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Sesquiterpene Lactones from *Gonospermum gomerae* and *G. fruticosum* and Their Cytotoxic Activities

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Four new sesquiterpene lactones (**1**−**4**) and a new sesquiterpene (**5**) together with 20 known compounds were isolated from two *Gonospermum* species (*G. gomerae* Bolle and *G. fruticosum* Less). Their structures were determined by analysis of spectroscopic data, including 1D and 2D NMR. The cytotoxicity of several new and known natural and semisynthetic sesquiterpene lactones was also assessed against human myeloid leukemia cell lines (HL-60 and U937), human melanoma cells (SK-MEL-1), and human adenocarcinoma (A549).

The genus *Gonospermum*, which belongs to the subtribe Gonosperminae (Anthemideae, family Asteraceae) and is endemic to the Canary Islands, contains four species distributed throughout the most westerly islands. This genus has been the subject of previous chemical research in which the presence of sesquiterpenes and sesquiterpene lactones was described.[1−3]

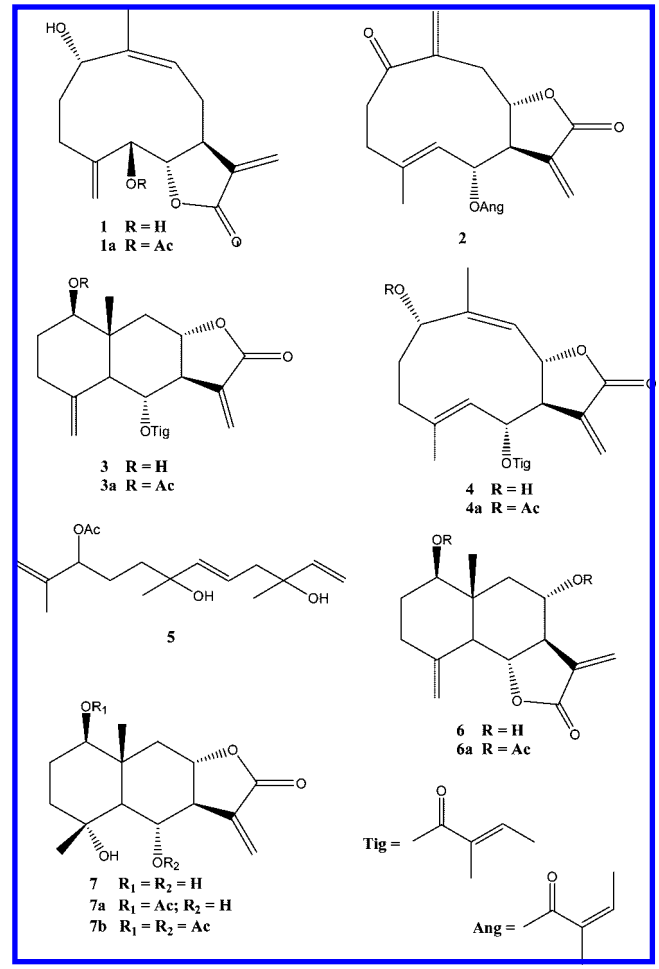
The present work describes the isolation and structural elucidation of four new sesquiterpene lactones, two from *G. gomerae* (**1** and **2**) and the other ones from *G. fruticosum* (**3** and **4**), as well as a new sesquiterpene (**5**) along with 20 known compounds (**6**−**25**). The structures of the known compounds were confirmed by comparison of their spectroscopic properties with data published in the literature.

Compounds containinig the α-methylene-γ-lactone functional group, such as sesquiterpene lactones, have attracted much attention during the last 50 years because they display a wide range of biological activities, including antitumor properties.[4] For this reason, the sesquiterpene lactones **1a**, **6a**, **7a**, and **7b** were assessed for cytotoxicity against several human tumor cell lines and for apoptosis induction in human myeloid leukemia cells.

Results and Discussion

Purification of an ethanolic extract of aerial parts of *G. gomerae* gave two new sesquiterpene lactones with a germacrane skeleton (**1** and **2**), as well as 18 known compounds, dentatin A (**6**),[5] 1β,4α,6α-trihydroxyeudesm-11-en-8α,12-olide (**7**),[6] 1α-hydroxydeacetyltulirinol-4α,5β-epoxide (**8**),[7] tatridin A (**9**),[8] tatridin B (**10**),[8] tamirin (**11**),[9] β-cyclopyrethrosin (**12**),[10] desacetyl-β-cyclopyrethrosin (**13**),[11] desacetyl-β-cyclopyrethrosin 6-*O*-angelate (**14**),[7] spiciformin (**15**),[12] 5β-hydroxycostic acid (**16**),[13] crocinervolide (**17**),[14] stigmasterol (**18**),[15] scoparone (**19**),[16] scopoletin (**20**),[2] sesamin (**21**),[17] axillarin (**22**),[18] and 5,7,4′-trihydroxy-3,6-dimethoxyflavone (**23**).[19]

An ethanolic extract of *G. fruticosum* yielded two new sesquiterpene lactones (**3** and **4**), one new sesquiterpene (**5**), and the known compounds **8**−**11**, **13**, **17**, **19**, **20**, **23**, desacetyl-β-cyclopyrethrosin 6-*O*-angelate acetate (**24**),[20] and 6-hydroxy-6-methylocta-3,7-dien-2-one (**25**)[21] (see Supporting Information for structures of known compounds).



Compound **1** was acetylated for the purpose of purification, affording the acetyl derivative **1a** as colorless oil. Its IR spectrum showed the presence of γ-lactone (1770 cm^{-1}), carbonyl ester (1742 cm^{-1}), and double-bond (1648 and 967 cm^{-1}) absorptions. Its

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Table 1. ^{1}H NMR Data of Compounds **1a**, **2**, **3**, **4a**, and **5** (300 MHz in $CDCl_3$)[a]

H	1a	2	3	4a	5
1	5.86 dd (4.0, 11.6)		3.59 dd (4.6, 11.3)	5.48 dd (4.2, 10.0)	5.05 d (10.7); 5.20 *d* (17.2)
2α	1.70 m	3.26 m	1.91 m		5.93 dd (10.7, 17.0)
2*β*	2.23 m		1.60 m		
3α	2.59 m	2.30−2.60 m	2.07 m		
3*β*	1.67 m		2.31 m		
4					2.28 ddd (5.5, 13.3, 19)
5	4.85 d (9.3)	4.99 d (5.9)	2.24 d (10.5)	4.90 d (11.3)	5.61 m
6	5.07 dd (9.3, 10.5)	5.21 dd (10.0, 10.0)	5.58 dd (10.5, 10.5)	5.48 dd (9.7, 10.5)	5.59 s
7	2.92 d (10.6)	2.99 m	2.79 m	2.78 m	
8	5.34 d (10.7)	4.05 m	4.08 ddd (3.5, 10.5, 10.5)	4.80 t (9.2)	1.49 m
9α	5.28 d (11.4)	3.44 d (11.0)	2.58 dd (3.5,12.0)	5.39 d (9.7)	1.65 m
9*β*		2.16 m	1.60 m		
10					5.14 t (6.6)
12a					4.88 s
12b					4.93 s
13a	5.72 s	5.80 d (2.4)	5.39 d (3.0)	5.71 d (3.3)	1.70 s
13b	6.37 s	6.34 d (2.7)	6.11 d (3.0)	6.24 d (2.9)	
14a	1.76 s	5.81 s	0.87 s	1.82 s	1.27 s
14b		5.85 d (2.4)			
15a	5.61 d (1.8)	1.79 s	4.63 d (4.5)	1.95 s	1.27 s
15b	5.63 d (1.8)		4.86 d (4.5)		
OAc	2.00 s			2.01 s	2.05
	2.03 s				
	2.08 s				
OAng		6.13 m			
		1.98 d (7.1)			
		1.91 s			
OTigl			6.84 m	6.97 m	
			1.81 s	1.83 s	
			1.88 s	1.88 s	

[a] Spectra were run at room temperature, and TMS was used as internal standard. Chemical shifts are recorded in ppm to TMS. Coupling constants (*J*) are given in parentheses.

molecular formula was established as $C_{21}H_{26}O_8$ from the EIMS at *m/z* 364.15 $[M - C_2H_2O]^+$ and the ^{1}H, ^{13}C, and DEPT NMR data. The ^{1}H NMR spectrum of **1a** (Table 1) exhibited three acetyl methyl singlets (δ_H 2.00, 2.03, and 2.08), a vinyl methyl singlet (δ_H 1.76), and signals consistent with two exocyclic double bonds [δ_H 5.61 (1H, d, J = 1.8 Hz, H-15a), 5.63 (1H, d, J = 1.8 Hz, H-15b), 5.72 (1H, s, H-13a), and 6.37 (1H, s, H-13b)]. The ^{13}C NMR and DEPT data indicated the presence of one carbonyl group corresponding to a γ-lactone, three ester carbonyl groups, six olefinic carbons, and five methine carbons. The relationships between the proton signals in **1a** were established from its ^{1}H−^{1}H COSY spectrum, which disclosed the following connectivities: H-1/H-2, H-5/H-6, H-6/H-7, H-7/H-8, and H-8/H-9. The above data, together with the HMBC experiment, placed the acetate groups at C-1, C-5, and C-8 and the lactone group at C-6. The relative configuration of **1a** was confirmed by a ROESY experiment (see Supporting Information) in which correlations observed between H-1 and H-8 and between H-6 and H-8 clearly showed that these protons were on the same face. Correlations were also observed between H-5 and H-7 and between Me-14 and H-9. Thus, the structure and relative configuration of **1** was proposed as 1α,5*β*,8α-trihydroxygermacra-4(15),9(*Z*),11(13)-trien-6α,12-olide.

Compound **2** showed IR absorption bands due to the carbonyl of a γ-lactone (1769 cm^{-1}) and olefinic bonds (1660 and 961 cm^{-1}). The EIMS did not show the molecular ion, but a fragment was observed at *m/z* 244 $[M - HOAng]^+$, corresponding to the molecular formula $C_{20}H_{24}O_5$. The ^{1}H NMR spectrum of compound **2** (Table 1) exhibited six olefinic proton signals at δ_H 4.99 (1H, d, J = 5.9 Hz, H-5), 5.80 (1H, d, J = 2.4 Hz, H-13a), 5.81 (1H, brs, H-14a), 5.85 (1H, d, J = 2.4 Hz, H-14b), 6.13 (1H, m, H-Ang), and 6.34 (1H, d, J = 2.7 Hz, H-13b). These assignments were similar to those of the known germacranolide tamirin,[9] while the low-field resonance of the C-6 proton (δ_H 5.21) located the angeloxy group at this carbon. Thus, **2** was deduced to be tamirin 6-*O*-angelate.

The IR spectrum of compound **3** showed absorption bands due to an OH group (3458 cm^{-1}) and two carbonyl bands from a γ-lactone (1770 cm^{-1}) and from an ester (1715 cm^{-1}). The EIMS showed a molecular ion peak at *m/z* 346 in agreement with the empirical formula $C_{20}H_{26}O_5$. The ^{1}H NMR spectrum of **3** (Table 1) exhibited five olefinic proton signals at δ_H 4.63 (1H, d, J = 4.5 Hz, H-15a), 4.86 (1H, d, J = 4.5 Hz, H-15b), 5.39 (1H, d, J = 3.0 Hz, H-13a), 6.11 (1H, d, J = 3.8 Hz, H-13b), and 6.84 (1H, m, tiglate). These assignments were similar to those of deacetyl-*β*-cyclopyrethrosin[11] with the exception of the signal of H-6 at δ_H 5.58 (1H, dd, J = 10.5, 10.5 Hz, H-6), which allowed assignment of the tigloyloxy group to C-6. Treatment of **3** with Ac_2O and pyridine afforded the acetyl derivative **3a**. Its NMR spectrum showed two doublets at δ_H 6.12 (J = 2.7 Hz) and 5.38 (J = 2.7 Hz) (methylene lactone), a multiplet at δ_H 6.84 and two singlets at δ_H 1.88 and 1.81 characteristic of a tiglate group, two protons geminal to tiglate, and acetate groups were assigned to the signals at δ_H 5.60 (m) and 4.83 (dd, J = 4.6 and 4.7 Hz), respectively. In addition, a singlet at δ_H 2.06 confirmed the presence of the acetyl methyl group. An exocyclic methylene group was indicated by doublets at δ_H 4.88 (J = 5.0 Hz) and 4.65 (J = 5.9 Hz) and assigned to C-15. Thus, **3** was deduced to be 6α-tigloyloxy-1*β*-hydroxy-4(15),11-eudesmadien-8α,12-olide.

Compound **4** was purified by acetylation, affording compound **4a**. Its IR and ^{1}H NMR spectra were quite similar to those of tatridin A,[8] indicating the presence of α-methylene-γ-lactone (1754 cm^{-1}), ester (1735 cm^{-1}), and olefinic groups (1139, 999, and 960 cm^{-1}). Prominent fragments at *m/z* 328 $[M - CH_3COOH]^+$ and 228 $[M - CH_3COOH - C_5H_8O_2]^+$ in the MS, in addition to a molecular ion at *m/z* 388, were consistent with the molecular formula $C_{22}H_{28}O_6$ and indicated that **4a** was a sesquiterpene lactone bearing an acetate and a tiglate group. The NMR spectrum of **4a** (Table 1) exhibited four olefinic proton signals at δ_H 4.90 (d, J = 11.3 Hz, H-5), 5.39 (d, J = 9.7 Hz, H-9), 5.71 (d, J = 3.3 Hz, H-13a), and 6.24 (d, J = 2.9 Hz, H-13b). Four vinylic methyl singlets (δ_H 1.82, 1.83, 1.88, and 1.95) and an acetyl methyl singlet (δ_H 2.01) were also observed. These assignments were similar to those of tatridin A acetate angelate[22] with the exception of H-3′ (m, δ_H 6.97) and Me-4′ (s,

Table 2. Effects of Lactones Isolated from *Gonospermum* on the Growth of Human Tumor Cell Lines[a]

	IC_{50} (μM)			
lactone	HL-60	U937	SK-MEL-1	A549
1a	16.0 ± 3.6	10.0 ± 0.3	19.5 ± 3.7	56.6 ± 15.1
6a	9.4 ± 0.7	9.2 ± 1.1	14.3 ± 3.2	72.4 ± 13.7
7a	>100	>100	>100	>100
7b	13.3 ± 2.5	10.4 ± 1.0	27.9 ± 4.1	>100
etoposide	0.25 ± 0.04	0.92 ± 0.24	>30	5.9 ± 1.7

[a] Cells were cultured for 72 h, and the IC_{50} values were calculated as described in the Experimental Section. The data shown represent the mean ± SEM of three independent experiments with three determinations in each.

δ_H 1.83). Thus, **4** was deduced to be 6α-tigloyloxy-1α-hydroxygermacra-4*E*,9*Z*,11-trien-8α,12-olide.

Compound **5** was assigned the molecular formula $C_{17}H_{26}O_3$ on the basis of the mass fragment $[M - H_2O]^+$ at *m/z* 278 in the EIMS. The IR spectrum showed OH (3429 cm^{-1}), acetoxy group (1738 cm^{-1}), and double-bond (1651 cm^{-1}) absorptions. The ^1H NMR spectrum (Table 1) showed seven olefinic protons [δ_H 4.88 (1H, s, H-12a), 4.93 (1H, s, H-12b), 5.05 (1H, d, J = 10.7 Hz, H-1a), 5.20 (1H, d, J = 17.2 Hz, H-1b), 5.59 (1H, m, H-6), 5.61 (1H, m, H-5), and 5.93 (1H, dd, J = 17.0 and 10.7 Hz, H-2)], an oxygenated methine [δ_H 5.14 (1H, t, J = 6.6 Hz, H-10)], and four methyl signals [δ_H 1.27 (6H, s, CH_3-14 and CH_3-15), 1.70 (3H, s, CH_3-13), and 2.05 (3H, s, OAc)]. Considering the above data, **5** was an acyclic geranylgeraniol-derived sesquiterpene with a structure similar to that of crocinervolide.[23] These observations together with the $^1H-^1H$ COSY and HMBC correlations established the structure of **5**, which was characterized as 10-acetoxy-3,7,11-trimethyldodeca-1,5,11-triene-3,7-diol.

It is widely believed that sesquiterpene lactones exert their biological effect by acting as alkylating agents. These compounds can form adducts *in vivo* with proteins and other nucleophilic biomolecules, via a Michael-type addition of a free sulfhydryl or amine group. Sesquiterpene lactones can overcome the cells' protection by high intracellular glutathione concentrations to alkylate protein molecules, leading to their numerous biological effects. Although under intracellular conditions (i.e., at neutral pH and high glutathione concentration) sesquiterpene lactones are largely transformed into the glutathione adducts, their inhibitory activity can be explained by instability of the thioether groups at physiological pH. Previous studies have demonstrated how sesquiterpene lactones are able to exert their biological activity in spite of the presence of glutathione, and this can be explained by the reversibility of glutathione addition.[24]

We also were interested in determining whether these compounds display cytotoxic properties in a cell-type specific manner. For this reason, we examined the effects of several sesquiterpene lactones on the growth of four human tumor cell lines: human leukemia cells (HL-60 and U-937), A549 lung cancer cells, and human melanoma SK-MEL-1 cells. We used the leukemia cells because they are prone to apoptosis (especially HL-60). The cell line SK-MEL-1 was used since melanoma is the most aggressive form of skin cancer and frequently resists chemotherapy, and the A549 lung cancer cells were selected since they are apoptotic-reluctant. Initial studies showed that compounds **1a**, **6a**, and **7b** were relatively cytotoxic agents against human myeloid leukemia HL-60 and U937 cells. Interestingly, compounds **1a** and **6a** were equally potent against human SK-MEL-1 melanoma cells. In the present report we describe the effect of these compounds on the growth of human myeloid (HL-60 and U937) and melanoma (SK-MEL-1) cell lines (Table 2). Growth inhibition of human tumor cells in culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) dye-reduction assay. Etoposide was used as a positive control. Antiproliferative studies on compounds **1a** and **6a** indicated that they were cytotoxic against human myeloid (HL-60 and U937) and melanoma (SK-MEL-1) cell lines, with an IC_{50} about 10 μM. However, **7b** was less potent than **1a** and **6a** only against SK-MEL-1 cells. Human adenocarcinoma A549 cells were resistant to all lactones tested.

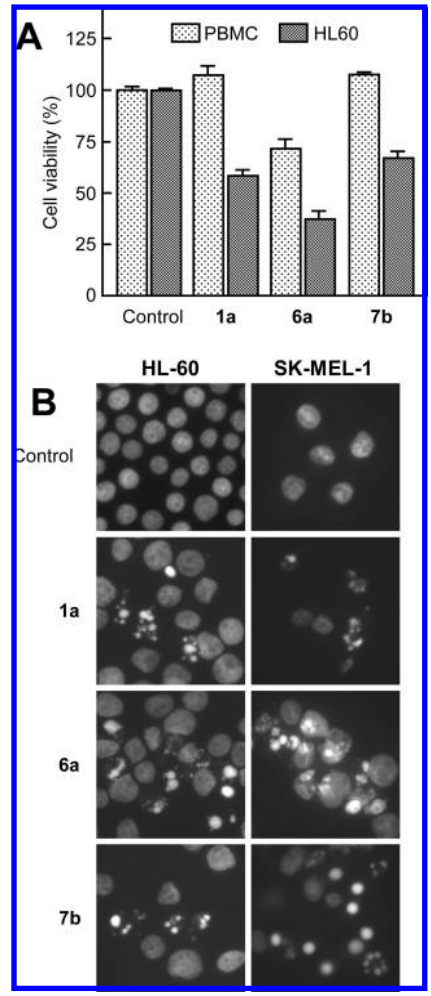

Figure 1. (A) Differential effect of compounds **1a**, **6a**, and **7b** on proliferation of normal peripheral blood mononuclear cells (PBMC) versus HL-60 cells. Proliferation of PBMC and HL-60 cells cultured in the presence of 30 μM of each compound for 24 h. Values represent means ± SE of three independent experiments, each performed in triplicate. (B) Photomicrographs of representative fields of HL-60 and SK-MEL-1 cells stained with bisbenzimide trihydrochloride to evaluate nuclear chromatin condensation (i.e., apoptosis) after treatment with compounds **1a**, **6a**, and **7b**.

Control experiments with normal lymphocytes showed no appreciable toxicity of compounds **1a** and **7b** and only weak toxicity with **6a** at 30 μM. As a positive control, HL-60 cells were also included in the experiment, and as expected, there was an important reduction in the proliferation of these cells (Figure 1A).

Inspection of Table 2 revealed that the growth of leukemia cells was, in general, highly susceptible to the cytotoxicity induced by sesquiterpene lactones. Therefore, further studies were performed on HL-60 cells. Only those compounds with low IC_{50} (≤15 μM)

Table 3. Effects of Sesquiterpene Lactones Isolated from *Gonospermum* on Cell Cycle Distribution of HL-60 Cells[a]

	% sub G_0/G_1	% G_0/G_1	%S	%G_2-M
control	2.9 ± 0.3	47.9 ± 0.6	21.4 ± 1.1	27.8 ± 0.8
1a	12.2 ± 2.8	35.6 ± 5.8	20.0 ± 1.0	29.6 ± 1.5
6a	30.0 ± 3.5	26.8 ± 6.7	22.0 ± 0.4	18.5 ± 2.6
7b	11.8 ± 1.9	39.3 ± 3.6	21.5 ± 0.7	21.8 ± 2.7
etoposide	47.7 ± 2.5	29.4 ± 1.5	17.1 ± 2.1	2.6 ± 0.9

[a] The cells were cultured with 30 μM of the indicated compounds for 48 h, and the cell cycle distribution was determined by flow cytometry. The results are expressed as mean ± SEM of two different experiments with two determinations in each.

were selected to explore the mechanism through which these selected sesquiterpenes lactones decrease cell viability.

We first analyzed whether cell growth inhibition induced by these compounds was mediated via alteration in cell cycle progression. Consistent with growth inhibitory effects, the cytometric flow studies (Table 3) revealed that compounds **6a** and **7b** induce significant S arrest at the expense of G1 phase cell population following treatment (over 24−48 h) with concentrations of 30 μM. Concentrations of 30 μM were used to demonstrate that the effects on cell cycle progression were dose dependent. Concentrations higher than the antiproliferative IC_{50} were used to identify the primary targets and early mechanism of action of sesquiterpene lactones. The IC_{50} values were determined at 72 h of treatment, and the flow cytometry experiments were analyzed after a short incubation time. However, the sesquiterpene lactone **1a** induced G_2-M arrest at the expense of G_1 phase cell number.

Taken together, the results indicated that compound **1a** versus **6a** and **7b** displayed antiproliferative activities through different mechanisms that involve cell cycle alteration. Selective modulation of different cell cycle-regulatory proteins could explain the differences among them, although other possibilities cannot be ruled out.

Although we did not identify the targets of these compounds, we showed that compounds **6a** and **7b** induce significant S arrest and that **1a** induced G2-M arrest. These results show that **6a** and **7b** have different targets than **1a**. The progression through the cell cycle is due to the timely regulated activation of serine/threonine cyclin-dependent kinases, the CDKs, a family of protein kinases that are in turn controlled by a complex array of proteins including the cyclins. These compounds could affect different sets of CDKs or CDK inhibitors. Further studies are needed to determine the effect of these compounds on specific regulators of each phase (S or G2-M) of the cell cycle.

To elucidate the possible mechanism(s) of action-mediated cell growth inhibition, we tested the effects of **1a**, **6a**, and **7b** to induce apoptosis using HL-60 cells. As shown in Figure 1B, compounds **1a**, **6a**, and **7b** induced morphological changes characteristic of apoptotic cells (fragmented and condensed chromatin), as visualized by fluorescence microscopy. In the left panel, the control HL-60 cells appeared normal, with the nuclei round and homogeneous, while cells exposed to compounds **1a**, **6a**, and **7b** displayed condensation of chromatin and the appearance of apoptotic bodies by fluorescence microscopy after DNA staining with Hoechst 33258. Similar results were obtained on SK-MEL-1 cells.

A biochemical hallmark of apoptosis is the fragmentation of genomic DNA into integer multiples of 180-bp units, resulting in a characteristic ladder on agarose gel electrophoresis. Therefore, we also examined whether these compounds induced chromosomal DNA fragmentation, which is considered the end point of the apoptotic pathway. The results demonstrated that exposure to 30 μM **1a**, **6a**, and **7b** resulted in endonucleolytic DNA cleavage, which then leads to DNA ladder formation in HL-60 cells (Figure 2A). Using QFM (quantitative fluorescence microscopy) the least potent compound was **7b**, while compound **6a** was the most potent (Figure 2B) at the times and concentrations assayed.

To confirm that the selected sesquiterpene lactones decrease HL-60 cell viability through apoptosis activation, quantification of the

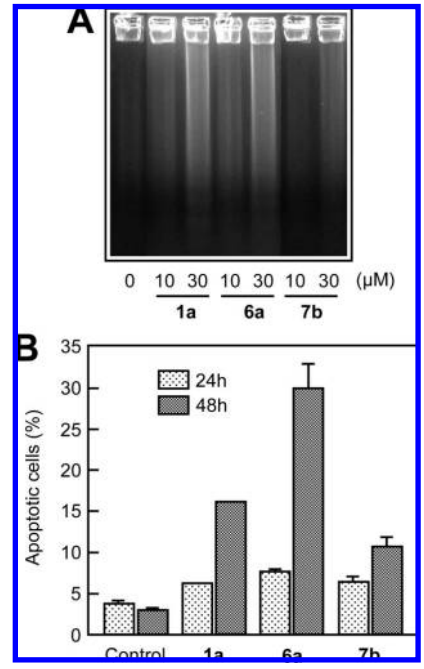

Figure 2. Induction of apoptosis in HL-60 cells by sesquiterpene lactones. (A) HL-60 cells were treated with the indicated compounds (30 μM) for 24 h, and total cellular DNA was isolated and stained with ethidium bromide after electrophoresis on a 2% agarose gel. Internucleosomal DNA fragmentation was visualized under UV light. (B) Cells were incubated with the indicated compounds (for 16 h), and the percentages of apoptotic cells were determined by flow cytometry. The results of a representative experiment are shown, and each point represents the average ± SE of triplicate determinations.

number of hypodiploid cells (i.e., apoptotic cells) by flow cytometry was performed. The results indicated that the percentage of apoptotic cells increased from 4 ± 1% (control) to 16 ± 0.5% (4-fold increase), 30 ± 0.5% (~8-fold increase), and 12 ± 0.5% (~3-fold) after 48 h of treatment with 30 μM **1a**, **6a**, and **7b**, respectively (Figure 2B). The other sesquiterpene lactones described in this paper did not induce apoptosis at either concentration tested (up 30 μM).

Next, we examined whether compounds **1a**, **6a**, and **7b** induce pro-caspase-3, -6, and -7 cleavage. Caspases have been shown to be key mediators of cell death in biological and biochemical analyses. Downstream effector caspases include caspases-3, -6, and -7. Since the proteolytic processing of caspases is an important event in caspase-dependent apoptotic cell death, we evaluated the effect of selected sesquiterpene lactones on executioner caspases by Western blot using specific antibodies. Cleavage of procaspases-3, -6, and -7 by sesquiterpene lactones was determined by immunoblotting using a polyclonal anti-human caspase-3 antibody (Stressgen) that recognized the M_r 32 000 proenzyme (procaspase-3), a monoclonal anti-human caspase-7 antibody (BD Pharmingen), and an anti-caspase-6 monoclonal antibody (MBL). The results indicate that these compounds (at 30 μM) promote an important cleavage of procaspases-3, -6, and -7 (Figure 3). Caspase-3 is the most active effector caspase to be involved in apoptosis induced by cytotoxic agents.

Since poly(ADP-ribose)polymerase (PARP) is a typical substrate for caspases-3 and -7, we also examined whether these compounds induce poly(ADP-ribose) polymerase cleavage. Western blot analysis using a polyclonal antibody that recognizes the M_r 85 000 cleaved form of PARP showed the generation of the 85 kDa fragment in sesquiterpene lactones-treated cells after 16 h of treatment (Figure 5). Equal protein loading was controlled by staining membranes with Ponceau S. Control lane (C) refers to untreated cells.

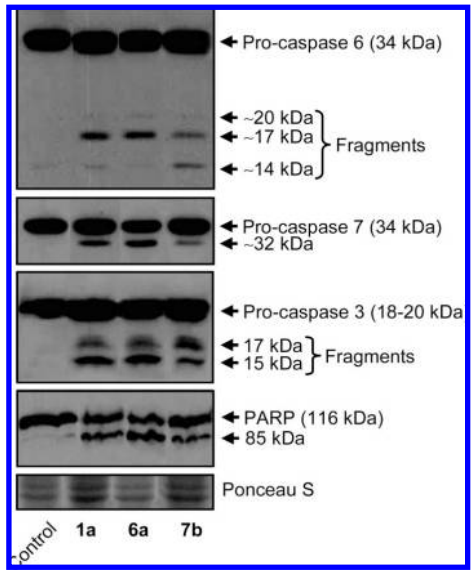

Figure 3. Cells were incubated with the indicated compounds, and cell lysates were assayed by immunoblotting for the cleavage of pro-caspases-6, -7, and -3 and PARP. One representative blot of three is shown. Equal protein loading was controlled by staining membranes with Ponceau S (a representative section of the stained membrane is shown).

In conclusion, this study demonstrates the cytotoxic activities of several sesquiterpene lactones against human myeloid leukemia HL-60 and U937 cells. Interestingly, compounds **1a** and **6a** were equally potent against human SK-MEL-1 melanoma cells. However, human lung carcinoma A549 cells were resistant to all lactones tested. Cell growth inhibition in HL-60 cells was associated with alterations in cell cycle progression and induction of apoptosis through a caspase-dependent mechanism.

Experimental Section

General Experimental Procedures. Optical rotations were recorded in a Perkin-Elmer model 343 polarimeter. 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-300 spectrometer with standard pulse sequences operating at 300 MHz in ^{1}H and 75 MHz in ^{13}C NMR. $CDCl_3$ was used as solvent. EIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Open column chromatography (1500 × 85 mm, fraction volumes 1 L) was performed using Si gel (particle size 0.2−0.5 mm, Merck Co.), and the low-pressure liquid chromatography was carried out using Ace Glass columns (300 × 21 and 250 × 8 mm, fraction volumes 50 and 25 mL, respectively) packed with Si gel (particle size 0.04−0.60 mm, Merck Co.) with a FMI QSY (Fluid Metering Inc.) pump. Preparative TLC used silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness, Merck Co.).

Plant Material. The aerial parts of *Gonospermum gomerae* Bolle and *G fruticosum* Less were collected by Prof. José L. Eiroa in Barranco de Agulo and Alojera, respectively (La Gomera, Canary Islands), in April 1999. The plant material was identified by Dra. Rosa Febles, and a voucher specimen has been deposited at the Herbarium of the Viera y Clavijo Botanical Garden (Gran Canaria) (*G. gomerae* No. 19422 and *G. fruticosum* No. 19421).

Extraction and Isolation. The aerial parts of *G. gomerae* (2950 g) were exhaustively extracted with 95% EtOH in a Soxhlet apparatus for 72 h. The solvent was removed *in vacuo* to yield 410 g of a viscous mass, which was submitted to silica gel CC using *n*-hexane−EtOAc mixtures of increasing polarity (7:3; 3:2, and 1:1 v/v), affording three fractions (1−3). Fraction 1, eluted with *n*-hexane−EtOAc (4:1), gave two fractions (1A and 1B). Fraction 1A yielded **14** (25 mg), **10** (50 mg), **8** (35 mg), and **18** (30 mg) and a nonseparable mixture (125 mg), which was treated with pyridine (1 mL) and acetic anhydride (2 mL) at room temperature for 12 h. Purification of the acetylated mixture by preparative TLC afforded **6a** (15 mg), **7a** (12 mg), **7b** (17 mg), and **1a** (21 mg). Fraction 1B was chromatographed on silica gel (*n*-hexane−EtOAc, 4:1) to give **20** (25 mg), **19** (30 mg), and a residue that was purified after acetylation under the usual conditions, yielding **15a** (22 mg). Fraction 2 was eluted with *n*-hexane−EtOAc (4:1) to give subfractions 2A, 2B, and 2C. Fraction 2A eluted with benzene−EtOAc (4:1) yielded **12** (23 mg) and **22** (35 mg). Fraction 2B eluted with benzene−EtOAc (4:1) gave **2** (10 mg) and **21** (12 mg). Fraction 2C was purified using preparative TLC with *n*-hexane−EtOAc (7:3) to afford **16** (30 mg). Fraction 3, eluting with *n*-hexane−EtOAc (7:3), yielded **17** (45 mg) and **23** (29 mg) along with a mixture that was submitted to preparative TLC with *n*-hexane−EtOAc (4:1) to yield **13** (40 mg), **9** (15 mg), and **11** (28 mg). See Supporting Information for structures of compounds **6**−**25**.

Compound 1a: colorless oil; $[\alpha]^{25}_D$ +18.3 (*c* 0.06, $CHCl_3$); IR (KBr) ν_{max} 1770, 1742, 1648, 1276, 1242, 967 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 69.9 (C-1), 26.5 (C-2), 29.7 (C-3), 142.5 (C-4), 77.2 (C-5), 73.2 (C-6), 47.3 (C-7), 74.0 (C-8), 125.9 (C-9), 137.3 (C-10), 133.9 (C-11), 170.0 (C-12), 127.3 (C-13), 17.7 (C-14), 114.8 (C-15), 20.7, 20.9, 21.1 (3 × CH_3CO), 169.0, 169.1, 169.7 (3 × CH_3CO); EIMS *m/z* (%) 364 $[M - C_2H_2O]^+$ (7.4), 347 $[M - C_2H_3O_2]^+$ (100), 304 $[M - C_2H_3O_2 - C_2H_2O]^+$ (51); HRESIMS *m/z* 364.1513 $[M - C_2H_2O]^+$ (calcd for $C_{19}H_{24}O_7$, 364.1522).

Compound 2: colorless oil; $[\alpha]^{25}_D$ +10.2 (*c* 0.40, $CHCl_3$); IR (KBr) ν_{max} 1769, 1732, 1660, 1229, 961 cm^{-1}; ^{1}H NMR, see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 203.0 (C-1), 31.9 (C-3), 139.6 (C-4), 135.4 (C-5), 71.6 (C-6), 76.6 (C-8), 40.2 (C-9), 147.0 (C-10), 139.1 (C-11), 169.2 (C-12), 126.0 (C-13), 126.0 (C-14), 17.3 (C-15), 169.2, 139.6, 127.0, 20.6, and 15.9 (Ang); EIMS *m/z* (%): 244 $[M - HOAng]^+$ (21.6); HRESIMS *m/z* 344.1624 $[M]^+$ (calcd for $C_{20}H_{24}O_5$, 344.1624).

The extraction of *G. fruticosum* (1295 g) gave 200 g of a viscous mass that afforded, by CC on silica gel using *n*-hexane−EtOAc in increasing polarity, five fractions. Fraction 1 eluted with *n*-hexane−EtOAc (9:1) to give **20** (52 mg), **19** (12 mg), and **25** (23 mg). Fraction 2, using *n*-hexane−EtOAc (7:3), gave **17** (26 mg) and **23** (19 mg). Fraction 3, using *n*-hexane−EtOAc (7:3), yielded **9** (24 mg) and **8** (16 mg). Fraction 4, using *n*-hexane−EtOAc (3:2), afforded **10** (21 mg), **13** (13 mg), and **11** (34 mg) and, by preparative TLC using *n*-hexane−EtOAc (3:2), **3** (5.3 mg). Finally, fraction 5 eluted with *n*-hexane−EtOAc (1:1) gave two subfractions. The less polar fraction was subjected to CC using *n*-hexane−EtOAc (2:3) to give **5** (40 mg). The other fraction was acetylated with pyridine (1 mL) and Ac_2O (2 mL) overnight to afford a mixture, which was separated on CC with *n*-hexane−EtOAc (9:1), giving **4a** (4 mg) and **24** (30 mg).

Compound 3: colorless oil, $[\alpha]_D$ +31.5 (*c* 0.13, $CHCl_3$); IR (KBr) ν_{max} 3458, 1770, 1715, 1645, 1265, 973 cm^{-1}; ^{1}H NMR, see Table 1; EIMS *m/z* (%) 346 $[M]^+$ (1.1).

Compound 3a: Compound **3** (4 mg) was acetylated with acetic anhydride (2 mL) and pyridine (2 mL) at room temperature overnight. Evaporation of the reagents under vacuum yielded the corresponding monoacetate **3a** (2.2 mg) as a colorless oil: IR (KBr) ν_{max} 1776, 1731, 1651, 1242, 977, 907, 814, 756, 667, 607 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.95 (3H, s, CH_3-14), 1.60 (1H, m, H-2β), 1.60 (1H, m, H-9β), 1.81 (3H, s, OTigl), 1.87 (1H, m, H-2α), 1.88 (3H, s, OTigl), 2.05 (1H, m, H-3α), 2.06 (3H, s OAc), 2.30 (1H, m, H-3β), 2.30 (1H, m, H-9α), 2.34 (1H, d J = 10.0 Hz, H-5), 2.78 (1H, m, H-7), 4.05 (1H, m, H-8), 4.65 (1H, d, J = 5.9 Hz, H-15a), 4.83 (1H, dd, J = 4.6, 11.5 Hz, H-1), 4.88 (1H, d, J = 5.0 Hz, H-15b), 5.38 (1H, d, J = 2.7 Hz, H-13a), 5.60 (1H, m, H-6), 6.12 (1H, d, J = 2.7 Hz, H-13b), 6.84 (1H, m, OTigl); EIMS *m/z* (%) 388 (0.7); HRESIMS *m/z* 288.1338 [M − TigOH]$^+$ (calcd for $C_{17}H_{20}O_4$, 288.1362).

Compound 4a: colorless oil, $[\alpha]^{25}$ −8.3 (*c* 0.15, $CHCl_3$); IR (KBr) ν_{max} 1754, 1735, 1241, 1139, 999, 960 cm^{-1}; ^{1}H NMR, see Table 1. ^{13}C NMR (75 MHz, $CDCl_3$) δ 68.0 (C-1), 24.5 (C-2), 34.8 (C-3), 138.0 (C-4), 132.0 (C-5), 72.7 (C-6), 49.2 (C-7), 74.2 (C-8), 128.0 (C-9), 137.0 (C-10), 138.0 (C-11), 169.5 (C-12), 122.5 (C-13), 17.3 (C-14), 15.3 (C-15), OAc: 168.0 and 20.9; OTigl: 162,0; 140.0; 128.2; 14.3 and 11.8; EIMS *m/z* (%) 388 $[M]^+$ (3.6); HRESIMS *m/z* 411.1787 [M + Na]$^+$ (calcd for $C_{22}H_{28}O_6Na$, 411.1784).

Compound 5: colorless oil, $[\alpha]^{25}_D$ +2.0 (*c* 0.30, $CHCl_3$); IR (KBr) ν_{max} 3429, 1738, 1651, 1242, 920 cm^{-1}; ^{1}H NMR see Table 1; ^{13}C NMR (75 MHz, $CDCl_3$) δ 18.00 (C-13), 21.20 (CH_3CO), 27.07 (C-4), 27.40 (C-14), 28.46 (C-15), 37.76 (C-5), 45.09 (C-9), 72.44 (C-6), 72.58 (C-10), 77.40 (C-3), 111.97 (C-12), 112.90 (C-1), 122.87 (C-8), 140.91 (C-7), 142.81 (C-2), 144.68 (C-11), 170.31 ($COCH_3$); EIMS *m/z* (%)

278 $[M - H_2O]^+$ (1.7); HRESIMS *m/z* 218.1671 $[M - C_2H_2O - 2\times H_2O]^+$ (calcd for $C_{15}H_{22}O$, 218.1671).

Cell Culture. Human HL-60 and U937 myeloid leukemia cells and human SK-MEL-1 melanoma cells were grown in RPMI 1640 (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) and 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The cell numbers were counted by a hematocytometer, and the viability was greater than 95% in all experiments as assayed by the 0.025% trypan blue exclusion method. Stock solutions of 100 mM sesquiterpene lactones were made in dimethyl sulfoxide (DMSO), and aliquots were frozen at −20 °C. Human peripheral blood mononuclear cells (PBMC) were isolated from heparin-anticoagulated blood of healthy volunteers by centrifugation with Ficoll-Paque Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

Assay for Growth Inhibition and Cell Viability. Cytotoxicity was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay. Briefly, 1×10^4 exponentially growing cells were seeded in 96-well microculture plates with various sesquiterpene lactone concentrations (0.3−100 μM) in a volume of 200 μL. DMSO concentration was the same in all the treatments and did not exceed 0.1% (v/v). After 72 h, surviving cells were detected on the basis of their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma) into formazan crystals. Optical density was read with an ELISA reader at 570 nm and 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: cell survival (%) = mean absorbance in treated cells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically using the curve-fitting algorithm of the computer software Prism 2.0 (GraphPad). Values are means ± SE from three independent experiments, each performed in triplicate.

Flow Cytometry Analysis for Cell Cycle Distribution. After 24−48 h of treatment, cells were harvested and quickly washed twice with ice-cold phosphate-buffered saline (PBS), and cell pellets were collected and resuspended in 50 μL of PBS. Following dropwise addition of 1 mL of ice-cold 75% ethanol, fixed cells were stored at −20 °C for 1 h. Samples were then centrifuged at 500*g* for 10 min at 4 °C and washed with PBS before resuspension in 1 mL containing 50 μg/mL propidium iodide and 100 μg/mL RNase A and incubation for 1 h at 37 °C in the dark. Cell cycle distribution was then analyzed by flow cytometry using a Coulter EPICS cytometer (Beckman Coulter). Histograms were analyzed with the Expo 32 ADC Software (Beckman Coulter). The quantitative data presented are mean ± SE of percentage of cells in different phases of cell cycle from triplicate samples in each treatment and were reproducible in two independent experiments.

Immunoblot Analysis of Procaspases-3, -6, and -7. HL-60 or U937 cells (1×10^6) were treated with sesquiterpene lactone derivatives at the indicated concentrations in RPMI 1640 medium. Cells were pelleted by centrifugation, washed with phosphate-buffered saline, lysed in lysis buffer containing 125 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol, and 1% β-mercaptoethanol, and boiled for 5 min. The samples were separated on 12% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane. The membrane was probed first with a polyclonal anti-procaspase-3 (Stressgen, 1:2000 dilution), anti-caspase-6 monoclonal antibody (MBL), anti-caspase-7 monoclonal antibody (BD Pharmingen), or procaspase-9 (Stressgen, 1:1,000) and then with anti-rabbit (pro-caspase-3) or anti-mouse (caspase-6, caspase-7, pro-caspase-9) antibody conjugated to horseradish peroxidase (HRP). Protein bands were detected by chemiluminescence (SuperSignal West Pico chemiluminescent substrate, Pierce) using the manufacturer's protocol.

Immunoblotting of Poly(ADP-ribose) Polymerase. Induction of apoptosis was also examined by proteolytic cleavage of poly(ADP-ribose) polymerase. Briefly, 1×10^6 exponentially growing HL-60 cells were treated with the selected sesquiterpene lactones at the indicated concentrations for 16 h at 37 °C. Cells were pelleted by centrifugation, washed twice with phosphate-buffered saline, resuspended in lysis buffer, and subjected to Western blot analysis. Proteins were separated on 7.5% sodium dodecyl sulfate-polyacrylamide minigels and electrotransferred to polyvinylidene difluoride membrane. The membrane was probed with polyclonal anti-poly(ADP-ribose) polymerase (Stressgen, 1:3000 dilution) and then with anti-rabbit antibody conjugated to horseradish peroxidase (HRP). Protein bands were detected by chemiluminescence (SuperSignal West Pico chemiluminescent substrate, Pierce) as described above.

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Supporting Information Available: ^1H and ^{13}C NMR, COSY, HMBC, and ROESY spectra of compounds **1**−**5**. Structures of known compounds **6**−**25**. This material is available free of charge via the Internet at http://pubs.acs.org.

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