New Lanostanoids from the Fungus *Ganoderma concinna*

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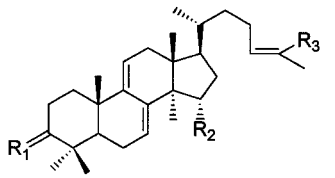
Three new compounds, 5α-lanosta-7,9(11),24-triene-3*β*-hydroxy-26-al (**1**), 5α-lanosta-7,9(11),24-triene-15α-26-dihydroxy-3-one (**2**), and 8α,9α-epoxy-4,4,14α-trimethyl-3,7,11,15,20-pentaoxo-5α-pregnane (**3**), were isolated from *Ganoderma concinna* along with 12 known compounds. The structures of compounds **1** and **2** were determined on the basis of MS and NMR studies. The structure of **3** was determined by MS, NMR, and single-crystal X-ray diffraction. Compounds **1**, **2**, and **3** induce apoptosis in human promyelocytic leukemia HL-60 cells, as indicated by examining the morphological features of cells and detection of DNA fragmentation by gel electrophoresis.

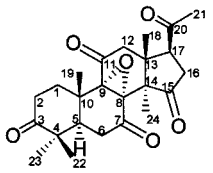
In connection with our earlier studies[1,2] of Polyporaceae (Basidiomycetes) metabolites, we have investigated the constituents of the EtOAc extract of the fruit bodies of *Ganoderma concinna* Ryv. Nov. sp. (Ganodermataceae). After extraction by the usual methods, the triterpenoids and the sterols in the EtOAc extract were separated by column chromatography, Sephadex LH-20, and preparative TLC methods. Twelve compounds of the 15 isolates were identified as known compounds. These include ganoderal A[3] (**4**), whose ^{13}C NMR spectral data (Table 2) have not previously been reported in the literature, ganodermenonol,[4] ganodermadiol,[5] ganoderic acid Y,[6] ganoderiol F,[7] ganodermatriol,[8] ganodermanontriol,[8] ganoderiol A,[8] ganoderiol B,[8] ergosta-7,22-dien-3-one,[9] fungisterol,[10] and ergosterol peroxide.[11] The structures of the known compounds were confirmed by comparison of their spectroscopic data (MS, ^{1}H and ^{13}C NMR) with literature references.

Two of the remaining three compounds were new lanostanoid triterpenes, which we named 5α-lanosta-7,9(11),24-triene-3*β*-hydroxy-26-al (**1**), 5α-lanosta-7,9(11),24-triene-15α-26-dihydroxy-3-one (**2**), and 8α,9α-epoxy-4,4,14α-trimethyl-3,7,11,15,20-pentaoxo-5α-pregnane (**3**).

Compound **1** showed a positive Lieberman-Burchard (LB) reaction, and a hydroxyl (3406 cm^{-1}) and an α-*β*-unsaturated aldehyde (1688 cm^{-1}) absorption were observed in its IR spectrum. The HREIMS spectrum of **1** showed a molecular ion at *m*/*z* 438.3426 corresponding to the molecular formula $C_{30}H_{46}O_2$ (calcd 438.3497). The ^{1}H NMR spectrum of **1** (Table 1) showed signals for tertiary methyl groups at *δ* 0.55, 2 × 0.86, 0.96, and 0.98 and a secondary methyl group at *δ* 0.93 (d, *J* = 6.6 Hz), as required by the lanostane skeleton. A vinyl methyl and olefinic signals were observed at *δ* 1.73, 5.30, 5.46, and 6.47, respectively. The ^{13}C NMR spectrum of **1** (Table 2) showed signals due a conjugated diene group at *δ* 120.3, 142.4, 145.9, 116.0, which suggested a 7,9(11)-diene lanostane skeleton. Comparison of the ^{13}C NMR spectral data with those reported for ganodermadiol[5] allowed the location of the aldehyde group.[2] These data established the structure of **1** as 5α-lanosta-7,9(11),24-triene-3*β*-hydroxy-26-al.



(1) R_1= β–OH, H ;R_2= H; R_3=CHO
(2) R_1= O; R_2=αOH; R_3= CH_2OH
(4) R_1= O; R_2=H; R_3= CHO



(3)

HREIMS and ^{13}C NMR data of **2** indicated the molecular formula $C_{30}H_{46}O_3$. The IR spectrum of this compound showed the presence of a hydroxyl group (3444 cm^{-1}), a carbonyl group (1703 cm^{-1}), and an unsaturated carbon (2968 cm^{-1}). The UV, ^{1}H NMR, and ^{13}C NMR spectra of **2** were similar to those of **1**, suggesting that **2** may have a 5α-lanosta-7,9(11),24-triene structure. However in the ^{13}C NMR spectrum the carbonyl carbon signal was observed at low field at *δ* 216.6. Also, the ^{1}H NMR spectrum did not exhibit any signal around *δ* 3.23 (H-3 of **1**) for the presence of a hydroxyl group. These observations suggested the presence of a carbonyl group at C-3. A singlet at *δ* 3.98 integrated for two hydrogens and the signal at *δ* 4.28 (dd, *J* = 9.2, 4.9 Hz) integrated for one hydrogen, in accord with those reported in the literature.[2,6] The shifts correspond to the protons at C-26 and C-15, respectively, on the basic of NOESY and HMBC spectra (Figure 1). Thus, compound

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Table 1. ^{1}H NMR Data of **1**−**3**[a] (*J* in Hz)

proton	1	2	3
1α	1.41 m	1.33 m	2.08 ddd (8.2, 7.9, 13.5)
1β	1.97 dt (13.2, 3.6)	2.25 m	2.63 m
2α	1.98 m	2.33 m	2.57 m
2β	1.69 m	2.74 dt (14.3, 5.5)	2.57 m
3α	3.23 dd (4.2, 11.3)		
4			
5α	1.07 dd (4.8, 11.4)	1.52 dd (12.6, 4.6)	2.35 d (5.08)
6α	2.06 m	2.09 m	2.40 m
6β	2.06 m	2.09 m	2.40 m
7	5.46 d (6.0)	5.89 d (6.1)	
8			
9			
10			
11	5.30 d (6.0)	5.38 d (6.0)	
12α	2.18 m	2.24 m	2.91 d (14.6)
12β	2.08 m	2.25 m	2.57 d (14.6)
13			
14			
15α	1.38 m		
15β	1.38 m	4.28 dd (4.9, 9.2)	
16α	1.91 m	1.93 m	2.91 dd (8.58, 17.0)
16β	1.62 m	1.71 m	2.68 m
17	1.57 m	1.68 m	3.17 t (8.58)
18	0.55 s	0.61 s	0.92 s
19	0.96 s	1.17 s	1.26 s
20	1.42 m	1.40 m	
21	0.93 d (6.6)	0.90 (6.4)	2.17 s
22	1.20 m	1.75 m	1.05 s
23	2.26 2.37	1.92 m	1.43 s
24	6.47 t (7.2)	5.37 t (6.7)	
25			
26	9.39 s	3.98 s	
27	1.73 s	1.64 s	
28	0.86 s	0.91 s	
29	0.98 s	1.06 s	
30	0.86 s	1.10 s	

[a] Spectra were recorded in $CDCl_3$.

Table 2. ^{13}C NMR Data for **1**−**4**[a,b]

carbon	1	2	3	4
1	35.58	35.78	32.36	36.52
2	27.84	34.78	32.21	34.72
3	78.83	216.58	214.10	216.7
4	38.59	47.32	47.04	47.43
5	48.98	50.41	43.53	50.62
6	22.89	23.57	36.71	23.57
7	120.30	121.03	198.3	119.97
8	142.39	141.02	66.78	142.62
9	145.86	144.69	68.12	144.48
10	37.25	37.24	37.37	37.11
11	116.01	117.00	200.6	117.03
12	37.69	38.48	46.01	37.30
13	43.71	44.30	45.53	43.73
14	50.19	51.92	54.84	50.62
15	31.36	74.60	205.9	27.83
16	27.69	40.08	36.39	31.35
17	50.72	48.84	52.43	50.75
18	15.66	15.87	19.57	15.61
19	22.62	22.13	16.30	22.34
20	36.04	35.83	205.2	36.05
21	18.18	18.34	31.16	18.19
22	34.61	36.59	26.85	34.61
23	25.92	25.40	20.26	25.94
24	155.35	126.60	17.47	155.19
25	139.10	134.56		139.20
26	195.29	68.98		195.20
27	9.05	13.54		9.05
28	25.44	16.89		25.26
29	28.01	25.40		25.30
30	15.53	22.13		21.95

[a] The number of protons directly attached to each carbon was verified by DEPT experiments. [b] Signals obtained by HSQC and HMBC techniques.

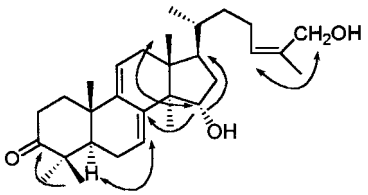

Figure 1. Correlations of **2**. Double-headed arrows indicate NOESY, and single-headed arrows indicate HMBC correlations.

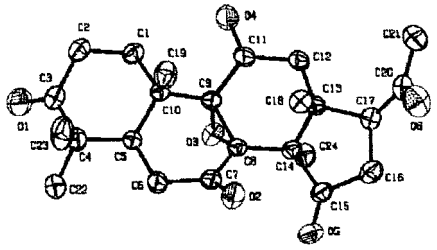

Figure 2. X-ray crystal structure of **3**.

2 was elucidated as 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one.

The third new triterpene (**3**) was obtained as a crystalline solid, mp 242−243 °C. The HREIMS of **3** showed the M^+ at *m/z* 414.1964, corresponding to the molecular formula $C_{24}H_{30}O_6$ (calcd 414.2042). The strong absorption in the IR spectrum showed the presence of one or more carbonyl groups (1710 cm^{-1}). The UV absorption at λ_{max} 289 was consistent with the presence of carbonyl groups in the molecule. The ^{1}H NMR spectrum of **3** closely resembled that of lucidone A,[12,13] showing six methyl groups (Table 1). In the ^{13}C NMR spectrum (Table 2), all the chemical shifts of the carbon signals were almost superimposable with those of lucidone A,[12] with the exception of the chemical shift of C-3, C-8, and C-9 signals. This clearly indicated a carbonyl group at C-3 and an epoxy group located between C-8 and C-9. The structure of **3** was confirmed by X-ray diffraction. Crystals are triclinic with $a = 6.8691(7)$ Å, $b = 8.4093(10)$ Å, $c = 9.6369(10)$ Å, $\alpha = 76.650(9)°$, $\beta = 78.793(9)°$, $\gamma = 74.977(9)°$. The asymmetric unit contained a molecule of composition $C_{24}H_{30}O_6$. All unique diffraction maxima with $2\theta < 60°$ were collected using ω-scans. Altogether, 3009 unique reflections were collected, and 2328 (77.4%) were judged observed [$I > 2\sigma(I)$] and used in calculations. The structure was solved by using direct methods and refined by full-matrix least-squares on F^2 to a final discrepancy index of 0.048 ($wR_2 = 0.1390$) for observed data. A view of the molecule with the atom-numbering scheme is given in Figure 2. Displacement ellipsoids for non-H atoms are drawn at the 50% probability level. The core of the molecule presents three six-membered rings and one five-membered ring, in addition to an epoxy group in α-configuration. The crystal structure is stabilized by C−H O═C intermolecular hydrogen bonds. We were unable to determine the absolute stereochemistry by X-ray

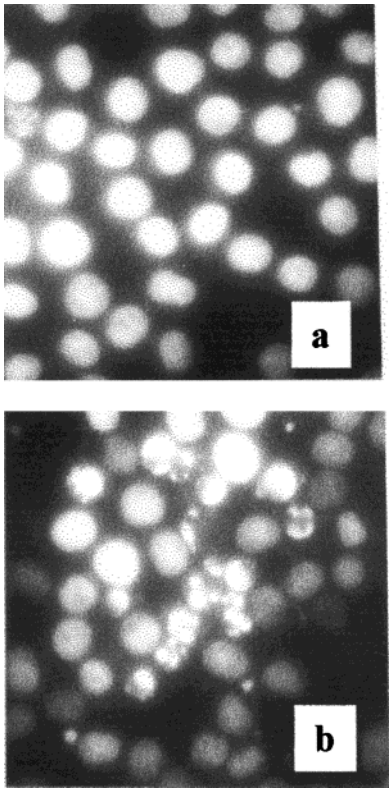

Figure 3. Morphological aspects of Hoechst 33258-stained cells: (a) untreated cells; (b) cells treated for 24 h with 30 μM of **2**.

methods, and the configuration shown here was chosen in accord with that reported in a previous chemical study of lucidone A.[12]

It has been recognized that apoptotic cell death in HL-60 cells is characterized by loss of proliferative capacity, double-stranded DNA damage, and profound alterations of cellular morphology.[14] To determine whether compounds **1**−**3** induce apoptosis, we treated HL-60 myeloid leukemia cells with these agents (10−30 μM) for 24 h. This treatment resulted in the appearance of typical morphological changes of apoptosis upon staining with the DNA-binding fluorochrome bis-benzimide trihydrochloride (Hoechst 33258) (Figure 3, a, untreated controls; b, treated cells). These morphological features associated with apoptosis include chromatin condensation, its compaction along the periphery of the nucleus, and nuclear segmentation into three or more chromatin fragments. Treatment of HL-60 cells with compounds **1** and **3** for 24 h induced apoptosis in 15 ± 2% and 12 ± 2% of the cells, respectively, which was greater than untreated controls (5 ± 1%). Meanwhile, treatment of HL-60 cells with **2** induced a significant increase in apoptosis in 62 ± 5% of the cells. This was significantly greater than was induced by compounds **1** and **3** (Figure 4a). Qualitative assessment of DNA damage on agarose gels demonstrated electrophoretic patterns of DNA fragments formed by intranucleosomal hydrolysis of chromatin in response to compounds **1**, **2**, and **3** (Figure 4b). Compound **2** not only induced intranucleosomal DNA fragmentation in HL-60 cells but also reduced survival (Figure 5), as determined by the 3-[4,5-dimethylthiazol-2-yl-] 2,5-diphenyltetrazolium bromide (MTT) dye-reduction assay. This inhibitory effect was already evident after 24 h of treatment. For compound **2**, the estimated dose of growth inhibition (IC_{50}) determined by the MTT assay method was 15.7 ± 4.6 μM after 24 h of treatment. In contrast, compounds **1** and **3** showed little effect on the growth of HL-60 cells after 24 h of treatment.

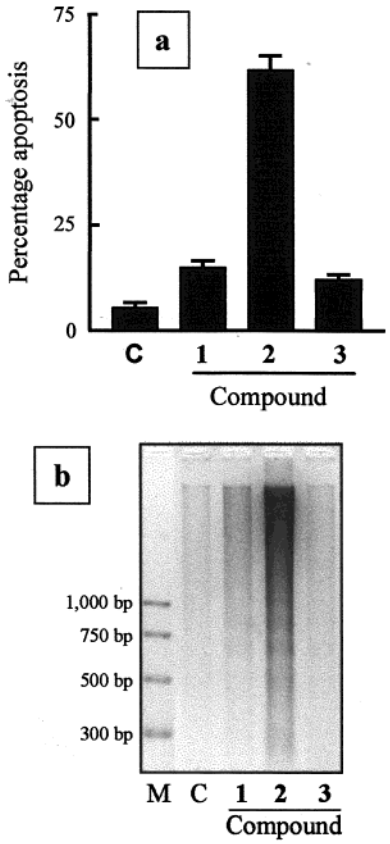

Figure 4. Induction of apoptosis in HL-60 cells. Apoptosis was determined by the quantitative fluorescent microscopy method showing typical condensation and fragmentation of nuclear chromatin (C, control; **1**, **2**, **3**, 30 μM) (a). Agarose gel electrophoresis of DNA extracted from untreated (C, control) and treated cells with 30 μM of compounds **1**−**3** for 24 h (M, molecular weight marker) (b).

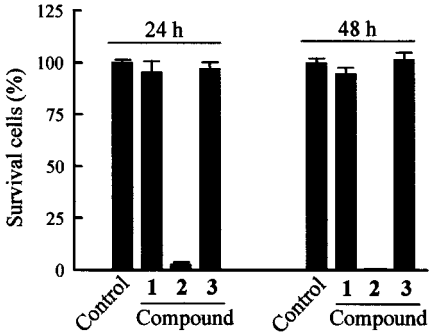

Figure 5. Cytotoxicity of compounds **1**−**3** (30 μM) by using the MTT-dye reduction assay.

Experimental Section

General Experimental Procedures. Melting points were determined on a Büchi B-540 apparatus and are uncorrected. Optical rotations were measured using a Perkin-Elmer model 343 polarimeter. UV spectra were recorded using a JASCO model V-560 spectrophotometer. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-500 spectrometer with standard pulse sequences, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. $CDCl_3$ was used as solvent and TMS as internal standard. EIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography was carried out on Si gel (70−230 mesh, Merck). Column fractions were monitored by TLC (Si gel 60 F_{254}), and preparative TLC was carried out on Si gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness).

Plant Material. The fungus *Ganoderma concinna* Ryv. Nov. sp. was collected in the Alto Sinú region of Córdoba, Colombia, in May 1999, and identified by Prof. Jaime Uribe, Instituto de Ciencias Naturales, Universidad Nacional de Colombia. A voucher specimen is deposited at the Herbario Nacional Colombiano with no. COL 343842.

Extraction and Isolation. The dried fungus (170 g) was ground and steeped in EtOH (96%) for a week. The ethanol extract was concentrated in vacuo, to yield a brown crude extract (6.3 g), and was partitioned between H_2O and EtOAc (1:1). The EtOAc extract (4.5 g) was chromatographed on a Si gel column packed in *n*-hexane and eluted with an *n*-hexane and EtOAc gradient. The fraction eluted with a 9:1 mixture gave ergosta-7,22-dien-3-one[9] (100 mg), ganoderal A[3] (6 mg), and ergosterol peroxide[11] (150 mg); a 4:1 mixture gave 5,6-dihydroergosterol[10] (130 mg), ganodermenonol[4] (30 mg), and 5α-lanosta-7,9(11),24-triene-3β-hydroxy-26-al (5 mg); a 7:3 mixture gave ganodermadiol[5] (30 mg); a 6:4 mixture gave ganoderic acid Y[6] (4 mg) and 5α-lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (5 mg); a 1:1 mixture gave ganoderiol F[7] (7 mg), ganodermatriol[8] (9 mg), ganodermanontriol[8] (15 mg), 8α-9α-epoxy-4,4,14α-trimethyl-3,7,11,15,20-pentaoxo-5α-pregnane (4 mg), ganoderiol A[8] (20 mg), and ganoderiol B[8] (17 mg). Sephadex LH-20 columns packed in *n*-hexane−$CHCl_3$−MeOH (2:1:1; 1:1:1) and/or preparative TLC (Si gel in thicknesses ranging from 1 to 10 mm) developed with *n*-hexanes−EtOAc (7:3, 3:2, 1:1, and 2:3) and acetone−CH_2Cl_2 (9:1, 4:1) were used in the purification of the indicated compounds.

5α-Lanosta-7,9(11),24-triene-3β-hydroxy-26-al (1): colorless solid (MeOH−EtOAc), mp 70−71 °C; $[\alpha]^{20}_D$ +21° (*c* 0.15, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 235 nm (3.3), 243 (3.8), 252 (3.5); IR (film) ν_{max} 3406 (OH), 2927 (unsaturated carbon), 1688 (C=C−C=O), 1455, 1373, 1188, 1076, 1036 cm^{-1}; ^1H, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m/z* 438 (28), 420 (42), 405 (71), 377 (47), 351 (8), 311 (24), 293 (20), 253 (20), 171 (20), 157 (27), 149 (80), 57 (100); HREIMS *m/z* 438.3426 $[M]^+$ (calcd for $C_{30}H_{46}O_2$, 438.3497).

5α-Lanosta-7,9(11),24-triene-15α,26-dihydroxy-3-one (2): colorless solid (MeOH), mp 124−125 °C; $[\alpha]^{20}_D$ +73° (*c* 0.20, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 236 nm (3.9), 244 (3.9), 252 (3.8); IR (film) ν_{max} 3444 (OH), 2968, 2935 (unsaturated carbon), 1703 (C=C−C=O), 1455, 1375, 1215, 1055 cm^{-1}; ^1H, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m/z* 454 (16), 436 (22), 418 (25), 354 (11), 337 (18), 325 (25), 309 (67), 255 (23), 223 (17), 209 (13), 197 (19), 185 (20), 171 (28), 157 (42), 147 (14), 109 (100); HREIMS *m/z* 454.3413 $[M]^+$ (calcd for $C_{30}H_{46}O_3$, 454.3446).

8α-9α-Epoxy-4,4,14α-trimethyl-3,7,11,15,20-pentaoxo-5α-pregnane (3): colorless solid (EtOAc−benzene), mp 242−243 °C; $[\alpha]^{20}_D$ +24° (*c* 0.05, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 289 nm (3.1), 251 (3.1); IR (film) ν_{max} 2927, 1753, 1710 (C=O), 1457, 1414, 1389, 1362, 1182, 756 cm^{-1}; ^1H, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m/z* 414 (10), 371 (14), 316 (30), 288 (20), 273 (33), 260 (10), 191 (11), 179 (25), 165 (100), 152 (22), 137 (28); HREIMS *m/z* 414.1964 $[M]^+$ (calcd for $C_{24}H_{30}O_6$, 414.2042).

Crystal Data and X-ray Crystal Structure Determination of Triterpene (3). Crystals of **3** suitable for X-ray diffraction analysis were obtained by recrystallization from EtOAc−benzene (1:1). $C_{24}H_{30}O_6$; M_r = 414.48, triclinic a = 6.8691(7) Å, b = 8.4093(10) Å, c = 9.6369(10) Å, α = 76.650(9)°, β = 78.793(9)°, γ = 74.977(9)°, V = 517.73(10) $Å^3$, space group *P*1 (no. 1), Z = 1, D_{calc} = 1.329 g/cm^3, μ(Mo Kα) = 0.095 mm^{-1}, $F(000)$ = 222; colorless crystals, dimensions 0.15 × 0.25 × 0.35 mm^3.

Data Collection. Enraf-Nonius MACH-S3 diffractometer, ω-scan technique, graphite-monochromated Mo Kα (λ = 0.71073 Å) radiation; 3243 reflections were measured ($3° < \theta < 30°$, $0 \le h \le 9$, $-11 \le k \le 11$, $-13 \le l \le 13$), 3009 were unique. Three standard reflections were measured after every 120 min, which showed no significant decay.

Structure Analysis and Refinement. The crystal structure was solved by direct methods[15] and refined by full-matrix least-squares on F^2 values.[16,17] Non-hydrogen atoms were refined anisotropically. Only the O4 present a lighter disorder and therefore a higher thermal factor than usual values. The methyl-H atoms were refined as rigid groups, which were allowed to rotate but not to tip, with $U_{iso}(H) = 1.5U_{eq}(C)$. All other H atoms were allowed to ride on their parent atoms with $U_{iso}(H) = 1.2U_{eq}(C)$. The absolute configuration of the structure of the crystal could not be determined reliably; refinement of the Flack parameter[18] gave an ambiguous result. Friedel opposite reflections were therefore merged.

The final values of the residual factors R and wR_2 [for 2328 reflections with $I > 2\sigma(I)$] were 0.0481 and 0.1390, respectively. In the final difference Fourier synthesis, the density fluctuated in the range 0.37 to −0.34 e $Å^3$.

Description of the Structure. The molecule 3 is formed by three six-membered rings and one five-membered ring. The conformations of the six-membered rings are chair, twist-boat, and half-boat for the A, B, and C rings, respectively. The five-membered ring has an envelope conformation on the C13 atom. The C8−C9 bond at 1.510(4) Å is long compared with the C−C bond in other epoxides. A search for two six-membered rings fused with epoxide was made in CSD version 5.21 (April 2001), finding a total of 12 organic structures without errors and disorder. The mean value was 1.472(6) Å, and the more frequent values were in the range 1.455−1.456 Å. This is according to the considerations made by Allen et al.[19] The increased distance is presumably due to the constraint imposed by the two fused six-membered rings where the epoxide lies across the ring junction. Other distances and angles are unexceptional.

Tissue Culture. HL-60 cells were maintained at a density of $<1 \times 10^6$ cells/mL. Cells were cultured in suspension in RPMI-1640 medium (Life Technologies, Inc) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (10 000 units mL^{-1}), and streptomycin (10 000 $\mu g\ ml^{-1}$) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Stock solutions of compounds **1**, **2**, and **3** in dimethyl sulfoxide (DMSO) were stored at −20 °C. Further dilutions were made in culture medium just before use. In all experiments, the final concentration of DMSO did not exceed 1% (v/v), a concentration that was nontoxic to the cells.

Cell viability was determined using the trypan blue exclusion test. To ensure an exponential growth, cells were resuspended in fresh medium 24 h before each treatment.

Cytotoxic assays were performed using an MTT assay.[20] Cells were continuously exposed to indicated concentrations of the compounds, and surviving cells were detected based on their ability to metabolize 3-[4,5-dimethylthiazol-2-yl-] 2,5-diphenyl tetrazolium bromide (MTT) into formazan crystals. The MTT dye reduction assay measures mitochondrial respiratory function and can detect the onset of cell death earlier than dye-exclusion methods. Cell survival was calculated as the fraction of cells alive relative to control for each point as cell survival (%) = mean absorbance in treated wells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment.

Quantitative Fluorescence Microscopy. For detection of apoptotic cells, cells were stained with Hoechst 33258 (10 μg/mL) for 30 min at 37 °C before fluorescence microscopy analysis. Apoptotic cells were identified by their fragmented chromatin.[21] Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery) or nuclei that were fragmented into multiple smaller dense bodies were considered as apoptotic. Nuclei with uncondensed and dispersed chromatin were considered as not apoptotic. A minimum of 500 cells were counted for each sample, and each experiment was done in triplicate.

Determination of DNA Fragmentation. The presence of apoptosis was evaluated by agarose gel electrophoresis of DNA extracted from HL-60 cells.[22] Each DNA sample was electrophoresed at 80 V for 4 h through a 2% agarose gel in TAE buffer (40 mM Tris-acetate and 1.0 mM EDTA, pH 8.3). DNA bands were visualized under UV light after staining with ethidium bromide.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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