1}) groups. In the UV spectrum, **1** shows absorption due to an α,β-unsaturated C=O group. The ^{1}H- and ^{13}C-NMR data (*Table 1*) of **1** indicated the presence of a C_{30} triterpenoid spirolactone. COSY, HSQC, and HMBC experiments allowed the complete assignment of all H- and C-atoms, and the ROESY data provided the configuration of compound **1**. Therefore, the structure of **1** was established as (23*S*,25*S*)-12α,23-epoxy-3β,15β, 20α-trihydroxy-7,11-dioxo-5α-lanosta-8,16-dien-23,26-olide. To the best of our knowledge, compound **1** is a novel triterpenoid, which we named *austrolactone* (**1**).

The ^{1}H-NMR spectrum of **1** (*Table 1*) exhibited 6*s* at δ 1.42, 1.24, 1.60, 0.91, 1.05, and 1.04 for Me(18), Me(19), Me(21), Me(28), Me(29), and Me(30). The *d* at δ 1.26 (J = 7.0 Hz) was ascribed to Me(27). A *dd* at δ 3.21 (J = 4.3 and 11.7 Hz), and *d* at δ 4.71 (J = 3.2 Hz) (H-atoms geminal to the OH group) was due to H–C(3) and H–C(15), respectively. A *s* at δ 3.97 was due to H–C(12).

The ^{13}C-NMR spectrum of **1** (*Table 1*) showed signals for 30 C-atoms. The DEPT spectra indicated the presence of 7 Me, 5 CH_2, 6 CH, and 12 quaternary C-atoms. The olefinic signals at δ(C) 146.2, 155.8, and 128.1, 157.6 corresponded to the endocyclic C=C bonds between C(8) and C(9), and C(16) and C(17), respectively. In addition, a quaternary C-atom signal at δ 106.8 was found, which corresponds to a spirolactone C-

Table 1. *^1H- (500 MHz) and ^{13}C-NMR (125 MHz) Data of Compounds* **1** *and* **2**[a])

Position	**1**[b])		**2**[c])	
	δ (C)	δ (H) (J in Hz)	δ (C)	δ (H) (J in Hz)
1	34.07	2.06 (*dt*, J = 3.7, 13.9, H_α), 2.20 (*dt*, J = 3.0, 13.9, H_β)	37.13	2.40 (*m*)
2	27.43	1.80 (*m*, H_α), 1.65 (*m*, H_β)	30.14	2.49 (*m*)
				2.73 (*dd*, J = 4.3, 12.5)
3	76.50	3.33 (*dd*, J = 4.3, 11.7, H_α)	175.61	
4	39.21		145.00	
5	50.25	1.81 (*dd*, J = 6.8, 10.6, H_α)	44.81	3.01 (*dd*, J = 6.3, 10.5, H_α)
6	36.88	2.67 (br. *s*, H_α), 2.65 (*d*, J = 3.8, H_β)	27.03	1.82 (*m*, H_α), 2.01 (*m*, H_β)
7	203.81		61.27	4.30 (*d*, J = 2.9, H_β)
8	146.17		66.54	
9	155.89		163.00	
10	40.37		43.78	
11	198.52		130.00	6.32 (*s*)
12	79.27	3.97 (*s*, H_β)	201.10	
13	54.06		59.05	
14	50.59		51.81	
15	80.14	4.71 (*d*, J = 3.2, H_α)	71.77	5.68 (*dd*, J = 7.0, 9.4, H_β)
16	128.10	6.09 (*d*, J = 3.2)	31.92	1.84 (*m*, H_α), 2.47 (*m*, H_β)
17	157.66		44.32	3.36 (*dd*, J = 7.6, 11.2, H_α)
18	26.01	1.42 (*s*)	17.65	1.06 (*s*)
19	20.82	1.24 (*s*)	23.01	1.02 (*s*)
20	71.15		139.90	
21	32.77	1.60 (*s*)	19.04	1.91 (s)
22	48.58	2.12 (*d*, J = 15.1, H_α), 2.41 (*d*, J = 15.1, H_β)	126.30	5.75 (*d*, J = 8.2)
23	106.84		75.17	5.42 (*ddd*, J = 5.1, 7.7, 12.8)
24	44.60	2.55 (*dd*, J = 8.2, 12.7, H_α), 1.93 (*dd*, J = 11.8, 12.7, H_β)	36.78	2.12 (*m*)
				2.00 (*m*)
25	34.07	3.06 (*m*, H_α)	34.31	2.82 (*sext.*, J = 7.4)
26	178.73		179.00	
27	14.50	1.26 (*d*, J = 7.0)	15.45	1.23 (*d*, J = 7.3)
28	29.61	1.05 (*s*)	115.21	4.79 (*s*)
				4.88 (*s*)
29	15.10	0.91 (*s*)	23.01	1.67 (*s*)
30	29.67	1.04 (*s*)	15.07	1.40 (*s*)
AcO	–	–	20.69	2.05 (*s*)
			169.9	

[a]) Assignments confirmed by ^1H,^1H-COSY, HMQC, HMBC, DEPT, and ROESY spectra. [b]) $CDCl_3$. [c]) C_5D_5N.

atom. The latter showed a long-range coupling (HMBC) with the H-atoms H–C(25), CH_2(22), and with H–C(12). This led to the conclusion that formation of a spirolactone had taken place, involving the O-atom at C(12), which adds to C(23), and this, in turn, with the C(26) OO, generating the formation of the lactone ring.

The relative configuration of **1**, determined by the ROE data, displays the important ROEs on the energy-minimized model of austrolactone (**1**; see *Fig. 1*). The cross-peak observed between H–C(5) and H–C(3) established the β-hydroxylation at C(3). The configurations at the other stereogenic centers were confirmed by the ROESY correlations between H–C(12)/Me(21)/Me(18) and H_β–C(22), H–C(15)/

Me(30), and H–C(16), Me(21)/Me(18) and H–C(16), H_β–C(24) and H_α–C(22), as well as between Me(27) and H_β–C(24).

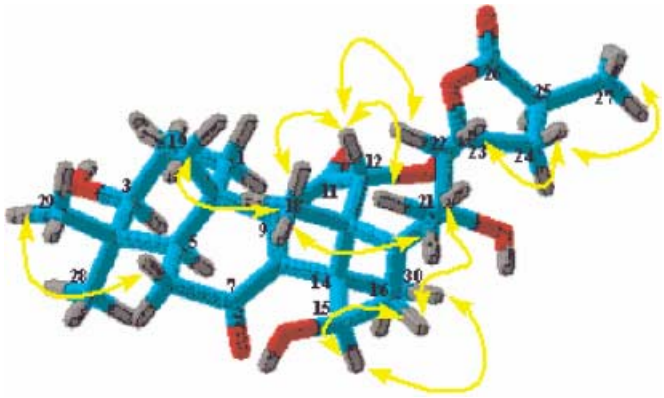


Fig. 1. *Key ROEs of austrolactone* (**1**)

Australic acid (**2**) was isolated as a colorless amorphous solid. The HR-EI-MS showed M^+ at *m/z* 554.2826, which corresponds to $C_{32}H_{42}O_8$ (calc. 554.2879). Compound **2** had structural features similar to those of the known triterpenoid elfvingic acid H [4]. The difference included the presence of a γ-lactone ring at C(23). Thus, the structure (20*Z*,23*R*,25*R*)-15α-acetyl-7α,8α-epoxy-12-oxo-3,4-seco-5α-lanosta-4(28),9,20(22)-trien-23,26-olid-3-oic acid (**2**) was deduced for the new compound.

The IR of **2** exhibited absorptions at 2918, 2850 (unsaturated C-atom), 1770 (lactone), and 1730 (acetate) cm^{-1}, and an UV absorption at 250 nm. The ^{1}H-NMR spectrum (*Table 1*) exhibited 5*s* at δ 1.02, 1.06, 1.40, 1.67, and 1.91 for Me(19), Me(18), Me(30), Me(29), and Me(21), respectively, while a *d* at δ 1.23 ($J = 7.3$ Hz) and a *s* at δ 2.05 were attributed to Me(27) and an Ac group, respectively; three oxymethine signals at δ 4.30 (*d*, $J = 2.9$ Hz), 5.42 (*ddd*, $J = 5.1$, 7.7, and 12.9 Hz) and 5.68 (*dd*, $J = 7.0$ and 9.4 Hz) were attributed to H–C(7), H–C(23), and H–C(15), respectively. Two *s* at δ 4.79 and 4.88, characteristic exomethylene signals, were assigned to 2 H–C(28), and two olefinic H-signals at δ 5.75 (*d*, $J = 8.2$ Hz) and 6.32 (*s*) to H–C(22) and H–C(11), respectively. The structure of **2** was determined by a combination of COSY, DEPT, HSQC, HMBC, and ROESY experiments. The above data implied the presence of C=C bonds at C(4(28)), C(9), and C(20), of epoxy group at C(7) and C(8), of an Ac group at C(15), a carboxylic acid at C(3), and a γ-lactone at C(26) and C(23). The relative configuration of **2** was confirmed by ROESY correlations between Me(18) and H–C(15), which indicated an α-orientation of the Ac group at C(15). The configuration of the epoxy group was determined to be α by the ROESY experiment, in which a cross-peak was observed between H–C(7), Me(18), and H–C(15). Correlations were also observed between Me(21) and H–C(22), indicating a (*Z*)-configuration of the C=C bond at C(20). The configuration at C(23) and C(25) was determined as (*R*) by comparison with the ^{1}H- and ^{13}C-NMR spectra of abiesolidic acid and its analogues [5][6].

Comparison of the spectroscopic properties (^{1}H- and ^{13}C-NMR) with the reported data allowed the following compounds to be identified: ergosterol [7], 5,6-dehydroergosterol [8], ergosterol peroxide [9], and applanoxidic acids A, C, F, and G [10][11].

Biological Activity. Compounds **1**–**6** were found to inhibit the viability and growth of HL-60 cells in a dose-dependent manner as determined by the MTT assay. The results presented in *Table 2* are a summary of several experiments in which the inhibitory concentrations 50 (IC_{50}) were determined. The results obtained show that compounds **2** ($IC_{50} = 94 \pm 6$ μM) and **3** ($IC_{50} = 132 \pm 22$ μM) were the most-active compounds among them. To determine whether the isolated compounds induce apoptosis, we incubated HL-60 cells with 30 μM of these agents for 12 h and performed a quantitative analysis. The treatment resulted in the appearance of typical morphological changes that include chromatin condensation, its compaction along the periphery of the nucleus, and nuclear segmentation into three or more chromatin fragments, as visualized by fluorescence microscopy. As shown in *Fig. 2*, all compounds were able to induce moderate levels of apoptosis ($P < 0.05$). The percentage of apoptotic cells ranges from 18 ± 3 (compound **5**) to 11 ± 0 (compound **6**), and all values were greater than controls ($4 \pm 2\%$). Tumor necrosis factor alpha (TNFα, 40 ng/ml) was used as a positive control and induced $47 \pm 6\%$ of apoptotic cells (data not shown). We also examined whether DNA fragmentation, which is considered the end point of the apoptotic pathway, is also affected under the same conditions as above. The qualitative analysis, as determined by agarose gel electrophoresis, indicated that intranucleosomal hydrolysis of chromatin was already detected after 12 h of treatment (*Fig. 3*). Although **2** and **3** were the most-potent cytotoxic compounds on HL-60 cells (see *Table 2*) by using the MTT assay, they increased the level of apoptosis, however, to a degree similar to that of the less-cytotoxic compounds (see *Figs. 2* and *3*). All these data show that compounds **2** and **3** induce inhibition of cell growth, partially through apoptosis activation, and suggest that other factors related to cell growth (*i.e.*, cell cycle) are probably affected by these compounds.

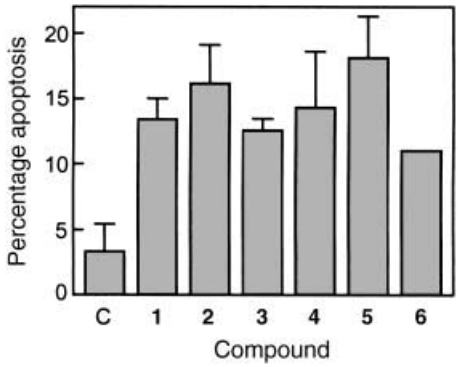


Fig. 2. *Quantitative analysis of apoptotic HL-60 cells after treatment with the isolated compounds.* Cells were incubated with the indicated compounds (30 μM) for 12 h, and the apoptotic morphology was determined by fluorescence microscopy after staining with bisbenzimide trihydrochloride as described in *Exper. Part*. The results of a representative experiment are shown, and each point represents the average ± SE of triplicate determinations (C: control).

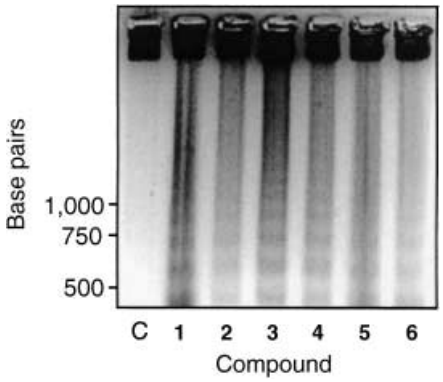


Fig. 3. *Qualitative analysis of genomic DNA fragmentation in HL-60 cells.* Cells were incubated in absence (C: control) or presence of 100 μM of the indicated compounds for 12 h; DNA was extracted, and fragmentation was assessed by agarose gel electrophoresis.

Table 2. *Effects of the Isolated Compounds on the Growth of HL-60 Cells Cultured for 96 h* [a])

Compound	IC_{50} [μM]
1	483 ± 83
2	94 ± 6
3	132 ± 22
4	334 ± 90
5	315 ± 52
6	404 ± 80

[a]) The data shown represent the mean ± SEM of two independent experiments with three determinations in each.

In summary, we found that compounds **1**–**6** inhibit the growth of HL-60 myeloid leukemia cells in culture. The morphological changes observed in addition to internucleosomal DNA fragmentation indicate that the cytotoxic effects are mediated, at least in part, by apoptosis activation.

Experimental Part

General. TLC: Pre-coated aluminium foil silica gel *60* F_{254} (*Merck*). Column chromatography (CC): silica gel *60* (*Merck*, 230–400 mesh), *Sephadex LH-20* (*Aldrich*). Optical rotations: *Perkin-Elmer* model *343* polarimeter; in $CHCl_3$. UV Spectra: *JASCO* model *V-560* spectrophotometer. IR Spectra: *Bruker* model *IFS-55* spectrophotometer. NMR Spectra: *Bruker* model *AMX-400* and *AMX-500* spectrometers; chemical shifts δ in ppm, TMS as internal standard, *J* in Hz. EI-MS and HR-EI-MS: *Micromass* model *Autospec* (70 eV) mass spectrometer.

Plant Material. Ganoderma australe (Fr.) PAT. BULL. was collected in the Nuquí region, Departamento del Choco, Colombia, in January 2000. The fungus was identified by Drs. *Jaime Uribe* and *Luis G. Henao* of the Instituto de Ciencias Naturales, Universidad Nacional de Colombia. A voucher specimen (COL LH-1185) was deposited at the *Herbario Nacional Colombiano*.

Extraction and Isolation. The dried fungus (0.7 kg) was ground and immersed in EtOH at r.t. for several days. The extract was then decanted and evaporated, and the residue was extracted with $CHCl_3$. The $CHCl_3$ extract (2.25 g) was fractionated by CC (silica gel; hexane/AcOEt step gradients): *Fractions 1–6*. *Fr. 1* (hexane/AcOEt 4:1; 122 mg) was subjected to CC (silica gel; hexane/AcOEt 4:1, and Sephadex LH-20; hexane/CH_2Cl_2/

MeOH 2:2:1) to give *ergosterol* (11 mg), *ergosta-7,22-dien-3β-ol* (12 mg), and *ergosterol peroxide* (11 mg). Repeated purification of *Fr. 2* (hexane/AcOEt 7:3; 128 mg) by CC (*Sephadex LH-20*; hexane/CH_2Cl_2/MeOH 1:1:1) gave **1** (1.5 mg). *Fr. 4* (hexane/AcOEt 3:2; 140 mg) was subjected to CC (silica gel; $CHCl_3$; and *Sephadex LH-20*; hexane/CH_2Cl_2/MeOH 1:1:1) and prep. TLC ($CHCl_3$/MeOH 100:1; two elutions) to give **2** (2.8 mg). *Fr. 5* (hexane/AcOEt 1:1; 264 mg) was subjected to CC (*Sephadex LH-20*; hexane/CH_2Cl_2/MeOH 1:1:1) and prep. TLC ($CHCl_3$/MeOH 98:2; several elutions) to give *applanoxidic acid* F (**5**; 7 mg) and *applanoxidic acid C* (**4**; 6 mg). *Fr. 6* (hexane/AcOEt 2:3; 740 mg) was purified by CC (*Sephadex LH-20*; hexane/CH_2Cl_2/MeOH 1:1:1) and repeated prep. TLC ($CHCl_3$/MeOH 95:5) to furnish *applanoxidic acid G* (**6**; 8 mg) and *applanoxidic acid A* (**3**; 12 mg).

Austrolactone (*=(23*S*,25*S*)-12α,23-Epoxy-3β,15β,20α-trihydroxy-7,11-dioxo-5α-lanosta-8,16-dien-23,26-olide*; **1**). Colorless amorphous solid (1.5 mg). $[\alpha]_D^{20} = -101$ ($c = 0.15$, $CHCl_3$). UV (EtOH; λ_{max} (log ε)): 275 (3.75). IR (KBr) 3444 (OH), 2917, 2849 (unsat. C), 1770 (γ-lactone), 1696, 1660, 1455, 1378, 1311, 1165, 1088, 1032, 964, 614, 886, 756. ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 528 (26), 511 (11), 510 (22), 492 (28), 477 (10), 468 (26), 466 (29), 450 (12), 449 (18), 448 (12), 431 (10), 416 (10), 401 (14), 398 (62), 381 (25), 373 (24), 369 (17), 355 (26), 341 (21), 337 (17), 327 (18), 292 (100). HR-EI-MS: 528.2767 (M^+, $C_{30}H_{40}O_8^+$; calc. 528.2723).

Australic acid (*=(20*Z*,23*R*,25*R*)-15α-Acetyl-7α,8α-epoxy-12-oxo-3,4-seco-5α-lanosta-4(28),9,20(22)-trien-23,26-olid-3-oic Acid*; **2**). Colorless amorphous solid (2.8 mg). $[\alpha]_D^{20} = +42$ ($c = 1.43$, $CHCl_3$). UV (EtOH): 250 (3.73). IR (film): 2918, 2850 (unsat. C), 1770 (γ-lactone), 1732, 1683, 1456, 1377, 1245, 1175, 1042, 996. ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 554 (12), 495 (11), 494 (25), 479 (15), 465 (10), 461 (14), 448 (13), 427 (16), 421 (12), 403 (17), 318 (12), 317 (55), 301 (35). HR-EI-MS: 554.2826 (M^+, $C_{32}H_{42}O_8^+$; calc. 554.2879).

Cell Culture. The human promyelocytic leukemia HL-60 cell line established by *Gallagher et al.* [12] was used in this study. Cell culture of HL-60 cells was performed as reported in [13].

Assay for Growth Inhibition/Reduction of Cell Viability. Cytotoxic assays were performed with an MTT assay [14]. Surviving cells were detected based on their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) into formazan crystals. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment by the curve-fitting routine of the computer software *Prism 2.0™* (*GraphPad*) and the equation derived by *DeLean* and co-workers [15].

Quantitative Fluorescence Microscopy. The apoptotic morphology was determined by fluorescence microscopy after staining with bisbenzimide trihydrochloride (*Hoechst* 33258) as described in [16]. Data shown in *Fig. 2* represent the means ± S.E., and data sets were compared using the *Student*'s *t* test.

Analysis of DNA Fragmentation [16]. Cellular DNA from whole cells was extracted and separated as described in [17]. DNA Bands were visualized under UV light, and the images were captured by a digital camera (*Kodak*).

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