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Synthesis of novel spirostanic saponins and their cytotoxic activity

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Dedicated to the memory of Professor Antonio G. González.

Abstract—This study was carried out to assess the cytotoxicity of several new synthetic steroidal saponins against the human myeloid leukemia cell lines (HL-60 and U937) and against human melanoma cells (SK-MEL-1). Several diosgenyl glycosides analyzed showed strong cell growth inhibition which was associated with alterations in cell cycle progression and induction of apoptosis. Studies of cytochrome *c* release and caspase-9 activation suggest a main role of the intrinsic pathway of apoptosis in the mechanism of cytotoxicity caused by this kind of compounds.

1. Introduction

Saponins are a very large group of glycosylated secondary metabolites isolated primarily from plants, but also found in lower marine organisms.[1] Saponins are believed to be some of the principal constituents of many plant drugs and traditional medicines worldwide, and are described as being responsible for most of the observed biological effects. Although the biological role of saponins in plants is not yet fully understood, they are considered to be a part of the natural chemical defense mechanism of plants against attack by pathogens.[2] Saponins consist of a hydrophobic aglycone, which may have a triterpenoid or steroid structure, linked by a glycosidic bond to a sugar moiety normally including glucose, rhamnose, galactose, or xylose, among others. Steroidal saponins are amphipathic molecules of special interest due to the biological activities they exhibit. In recent years several research groups have evaluated their cytotoxic activities.[3–11]

Consequently, various syntheses of steroidal saponins have been successfully developed.[12–15] As a part of our research focused on the cytotoxicity of several steroidal saponins isolated from *Dracaena draco*,[5,16] we designed the synthesis of ten analogs containing a diosgenyl α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside skeleton. Mimaki et al.,[17] employing molecular models and molecular dynamic studies, suggested that the three-dimensional structure of the diglycoside moiety contributed to the cytotoxic activity of the molecule. In that study, they revealed that in the diosgenyl α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside derivative, which is cytotoxic, the diglycoside exists in a vertically oriented conformation against the steroid plane. However, in the diosgenyl α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranoside derivative, which presents a conformation with the diglycoside and the steroid in the same plane, the activity was not relevant.

Starting from this hypothesis, we proposed the synthesis of several saponins analogous to those obtained from *Dracaena draco*,[5,16] always having the diosgenyl β-D-glucopyranoside basic structure and including different monosaccharides (Glc, Gal, Man, Ara, Xyl, and Fuc) as well as the same derivative with rhamnose, linked through the C-2′ of the glucose. Then, the most potent

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cytotoxic compound was submitted to additional modifications.

Some chemotherapeutic drugs have been shown to use apoptotic pathways to mediate their cytotoxic effects. An important goal in chemotherapy is to find new cytotoxic agents that are able to increase or restore the ability of tumor cells to undergo apoptosis. Apoptotic cell death is characterized by morphological and biochemical changes, which largely result from the activation of a family of cysteine proteases known as caspases. There are two primary caspase activation pathways, involving either stimulation of cell surface death receptors (the extrinsic pathway) or perturbation of mitochondria (the intrinsic pathway). Many anti-cancer drugs induce apoptosis by activating the intrinsic pathway. This cell death pathway involves the release of cytochrome *c* and the activation of the apoptosome-catalyzed caspase cascade. The apoptosome is a protein complex consisting of cytochrome *c*, apoptotic protease-activating factor 1, and caspase-9. Apoptosome assembly processes and activates the initiator caspase-9. Active caspase-9 then triggers a caspase cascade to induce apoptosis.

This study was undertaken to investigate the effects of synthetic diosgenyl glycosides on cell growth and death in human tumor cells, and associated biological events such as induction of apoptosis and impairment of cell cycle progression.

2. Results and discussion

Chemistry: In order to develop the synthesis of a set of new steroidal saponins, we initially decided to prepare the common precursor diosgenyl β-D-glucopyranoside, known as trillin (**3**), suitably protected as 4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside **4a**. Compound **3**, as shown in Scheme 1, was prepared starting from 2,3,4,6-tetra-*O*-benzoyl-α-D-glucopyranosyl bromide (**1**) and commercially available diosgenin, using AgOTf as coupling promoter with high stereoselectivity and was prepared in good yield (67%). Hydrolysis of the ester groups of **2** under basic conditions yielded **3**, which was protected as the *O*-benzylidene derivative **4**. Attempts at selective monoprotection of the hydroxyl group located at C-3′ under conditions described by Li et al.[18] using TBDMSCl and imidazole in DMF were unrewarded in our case because starting material was recovered.

A report was found in the literature on the difficulty of accomplishing the selective monoprotection of the hydroxyl groups at C-2′ and C-3′ of a glucopyranosyl unit.[18,19] As an alternative, treatment of the above-mentioned *O*-benzylidene derivative **4** with pivaloyl chloride[19] provided a mixture of protected alcohols at the desired C-3′ position (45%, **4a**), at C-2′ (9.5%, **4b**), and at both C-2′ and C-3′ (5%, **4c**). Compound **4a**, which has the free –OH group at C-2′, was submitted to glycosylation reaction with the trichloroacetamidate partners (**5-11**, Table 1) obtained from their corresponding natural monosaccharides.[20]

The use of boron trifluoride-diethyl etherate complex as Lewis acid[18] and trichloroacetonitrile as leaving group allowed the coupling of the monosaccharides in a stereoselective manner and with acceptable yields affording the corresponding coupling products **12–18** (Scheme 2).

Finally, a two-step deprotection protocol employing AcOH (80%), followed by basic deacetylation with NaOMe in MeOH, gave the desired new spirostane-type steroidal saponins **20**, **21**, **23**, and **24**, and the known natural ones **19**, **22**, and **25**. The physical data of the known compounds were in accordance with those reported in the literature.[21–23] Analysis of the ^{1}H and ^{13}C NMR spectra of **20**, **21**, **23** and **24** together with homonuclear correlation (COSY) and heteronuclear multiple coherence (HMQC and HMBC) and TROESY experiments allowed the structure of these new compounds to be unambiguously deduced. Correlations between C-2′ of the glucopyranoside unit and H-1″ of the second monosaccharide unit as well as the ROE effect between H-2′ and H-1″ confirmed the interglycosidic linkage. The connection between the aglycone moiety and the saccharide portion was confirmed by a correlation between the C-1′ of glucose and H-3 of the aglycone and the ROE effect between H-1′ and H-3.

Preliminary results on cytotoxicity bioassays showed that compound **25** was the most potent cytotoxic agent against the three tumor cell lines tested (Table 2), and thus subsequent modifications were developed over this structure. It has been reported that the presence of acetyl groups usually increases cytotoxic potency.[17]

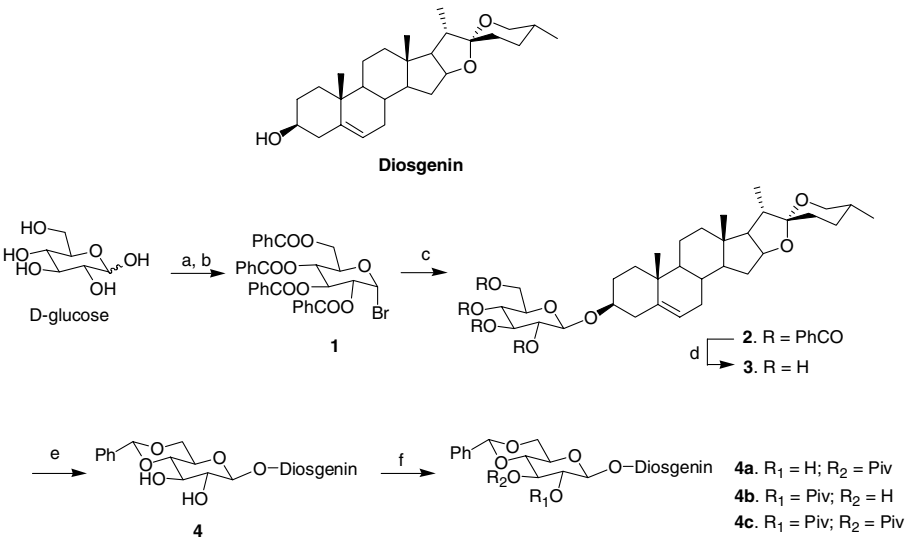
Thus, following the methodology described by Yu et al.,[24] the selective introduction of acetyl groups employing the lipase Novozyme 435 was performed (Scheme 3). This procedure afforded a mono- and a diacetyl derivative named diosgenyl α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-glucopyranoside (**27**) and diosgenyl 4-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-glucopyranoside (**26**), respectively.

It is noteworthy that trisaccharides with pharmacological activity are reported in the literature.[3,5] We therefore synthesized, starting from **4a**, a novel derivative **30**, containing a trisaccharide core attached to diosgenin, taking advantage of a 'one-pot' procedure.[25] In this method, trichloroacetamidate glycosyl and thioglycoside were used as sequential glycosyl donors and two glycosidic linkages (Scheme 4).[26]

3. Biological evaluation

3.1. Diosgenyl glycosides inhibit growth of human tumor cell lines

Initial studies on the cytotoxicity of diosgenyl α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (**25**) showed that this compound was a potent cytotoxic agent against HL-60 cells.[17] In the present report, we describe the chemical modifications that were developed



Scheme 1. Reagents and conditions: (a) PhCOCl, DMAP, Py; (b) HBr/AcOH (30:70), CH_2Cl_2; (c) diosgenin, AgOTf, CH_2Cl_2, −20 °C–rt, 4 Å MS; (d) NaOMe in MeOH 0.5 M; (e) $PhCH(OMe)_2$, CSA, DMF, 50 °C; (f) PivCl, Py, rt.

over this basic structure to obtain new semi-synthetic diosgenyl glycosides and the effect of these chemical changes on the growth of human myeloid (HL-60 and U937) and melanoma (SK-MEL-1) cell lines (Table 2).

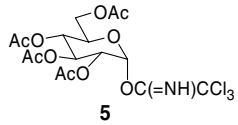
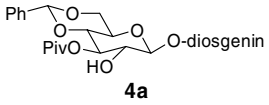
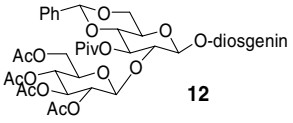
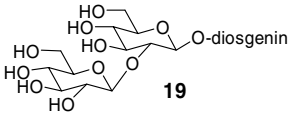
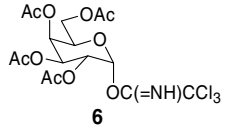
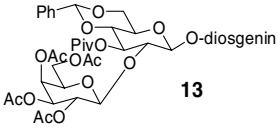
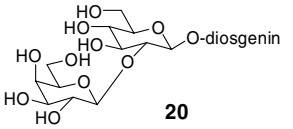
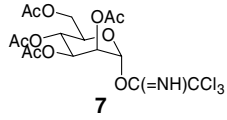
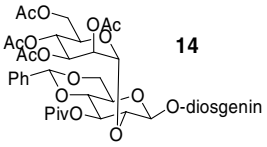
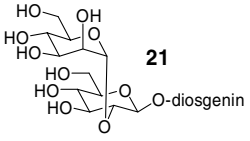
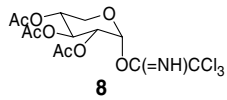
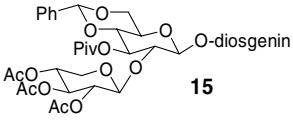
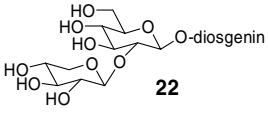
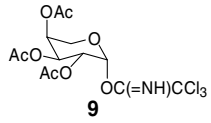
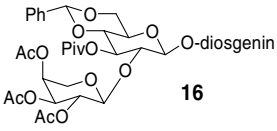
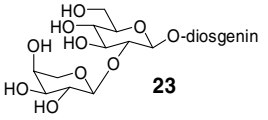
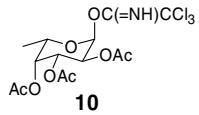
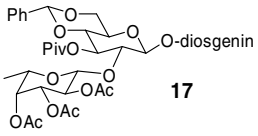
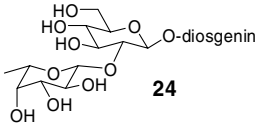
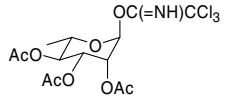
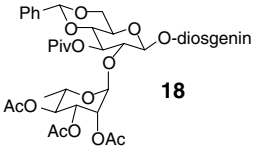
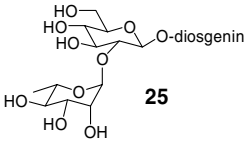
Growth inhibition of human tumor cells in culture was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) dye-reduction assay. Antiproliferative studies of compound **25** indicated that these diosgenyl glycosides displayed strong cytotoxic properties in all assayed cell lines, with an IC_{50} of about 5–7 μM. Interestingly this compound was more potent than etoposide, a chemotherapy drug for the treatment of some types of cancer, against SK-MEL-1 melanoma cells (results not shown). Since an important structural component of compound **25** is rhamnose, we next decided to evaluate the role of this monosaccharide. Therefore, saponin glycosides containing different hexoses were synthesized. The results indicated that those compounds containing glucose (**19**), galactose (**20**), mannose (**21**) or fucose (**24**) instead of rhamnose were, in general, less cytotoxic in all cell lines tested. However, a certain cell-type dependence was also observed with some chemical changes. For example, compounds **21** and **24** presented IC_{50}s similar to those of compound **25** ($IC_{50} \sim 5$ to 11 μM) on HL-60 cells. However, these substances were completely ineffective on SK-MEL-1 cells if compared to **25** ($IC_{50} > 100$ μM vs $IC_{50} = 7$ μM). Selective acetylation was also performed on compound **25** to yield the mono- and diacetylated derivatives **27** and **26**, respectively. Compound **27** was highly cytotoxic (Fig. 1A and B) and no impact on the IC_{50}s was observed when compared with its deacetylated counterpart **25**. This result indicated that the hydroxyl group at C-6′ of glucose may not play a significant role in the cytotoxicity of this compound. Contrarily, a significant decrease in cytotoxic potency was observed for the diacetylated derivative **26** in all cell lines tested, which suggests that the free hydroxyl group linked to C-4″ on the rhamnose unit is relevant for antiproliferative activity. In consonance with these results, compound **30**, which contains the hydroxyl group at C-4″ attached to a second rhamnose, was also significantly less cytotoxic than **25** but similar to **26**, with no noteworthy differences among cell lines. Next, we decided to determine the influence of a pentose instead of rhamnose on the cytotoxicity of the diosgenyl glycosides. Therefore, compounds **22** and **23**, containing arabinose and xylose, respectively, were synthesized. The results indicated that the presence of these pentoses did not improve cytotoxicity. Contrarily, the antiproliferative capability of both compounds was decreased, as compared to compound **25**, in all human cell lines. As occurs with other substitutions, these chemical changes affected the cell lines under study to different degrees. A dramatic decrease in growth inhibitory activity was specifically observed on SK-MEL-1 in response to the above compounds ($IC_{50} > 100$ μM). These results support the role of rhamnose as a necessary structural requirement for diosgenyl glycosides' cytotoxicity.

3.2. Effect of diosgenyl glycosides on cell cycle progression

A detailed inspection of Table 2 reveals that of the three human cell lines used in this study, the growth of promyelocytic HL-60 cells was, in general, highly susceptible to the cytotoxicity induced by diosgenyl glycosides and, therefore, further studies were performed in this cell line. Thus, only those compounds with low IC_{50} ($\leqslant 15$ μM) were selected to explore the mechanism through which diosgenyl glycosides decrease cell viability. Therefore, we first analyzed whether cell growth inhibition induced by compounds **20**, **21**, **24**, **25**, and **27** is mediated via alteration in cell cycle progression.

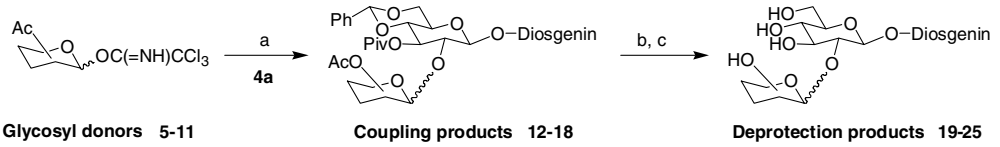
Consistent with growth inhibitory effects, the cytometric flow studies (Table 3) reveal that compounds **21** and **24** in-

Table 1. Synthesis of disaccharide saponins by coupling reaction

Donor	Acceptor	Coupling product	Yield	Deprotection product	Yield
 **5**	 **4a**	 **12**	**77**	 **19**	**58**
 **6**	**4a**	 **13**	**50**	 **20**	**38**
 **7**	**4a**	 **14**	**52**	 **21**	**41**
 **8**	**4a**	 **15**	**64**	 **22**	**49**
 **9**	**4a**	 **16**	**56**	 **23**	**47**
 **10**	**4a**	 **17**	**58**	 **24**	**40**
 **11**	**4a**	 **18**	**49**	 **25**	**41**

duce significant G_1 arrest at the expense of S- and G_2/M-phase cell population following treatment over 16 h with concentrations from 10 to 30 μM. Concentrations of 30 μM were used to demonstrate that the effects on cell cycle progression were dose-dependent. Doses higher than the antiproliferative IC_{50} were used to identify the primary targets and early mechanism of action of diosgenyl glycosides.

Moreover, the IC_{50} values were determined at 72 h of treatment, while the experiments of flow cytometry were analyzed after a short incubation time. Interestingly, compounds **25** and **27** also produced alterations in cell cycle progression although they induced G_2–M arrest at the expense of G_1 phase cell number. A significant increase in the S-phase was also observed at 30 μM of compounds. Taken together, the results indicated that compounds **21** and **24** versus **25** and **27** displayed antiproliferative activities through different mechanisms that involve cell cycle alteration. Selective modulation of different cell cycle-regulatory proteins could explain the differences among them, although other possibilities cannot be ruled out. No evidence of change in the phases of the cell cycle was observed in response to compound **20**. In this context it is important to note that galactose present in **20** is a diastereomer of mannose, which is the monosaccharide present in **21**. The results suggest that not only the configuration of the hydroxyl group in compound **21** plays an important role in the cell cycle arrest but also the configuration of the glycosidic bond between the monosaccharides.



Scheme 2. Reagents: (a) $BF_3{\cdot}Et_2O$; (b) AcOH (80%); (c) NaOMe, MeOH.

Table 2. Effects of synthetic steroidal saponins on the growth of human tumor cell lines[a]

Compound	IC_{50} (μM)		
	HL-60	U937	SK-MEL-1
19	88 ± 12	>100	>100
20	15 ± 3	43 ± 12	>100
21	11 ± 4	12 ± 1	>100
22	38 ± 10	56 ± 4	>100
23	41 ± 4	80 ± 21	>100
24	10 ± 4	28 ± 8	>100
25	5 ± 1	6 ± 1	7 ± 2
26	33 ± 8	18 ± 3	17 ± 4
27	7 ± 2	5 ± 1	4 ± 2
30	22 ± 3	21 ± 2	37 ± 8

[a] Cells were cultured for 72 h and the IC_{50} values were calculated as described in Section 5. The data shown represent means ± SEM of 3–5-independent experiments with three determinations in each.

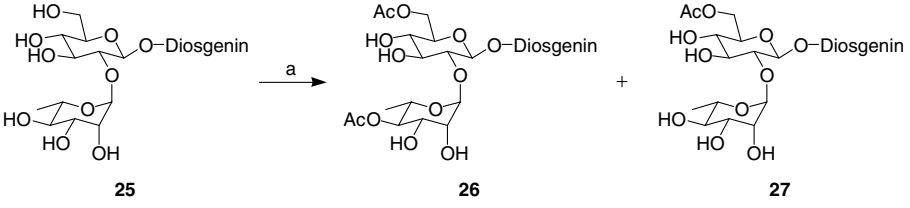
These data indicate a considerable effect on the extent as well as the nature of the cell cycle arrest in HL-60 cells when the diosgenyl β-D-glucopyranoside skeleton is modified by substitution at C-2′ with different glycosyl moieties.

3.3. Diosgenyl glycosides induce apoptosis in human myeloid leukemia cells

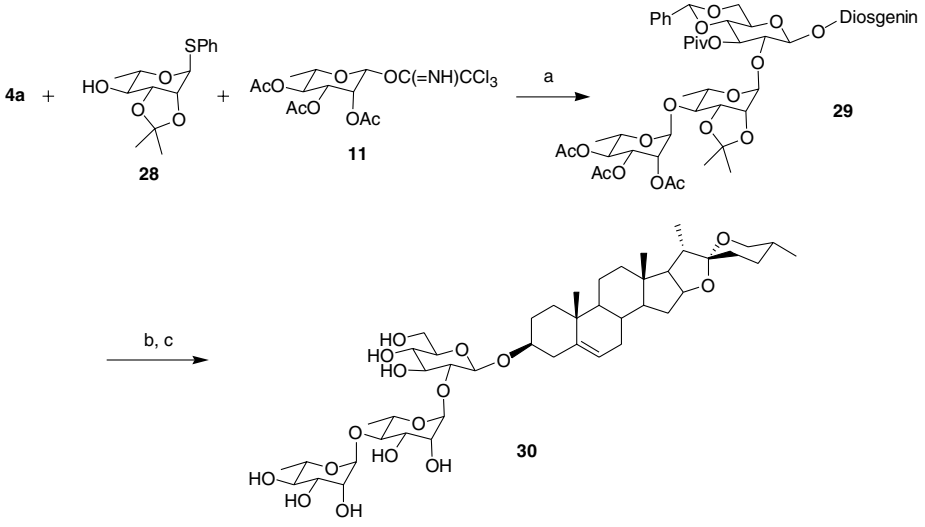
To determine whether diosgenyl glycosides decrease HL-60 cell viability through apoptosis activation, quantification of the number of hypodiploid cells (i.e., apoptotic cells) by flow cytometry was performed.

The results indicate that the percentage of apoptotic cells increased from 5 ± 1% (control) to 12.5 ± 0.5% (2.5-fold increase) and 18 ± 0.5% (3.6-fold increase) after 16 h of treatment with 10 μM of diosgenyl glycosides **25** and **27**, respectively (Fig. 2A). Using a higher concentration (30 μM) of compounds **21** and **24** the percentage of apoptotic cells increased from 4.5 ± 1% to 22 ± 1% (5-fold) and to 18 ± 2% (fourfold), respectively (Fig. 2B and C). Doses of 10–30 μM were used to demonstrate that the induction of apoptosis was dose-dependent.

Compound **20** did not induce apoptosis at either concentration tested (Fig. 2A and B). Etoposide, which induces morphological changes and internucleosomal DNA fragmentation characteristic of apoptotic cell death in human leukemia cells, was used as a positive



Scheme 3. Reagents: (a) Novozyme 435, vinyl acetate, THF.



Scheme 4. Reagents and conditions: (a) 4 Å MS, TMSOTf, NIS, CH_2Cl_2, −20 °C; (b) AcOH (80%), 70 °C; (c) NaOH, MeOH/THF/H_2O (1:1:1).

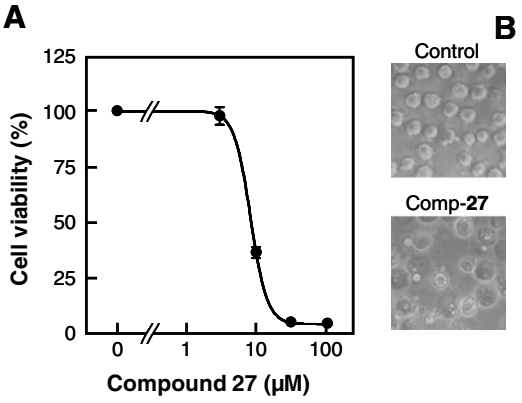


Figure 1. (A) Effect of diosgenyl glycoside **27** on human HL-60 cell viability. Cells were cultured in the presence of the indicated doses of compound **27** for 72 h, and thereafter cell viability was determined by the MTT assay as described in Section 5. The results of a representative experiment are shown. Each point represents the average of triplicate experiments. (B) Effects of diosgenyl glycoside **27** on the morphology of HL-60 cells as visualized by phase contrast microscopy. The cells were untreated (control) or treated with 10 μM of compound **27** (lower panel) for 16 h.

control and induced 48.6 ± 2.6% of apoptotic cells (data not shown). Morphological changes characteristic of apoptotic cells (fragmented and condensed chromatin) were visualized by fluorescent microscopy. Figure 2D shows a representative field of cells exposed to 10 μM of compound **25** for 6 h displaying such morphological changes and etoposide was also included as a positive control.

3.4. Involvement of caspase-3 activation in diosgenyl glycoside induced cell death

Next, we examined whether these diosgenyl glycosides (compounds **21**, **24**, **25**, and **27**) induce pro-caspase-3 cleavage since caspase-3 is the most active effector caspase to be involved in apoptosis induced by cytotoxic agents. Cleavage of procaspase-3 by diosgenyl glycosides was determined by immunoblotting using a polyclonal antihuman caspase-3 antibody that recognized the M_r 32,000 proenzyme (procaspase-3). The results indicate that these compounds at 10 μM promote the cleavage of procaspase-3 (Fig. 3, upper panel). An

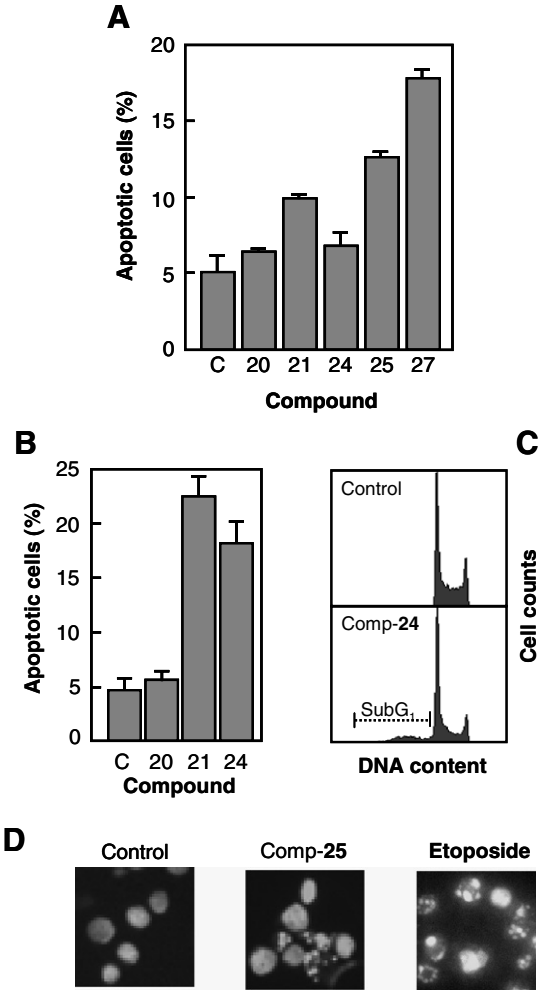


Figure 2. Induction of apoptosis in HL-60 cells by diosgenyl glycosides. (A) Cells were incubated with 10 μM of the indicated compounds for 16 h, and the percentages of apoptotic cells were determined by flow cytometry. The results of a representative experiment are shown, and each point represents the average ± SE of triplicate determinations. (B) Cells were treated with 30 μM of the indicated compounds and the percentages of apoptotic cells were determined by flow cytometry as above. (C) HL-60 cells were treated with 30 μM of **24**, harvested at 16 h, and subjected to DNA flow cytometry. (D) Cells were cultured in the absence (control) or presence of 10 μM of compound **25** for 6 h; then they were stained with Hoechst 33258 and nuclei were visualized using fluorescence microscopy. Etoposide was used as a positive control.

Table 3. Effect of synthetic steroidal saponins on cell cycle distribution of HL-60 cells

		%G_0/G_1	%S	%G_2-M
Control		49.1 ± 1.2	22.4 ± 1.0	28.5 ± 1.8
20	10 μM	50.8 ± 0.1	21.7 ± 0.8	27.5 ± 0.5
	30 μM	49.9 ± 1.2	22.1 ± 0.8	28.0 ± 0.7
21	10 μM	52.0 ± 0.2	20.8 ± 0.2	27.1 ± 0.7
	30 μM	61.5 ± 1.0	17.4 ± 2.0	21.1 ± 3.5
24	10 μM	52.2 ± 0.7	21.6 ± 0.5	26.2 ± 0.1
	30 μM	58.9 ± 4.5	17.4 ± 0.1	23.7 ± 0.9
25	10 μM	46.7 ± 0.2	22.7 ± 1.1	30.5 ± 0.1
	30 μM	41.7 ± 3.1	23.1 ± 0.1	35.2 ± 0.4
27	10 μM	44.9 ± 0.4	22.8 ± 0.1	32.3 ± 0.4
	30 μM	33.8 ± 4.5	25.2 ± 3.5	41.0 ± 6.4

The cells were cultured with the indicated compounds for 16 h and the cell cycle distribution was determined by flow cytometry. The results of a representative experiment are expressed as means ± SE of duplicate determinations

important processing of procaspase-3 was observed at 30 μM concentration, indicating that this effect was dose-dependent (results not shown).

Since poly(ADP-ribose) polymerase (PARP) is a typical substrate for caspase-3, we also examined whether these compounds induce PARP cleavage. Western blot analysis using a polyclonal antibody which recognizes the M_r 85,000 cleaved form of PARP shows the generation of the 85 kDa fragment in diosgenyl glycoside-treated cells after 6 h of treatment (Fig. 3, middle panel). Membranes were stripped and reprobed with β-actin antibody as loading control (Fig. 3, lower panel).

3.5. Diosgenyl glycoside-induced apoptosis involves mitochondrial cytochrome *c* release, but does not affect the expression of *Bcl*-2

The vast majority of conventional anti-cancer agents indirectly exploit mitochondria to exert their cytotoxic action. In order to evaluate whether apoptosis induced by diosgenyl glycosides **21**, **24**, **25**, and **27** in HL-60 and U937 cells involves cytochrome *c* release from mitochondria to cytosol, we subjected cytosolic preparations to immunoblot analysis. The exposure of HL-60 cells to these diosgenyl glycosides resulted in increased cytosolic cytochrome *c* and this response was observed at 6 h of treatment (Fig. 4A). Similar results were obtained with compounds **21** and **27** on U937 cells (Fig. 4B). Since the Bcl-2 family proteins are the central regulator of cytochrome *c* release and caspase activation,[27] we tested whether Bcl-2 is involved in the apoptosis induction by diosgenyl glycosides. The results indicate that the expression of Bcl-2 was unchanged after exposure to compounds **21**, **24**, **25**, and **27** (results not shown).

3.6. Diosgenyl glycoside-induced cell death involves caspase-9 activation

Once cytochrome *c* is in the cytoplasm, it binds to apoptotic protease-activating factor-1 (Apaf-1), which then permits recruitment of procaspase-9. Oligomerization results in autoactivation of procaspase-9. In the mitochondrial pathway, the complex of cytochrome *c*, Apaf-1, and caspase-9, called the 'apoptosome', is a critical activator of effector caspases. Incubation of HL-60 cells with 10 μM diosgenyl glycosides **21**, **24**, **25**, and **27** for 6 h promoted cleavage of the inactive pro-caspase-9 to the active 37 kDa fragment (Fig. 5).

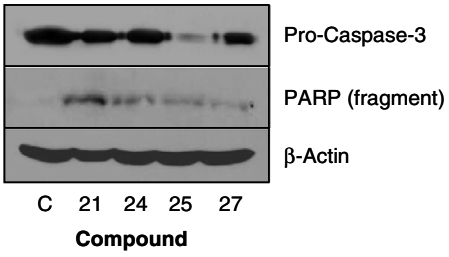


Figure 3. Western blot analysis for the cleavage of procaspase-3 and for the cleavage of poly (ADP-ribose) polymerase. Cells were treated with 10 μM of the indicated diosgenyl glycosides for 6 h and whole cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by blotting with an anti-procaspase-3 antibody (upper panel) or with an anti-poly(ADP-ribose) polymerase (PARP) antibody which recognizes the 85 kDa fragment (middle panel). The migration positions of full-length procaspase-3, as well as of PARP cleavage product p85, are indicated. Data shown are representative of two experiments performed. β-Actin was used as loading control (lower panel). Control lane (C) refers to untreated cells.

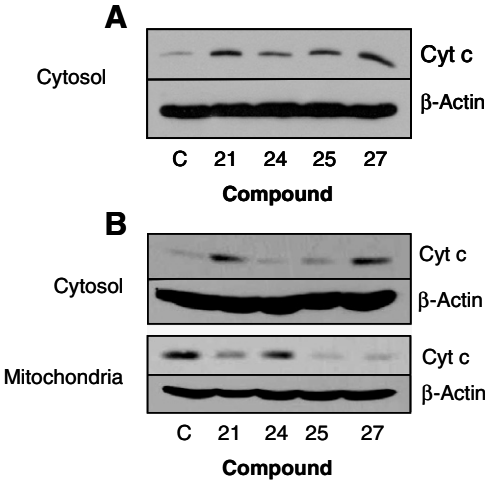


Figure 4. Analysis of cytochrome *c* release from mitochondria induced by diosgenyl glycosides. (A) HL-60 cells were treated with 10 μM of the indicated diosgenyl glycosides and harvested at 6 h. After treatment, cytosolic extracts were prepared, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and cytochrome *c* was detected by immunoblotting. β-Actin was used as loading control. (B) U937 cells were treated as above and cytosolic or mitochondrial lysates were analyzed by immunoblotting with an anti-cytochrome *c* antibody.

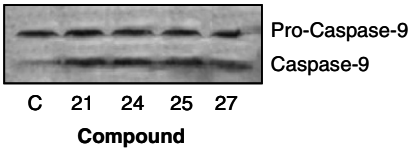


Figure 5. Western blot analysis for the cleavage of procaspase-9. HL-60 cells were cultured in the presence of 10 μM of the indicated diosgenyl glycosides and harvested at 6 h. Total cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by immunoblotting with an anti-procaspase-9 antibody that also recognizes the proteolytic fragments generated.

Taken together, these results allow us to conclude that the mitochondria play an important role in the activation of caspase-3, proteolytic cleavage of poly(ADP-ribose) polymerase, and induction of apoptosis triggered by diosgenyl glycosides in human myeloid cells.

4. Conclusions

In conclusion, we have synthesized a series of diosgenyl glycosides binding different monosaccharide units to

diosgenyl β-glucopyranoside (trillin) and evaluated the cytotoxicity against three different human cell lines (HL-60, U937, and SK-MEL-1). Not all diosgenyl glycosides were equally cytotoxic to the three cell lines. SK-MEL-1 cells were resistant to compounds **19**–**24**, while compounds **25** and **27** were the most cytotoxic toward all cell lines assayed. Induction of cell death by diosgenyl glycosides **21** and **24** in human leukemia cells was associated with cell cycle delays at the G_1 (and S) phase. However, compounds **25** and **27** induced G_2/M arrest like most DNA-damaging agents that arrest cells at the G_2/M transition. Cell death induced by diosgenyl glycosides **21**, **24**, **25**, and **27** was preceded by a rapid release of cytochrome *c* from mitochondria into the cytosol and subsequent caspase activation involving caspase-9 and -3 to cleave poly (ADP-ribose) polymerase. These findings suggest that these diosgenyl glycosides present anti-tumor activity, which may be mediated by apoptosis caused by cytochrome *c* release and caspase activation in human leukemia cells. Our data should contribute to the development of diosgenyl glycosides or related drugs as potential cancer chemotherapeutic or chemopreventive agents.

5. Experimental

5.1. General experimental procedures

Optical rotations were recorded in a Perkin-Elmer 343 polarimeter. ^{1}H NMR and ^{13}C NMR spectra were obtained on Bruker AMX-400, Avance 400, and Avance 300 spectrometers with standard pulse sequences operating at 400, 300 MHz in ^{1}H NMR and 100, 75 MHz in ^{13}C NMR. Chemical shifts are given in δ values (ppm) using tetramethylsilane as the internal standard. EIMS, HREIMS, FABS, and HRFABS were taken on a Micromass Autospec (70 eV) spectrometer. Column chromatography was carried out on silica gel 60 (Merck 230–400 mesh), and preparative TLC on silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thickness), and Sephadex LH-20 (Aldrich). IR data reported in cm^{-1} were obtained using a Bruker IFS 55 spectrophotometer. Elemental analyses (C, H, N) were performed on a Fisons EA 1108 analyzer.

5.2. General conditions for coupling

To a mixture of diosgenyl 4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside **4a** (360 mg, 0.48 mmol) and 4 Å MS (0.6 g) in dry CH_2Cl_2 (12 mL) at −78 °C, under N_2, $BF_3{\cdot}OEt_2$ (0.07 mL, 0.57 mmol) was added, followed by a solution of imidate **5** (349 mg, 0.71 mmol) in dry CH_2Cl_2 (4 mL). The mixture, warmed up to room temperature for 7 h, was neutralized with Et_3N (≈0.1 mL) to yield saponin **12** (Table 1).

5.2.1. Diosgenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (12). White amorphous solid, $[\alpha]_D^{20}$ −44° (*c* 0.100, $CHCl_3$). ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 0.76 (6H, CH_3-18, CH_3-27), 0.95 (d, 3H, *J* = 6.7 Hz, CH_3-21), 1.00 (s, 3H, CH_3-19), 1.22 (s, 9H, CH_3-Piv), 1.97 (s, 3H, –OAc), 1.99 (s, 3H, –OAc), 2.03 (s, 3H, –OAc), 2.05 (s, 3H, –OAc), 3.38 (t, 1H, *J* = 10.8 Hz, H-26), 3.45 (m, 3H, H-26, H-3, H-5′), 3.57 (t, 1H, *J* = 9.4 Hz, H-4′), 3.67 (m, 2H, H-5″, H-2′), 3.71 (m, 1H, H-6′), 4.09 (m, 1H, H-6″), 4.29–4.42 (m, 3H, H-6′, H-6″, H-16), 4.61 (d, 1H, *J* = 8.0 Hz, H-1′), 4.67 (d, 1H, *J* = 7.9 Hz, H-1″), 4.97 (t, 1H, *J* = 7.2 Hz, H-2″), 5.08 (m, 2H, H-3″, H-4″), 5.26 (t, 1H, *J* = 7.9 Hz, H-3′), 5.35 (br d, 1H, H-6), 5.43 (s, 1H, Ph-*CH*), 7.29–7.35 (m, 5H, Ph). ^{13}C NMR (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 170.7 (*C*O-Ac), 170.2 (*C*O-Ac), 169.8 (*C*O-Ac), 169.4 (*C*O-Ac), 140.4 (C-5), 128.2 (Ph), 125.8 (Ph), 121.8 (C-6), 109.3 (C-22), 101.1 (Ph*C*H), 100.7 (C-1″), 100.2 (C-1′), 80.80 (C-16), 80.20 (C-3), 78.9 (C-4′), 78.7 (C-2′), 73.6 (C-3′), 73.0 (C-3″), 71.0 (C-5″), 71.0 (C-2″), 68.8 (C-6′), 68.4 (C-4″), 66.85 (C-26), 65.4 (C-5′), 62.19 (C-17), 62.0 (C-6″), 56.51 (C-14), 50.07 (C-9), 41.62 (C-20), 40.27 (C-13), 39.78 (C-12), 38.69 (C-4), 37.12 (C-1), 36.81 (C-10), 32.05 (C-7), 31.83 (C-2), 31.42 (C-15), 31.40 (C-8), 30.30 (C-25), 29.56 (C-23), 28.81 (C-24), 27.16 (CH_3-Piv), 20.86 (C-11), 20.76 (*C*H_3-Ac), 20.55 (*C*H_3-Ac) and 19.36 (C-19), 17.12 (C-27), 16.28 (C-18), 14.51 (C-21). HRFABMS: 1079.5514 $(M+H)^+$ (calcd for $C_{59}H_{83}O_{18}$, 1079.5579) (5), 665 (12), 398 (14), 397 (56), 331 (36). Anal. Calcd for $C_{59}H_{82}O_{18}$: C, 65.66; H, 7.66. Found: C, 65.66; H, 7.28.

5.2.2. Diosgenyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (13). White amorphous solid, $[\alpha]_D^{20}$ −38° (*c* 0.102, $CHCl_3$). ^{1}H NMR (δ, $CDCl_3$, 400 MHz): 0.76 (m, 6H, CH_3-18, CH_3-27), 0.95 (d, 3H, *J* = 6.7 Hz, CH_3-21), 1.00 (s, 3H, CH_3-19), 1.20 (s, 9H, CH_3-Piv), 1.95 (s, 3H, –OAc), 2.02 (s, 3H, –OAc), 2.04 (s, 3H, –OAc), 2.11 (s, 3H, –OAc), 3.35 (t, 1H, *J* = 10.9 Hz, H-26), 3.48 (m, 3H, H-26, H-3, H-5′), 3.59 (t, 1H, *J* = 9.5 Hz, H-4′), 3.68–3.73 (m, 2H, H-2′, H-6′), 3.86 (t, 1H, *J* = 7.0 Hz, H-5″), 4.0 (dd, 1H, *J* = 10.5; 5.0 Hz, H-6′), 4.12 (m, 2H, H-6″), 4.39 (dd, 1H, *J* = 14.2; 6.6 Hz, H-16), 4.63 (m, 2H, H-1′, H-1″), 4.89 (dd, 1H, *J* = 10.5; 3.3 Hz, H-3″), 5.14 (dd, 1H, *J* = 10.4; 8.0 Hz, H-2″), 5.26 (t, 1H, *J* = 8.5 Hz, H-3′), 5.35 (m, 2H, H-6, H-4″), 5.43 (s, 1H, Ph-C*H*), 7.29–7.35 (m, 5H, Ph). ^{13}C NMR (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 170.4, 170.2 and 169.9 (*C*O-Ac), 140.6 (C-5), 128.2 (Ph), 125.8 (Ph), 121.7 (C-6), 109.3 (C-22), 101.1 (Ph*C*H), 100.9 (C-1″), 100.3 (C-1′), 80.82 (C-16), 80.16 (C-3), 78.80 (C-4′), 78.23 (C-2′), 73.58 (C-3′), 71.05 (C-3″), 70.41 (C-5″), 68.88 (C-6′), 68.73 (C-2″), 66.88 (C-4″), 66.88 (C-26), 65.50 (C-5′), 62.15 (C-17), 60.9 (C-6″), 56.50 (C-14), 50.07 (C-9), 41.63 (C-20), 40.28 (C-13), 39.77 (C-12), 38.79 (C-4), 37.07 (C-1), 36.84 (C-10), 32.06 (C-7), 31.85 (C-2), 31.47 (C-8), 31.41 (C-15), 30.30 (C-25), 29.51 (C-23), 28.82 (C-24), 27.16 (*C*H_3-Piv), 20.86 (C-11), 20.73, 20.65 and 20.54 (*C*H_3-Ac), 19.40 (C-19), 17.12 (C-27), 16.28 (C-18), 14.51 (C-21). HRFABMS: 1079 $(M+H)^+$, 1078.5470 (M^+) (calcd. for $C_{59}H_{83}O_{18}$, 1078.5501) (5), 665 (14), 398 (17), 397 (57), 331 (74). Anal. Calcd for $C_{59}H_{82}O_{18}$: C, 65.66; H, 7.66. Found: C, 65.91; H, 7.40.

5.2.3. Diosgenyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (14). White amorphous solid, $[\alpha]_D^{20}$ −43° (*c*

0.020, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.76 (m, 6H, CH_3-18, CH_3-27), 0.89 (d, 3H, J = 5.5 Hz, CH_3-21), 0.99 (s, 3H, CH_3-19), 1.16 (s, 9H, CH_3-Piv), 3.35 (t, 1H, J = 10.9 Hz, H-26), 3.44 (d, 1H, J = 3.4 Hz, H-26), 3.50 (m, 1H, H-5′), 3.56 (t, 1H, J = 9.6 Hz, H-4′), 3.61 (m, 1H, H-3), 3.70 (m, 1H, H-2′), 3.56 (m, 1H, H-4′), 3.74 (t, 1H, J = 10.2 Hz, H-6′$_b$), 3.99 (m, 1H, H-5′), 4.33 (dd, 1H, J = 10.4; 4.8 Hz, H − 6′$_a$), 4.38 (dd, 1H, J = 14.4, 7.1 Hz, H-16), 4.71 (d, 1H, J = 7.5 Hz, H-1′), 5.22 (dd, 1H, J = 10.1; 2.7 Hz, H-3″), 5.29 (m, 1H, H-2″), 5.30 (m, 1H, H-4″), 5.33 (t, 1H, J = 9.7 Hz, H-3′), 5.36 (br d, 1H, H-6), 5.44 (s, 1H, H-1″), 5.45 (s, 1H, PhC*H*), 7.33–7.40 (m, 5H, Ph). ^{13}C RMN (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 170.7, 169.7, 169.6 and 169.5 (*C*O-Ac), 140.0 (C-5), 125.8 and 128.2 (Ph), 122.1 (C-6), 109.3 (C-22), 101.5 (C-1′), 101.1 (Ph*C*H), 97.2 (C-1″), 80.81 (C-16), 79.25 (C-4′), 79.25 (C-3), 76.48 (C-2′), 72.43 (C-3′), 69.33 (C-2″), 68.89 (C-3″), 68.63 (C-6′), 68.63 (C-5″), 66.82 (C-26), 65.95 (C-5′), 65.79 (C-4″), 62.13 (C-17), 62.13 (C-6″), 56.47 (C-14), 50.07 (C-9), 41.62 (C-20), 40.26 (C-13), 39.79 (C-12), 38.07 (C-4), 37.17 (C-1), 36.85 (C-10), 32.10 (C-7), 31.84 (C-2), 31.84 (C-15), 31.40 (C-8), 30.29 (C-25), 29.63 (C-23), 28.81 (C-24), 27.16 (*C*H_3-Piv), 20.80 (C-11), 20.71 and 20.63 (*C*H_3-Ac), 19.18 (C-19), 17.12 (C-27), 16.24 (C-18), 14.51 (C-21). HRFABMS: 1079.5623 $(M+H)^+$ (calcd for $C_{59}H_{83}O_{18}$, 1079.5579), 661 (6), 398 (9), 397 (39), 331 (32). Anal. Calcd for $C_{59}H_{82}O_{18}$: C, 65.66; H, 7.66. Found: C, 65.35; H, 7.61.

5.2.4. Diosgenyl 2,3,4-tri-*O*-acetyl-β-D-xilopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (15). White amorphous solid, $[\alpha]_D^{20}$ −57° (*c* 0.100, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.77 (m, 6H, CH_3-18, CH_3-27), 0.96 (d, 3H, J = 6.7 Hz, CH_3-21), 1.02 (s, 3H, CH_3-19), 1.21 (s, 9H, CH_3-Piv), 2.05 (s, 3H, –OAc), 2.08 (s, 3H, –OAc), 2.16 (s, 3H, –OAc), 3.35 (m, 1H, H-26), 3.38 (1H, H-5″), 3.44–3.55 (m, 4H, H-26, H-3, H-5′, H-4′), 3.69–3.76 (m, 2H, H-2′, H-6′), 4.25 (dd, 1H, J = 12.2; 4.7 Hz, H-5″), 4.31 (dd, 1H, J = 10.5; 4.9 Hz, H-6′), 4.40 (dd, 1H, J = 14.7; 6.9 Hz, H-16), 4.62 (d, 1H, J = 7.2 Hz, H-1′), 4.75 (d, 1H, J = 6.1 Hz, H-1″), 4.85–4.93 (m, 2H, H-2″, H-4″), 5.06 (t, 1H, J = 7.9 Hz, H-3″), 5.29 (t, 1H, J = 8.9 Hz, H-3′), 5.37 (br d, 1H, H-6), 5.44 (s, 1H, Ph-*CH*), 7.29–7.35 (m, 5H, Ph). ^{13}C RMN (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 169.9 and 169.6 (*C*O-Ac), 140.2 (C-5), 128.2 and 125.8 (Ph), 121.9 (C-6), 109.3 (C-22), 101.1 (Ph*C*H), 101.0 (C-1″), 100.1 (C-1′), 80.80 (C-16), 79.73 (C-3), 78.91 (C-4′), 77.25 (C-2′), 73.74 (C-3′), 70.89 (C-3″), 70.19 (C-2″), 69.05 (C-4″), 68.79 (C-6′), 66.81 (C-26), 65.59 (C-5′), 62.06 (C-17), 61.78 (C-5″), 56.47 (C-14), 50.04 (C-9), 41.58 (C-20), 40.24 (C-13), 39.74 (C-12), 38.63 (C-4), 37.12 (C-1), 36.82 (C-10), 32.04 (C-7), 31.81 (C-2), 31.37 (C-8), 31.37 (C-15), 30.27 (C-25), 29.53 (C-23), 28.77 (C-24), 27.16 (CH_3-Piv), 20.83 (C-11), 20.77, 20.70 and 20.63 (*C*H_3-Ac), 19.40 (C-19), 17.10 (C-27), 16.27 (C-18), 14.49 (C-21). FABMS: 1008 $(M+H)^+$($C_{56}H_{79}O_{16}$), 1005 $(M-H)^+$, 604 (10), 593 (16), 523 (24), 398 (24), 397 (54), 396 (21). Anal. Calcd for $C_{56}H_{79}O_{16}$: C, 66.78; H, 7.81. Found: C, 66.91; H, 7.35.

5.2.5. Diosgenyl 2,3,4-tri-*O*-acetyl-β-L-arabinopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (16). White amorphous solid, $[\alpha]_D^{20}$ −33° (*c* 0.105, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.75 (m, 6H, CH_3-18, CH_3-27), 0.94 (d, 3H, J = 6.7 Hz, CH_3-21), 0.98 (s, 3H, CH_3-19), 1.17 (s, 9H, CH_3-Piv), 2.00 (s, 3H, –OAc), 2.03 (s, 3H, –OAc), 2.05 (s, 3H, –OAc), 3.35 (t, 1H, J = 10.9 Hz, H-26), 3.43–3.60 (m, 5H, H-26, H-3, H-5′, H-5″, H-4′), 3.66–3.73 (m, 2H, H-2′, H-6′), 4.12 (dd, 1H, J = 12.7; 4.8 Hz, H-5″), 4.29 (dd, 1H, J = 10.5; 4.9 Hz, H-6′), 4.40 (dd, 1H, J = 14.7; 6.9 Hz, H-16), 4.63 (d, 1H, J = 7.0 Hz, H-1′), 4.65 (d, 1H, J = 5.7 Hz, H-1″), 4.98 (dd, 1H, J = 8.2; 3.4 Hz, H-3″), 5.04 (dd, 1H, J = 8.1; 5.8 Hz, H-2″), 5.16 (m, 1H, H-4″), 5.25 (t, 1H, J = 8.5 Hz, H-3′), 5.36 (br d, 1H, H-6), 5.42 (s, 1H, Ph-*CH*), 7.29–7.35 (m, 5H, Ph). ^{13}C RMN (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 170.2 and 170.1 (*C*O-Ac), 140.5 (C-5), 128.2 (Ph) and 125.8 (Ph), 121.8 (C-6), 109.3 (C-22), 101.1 (Ph*C*H), 100.2 (C-1′), 99.9 (C-1″), 80.78 (C-16), 79.70 (C-3), 78.87 (C-4′), 77.40 (C-2′), 73.63 (C-3′), 69.46 (C-3″), 69.10 (C-2″), 68.87 (C-6′), 66.99 (C-4″), 66.81 (C-26), 65.54 (C-5′), 62.10 (C-17), 61.73 (C-5″), 56.47 (C-14), 50.07 (C-9), 41.60 (C-20), 40.25 (C-13), 39.74 (C-12), 38.79 (C-4), 37.09 (C-1), 36.83 (C-10), 32.05 (C-7), 31.82 (C-2), 31.43 (C-8), 31.38 (C-15), 30.27 (C-25), 29.51 (C-23), 28.79 (C-24), 27.16 (CH_3-Piv), 20.86 (C-11), 20.74 and 20.63 (*C*H_3-Ac), 19.34 (C-19), 17.13 (C-27), 16.29 (C-18), 14.52 (C-21) FABMS: 1008 $(M+H)^+$ ($C_{56}H_{79}O_{16}$), 397 (64). Anal. Calcd for $C_{56}H_{79}O_{16}$: C, 66.78; H, 7.81. Found: C, 66.85; H, 7.43.

5.2.6. Diosgenyl 2,3,4-tri-*O*-acetyl-β-L-fucopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (17). White amorphous solid, $[\alpha]_D^{20}$ −34° (*c* 0.095, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.73 (m, 6H, CH_3-18, CH_3-27), 0.91 (d, 3H, J = 6.7 Hz, CH_3-21), 1.00 (s, 3H, CH_3-19), 1.11 (d, 3H, J = 6.2 Hz, H-6″), 1.19 (s, 9H, CH_3-Piv), 1.91 (s, 3H, –OAc), 1.97 (s, 3H, –OAc), 2.05 (s, 3H, –OAc), 3.31 (t, 1H, J = 10.9 Hz, H-26), 3.41–3.56 (m, 4H, H-26, H-3, H-5′, H-4′), 3.66–3.73 (m, 3H, H-2′, H-6′, H-5″), 4.26 (dd, 1H, J = 10.5; 4.8 Hz, H-6′), 4.36 (dd, 1H, J = 14.7; 6.9 Hz, H-16), 4.59 (d, 1H, J = 7.9 Hz, H-1′), 4.86 (d, 1H, J = 7.6 Hz, H-1″), 4.90–4.98 (m, 2H, H-3″, H-4″), 5.12–5.19 (m, 2H, H-2″, H-3′), 5.32 (br d, 1H, H-6), 5.41 (s, 1H, Ph-C*H*), 7.29–7.35 (m, 5H, Ph). ^{13}C RMN (δ, $CDCl_3$, 75 MHz) 177.0 (*C*O-Piv), 170.7, 170.2 169.8 and 169.4 (*C*O-Ac), 140.4 (C-5), 128.2 and 125.8 (Ph). 121.8 (C-6), 109.3 (C-22), 101.1 (Ph*C*H), 100.97 (C-1′), 99.08 (C-1″), 80.80 (C-16), 79.90 (C-3), 78.92 (C-4′), 75.49 (C-2′), 71.79 (C-4″), 70.94 (C-2″), 70.28 (C-3′), 69.56 (C-3″), 68.80 (C-5″), 68.66 (C-6′), 66.81 (C-26), 66.11 (C-5′), 62.10 (C-17), 56.51 (C-14), 50.07 (C-9), 41.62 (C-20), 40.27 (C-13), 39.78 (C-12), 38.80 (C-4), 37.10 (C-1), 36.81 (C-10), 32.05 (C-7), 31.79 (C-2), 31.42 (C-8), 31.42 (C-15), 30.30 (C-25), 29.56 (C-23), 28.81 (C-24), 27.16 (*C*H_3-Piv), 20.97 (C-6″), 20.86 (C-11), 20.76 and 20.55 (*C*H_3-Ac), 19.39 (C-19), 17.12 (C-27), 16.28 (C-18), 14.51 (C-21). HRFABMS: 1021.5515 $(M+H)^+$ (calcd. for $C_{57}H_{81}O_{16}$, 1021.5525) (5), 607 (14), 398 (14), 397 (50), 396 (13). Anal. Calcd for $C_{57}H_{80}O_{16}$: C, 67.04; H, 7.90. Found: C, 67.05; H, 7.93.

5.2.7. Diosgenyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (18). White amorphous solid, $[\alpha]_D^{20}$ −45° (*c* 0.090, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.77 (m, 6H, CH_3-18, CH_3-27), 0.96 (d, 3H, *J* = 6.7 Hz, CH_3-21), 1.01 (s, 3H, CH_3-19), 1.19 (d, 3H, *J* = 6.1 Hz, CH_3-6′), 1.12 (s, 9H, CH_3-Piv), 1.95 (s, 3H, –OAc), 2.00 (s, 3H, –OAc), 2.09 (s, 3H, –OAc), 3.36 (t, 1H, *J* = 10.8 Hz, H-26), 3.45–3.54 (m, 3H, H-26, H-5′, H-4′), 3.63 (m, 1H, H-3), 3.71–3.80 (m, 2H, H-2′, H-6′), 4.32 (dd, 1H, *J* = 10.5; 4.8 Hz, H-6′), 4.37–4.48 (m, 2H, H-16, H-5″), 4.70 (d, 1H, *J* = 7.6 Hz, H-1′), 4.93 (s, 1H, H-1″), 5.04 (t, 1H, *J* = 9.9 Hz, H-4″), 5.17 (d, 1H, *J* = 1.4 Hz, H-2″), 5.23 (dd, 1H, *J* = 9.9; 3.3 Hz, H-3″), 5.37–5.41 (m, 2H, H-6, H-3′), 5.43 (s, 1H, Ph-C*H*), 7.29–7.35 (m, 5H, Ph). ^{13}C RMN (δ, $CDCl_3$, 75 MHz): 177.0 (*C*O-Piv), 170.55, 170.27 and 169.27 (*C*O-Ac), 140.09 (C-5), 128.2 and 125.8 (Ph), 122.03 (C-6), 109.22 (C-22), 101.1 (Ph*C*H), 99.6 (C-1′), 97.6 (C-1″), 80.74 (C-16), 79.04 (C-4′), 78.61 (C-3), 75.39 (C-2′), 73.84 (C-3′), 71.22 (C-4″), 69.60 (C-2″), 68.84 (C-3″), 68.65 (C-6′), 66.77 (C-26), 66.40 (C-5″), 66.02 (C-5′), 62.09 (C-17), 56.44 (C-14), 50.06 (C-9), 41.57 (C-20), 40.26 (C-13), 39.72 (C-12), 38.15 (C-4), 37.16 (C-1), 36.82 (C-10), 32.03 (C-7), 31.83 (C-2), 31.40 (C-15), 31.35 (C-8), 30.24 (C-25), 29.74 (C-23), 28.78 (C-24), 27.16 (CH_3-Piv), 20.89 (C-11), 20.97, 20.89 and 20.52 (*C*H_3-Ac), 19.41 (C-19), 17.21 (C-6″), 17.13 (C-27), 16.00 (C-18), 14.52 (C-21). HRFABMS: 1021.5549 $(M+H)^+$ (calcd for $C_{57}H_{81}O_{16}$, 1021.5525) (4), 607 (17), 398 (19), 397 (65), 396 (17). Anal. Calcd for $C_{57}H_{81}O_{16}$: C, 67.04; H, 7.90. Found: C, 67.09; H, 7.89.

5.3. General procedure to obtain compounds 19–25

A solution of **12** (269.5 mg, 0.25 mmol) in AcOH (20 mL, 80%) was stirred at 70 °C for 2 h, then concentrated and dissolved in CH_2Cl_2/MeOH (1:1) (8 mL) and NaOH (0.1 equiv for each ester) was added and stirred overnight at 40 °C. The mixture was neutralized with acid resin Dowex-50 (H^+), the residue was filtered, and the solvent was evaporated to yield compound **19** (Table 1).

5.3.1. Diosgenyl β-D-glucopyranosyl-(1 → 2)-β-D-glucopyranoside (19). White amorphous solid, $[\alpha]_D^{20}$ −38° (*c* 0.011, EtOH); IR ν_{max} (film, NaCl) 3363, 2926, 1376, 1075, 982, 898 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.66 (d, 3H, *J* = 4.3 Hz, CH_3-27), 0.78 (s, 3H, CH_3-18), 0.95 (s, 3H, CH_3-19), 1.10 (d, 3H, *J* = 6.8 Hz, CH_3-21), 1.77 (m, 1H, H-17), 3.45 (t, 1H, *J* = 9.9 Hz, H-26), 3.54 (m, 1H, H-26), 3.80 (m, 1H, H-3), 3.87 (m, 1H, H-5′), 3.95 (m, 1H, H-5″), 4.11 (m, 2H, H-2′, H-2″), 4.21 (m, 2H, H-3″, H-4″), 4.31 (m, 2H, H-3′, H-4′), 4.31–4.50 (dd, 1H, *J* = 14.4, 7.1 Hz, H-16), 4.51 (m, 2H, H-6′, H-6″), 5.03 (d, 1H, *J* = 7.5 Hz, H-1′), 5.24 (d, 1H, *J* = 7.6 Hz, H-1″), 5.30 (br d, 1H, *J* = 4.6 Hz, H-6). ^{13}C NMR (δ, pyridine, 75 MHz): 141.0 (C-5), 121.6 (C-6), 109.3 (C-22), 106.5 (C-1″), 101.4 (C-1′), 84.42 (C-2′), 81.83 (C-16), 79.35 (C-3), 78.73 (C-5′), 78.16 (C-5″), 77.96 (C-3″), 77.84 (C-3′), 76.95 (C-2″), 71.42 (C-4′), 71.36 (C-4″), 66.84 (C-26), 62.84 (C-17), 62.84 (C-6″), 62.62 (C-6′), 56.65 (C-14), 50.24 (C-9), 41.97 (C-20), 40.45 (C-13), 39.89 (C-12), 39.22 (C-4), 37.32 (C-1), 37.02 (C-10), 32.18 (C-2), 32.18 (C-7), 31.79 (C-8), 31.61 (C-15), 30.58 (C-25), 30.21 (C-23), 29.24 (C-24), 21.11 (C-11), 19.49 (C-19), 17.34 (C-27), 16.37 (C-18), 15.05 (C-21). FABMS *m*/*z* 761 $(M+Na)^+$, 413, 397. HRFABMS *m*/*z* 761.4117, calcd for $C_{39}H_{62}O_{13}Na$, 761.4088; Anal. Calcd for $C_{39}H_{62}O_{13}$: C, 63.39; H, 8.46. Found: C, 63.32; H, 8.74.

5.3.2. Diosgenyl β-D-galactopyranosyl-(1 → 2)-β-D-glucopyranoside (20). White amorphous solid, $[\alpha]_D^{20}$ −27° (*c* 0.041, EtOH); IR ν_{max} (film, NaCl) 3363, 2925, 1462, 1080, 897 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.65 (d, 3H, *J* = 4.7 Hz, CH_3-27), 0.78 (s, 3H, CH_3-18), 0.94 (s, 3H, CH_3-19), 1.09 (d, 3H, *J* = 6.8 Hz, CH_3-21), 3.45 (t, 1H, *J* = 9.9 Hz, H-26), 3.53 (m, 1H, H-26), 3.79–3.84 (m, 2H, H-3, H-5′), 3.95 (m, 1H, H-5″), 4.08 (m, 2H, H-2′, H-2″), 4.15 (m, 1H, H-3″), 4.19 (m, 1H, H-4′), 4.27–4.33 (m, 2H, H-3′, H-6″), 4.44–4.54 (m, 3H, H-6′, H-6″) 4.55 (m, 1H, H-5″), 4.58 (m, 1H, H-2″), 5.02 (d, 1H, *J* = 7.5 Hz, H-1′), 5.11 (d, 1H, *J* = 7.7 Hz, H-1″), 5.33 (br d, 1H, H-6). ^{13}C NMR (δ, pyridine, 75 MHz): 140.8 (C-5), 121.3 (C-6), 109.0 (C-22), 107.3 (C-1″), 101.2 (C-1′), 85.00 (C-2′), 81.87 (C-16), 79.12 (C-3), 77.93 (C-5′), 77.67 (C-3′), 76.97 (C-4″), 74.80 (C-3″), 74.35 (C-2″), 71.12 (C-4′), 69.64 (C-5″), 62.65 (C-17), 66.64 (C-26), 62.64 (C-6″), 62.33 (C-6′), 56.42 (C-14), 50.01 (C-9), 41.73 (C-20), 40.21 (C-13), 39.65 (C-12), 39.05 (C-4), 37.18 (C-1), 36.81 (C-10), 32.04 (C-2), 32.04 (C-7), 31.58 (C-8), 31.40 (C-15), 30.36 (C-25), 30.00 (C-23), 29.03 (C-24), 20.88 (C-11), 19.27 (C-19), 17.10 (C-27), 16.12 (C-18), 14.80 (C-21). FABMS *m*/*z* 761 $(M+Na)^+$, 397; HRFABMS *m*/*z* 761.4053, calcd for $C_{39}H_{62}O_{13}Na$, 761.4088; Anal. Calcd for $C_{39}H_{62}O_{13}$: C, 63.39; H, 8.46. Found: C, 63.34; H, 8.71.

5.3.3. Diosgenyl α-D-mannopyranosyl-(1 → 2)-β-D- glucopyranoside (21). White amorphous solid, $[\alpha]_D^{20}$ −34 ° (*c* 0.013, EtOH); IR ν_{max} (film, NaCl) 3427, 2930, 1645, 979 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.65 (d, 3H, *J* = 4.3 Hz, CH_3-27), 0.79 (s, 3H, CH_3-18), 0.81 (s, 3H, CH_3-19), 1.09 (d, 3H, *J* = 6.9 Hz, CH_3-21), 3.45 (t, 1H, *J* = 10.4 Hz, H-26), 3.54 (m, 1H, H-26), 3.76 (m, 1H, H-5′), 3.85 (m, 1H, H-3), 3.95 (m, 1H, H-5″), 4.10–4.17 (m, 3H, H-2′, H-3′, H-4′), 4.27–4.55 (m, 3H, H-6″, H-6′, H-16), 4.68 (dd, 1H, *J* = 3.2; 9.1 Hz, H-3″), 4.75–4.79 (m, 2H, H-2″, H-4″), 4.94 (d, 1H, *J* = 7.3 Hz, H-1′), 5.13 (m, 1H, H-5″), 5.19 (br d, 1H, *J* = 4.8 Hz, H-6), 6.07 (s, 1H, H-1″). ^{13}C NMR (δ, pyridine, 75 MHz): 141.0 (C-5), 121.6 (C-6), 109.3 (C-22), 106.5 (C-1″), 101.4 (C-1′), 84.42 (C-2′), 81.83 (C-16), 79.35 (C-3), 78.73 (C-5′), 78.16 (C-5″), 77.96 (C-3″), 77.84 (C-3′), 76.95 (C-2″), 71.42 (C-4′), 71.36 (C-4″), 66.84 (C-26), 62.84 (C-17), 62.84 (C-6″), 62.80 (C-6′), 56.65 (C-14), 50.24 (C-9), 41.97 (C-20), 40.45 (C-13), 39.89 (C-12), 39.22 (C-4), 38.32 (C-1), 37.02 (C-10), 32.18 (C-2), 32.18 (C-7), 31.79 (C-8), 31.61 (C-15), 30.58 (C-25), 30.21 (C-23), 29.24 (C-24), 21.11 (C-11), 19.49 (C-19), 17.34 (C-27), 16.37 (C-18), 15.05 (C-21). FABMS *m*/*z* 761 $(M+Na)^+$, 513, 397; HRFABMS *m*/*z*

761.4088, calcd for $C_{39}H_{62}O_{13}Na$, 761.4088; Anal. Calcd for $C_{39}H_{62}O_{13}$: C, 63.39; H, 8.46. Found: C, 63.41; H, 8.42.

5.3.4. Diosgenyl β-D-xylopyranosyl-(1 → 2)-β-D-glucopyranoside (22). White amorphous solid, $[\alpha]_D^{20}$ −39 ° (*c* 0.022, EtOH); IR ν_{max} (film, NaCl) 3271, 2928, 1046, 982, 833 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.66 (d, 3H, *J* = 4.3 Hz, CH_3-27), 0.80 (s, 3H, CH_3-18), 0.92 (s, 3H, CH_3-19), 1.10 (d, 3H, *J* = 6.8 Hz, CH_3-21), 3.45 (t, 1H, *J* = 9.5 Hz, H-26), 3.55 (m, 1H, H-26), 3.72 (t, 1H, *J* = 10.2 Hz, H-5″), 3.86 (m, 2H, H-3, H-5′), 4.04–4.10 (m, 2H, H-2′, H-2″), 4.14–4.19 (m, 3H, H-4′, H-3″, H-4″), 4.29 (m, 2H, H-3′, H-6′), 4.40–4.51 (m, 3H, H-6′, H-5″, H-16), 5.00 (d, 1H, *J* = 7.4 Hz, H-1′), 5.18 (m, 1H, H-1″), 5.29 (br d, 1H, H-6). ^{13}C NMR (δ, pyridine, 75 MHz): 140.8 (C-5), 121.46 (C-6), 109.0 (C-22), 106.5 (C-1″), 100.7 (C-1′), 83.32 (C-2′), 81.86 (C-16), 78.80 (C-3), 78.04 (C-5′), 77.95 (C-3′), 77.43 (C-3″), 75.79 (C-2″), 71.09 (C-4′), 70.79 (C-4″), 67.12 (C-5″), 62.65 (C-17), 66.62 (C-26), 62.33 (C-6′), 56.44 (C-14), 50.02 (C-9), 41.73 (C-20), 40.23 (C-13), 39.66 (C-12), 39.00 (C-4), 37.16 (C-1), 36.84 (C-10), 32.01 (C-2), 31.96 (C-7), 31.58 (C-8), 31.43 (C-15), 30.36 (C-25), 29.90 (C-23), 29.03 (C-24), 20.89 (C-11), 19.20 (C-19), 17.10 (C-27), 16.14 (C-18), 14.81 (C-21); FABMS *m*/*z* 731 $(M+Na)^+$, 415, 397. HRFABMS *m*/*z* 731.3962, calcd for $C_{38}H_{60}O_{12}Na$, 731.3982; Anal. Calcd for $C_{38}H_{60}O_{12}$: C, 64.38; H, 8.53. Found: C, 64.45; H, 8.46.

5.3.5. Diosgenyl β-L-arabinopyranosyl-(1 → 2)-β-D-glucopyranoside (23). White amorphous solid, $[\alpha]_D^{20}$ −21° (*c* 0.038, EtOH); IR ν_{max} (film, NaCl) 3442, 2360, 1054, 835 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.72 (d, 3H, *J* = 4.3 Hz, CH_3-27), 0.86 (s, 3H, CH_3-18), 0.98 (s, 3H, CH_3-19), 1.16 (d, 3H, *J* = 6.8 Hz, CH_3-21), 3.52 (t, 1H, *J* = 9.5 Hz, H-26), 3.62 (m, 1H, H-26), 3.84–3.91 (m, 3H, H-5′, H-6″, H-3), 4.22 (t, 1H, *J* = 10.2 Hz, H-4′), 4.27–4.37 (m, 3H, H-3′, H-3″, H-6′), 4.44–4.52 (m, 2H, H-6′, H-6″), 4.56–4.62 (m, 2H, H-2″, H-16), 5.05 (d, 1H, *J* = 7.2 Hz, H-1′), 5.30 (d, 1H, *J* = 5.8 Hz, H-1″), 5.34 (br d, 1H, H-6). ^{13}C NMR (δ, pyridine, 75 MHz): 140.9 (C-5), 121.76 (C-6), 109.3 (C-22), 105.7 (C-1″), 100.9 (C-1′), 82.85 (C-2′), 81.12 (C-16), 78.92 (C-3), 78.13 (C-3′), 78.13 (C-5′), 74.06 (C-3″), 73.09 (C-2″), 71.30 (C-4′), 68.58 (C-4″), 66.85 (C-26), 66.14 (C-5″), 62.79 (C-17), 62.48 (C-6′), 56.64 (C-14), 50.22 (C-9), 41.94 (C-20), 40.44 (C-13), 39.86 (C-12), 39.12 (C-4), 37.40 (C-1), 37.02 (C-10), 32.15 (C-2), 32.15 (C-7), 31.76 (C-8), 31.62 (C-15), 30.55 (C-25), 29.94 (C-23), 29.21 (C-24), 21.09 (C-11), 19.42 (C-19), 17.31 (C-27), 16.36 (C-18), 15.02 (C-21). FABMS *m*/*z* 731 $(M+Na)^+$ 415, 397. HRFABMS *m*/*z* 731.3977, calcd for $C_{38}H_{60}O_{12}Na$, 731.3982; Anal. Calcd for $C_{38}H_{60}O_{12}$: C, 64.38; H, 8.53. Found: C, 64.25; H, 8.59.

5.3.6. Diosgenyl β-L-fucopyranosyl-(1 → 2)-β-D-glucopyranoside (24). White amorphous solid, $[\alpha]_D^{20}$ −54° (*c* 0.024, EtOH); IR ν_{max} (film, NaCl) 3388, 2940, 1052, 900, 865 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.65 (d, 3H, *J* = 4.3 Hz, CH_3-27), 0.79 (s, 3H, CH_3-18), 0.94 (s, 3H, CH_3-19), 1.10 (d, 3H, *J* = 6.7 Hz, CH_3-21), 1.45 (d, 3H, *J* = 6.2 Hz, CH_3-6″), 3.45 (t, 1H, *J* = 9.5 Hz, H-26), 3.55 (m, 1H, H-26), 3.78–3.86 (m, 3H, H-5″, H-5′, H-3), 3.98–4.02 (m, 2H, H-2′, H-2″), 4.05 (m, 1H, H-3″), 4.13 (t, 1H, *J* = 8.8 Hz, H-3′), 4.21 (t, 1H, *J* = 8.9 Hz, H-4′), 4.31–4.38 (m, 1H, H-16), 4.47–4.58 (m, 2H, H-16, H-6′), 4.96 (d, 1H, *J* = 7.7 Hz, H-1′), 5.14 (d, 1H, *J* = 7.7 Hz, H-1″), 5.31 (br d, 1H, H-6). ^{13}C NMR δ (δ, pyridine, 75 MHz): 140.9 (C-5), 121.4 (C-6), 109.0 (C-22), 104.6 (C-1″), 100.4 (C-1′), 82.30 (C-2′), 81.86 (C-16), 78.94 (C-3), 77.95 (C-5′), 76.63 (C-3′), 74.95 (C-3″), 72.33 (C-4″), 72.23 (C-2″), 71.49 (C-5″), 71.16 (C-4′), 62.65 (C-17), 66.62 (C-26), 62.36 (C-6′), 56.46 (C-14), 50.06 (C-9), 41.73 (C-20), 40.23 (C-13), 39.66 (C-12), 39.01 (C-4), 37.21 (C-1), 36.82 (C-10), 32.07 (C-2), 31.96 (C-7), 31.58 (C-8), 31.45 (C-15), 30.36 (C-25), 30.08 (C-23), 29.02 (C-24), 20.89 (C-11), 19.25 (C-19), 17.09 (C-27), 16.90 (C-6″), 16.14 (C-18), 14.81 (C-21); FABMS *m*/*z* 723 $(M+H)^+$, 416, 397. HRFABMS *m*/*z* 723.4362, calcd for $C_{39}H_{63}O_{12}$, 723.4320; Anal. Calcd for $C_{39}H_{62}O_{12}$: C, 64.80; H, 8.64. Found: C, 64.39; H, 8.98.

5.3.7. Diosgenyl α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (25). White amorphous solid, $[\alpha]_D^{20}$ −56° (*c* 0.034, EtOH); IR ν_{max} (film, NaCl) 3365, 2936, 1455, 1050, 900, 873 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz) 0.66 (d, 3H, *J* = 3.8 Hz, CH_3-27), 0.78 (s, 3H, CH_3-18), 1.01 (s, 3H, CH_3-19), 1.10 (d, 3H, *J* = 6.7 Hz, CH_3-21), 1.73 (d, 3H, *J* = 6.7 Hz, CH_3-6″), 3.46 (t, 1H, *J* = 9.5 Hz, H-26), 3.55 (m, 1H, H-26), 3.81–3.91 (m, 2H, H-3, H-5′), 4.13 (m, 1H, H-3′), 4.24–4.28 (m, 3H, H-2′, H-4′, H-4″), 4.45–4.53 (m, 2H, H-16, H-5″), 4.59 (dd, 1H, *J* = 9.1; 3.1 Hz, H-3″), 4.76 (br s, 1H, H-2″), 5.00 (m, 1H, H-1′), 5.27 (br d, 1H, *J* = 3.8 Hz, H-6), 6.34 (s, 1H, H-1″). ^{13}C NMR (δ, pyridine, 75 MHz): 140.9 (C-5), 121.8 (C-6), 109.0 (C-22), 101.8 (C-1″), 100.1 (C-1′), 79.50 (C-16), 79.32 (C-2′), 77.96 (C-5′), 77.65 (C-3′), 77.65 (C-3), 73.87 (C-4″), 72.54 (C-3″), 72.27 (C-2″), 71.53 (C-4′), 69.23 (C-5″), 66.58 (C-26), 62.59 (C-17), 62.36 (C-6′), 56.35 (C-14), 49.99 (C-9), 41.69 (C-20), 40.18 (C-13), 39.58 (C-12), 38.70 (C-4), 37.23 (C-1), 36.86 (C-10), 32.03 (C-2), 31.53 (C-7), 31.40 (C-8), 30.31 (C-15), 29.90 (C-25), 29.69 (C-23), 28.97 (C-24), 20.82 (C-11), 19.14 (C-19), 18.39 (C-6″), 17.05 (C-27), 16.06 (C-18), 14.76 (C-21). FABMS *m*/*z* 723 $(M+H)^+$, 413, 397; Anal. Calcd for $C_{39}H_{62}O_{12}$: C, 64.80; H, 8.64. Found: C, 64.74; H, 8.67.

5.3.8. Diosgenyl 4-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-glucopyranoside (26)[24]. White amorphous solid, $[\alpha]_D^{20}$ −29° (*c* 0.015, EtOH); IR ν_{max} (film, NaCl) 3421, 2934, 1734, 1041, 900, 872 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz) 0.66 (d, 3H, *J* = 5.2 Hz, CH_3-27), 0.83 (s, 3H, CH_3-18), 1.06 (s, 3H, CH_3-19), 1.23 (d, 3H, *J* = 6.9 Hz, CH_3-21), 1.46 (d, 3H, *J* = 6.3 Hz, CH_3-6″), 1.88 (s, 3H, Ac), 2.06 (s, 3H, Ac), 3.46 (t, 1H, *J* = 9.5 Hz, H-26), 3.55 (m, 1H, H-26), 3.92–4.01 (m, 3H, H-3, H-4′, H-5′), 4.12–4.24 (m, 2H, H-2′, H-3′), 4.53 (dd, 1H, *J* = 14.5; 7.4 Hz, H-16), 4.63 (dd, 1H, *J* = 9.5; 3.1 Hz, H-3″), 4.69–4.84 (m, 3H, H-6′, H-2″), 4.99–5.01 (m, 1H, H-1′), 5.85 (t, 1H, *J* = 9.8 Hz, H-4″), 6.37 (s, 1H, H-1″). ^{13}C NMR (δ, pyridine, 75 MHz): 171.581 and 171.51 (*C*O-Ac), 140.50

(C-5), 121.81 (C-6), 109.0 (C-22), 101.30 (C-1′), 99.83 (C-1″), 81.87 (C-16), 79.05 (C-2′), 77.77 (C-3), 76.52 (C-3′), 75.94 (C-4″), 74.68 (C-4′), 72.24 (C-2″), 71.16 (C-5′), 69.96 (C-3″), 66.63 (C-26), 65.23 (C-5″), 64.23 (C-6′), 62.66 (C-17), 56.41 (C-14), 50.08 (C-9), 41.74 (C-20), 40.25 (C-13), 39.63 (C-12), 38.78 (C-4), 37.20 (C-1), 36.92 (C-10), 32.08 (C-2), 31.99 (C-7), 31.58 (C-8), 31.50 (C-15), 30.36 (C-25), 29.03 (C-23), 29.03 (C-24), 20.93 (C-11), 20.93 and 20.48 (–*OAc*), 19.18 (C-19), 17.77 (C-6″), 17.09 (C-27), 16.17 (C-18), 14.82 (C-21). FABMS *m*/*z* 829 $(M+Na)^+$ 413, 397. Anal. Calcd for $C_{43}H_{66}O_{14}$: C, 64.00; H, 8.24. Found: C, 64.10; H, 8.33.

5.3.9. Diosgenyl α-L-rhamnopyranosyl-(1 → 2)-6-*O*-acetyl-β-D-glucopyranoside (27)[24]. White amorphous solid, $[\alpha]_D^{20}$ −22 ° (*c* 0.009, EtOH); IR ν_{max} (film, NaCl) 3277, 2934, 1648, 1419, 1052, 872 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.66 (d, 3H, *J* = 4.9 Hz, CH_3-27), 0.85 (s, 3H, CH_3-18), 1.02 (s, 3H, CH_3-19), 1.12 (d, 3H, *J* = 6.9 Hz, CH_3-21), 1.75 (d, 3H, *J* = 6.3 Hz, CH_3-6″), 1.91 (s, 3H, Ac), 3.47 (t, 1H, *J* = 9.5 Hz, H-26), 3.56 (m, 1H, H-26), 3.93–3.99 (m, 3H, H-3, H-4′, H-5′), 4.21–4.26 (m, 2H, H-2′, H-3′), 4.34 (t, 1H,*J* = 9.5 Hz, H-4″), 4.53 (dd, 1H, *J* = 14.5; 7.4 Hz, H-16), 4.62 (dd, 1H, *J* = 9.3; 3.0 Hz, H-3″), 4.75 (dd, 1H, *J* = 11.7; 5.1 Hz, H-6′), 4.79 (br s, 1H, H-2″), 4.84 (m, 1H, H-6′), 4.95 (m, 1H, H-1′), 4.99 (m, 1H, H-5″), 5.28 (d, 1H, *J* = 4.5 Hz, H-6), 6.38 (s, 1H, H-1″). ^{13}C NMR (δ, pyridine, 75 MHz): 171.0 (*C*O-Ac), 141.0 (C-5), 121.9 (C-6), 109.4 (C-22), 102.7 (C-1′), 102.3 (C-1″), 81.3 (C-16), 79.5 (C-2′), 77.6 (C-3), 76.8 (C-3′), 75.9 (C-4′), 73.87 (C-4″), 72.7 (C-2″), 70.5 (C-5′), 70.5 (C-3″), 67.0 (C-26), 67.0 (C-5″), 64.7 (C-6′), 61.5 (C-17), 56.8 (C-14), 50.4 (C-9), 40.6 (C-20), 40.0 (C-13), 39.5 (C-4), 39.2 (C-12), 37.2 (C-1), 37.2 (C-10), 32.0 (C-2), 32.0 (C-7), 31.9 (C-15), 31.8 (C-8), 30.0 (C-25), 29.4 (C-24), 29.3 (C-23), 21.3 (C-11), 20.9 (*C*H_3-Ac), 19.6 (C-19), 17.5 (C-27), 117.5 (C-6″), 6.5 (C-18), 15.2 (C-21). FABMS *m*/*z* 787 $(M+Na)^+$. HRFABMS *m*/*z* 787.4210, calcd for $C_{41}H_{64}O_{13}Na$, 787.4245; Anal. Calcd for $C_{41}H_{64}O_{13}$: C, 64.38; H, 8.43. Found: C, 64.05; H, 8.72.

5.3.10. Diosgenyl 2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1 → 4)-2,3-*O*-isopropylidene-α-L-rhamnopyranosyl-(1 → 2)-4,6-*O*-benzylidene-3-*O*-pivaloyl-β-D-glucopyranoside (29). To a stirred mixture of imidate (350 mg, 0.80 mmol), **28**[20] (158 mg, 0.53 mmol), and 4 Å MS in dry CH_2Cl_2 (12 mL) at −70 °C under N_2, TMSOTf (0.05 M in CH_2Cl_2, 1.0 mL) was added dropwise. After stirring for 1 h, compound **4a** (258 mg, 0.34 mmol) was added. The resulting mixture was stirred at −20 °C for 1.5 h, before the addition of Et_3N to quench the reaction. The mixture was filtered through a pad of Celite. The filtrate was concentrated and applied to a silica gel column chromatography (CH_2Cl_2/acetone; 40:1) to provide the desired saponin **29** as a white solid (0.19 mmol, 55%). White amorphous solid, $[\alpha]_D$ −88° (*c* 0.095, $CHCl_3$). ^1H NMR (δ, $CDCl_3$, 400 MHz): 0.80 (m, 6H, CH_3-18, CH_3-27), 0.98 (d, 3H, *J* = 6.9 Hz, CH_3-21), 1.04 (s, 3H, CH_3-19), 1.21 (d, 3H, *J* = 6.3 Hz, CH_3-6‴), 1.22 (s, 9H, CH_3-Piv), 1.33 (d, 3H, *J* = 6.2 Hz, CH_3-6″), 1.51 (s, 3H, (CH_3)$_2$–C), 1.67 (s, 3H, (CH_3)$_2$–C), 1.96 (s, 3H, –OAc), 2.03 (s, 3H, –OAc), 2.11 (s, 3H, –OAc), 3.38 (t, 1H, *J* = 10.9 Hz, H-26), 3.47–3.53 (m, 3H, H-26, H-5′, H-2″), 3.60–3.65 (m, 2H, H-3, H-4′), 3.78–3.87 (m, 3H, H-2′, H-6′, H-5‴), 4.03 (d, 1H, *J* = 5.5 Hz, H-2″), 4.14–4.17 (m, 2H, H-3″, H-5″), 4.35 (dd, 1H, *J* = 10.9, 5.5 Hz, H-6′), 4.41 (dd, 1H, *J* = 14.4, 7.1 Hz, H-16), 4.66 (d, 1H, *J* = 7.6 Hz, H-1′), 5.09 (t, 1H, *J* = 9.8 Hz, H-4″), 5.15 (s, 1H, H-1‴), 5.23 (dd, 1H, *J* = 10.0; 3.4 Hz, H-3″), 5.28 (m, 1H, H-2″), 5.33 (d, 1H, *J* = 1.5 Hz, H-1″), 5.39 (t, 1H, *J* = 9.4 Hz, H-3′), 5.44 (br d, 1H, H-6), 5.49 (s, 1H, Ph-CH), 7.33–7.42 (m, 5H, Ph). ^{13}C NMR (δ, $CDCl_3$, 75 MHz): 177.1 (*C*O-Piv), 169.7 and 169.3 (*C*O-Ac), 139.6 (C-5), 136.7, 128.6 and 125.6 (Ph), 122.1 (C-6), 109.2 ((CH_3)$_2$-*C*), 109.0 (C-22), 100.7 (Ph*CH*), 99.7 (C-1′), 97.4 (C-1‴), 95.8 (C-1″), 80.58 (C-16), 78.58 (C-3), 78.58 (C-4′), 77.59 (C-3″), 76.79 (C-5′), 75.96 (C-2′), 75.64 (C-4″), 73.81 (C-3′), 70.90 (C-4‴), 69.63 (C-2‴), 69.03 (C-3‴), 66.53 (C-5‴), 68.67 (C-6′), 65.91 (C-2″), 64.02 (C-5″), 61.87 (C-17), 60.60 (C-26), 56.62 (C-14), 49.83 (C-9), 41.37 (C-20), 40.03 (C-13), 39.55 (C-12), 38.70 (C-Piv), 38.05 (C-4), 36.88 (C-1), 36.64 (C-10), 31.85 (C-2), 31.62 (C-7), 31.14 (C-8), 31.14 (C-15), 30.06 (C-25), 29.30 (C-23), 28.56 (C-24), 27.61 and 26.10 ((CH_3)$_2$–C), 26.86 (*C*H_3-Piv), 20.8 (C-11), 20.72, 20.57 and 20.40 (*C*H_3-Ac), 18.93 (C-19), 17.82 (C-6‴), 17.13 (C-6″), 16.89 (C-27), 15.99 (C-18), 14.28 (C-21). HRFABMS: 1207.6401 $(M+H)^+$ (calcd for $C_{66}H_{95}O_{20}$, 1207.6417), 793 (6), 397 (17), 395 (9). Anal. ($C_{66}H_{94}O_{20}$) C, H. Anal. Calcd for $C_{66}H_{95}O_{20}$: C, 65.65; H, 7.85. Found: C, 65.70; H, 7.36.

5.3.11. Diosgenyl α-L-rhamnopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranoside (30). 120.0 mg (0.09 mmol) of **29** in 10 mL of AcOH (80%) was heated for 3 h at 70 °C. The residue was concentrated, dissolved in a mixture of MeOH/THF/H_2O (1:1:1) and NaOH (38 mg, 0.95 mmol) was added. The solution was heated at 40 °C for 7 h, then neutralized with an acid resin Dowex-50 (H^+), filtered, concentrated, and purified in CC using CH_2Cl_2/MeOH (8:2) to yield **30** (15.1 mg, 0.017 mmol, 19%). White amorphous solid, $[\alpha]_D^{20}$ −48° (*c* 0.015, EtOH); IR ν_{max} (film, NaCl) 3418, 2360, 1050, 891 cm^{-1}; ^1H NMR (δ, pyridine, 400 MHz): 0.65 (d, 3H, *J* = 4.9 Hz, CH_3-27), 0.76 (s, 3H, CH_3-18), 1.10 (d, 3H, *J* = 6.9 Hz, CH_3-21), 1.15 (s, 3H, CH_3-19), 1.59 (d, 3H, *J* = 6.3 Hz, CH_3-6″), 1.69 (d, 3H, *J* = 6.3 Hz, CH_3-6‴), 3.46 (t, 1H, *J* = 9.5 Hz, H-26), 3.55 (m, 1H, H-26), 3.83–3.93 (m, 2H, H-3, H-5′), 4.13 (t, 1H,*J* = 9.0 Hz, H-4′), 4.21–4.25 (m, 2H, H-2′, H-3′), 4.29 (m, 1H, H-6′), 4.39 (m, 1H, H-5‴), 4.45–4.52 (m, 5H, H-6′, H-3″, H-4‴, H-3‴, H-16), 4.65 (br s, 1H, H-2″), 4.80 (br s, 1H, H-2‴), 4.99 (m, 1H, H-1′), 5.05 (m, 1H, H-5′), 5.33 (d, 1H, *J* = 3.7 Hz, H-6), 6.34 (s, 1H, H-1″), 6.36 (s, 1H, H-1‴). ^{13}C NMR (δ, pyridine, 75 MHz): 140.48 (C-5), 121.66 (C-6), 109.0 (C-22), 102.71 (C-1‴), 101.24 (C-1″), 99.71 (C-1′), 80.88 (C-16), 79.36 (C-2′), 79.05 (C-4″), 78.05 (C-5′), 77.72 (C-3′), 77.48 (C-3), 73.80 (C-4‴), 73.39 (C-2‴), 72.93 (C-3‴), 72.67 (C-3″), 72.28 (C-2″), 71.53 (C-4′), 70.07 (C-5″), 67.40 (C-5‴), 66.63 (C-26), 62.65 (C-17), 62.37 (C-6′), 56.50 (C-14), 50.20 (C-9), 41.74 (C-20), 40.22 (C-13),

39.67 (C-12), 38.73 (C-4), 37.26 (C-1), 36.92 (C-10), 32.07 (C-2), 31.96 (C-7), 31.60 (C-8), 30.39 (C-15), 30.36 (C-25), 29.90 (C-23), 29.03 (C-24), 20.94 (C-11), 19.39 (C-19), 18.91 (C-6‴), 18.25 (C-6″), 17.09 (C-27), 16.22 (C-18), 14.76 (C-21). FABMS *m/z* 891 $(M+Na)^+$, 413 (3), 397 (4). Anal. ($C_{45}H_{72}O_{16}$) C, H. Anal. Calcd for $C_{45}H_{72}O_{16}$: C, 62.19; H, 8.35. Found: C, 62.15; H, 8.54.

5.4. Biological activity

5.4.1. Cell culture. Human HL-60 and U937 myeloid leukemia cells and human SK-MEL-1 melanoma cells were grown in RPMI 1640 (Sigma) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Sigma) and 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The cell numbers were counted by a hematocytometer, and the viability was always greater than 95% in all experiments as assayed by the 0.025% Trypan blue exclusion method. Stock solutions of 100 mM diosgenyl glycosides were made in dimethylsulfoxide (DMSO), and aliquots were frozen at −20 °C.

5.4.2. Assay for growth inhibition and cell viability. The cytotoxicity of diosgenyl glycosides was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.[28] Briefly, 1×10^4 exponentially growing cells were seeded in 96-well microculture plates with various diosgenyl glycoside concentrations (0.3–100 μM) in a volume of 200 μL. DMSO concentration was the same in all the treatments and did not exceed 0.1% (v/v). After 72 h, surviving cells were detected based on their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide (Sigma) into formazan crystals. Optical density was read with an ELISA reader at a wavelength of 570 nm and was used as a measure of cell viability. The MTT dye-reduction assay measures mitochondrial respiratory function and can detect the onset of cell death earlier than dye-exclusion methods. Cell survival was calculated as the fraction of cells alive relative to control for each point: cell survival (%) = mean absorbance in treated cells/mean absorbance in control wells × 100. Concentrations inducing a 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment. Parameters describing the concentration–response curves (IC_{50}) were determined using the curve fitting routine of the computer software Prism™ (GraphPad) and the equation derived by DeLean et al.[29]

5.4.3. Flow cytometry analysis for cell cycle distribution. After 16 h of treatment, cells were harvested and quickly washed twice with ice-cold phosphate-buffered saline (PBS), and cell pellets were collected and resuspended in 50 μL of PBS. Following dropwise addition of 1 mL of ice-cold 75% ethanol, fixed cells were stored at −20 °C for 1 h. Samples were then centrifuged at 500*g* for 10 min at 4 °C and washed with PBS before resuspension in 1 mL containing 50 μg/mL propidium iodide and 100 μg/mL RNase A and incubation for 1 h at 37 °C in the dark. Cell cycle distribution was then analyzed by flow cytometry using a Coulter EPICS™ cytometer (Beckman–Coulter). Histograms were analyzed with the Expo 32 ADC Software™ (Beckman–Coulter). The quantitative data presented are means ± SE of percentage of cells in different phases of cell cycle from triplicate samples in each treatment, and were reproducible in 2–3-independent experiments.

5.4.4. Immunoblot analysis of procaspase-3, procaspase-9, and Bcl-2. HL-60 or U937 cells (1×10^6) were treated with diosgenyl glycosides at the indicated concentrations in RPMI 1640 medium. Cells were pelleted by centrifugation, washed with phosphate-buffered saline and lysed in lysis buffer containing 125 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 5% glycerol, and 1% β-mercaptoethanol, and boiled for 5 min. The samples were separated on 12% sodium dodecyl sulfate–polyacrylamide gel, electrotransferred to a polyvinylidene difluoride membrane. The membrane was probed first with a polyclonal anti-procaspase-3 (Stressgen, 1:2000 dilution), anti-procaspase-9 (Stressgen, 1:1000) or anti-Bcl-2 (Santa Cruz Biotechnology, 1:1000) and then with anti-rabbit (pro-caspase-3) or anti-mouse (pro-caspase-9 and Bcl-2) antibody conjugated to horseradish peroxidase (HRP). Protein bands were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce) using the manufacturer's protocol.

5.4.5. Immunoblotting of poly(ADP-ribose) polymerase. Induction of apoptosis was also examined by proteolytic cleavage of poly(ADP-ribose) polymerase. Briefly, 1×10^6 exponentially growing HL-60 or U937 cells were treated with diosgenyl glycosides at the indicated concentrations for 6 h at 37 °C. Cells were pelleted by centrifugation, washed twice with phosphate-buffered saline resuspended in lysis buffer, and subjected to Western blot analysis as described previously.[28] Proteins were separated on 7.5% sodium dodecyl sulfate–polyacrylamide mini gels and electrotransferred to polyvinylidene difluoride membrane. The membrane was probed with polyclonal anti-poly(ADP-ribose) polymerase (Stressgen, 1:1000 dilution) and then with anti-rabbit antibody conjugated to horseradish peroxidase (HRP). Protein bands were detected by chemiluminescence (SuperSignal West Pico Chemiluminescent Substrate, Pierce) as described above.

5.4.6. Release of cytochrome *c* from mitochondria in diosgenyl glycoside-treated cells. Untreated and diosgenyl glycosides-treated cells were harvested by centrifugation at 1000*g* for 5 min at 4 °C. Cell pellets were washed twice with ice-cold phosphate-buffered saline and resuspended with 20 mM Hepes–KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride (PMSF), and 250 mM sucrose. After 15-min incubation on ice, cells were lysed by pushing them several times through a 22-gauge needle and the lysate spun down at 1000*g* for 5 min at 4 °C. The supernatant fraction was centrifuged at 105,000*g* for 45 min at 4 °C and the resulting supernatant was used as the soluble cytosolic fraction. Cytosolic proteins (50 μg) were resolved on a sodium dodecyl sulfate/15% polyacrylamide gel and elec-

trotransferred onto a polyvinylidene difluoride membrane (Millipore). Membrane was blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h, followed by incubation with anti-cytochrome *c* monoclonal antibody (BD Pharmingen; 1:1000) overnight. After washing and incubation with horseradish peroxidase-conjugated anti-mouse antibody (Amersham Biosciences), the antigen–antibody complexes were visualized by SuperSignal West Pico Chemiluminescent Substrate (Pierce) as described above. The same membrane was probed with monoclonal anti-β-actin (Sigma, 1:3000 dilution) as loading control.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.10.089.

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