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Synthesis and biological evaluation of the glycoside (25*R*)-3β,16β-diacetoxy -22-oxocholest-5-en-26-yl β-D-glucopyranoside: A selective anticancer agent in cervicouterine cell lines

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ABSTRACT

The synthesis and biological evaluation of the new cholestane glycoside (25*R*)-3β,16β-diacetoxy-22-oxocholest-5-en-26-yl β-D-glucopyranoside starting from diosgenin is described. This compound showed selective antiproliferative activity against CaSki, ViBo, and HeLa cervicouterine cancer cells. Its effect on the cell-cycle was determined. The cytotoxic effects of the title compound on cervicouterine cancer cell lines and human lymphocytes indicate that the main cell death process is not necrosis; hence it is not cytotoxic. The title compound induced apoptosis in cervicouterine cancer cells. Importantly, the antiproliferative activity on tumor cells did not affect the proliferative potential of peripheral blood lymphocytes. The title compound showed selective antitumor activity and greater antiproliferative activity than its aglycon, and therefore serves as a promising lead candidate for further optimization.

1. Introduction

Steroidal saponins are naturally occurring glycosides. Some saponin-containing plants have been widely employed in folk medicine because of the extended properties, and consequently, great interest has been shown in the pharmacological and biological characterization, as well as the chemical synthesis of the active components [1]. Some of the steroidal saponins isolated recently have shown antidiabetic [2], platelet aggregation inhibitory [3], antifungal [4], antiinflammatory [5], and anticancer [6] properties. We have systematically focused on the antitumor activity of these agents, since several compounds currently used in cancer therapies display problems due to the lack of selectivity, and trigger unwanted side effects. Consequently, it is of interest to search for new scaffolds with selective antiproliferative and anticancer activity. Steroidal sapogenins have become ideal synthons for the elaboration of diverse saponins and derivatives because of their rigid framework and potential for varying levels of functionalization, broad biological activity profile, and ability to penetrate cell membranes and bind to specific receptors [7]. Transformations of the spiroketal moiety of sapogenins have yielded interesting steroidal structures for partial synthesis [8]. We have described in previous work an efficient process to open spirostanic side-chains, the glycosylation of the resultant product, and the anticancer activities of the glyco-adducts [9]. In an extension of this work, we now report the synthesis, characterization, and biological evaluation of a new cholestanic saponin *versus* CaSki, ViBo, and HeLa cell lines, starting from diosgenin.

2. Results and discussion

2.1. Chemical synthesis

For the synthesis of the aglycon, the side chain of diosgenin (**1**) was opened, as previously described [9a] to give (25*R*)-26-hydroxy-

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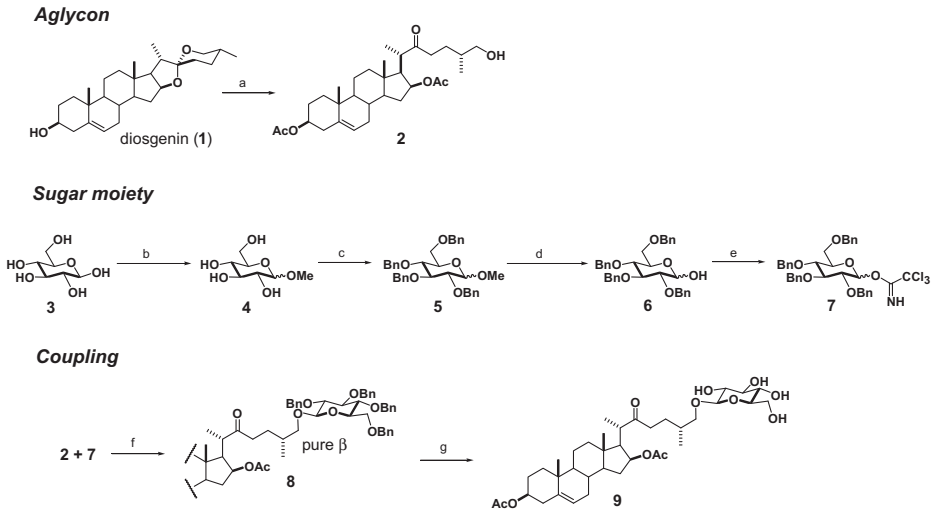

Reagents and conditions: (a) Ac_2O, $BF_3{\cdot}OEt_2$, 0 °C, 84% [9a]; (b) AcCl, MeOH, room temperature, quantitative [9b]; (c) BnBr, NaH, DMF, room temperature, 87% [9b]; (d) AcOH, 1 M H_2SO_4, reflux, 55% [9b]; (e) CCl_3CN, DBU, CH_2Cl_2, room temperature, 89% [10]; (f) TMSOTf (0.1 equiv), 4 Å MS, CH_2Cl_2, -20 °C, 89% [11]; (g) 10% Pd-C, H_2, AcOEt-MeOH (v:v, 7:3), 89%.

Scheme 1. Synthesis of the glycoside **9**.

22-oxocholest-5-en-3β,16β-diyl diacetate (**2**) in excellent yield (84%, Scheme 1). With regard to the configuration at C-16, the substituent at this position is attached to a five-membered ring, this can be only alpha or beta. A recent study based on ROESY correlations (interaction between H-16α and H-17α), and supported by X-ray crystallographic data [9c], demonstrated that the substituent at C-16 is beta oriented. The assignment is also supported by the mechanism of opening of the side chain [9a,c]. The synthesis of the sugar moiety was performed starting from glucose (**3**) as described for the corresponding hecogenin analogue [9b] (Scheme 1). The hemiacetal **6**,

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data (δ in ppm, d^4-methanol) for the glycoside **9**.

Position	^{13}C	^{1}H	Mult.	J (Hz)	HMBC	COSY
Aglycon						
3	75.1	4.53	*m*		C-1, C-4, C=O-acetate-3	H-2*eq*, H-2*ax*, H-4*eq*, H-4*ax*
4	38.8	2.31	*d*	7.6	C-1, C-2,	H-2*eq*, H-2*ax*,
		2.31	*m*		C-3, C-5, C-6	H-3
5	140.6				H-4*eq*, H-4*ax*, CH_3–19	
6	123.1	5.37	m_{broad}		H-4*eq*, H-4*ax*, C-7, C-8, C-10	H-4*eq*, H-4*ax*, H-7*eq*, H-7*ax*
15	35.5	2.36	*dd*	6.8, 6.8	C-13, C-17	H-16
16	76.7	4.98	*m*		C-13, C-14, C=O-acetate-16	H-15, H-17
18	13.7	0.90	*s*		C-12, C-13, C-14, C-17	
19	19.7	1.04	*s*		C-5, C-9, C-10	
20	44.5	3.01	*dq*	10.5, 7.1	C-17, C-21, C-22	H-17, H-21
21	17.3	1.15	*d*	7.1	C-17, C-20, C-22	H-20
22	216.0				H-20, CH_3–21,H–23	
23	39.4	2.72	*m*		C-22, C-24, C-25	H-24
		2.42	*m*			
26	75.7	3.73	*dd*	9.6, 6.0	C-27, C-1′	H-25
		3.35	*m*			
27	17.1	0.92	*d*	6.3	C-24, C-25, C-26	H-25
Me-acetate-3	21.5	2.01	*s*		C=O-acetate-3	
Me-acetate-16	21.4	1.97	*s*		C=O-acetate-16	
C=O-acetate-3	172.2					
C=O-acetate-16	171.6					
26-*O*-β-D-glucopyranoside						
1′	104.2	4.23	*d*	7.7	C-26, H-26	H-2′
2′	74.7	3.20	*dd*	8.4, 7.7		H-1′, H-3′
3′	77.7	3.35	*m*			H-2′, H-4′
4′	71.3	3.31	*m*			H-3′, H-5′
5′	77.4	3.26	*m*			H-4′, H-6′
6′	62.5	3.86	*d*	12.0		H-5′
		3.66	*dd*	12.0, 5.4		

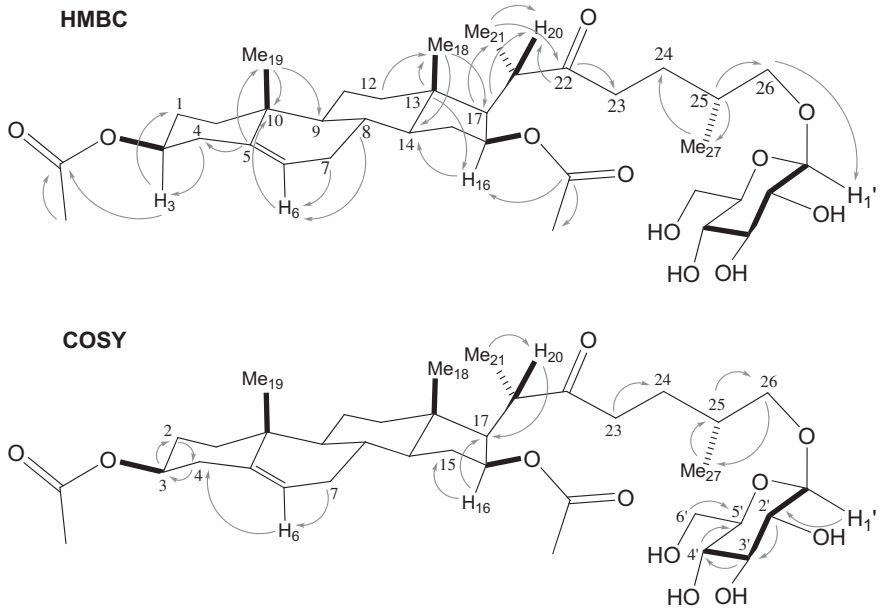

Fig. 1. Selected HMBC and COSY correlations for the glycoside **9**.

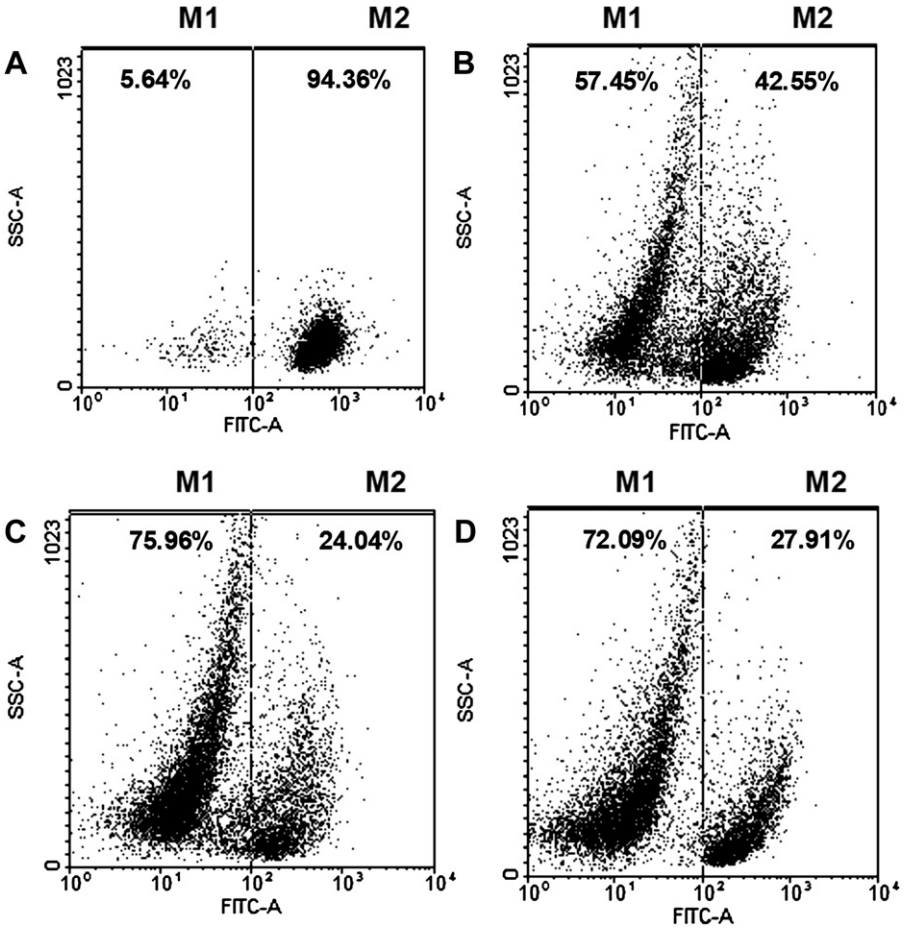

Fig. 2. Effect of compound **9** on lymphocyte proliferation. M1 is the proliferating cells region, and M2 is the non-proliferating cells region. (A) ELPs untreated. (B) ELPs in the presence of PHA stimulation. (C) ELPs treated with 10 μL of ethanol (1%). (D) ELPs treated with 33.9 μM of **9**.

obtained via **4** and **5**, was treated with CCl_3CN [10] to give the trichloroacetimidate donor **7** which was used directly in the glycosylation reaction. Trimethylsilyl trifluoromethanesulfonate (TMSOTf)-promoted glycosylation [11] of the trichloroacetimidate **7** with the cholestanic alcohol **2**, proceeded smoothly to afford the pure β-anomer (**8**) in 89% yield. Such glycosylation reaction conditions generally afford a mixture of α and β anomers. Interestingly, the α-anomer was not formed despite the absence of a participating group at O-2, presumably because of the steric requirements of the bulky aglycon. Debenzylation of compound **8** with 10% Pd–C and hydrogen at atmospheric pressure afforded compound **9**. It is significant that cytotoxicity bioassays of some steroidal glycosides have shown that the β-anomers exhibited higher activities than the corresponding α-anomers [12].

2.2. NMR analysis

Analysis of the ^{1}H NMR spectrum of **9** showed the signal for the anomeric hydrogen H-1′ at δ 4.26 ppm presenting a coupling constant of 7.7 Hz, indicative of a β-linkage between the sugar moiety and the aglycon. Table 1 shows selected ^{1}H and ^{13}C chemical shifts observed for the glycoside **9**.

In addition, selected HMBC and COSY correlations are displayed in Fig. 1. Correlations in the HMBC spectrum were found between H-3 and H-16 with their corresponding acetate carbonyl groups. H-20, Me-21 and H-23 showed correlations with the carbonyl carbon at C-22. Correlations between the anomeric carbon (C-1′) and both protons H-26, and between H-1′ and C-26, established the aglycon-sugar linkage. ^{1}H-^{1}H-COSY correlations were observed from H-3 to H-2 and H-4, from the vinylic H-6 to H-4 and H-7, from H-16 to peaks at δ 2.36, 1.06 and 1.87 ppm, corresponding to three different proton signals from CH_2-15 and CH-17. In addition, Me-21 and Me-27 showed typical correlations with H-20 and H-25, respectively.

3. Biological evaluation

3.1. In vitro antiproliferative activity on CaSki, ViBo, and HeLa cervical cancer cell lines

In order to determine the antiproliferative activity of compound **9**, cervicouterine cancer cell lines CaSki, ViBo, and HeLa were treated in a range of concentrations using ethanol as a vehicle, as measured by a decrease in cell population (IC_{50}). The antiproliferative activity was determined after 24 h by crystal violet staining [13]. The inhibitory effect of **9** on the proliferation of CaSki, ViBo, and HeLa cells was observed to occur in a dose-dependent manner with an IC_{50} value of 33.9 μM (23 μg/mL) for CaSki, and 29.5 μM (20 μg/mL) for ViBo and HeLa cells. Compared to the IC_{50} of the aglycon itself (compound **2**) in CaSki cells [9a], the activity of the glycoside **9**, was enhanced (47.1 vs. 33.9 μM). It is well known that unlike sapogenins, saponins more easily permeate the cell wall [1d–f], and presumably, glucosylation of **2** is also beneficial by the same mechanism.

3.2. In vitro antiproliferative activity on human lymphocytes

Major compounds used currently in chemotherapy present problems for selective activity towards malignant cells and produce undesirable secondary effects. For this reason, the effect of **9** on the proliferation of peripheral blood lymphocytes was assessed. It is well known that during chemotherapy the immune system is usually affected; thus, the proliferation of enriched lymphocyte population (ELP) was evaluated with compound **9**. ELPs from a healthy blood donor were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), stimulated with phytohemagglutinin (PHA), and/or treated with **9**, and cultured for 72 h [14]. Cells were harvested and their proliferative potential was analyzed by flow cytometry. The effect of **9** on the proliferative potential of ELPs is shown in Fig. 2, indicating that under normal conditions, proliferating cells were 58% of the total population (Fig. 2B). When lymphocytes were treated with 33.9 μM of **9** (the highest concentration found and described in the section 3.1), proliferating cells were 72% (Fig. 2D). Thus, in the presence of compound **9**, proliferative potential was surprisingly not negatively affected. These results suggest a greater degree of antiproliferative selectivity towards malignant cell lines than with lymphocytes. This selective promotion in lymphocyte population will be beneficial in *in vivo* assays.

3.3. 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. Cells progress through the cell-cycle in several well-

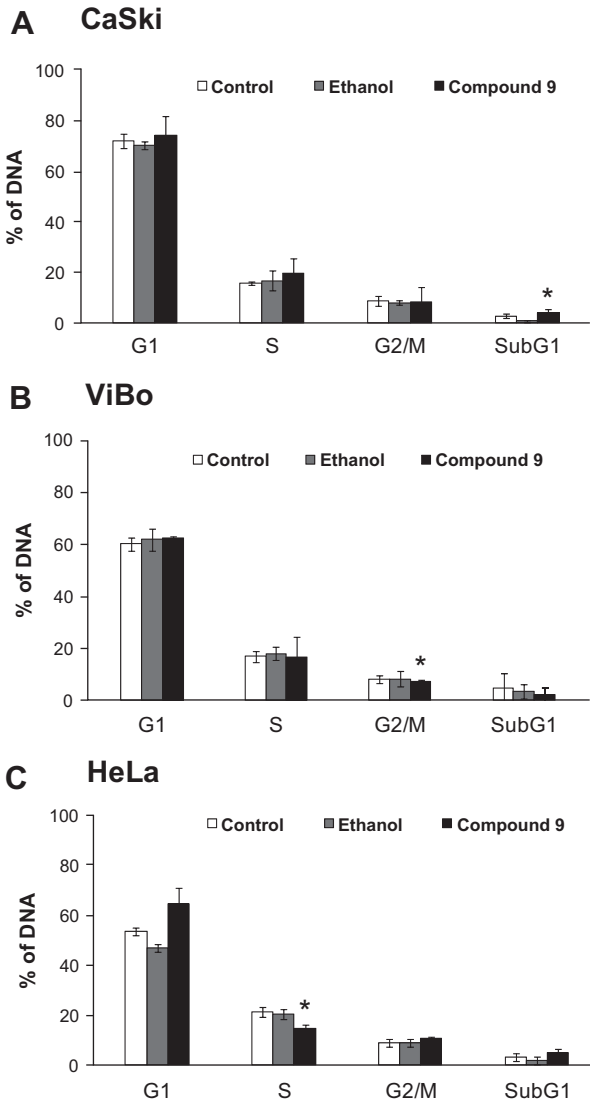

Fig. 3. Cell-cycle analysis of (A) CaSki, (B) ViBo, and (C) HeLa cells upon treatment with compound **9** after 24 h. Values are expressed in distribution of % DNA, $^*p < 0.05$ *versus* ethanol.

controlled phases [15]. Entry into each phase of the cell-cycle is carefully regulated by cell-cycle checkpoints (G_1/S and G_2/M transitions) [16].

One theme emerging in drug discovery is the development of agents that target the cell-cycle checkpoints that are responsible for the control of cell-cycle phase progression. It is clear that cell-cycle checkpoints can regulate the quality and rate of cell division and several agents are now under development [17]. In cancer cell lines, inducing cells to leave the cycle or cell-cycle arrest constitutes one of the most prevalent strategies used to stop or limit cancer spreading. In CaSki cells, compound **9** did not affect the cell-cycle, implying that its antiproliferative activity is independent of the cell-cycle phases (Fig. 3A), only showing 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. Regarding ViBo (Fig. 3B) and HeLa (Fig. 3C) cells, arrest in the G_2/M and S phases respectively was identified, suggesting that the activity is slightly dependent on the phases [18].

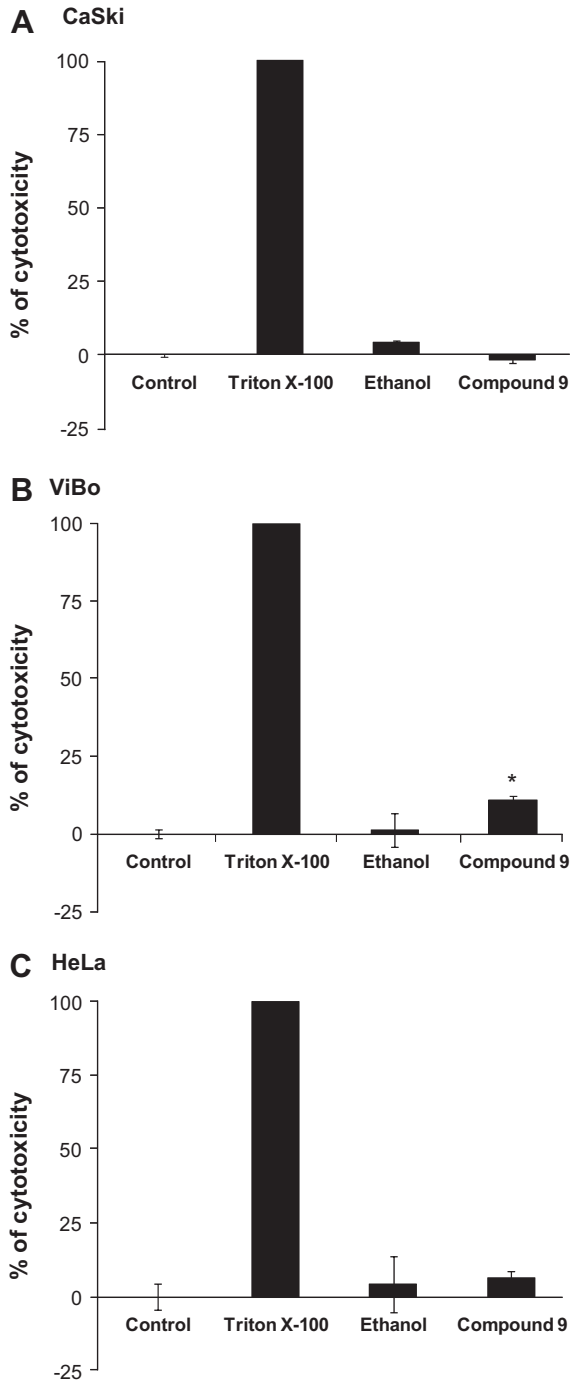

Fig. 4. Evaluation of cytotoxicity of compound **9** on (A) CaSki, (B) ViBo, and (C) HeLa cultures. 7500 cells/well were seeded in 96-well tissue culture plates. After 24 h the medium was removed and cells were exposed to compound **9** at the respective IC_{50} concentrations or with ethanol (10 μL/mL) and evaluated after 24 h by the amount of LDH released in the culture supernatant. Experimental data are presented as the mean ± S.D. of three independent experiments with three repetitions, $^*p < 0.05$ ethanol *versus* compound **9** (Student's *t*-test).

3.4. Determination of cytotoxic activity of compound 9 on cervicouterine cancer cells and human lymphocytes

It is crucial to determine the selectivity using the cytotoxicity and apoptosis assays in order to derive any conclusions on the potential for anticancer treatment. In order to evaluate if necrosis was induced, the cytotoxic activity of compound **9** was evaluated. CaSki, ViBo, and HeLa cultures were exposed to compound **9** at the respective IC_{50} concentration, and the amount of lactate dehydrogenase (LDH) released in the culture supernatant was used as a measure of loss of plasma-membrane integrity. The three cancer lines were treated with the nonionic surfactant Triton X-100 in independent experiments, and the released LDH was adjusted to 100% as a control [19]. Compound **9** induced weak cytotoxicity, 1% of the cases for CaSki, 11% for ViBo, and 6% for HeLa cells (Fig. 4A–C). These results suggest that compound **9** presents null cytotoxicity in all three lines, especially CaSki and HeLa, indicating that the cell death induced by compound **9** is a process different from necrosis.

Cytotoxicity was evaluated next on human lymphocytes in order to determine if necrosis is induced by **9** in non-tumor cells (Fig. 5). Lymphocytes were activated with PHA and stimulated with 33.9 μM of **9**. Results indicated that such concentrations are not cytotoxic to human lymphocytes, and suggested a selective activity through a different pathway than necrosis. Fig. 5.

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

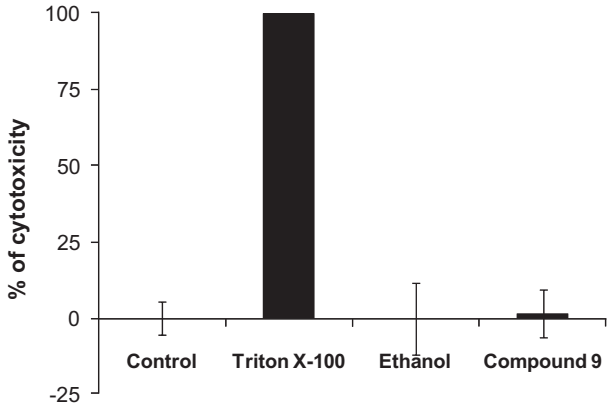

Fig. 5. Evaluation of cytotoxicity of compound **9** on human lymphocyte cultures. Lymphocytes were seeded in 96-well tissue culture plates, exposed to compound **9** at the respective IC_{50} concentrations or with ethanol (10 μL/mL) and evaluated after 72 h by the amount of LDH released in the culture supernatant. Activated lymphocytes with PHA (15 μL/mL) were used as a control. Experimental data are presented as the mean ± S.D. of three independent experiments with three repetitions.

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 [20].

3.5.1. Expression 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 [21]. Active caspase-3 expression was determined by immunocytochemistry. Fig. 6 shows that **9** induced the expression of active caspase-3 in all cell lines, implying that apoptosis could be triggered.

3.5.2. DNA fragmentation assessments

One of the characteristics of apoptosis is the degradation of DNA. The TUNEL method identifies apoptotic cells *in situ* by using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to these strand breaks of cleaved DNA [22]. Fig. 7

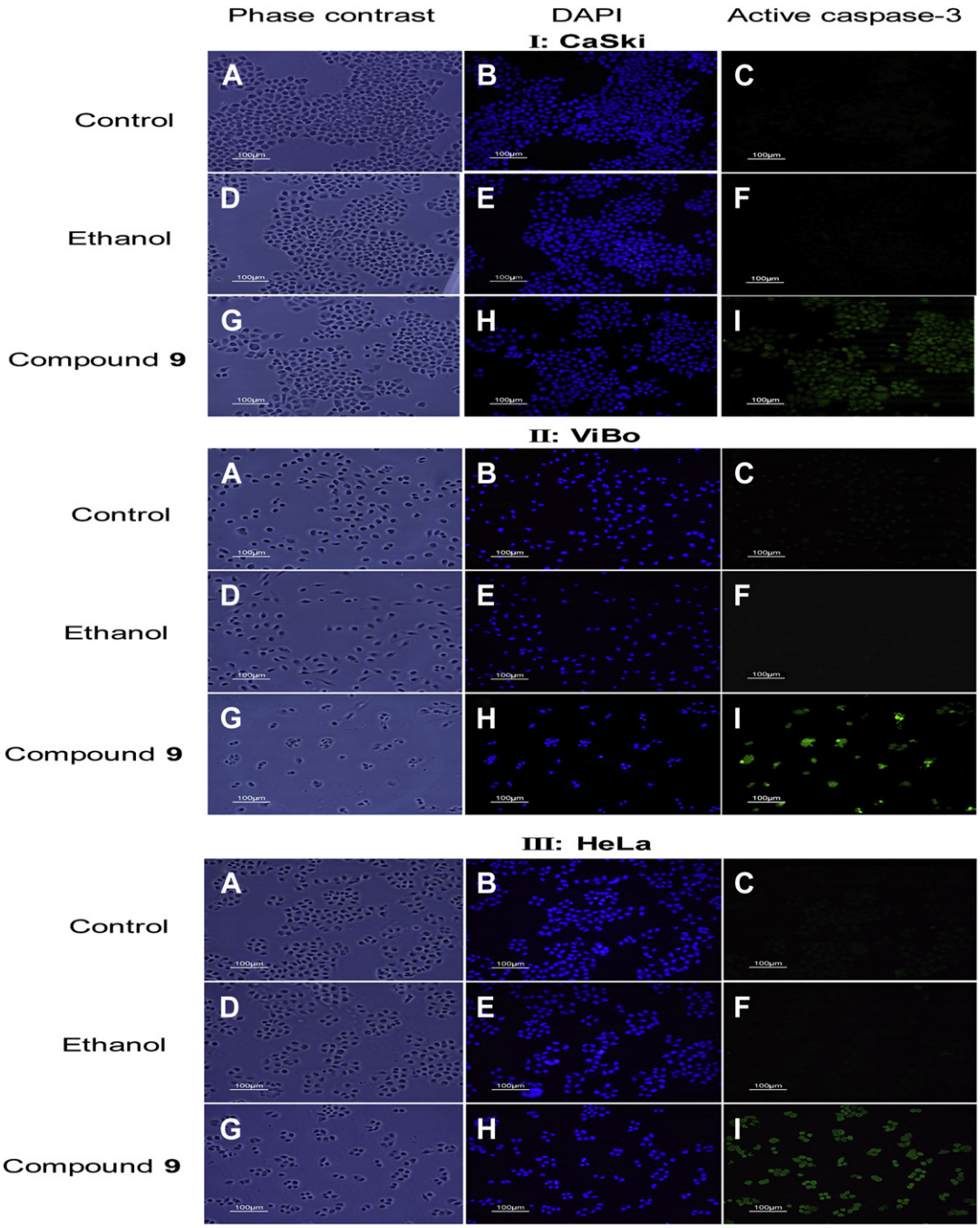

Fig. 6. Immunodetection of active caspase-3 on CaSki (I), ViBo (II), and HeLa (III) cultures. (A), (D) and (G) Cells in phase contrast. (B), (E) and (H) Blue fluorescence indicates cells counterstained with DAPI. (C), (F) and (I) Green fluorescence indicates the presence of active caspase-3 distributed in the cytoplasm of apoptotic cells, induced by the glycoside **9**. The images were obtained using an epifluorescence microscope, and correspond to an experiment representative of three independent assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

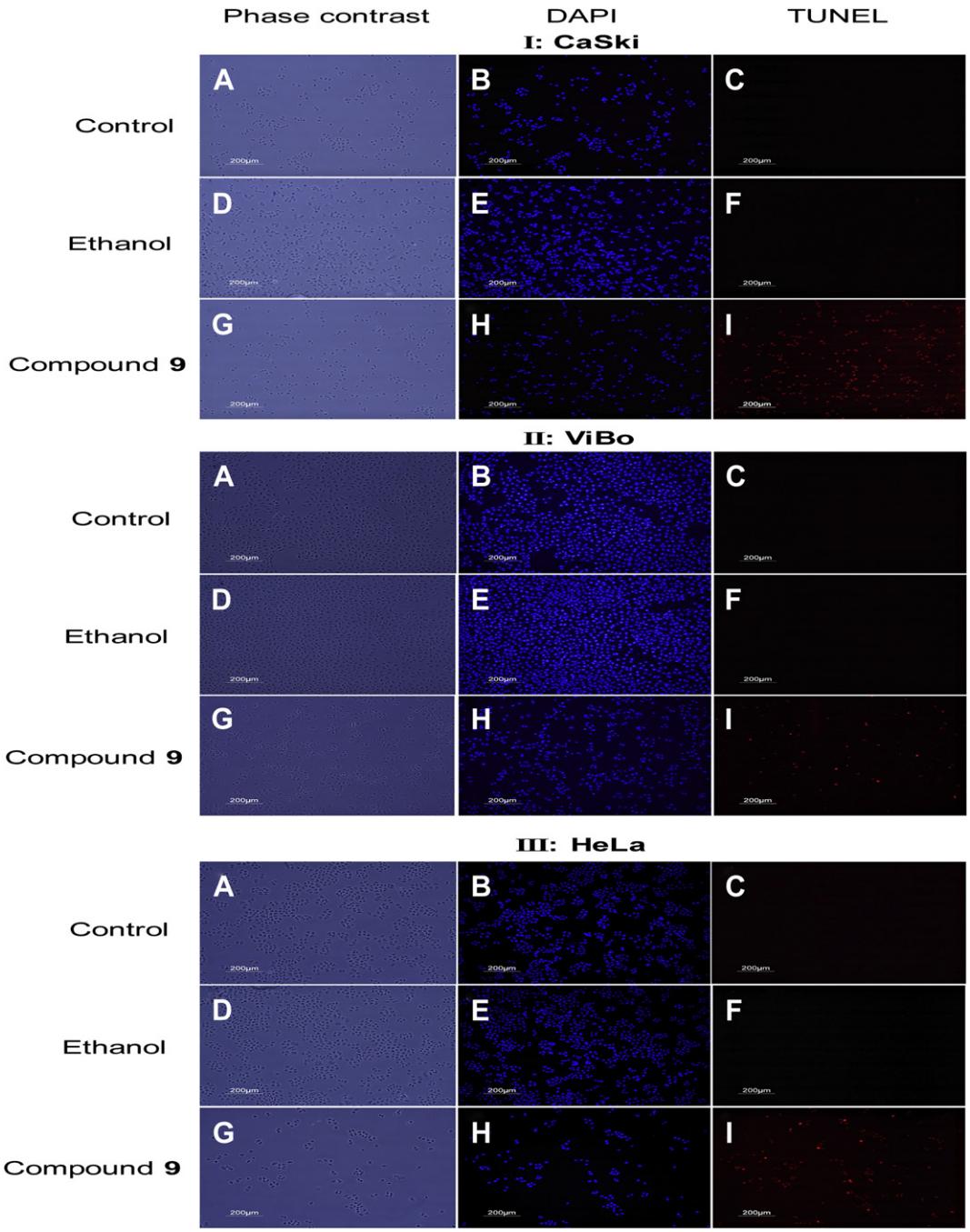

Fig. 7. Compound **9** induces apoptotic death. CaSki (I), ViBo (II) and HeLa (III) cells were cultured with and without compound **9** (IC_{50}) for 24 h. Ethanol (10 μL/mL) was used as a control. DNA fragmentation was detected by TUNEL assay as described in Experimental procedures. (A), (D) and (G) Cells in phase contrast. (B), (E) and (H) Blue fluorescence indicates cells counterstained with DAPI. (C), (F) and (I) Red fluorescence indicates cells positive to the technique. The images were obtained using an epifluorescence microscope, and correspond to an experiment representative of three independent assays. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

shows that fragmentation of DNA in CaSki, ViBo, and HeLa cells is induced by compound **9**. These results indicate that the apoptotic event induced by **9** is completed in a 24 h period, suggesting a short period for the completion of the apoptosis induced by **9**.

4. Conclusions

In summary, the cholestanic 22-keto-26-glycoside **9** was synthesized from the steroidal sapogenin diosgenin, through an excellent method to open spirostanic skeletons. Compound **9** showed anticancer and selective activity against CaSki, ViBo, and HeLa cervicouterine cancer cell lines, with greater antiproliferative activity against CaSki cells than the de-*O*-glucosylated compound **2**. The biological evaluations suggest that **9** is a potent apoptosis inducer with a null cytotoxic consequence. In addition, the proliferation of peripheral blood lymphocytes was not affected, indicative of an immunostimulatory effect. These *in vitro* results certainly augur well for testing in other cancer cell lines and *in vivo* assays in the next stage of the research. We believe, therefore, that this glycoside serves as a promising lead candidate for further evaluation.

5. Experimental section

5.1. Materials

Optical rotations were measured at 24 °C on 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 1D and 2D experiments (APT, 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. (25R)-3β,16β-Diacetoxy-22-oxocholest-5-en-26-yl 2,3,4,6-tetra-O-benzyl-β-D-glucopyranoside (**8**)

A mixture of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose **6** (513 mg, 0.95 mmol) [9b,10], CCl_3CN (0.48 mL, 4.74 mmol), and DBU (15 μL, 0.1 mmol) in CH_2Cl_2 (5 mL) was stirred at room temperature for 3 h. The mixture was concentrated under vacuum and the resulting residue was purified by flash column chromatography with hexane/ethyl acetate/triethylamine (90:9:1) as eluent to give the corresponding trichloroacetimidate **7** as a syrup (575 mg, 89%). This compound was used immediately in the glycosylation reaction without any further purification. A mixture of the donor **7** (409 mg, 0.60 mmol), aglycon **2** (258 mg, 0.50 mmol) and 4 Å MS (200 mg) in dry CH_2Cl_2 (10 mL) was stirred at room temperature for 15 min and then cooled to −20 °C. A solution of TMSOTf in CH_2Cl_2 (10 μL, 0.05 mmol) was added slowly to the reaction mixture. After stirring for 1 h, the reaction was quenched by addition of triethylamine (0.1 mL), and filtered. The filtrate was concentrated under vacuum to give a residue of the crude β-anomer **8** which was purified by flash column chromatography with hexane/ethyl acetate (7:3) as eluent to afford a colorless foam (462 mg, 89%). $[\alpha]_D$ + 7.0° (*c* 1.1, $CHCl_3$). IR: 2938 (CH, aliphatic), 1727 (C=O, acetate), 1710 (C=O, ketone), 1505, 1086 and 3067 (CH aromatics). ^{1}H NMR ($CDCl_3$): δ 7.34-7.12 (20H, m, aromatics), 5.34 (1H, d, $J_{6,7eq}$ = 4.8 Hz, H-6), 4.95 (1H, m, H-16), 4.93 (1H, d, J_{gem} = 11.0 Hz, PhC$\underline{H}_2$-O-2′a), 4.90 (1H, d, J_{gem} = 10.9 Hz, PhC$\underline{H}_2$-O-3′a), 4.79 (1H, d, J_{gem} = 10.8 Hz, PhC$\underline{H}_2$-O-4′a), 4.76 (1H, d, J_{gem} = 10.9 Hz, PhC$\underline{H}_2$-O-3′b), 4.69 (1H, d, J_{gem} = 11.0 Hz, PhC$\underline{H}_2$-O-2′b), 4.59 (1H, d, J_{gem} = 12.6 Hz, PhC$\underline{H}_2$-O-6′a), 4.58 (1H, m, H-3), 4.53 (1H, d, J_{gem} = 12.6 Hz, PhC$\underline{H}_2$-O-6′b), 4.51 (1H, d, J_{gem} = 10.8 Hz, PhC$\underline{H}_2$-O-4′b), 4.35 (1H, d, $J_{1',2'}$ = 7.8 Hz, H-1′), 3.75 (1H, dd, $J_{26a,25}$ = 6.2 Hz, J_{gem} = 9.5 Hz, H-26a), 3.72 (1H, dd, J_{gem} = 10.8 Hz, $J_{6'a,5'}$ = 1.9 Hz, H-6′a), 3.66 (1H, dd, J_{gem} = 10.8 Hz, $J_{6'b,5'}$ = 4.9 Hz, H-6′b), 3.62 (1H, dd, $J_{3',2'}$ = 9.0 Hz, $J_{3',4'}$ = 11.2 Hz, H-3′), 3.56 (1H, dd, $J_{4',3'}$ = 11.2 Hz, $J_{4',5'}$ = 9.6 Hz, H-4′), 3.43 (1H, m, H-5′), 3.42 (1H, dd, $J_{2',3'}$ = 9.0 Hz, $J_{2',1'}$ = 7.8 Hz, H-2′), 3.38 (1H, dd, $J_{26b,25}$ = 5.5 Hz, J_{gem} = 9.5 Hz, H-26b), 2.91 (1H, dq, $J_{20,21}$ = 7.1 Hz, $J_{20,17}$ = 10.9 Hz, H-20), 2.61 (1H, ddd, J_{gem} = 17.7 Hz, $J_{23a,24b}$ = 10.0 Hz, $J_{23a,24a}$ = 5.5 Hz, 23a), 2.39 (1H, m, H-15b), 2.33 (1H, m, H-23b), 2.29 (2H, m, 4*ax* and 4*eq*), 2.01 (3H, s, C$\underline{H}_3CO_2$-3), 1.92 (3H, s, C$\underline{H}_3CO_2$-16), 1.35 (1H, m, H-24b), 1.24 (1H, ddd, $J_{12ax,11ax}$ = J_{gem} = 12.7 Hz, $J_{12ax,11eq}$ = 4.0 Hz, H-12*ax*), 1.08 (3H, d, $J_{21,20}$ = 7.2 Hz, CH_3-21), 1.00 (3H, s, CH_3-19), 0.95 (3H, d, $J_{27,25}$ = 6.6 Hz, CH_3-27), 0.83 (3H, s, CH_3-18). ^{13}C NMR ($CDCl_3$): δ 36.8 (C-1), 27.7 (C-2), 73.8 (C-3), 38.0 (C-4), 139.6 (C-5), 122.3 (C-6), 31.5 (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), 53.8 (C-14), 34.8 (C-15), 75.7 (C-16), 55.0 (C-17), 13.2 (C-18), 19.2 (C-19), 43.5 (C-20), 16.7 (C-21), 212.8 (C-22), 38.7 (C-23), 27.1 (C-24), 33.2 (C-25), 74.8 (C-26), 17.0 (C-27), 103.6 (C-1′), 82.2 (C-2′), 84.7 (C-3′), 77.9 (C-4′), 74.8 (C-5′), 68.9 (C-6′), 170.5 ($CH_3\underline{C}O_2$-3), 169.7 ($CH_3\underline{C}O_2$-16), 21.4 (C$\underline{H}_3CO_2$-3), 21.1 (C$\underline{H}_3CO_2$-16), 128.3–127.5 (C$\underline{H}$-aromatics), 138.6 ($\underline{C}_{ipso}$-PhCH$_2$-O-3′), 138.4 ($\underline{C}_{ipso}$-PhCH$_2$-O-2′), 138.1 ($\underline{C}_{ipso}$-PhCH$_2$-O-6′), 138.0 ($\underline{C}_{ipso}$-PhCH$_2$-O-4′), 74.8 (Ph$\underline{C}$H$_2$-O-2′), 75.6 (Ph$\underline{C}$H$_2$-O-3′), 75.0 (Ph$\underline{C}$H$_2$-O-4′), 73.4 (Ph$\underline{C}$H$_2$-O-6′). HRMS Calcd for $C_{65}H_{83}O_{11}$: 1039.5935, $[M + H]^+$. Found: 1039.5927.

5.2.2. (25R)-3β,16β-Diacetoxy-22-oxocholest-5-en-26-yl β-D-glucopyranoside (**9**)

To a solution of **8** (300 mg, 0.29 mmol) in MeOH:AcOEt (7:3) was added 10% Pd on carbon (200 mg) and the mixture was stirred under hydrogen for 2 h at room temperature. The catalyst was removed by filtration and the filtrate was evaporated to dryness. The residue was purified by flash column chromatography using $CHCl_3$/MeOH (93:7) as eluent to give **9** as a white powder (196 mg, 89%). $[\alpha]_D$ + 10.0° (*c* 0.3, $CHCl_3$). IR: 3390 (OH), 2939 (CH, aliphatic), 1724 (C=O, acetate), 1711 (C=O, ketone). ^{1}H NMR (CD_3OD): δ 5.37 (1H, m_{broad}, H-6), 4.98 (1H, m, H-16), 4.53 (1H, m, H-3), 4.23 (1H, d, $J_{1',2'}$ = 7.7 Hz, H-1′), 3.86 (1H, d, J_{gem} = 12.0 Hz, H-6′a), 3.73 (1H, dd, $J_{26a,25}$ = 6.0 Hz, J_{gem} = 9.6 Hz, H-26a), 3.66 (1H, dd, J_{gem} = 12.0 Hz, $J_{6'a,5'}$ = 5.4 Hz, H-6′b), 3.35 (1H, m, H-3′), 3.35 (1H, m, H-26b), 3.31 (1H, m, H-4′), 3.26 (1H, m, H-5′), 3.20 (1H, dd, $J_{2',3'}$ = 8.4 Hz, $J_{2',1'}$ = 7.7 Hz, H-2′), 3.01 (1H, dq, $J_{20,21}$ = 7.1 Hz, $J_{20,17}$ = 10.5 Hz, H-20), 2.72 (1H, m, 23a), 2.42 (1H, m, H-23b), 2.36 (1H, dd, $J_{15a,16}$ = $J_{15a,14}$ = 6.8 Hz, H-15a), 2.31 (1H, d, $J_{4ax,3}$ = 7.6 Hz, H-4*ax*), 2.31 (1H, m, H-4*eq*), 2.01 (3H, s, C$\underline{H}_3CO_2$-3), 1.97 (3H, s, C$\underline{H}_3CO_2$-16), 1.15 (3H, d, $J_{21,20}$ = 7.1 Hz, CH_3-21), 1.04 (3H, s, CH_3-19), 0.92 (3H, d, $J_{27,25}$ = 6.3 Hz, CH_3-27), 0.90 (3H, s, CH_3-18). ^{13}C NMR (CD_3OD): δ 37.8 (C-1), 28.5 (C-2), 75.1 (C-3), 38.8 (C-4), 140.6 (C-5), 123.1 (C-6), 32.5 (C-7), 32.2 (C-8), 50.9 (C-9), 37.4 (C-10), 21.6 (C-11), 40.6 (C-12), 42.8 (C-13), 54.9 (C-14), 35.5 (C-15), 76.7 (C-16), 56.3 (C-17), 13.7 (C-18), 19.7 (C-19), 44.5 (C-20), 17.3 (C-21), 216.0 (C-22), 39.4 (C-23), 28.0 (C-24), 33.9 (C-25), 75.7 (C-26), 17.1 (C-27), 104.2 (C-1′), 74.7 (C-2′), 77.7 (C-3′), 71.3 (C-4′), 77.4 (C-5′), 62.5 (C-6′), 172.2 ($CH_3\underline{C}O_2$-3), 171.6 ($CH_3\underline{C}O_2$-16), 21.5 (C$\underline{H}_3CO_2$-3), 21.4 (C$\underline{H}_3CO_2$-16). HRMS Calcd for $C_{37}H_{59}O_{11}$: 679.4057, $[M + H]^+$. Found: 679.4051.

5.3. Biological activity

5.3.1. Cell culture

CaSki, ViBo, and HeLa cell lines were purchased from the American Type Culture Collection (ATCC Rockville, MD) and were cultured in RPMI-1640 medium (GIBCO, USA) containing 5% Newborn Calf Serum (NCS, GIBCO, USA) with phenol red 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 33.9 μM or 29.5 μM of **9**. 1% of vehicle (ethanol) was added to control cells. Antiproliferative activity (IC_{50}) was determined after 24 h by crystal violet staining [13]. 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. 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 adherent cells (cells were incubated at 37 °C, 5% CO_2 for 1 h, and non-adherent cells were harvested). ELPs were re-suspended in 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 5 times with sterile PBS containing 10% NCS, counted, and re-suspended in RPMI-1640 medium at 1×10^6 cells/mL [14]. 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 CellQuest (Becton–Dickinson) software.

5.3.4. Cell-cycle analysis

CaSki, ViBo, and HeLa 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 33.9 μM or 29.5 μM of **9**. Cells were harvested with versene 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 (FACS Aria II, B.D., USA) [18].

5.3.5. Determination of cytotoxicity

The cytotoxic activity was determined by means of the 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].

5.3.6. Detection of active caspase-3 from apoptotic cells

CaSki, ViBo, and HeLa cells were seeded at 5×10^4 cells/mL in 300 μL of RPMI-1640 containing 5% NCS for 24 h. Cells were treated with 33.9 μM or 29.5 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). Cells were then washed with PBS and blocked with PBS-SAA-tween. Anti-active caspase-3, antibody (rabbit polyclonal antibody 1:50 in PBS, Sigma–Aldrich, USA) was added. Samples were washed with PBS and the secondary goat anti-rabbit antibody with fluorescein isothiocyanate (FITC) 1:200 in PBS was added. Samples were washed again with PBS and DAPI fluorochrome was added. Analyses were performed by epifluorescence microscopy (Nikon) [23].

5.3.7. DNA fragmentation by TUNEL assay

Detection of DNA fragmentation was performed by TUNEL assay using the Apoptag Red *in situ* apoptosis detection kit [22]. 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 **9** for 24 h. The cells were then 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.

5.3.8. 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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References

[1] (a) K. Hostettmann, A. Marston, Saponins: Chemistry and Pharmacology of Natural Products. Cambridge University Press, London, 1995;
(b) G.R. Waller, K. Yamasaki (Eds.), Saponins Used in Food and Agriculture, Plenum Press, New York, 1996;
(c) G.R. Waller, K. Yamasaki (Eds.), Saponins Used in Traditional and Modern Medicine, Plenum Press, New York, 1996;
(d) H. Pellissier, Tetrahedron 60 (2004) 5123–5162;
(e) D.B. Salunke, B.G. Hazra, V.S. Pore, Curr. Med. Chem. 13 (2006) 813–847;
(f) G. Francis, Z. Kerem, H.P.S. Makkar, K. Becker, Br. J. Nutr. 88 (2002) 587–605.
[2] N. Nakashima, I. Kimura, M. Kimura, H. Matsuura, J. Nat. Prod. 56 (1993) 345–350.
[3] (a) A. Niwa, O. Takeda, M. Ishimaru, Y. Nakamoto, K. Yamasaki, H. Kohda, H. Nishio, T. Segawa, K. Fujimura, A. Kuramoto, Yakugaku Zasshi 108 (1988) 555–561;
(b) D. Jun-Xing, H.G. Yu, Planta Med. 57 (1991) 460–462.
[4] C.R. Yang, Y. Zhang, M.R. Jacob, S.I. Khan, Y.J. Zhang, X.C. Li, Antimicrob. Agents Chemother. 50 (2006) 1710–1714.
[5] L.A. Tapondjou, K.B. Ponou, R.B. Teponno, M. Mbiantcha, J.D. Djoukeng, T.B. Nguelefack, P. Watcho, A. Gomez-Cadenas, H.J. Park, Arch. Pharm. Res. 31 (2008) 653–658.
[6] J. Cai, M. Liu, Z. Wang, Y. Ju, Biol. Pharm. Bull. 25 (2002) 193–196.
[7] (a) G. Mehta, V. Singh, Chem. Soc. Rev. 31 (2002) 324–334;
(b) L.F. Tietze, H.P. Bell, S. Chandrasekhar, Angew. Chem. Int. Ed. 42 (2003) 3996–4028.
[8] (a) J. Sandoval-Ramírez, A. Castro-Méndez, S. Meza-Reyes, F. Reyes-Vázquez, R. Santillán, N. Farfán, Tetrahedron Lett. 40 (1999) 5143–5146;
(b) S. Rincón, R.E. del Río, J. Sandoval-Ramírez, S. Meza-Reyes, S. Montiel-Smith, M.A. Fernández-Herrera, N. Farfán, R. Santillán, Tetrahedron 62 (2006) 2594–2602;
(c) M.A. Fernández-Herrera, J. Sandoval-Ramírez, S. Meza-Reyes, S. Montiel-Smith, J. Mex, Chem. Soc. 53 (2009) 126–130;
(d) M.A. Fernández-Herrera, J. Sandoval-Ramírez, H. López-Muñoz, L. Sánchez-Sánchez, Arkivoc xiii (2009) 170–184;
(e) M.G. Hernández-Linares, J. Sandoval-Ramírez, S. Meza-Reyes, S. Montiel-Smith, M.A. Fernández-Herrera, S. Bernès, Steroids 75 (2010) 240–244.
[9] (a) M.A. Fernández-Herrera, H. López-Muñoz, J.M.V. Hernández-Vázquez, M. López-Dávila, M.L. Escobar-Sánchez, L. Sánchez-Sánchez, B.M. Pinto, J. Sandoval-Ramírez, Bioorg. Med. Chem. 18 (2010) 2474–2484;
(b) M.A. Fernández-Herrera, S. Mohan, H. López-Muñoz, J.M.V. Hernández-Vázquez, E. Pérez-Cervantes, M.L. Escobar-Sánchez, L. Sánchez-Sánchez, I. Regla, B.M. Pinto, J. Sandoval-Ramírez, Eur. J. Med. Chem. 45 (2010) 4827–4837;
(c) J.O.H. Pérez-Díaz, J.L. Vega-Baez, J. Sandoval-Ramírez, S. Meza-Reyes, S. Montiel-Smith, N. Farfán, R. Santillán, Steroids 75 (2010) 1127–1136.
[10] M. Numata, M. Sugimoto, K. Koike, T. Ogawa, Carbohydr. Res. 163 (1987) 209–225.
[11] R.R. Schmidt, A. Toepfer, Tetrahedron Lett. 32 (1991) 3353–3356.
[12] H. Miyashita, Y. Kay, T. Nohara, T. Ikeda, Carbohydr. Res. 343 (2008) 1309–1315.

[13] W. Kueng, E. Silber, U. Eppenberger, Anal. Biochem. 182 (1989) 16–19.
[14] A.B. Lyons, J. Hasbold, P.D. Hodgkin, Methods Cell Biol. 63 (2001) 375–398.
[15] C.J. Sherr, Cancer Res. 60 (2000) 3689–3695.
[16] F. McLaughlin, P. Finn, N.B. La Thangue, Drug Discov. Today 8 (2003) 793–802.
[17] (a) A.M. Senderowicz, E.A. Sausville, J. Natl. Cancer Inst. 92 (2000) 376–387;
(b) J.R. Jackson, A. Gilmartin, C. Imburgia, J.D. Winkler, L.A. Marshall, A. Roshak, Cancer Res. 60 (2000) 566–572;
(c) Y. Hirose, M.S. Berger, R.O. Pieper, Cancer Res. 61 (2001) 5843–5849;
(d) B. Carlson, T. Lahusen, S. Singh, A. Loaiza-Perez, P.J. Worland, R. Pestell, C. Albanese, E.A. Sausville, A.M. Senderowicz, Cancer Res. 59 (1999) 4634–4641;
(e) L.H. Hartwell, T.A. Weinert, Science 246 (1989) 629–634.
[18] (a) H.M. Shapiro, Practical Flow Cytometry, second ed.. Alan R. Liss, Inc, New York, 1988, 353;
(b) Z. Darzynkiewicz, Nucleic acid analysis. in: J.P. Robinson (Ed.), Current Protocols in Cytometry. John Wiley & Sons, Inc, New York, 1997 (Chapter 7).
[19] C. Legrand, J.M. Bour, C. Jacob, J. Capiaumont, A. Martial, A. Marc, M. Wudtke, G. Kretzmer, C. Demangel, D. Duval, J. Hache, J. Biotechnol. 25 (1992) 231–243.
[20] (a) M.O. Hengartner, Nature 407 (2000) 770–776;
(b) S. Roy, D.W. Nicholson, J. Exp. Med. 192 (2000) F21–F26;
(c) C.B. Thompson, Science 267 (1995) 1456–1462;
(d) M.D. Jacobson, M. Weil, M.C. Raff, Cell 88 (1997) 347–354.
[21] (a) D.W. Nicholson, N.A. Thornberry, Trends Biochem. Sci. 22 (1997) 299–306;
(b) V. Cryns, J. Yuan, Genes Dev. 12 (1998) 1551–1570;
(c) N.A. Thornberry, Y. Lazebnik, Science 281 (1998) 1312–1316;
(d) H.R. Stennicke, J.M. Jürgensmeier, H. Shin, Q. Deveraux, B.B. Wolf, X. Yang, Q. Zhou, H.M. Ellerby, L.M. Ellerby, D. Bredesen, D.R. Green, J.C. Reed, C.J. Froelich, G.S. Salvesen, J. Biol. Chem. 273 (1998) 27084–27090;
(e) A.G. Porter, R.U. Jänicke, Cell. Death Differ. 6 (1999) 99–104.
[22] D.T. Loo, TUNEL assay: an overview of techniques. in: V.V. Didenko (Ed.), *In situ* Detection of DNA Damage: Methods and Protocols. Humana Press, Totowa, 2002, p. 21.
[23] M. Krajewska, H.G. Wang, S. Krajewski, J.M. Zapata, A. Shabaik, R. Gascoyne, J.C. Reed, Cancer Res. 57 (1997) 1605–1613.