European Journal of Medicinal Chemistry 45 (2010) 4827–4837

Contents lists available at ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Synthesis of the steroidal glycoside (25*R*)-3β,16β-diacetoxy-12, 22-dioxo-5α-cholestan-26-yl β-D-glucopyranoside and its anti-cancer properties on cervicouterine HeLa, CaSki, and ViBo cells

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ARTICLE INFO

Article history:
Received 8 June 2010
Received in revised form
26 July 2010
Accepted 27 July 2010
Available online 11 August 2010

Keywords:
Steroidal glycoside
HeLa
CaSki
ViBo
Antiproliferative activity
Apoptosis

ABSTRACT

The synthesis of the new glycoside (25*R*)-3β,16β-diacetoxy-12,22-dioxo-5α-cholestan-26-yl β-D-glucopyranoside starting from hecogenin is described. This compound showed anti-cancer activity against cervicouterine cancer cells HeLa, CaSki and ViBo in the micromolar range. Its effect on cell proliferation, cell cycle and cell death is also described. The cytotoxic effect of the title compound on HeLa, CaSki and ViBo cells and human lymphocytes was evaluated through the LDH released in the culture supernatant, indicating that the main cell death process is not necrosis; the null effect on lymphocytes implies that it is not cytotoxic. The ability of this novel glycoside to induce apoptosis was investigated; several apoptosis events like chromatin condensation, formation of apoptotic bodies, as well as the increase in the expression of active caspase-3 and the fragmentation of DNA confirmed that the compound induced apoptosis in cervicouterine cancer cells. Significantly, the antiproliferative activity on tumor cells did not affect the proliferative potential of normal fibroblasts from cervix and peripheral blood lymphocytes. The glycoside showed selective antitumor activity and greater antiproliferative activity than its aglycon; it therefore serves as a promising lead candidate for further optimization.

1. Introduction

Cancer is responsible for about 25% of deaths in developed countries and for 15% of all deaths worldwide. It can therefore be considered as one of the foremost health problems, with about 1.45 million new cancer cases being expected yearly [1]. Tumorigenesis is a multistep process that involves the accumulation of successive mutations in oncogenes and suppressor genes that deregulate the cell cycle [2]. Cancer therapy is based on surgery, radiation therapy, and chemotherapy, which to date are not completely successful interventions. Antitumor research is a very active field, and a large amount of information dealing with clinical aspects of cancer chemotherapy is generated; there is, however, a continuing need for new treatments inspired by medicinal chemistry and drug design. The aim of most cancer chemotherapeutic drugs currently in clinical use is to kill malignant tumor cells by inhibiting some of the mechanisms implied in cellular division. Accordingly, the antitumor compounds developed through this approach are cytostatic or cytotoxic [3].

Chemistry has played important roles in the discovery and development of anti-cancer drugs since the beginning of cancer therapies [4]. Synthetic chemistry has been used extensively to modify drug targets, especially those of natural origin, and to solve the problem of the scarce supply of natural products by developing synthetic strategies. The rationale for the use of conventional cytotoxic antitumor drugs is based on the theory that rapidly proliferating and dividing cells are more sensitive to these compounds than normal cells. About half the drugs currently in clinical use for anti-cancer therapy are of natural origin, and it has been estimated that about 60% of new chemical entities introduced in the 1981–2002 period in this field were natural products or

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doi:10.1016/j.ejmech.2010.07.051

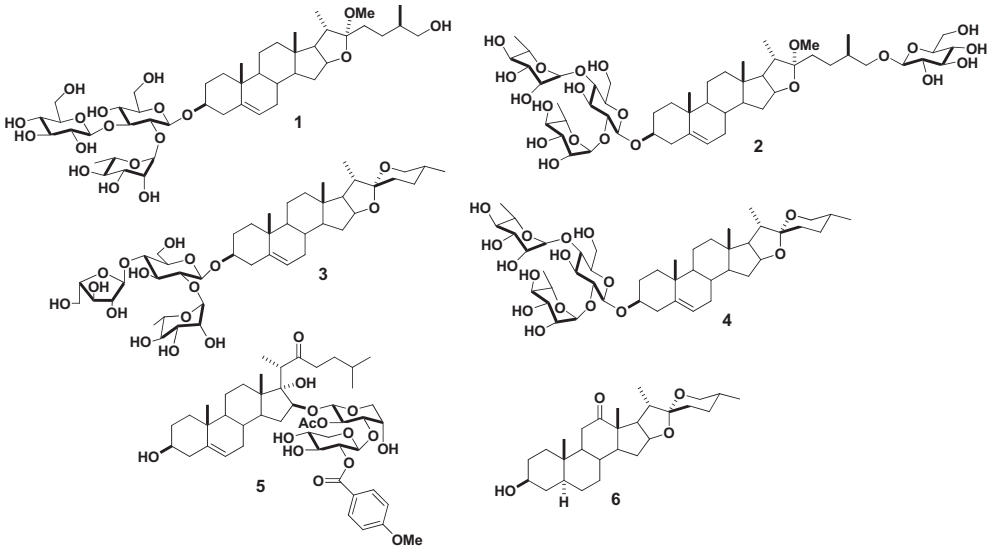
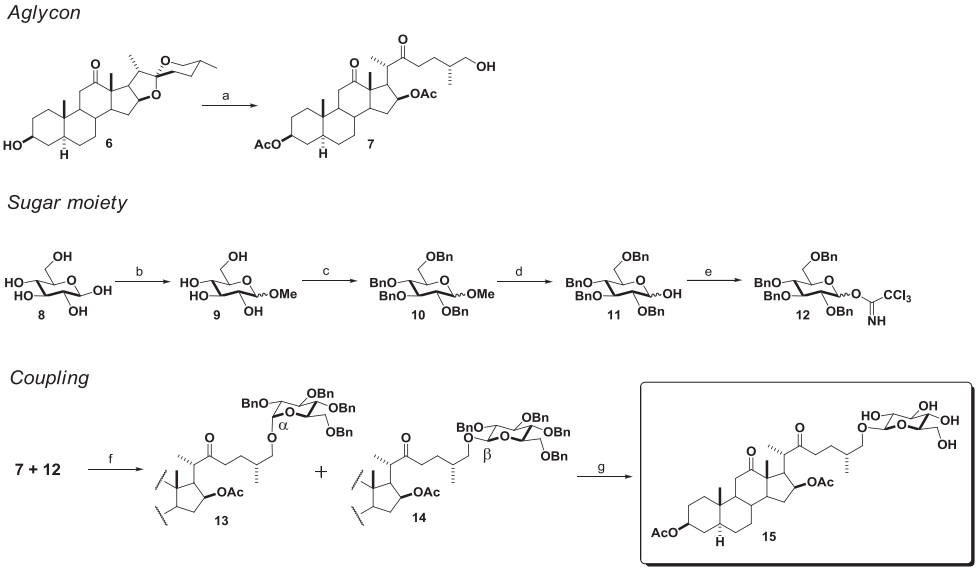


Fig. 1. Steroidal compounds with significant antiproliferative activity.

derived from a natural lead compound [5]. In general, natural products have been optimized to present several functional groups which have specific interactions with target molecules. Consequently, they may be considered as highly advanced lead compounds in which further optimization of activity is difficult. In many cases, some parts of the complex structure of a natural product act only as a framework to position certain functionality, and more simple analogues may be developed without considerable loss of activity. For this reason, structural modification of natural products is often aimed at finding the simplest portion that maintains most of the biological activity, that is, its pharmacophoric unit. Plants, microorganisms, and marine organisms of various types have traditionally represented the main source of cytotoxic anti-cancer agents since the beginning of chemotherapy [6]. Glycosides consist of a sugar moiety linked to an aglycon (frequently steroidal or triterpenic), and occur as constituents of many herbal treatments used in folk medicine, especially in ancient cultures. Consequently, their characterization and investigation of their pharmacological and biological properties is of interest. Steroidal saponins possess varied biological activity such as antimicrobial, piscicidal, molluscicidal, insecticidal, antifeedant, antifertility, plant growth inhibition, antiinflammatory, cytotoxic, and antitumoral. Some steroidal glycosides (Fig. 1), such as icogenin (**1**) [7], methyl protodioscin (**2**) [8], polyphyllin D (**3**) [9] and dioscin (**4**) [10] exhibit high anti-cancer activities. At the end of the last century it was reported that the naturally occurring glycoside OSW-1 [**5**, 3β,17α-dihydroxy-22-oxocholest-5-en-16β-yl 2-*O*-(4-methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2-*O*-acetyl-α-L-arabinopyranoside] [11], a major component of a small group of cholestanic saponins isolated from the bulbs of *Ornithogalum saundersiae*, exhibited 100 times greater anti-cancer activity than taxol [12]. More recently, it was demonstrated that the sapogenins, for example, hecogenin (**6**) [13] exhibit antiproliferative activity and induce apoptosis in several cell lines.



Scheme 1. Synthesis of the aglycon (from hecogenin) and sugar moiety (from glucose) and their coupling to obtain the glycoside **15**. Reagents and conditions: (a) Ac_2O, $BF_3{\cdot}OEt_2$, 0 °C, 86%; (b) AcCl, MeOH, rt, quantitative; (c) BnBr, NaH, DMF, rt, 87%; (d) AcOH, 1 M H_2SO_4, reflux, 55%; (e) CCl_3CN, DBU, CH_2Cl_2, rt, 89%; (f) TMSOTf (0.1 equiv), 4 Å MS, CH_2Cl_2, −20 °C, 93%; (g) 10% Pd/C, H_2, AcOEt-MeOH (v:v, 7:3), 95%.

Table 1
^{1}H (600 MHz) and ^{13}C (150 MHz) NMR spectral data (δ in ppm, d^4-methanol) for the glycoside **15**.

Position	^{1}H	*Mult.*	*J* (Hz)	^{13}C	HMBC	COSY
2*eq*	1.11	*m*		28.3	C-4	H-2*ax*, H-3
2*ax*	1.53	*ddd*	17.0, 4.0, 13.1			H-2*eq*, H-3
3	4.66	*m*		74.8	C-1, C-4, C=O–acetate-3	H-2*eq*, H-2*ax*, H-4*eq*, H-4*ax*
8	1.95	*ddd*	7.2, 3.6, 21.6	36.0	C-6	H-9, H-14, H-7*eq*
9	1.18	*ddd*	13.2, 4.8, 10.8	58.5	C-19	H-8, H-11*eq*, H-11*ax*
10				37.4		
11*eq*	2.16	*dd*	4.8, 12.9	39.3	C-12, C-9, C-8	H-9, H-11*ax*
11*ax*	2.68	*dd*	12.9, 13.2			H-9, H-11*eq*
12				215.6		
13				57.7		
15a	2.46	*m*		35.3	C-13, C-14	H-14, H-16, H-17
15b	1.32	*m*				H-14, H-16, H-17
16	4.98	*ddd*	8.2, 8.2, 8.3	75.6	C-13, C-14, C=O–acetate-16	H-15a, H-15b, H-17
17	2.74	*dd*	14.6, 14.6	47.8	C-22	H-16, H-20
18	1.23	*s*		13.2	C-13, C-14, C-12	
19	0.98	*s*		12.2	C-5, C-9, C-10	
20	2.91	*dq*	7.3, 10.5	45.0	C-22	H-17, H-21
21	1.08	*d*	7.2	17.7	C-22	H-20
22				216.2		
23a	2.77	*m*		39.5	C-22	H-24a, H-24b, H-23b
23b	2.46	*m*				H-24a, H-24b, H-23a
26a	3.75	*dd*	5.8, 9.6	75.9	C-27, C-1′	H-25, H-26a
26b	3.36	*dd*	6.0, 9.6			H-25, H-26b
27	0.94	*d*	6.6	17.3	C-24, C-25, C-26	H-25
Me-acetate-3	1.98	*s*		21.3	C=O–acetate-3	
Me–acetate-16	1.95	*s*		21.2	C=O–acetate-16	
C=O–acetate-3				172.5		
C=O–acetate-16				171.7		
26-*O*-β-D-glucopyranoside						
1′	4.76	*d*	7.8	104.6	C-26	H-2′
2′	3.18	*dd*	9.1, 7.8	75.2		H-1′, H-3′
3′	3.35	*dd*	9.1, 9.1	78.2		H-2′, H-4′
4′	3.28	*dd*	9.1, 9.1	71.7		H-3′, H-5′
5′	3.26	*m*		77.9		H-4′, H-6′a, H-6′b
6′a	3.86	*dd*	1.9, 11.9	62.9		H-5′, H-6′b
6′b	3.66	*dd*	5.3, 11.9			H-5′, H-6′a

This fact has prompted the search for new routes to synthesize OSW-1 on a large scale, and to synthesize analogues for optimization of biological activity. The partial synthesis of OSW-1 (**5**) and analogues has been accomplished in low yields, due to the difficulty in building the 22-oxocholestanic side-chain. Although the aglycon plays the main role on biological activity, it is well known that the sugar moieties also play an important role in the activity of steroidal glycosides, for example, by increasing the solubility in physiological media, assisting with cell permeability and lifetime, and in directing the molecule to the active site [14]. It is of interest, therefore, to search for new variants of steroidal glycosides with selective anti-cancer activity against malignant cells. Transformations of the spiroketal moiety of sapogenins have yielded interesting steroidal structures for partial synthesis [15]. In most cases, ring F of the spiroketal side-chain has been opened to produce pseudosapogenins or furostanes, but less work has been reported on the regioselective fission of ring E. The 22-oxocholestanic side-chain has become a useful target for the preparation of analogues of the potent anti-cancer saponin OSW-1. Recently, we reported an efficient process to open such spirostanic side-chains to produce 22-oxocholestanic frameworks, and synthesized compound **7** (Scheme 1) [16]. We report now the effect of glucosylation at C-26 of **7** on anti-cancer activity against cervicouterine cancer cells.

2. Results and discussion

2.1. Chemical synthesis

For the synthesis of the aglycon, the side-chain of hecogenin (**5**) was opened by treatment with Ac_2O, $BF_3 \cdot OEt_2$, at 0 °C, followed by quenching with water; thus, the (25*R*)-26-hydroxy-12,22-dioxo-5α-cholestan-3β,16β-diyl diacetate (**7**) was obtained in excellent

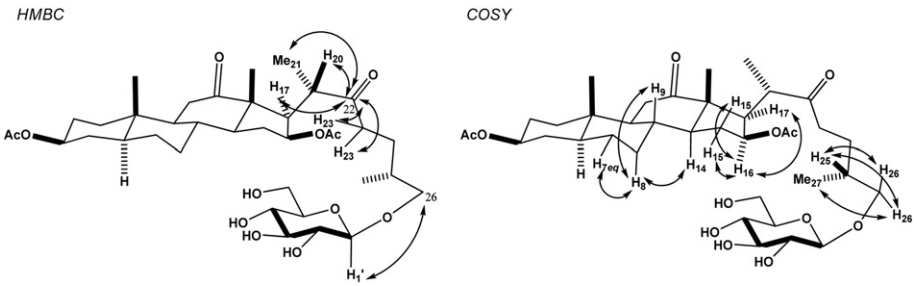


Fig. 2. HMBC and COSY correlations for the glycoside **15**.

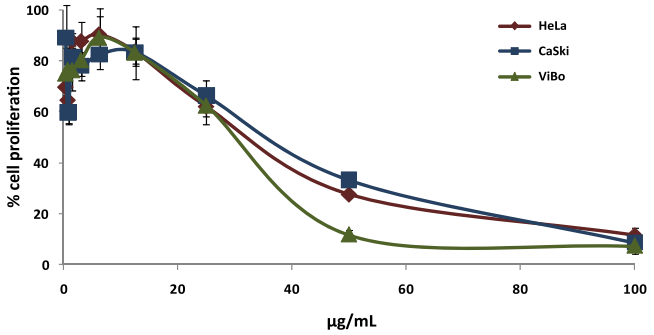


Fig. 3. Dose-response curves of the antiproliferative effect of compound **15** on HeLa, CaSki, and ViBo cells.

yield (86%). For the preparation of the sugar moiety, Fischer glycosidation yielded methyl α-D-glucopyranoside **9** which was benzylated with benzyl bromide and sodium hydride to give **10**. Acidic hydrolysis of the acetal then gave the hemiacetal **11** which was treated with CCl_3CN/DBU [17] to afford the trichloroacetimidate donor **12**, which was used directly in the glycosylation reaction. Trimethylsilyl trifluoromethanesulfonate (TMSOTf)-promoted glycosylation [18] of the trichloroacetimidate **12** with **7** proceeded smoothly to afford a mixture of epimers **13** and **14** in 95% yield. Interestingly, 90% of the mixture corresponded to the β-anomer despite the absence of a participating group at O-2, presumably because of the steric requirements of the aglycon. The mixture was separated by column chromatography to obtain the pure β-anomer (**14**), and a fraction containing an unresolved mixture of α- and β-anomers. Debenzylation of the pure β-isomer (**14**) with 10% Pd/C and hydrogen at atmospheric pressure afforded compound **15**. It is of significance that cytotoxicity bioassays of some steroidal glycosides have shown that the β-anomers exhibited higher activities than the corresponding α-anomers [19] (Scheme 1).

2.2. NMR analysis

Analysis of the ^{1}H NMR spectrum of **15** showed that the signal for the anomeric hydrogen H-1′ at δ 4.76 ppm had a coupling

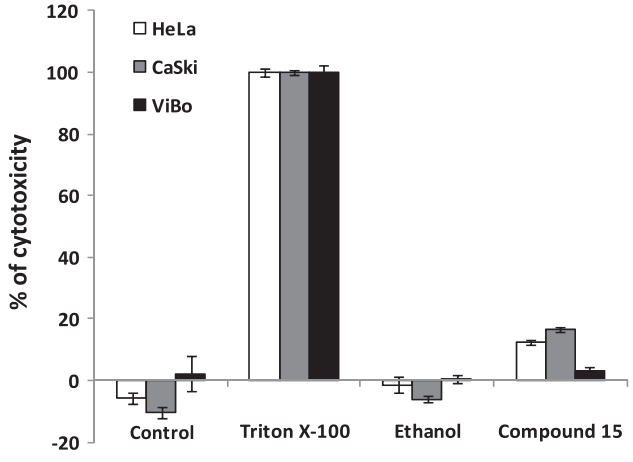


Fig. 4. Evaluation of cytotoxicity of compound **15** on HeLa, CaSki and ViBo cultures. 7500 cells/well were seeded in 96-well tissue culture plates. After 24 h the medium was removed and cells were exposed to compound **15** at the respective IC_{50} concentrations or with ethanol (10 µL/mL) and evaluated 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. $^*p < 0.05$ *versus* ethanol (Student's *t*-test).

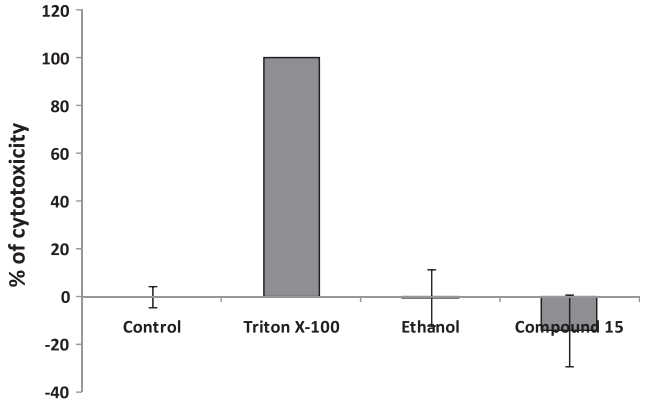


Fig. 5. Evaluation of cytotoxicity of compound **15** on human lymphocyte cultures. Lymphocytes were seeded in 96-well tissue culture plates, exposed to compound **15** at the respective IC_{50} concentrations or with ethanol (10 µL/mL) and evaluated after 72 h by the amount of LDH released in the culture 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. $^*p < 0.05$ *versus* 0 µg/mL (Student's *t*-test).

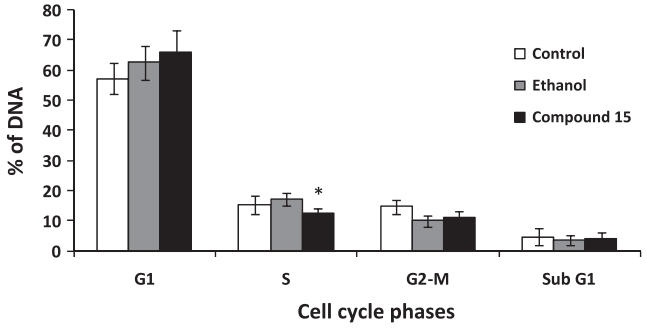


Fig. 6. Cell cycle analysis of HeLa cells upon treatment with compound **15** after 24 h. Values are expressed in distribution of % DNA, $^*p < 0.05$ *versus* ethanol.

constant of 7.8 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 **15**. In addition, selected HMBC and COSY correlations are displayed in Fig. 2. Correlations in the HMBC spectrum were found between H-3 and H-16 with their corresponding acetate carbonyl groups, and H-17, H-20, H-21 and H-23 with C-22. Furthermore, there were correlations between the anomeric carbon (C-1′) and both hydrogens H-26, and between H-1′ and C-26. ^{1}H–^{1}H-COSY correlations were observed between H-16 and peaks at δ 2.46, 1.32 and 2.74 ppm, corresponding to three

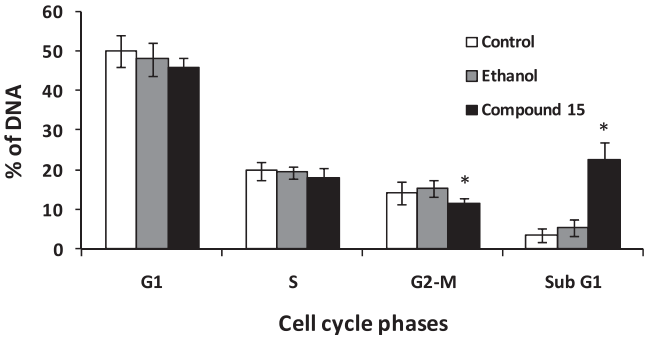


Fig. 7. Cell cycle analysis of CaSki cells upon treatment with compound **15** after 24 h. Values are expressed in distribution of % DNA, $^*p < 0.05$ *versus* ethanol.

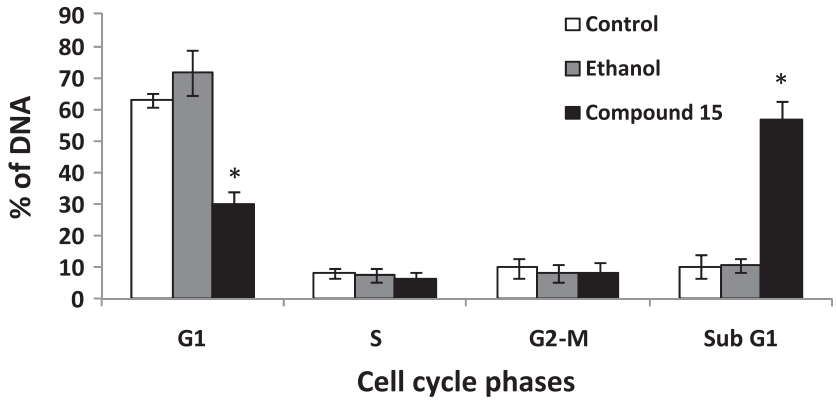


Fig. 8. Cell cycle analysis of ViBo cells during compound **15** treatment after 24 h. Values are expressed in distribution of % DNA, $^{*}p < 0.05$ *versus* ethanol.

different proton signals from CH_2-15 and CH-17. In addition, two correlations were observed between the axial H-8, H-9 and H-14; an additional correlation with H-7*eq* ($J = 3.6$ Hz) was detected. Finally, CH_2-26 and CH_3-27 were coupled with CH-25.

3. Biological evaluation

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

In order to determine the antiproliferative activity of compound **15**, cervicouterine cancer cell lines HeLa, CaSki and ViBo were treated with **15** 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 [20] and the dose–response curves are shown in Fig. 3. The inhibitory effect of **15** on the proliferation of HeLa, CaSki and ViBo cells was observed to occur in a dose-dependent manner with an IC_{50} value of 65 μM (45 μg/mL) for HeLa and CaSki and 42 μM (29 μg/mL) for ViBo cells. Compared to the IC_{50} of the aglycon itself (compound **7**) in CaSki cells [16], the activity of the glycoside **15**, was enhanced (188.0 vs. 42 μM), indicating that glucosylation of **7** promoted bioavailability.

3.2. Determination of cytotoxic activity of compound *15* on cervicouterine cancer cells and human lymphocytes

In order to evaluate whether necrosis was induced, the cytotoxic activity of compound **15** was evaluated (Fig. 4). HeLa, CaSki and ViBo cultures were exposed to compound **15** 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 Triton X-100 in independent experiments, and the released LDH was adjusted to 100% as a control [21]. Compound **15** induced cytotoxicity in 13% of the cases for HeLa, 17% for CaSki and 3% for

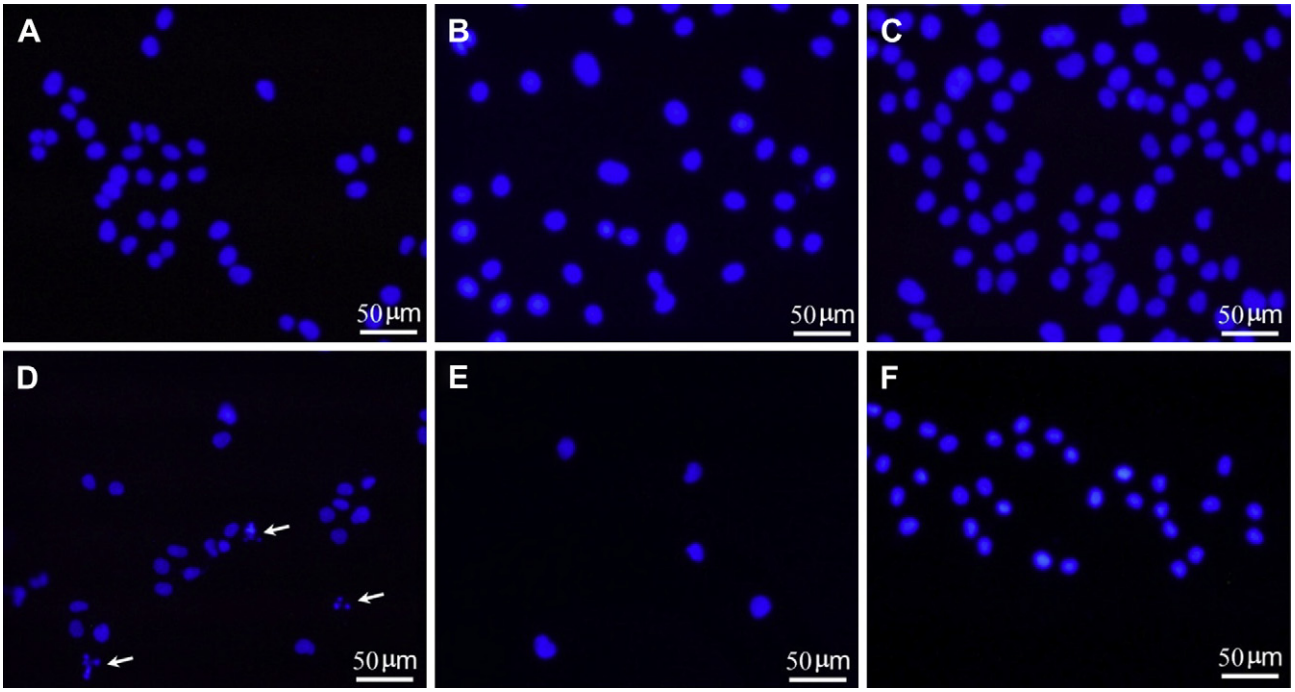


Fig. 9. Chromatin condensation and observation of apoptotic bodies on cell cultures evaluated after 24 h, stained with DAPI. A) Control CaSki cells. B) Control HeLa cells. C) Control ViBo cells. D) CaSki cells treated with **15** (65 μM). E) HeLa cells treated with **15** (65 μM). F) ViBo cells treated with **15** (42 μM). Arrows show classical apoptotic bodies.

ViBo cells. These results suggest that compound **15** has relatively low cytotoxicity in all three lines, especially ViBo. For comparison, in CaSki cells the aglycon **7** induced necrosis [16] in 28% of the cases, implying that the presence of the sugar unit decreases the cytotoxic activity.

Cytotoxicity was evaluated next on human lymphocytes in order to determine if necrosis is induced by **15** in non-tumor cells (Fig. 5). Lymphocytes were activated with phytohemagglutinin (PHA) and stimulated at the level of the determined IC_{50} values of **15**. Results indicated that such concentrations are not cytotoxic to human lymphocytes, and suggested a selective activity through a different pathway than necrosis.

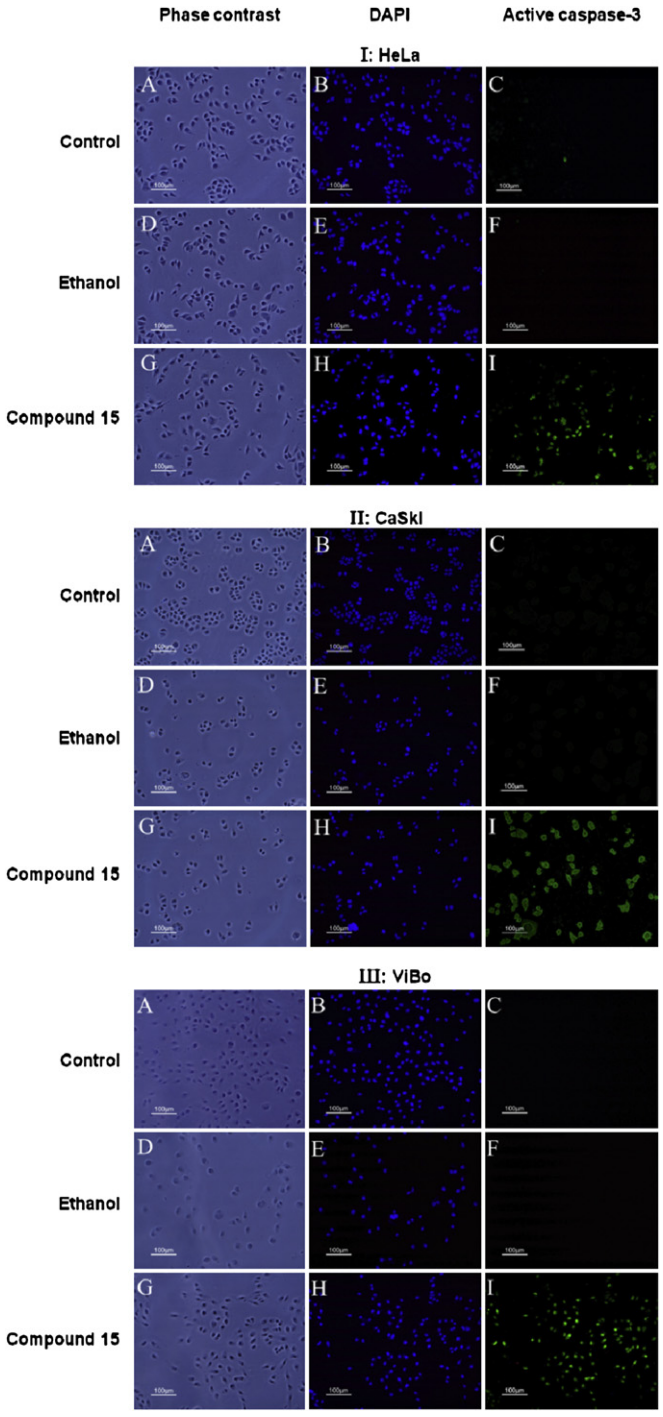


Fig. 10. Immunodetection of active caspase-3 on HeLa (I), CaSki (II) and ViBo (III) cultures. A), D) and G) Cells in phase contrast. B), E) and H) Blue fluorescence indicates cells counterstained with DAPI. C), F) and I) Green fluorescence indicates the presence of active caspase-3 distributed in the cytoplasm of apoptotic cells. The images were obtained using an epifluorescence microscope, and correspond to an experiment representative of three independent assays.

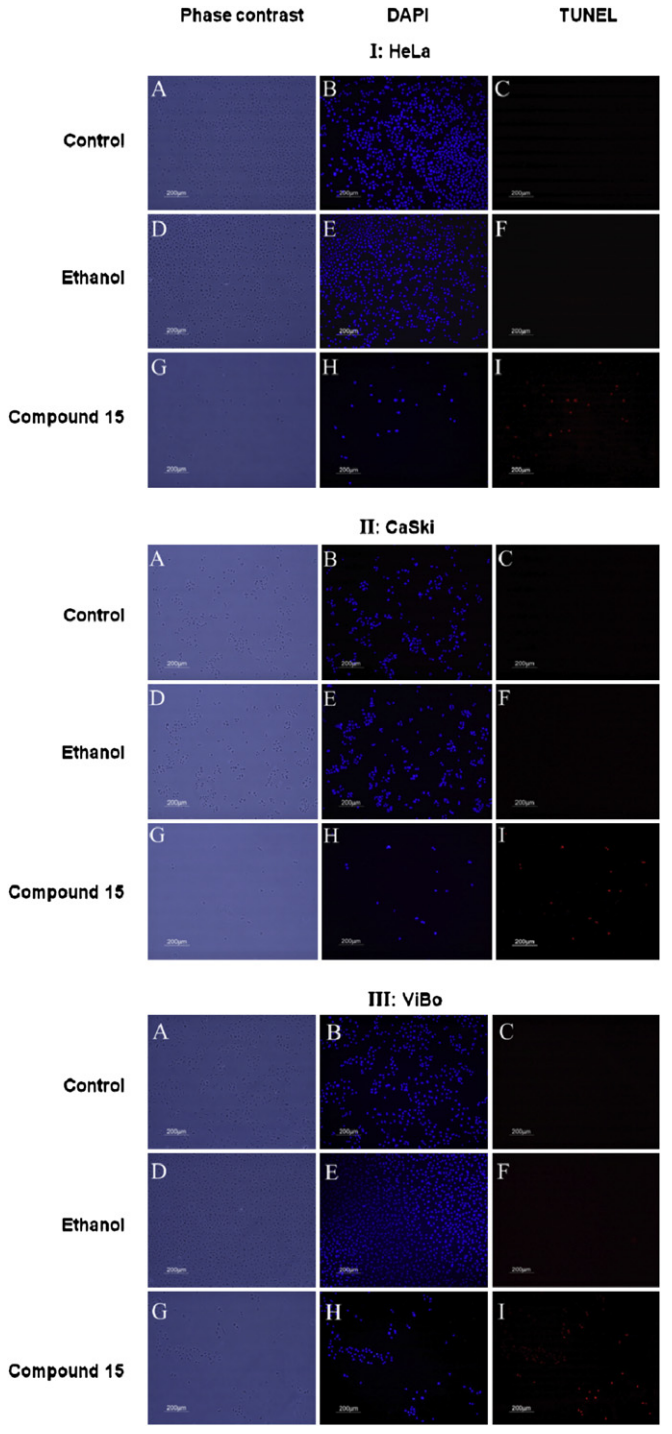


Fig. 11. Compound **15** induces apoptotic death. HeLa (I), CaSki (II) and ViBo (III) cells were cultured with and without Compound **15** (IC_{50}) for 24 h. Ethanol (10 μL/mL) was used as a control. DNA fragmentation was detected by TUNEL assay as described in Experimental procedures. A), D) and G) 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.

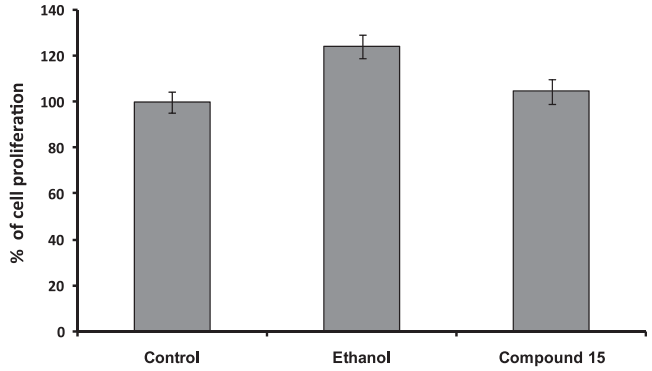


Fig. 12. Effect of compound **15** on the proliferative potential of non-tumor cervical fibroblast cells, $^{*}p < 0.05$ *versus* ethanol.

3.3. Effect on cell cycle

Cells progress through the cell cycle in several well-controlled phases [22]. In the G_1 phase, cells commit to enter the cell cycle and prepare to duplicate their DNA in the S phase. After the S phase, cells enter the G_2 phase, where repair might occur along with preparation for mitosis in the M phase. In the M phase, chromatids and daughter cells separate. After the M phase, the cells can enter the G_1 or G_0 quiescent phase. Entry into each phase of the cell cycle is carefully regulated by cell cycle checkpoints [23].

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; several agents are now under development [24,25]. In cancer cell lines, inducing cells to arrest in the cell cycle constitutes one of the most prevalent strategies used to stop or limit cancer spreading. Compound **15** induced HeLa cells arrest in the S phase of the cell cycle, CaSki cells in the G_2-M phase, and ViBo cells in the G_1 phase, suggesting that the activity is dependent on the phases (Figs. 6–8, respectively). Regarding CaSki and ViBo cells, this decrease in G_2-M and G_1 phases, respectively 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 [26].

3.4. Apoptosis

Apoptosis is a fundamental and complex biological process that enables an organism to kill and remove unwanted cells during development, normal homeostasis, and disease. 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 [27,28]. Hallmarks of the terminal stages of apoptosis are genomic DNA fragmentation and chromatin condensation among others. Chromatin condensation causes compact and smaller nuclei and/or the formation of apoptotic bodies. HeLa, CaSki and ViBo cultures were stimulated at the level of their found IC_{50}, and the chromatin condensation, including the formation of apoptotic bodies, were determined through staining with fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) [29]. Compact and smaller nuclei can be noticed (indicating chromatin condensation) for HeLa and ViBo cultures (Fig. 9E and F); on the other hand, the presence of apoptotic bodies was clearly observed in CaSki cultures (Fig. 9D), which suggested that **15** induced death by apoptosis in this cell line.

Nevertheless, chromatin condensation is often found in necrosis as well, and the presence of apoptotic bodies do not ensure that apoptosis is the mechanism of cell death. Hence, the evaluation of the proteins implicated in the activation and execution of apoptosis is required. Caspases (14 so far identified in humans) stand out as being crucial for this process in diverse metazoan organisms [30]. In mammals, caspases (principally caspase-3) appear to be activated in a protease cascade that leads to inappropriate activation or rapid disabling of key structural proteins and important signaling, homeostatic and repair enzymes. Caspase-3 is an activated death protease which is important for cell death in a remarkable tissue-, cell type- or death stimulus-specific manner, and is essential for some of the characteristic changes in cell morphology and certain biochemical events associated with the execution and completion of apoptosis [31]. Active caspase-3 expression was determined by

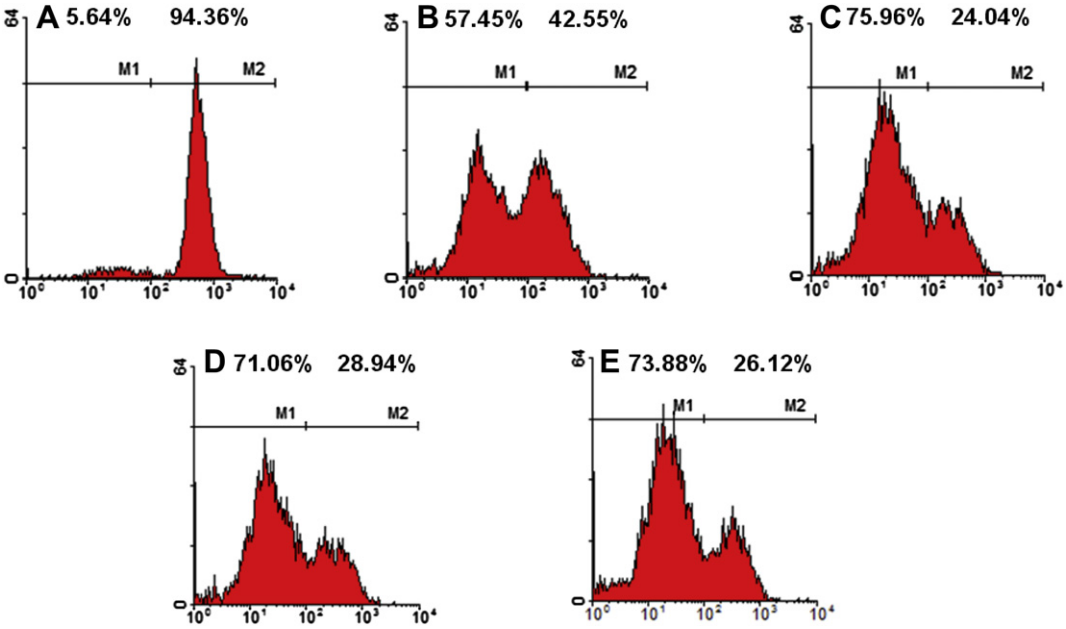


Fig. 13. Effect of compound **15** on lymphocyte proliferation. A) ELPs untreated. B) ELPs in the presence of PHA stimulation. C) ELPs treated with 10 μL of ethanol. D) ELPs treated with 65 μM of **15**. E) ELPs treated with 42 μM of **15**. M1 is the proliferating cells region, and M2 is the non-proliferating cells region.

immunocytochemistry and quantified through emitted fluorescence [32]. Fig. 10 shows that compound **15** induced the expression of active caspase-3 in CaSki, HeLa and ViBo cultures, implying that apoptosis could be triggered.

One of the characteristics of the final stage of apoptosis is the degradation of DNA. The terminal deoxynucleotidyl transferase (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 [33]. Fig. 11 shows that fragmentation of DNA in HeLa, CaSki and ViBo cells is induced by compound **15**. These results indicate that the full apoptotic event induced by **15** is completed in a 24 h period for all cell lines.

3.5. Evaluation of antiproliferative activity on non-tumor cells

Major compounds used currently in chemotherapy present problems for selective activity towards malignant cells and produce undesirable secondary effects. It is crucial to determine the selectivity of tested compounds using the antiproliferative and apoptosis assays in order to derive any conclusions on the potential for anticancer treatment. For this reason, the effect of **15** on the proliferation of non-tumor cervical fibroblastic cells [34], and peripheral blood lymphocytes was assessed. Non-tumor fibroblast cultures were treated with compound **15** and the antiproliferative activity was determined by violet crystal staining (Fig. 12). Compound **15** affected the proliferative potential of fibroblastic cells below 20%, suggesting selectivity towards the malignant cultures.

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 **15**. ELPs from a healthy blood donor were labeled with 5(6)-carboxyfluorescein diacetate *N*-succinimidyl ester (CFSE), stimulated with PHA, and/or treated with **15**, and cultured for 72 h [35]. Cells were harvested and their proliferative potential was analyzed by flow cytometry. The effect of **15** on proliferative potential of ELPs is shown in Fig. 13, indicating that under normal conditions, proliferating cells were 58% of the total population (Fig. 13B). When lymphocytes were treated with 65 μM of **15**, proliferating cells were 71% (Fig. 13D), and with 42 μM of **15**, proliferating cells were 74% (Fig. 13E). Thus, with compound **15**, proliferative potential was not negatively affected. These results suggest a greater degree of antiproliferative selectivity towards malignant cell lines than with fibroblasts and lymphocytes.

4. Conclusions

In conclusion, the 22-oxo-26-glycoside **15** was synthesized from hecogenin and showed anti-cancer and some selective activity against HeLa, CaSki and ViBo cervicouterine cancer cell lines, with greater antiproliferative activity against CaSki cells than the de-*O*-glucosylated compound **7**. Our biological evaluations suggest that **15** is a potent apoptosis inducer with a null cytotoxic consequence. In addition, the proliferation of fibroblast cells and peripheral blood lymphocytes was not affected significantly. These in vitro results certainly augur well for *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 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 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)-26-Hydroxy-12,22-dioxo-5α-cholestan-3β,16β-diyl diacetate (**7**)

Hecogenin **6** (3 g, 6.97 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 dropped into ice water (50 mL). The organic phase was washed with a saturated solution of $NaHCO_3$ (4 × 100 mL), and dried over Na_2SO_4, then concentrated under reduced pressure. The crude product was purified by chromatography on silica gel, with hexane/ethyl acetate (65:35) as eluent, to afford compound **7** as a colorless solid (3.19 g, 86%). Mp 125–127 °C $[\alpha]_D = +82.1°$ (0.3, $CHCl_3$). IR: 3735 (OH), 2933 (CH, aliphatic), 1725 and 1721 (C=O, acetate), 1706 (C=O, ketone). ^{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, $J_{gem} = 8.4$ 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, $J_{gem} = 8.4$ Hz, H-11*eq*), 1.99 (3H, s, $C\underline{H}_3CO_2$-3), 1.93 (3H, s, $C\underline{H}_3CO_2$-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 ($CH_3\underline{C}O_2$-3), 169.6 ($CH_3\underline{C}O_2$-16), 21.3 ($\underline{C}H_3CO_2$-3), 20.9 ($\underline{C}H_3CO_2$-16). HRMS Calcd. for $C_{31}H_{49}O_7$: 533.3478 $[M + H]^+$. Found: 533.3471.

5.2.2. Methyl 2,3,4,6-tetra-O-benzyl-α-D-glucopyranoside (***10***)

Glucose **8** (2.0 g, 11.1 mmol) was suspended in MeOH (50 mL) and acetyl chloride (1.78 mL, 25 mmol) was added dropwise. The solution was stirred continuously for 2 h until glucose had been consumed, and the reaction was quenched by addition of triethylamine (3.48 mL, 25 mmol). The solvent was evaporated under reduced pressure and the methyl glucoside **9** was dried under vacuum and was immediately dissolved in dry DMF and cooled to 0 °C. NaH from 60% dispersion in mineral oil (3.6 g, 88.8 mmol) was added over a period of 30 min followed by the addition of BnBr (10.5 mL, 88.8 mmol); the reaction was allowed to reach room temperature and stirring was continued overnight. After completion of the reaction, the mixture was cooled in a water bath and quenched with MeOH. The organic layer was extracted four times with CH_2Cl_2 and the combined extracts were washed with brine (3 × 100 mL) and water (3 × 100 mL), dried over Na_2SO_4, and concentrated under vacuum. The product was purified by column chromatography with hexane/ethyl acetate (9:1) as eluent to give **10** as a syrup (5.4 g, 87%), ^{1}H NMR ($CDCl_3$); data for α-anomer: δ 7.37–7.10 (20H, m, Ar), 4.98–4.49 (8H, m, CH_2-benzyl), 4.63 (1H, d, $J_{1,2} = 3.6$ Hz, H-1), 3.99 (1H, m, H-3), 3.75 (1H, m, H-5), 3.71–3.65 (2H, m, CH_2-6), 3.63 (1H, m, H-4), 3.56 (1H, dd, $J_{2,1} = 3.6$ Hz $J_{2,3} = 9.6$ Hz, H-2), 3.37 (3H, s, $OC\underline{H}_3$). ^{13}C NMR ($CDCl_3$): δ 98.0 (C-1), 79.7 (C-2), 82.0 (C-3), 77.5 (C-4), 69.9 (C-5), 68.3 (C-6), 75.6, 74.9,

73.3, 73.2 (CH_2-benzyl), 138.6, 138.1, 138.0, 137.7 (*Cipso*), 128.3–127.4 (Ar), 55.0 (OCH_3).

*5.2.3. 2,3,4,6-tetra-O-Benzyl-D-glucopyranose (**11**)*

Compound **10** (1.0 g, 1.8 mmol) was dissolved in acetic acid (20 mL) and 1N H_2SO_4 (2.5 mL) was added. The mixture was refluxed for a period of 2 h and the organic phase was extracted four times with CH_2Cl_2 and the combined extracts were washed with a saturated solution of $NaHCO_3$ (5 x 100 mL) and water (3 x 100 mL), dried over Na_2SO_4, and concentrated under vacuum. The product was purified by column chromatography with hexane/ethyl acetate (85:15) as eluent to give **11** as a solid (mixture of α and β anomers; 588 mg, 55%). ^{1}H NMR ($CDCl_3$): δ 7.49–7.09 (Ar), 4.98–4.45 (CH_2-benzyl), 5.23 (1H, d, $J_{1,2}$ = 3.7 Hz, H-1 β-anomer), 4.72 (1H, d, $J_{1,2}$ = 7.8 Hz, H-1 α-anomer), 4.07–3.37 (H-2-6). ^{13}C NMR ($CDCl_3$): δ 91.3 (C-1 α-anomer), 97.5 (C-1 β-anomer), 84.6–68.4 (C-2-6), 75.7–73.2 (CH_2-benzyl), 138.7–137.7 (*Cipso*), 128.5–127.6 (Ar).

*5.2.4. (25R)-3β,16β-Diacetoxy-12,22-dioxo-5α-cholestan-26-yl 2,3,4,6-tetra-O-benzyl-β-D-glucopyranoside (**14**)*

A solution of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (**11**, 513 mg, 0.95 mmol), 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 **12** as a syrup (575 mg, 89%). This compound was used immediately in the glycosylation reaction. A mixture of the donor **12** (409 mg, 0.60 mmol), aglycon **7** (265 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 α-(**13**) and β-(**14**) anomers in a 5:95 ratio (HPLC acetonitrile/water 9:1) which was purified by flash column chromatography with hexane/ethyl acetate (7:3) as eluent to afford the pure β-anomer as a colorless foam (498 mg, 93%). $[\alpha]_D$ +40.1° (*c* 0.5, $CHCl_3$). IR: 2939 (CH, aliphatic), 1726 and 1729 (C=O, acetate), 1709 and 1702 (C=O, ketone), 1506, 1086 and 3068 (CH aromatics). ^{1}H NMR ($CDCl_3$): δ 7.34–7.15 (20H, m, Ar), 5.00 (1H, m, H-16), 4.94 (1H, d, J_{gem} = 10.8 Hz, PhCH_2-O-2′a), 4.92 (1H, d, J_{gem} = 11.4 Hz, PhCH_2-O-3′a), 4.81 (1H, d, J_{gem} = 10.8 Hz, PhCH_2-O-4′a), 4.78 (1H, d, J_{gem} = 10.8 Hz, PhCH_2-O-3′b), 4.71 (1H, d, J_{gem} = 11.0 Hz, PhCH_2-O-2′b), 4.67 (1H, m, H-3), 4.61 (1H, d, J_{gem} = 12.6 Hz, PhCH_2-O-6′a), 4.55 (1H, d, J_{gem} = 12.6 Hz, PhCH_2-O-6′b), 4.53 (1H, d, J_{gem} = 10.8 Hz, PhCH_2-O-4′b), 4.37 (1H, d, $J_{1′,2′}$ = 7.8 Hz, H-1′), 3.76 (1H, dd, $J_{26a,25}$ = $J_{26a,26b}$ = 9.6 Hz, H-26a), 3.74 (1H, dd, $J_{6′a,6′b}$ = 10.8 Hz, $J_{6′a,5′}$ = 1.8 Hz, H-6′a), 3.67 (1H, dd, $J_{6′b,6′a}$ = 10.8 Hz, $J_{6′b,5′}$ = 5.4 Hz, H-6′b), 3.63 (1H, dd, $J_{3′,2′}$ = $J_{3′,4′}$ = 9.0 Hz, H-3′), 3.57 (1H, dd, $J_{4′,3′}$ = 9.0 Hz, $J_{4′,5′}$ = 9.6 Hz, H-4′), 3.45 (1H, m, H-5′), 3.44 (1H, dd, $J_{2′,3′}$ = 9.0 Hz, $J_{2′,1′}$ = 7.8 Hz, H-2′), 3.40 (1H, dd, $J_{26b,25}$ = 5.4 Hz, $J_{26b,26a}$ = 9.6 Hz, H-26b), 2.79 (1H, m, H-20), 2.79 (1H, m, H-17), 2.62 (1H, m, 23a), 2.53 (1H, m, H-15a), 2.53 (1H, dd, $J_{11ax,9}$ = J_{gem} = 12.6 Hz, H-11*ax*), 2.34 (1H, m, H-23b), 2.19 (1H, dd, $J_{11eq,9}$ = 4.8 Hz, J_{gem} = 12.6 Hz, H-11*eq*), 2.02 (3H, s, CH_3CO_2-3), 1.93 (3H, s, CH_3CO_2-16), 1.58 (1H, ddd, $J_{1eq,2ax}$ = 4.2 Hz, $J_{1eq,2ec}$ = 3.0 Hz, J_{gem} = 13.2 Hz, H-1*eq*), 1.49 (1H, ddd, $J_{2ax,1ax}$ = 13.2 Hz, $J_{2ax,1eq}$ = 4.2 Hz, J_{gem} = 16.2 Hz, H-2*ax*), 1.15 (3H, s, CH_3-18), 1.08 (3H, d, $J_{21,20}$ = 6.6 Hz, CH_3-21), 0.96 (3H, d, $J_{27,25}$ = 6.6 Hz, CH_3-27), 0.92 (3H, s, CH_3-19). ^{13}C NMR ($CDCl_3$): δ 36.2 (C-1), 27.1 (C-2), 73.1 (C-3), 28.1 (C-4), 44.3 (C-5), 30.1 (C-6), 33.7 (C-7), 34.7 (C-8), 56.6 (C-9), 36.2 (C-10), 38.1 (C-11), 212.9 (C-12), 56.3 (C-13), 54.8 (C-14), 34.5 (C-15), 74.4 (C-16), 46.2 (C-17), 12.8 (C-18), 11.8 (C-19), 43.9 (C-20), 17.0 (C-21), 212.5 (C-22), 38.2 (C-23), 27.2 (C-24), 33.1 (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′), 170.6 (CH_3CO_2-3), 169.6 (CH_3CO_2-16), 21.4 (CH_3CO_2-3), 21.0 (CH_3CO_2-16), 128.3–127.5 (Ar), 138.4 (C_{ipso}-PhCH$_2$-O-2′), 138.6 (C_{ipso}-PhCH$_2$-O-3′), 138.1 (C_{ipso}-PhCH$_2$-O-4′), 138.2 (C_{ipso}-PhCH$_2$-O-6′), 74.8 (PhCH$_2$-O-2′), 75.6 (PhCH$_2$-O-3′), 75.0 (PhCH$_2$-O-4′), 73.4 (PhCH$_2$-O-6′). HRMS Calcd for $C_{65}H_{83}O_{12}$: 1055.5885, $[M + H]^+$. Found: 1055.5878.

*5.2.5. (25R)-3β,16β-Diacetoxy-12,22-dioxo-5α-cholestan-26-yl β-D-glucopyranoside (**15**)*

To a solution of **14** (300 mg, 0.29 mmol) in MeOH:AcOEt (7:3) was added 10% Pd/C (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 ($CHCl_3$/MeOH (95:5)) to give **15** as a pale yellow powder (198 mg, 95%). $[\alpha]_D$ +57.5° (*c* 0.4, MeOH). IR: 3391 (OH), 2938 (CH, aliphatic), 1723 and 1726 (C=O, acetate), 1709 and 1711 (C=O, ketone). ^{1}H NMR ($CDCl_3$/CD_3OD): δ 4.98 (1H, ddd, J_{16-17} = J_{16-15a} = J_{16-15b} = 8.2 Hz, H-16), 4.66 (1H, m, H-3), 4.23 (1H, d, $J_{1′,2′}$ = 7.8 Hz, H-1′), 3.86 (1H, dd, $J_{6′a,5′}$ = 1.9 Hz, J_{gem} = 11.9 Hz, H-6′a), 3.75 (1H, dd, $J_{26a,25}$ = 5.8 Hz, J_{gem} = 9.6 Hz, H-26a), 3.66 (1H, dd, $J_{6′b,5′}$ = 5.3 Hz, J_{gem} = 11.9 Hz, H-6′b), 3.36 (1H, dd, $J_{26b,25}$ = 6.0 Hz, J_{gem} = 9.6 Hz, H-26b), 3.35 (1H, dd, $J_{3′,2′}$ = $J_{3′,4′}$ = 9.1 Hz, H-3′), 3.28 (1H, dd, $J_{4′,3′}$ = $J_{4′,5′}$ = 9.1 Hz, H-4′), 3.26 (1H, m, H-5′), 3.18 (1H, dd, $J_{2′,3′}$ = 9.1 Hz, $J_{2′,1′}$ = 7.8 Hz, H-2′), 2.91 (1H, dq, $J_{20,21}$ = 7.3 Hz, $J_{20,17}$ = 10.5 Hz, H-20), 2.77 (1H, m, 23a), 2.74 (1H, dd, $J_{17,20}$ = $J_{17,16}$ = 8.2 Hz, H-17), 2.68 (1H, dd, $J_{11ax,9}$ = J_{gem} = 12.9 Hz, H-11*ax*), 2.46 (1H, m, 15a), 2.46 (1H, m, H-23b), 2.16 (1H, dd, $J_{11eq,9}$ = 4.8 Hz, J_{gem} = 12.9 Hz, H-11*eq*), 2.00 (3H, s, CH_3CO_2-3), 1.98 (3H, s, CH_3CO_2-16), 1.95 (1H, ddd, $J_{8,7ax}$ = 7.2 Hz, $J_{8,7eq}$ = 3.6 Hz, $J_{8,9}$ = 10.8 Hz, H-8), 1.53 (1H, ddd, $J_{2ax,1ax}$ = 17.0 Hz, $J_{2ax,1eq}$ = 4.0 Hz, J_{gem} = 13.1 Hz, H-2*ax*), 1.23 (3H, s, CH_3-18), 1.18 (1H, ddd, $J_{9,11ax}$ = 12.9 Hz, $J_{9,11eq}$ = 4.8 Hz, $J_{9,8}$ = 10.8 Hz, H-9), 1.08 (3H, d, $J_{21,20}$ = 7.3 Hz, CH_3-21), 0.98 (3H, s, CH_3-19), 0.94 (3H, d, $J_{27,25}$ = 6.6 Hz, CH_3-27). ^{13}C NMR ($CDCl_3$/CD_3OD): δ 37.5 (C-1), 28.3 (C-2), 74.8 (C-3), 29.4 (C-4), 45.7 (C-5), 32.3 (C-6), 34.9 (C-7), 36.0 (C-8), 58.5 (C-9), 37.4 (C-10), 39.3 (C-11), 215.6 (C-12), 57.7 (C-13), 56.6 (C-14), 35.3 (C-15), 75.6 (C-16), 47.8 (C-17), 13.2 (C-18), 12.2 (C-19), 45.0 (C-20), 17.7 (C-21), 216.2 (C-22), 39.5 (C-23), 28.5 (C-24), 34.3 (C-25), 75.9 (C-26), 17.3 (C-27), 104.6 (C-1′), 75.2 (C-2′), 78.2 (C-3′), 71.7 (C-4′), 77.9 (C-5′), 62.9 (C-6′), 172.5 (CH_3CO_2-3), 171.7 (CH_3CO_2-16), 21.3 (CH_3CO_2-3), 21.2 (CH_3CO_2-16). HRMS Calcd for $C_{37}H_{59}O_{12}$: 695.4006, $[M + H]^+$. Found: 695.3998.

5.3. Biological activity

5.3.1. Cell culture

HeLa, CaSki and ViBo 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 42 μM or 65 μM of **15**. 1% of vehicle (ethanol) was added to control cells. Antiproliferative activity (IC_{50}) was determined after 24 h by crystal violet staining [20]. 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 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 [21].

5.3.4. Cell cycle analysis

HeLa, CaSki, and ViBo 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 42 μM or 65 μM of **15**. 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 (Coulter, USA) [26].

5.3.5. Detection of active caspase-3 from apoptotic cells

HeLa, CaSki and ViBo cells were seeded at 5^4 cells/mL in 300 μL of RPMI-1640 containing 5% NCS for 24 h. Cells were treated with 42 μM or 65 μM of **15**. 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 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. Samples were washed again with PBS and DAPI fluorochrome was added. Analyses were performed by epifluorescence microscopy (Nikon) [32].

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 [33]. 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 **15** 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.

5.3.7. Assay on non-tumor 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 subcultured once again. Tissue samples were maintained in RPMI-1640 medium containing 20% NCS at 4 °C and processed after 3h. 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 [34]. Cell cultures were exposed to 42 μM or 65 μM of **15**. 1% of vehicle (ethanol) was added to control cells. Finally, the cell numbers were determined 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 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 [35]. 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.

5.3.9. 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.

Acknowledgments

The authors gratefully thank CONACYT for the scholarships to MAFH and HLM and for grant 84380 to JSR. We thank PAPIME for grant PE204609 and the Natural Sciences and Engineering Research Council of Canada for a grant to BMP. We thank Dr. Dionisio Parra (Gyneco-perinatology Department) and Dr. René García (Teaching Department) from Hospital General "Ignacio Zaragoza," ISSSTE for technical assistance.

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