Sesquiterpenoid Derivatives from *Gonospermum elegans* and Their Cytotoxic Activity for HL-60 Human Promyelocytic Cells[#]

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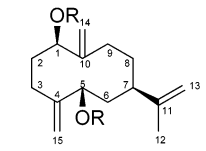
Four new compounds, a sesquiterpene, eleganodiol (**1**), and three sesquiterpene lactones, eleganolactone A (**2**), eleganolactone B (**3**), and elegain (**4**), were isolated from *Gonospermum elegans* along with 16 known compounds. The structures of **1**, **2**, and **4** were determined on the basis of MS and NMR studies of their acetate derivatives (**1a**, **2a**, **4a**). The structure of the acetate derivative (**3a**) of **3** was determined on the basis of spectroscopic data interpretation and by single-crystal X-ray diffraction. Compounds **2a** and **3a** were used to study their biological activities on the HL-60 human promyelocytic leukemia cell line. These compounds induced morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death.

The genus *Gonospermum* Less (Compositae) is endemic to the Canary Islands and is represented by four species distributed throughout the most westerly islands, to which the single species *Lugoa revoluta* D.C. was recently added, in accordance with cytogenetic and evolutionary studies,[1] supported by the presence of similar sesquiterpene lactones, as deduced from earlier chemical studies.[2,3] In a continuation of our studies of species of the genus *Gonospermum*, we have investigated the constituents of an EtOH extract of the aerial parts of *G. elegans* (Cass) Less. The constituents of the EtOH extract were separated by column chromatography, medium-pressure column chromatography (MPLC), and preparative TLC. Sixteen known compounds were identified including the coumarin scopoletin,[4] the flavonoid axillarin,[5] the sesquiterpene lactones spiciformin,[6] reynosin,[7] 4α,5β-epoxydeactyllaurenolide,[8] deacetyl-β-cyclopyrethrosin,[8] tatridin A,[9] artemorin,[10] 1α-hydroxydeacetyltulirinol-4α,5β-epoxide,[11] tatridin B,[9] 1-*epi*-tatridin B,[9] 11,13-dihydrotatridin B,[12] sivasinolide,[13] and 1α-hydroxy-1-desoxotamirin-4α,5β-epoxide,[11] and the sterol β-sitosterol-β-D-glucoside.[14] The structures of these compounds were confirmed by comparing their ^{1}H NMR and mass spectra with literature values. The remaining four compounds were new and were assigned as eleganodiol (**1**), eleganolactone A (**2**), eleganolactone B (**3**), and elegain (**4**), respectively.

In the present study, the cytotoxicity of **2**, **2a**, and **3a** was assessed against HL-60 cells. We observed that derivatives **2a** and **3a** suppress survival and proliferation by triggering typical apoptosis in human promyelocytic leukemia HL-60 cells, as judged by classical morphological as well as biochemical criteria.



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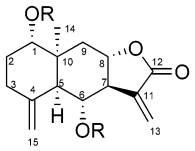
1a R=Ac



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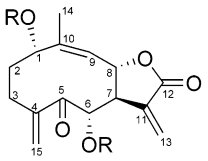

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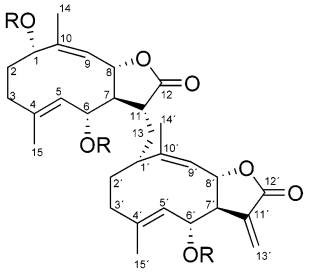

4 R=H
4a R=Ac

Results and Discussion

Compound **1** was purified by treatment with Ac_2O and pyridine to give the acetyl derivative, **1a**. Its IR spectrum showed characteristic bands for acetate groups (1737 and 1273 cm^{-1}) and for one or more terminal double bonds (1648 and 898 cm^{-1}), with no bands for a γ-lactone being observed. The HREIMS of **1a** showed a molecular ion at *m*/*z* 320.1969 corresponding to the molecular formula, $C_{19}H_{28}O_4$ (calcd 320.1987). The ^{1}H NMR spectrum of **1a** (Table 1) exhibited signals for three terminal double bonds at δ 5.17 (1H, s), 5.20 (1H, s), 5.14 (1H, s), 5.25 (1H, s), 4.73 (1H, br s), and 4.75 (1H, br s), which are typical of exomethylene resonances at C-4, C-10, and C-11 of a germacrane. The ^{1}H NMR patterns of **1a** were similar to those of the known germacrane triacetylageratriol, with

[#] Dedicated to the memory of Professor Antonio G. González.

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Table 1. ^{1}H NMR Data of Compounds **1a**−**3a**[a]

proton	1a	2a	3a
1	5.39 dd (α; 4.9, 8.4)	5.05 dd (β; 4.7, 11.5)	5.72 dd (β; 5.4, 10.5)
2	2.06 m	1.62 m (α)	2.17 m
	2.33 m	1.85 m (β)	1.92 m
3	2.30 m	2.25 m (α)	2.35 m
	2.10 m	2.43 td (β; 5.3, 14.4)	2.26 dd (4.3, 12.9)
5	5.08 dd (α; 3.9, 11.7)	2.17 d br (α; 10.44)	
6	1.87 td (α; 3.8, 15.3)	5.44 t (β; 10.6)	5.19 s br (β)
	1.68 m (β)		
7	2.21 m (α)	2.70 ddd (α; 2.9, 5.8, 10.9)	3.08 m (α)
8	1.70 m	4.19 td	5.60 d
	1.80 m	(β; 3.5, 12.1)	(β; 10.3)
9	2.33 m	1.37 t (α; 12.6)	5.20 s
	2.00 m	2.31 dd (β; 3.7, 12.8)	
12	1.68 s		
13a	4.75 br s	6.11 d (2.8)	6.38 d (2.6)
13b	4.73 br s	5.39 d (2.8)	5.96 d (2.6)
14a	5.25 s	1.09 s	1.74 s
14b	5.14 s		
15a	5.20 s	4.91 s	6.61 s
15b	5.17 s	4.77 s	6.14 s
OAc	1.99	2.07	2.03
OAc	2.04	2.04	2.17

[a] Spectra were recorded in $CDCl_3$; *J* values in Hz as shown in parentheses.

Table 2. ^{13}C NMR Data of Compounds **1a**−**3a**[a,b]

carbon	1a	2a	3a
1	76.0	71.0	68.4
2	29.6	27.4	29.2
3	24.8	29.4	23.6
4	145.0	142.2	146.0
5	78.5	60.0	199.2
6	34.4	68.5	72.1
7	40.5	51.8	47.5
8	31.2	75.6	74.7
9	30.4	37.5	125.9
10	145.1	40.7	140.3
11	147.0	136.6	136.0
12	19.4	169.5	170.0
13	110.6	119.4	125.0
14	116.7	22.2	17.3
15	117.0	115.3	128.7
OAc	21.3	20.6	20.3
	21.3	21.0	21.0
	170.0	169.7	170.0
	170.0	170.5	170.0

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

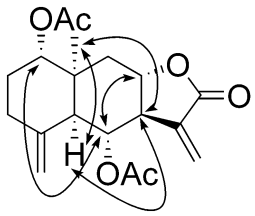

Figure 1. Representative ROESY correlations of **2a**.

the exception of the signal for a CH_2 group instead of a hydroxyl group at C-9.[15] Irradiation of the signal at δ 5.08 (H-5α) belonging to a proton geminal to the acetyl group collapsed the signal at δ 1.87 (H-6α), but this irradiation had no effect on the signal at δ 2.21 (H-7α). The ^{13}C NMR spectrum and DEPT experiments showed 19 carbons consisting of three methyls at δ 19.4 and 21.3 (two acetyl groups), eight methylenes at δ 29.6, 24.8, 34.4, 31.2, 30.4, and 110.6, 116.7, 117.0 (three terminal double-bond CH_2 carbons), three methines at δ 40.5, 76.0, and 78.5, and three quaternary carbons at δ 145.0, 145.1, and 147.0, consistent with signals typical for a germacrane skeleton. The stereochemistry of the C-7 isopropenyl group has been found to be β in germacranes and biogenetically related sesquiterpenes based on Hendrickson's biogenetic rule,[16] which has been confirmed by several X-ray crystallographic studies of germacranes.[17] The relative stereochemistry in **1** was determined by ROESY difference experiments. ROEs were observed between H-7 and H-1 and H-5, and between H-9 and H-7. Upon consideration of all the above data, **1** was deduced from **1a** as 1β,5β-dihydroxygermacra-4(15),10(14), 11(13)-triene and was assigned the trivial name eleganodiol.

Compound **2** was obtained as a colorless oil. Its IR spectrum showed absorption bands due to a hydroxyl (3467 cm^{-1}), the carbonyl of a γ-lactone (1737 cm^{-1}), and olefinic bonds (982 cm^{-1}). The EIMS showed a molecular ion peak at *m/z* 264 in accord with the empirical formula, $C_{15}H_{20}O_4$. The ^{1}H NMR spectrum of compound **2** exhibited four olefinic proton signals at δ_H 6.11 (d, J = 2.8 Hz, H-13a), 5.39 (d, J = 2.8 Hz, H-13b), 4.91 (s, H-15a), and 4.77 (s, H-15b). These assignments were similar to those of the known eudesmanolide deacetyl-β-cyclopyrethrosin,[8] with the exceptions of the signals of H-1 (δ 3.78) and Me-14 (δ 1.04), which were in agreement with those of 1α,8α-dihydroxy-10-*epi*-β-cyclocostunolide.[18]

Treatment of **2** with Ac_2O and pyridine gave the acetyl derivative **2a** as an oil, $[\alpha]^{29}_D$ −51°. Its HREIMS at *m/z* 348.1590 was consistent with the molecular formula, $C_{19}H_{24}O_6$. Further fragment ions at *m/z* 288 $[M - 60]^+$ and 228 $[M - 60 - 60]^+$ revealed the presence of two acetyl groups. The ^{13}C NMR spectrum (Table 2) and DEPT experiments of the acetyl derivative **2a** showed 19 carbons consisting of three methyls at δ 22.2, 21.0, and 20.6 (two acetyls); five methylenes at δ 27.4, 29.4, 37.5, 115.3, and 119.4 (two terminal double bond CH_2 carbons); five methines at δ 51.8, 60.0, 68.5, 71.0, and 75.6; and six quaternary carbons at δ 40.7, 136.6, 142.2, 169.5 (a carbonyl carbon), and 169.7 and 170.5 (two carbonyl acetates), consistent with a eudesmanolide skeleton. The *cis* A/B ring junction of **2a** was confirmed by the observation of ROESY cross-peaks between δ 1.09 (Me-14)/2.70 (H-7α), 1.09 (Me-14)/1.62 (H-2α), 2.17 (H-5α)/2.70 (H-7α), as well as between δ 5.05 (H-1β)/4.19 (H-8β), 4.19 (H-8β)/5.44 (H-6β) (Figure 1). Thus, **2** was deduced to be 1α,6α-dihydroxy-14α-methyl-4(15),11(13)-dien-eudesman-8α,12-olide and was assigned the trivial name eleganolactone A.

Compound **3** was acetylated for the purpose of purification, affording the acetyl derivative **3a** as colorless crystals (hexane−EtOAc), $[\alpha]^{29}_D$ +25°. Its IR spectrum showed the presence of a carbonyl ester (1746 cm^{-1}), a γ-lactone (1760 cm^{-1}), and one or more double bonds (1681 cm^{-1}). Its molecular formula was established as $C_{19}H_{22}O_7$ from the HREIMS (*m/z* 362.1394) and the ^{1}H, ^{13}C, and DEPT NMR data. Its UV spectrum showed an absorption maximum at 281 nm due to a n → π* transition of a conjugated carbonyl group. The ^{1}H NMR (Table 1) spectrum of **3a** exhibited the presence of two acetyl methyl groups at δ 2.17 (3H, s) and 2.03 (3H, s), a vinyl methyl group at δ 1.74 (3H, s), and two terminal double bonds at δ 6.38 (1H, d, J = 2.6 Hz), 5.96 (1H, d, J = 2.6 Hz) and 6.61 (1H, s), 6.14 (1H, s),

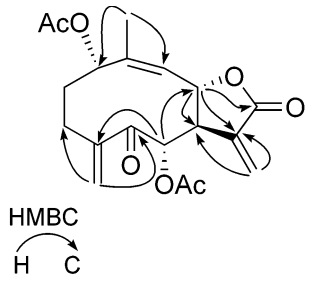

Figure 2. Representative HMBC correlations of **3a**.

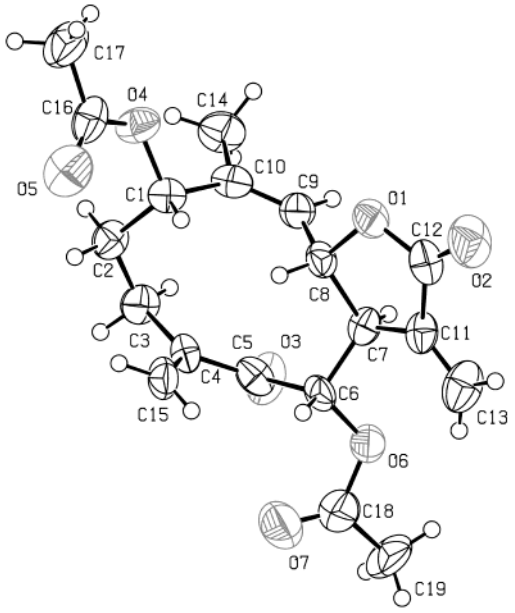

Figure 3. View of compound **3a** with the non-H atom numbering scheme. Displacement ellipsoids for non-H atoms are drawn at the 50% probability level.

typical of a germacranolide. The relationships between the proton signals in **3a** were established from the $^1H-^1H$ COSY spectrum, which disclosed the following connectivities: H-1/H-2, H-6/H-7, H-7/H_2-13, H-7/H-8, and H-8/H-9. The ^{13}C NMR (Table 2) and DEPT spectral data indicated the presence of two carbonyl groups, one of them corresponding to a γ-lactone, as well as two ester carbonyl groups, six olefinic carbons, three methyl carbons, two methylene carbons, and four methine carbons. The ^1H, ^{13}C, and DEPT NMR spectral data suggested that **3a** has a germacranolide skeleton lactonized at C-8,[9] and HMBC experiments established the following connectivities: H-14/C-1, C-9, and C-10; H-8/C-7, C-12, and C-11; H-15/C-3, C-5, and C-4; H-6/C-8 and C-4; and H-13/C-7, C-11, and C-12, as shown in Figure 2. The structural model of compound **3a** (Figure 3) was confirmed by X-ray diffraction analysis, but we were unable to determine the absolute stereochemistry by this method. The configuration shown here was chosen because it was in accord with the data reported in a previous chemical study.[19] In conclusion, the structure of the new compound **3** corresponded to 1α,6α-dihydroxy-5-oxo-4(15),9,11(13)-trien-germacra-8α,12-olide and was named eleganolactone B.

The acetyl derivative of compound **4**, **4a**, was obtained as a colorless oil. Its HREIMS showed a molecular ion peak at *m/z* 638.3123, which indicated a molecular formula of $C_{36}H_{46}O_{10}$ (calcd 638.3091). Its IR spectrum showed absorptions at 1767 cm^{-1} (α,β-unsaturated lactone), and 1736 and 1236 cm^{-1} (acetyl group). The ^1H NMR spectrum (Table 3) of compound **4a** displayed the presence of six olefinic protons at δ 4.90 (1H, d, J = 9.8 Hz, H-5), 5.40 (1H, m, H-9), 4.87 (1H, d, J = 9.6 Hz, H-5′), 5.40 (1H, m, H-9′), 6.24 (1H, d, J = 2.9 Hz, H-13′a), and 5.70 (1H, d, J = 2.9 Hz, H-13′b), five oxygenated methines at δ 5.37 (1H, t, J = 5.1 Hz, H-1), 5.40 (1H, m, H-6), 4.73 (1H, t, J = 9.8 Hz, H-8), 5.44 (1H, t, J = 5.2 Hz, H-6′), and 4.66 (1H, t, J = 9.4 Hz, H-8′), and four vinyl methyls at δ 1.80 (3H, d, J = 1.2 Hz, H-14), 1.88 (3H, s, H-15), 1.76 (3H, d, J = 1.2 Hz, H-14′), and 1.92 (3H, s, H-15′), as well as other signals belonging to additional methylene and methine protons. The ^{13}C NMR spectrum (Table 3) exhibited characteristic signals at δ 68.7 (C-1), 138.1 (C-4), 125.9 (C-5), 128.9 (C-9), 137.3 (C-10), 34.3 (C-1′), 137.5 (C-4′), 126.3 (C-5′), 126.5 (C-9′), and 143.8 (C-10′) and suggested the presence of two germacranolide sesquiterpene monomeric units in **4a**.

The $^1H-^1H$ COSY spectrum of **4a** showed correlations from H-1 to H-2, H-5 to H-6, H-7 to H-8 and H-11, H-1′ to

Table 3. NMR Data for Compound **4a** (75 and 400 MHz, $CDCl_3$)[a,b]

position	δ_C	δ_H	HMBC C−H
1	68.7	5.37 t (β; 5.12)	Me-14
2	24.4	1.90 m 1.82 m	H_2-3
3	37.0	2.15 m 1.92 m	H-5, Me-15
4	138.1		Me-15, H_2-3
5	125.9	4.90 d (9.76)	Me-15
6	74.5	5.40 m (β)	H-8, H-11
7	50.9	2.30 m (α)	H-11, H-9, H-8
8	74.6	4.73 t (β; 9.81)	H-11, H-6
9	128.9	5.40 m	H-7, Me-14
10	137.3		Me-14, H-8
11	44.2	2.58 q (β; 4.82)	H-6, H-1′, H_2-13
12	176.8		H-11, H-1′
13	33.1	1.75 m	H-11, H-1′
14	17.3	1.80 d (1.20)	H-1, H-9
15	15.5	1.88 s	H-5
1′	34.3	2.86 m (β)	H-11, H_2-3, Me-14′
2′	25.8	1.70 m 1.40 m	
3′	34.9	2.20 m 1.90 m	Me-15′, H-6′
4′	137.5		Me-15′, H-6′
5′	126.3	4.87 d (9.60)	Me-15′, H-7′
6′	73.3	5.44 t (β; 5.17)	H-8′
7′	49.5	2.98 dd (α; 6.0, 9.34)	H_2-13′, H-8′
8′	74.9	4.66 t (β; 9.38)	
9′	126.5	5.40 m	H-8′
10′	143.8		H-8′, Me-14′
11′	137.3		H_2-13′, H-6′
12′	169.9		H_2-13′
13′	122.1	6.24 d (2.90) 5.70 d (2.90)	
14′	18.0	1.76 d (1.20)	H-1′, H-9′
15′	15.4	1.92 s	H-5′
OAc	169.9	2.00, 2.04, 2.08	

[a] Assignments confirmed by decoupling, H/H COSY, HMQC, and ROESY spectra. [b] J values are given in Hz in parentheses.

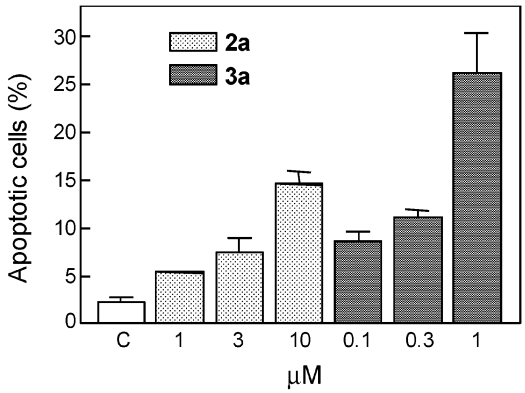

Figure 4. Quantitative analysis of apoptotic HL-60 cells after treatment with the sesquiterpene lactones **2a** and **3a**. Cells were incubated with the indicated doses for 12 h, and the apoptotic morphology was determined by fluorescence microscopy after staining with bisbenzimide trihydrochloride as described in the Experimental Section. The results of a representative experiment are shown, and each point represents the average ± SE of triplicate determinations.

H-13 and H-2′, H-5′ to H-6′, H-7′ to H-8′ and H-6′, and H-8′ to H-9′. Combined with the observed HMBC correlations (Table 3), the structure of the units of the molecule **4a** were found to be the same as those of acetyltatridin A.[8] The positions of linkage of the two units of tatridin A were established according to the following key correlations. In the $^1H-^1H$ COSY spectrum, H-13 correlated to H-11 and H-1′; in the HMBC spectrum (Table 3), C-1′ was correlated to H-11 and H-13. Thus, the two sesquiterpene units were connected between C-13 and C-1′. In the ROESY spectrum of **4a**, H-8 was correlated to H-14 and H-1β (δ 5.37, t, J = 5.1 Hz), H-5 correlated to H-6β (δ 5.4, m), H-1′ correlated to H-8′ (δ 4.66, t, J = 9.3 Hz), H-9′ correlated to H-7′ (δ 2.98, dd, J = 6.0 and 9.3 Hz), and H-7′ correlated to H-5′ (δ 4.87, d, J = 9.6 Hz). The relative configuration of **4a** was determined as shown according to these ROESY correlations as well as from the coupling constants and chemical shifts observed. Thus, it was concluded that **4** is a germacrane-type sesquiterpene dimer, and it was named elegain.

Compounds **2a** and **3a** were found to inhibit the proliferation of HL-60 cells in a dose-dependent manner, as determined by the MTT assay. The results showed that the decreasing order of potency was **3a** > **2a** > **2**. Compound **3a** (IC_{50} = 0.25 ± 0.0 μM) was at least 1 order of magnitude more potent than **2a** (IC_{50} = 5.3 ± 0.1 μM) and at least 2 orders of magnitude more potent than **2** (IC_{50} = 55.1 ± 7.5 μM).

To determine whether the two more active compounds, **2a** and **3a**, induced apoptosis, HL-60 cells were incubated with increasing concentrations of these agents for up to 12 h. This treatment resulted in the appearance of typical morphological changes that included chromatin condensation, compaction along the periphery of the nucleus, and nuclear segmentation into three or more chromatin fragments, as visualized by fluorescence microscopy. As shown in Figure 4, compounds **2a** (1−10 μM) and **3a** (0.1−1 μM) were able to induce apoptosis in a dose-dependent manner. At the higher concentrations used (10 μM for **2a** and 1 μM for **3a**) the percentages of apoptotic cells were 14.3 ± 1.3% and 25.9 ± 5.8%, respectively, and were greater than controls (2.2 ± 0.6%). Figure 5 shows the morphological changes associated with the cells in response to different doses of compound **3a**, the more potent apoptotic inducer. These sesquiterpene lactones also induced poly(ADP-ribose) polymerase (PARP) cleavage, a hallmark of apoptosis that

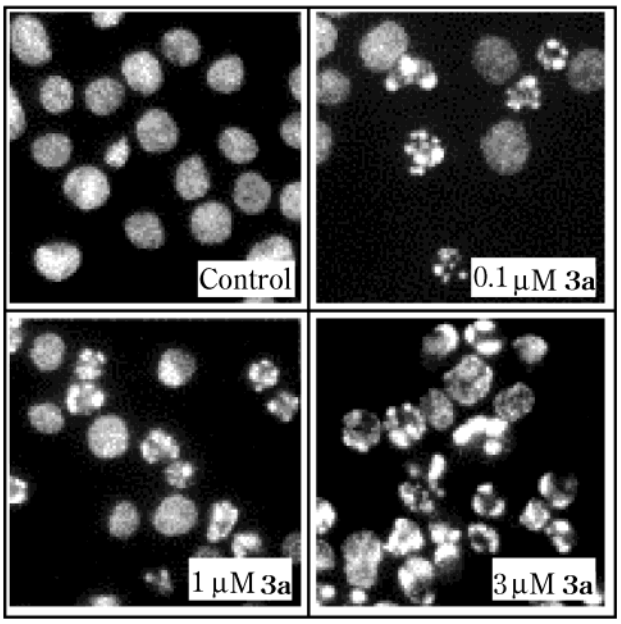

Figure 5. Photomicrographs of representative fields of HL-60 cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation (i.e., apoptosis) after treatment with no drug (control) or the indicated concentrations of **3a** for 12 h. Nuclei were visualized using a fluorescent microscope.

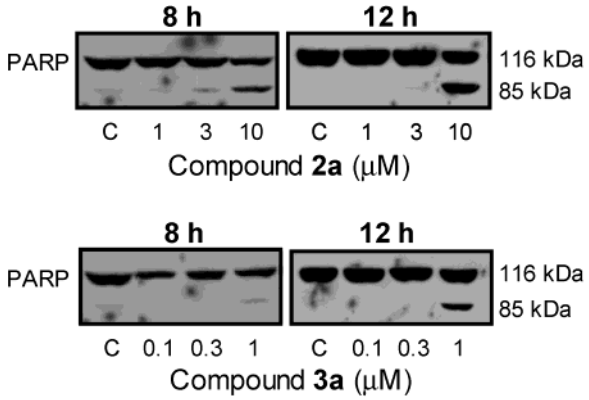

Figure 6. Western blot analysis of poly(ADP-ribose) polymerase (PARP) cleavage in HL-60 cells. Cells were incubated in the absence (C, control) or presence of the indicated concentrations of sesquiterpene lactones **2a** (upper figure) or **3a** (lower figure) for 8 and 12 h. Cell lysates were subjected to SDS-PAGE, and the PARP level was determined by immunoblotting with a monoclonal antibody that also recognizes the 85 kDa fragment.

indicates activation of caspase. PARP 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 inactivates the enzyme, thereby making DNA repair impossible. As expected, two fragments corresponding to the remaining intact PARP protein (116 kDa) and the typical apoptotic 85 kDa fragments were visualized (Figure 6). We also examined whether these sesquiterpene lactones induced fragmentation of DNA, which is considered the end point of the apoptotic pathway. DNA fragments formed by intranucleosomal hydrolysis of chromatin were evident after 12 h of treatment with 10 μM of **2a** or with 1 μM of **3a** (data not shown).

Accordingly, it has been found that compounds **2a** and **3a** inhibited the growth of HL-60 promyelocytic leukemia cells in culture and that the mechanism involved is apoptosis activation. Treatment of HL-60 cells with compounds **2a** and **3a** induced morphological changes and

internucleosomal DNA fragmentation characteristic of apoptotic cell death and was also associated with PARP cleavage.

Experimental Section

General Experimental Procedures. Melting points were determined on a Gallenkamp 4A 0865 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-400 spectrometer with standard pulse sequences, operating at 400 MHz for ^{1}H and 75 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. Medium-pressure column chromatography (MPLC) was carried out with Si gel Merck (40−63 μm), and column fractions were monitored by TLC (Si gel 60 F_{254}). Preparative TLC was carried out on Si gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness).

Plant Material. *Gonospermum elegans* was collected along the road running from El Pinar to La Restinga (El Hierro, Canary Islands) in May 1996. A voucher specimen has been deposited at the Herbarium of the Viera y Clavijo Botanical Garden in Gran Canaria. (no. 19420).

Extraction and Isolation. The aerial parts (783 g) were extracted with ethanol according to a previously described method.[20] The extracts were combined and the solvent removed in vacuo to yield 261 g of a gummy residue, which was fractionated by column chromatography on silica gel using mixtures of hexane and EtOAc of increasing polarity for elution. From the fractions eluted with hexane−EtOAc (7:3), spiciformin (124 mg) was isolated. The fractions eluted with hexane−EtOAc (6:4) were chromatographed by MPLC on Si gel with benzene−EtOAc (4:1), yielding reynosin (81 mg) and 4α,5β-epoxydesacetyllaurenobiolide (170 mg). The fractions eluted with hexane−EtOAc (1:1) afforded, after chromatography by MPLC with benzene−EtOAc (7:3), scopoletin (176 mg), axillarin (89 mg), and a mixture of substances (800 mg). This mixture was separated after acetylation (Ac_2O, 0.5 mL and C_5H_5N, 0.5 mL, room temperature for 12 h) by MPLC with hexane−EtOAc (9:1), yielding the acetyl derivative of eleganodiol (**1a**, 32 mg). Further elution with hexane−EtOAc (8:2) gave deacetyl-β-cyclopyrethrosin (71 mg) and tatridin A (619 mg). The fractions that eluted with hexane−EtOAc (4:6) were further chromatographed with hexane−EtOAc (7:3) to give by preparative TLC the following substances: tamirin (66 mg) and tatridin B (1.06 g) (benzene−EtOAc, 1:1), 1α-hydroxy-deacetyltulirinol-4α,5β-epoxide (19 mg) (hexane−EtOAc, 4:6), 1-*epi*-tatridin B (56 mg) (benzene−acetonitrile, 4:6), eleganolactone A (**2**, 36 mg) (benzene−EtOAc, 3:1), and artemorin (11 mg) (hexane−EtOAc, 1:1). Finally, a complex mixture of substances was obtained (250 mg) which, after acetylation (Ac_2O, 0.3 mL and C_5H_5N, 0.3 mL, room temperature for 12 h), was chromatographed by MPLC on Si gel with hexane−EtOAc (7:3), yielding acetyl derivatives of β-sitosterol-β-D-glucoside (15 mg), 11,13-dihydrotatridin B (37 mg), 1α-hydroxy-1-desoxotamirin-4α,5β-epoxide (21 mg), sivasinolide (35 mg), eleganolactone B (**3a**, 87 mg), and, after purification by TLC with benzene−acetonitrile (3:1), elegain (**4a**, 64 mg).

Diacetyleleganodiol (1a): oil; $[\alpha]^{29}_D$ +9° (*c* 0.005, $CHCl_3$); IR (film) ν_{max} 2918, 1737, 1648, 1344, 1237, 1018, 898 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m*/*z* 320 (2), 278 (10), 262 (17), 260 (15), 218 (41), 202 (52), 200 (100), 190 (13), 185 (54), 172 (40), 159 (52), 157 (55), 149 (60), 145 (45), 134 (60), 131 (68), 119 (69), 105 (65), 93 (60), 79 (50); HREIMS *m*/*z* 320.1969 $[M]^+$ (calcd for $C_{19}H_{28}O_4$, 320.1987).

Eleganolactone A (2): oil; IR (film) ν_{max} 3467, 2925, 1733, 1630, 1456, 1261, 1136, 1041, 982 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.17 (1H, d, J = 3 Hz, H-13a), 6.00 (1H, d, J = 3 Hz, H-13b), 5.08 (1H, s, H-15a), 4.92 (1H, s, H-15b), 4.08 (1H, ddd, J = 12, 12, 3.5 Hz, H-8β), 3.85 (1H, dd, J = 10, 10 Hz, H-6β), 3.78 (1H, dd, J = 11.5, 5.0 Hz, H-1β), 2.53 (1H, m, H-7α), 2.02 (1H, d, J = 10 Hz, H-5α), 1.04 (3H, s, CH_3-14); EIMS (70 eV) *m*/*z* 264 (18), 246 (11), 228 (12), 220 (21), 150 (20), 133 (20), 121 (49), 107 (100), 96 (77), 81 (49), 69 (95), 55 (37).

Acetylation of **2** (25 mg) in Ac_2O−pyridine for 24 h at room temperature and workup in the usual manner gave **2a** (18 mg) as a colorless gum, $[\alpha]^{29}_D$ −51° (*c* 0.13, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 282 (2.3), 218 nm (3.6); IR (film) ν_{max} 2924, 1778, 1738, 1651, 1371, 1234, 1131, 1031, 994, 904, 757 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m*/*z* 348 (7), 288 (91), 246 (25), 228 (99), 213 (27), 185 (10), 167 (17), 157 (18), 149 (50), 143 (18), 133 (35), 121 (82), 119 (43), 111 (20), 108 (100), 95 (24); HREIMS *m*/*z* 348.1590 $[M]^+$ (calcd for $C_{19}H_{24}O_6$, 348.1572).

Diacetyleleganolactone B (3a): colorless solid (hexane−EtOAc) mp 218−220 °C; $[\alpha]^{29}_D$ +25° (*c* 0.03, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 319 (3.2), 281 (3.5), 219 (4.0) nm; IR (film) ν_{max} 1760, 1746, 1734, 1681, 1374, 1241, 1142, 995, 968 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR, see Table 2; EIMS (70 eV) *m*/*z* 362 (21), 320 (64), 303 (16), 302 (35), 261 (19), 260 (100), 242 (49), 232 (27), 231 (34), 214 (47), 185 (26), 175 (23), 164 (48), 149 (18), 147 (42), 136 (24), 123 (20), 121 (24), 119 (28), 111 (26), 107 (31), 96 (34); HREIMS *m*/*z* 362.1394 $[M]^+$ (calcd for $C_{19}H_{22}O_7$, 362.1365).

Triacetylelegain (4a): oil; $[\alpha]^{25}_D$ −15° (*c* 0.02, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 205 (1.4); IR ν_{max} (film) 2925, 1767, 1736, 1667, 1372, 1236, 1137, 1019, 990, 956 cm^{-1}; ^{1}H and ^{13}C NMR, see Table 3; EIMS (70 eV) *m*/*z* 638 (2), 578 (10), 518 (15), 476 (10), 458 (20), 243 (11), 216 (19), 199 (52), 183 (16), 181 (46), 175 (20), 157 (48), 119 (62), 105 (60), 91 (100), 57 (91), 55 (77); HREIMS *m*/*z* $[M]^+$ 638.3123 (calcd for $C_{36}H_{46}O_{10}$, 638.3091).

X-ray Crystal Structure Determination of 3a.[21] A suitable crystal for an X-ray experiment was mounted in an Enraf-Nonius MACH-S3 diffractometer with monochromatic Mo Kα radiation (λ = 0.71073 Å). Accurate cell parameters were determined by refinement from the setting of 16 reflections and diffraction intensities measured at 293 K using the ω-scanning method. Crystal size 0.08 × 0.04 × 0.01 mm; colorless plate; orthorhombic system; space group $P2_12_12_1$ with unit cell parameters a = 5.612(2) Å, b = 13.574(3) Å, c = 24.628(4) Å, V = 1876.1(8) Å^3, Z = 4, $F(000)$ = 768, D_{cal} = 1.283 g/cm^3; μ/mm^{-1} = 0.098. The intensities of three standard reflections, recorded every 120 min, showed a decay of 2%. All data sets were collected for Lorentz−polarization effects, but no absorption corrections were applied. The structure was determined by direct methods (SIR97)[22] and refined by full-matrix least-squares methods using SHELX97.[23] Reflections and Friedel opposites were combined and merged before refinement. All H atoms were placed in geometrically calculated positions. 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 rest of the atoms were refined with anisotropic thermal parameters.

Cell Culture. The human promyelocytic leukemia HL-60 cell line established by Gallagher et al.[24] was used in this study. Cells were cultured in suspension in RPMI-1640 medium (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum, penicillin (10 000 units mL^{-1}), and streptomycin (10 000 μg mL^{-1}) in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. Cells were maintained at a density of <1 × 10^6 cells/mL. Sesquiterpene lactones were dissolved in dimethyl sulfoxide (DMSO) and stored as stock solutions at −20 °C. Further dilutions were made in culture medium immediately prior to 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.

Assay for Growth Inhibition and Cell Viability. Cytotoxic assays were performed using an MTT assay.[25] Cells (1 × 10^4/well) were continuously exposed to different concentrations of the compounds in 96-well plates for 24 h at 37 °C. Sesquiterpene lactones were applied in DMSO. Controls were always treated with the same amount of DMSO as used in

the corresponding experiments. Surviving cells were detected on the basis of their ability to metabolize 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) into formazan crystals. Optical density at 570 nm was used as a measure of cell viability. 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 using the curve-fitting routine of the computer software Prism 2.0 (GraphPad) and the equation derived by De Lean and coworkers.[26]

Quantitative Fluorescence Microscopy. For detection of apoptotic cells, cells were fixed in 3% paraformaldehyde for 10 min at room temperature and then stained with 10 μg/mL bisbenzimide trihydrochloride (Hoechst 33258) for 30 min at 37 °C before fluorescence microscopic analysis. Apoptotic cells were identified by their fragmented chromatin.[27] Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery) or nuclei that were fragmented into multiple smaller dense bodies were considered to be apoptotic. Nuclei with uncondensed and dispersed chromatin were considered not to be 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.[28] Briefly, cells (2×10^6) were washed with phosphate buffer saline and incubated in 100 μL of hypotonic detergent buffer (10 mM Tris-HCl, 1 mM EDTA, 0.2% Triton X-100, pH 7.5) for 30 min at 4 °C. The supernatants obtained after centrifugation were sequentially incubated with 40 μg of DNase-free RNase and 40 μg of proteinase K at 37 °C for 30 min. The samples were mixed with 100 μL of phenol, and the aqueous phase, containing the fragmented DNA, was incubated with 250 μL of ethanol in the presence of 5 μg of t-RNA for 24 h at −20 °C. After the centrifugation the DNA precipitate was resuspended in 20 μL of 10 mM Tris-HCl (pH 7.5) and aliquots (10 μL) were subjected to electrophoresis at 40 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 (0.5 μg/mL), and the images were captured by a digital camera (Kodak).

Western Blot Analysis of PARP Hydrolysis. Induction of apoptosis was also examined by proteolytic cleavage of PARP.[29] Exponentially growing HL-60 cells ($\sim 7 \times 10^5$) were treated with sesquiterpene lactones at the indicated concentrations for different time periods at 37 °C. Cell lysates were prepared in a buffer containing 20 mM Hepes (pH 7.4), 250 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, aprotinin, and pepstatin A. Insoluble materials were removed by centrifugation at 10000*g* for 10 min. Extracted proteins (50 μg/well) were separated by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5% polyacrylamide gels and were electrophoretically transferred onto Immobilon-P membrane. The loading and transfer of equal amounts of protein was confirmed by staining the membrane with Ponceau S. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h, followed by incubation with anti-PARP monoclonal antibody (BD PharMingen; 1:1000 dilution in TBST supplemented with 3% nonfat milk) overnight. After washing and incubation with anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Amersham Pharmacia Biotech), the antigen−antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) using the manufacturer's protocol. The appearance of an 85 kDa cleavage product was used as a measure of apoptosis.

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