Lanostanoid Triterpenes from *Laetiporus sulphureus* and Apoptosis Induction on HL-60 Human Myeloid Leukemia Cells

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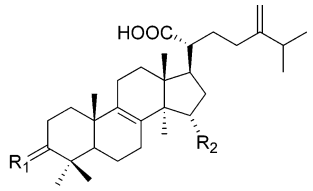
Received July 20, 2004

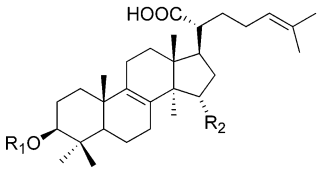
A new lanostanoid triterpene, 3-oxosulfurenic acid (**1**), together with three known triterpenes (**3**, **4**, and **7**) were isolated from the fruit bodies of *Laetiporus sulphureus*. Cytotoxicity of these compounds and their derivatives (**2**, **5**, and **6**) was evaluated on HL-60 cells. Further studies revealed that acetyl eburicoic acid (**5**) was the most potent apoptosis inducer. Apoptosis was accompanied by both the activation of caspase-3 and the fragmentation of poly(ADP-ribose) polymerase-1 and was also associated with an early release of cytochrome *c* from the mitochondria.

As a part of a research program on biologically active compounds isolated from Basidiomycetes from Colombia,[1] we have studied *Laetiporus sulphureus* (Bull.: Fr.) Murr. (Polyporaceae). Commonly known as "chicken of the woods",[2] it is used in wine production and as a source of gibberellic acid and cytokinin.[3] It has been reported to show antithrombin[4] and antioxidant activities.[5] Previous biological studies have also revealed that secondary metabolites isolated from *Laetiporus sulphureus* exhibit antimicrobial, cytotoxic, and dopamine D_2 receptor agonistic activities.[6]

We report herein on the isolation and characterization of a new lanostane triterpene, 3-oxo-sulfurenic acid (**1**), along with the known compounds eburicoic acid (**3**),[7] sulfurenic acid (**4**),[7] acetyl eburicoic acid (**5**),[7] acetyl trametenolic acid (**6**),[8] 15α-hydroxytrametenolic acid (**7**),[9] the isoprenoid ubiquinone Q9,[10] and the sterols ergosterol peroxide[11] and cerevisterol.[12] The EtOH extract of the fresh fruiting bodies of the fungus was separated by column chromatography on silica gel, to give the new compound **1** in addition to eight already known compounds. Compounds **5** and **6** were obtained by acetylation of a mixture of products from fraction A_5. The structures of the known compounds were determined by a combination of spectroscopic analysis and comparison with reported data. Programmed cell death, or apoptosis, is triggered by a variety of stimuli, including cell surface receptors such as FAS, the mitochondrial response to stress, and factors released from cytotoxic T cells. Release of cytochrome *c* from the mitochondria provides the signal for the initiation of the assembly of the apoptosome complex. The apoptosome sequentially recruits, processes, and activates caspase-9 as the initiator caspase, which then activates downstream effector caspases.[13] Caspase-3 is one of the key executioners of apoptosis, being responsible either partially or totally for the proteolytic cleavage of many key proteins, such as the nuclear enzyme poly(ADP-ribose)polymerase-1.[14,15]



1 R_1= O; R_2= OH
2 R_1= O; R_2= OAc
3 R_1= β-OH, H; R_2= H
4 R_1= β-OH, H; R_2= OH
5 R_1= β-OAc,H; R_2= H



6 R_1= Ac; R_2= H
7 R_1= H; R_2= OH

We have assessed the cytotoxicity of triterpenoids **1**−**7** on the HL-60 cell line and observed that these compounds suppress survival and proliferation by triggering morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death. The apoptosis induced by these compounds is mediated by caspase-3 activation and cytochrome *c* release.

Results and Discussion

3-Oxo-sulfurenic acid (**1**) was obtained as a colorless amorphous solid and showed a molecular ion peak at *m/z* 484.3522 $[M]^+$ by HREIMS, which corresponded to the molecular formula, $C_{31}H_{48}O_4$. The IR spectrum of **1** exhibited absorptions of hydroxyl (3333 cm^{-1}) and carbonyl (1709 cm^{-1}) groups. The ^1H NMR spectrum of **1** displayed signals due to five tertiary methyls (δ_H 1.30, 1.19, 1.12, 1.04, 1.03), two secondary methyls (δ_H 1.00, 1.01), two coupled broad singlets at δ_H 4.86 and 4.90 characteristic of 24-methyl-

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10.1021/np049762o CCC: $27.50
Published on Web 11/18/2004

Table 1. NMR Spectral Data for **1** in C_5D_5N[a]

position	δ_C	δ_H (J in Hz)
1	35.3	1.74 ddd (12.02; 7.20; 3.78)
		1.45 ddd (12.56; 11.65; 7.12)
2	33.8	β, 2.53 dd (3.81; 8.56)
		α, 2.59 m
3	215.4	
4	46.4	
5	50.3	1.65 (2.13; 12.51)
6	18.9	1.55 m
7	26.5	2.59 m
8	132.6	
9	134.0	
10	36.3	
11	20.3	1.95 m
12	29.2	2.20 m
		1.90 m
13	44.6	
14	51.3	
15	71.4	4.64 dd (5.70; 9.31)
16	38.4	2.25 m
17	45.8	2.73 q br (9.38)
18	16.0	1.19 s
19	17.7	1.04 s
20	48.2	2.65 dt (3.01; 10.82)
21	177.9	
22	30.9	2.11 m
23	31.8	2.40 m
		2.30 m
24	154.9	
25	33.3	2.31 m
26	20.9	1.01 d (6.78)
27	21.1	1.00 d (6.81)
28	25.5	1.03 s
29	20.4	1.12 s
30	17.2	1.30 s
31	106.2	4.86 s
		4.90 s

[a] ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz). Assignments confirmed by ^{1}H−^{1}H COSY, HSQC, HMBC, DEPT, and ROESY spectra.

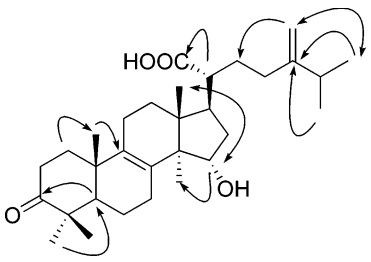

Figure 1. Relevant correlations of **1**. Double-headed arrows indicate ROESY, and single-headed arrows indicate HMBC correlations.

enelanostane,[16] and an oxygen-substituted methine at δ_H 4.64, while its ^{13}C NMR spectrum revealed the signals of a ketone and a carboxylic acid at δ_C 215.4 and 177.9, respectively (Table 1). The structure elucidation and NMR assignments were therefore based primarily on the results of HSQC, HMBC, and ROESY experiments. The most important HMBC and ROESY correlations are shown in Figure 1. Treatment of **1** with Ac_2O−pyridine gave an acetyl derivative (**2**) for which the ^{1}H NMR spectrum showed chemical shifts identical to those reported for versisponic acid C ($C_{33}H_{50}O_5$, $[\alpha]^{25}_D$ +58.0°, *c* 0.07, $CHCl_3$).[16] From the above findings, the structure of 15α-hydroxy-3-oxo-24-methylenelanost-8-en-21-oic acid was assigned to **1**.

Five known triterpenoids were identified, respectively, as eburicoic acid (**3**) ($C_{31}H_{50}O_3$, $[\alpha]^{25}_D$ +37.0°, *c* 0.9, pyridine),[7] sulfurenic acid (**4**) ($C_{31}H_{50}O_4$, $[\alpha]^{25}_D$ +39.7°, *c* 3.3, pyridine),[7] acetyl eburicoic acid (**5**) ($C_{33}H_{52}O_4$, $[\alpha]^{25}_D$ +46.6°, *c* 1.6, $CHCl_3$),[7] acetyl trametenolic acid (**6**) ($C_{32}H_{50}O_4$, $[\alpha]^{25}_D$ +61.1°, *c* 0.9, $CHCl_3$),[8] and 15α-hydroxytrametenolic acid (**7**) ($C_{30}H_{48}O_4$, $[\alpha]^{25}_D$ +37.1°, *c* 0.05, pyridine).[17]

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

compound	IC_{50} (μM)
1	407 ± 42
2	25 ± 1
3	25 ± 2
4	14 ± 1
5	15 ± 4
6	31 ± 7
7	12 ± 1
UA[b]	21 ± 4

[a] The data shown represent the mean ± SEM of two independent experiments with three determinations in each. The IC_{50} values were calculated using the methodology described in the Experimental Section. [b] UA, ursolic acid.

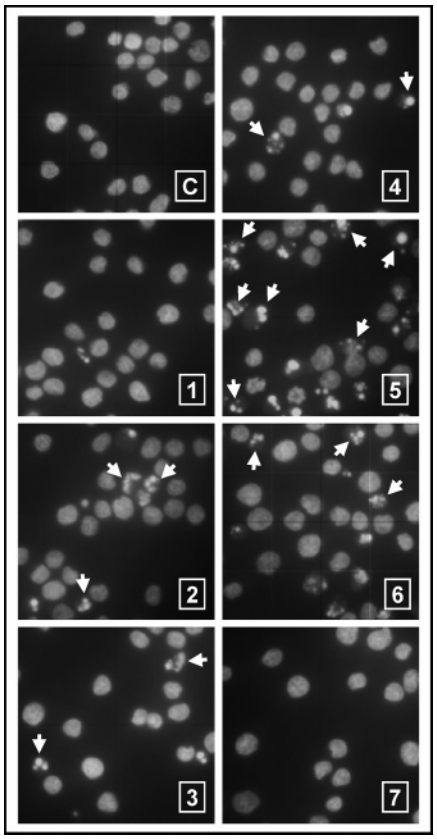

Figure 2. Induction of apoptosis in HL-60 cells by compounds **1**−**7**. Cells were cultured in the absence (C, control) or presence of 10 μM of the indicated compounds for 8 h. Then they were stained with Hoechst 33258 and nuclei were visualized using fluorescence microscopy. Arrows indicate apoptotic cells.

Triterpenoids **1**−**7** were found to inhibit the proliferation of human HL-60 myeloid leukemia cells in a dose-dependent manner as determined by the MTT assay (Table 2). The pentacyclic triterpene, ursolic acid (UA), which is known to decrease proliferation in HL-60 cells, was used as positive control.[18]

To determine whether this cytotoxic effect is due to apoptosis, we treated human HL-60 myeloid leukemia cells with these compounds and examined the typical appearance of this kind of cell death (i.e., shrinkage and apoptotic bodies as well as fragmented apoptotic nuclei) by fluorescence microscopy after DNA staining with Hoechst 33258. As shown in Figure 2, triterpenoids **2**, **3**, **5**, and **6** (10 μM, 8 h) induced morphological changes typical of apoptotic cells. While untreated cells exhibited a typically nonad-

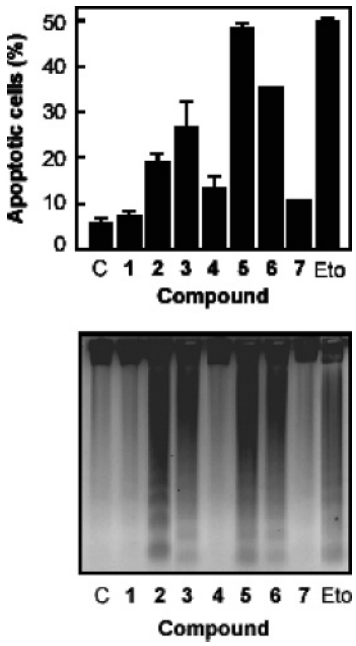

Figure 3. Induction of apoptosis in HL-60 cells by compounds **1**−**7**. Upper panel: Cells were cultured in the absence (C, control) or presence of 30 μM of the indicated compounds for 8 h. Then they were stained with Hoechst 33258 and apoptotic nuclei were quantified by fluorescence microscopy. Values represent the mean ± standard error of a single experiment run in triplicate; similar results were obtained in a separate experiment; etoposide (Eto) was used as positive control. Lower panel: Cells were treated with 30 μM of compounds for 16 h; total cellular DNA was isolated and stained with ethidium bromide after electrophoresis on a 2% agarose gel. Internucleosomal DNA fragmentation was visualized under UV light.

herent, fairly round morphology (Figure 2, C), cells exposed to 10 μM triterpenes for 8 h displayed condensation of chromatin and the appearance of apoptotic bodies. Triterpenoids **5** and **6** were the most potent apoptosis inducers (Figure 3, upper panel), as determined by quantitative fluorescence microscopy. Etoposide (10 μM), which induces cell death through apoptosis, was used as a positive control.

We also examined whether these triterpenoids induced DNA fragmentation, which is considered the end point of the apoptotic pathway. DNA fragments formed by internucleosomal hydrolysis of chromatin was evident after 16 h of treatment with 30 μM **2**, **3**, **5**, and **6** (Figure 3, lower panel). Etoposide (3 μM) was used as a positive control. To define which caspases are involved during apoptosis induced by the triterpenes, we analyzed the enzymatic activities of HL-60 cell lysates against tetrapeptide substrate DEVD-pNA (for caspase-3) after 6 h exposure to 10 μM of each compound. The relative hydrolytic activity toward the tetrapeptide substrate increased significantly (Figure 4, upper panel) in a manner dependent on triterpene concentration (results not shown). Maximal caspase-3 activity was obtained with 10 μM of compound **5** (7-fold compared to untreated cells; Figure 4, upper panel). As a positive control, etoposide (10 μM) was also included.

These triterpenoids also induced poly(ADP-ribose) polymerase-1 (PARP-1) cleavage, a hallmark of apoptosis that indicates activation of caspase. PARP-1 catalyzes the transfer of the ADP ribose moiety from its substrate, NAD^+, to a limited number of protein acceptors involved in chromatin architecture or in DNA metabolism. The cleavage of PARP-1 inactivates the enzyme, thereby making DNA repair impossible. As expected, two bands corresponding to the remaining intact PARP-1 protein (116 kDa) and the typical apoptotic 85 kDa fragment were visualized

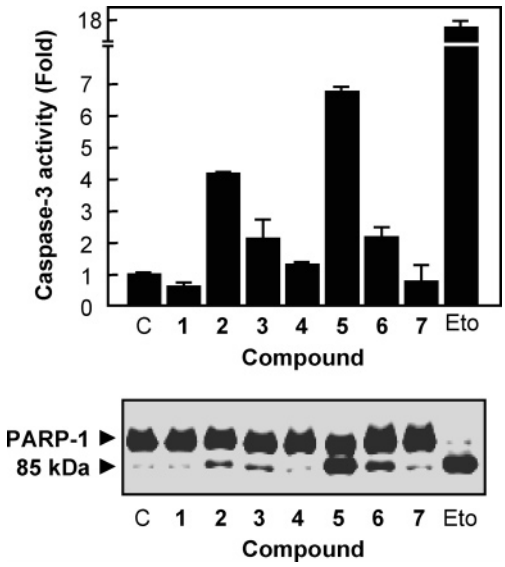

Figure 4. Activation of caspase-3 and cleavage of PARP-1. Upper panel: The cells were incubated in the absence (C, control) or presence of 10 μM of the indicated compounds for 6 h, and total cell lysate was assayed for caspase-3 activity using the DEVD-pNA colorimetric substrate. The results from a representative experiment performed in duplicate are shown and expressed as fold-increase in caspase activity compared with control. Lower panel: The cells were treated as above and equal amounts of proteins from whole cell lysates were loaded in each lane and subjected to SDS-PAGE followed by blotting with an anti-PARP-1 monoclonal antibody. Etoposide (Eto) was used as a positive control.

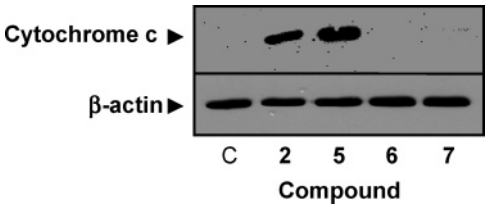

Figure 5. Western blot analysis of cytochrome *c* release. HL-60 cells were treated with 10 μM of triterpene compounds **2** and **5**−**7** and harvested at 6 h. Cytosolic lysates were analyzed by immunoblotting with an anti-cytochrome *c* antibody. β-Actin was used as loading control.

(Figure 4, lower panel), and this effect was evident for compound **5** (10 μM) at 6 h of treatment. Etoposide, a cancer chemotherapeutic drug that induces apoptosis in most cell lines, was used as positive control (Figure 4, lower panel).

To better understand the mechanisms underlying triterpene-induced apoptosis in human HL-60 myeloid leukemia cells, we examined the effect of triterpenes on cytochrome *c* translocation from the mitochondria into the cytosol. The representative Western blot analysis showed that the monoclonal antibody for cytochrome *c* detected a single band at the expected size of 15 kDa (Figure 5). Cytochrome *c* release was observed in the presence of doses as low as 10 μM of compounds **2** and **5**.

To our knowledge, no cytotoxicity study of the known compounds **2**−**7** has been reported. However, trametenolic acid has been tested against Walker-256 and MCF-7 cells, exhibiting moderate activity.[19] In this study we show a notable difference in cytotoxicity between **1** and **4** (Table 2), indicating that reduction of the carbonyl group at C-3 implies important cytotoxicity against HL-60 cells.

In conclusion, our results point to the fact that the triterpenoids investigated induce apoptosis via activation of caspase-3. Although further investigations are necessary to determine the detailed pathway of programmed cell

death by these compounds, triterpenoid **5** may be an interesting lead compound for the development of antitumor agents.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Optical rotations were recorded in a Perkin-Elmer model 343 polarimeter, and UV spectra were recorded using a JASCO model V-560 spectrophotometer. ^{1}H NMR and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 spectrometer with standard pulse sequences operating at 400 MHz in ^{1}H NMR and 100 MHz in ^{13}C NMR. $CDCl_3$, C_5D_5N, and DMSO-d_6 were used as solvents. EIMS and HREIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography was carried out on silica gel 60 (Merck 230−400 mesh) and preparative TLC on silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness) and Sephadex LH-20 (Aldrich).

Plant Material. The fungus *Laetiporus sulphureus* (Bull.: Fr.) Murr. was collected in the region of Tequendama, Cundinamarca Department, Colombia, in March 2001. The fungus was identified by Professor Luis G. Henao of the Instituto de Ciencias Naturales, Universidad Nacional de Colombia, where a voucher specimen is deposited (Col. 484-328).

Extraction and Isolation. The body fungi (500 g) were ground and steeped in EtOH (96%) for a week. The ethanol extract (20 g) was chromatographed on silica gel (6 × 50 cm, 230−400 mesh, 500 g), and the fractions were eluted with hexane, hexane−EtOAc mixtures (8:2; 7:3; 6:4; 1:1), EtOAc, and EtOAc−MeOH (8:2), each 2 L, yielding seven fractions, respectively (A_1−A_7). Fraction A_1 (220 mg) was rechromatographed over Sephadex LH-20 (2.5 × 70 cm, 150 g), eluted with hexane−$CHCl_3$−MeOH (2:2:1), to yield ubiquinone Q9 (12 mg, 6.0×10^{-3}%). Fractions A_2 (800 mg) and A_3 (250 mg) were further chromatographed by preparative TLC with hexane−EtOAc (6:4) and CH_2Cl_2−acetone (9:1), respectively, affording ergosterol peroxide (150 mg, 0.7%) and eburicoic acid **3** (190 mg, 0.9%). Fraction A_4 (134 mg) was rechromatographed on a silica gel column (2 × 20 cm, 230−400 mesh, 50 g), eluting with CH_2Cl_2−acetone (9:1), yielding 3-oxo-sulfurenic acid **1** (12 mg, 6.0×10^{-3}%) and sulfurenic acid **4** (30 mg, 0.15%). Fraction A_5 (350 mg), after purification by acetylation with Ac_2O−pyridine and subsequent chromatography by preparative TLC with CCl_4−dioxane (4:1, eluting three times), afforded acetyl eburicoic acid **5** (30 mg, 0.15%) and acetyl trametenolic acid **6** (10 mg, 0.05%). Fraction A_6 (350 mg) was chromatographed by preparative TLC with hexane−EtOAc (1:1) and $CHCl_3$−$(CH_3CH_2)_2O$ (4:1), yielding 15α-hydroxytrametenolic acid **7** (9 mg, 4.5×10^{-2}%). Fraction A_7 (200 mg) was rechromatographed over Sephadex LH-20 (2.5 × 70 cm, 150 g), eluted with hexane−$CHCl_3$−MeOH (1:1:1), and finally purified by preparative TLC with $CHCl_3$−MeOH (19:1), eluting twice, to yield cerevisterol (9 mg, 4.5×10^{-2}%).

3-Oxo-sulfurenic acid (1): colorless amorphous solid; mp 209−211°C; $[\alpha]^{25}_D$ +15° (*c* 0.4, EtOH); UV (EtOH) λ_{max} 278 nm (log ϵ 2.86); IR (KBr) ν_{max} 3333, 2920, 1709, 1674, 1454, 1377, 1279, 1053, 887 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 1; EIMS *m/z* 484 $[M]^+$ (100), 469 (32), 451 (66), 295 (24), 287 (40), 257 (20); HREIMS *m/z* 484.3522 (calcd for $C_{31}H_{48}O_4$ 484.3552).

Acetylation of 1. Compound **1** (5 mg) was treated in the usual manner with Ac_2O and pyridine to give the monoacetate **2** (5 mg): EIMS *m*/z 526 $[M]^+$ (52), 466 (73), 451 (67), 405 (21), 311 (85), 310 (65), 295 (31), 257 (100), 244 (51), 229 (20); HREIMS *m*/z 526.3685 (calcd for $C_{33}H_{50}O_5$ 526.3658).

Cell Culture. The human promyelocytic leukemia HL-60 cell line established by Gallagher et al.[20] was used in this study, and cell culture was performed as reported.[21]

Cytotoxicity Assays. Cytotoxic assays were performed using an MTT assay as described,[21] and concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment using the curve-fitting routine of the computer software Prism 2.0 (GraphPad).

Quantitative Fluorescence Microscopy. The apoptotic morphology was analyzed by fluorescence microscopy after staining with bisbenzimide trihydrochloride. Quantitation of apoptotic cells was performed as described previously.[21]

Analysis of DNA Fragmentation. DNA fragmentation analysis was carried out as described previously.[21] Briefly, cellular DNA from whole cells was extracted and separated by electrophoresis in agarose gels. After staining with ethidium bromide the images were captured by a digital camera.

Assay of Caspase-3 Activity. Activity of caspase-3 was determined as described previously[21] from cytosolic lysates, using the specific labeled substrate DEVD-pNA.

Briefly, cytosolic lysates containing the same amount of protein were incubated in the presence of the labeled substrate DEVD-pNA, and absorbance at 405 nm was determined.

Western Blot Analysis of PARP-1 Hydrolysis. Proteolytic cleavage of PARP-1 was analyzed as recently described.[21] Briefly, proteins were separated by SDS-PAGE and then transferred onto a membrane. A PARP-1 monoclonal antibody which recognizes the native form (116 kDa) and the hydrolyzed form (85 kDa) was used.

Isolation of the Cytosolic Fraction and Immunoblot Analyses of Cytochrome *c* Release. Cytochrome *c* release was analyzed from cytosolic proteins by immunoblotting with a monoclonal anti-cytochrome *c*, as described elsewhere.[21] β-Actin was used as loading control.

Acknowledgment. The authors thank the Dirección General de Universidades e Investigación (Canary Islands Government) for a Research Grant (ULPGC-INFR 2003/10 to F.E.) and FEDER funds (1FD 1997−1831 to J.B.). We thank José Estévez (University Hospital of Gran Canaria) for his collaboration in the Western blot assays and Sara Rubio for excellent technical assistance.

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NP049762O