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Synthesis of 26-hydroxy-22-oxocholestanic frameworks from diosgenin and hecogenin and their in vitro antiproliferative and apoptotic activity on human cervical cancer CaSki cells

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ABSTRACT

Certain steroidal compounds have demonstrated an antiproliferative effect against several tumor cell lines; however, their complete role on cancer cells is not currently established. Herein, we report the synthesis and evaluation of two new 26-hydroxy-22-oxocholestanic steroids on cervical cancer CaSki cells. The title compounds were prepared from diosgenin and hecogenin in excellent yields. We determined their effect on cell proliferation, cell cycle, and cell death. The cytotoxic effect of the title compounds on CaSki and human lymphocytes was also evaluated, indicating that the main cell death process is not necrosis; the null effect on lymphocytes implies that they are not cytotoxic. The observation of apoptotic bodies as well as the increase in the expression of active caspase-3 along with the fragmentation of DNA confirmed that such new cholestanic frameworks induced apoptosis in tumor cells. Significantly, their antiproliferative activity on tumor cells did not affect the proliferative potential of normal fibroblasts from cervix and peripheral blood lymphocytes. The title compounds show selective antitumor activity and therefore serve as promising lead candidates for further optimization.

1. Introduction

Today, cancer poses one of the most serious health problems, that is difficult to treat due to the successive mutations on oncogenes and suppressor genes that deregulate the cell cycle.[1,2] Significant progress in the development of novel drugs and therapies has occurred owing to the discovery of camptothecin,[3] taxanes,[4,5] and others. Currently, some forms of cancer have been successfully treated by modern therapies, although patients must often tolerate inconvenient side effects. Recently, it has been found that steroidal compounds exhibit antiproliferative activity against several cancer types, but their selective action toward malignant cells has not been properly established. At the end of the last century it was reported that the naturally occurring glycoside OSW-1 [**1**, 3β, 17α-dihydroxy-22-oxocholest-5-en-16β-yl 2-*O*-(4-methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside],[6] a major component of a small group of cholestane saponins isolated from the bulbs of *Ornithogalum saundersiae*, exhibited 100 times greater anticancer activity than taxol.[7,8] This fact has prompted the search for new pathways to synthesize OSW-1 on large scale, and to synthesize analogues for optimization of biological activity. The partial synthesis of OSW-1 and analogues has been accomplished starting from pregnanic or cholestanic skeletons, but in low yields, due to the difficulty in building the 22-oxocholestanic side-chain and the introduction of the C-17 hydroxyl group.[9] Other steroidal glycosides related to saponins (Fig. 1), such as icogenin (**2**),[10] methyl protodioscin (**3**),[11] polyphyllin D (**4**),[12] and dioscin (**5**)[13] also exhibit high anticancer activities. More recently, it was demonstrated that diosgenin (**6**)[14] and hecogenin (**7**)[15] exhibit antiproliferative activity and induce apoptosis in several cell lines.

Although the aglycon plays the main role on biological activity, it is well known that the sugar moieties play a very important role in the activity of steroidal glycosides, for example, by increasing the solubility in physiological media and assisting with cell permeability and lifetime, and in directing the molecule to the active site. After diverse biological tests, it was concluded that both, aglycon and sugar moiety are essential for activity.[16]

Fuchs and co-workers proposed that the high anticancer activity of OSW-1 and related compounds could be explained by their

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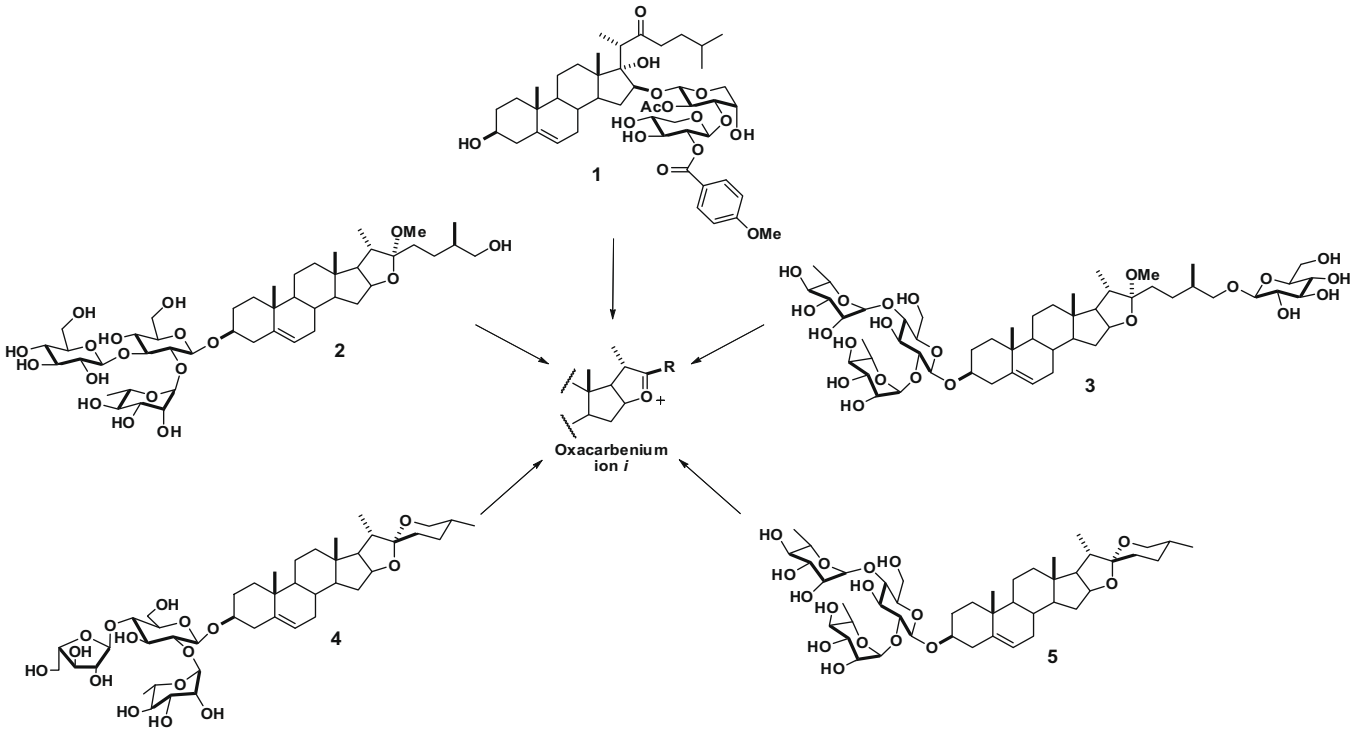


Figure 1. Steroidal saponins with significant antiproliferative activity.

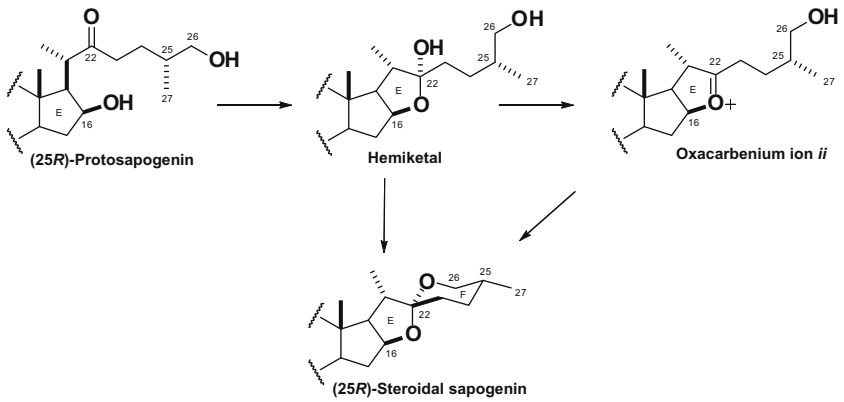
ability to form oxacarbenium-ions.[17] This hypothesis is valid for cephalostatins, ritterazines and the family of steroidal compounds described above. To date, most compounds currently used in chemotherapy display problems due to the lack of selectivity toward malignant cells, and provoke undesirable secondary effects. It is of interest, therefore, to search for new molecules with selective anticancer activity.

2. Results and discussion

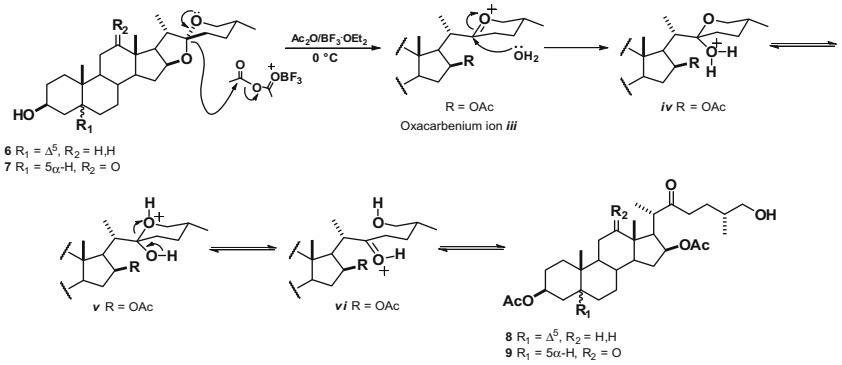
2.1. Chemical synthesis and characterization

New transformations of the spiroketal moiety of sapogenins have been undertaken to obtain interesting steroidal structures desirable for partial synthesis.[18–20] In most cases the ring F of the spiroketal side-chain has been opened to produce pseudosapogenins or furostanes. Less work is related to the regioselective fission of ring E. In accordance with the sapogenin biosynthetic pathway, we studied the best way to open such side-chains to produce the elusive protosapogenin framework (Scheme 1).

The spiroketal function is biosynthetically derived from the cholesterol side-chain by a series of oxidation steps: hydroxylation of C-16 and one of the terminal isopropyl methyls (C-26), and generation of a ketone function at C-22. This last structure, called protosapogenin, is transformed consecutively into the hemiketal (attack of the 16β-hydroxyl on the 22-oxo group), then to an oxacarbenium-ion ***ii*** and finally to the spiroketal. The chirality at C-22 is fixed by the selective attack from the less hindered face of the C-26-hydroxyl group on the hemiketal or to the oxacarbenium-ion ***ii***. The possible stereochemistry at C-25 is dictated by whether C-26 or C-27 is hydroxylated in the earlier step.[21] Based on the principle of microscopic reversibility, the spiroketal side-chain could be opened under the appropriate conditions to provide the desired protosapogenin, representing an ideal pathway for the formation of analogues of the OSW-1 side-chain. However such a system is very reactive under acidic and even neutral conditions, giving the thermodynamically favored spiroketal moiety in the equilibrium



Scheme 1. Biosynthetic pathway of the spiroketal side-chain in steroidal sapogenins.



Scheme 2. A 'one-pot' procedure to obtain 22-oxocholestanic side-chains.

mixture. To date, this transformation has been a difficult challenge and has resisted several attempts.[22,23]

Recently, we reported a novel and quantitative method to open regioselectively the E ring of steroidal sapogenins to obtain triacetylated protosapogenins (22-oxocholestane-3β,16β,26-triyl triacetate compounds).[24] We continued the search for conditions to avoid the acetylation of C-26 and found that when sapogenins **6** and **7** were treated with Ac_2O, $BF_3{\cdot}OEt_2$, at 0 °C, by controlling the time and the concentration of acetic anhydride, the reaction could be stopped, thus avoiding the acetylation at the C-26 hydroxy group (Scheme 2). Under these reaction conditions, the hydrolysis of the oxacarbenium-ion intermediate ***iii*** occurs by attack at C-22 by water used in the quenching step to give (***iv***) and then the hemiketal (***v***);[25] opening of the F ring then gives (***vi***) and the 26-hydroxy-22-ketone product. It is worth noting that under these conditions, the intramolecular cyclization from the C-26-OH on the 22-oxo group of the final products does not take place, and suitable cholestanic protosapogenin skeletons are readily produced in a 'one-pot' procedure. The resulting compounds **8** and **9** are 17-deoxy-26-hydroxylated analogues of the OSW-1 aglycon side-chain.

Analysis by ^{1}H NMR spectroscopy indicated that the transformation of the spiroketal moiety at C-22 of sapogenins **6** and **7** into the corresponding ketones **8** and **9** produced the expected downfield shifts for both methylene H-23 and the methyne H-20. In addition, the signal pattern for the H-26 protons changed: in **6** and **7** these signals are well separated but in **8** and **9** they have close δ values. Table 1 shows selected ^{1}H and ^{13}C chemical shifts observed for final compounds. The acetate methyl groups at positions 3 and 16 were assigned by means of HMBC experiments; the carbonyl groups of such acetates are long distance-coupled to their corresponding 3, 16 and 26 protons.

3. Biological evaluation of products

3.1. In vitro antiproliferative activity on cervical cancer cell lines

Compounds **8** and **9** were dissolved in ethyl acetate, and screened at a range of concentrations against cervical cancer cells CaSki. Antiproliferative activity (IC_{50}) was determined after 24 h by crystal violet staining,[26] and the dose–response curves are shown in Figure 2. The inhibitory effect of **8** and **9** on the proliferation of CaSki cells was observed to occur in a dose-dependent manner with IC_{50} values of 47.1 μM (24.34 μg/mL) and 188.0 μM (100 μg/mL), respectively.

3.2. Evaluation of cell morphology

The effect of compounds **8** and **9** in cell morphology was determined next. CaSki cultures were stimulated at the level of the IC_{50} values and evaluated after 24 h. Both compounds affected cell morphology; in the culture treated with compound **8**, smaller and spherical cells were found. These results suggest an effect on the cytoskeleton microfilaments or microtubules, involving the loss of cell adhesion. For compound **9**, cells were observed to be larger than normal, suggesting swelling. In addition, non-reflective small inclusion bodies were observed, indicative of the presence of cell

Table 1
Selected ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) data of **8** and **9** (*J* in Hz)

Position	8			9		
	δ_H	δ_C		δ_H	δ_C	
3	4.58, m	73.7		4.64, m	73.0	
5		139.3			44.3	
6	5.35, d (4.8)	122.0			31.0	
12		39.6			212.9	
16	4.96, m	75.6		4.98, m	74.3	
18	0.87, s	13.3		1.15, s	12.7	
19	1.02, s	19.3		0.89, s	11.8	
20	2.96, dq (6.8, 3.6)	43.5		2.78, m	43.8	
21	1.14, d (6.8)	16.7		1.07, d (5.7)	17.0	
22		213.3			213.3	
23	2.63, m, 2.39, m	38.5		2.60, m, 2.37, m	38.0	
26	3.41, d (6.4)	67.4		3.39, br s	67.4	
27	0.91, d (6.8)	16.9		0.88, d (4.8)	16.5	
	Me	C=O	Me	Me	C=O	Me
OAc-3	2.03, s	170.2	21.5	1.99, s	170.5	21.3
OAc-16	1.96, s	169.6	21.2	1.93, s	169.6	20.9

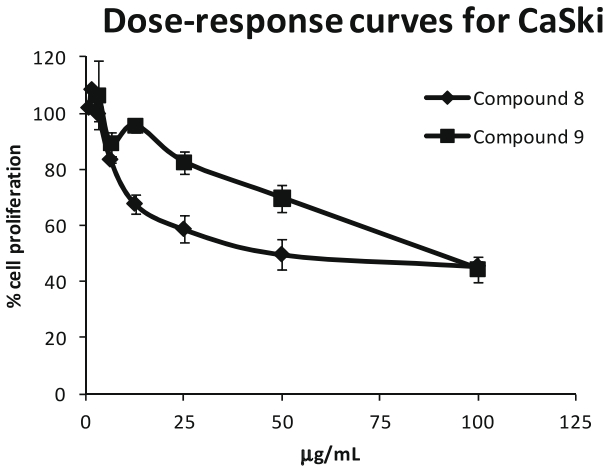


Figure 2. Dose–response curves of the antiproliferative effect of compounds **8** and **9** on CaSki.

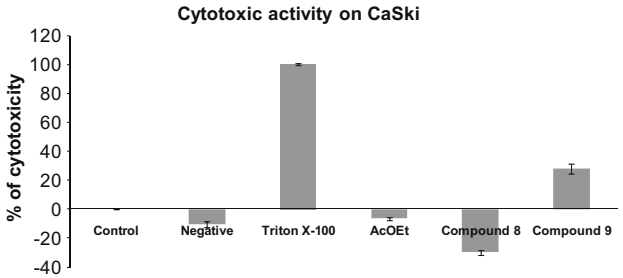


Figure 4. Evaluation of cytotoxicity of compounds **8** and **9** on CaSki cultures. 7500 cells/well were seeded in 96-well tissue culture plates, after 24 h the medium was removed and cells were stimulated at the level of IC_{50} values for **8**, **9** or ethyl acetate (10 µL/mL) and evaluated after 24 h by the amount of LDH released in the culture supernatant. Experimental data is presented as the mean ± SD of three independent experiments with three repetitions. $^{*}p$ <0.05 versus ethyl acetate (Student's *t*-test).

nuclei from broken cells (Fig. 3), suggesting a necrotic process which will be supported or refuted in cytotoxicity and apoptosis assays.

3.3. Cytotoxic activity determination on CaSki cells and human lymphocytes

In order to determine if necrosis was induced, the cytotoxic activity of compounds **8** and **9** was evaluated (Fig. 4). CaSki cultures were stimulated with **8** (47.1 µM) and **9** (188.0 µM). The amount of lactate dehydrogenase (LDH) released in the culture supernatant was used as a measure of loss of plasma-membrane integrity (Triton X-100 was used to induce mortality of cells by lysis).[27] For **8**, no cytotoxicity was detected, suggesting that the observed cell decrease in treated cultures is not a necrotic process. On the other hand, **9** induced cytotoxicity in only 28% of the cases; this value suggests that another cell death type different from necrosis decreases the cell number observed in cultures.

Cytotoxicity was evaluated next on human lymphocytes, in order to determine if necrosis is induced by **8** and **9** in non-tumoral cells (Fig. 5). Lymphocytes were activated with phytohemagglutinin (PHA) and stimulated at the level of IC_{50} of **8** or **9**. Results indicated that such concentrations are not cytotoxic to human lymphocytes, and suggested a selective activity through a different pathway than necrosis.

3.4. Effect on cell cycle

Cell cycle regulation ensures the fidelity of genomic replication and cell division in order to avoid impaired transmission of genetic information. The cell cycle is regulated by two major checkpoints at G_1/S and G_2/M transitions. These checkpoints allow cells to control any modifications in their DNA content. Checkpoint loss results in genomic instability and has been implicated in carcinogenesis. Inducing cells to leave the cycle or cell cycle arrest in cancer cell lines constitutes one of the most prevalent strategies used to stop or limit cancer spreading.[28] In CaSki cells, compound **8** did not affect the cell cycle, implying that its antiproliferative activity is independent of the phases (Fig. 6). In contrast, compound **9**

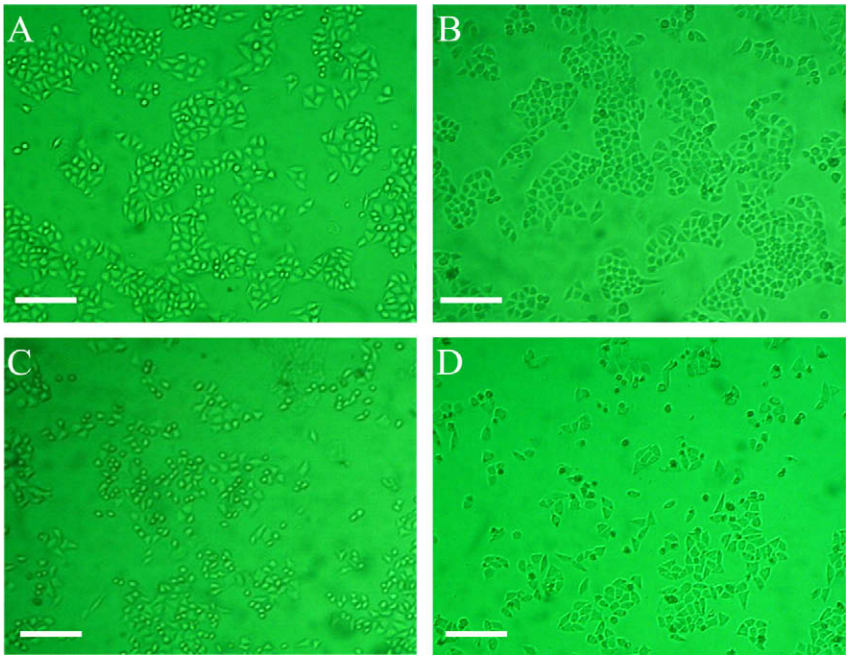


Figure 3. Effect on the morphology of CaSki cells after 24 h of treatment with **8** and **9**. (A) Cells untreated. (B) Cells treated with ethyl acetate. (C) Cells treated with **8** (47.1 µM). (D) Cells treated with **9** (188.0 µM). Scale bars 100 µm.

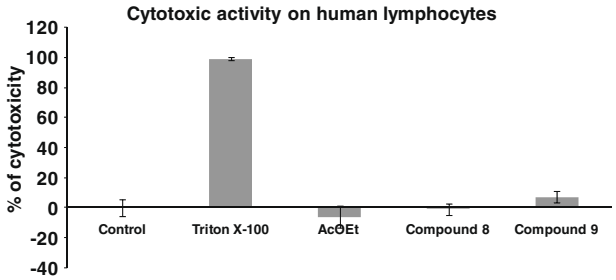


Figure 5. Evaluation of cytotoxicity of compounds **8** and **9** on human lymphocyte cultures. Lymphocytes were seeded in 96-well tissue culture plates, stimulated at the level of IC_{50} values for **8**, **9** or ethyl acetate (10 μL/mL) and evaluated after 72 h by the amount of LDH released in the culture supernatant. Activated lymphocytes with PHA (15 μL/mL) were used as a control. Experimental data is presented as the mean ± SD of three independent experiments with three repetitions. $^{*}p$ <0.05 versus 0 μg/mL (Student's *t*-test).

induced cells to leave the cell cycle in the G_1 and G_2/M phases, and probably to die, suggesting that its activity is dependent on the phases (Fig. 7). This decrease in abovementioned phases is associated with an increase in the percentage of cell nuclei from broken cells with a lower amount of DNA in the so-called sub-G_1 phase, thus indicating cell death.[29]

3.5. Apoptosis

Apoptosis is an important and well controlled form of cell death observed under a variety of physiological and pathological conditions. Inappropriate apoptosis may be involved in many diseases such as Alzheimer's disease, immune deficiency and autoimmune disorders, leukemias, lymphomas, and other malignancies. Therefore, the control of apoptosis is an important potential target for therapeutic intervention.[30,31]

3.5.1. Apoptotic bodies-DAPI staining

Apoptosis is characterized by chromatin condensation which causes compact and smaller nuclei and/or the formation of apoptotic bodies. CaSki cell cultures were stimulated with **8** and **9**, and the chromatin condensation including the formation of apoptotic bodies were determined through the staining with fluorochrome 4′,6-diamidino-2-phenylindole (DAPI).[32] Compact nuclei can be noticed for cultures treated with **8**, indicating chromatin condensation. For compound **9**, the presence of apoptotic bodies was observed which suggested that **8** and **9** induced death by apoptosis in CaSki cells (Fig. 8). Nevertheless, chromatin condensation is often found in necrosis as well, and the presence of apoptotic bodies do not assure that apoptosis is the mechanism of cell death. Thus, more conclusive experiments were performed as follows.

3.5.2. Detection of active caspase-3

Caspases, or cysteine-aspartic proteases, are a family of cysteine proteases, which are crucial mediators of apoptosis. Among them, caspase-3 is probably the best understood of the mammalian caspases in terms of its specificity and roles in apoptosis. Caspase-3 is also required for some typical hallmarks of apoptosis, and is indispensable for apoptotic chromatin condensation, and DNA fragmen-

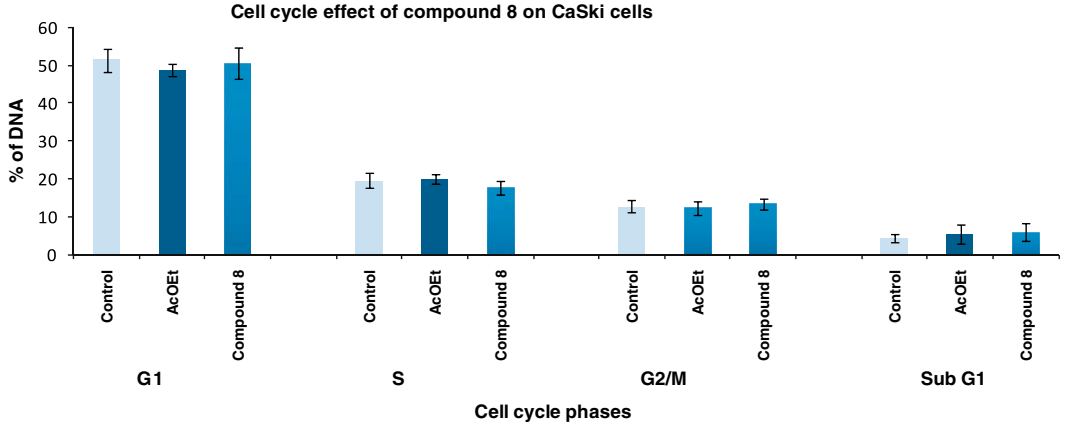


Figure 6. Cell cycle analysis of CaSki cells during compound **8** treatment after 24 h. Values are expressed in distribution of % DNA, $^{*}p$ <0.05 versus ethyl acetate.

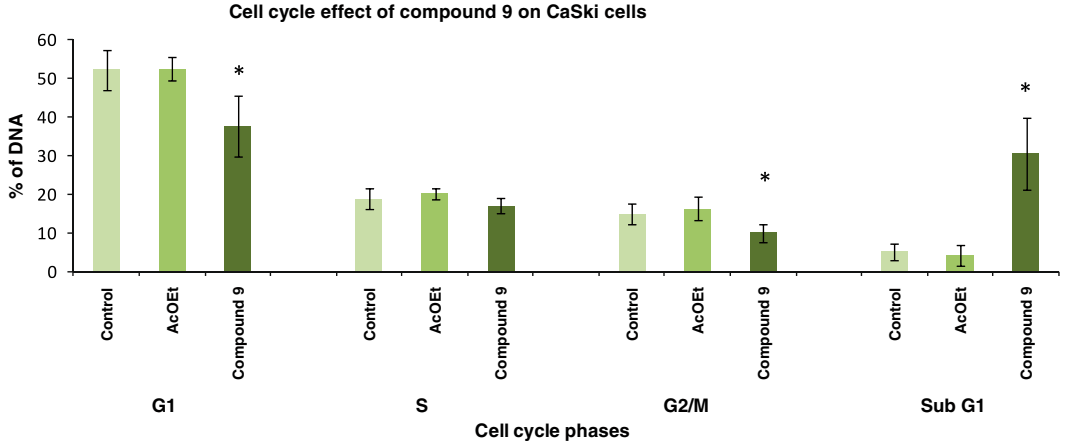


Figure 7. Cell cycle analysis of CaSki cells during compound **9** treatment after 24 h. Values are expressed in distribution of % DNA, $^{*}p$ <0.05 versus ethyl acetate.

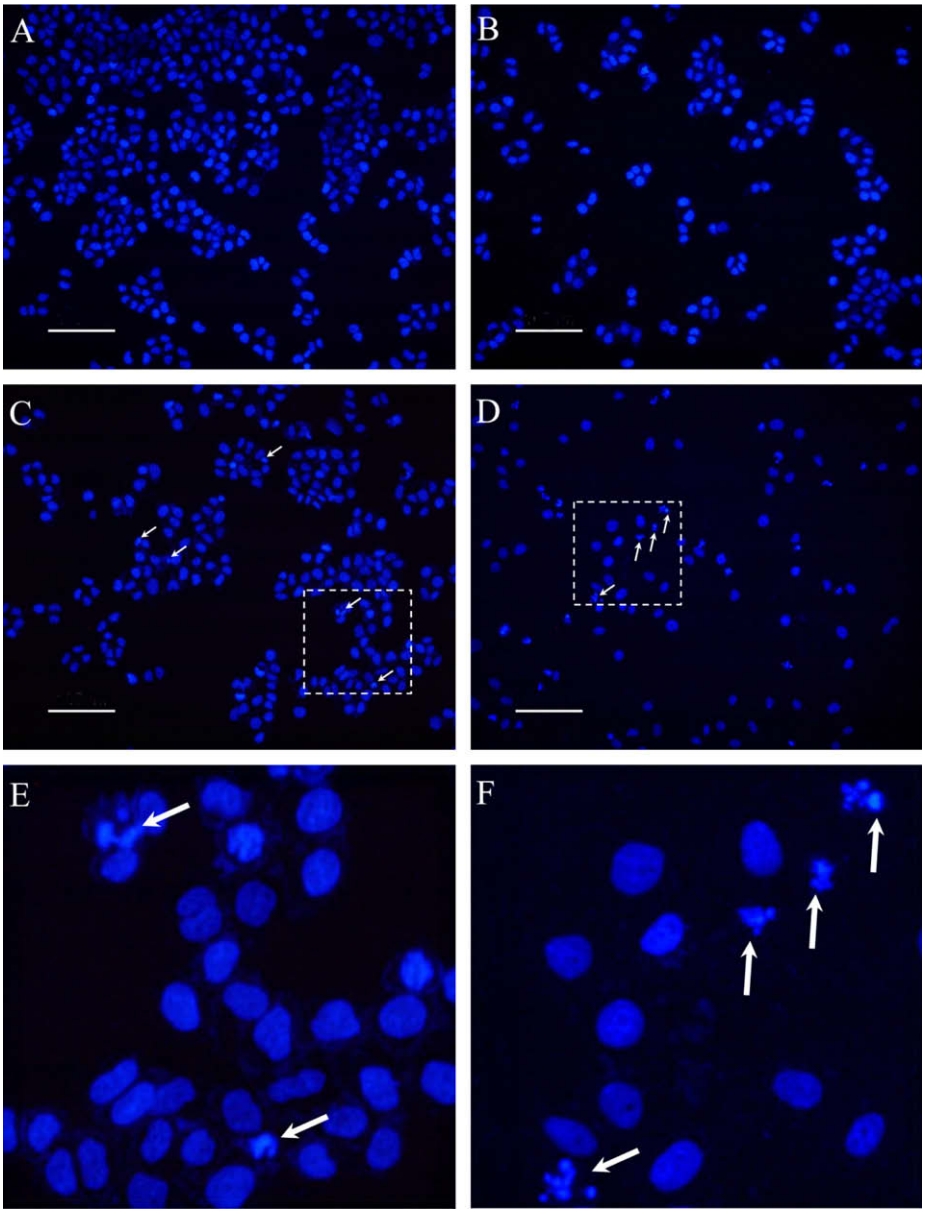


Figure 8. Chromatin condensation and observation of apoptotic bodies on CaSki cell cultures evaluated after 24 h, stained with DAPI. (A) Control. (B) Cells treated with ethyl acetate. (C) Cells treated with **8** (47.1 μM). (D) Cells treated with **9** (188.0 μM). (E) and (F) Are high magnifications of dotted squares in C and D, respectively. Arrows show classical apoptotic bodies. Scale bars 100 μm.

tation, and is essential for certain processes associated with the formation of apoptotic bodies in all cell types examined.[33] Active caspase-3 expression was determined by immunocytochemistry and quantified through emitted luminescence.[34] Figure 9 shows that **8** and **9** induced the expression of active caspase-3, implying that apoptosis could be triggered.

3.5.3. DNA fragmentation assays

One of the characteristics of apoptosis is the degradation of DNA. The Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) method identifies apoptotic cells in situ by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to these strand breaks of cleaved DNA.[35] Figure 10 shows that fragmentation of DNA in CaSki cells is only induced by compound **9**. In spite of the fact that **8** presented chromatin condensation and induced the expression of active caspase-3, it did not cause DNA fragmentation after 24 h. These results indicate that the apoptotic event induced by **9** is completed in a 24 h period, while for **8**, the apoptotic event at 24 h is completed until the activation of caspase-3 under the same conditions as for **9**, suggesting a longer period for the completion of the apoptosis induced by compound **8**.

3.6. Evaluation of antiproliferative activity on non-tumoral cells

Major compounds used currently in chemotherapy present problems for selective activity toward malignant cells and produce undesirable secondary effects. It is crucial to determine the selectivity of tested compounds along with the antiproliferative and apoptosis assays in order to derive any conclusions on anticancer activity. For this reason, the effect of **8** and **9** on the proliferation of non-tumoral cervical fibroblastic cells[36] and peripheral blood lymphocytes was assessed (Fig. 11). Non-tumoral fibroblast cultures were treated with compounds **8** and **9** and the antiproliferative

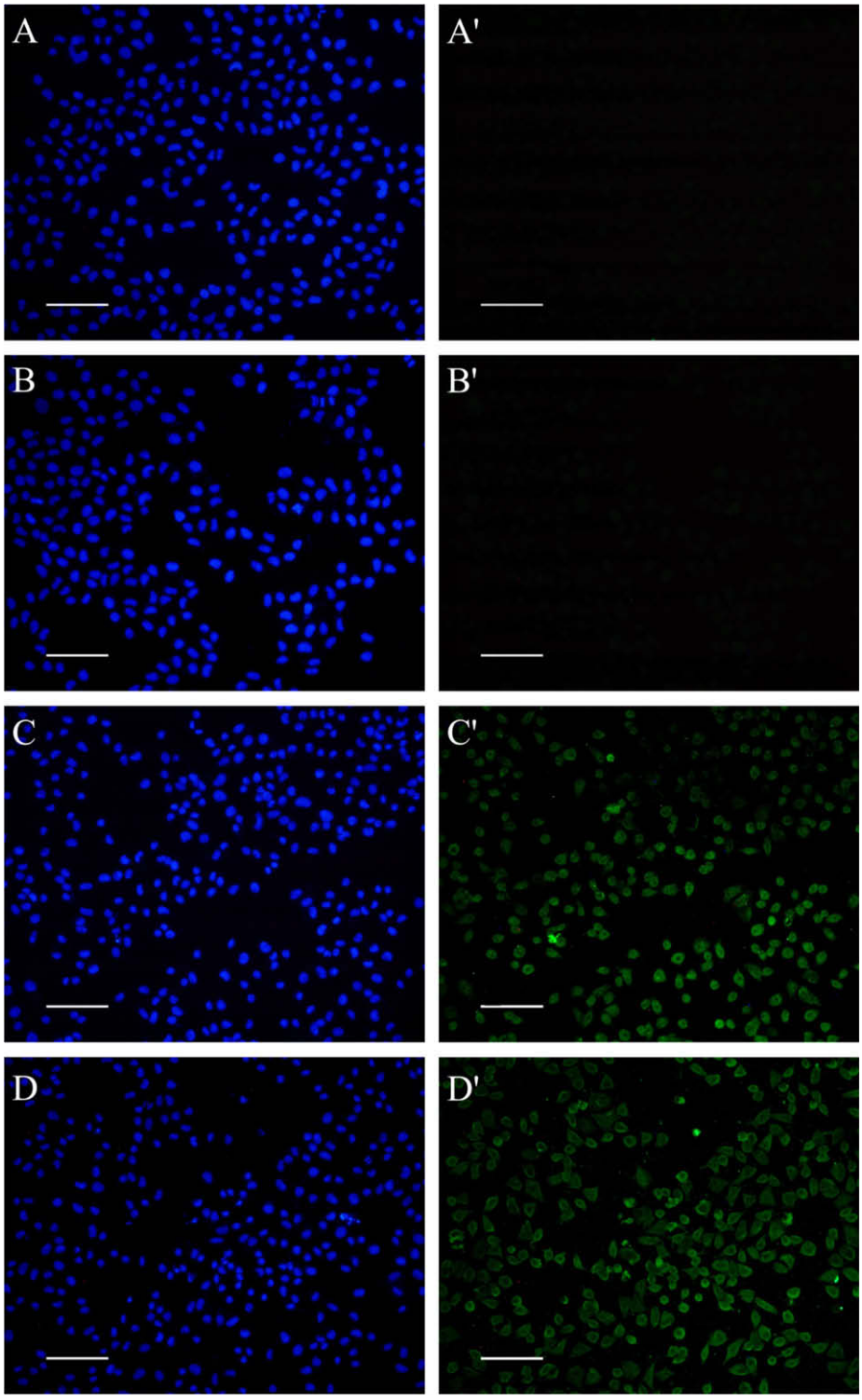


Figure 9. Immunodetection of active caspase-3 on CaSki cell culture. The blue fluorescence indicates the nucleus stained with DAPI (A, B, C, and D). The green fluorescence indicates the presence of active caspase-3 distributed in the cytoplasm of apoptotic cells (A′, B′, C′, and D′). (A) and (A′) Control. (B) and (B′) Cells treated with ethyl acetate. (C) and (C′) Cells treated with **8** (47.1 μM). (D) and (D′) Cells treated with **9** (188.0 μM). Scale bars 100 μm.

activity was determined by violet crystal staining. Compound **8** did not affect significantly the proliferation of fibroblastic cells; whereas with compound **9**, the proliferation was affected by 18%.

It is well known that during chemotherapy the immune system is usually affected; thus, the proliferation of enriched lymphocyte population (ELP) was evaluated with compounds **8** and **9**. ELPs from a normal blood donor were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE) and stimulated with PHA, and/or treated with **8** or **9**, and cultured for 72 h.[37] Cells were harvested and their proliferative potential was analyzed by flow cytometry. The effect of **8** and **9** on proliferative potential of ELPs is shown in Figure 12, indicating that under normal conditions, proliferating cells were 49.26% (Fig. 12B). When lymphocytes were treated at the level of the IC_{50} of **8** (47.1 μM), proliferating cells

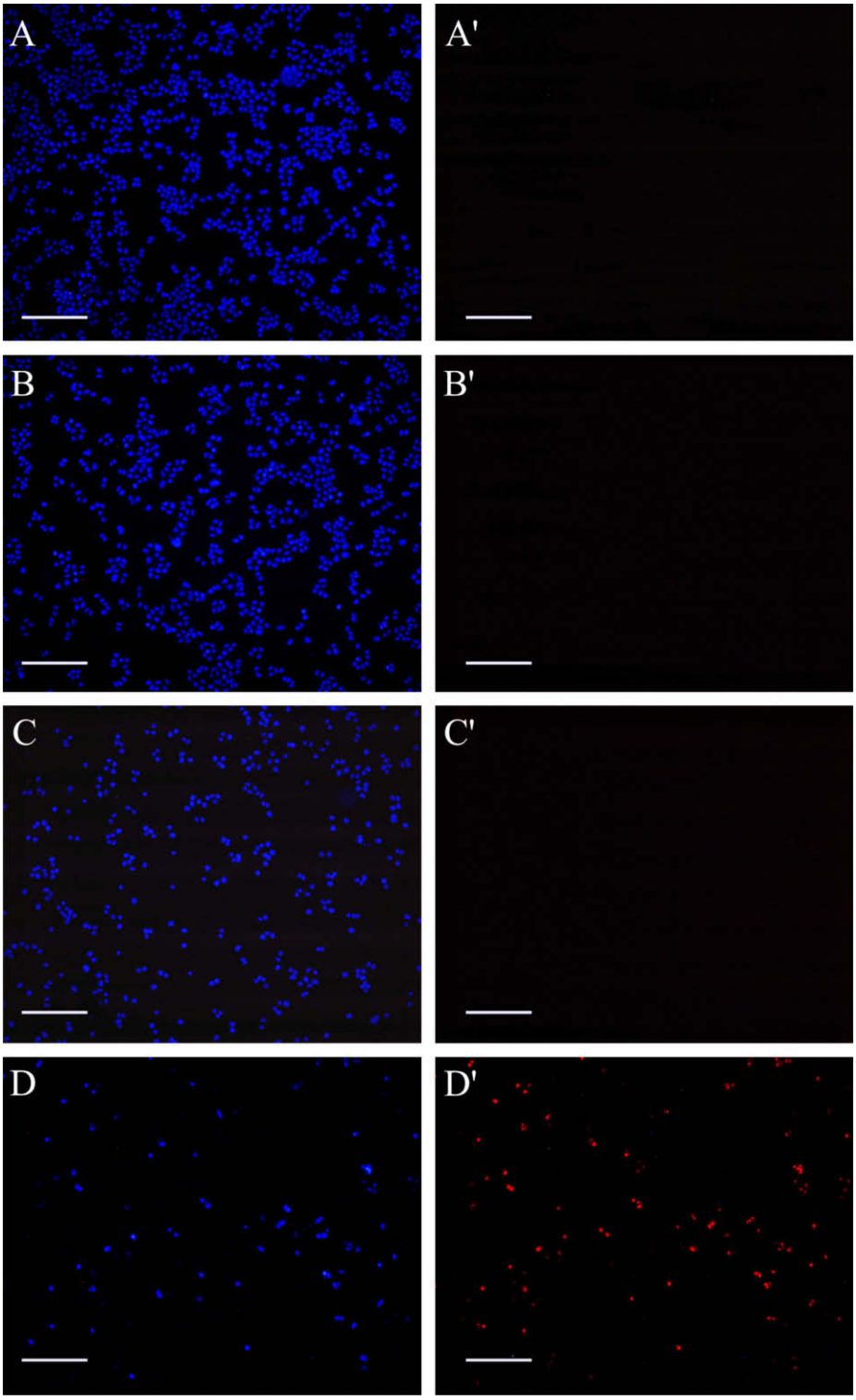


Figure 10. DNA fragmentation detected by the TUNEL assay on CaSki cell cultures, evaluated after 24 h. A, B, C, and D show the chromatin stained with DAPI. A′, B′, C′, and D′ show the TUNEL assay, the red fluorescence in D′ indicates positive cells to the technique. (A) and (A′) Control. (B) and (B′) Cells treated with ethyl acetate. (C) and (C′) Cells treated with **8** (47.1 μM). (D) and (D′) Cells treated with **9** (188.0 μM). Scale bars 200 μm.

were 76.15% (Fig. 12D). With compound **9** (188.0 μM), proliferating cells were 63.66% (Fig. 12E). For both compounds, proliferative potential was not negatively affected; on the contrary, surprisingly, cells were induced to proliferate, suggesting that **8** and **9** might have an immunostimulatory effect. These results on fibroblasts and lymphocytes indicate that the inhibition of the cancer cell proliferation was selective.

4. Conclusions

In conclusion, the method to synthesize 22-oxo-26-hydroxy skeletons developed here has the advantages of cheap starting material and reagents, simple manipulation and separation of products, high yield, and the provision of new core scaffolds for the synthesis of steroids with a cholestane structure such as

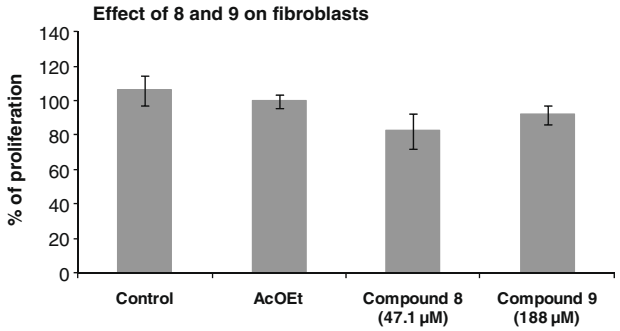


Figure 11. Effect of compounds **8** and **9** on the proliferative potential of non-tumoral cervical fibroblast cells, $^{*}p$ <0.05 versus ethyl acetate.

OSW-1. Our biological evaluations suggest that compounds **8** and **9** are potent apoptosis inducers in CaSki cells with a null cytotoxic consequence. In addition, both compounds did not affect significantly the proliferation of fibroblast cells, and, surprisingly, induced peripheral blood lymphocytes to proliferate, indicative of an immunostimulatory effect. We believe, therefore, that these compounds serve as promising lead candidates for further optimization.

5. Experimental section

5.1. Materials

Optical rotations were measured at 24 °C in a Perkin–Elmer 241 polarimeter. ^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 MHz, respectively, on a Bruker AVANCE NMR instrument. The spectra were referenced to residual protonated solvent. Coupling constants are expressed in hertz (Hz). All assignments were confirmed with the aid of two-dimensional experiments (COSY, HSQC, and HMBC). Processing of the spectra was performed using MestRec software. High resolution mass spectra were obtained by the electrospray ionization (ESI) technique, using an Agilent 6210 TOF LC/MS mass spectrometer. Column chromatography was performed using Merck Silica Gel 60 (230–400 mesh), and analytical thin-layer chromatography (TLC) was performed on aluminum plates precoated with Silica Gel 60F-254.

5.2. Chemical synthesis

5.2.1. (25*R*)-26-Hydroxy-22-oxocholest-5-en-3β,16β-diyl diacetate (8)

Diosgenin **6** (3 g, 7.2 mmol) was dissolved in a mixture of CH_2Cl_2 (20 mL) and Ac_2O (7 mL) and cooled to 0 °C; then, $BF_3{\cdot}OEt_2$; (6 mL, 48 mmol) was added dropwise. The mixture was stirred for 15 min and the resulting syrup was added to ice water (50 mL). The organic phase was washed with a saturated solution of $NaHCO_3$ (4×50 mL) and dried over Na_2SO_4, then concentrated under reduced pressure. The crude product was purified by chromatography on silica gel, with hexanes/ethyl acetate (7:3) as eluent, to afford compound **8** as a colorless solid (84%). Mp 208–210 °C. $[\alpha]_D = -0.3^{\circ}$ (c 1.0, $CHCl_3$). IR: 3497 (OH), 2936 (CH, aliphatic), 1728 (C=O, ketone), 1708 (C=O, acetate), 1596 (C=C). ^{1}H NMR ($CDCl_3$):δ 5.35 (1H, d, $J_{6,7eq}$ 4.8 Hz, H-6), 4.96 (1H, m, H-16), 4.58 (1H, m, H-3), 3.41 (2H, d, $J_{26,25}$ 6.4 Hz, CH_2-26), 2.96 (1H, dq, $J_{20,21}$ 6.8 Hz, $J_{20,17}$ 3.6 Hz, H-20), 2.63 (1H, m, H-23a), 2.39 (1H, m, H-23b), 2.03 (3H, s, C***H*****$_3$**CO_2-3), 1.96 (3H, s, C***H*****$_3$**CO_2-16), 1.14 (3H, d, $J_{21,20}$ 6.8 Hz, CH_3-21), 1.02 (3H, s, CH_3-19), 0.91 (3H, d, $J_{27,25}$ 6.8 Hz, CH_3-27), 0.87 (3H, s, CH_3-18). ^{13}C NMR ($CDCl_3$):δ 36.8 (C-1), 27.7 (C-2), 73.7 (C-3), 38.0 (C-4), 139.3 (C-5), 122.0 (C-6), 31.6 (C-7), 31.2 (C-8), 49.7 (C-9), 36.5 (C-10), 20.7 (C-11), 39.6 (C-12), 41.8 (C-13), 53.9 (C-14), 34.8 (C-15), 75.6 (C-16), 55.0 (C-17), 13.3 (C-18), 19.3 (C-19), 43.5 (C-20), 16.7 (C-21), 213.3 (C-22), 38.5 (C-23), 26.3 (C-24), 35.4 (C-25), 67.4 (C-26), 16.9 (C-27), 170.2 (CH_3CO_2-3), 169.6 (CH_3CO_2-16), 21.5 (CH_3CO_2-3), 21.2

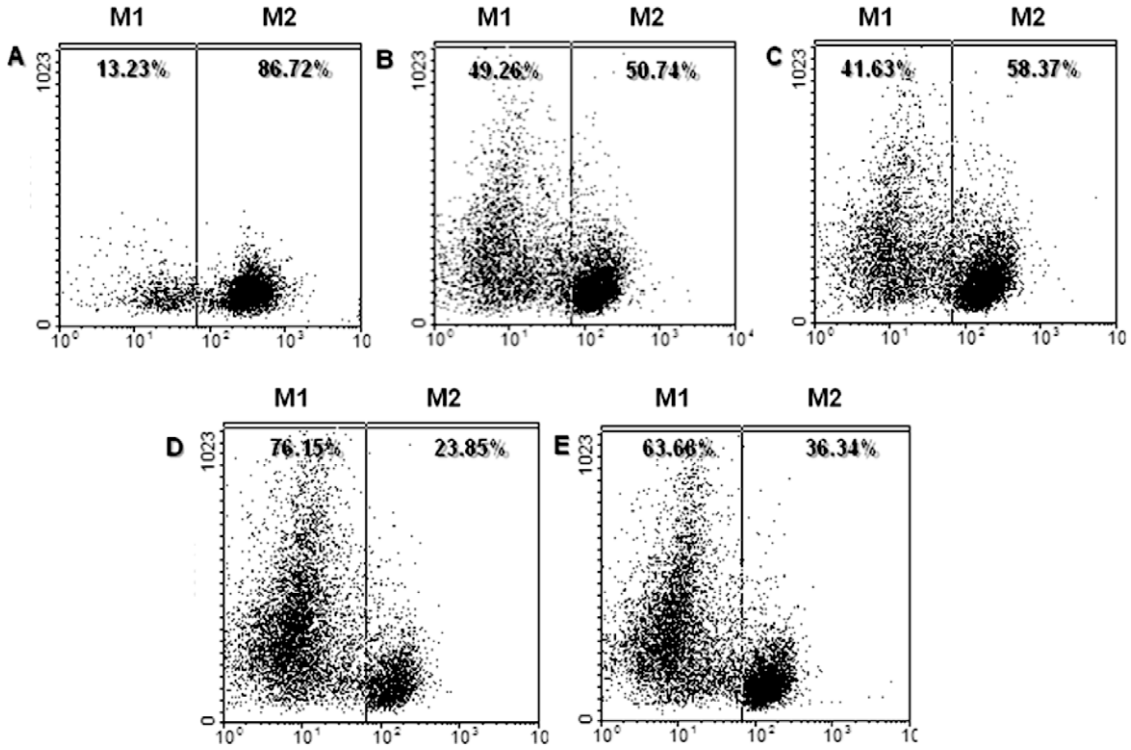


Figure 12. M1 is the proliferating cells region, and M2 is the non-proliferating cells region. (A) ELPs untreated. (B) ELPs in the presence of PHA stimulation. (C) ELPs treated with 10 μL of ethyl acetate. (D) ELPs treated with **8** (47.1 μM). (E) ELPs treated with **9** (188.0 μM).

(CH_3CO_2-16). HRMS Calcd for formula: $C_{31}H_{49}O_6$ 517.3529, $[M+H]^+$. Found: 517.3524.

5.2.2. (25*R*)-26-Hydroxy-12,22-dioxo-5α-cholestan-3β,16β-diyl diacetate (9)

The same procedure described above for the synthesis of **8** was followed to obtain compound **9** (86%) as a colorless solid after chromatography with hexanes/ethyl acetate (65:35). Mp 125–127 °C. $[\alpha]_D$ = +82.1° (*c* 1.3, $CHCl_3$). IR: 3735 (OH), 2933 (CH, aliphatic), 1725 (C=O, ketone), 1706 (C=O, acetate). ^{1}H NMR ($CDCl_3$):δ 4.98 (1H, m, H-16), 4.64 (1H, m, H-3), 3.39 (2H, br s, H-26), 2.78 (1H, dq, $J_{20,21}$ 5.7 Hz, $J_{20,17}$ 3.3 Hz, H-20), 2.78 (1H, m, H-17), 2.60 (1H, m, 23a), 2.52 (1H, dd, $J_{11ax,9}$ 8.8 Hz, $J_{11ax,11eq}$ 8.4 Hz, H-11*ax*), 2.50 (1H, m, H-15a), 2.37 (1H, m, H-23b), 2.16 (1H, dd, $J_{11eq,9}$ 3.2 Hz, $J_{11eq,11ax}$ 8.4 Hz, H-11*eq*), 1.99 (3H, s, CH_3CO_2-3), 1.93 (3H, s, CH_3CO_2-16), 1.47 (1H, m, H-2*ax*), 1.15 (3H, s, CH_3-18), 1.07 (3H, d, $J_{21,20}$ 5.7 Hz, CH_3-21), 0.89 (3H, s, CH_3-19), 0.88 (3H, d, $J_{27,25}$ 4.8 Hz, CH_3-27). ^{13}C NMR ($CDCl_3$):δ 36.2 (C-1), 27.1 (C-2), 73.0 (C-3), 26.3 (C-4), 44.3 (C-5), 31.0 (C-6), 33.6 (C-7), 34.7 (C-8), 56.6 (C-9), 36.2 (C-10), 38.1 (C-11), 212.9 (C-12), 56.2 (C-13), 54.8 (C-14), 34.4 (C-15), 74.3 (C-16), 46.2 (C-17), 12.7 (C-18), 11.8 (C-19), 43.8 (C-20), 17.0 (C-21), 213.3 (C-22), 38.0 (C-23), 28.1 (C-24), 35.3 (C-25), 67.4 (C-26), 16.5 (C-27), 170.5 (CH_3CO_2-3), 169.6 (CH_3CO_2-16), 21.3 (CH_3CO_2-3), 20.9 (CH_3CO_2-16). HRMS Calcd for formula: $C_{31}H_{49}O_7$ 533.3478 $[M+H]^+$. Found: 533.3471.

5.3. Biological activity

5.3.1. Cell culture

The CaSki cell line was purchased from the American Type Culture Collection (ATCC Rockville, MD) and was cultured in RPMI-1640 medium (GIBCO, USA) containing 5% Newborn Calf Serum (NCS, GIBCO, USA) with red phenol supplemented by benzylpenicillin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37 °C. All cell-based assays were performed using cells in the exponential growth phase.

5.3.2. Cell proliferation assay

Assays were performed by seeding 7500 cells/well in 96-well tissue culture plates in a volume of 100 μL of RPMI-1640 medium supplemented with 5% NCS per well. Cells were allowed to grow for 24 h in culture medium prior to exposure to 47.1 μM of **8** or 188.0 μM of **9**. 1% of vehicle (ethyl acetate) was added to control cells. Antiproliferative activity (IC_{50}) was determined after 24 h by crystal violet staining.[18] Growth inhibition was determined by measuring the absorbance at 590 nm in an Enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, USA).

5.3.3. Determination of cytotoxicity

The cytotoxic activity was determined by means of the Kit LDH-Cytotoxicity Assay Kit (BioVision, USA) according to the instructions of the manufacturer. LDH oxidizes lactate to pyruvate which then reacts with the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium (INT) to form formazan. The increase in the amount of formazan produced in culture supernatant directly correlates to the increase in the number of lysed cells. The formazan dye is water-soluble and can be detected with a spectrophotometer at 500 nm.[19]

5.3.4. Cell cycle analysis

CaSki cells were seeded at 10^5 cells/mL in 50 mm tissue culture plates and allowed to grow for 24 h in culture medium prior to exposure to 47.1 μM of **8** or 188.0 μM of **9**. Cells were harvested with versen solution. For DNA content analysis, cells were fixed and permeabilized in 50% methanol in phosphate-buffered saline (PBS), washed in PBS, treated with RNase (2.5 U/mL) and stained with propidium iodide (0.2 μg/mL). Finally, samples were analyzed by flow cytometry analysis (Coulter, USA).[21]

5.3.5. Detection of active caspase-3 from apoptotic cells

CaSki cells were seeded at 5^4 cells in 300 μL of RPMI-1640 containing 5% NCS for 24 h. Cells were treated with 47.1 μM of **8** and 188.0 μM of **9**. Cells were fixed with formaldehyde and 2% PBS for 15 min, then washed with PBS and permeabilized in 0.5% Triton X-100 (Gibco, USA). After that, cells were washed with PBS and blocked with PBS-SAA-tween. Anti-active caspase-3, antibody was added (rabbit polyclonal antibody 1:50 in PBS, Sigma–Aldrich, USA). Samples were washed with PBS and the secondary goat anti-rabbit antibody with fluorescein isothiocyanate (FITC) 1:200 in PBS was added, both assayed with a fluorochrome. Samples were washed again with PBS and DAPI fluorochrome was added. Analyses were performed by epifluorescence microscopy (Nikon).[26]

5.3.6. DNA fragmentation by TUNEL assay

Detection of DNA fragmentation was performed by TUNEL assay using the Apoptag Red in situ apoptosis detection kit.[27] TUNEL assay involves labeling of the 3′-hydroxyl DNA ends generated during DNA fragmentation by means of TdT and labeled dUTP. Cells were cultured on cover slips and treated with **8** or **9** for 24 h. Afterwards, the cells were fixed with 2% formaldehyde for 20 min, washed three times, permeabilized with 0.05% Triton X-100 for 5 min at 4 °C, washed three times, and labeled with biotin-dUTP by incubation with reaction buffer containing terminal deoxynucleotidyl transferase enzyme for 1 h at 37 °C. Biotinylated nucleotides were detected using rhodamine-conjugated streptavidin. Cells were counterstained using DAPI to determine DNA distribution. Cell fluorescence was determined using an E600 Nikon Eclipse microscope with red and blue filters.

5.3.7. Assay on non-tumoral cervical fibroblastic cells

Human fibroblasts were obtained from explants from cervixes taken from patients undergoing hysterectomy without a malignant disease diagnosis. The tissue was cut in 5 mm segments and incubated with trypsin at a concentration of 0.05% at 37 °C with constant stirring. The cellular suspension was filtered and cultured with RPM1-1640 medium and 10% NCS. Cells attached to the substrate were sub-cultured once again. Tissue samples were maintained in RPMI-1640 medium containing 20% NCS at 4 °C and processed after 3 h. Disaggregated epithelial tissue was processed and cultured in a humidified atmosphere with 5% CO_2 at 37 °C. Non-adherent cells were removed and adherent cells were treated with fresh RPMI-1640 medium containing 10% NCS.[28] Cell cultures were exposed to 47.1 μM of **8** or 188.0 μM of **9**. One percent of vehicle (ethyl acetate) was added to control cells. Finally, the cell numbers were determinated by crystal violet staining.

5.3.8. CSFE labeling assay

Heparinized blood samples were obtained from healthy human volunteers. Peripheral blood mononuclear cells (PBMCs) were isolated using standard Hypaque (Sigma–Aldrich, USA) density gradient centrifugation. PBMCs were washed twice with RPMI-1640 (GIBCO USA) medium containing 10% NCS, penicillin (100 U/mL), and streptomycin (100 U/mL). The lymphocyte population was further enriched (ELP) by the elimination of the adherent cells (cells were incubated at 37 °C, 5% CO_2 for 1 h, and non-adherent cells were harvested). ELPs were re-suspended into RPMI-1640 medium at a concentration of 1×10^6 cells/mL. CFSE (from Sigma–Aldrich, USA) was added to the cell suspension at a final concentration of 12 μM and incubated for 15 min at room temperature in the dark. Labeling was completed by adding, during 5 min at room temperature, the same volume of NCS to quench the free CFSE. Labeled

cells were washed five times with sterile PBS containing 10% NCS, counted and re-suspended into RPMI-1640 medium at 1×10^6 cells/mL.[29] Unstimulated, PHA-stimulated or treated cells were plated at 2×10^5 cells/well in 96-well flat-bottomed cell culture plates, and five replicate samples for each treated amount were prepared. The cells were incubated in a 5% CO_2 incubator at 37 °C for 72 h. Cultured cells were harvested, washed twice with PBS, fixed with 1% formaldehyde, then analyzed using flow cytometry, acquiring a minimal of 20,000 events from each sample; data analysis was performed using FACSDiva Software, FACSAria-II (Becton-Dickinson).

5.3.9. Statistical analysis

The median and standard deviation (SD) were calculated using Excel (Microsoft Office, Version 2007). Statistical analysis of differences was carried out by analysis of variance (ANOVA) using SPSS 10.0 for Windows. A *p*-value of less than 0.05 (Student's *t*-test) was considered to be significant.

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