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Icogenin, a new cytotoxic steroidal saponin isolated from *Dracaena draco*

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Abstract—This paper reports on the cytotoxic effect induced by a new natural steroidal saponin, icogenin, on the myeloid leukemia cell line HL-60. Icogenin was found to be a cytotoxic compound IC_{50} $2.6 \pm 0.9\,\mu M$ at 72 h, with growth inhibition caused by the induction of apoptosis, as determined by microscopy of nuclear changes and the fragmentation of poly(ADP-ribose) polymerase-1.

1. Introduction

The dragon tree is, undoubtedly, one of the most emblematic members of Canarian flora. Its scientific name is *Dracaena draco*, and it lives naturally in the archipelagoes of Madeira, Cape Verde, and the Canary Islands, being found in the last instance in La Palma, Tenerife, and Gran Canaria.

As a part of our systematic studies of the chemical constituents of the different parts of *D. draco*[1–3] in this paper we report on the isolation and structure determination of a new steroidal saponin, icogenin (**1**), along with 20 known compounds including sterols, carotenes, aromatic compounds, and steroidal saponins. The known compounds were identified by analysis of the ^1H and ^{13}C NMR spectra and comparison with published data. In particular, one of the isolated metabolites, icogenin **1**, showed highly potent cytotoxic activity.

The present report demonstrates that exposure to icogenin elicits apoptosis in HL-60 cells as manifested by the appearance of apoptotic morphology and the cleavage of poly(ADP-ribose) polymerase-1 (PARP-1).

2. Results and discussion

Icogenin **1** was obtained as an amorphous solid. The FAB experiment showed a fragment at m/z 907 $[M+Na-OMe]^+$. Anal. C 60.35%, H 8.25%, calcd for $C_{46}H_{76}O_{18}$, C 60.24%, H 8.35%. The IR (KBr) spectrum of **1** displayed the characteristic absorption for hydroxyl groups (3420, 1040 cm^{-1}). The spectrum of ^1H NMR (pyridine-d_5) showed signals for four steroid methyl groups at δ 0.67 (3H, br s), 0.80 (3H, s), 1.02 (3H, s), and 1.11 (3H, d, $J = 5.8$ Hz), a methoxyl group at δ 3.56 (3H, s), an olefinic proton at δ 5.33 (1H, br d), and signals for three anomeric protons at δ 4.87 (overlapped), 5.04 (1H, d, $J = 7.0$ Hz), and 6.27 (1H, br s), which gave correlations in the HSQC spectrum with carbon signals at δ 101.2 (C-1′), 105.7 (C-1‴), and 103.3 (C-1″). The proton and carbon signals due to the steroidal saponin structure in the ^1H and ^{13}C NMR spectra of **1** were similar to those of (**1a**),[4] with the exception of the signals assignable to ring F. A quaternary carbon signal at δ 110.5 (C-22), which showed a correlation in the heteronuclear multiple bond connectivity (HMBC) experiment (Fig. 1) with the hydrogens of a methoxyl

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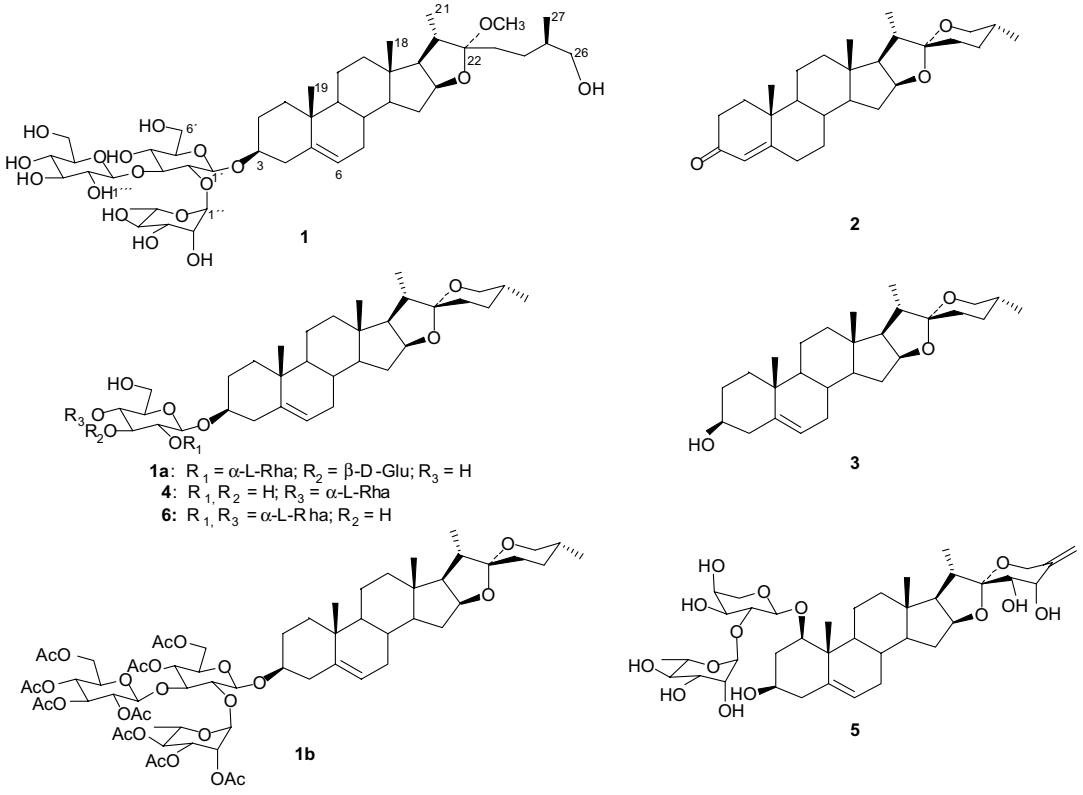

group suggested that **1** is a steroidal saponin with a furostanol skeleton.

In ring F, we also see a correlation between H-27 (CH_3) and C-26, which possesses a free hydroxyl group.

In regard to the glycosidic moiety, in the HMBC spectrum, the anomeric proton signals of glucose (δ 4.87, H-1′), rhamnose (δ 6.27, H-1″), and glucose (δ 5.04, H-1‴) showed correlations with C-3 of the aglycone (δ 79.56), C-2′ of glucose (δ 79.80), and C-3′ of glucose (δ 90.35), respectively (Fig. 1), leading to an *O*-α-L-rhamnopyranosyl(1 → 2)[β-D-glucopyranosyl(1 → 3)]-β-D-glucopyranoside structure attached to C-3 of the aglycone, which was confirmed by comparison with published ^{13}C NMR data.[4] Further confirmation of the above was observed on COSY, t-ROESY, HMBC, and HSQC experiments (Table 1). Compound **1** was derivatized via cyclization of the side chain under acetylation conditions to give gracillin nonaacetate (**1b**).[5a] Acid hydrolysis of **1** yielded gracillin **1a**.[5b] Hence, compound **1** was identified as (25*S*)-22-*O*-methyl-furost-5-en-3β,26-diol, 3-*O*-α-L-rhamnopyranosyl (1 → 2)[β-D-glucopyranosyl(1 → 3)]-β-D-glucopyranoside.

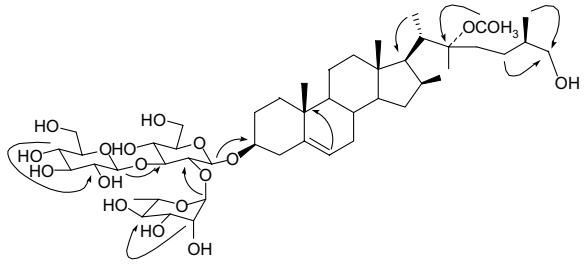

Figure 1. Key HMBC of icogenin **1**.

2.1. Icogenin induces apoptosis in human myeloid leukemia cells

Icogenin **1** was found to inhibit the growth and cell viability of HL-60 cells in a dose dependent manner as determined by the 3-[4,5-dimethylthiazol-2-yl-]2,5-diphenyl tetrazolium bromide (MTT) dye-reduction assay (Fig. 2). In order to compare the cytotoxic activity of icogenin against steroidal saponins **1a** and **1b**, these compounds were also included in the cytotoxic studies. The results obtained (Table 2) show that icogenin, **1a** and **1b** have a similar range of cytotoxic potency, with a 50% inhibitory concentration (IC_{50}) between 2.6 and 4 μM. Etoposide was used as a positive control ($IC_{50} = 0.17\ \mu M$).

The other compounds isolated show a wide range of potency. Only dioscin (**6**) displayed a similar cytotoxic activity to icogenin ($IC_{50} = 2.6 \pm 0.9\ \mu M$) while (**2**)–(**5**) displayed very weak cytotoxic activity with IC_{50}s higher than 30 μM.

Compounds **1** and **1a** are similar, but **1** possesses a furostanol ring instead a spirostanol ring in **1a**, indicating that the spirostanol structure does not play a critical role in the effects on HL-60 cells cytotoxicity and also that the acetyl groups in the sugar moiety do not play a crucial role in cytotoxicity (**1a** and **1b**, Table 2).

Diosgenone **2** and diosgenin **3** showed a very low cytotoxic activity against HL-60 cells (IC_{50}>50 μM). Also

Table 1. Data of icogenin **1** (δ ppm, J_{H-H} in Hz)[a,b,c]

Position	^{13}C	^{1}H	HMBC	t-ROESY
1	38.70 t	0.96 m	H-19	
		1.70 m		
2	31.28 t	1.85 $m_{(ax)}$		
		2.06 $m_{(eq)}$		
3	79.56 d	4.00	H-1′	
4	39.90 t	2.70 m		H-6
5	142.05 s	—	H-4, H-19	
6	123.15 d	5.33 d br	H-4	H-7
7	33.53 t	1.46 m		H-6, H-9
		1.85 m		
8	32.91 d	1.55 m	H-6	
9	51.49 d	1.15 m	H-7, H-19	H-7
10	38.35 s	—	H-4, H-6, H-19	—
11	22.31 t	1.41 m		
12	41.09 t	1.70	H-17	H-17
13	41.68 s	—	H-16, H-18	—
14	57.86 d	0.89 m	H-7, H-15, H-18	H-16
15	33.41 t	1.47 m		
		2.02 m		
16	82.35 d	4.51		H-14, H-17
17	64.06 d	1.79 m	H-18, H-20, H-21	H-16, H-21
18	17.55 q	0.80 s		H-20
19	20.61 q	1.02 s		
20	43.19 d	1.91 m	H-17, H-21	H-18, H-21
21	16.23 q	1.11 d (5.8)	H-17, H-20	H-17, H-20
22	110.50 s	—	OMe, H-21, H-24	—
23	33.02 t	1.60 m		
24	30.45 t	1.55 m	H-26, H-27	H-27
25	31.78 d	1.55 br s	H-27	H-27
26	68.10 t	3.48 $m_{(eq)}$	H-27	H-27
		4.56 $m_{(ax)}$		
27	18.53 q	0.67 s br		H-24, H-25, H-26
MeO	50.85 q	3.56 s		
1′	101.20 d	4.87		H-3, H-5′
2′	79.80 d	4.00	H-1″, H-4′	H-1″
3′	90.35 d	4.15	H-1‴	
4′	72.70 d	4.02		
5′	79.00 d	3.80	H-1′	H-1′
6′	63.60 t	4.38 m	H-4′	
		4.20 m	′	
1″	103.30 d	6.27 s br	H-2′	H-2′, H-2″
2″	73.55 d	4.83 s br	H-4″	H-1″
3″	73.90 d	4.53 d (9.3)	H-1″	H-5″
4″	75.19 d	4.28 t (8.8)	H-2″, H-3″	H-6″
5″	70.78 d	4.88 d (4.3)	H-1″, H-4″, H-6″	H-3″, H-6″
6″	19.65 q	1.70 d (5.3)	H-4″	H-4″, H-5″
1‴	105.7 d	5.04 d (7.0)	H-3′	H-3′
2‴	76.13 d	3.94	H-4‴	
3‴	78.32 d	4.14 s br	H-1‴	
4‴	70.83 d	4.04	H-6‴	
5‴	78.89 d	3.94		
6‴	63.60 t	4.53 m	H-4‴	
		4.38 m		

[a] Multiplicities were assigned from DEPT spectra.
[b] Spectra obtained at 75 MHz for ^{13}C and 400 MHz for ^{1}H NMR (C_5D_5N). The assignments were based on DEPT, HSQC, HMBC, COSY, and t-ROESY experiments.
[c] Overlapped proton signals are reported without designated multiplicity.

compound **4** did not show cytotoxic activity in the range of concentrations assayed. However **1a** and also **6** (dioscin), which have an attachment of an α-L-rhamnosyl group at C-2 of the glucosyl moiety showed important cytotoxic activity (IC_{50} 4.0 ± 0.4 and $2.3 \pm 0.8\,\mu M$, respectively). The position of the rhamnosyl substituent seems to be important because compound **4**, which has a rhamnosyl group at C-4 of the glucosyl moiety attached to the aglycone led to a decrease in the cytotoxicity.

To understand the mechanism of growth inhibition, HL-60 cells treated with icogenin were analyzed and quantified by fluorescent microscopy of nuclear changes. The

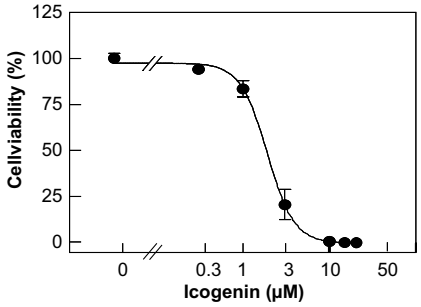

Figure 2. Effect of icogenin on human HL-60 cell viability. Cells were cultured in the presence of the indicated doses of icogenin for 72 h and thereafter cell viability was determined by the MTT assay as described in the Experimental section. The results of a representative experiment are shown. Each point represents the average of triplicate determinations.

Table 2. Effects of steroidal saponins on the growth of HL-60 cells

Compound	IC_{50} (μM)
Icogenin (**1**)	2.6 ± 0.9
1a	4.0 ± 0.4
1b	3.3 ± 0.5

The data shown represent the mean ± SEM of several independent experiments ($n = 2$–5) with three determinations in each. The IC_{50} values were calculated from experiments such as those shown in Figure 2 using the methodology described in Experimental section.

results showed that this compound induced morphological changes that are characteristic of apoptotic cells (Fig. 3A, middle). Whereas untreated cells exhibit a typically nonadherent, fairly round morphology (Fig. 3A, left), cells exposed to 1 μM icogenin for 2 h displayed condensation of chromatin (Fig. 3A, middle). Etoposide was used as a positive control (Fig. 3A, right). Using quantitative fluorescent microscopy, the percentage of apoptotic cells was 25 ± 2% after icogenin treatment (1 μM, 2 h) and was greater than controls (3.5 ± 1%) (Fig. 3B).

One of the downstream targets of caspase-3 is poly(ADP-ribose) polymerase-1. This enzyme has been implicated in DNA repair and maintenance of genomic integrity[6] and it is also a key enzyme in the apoptosis process.[7] The cleavage of poly(ADP-ribose) polymerase-1 inactivates the enzyme, thereby making DNA repair impossible. The western blot analysis in Figure 3C demonstrates that the 116 kDa poly(ADP-ribose) polymerase-1 protein was cleaved into its characteristic 85 kDa fragment after treatment of the cells with icogenin (1 μM, 4 h), thus confirming in vivo activation of caspase, the main protease responsible for poly(ADP-ribose) polymerase-1 cleavage.[8,9]

3. Conclusion

In conclusion, we demonstrate that the exposure of HL-60 cells to icogenin elicits a strong antiproliferative effect and induces apoptotic cell death. It was found that the induction of apoptosis by icogenin in HL-60 cells correlated with enhanced poly(ADP-ribose) polymerase-1 cleavage. The proteolytic cleavage of poly(ADP-ribose) polymerase-1 was verified by the formation of an 85 kDa fragment from an 116 kDa active poly(ADP-ribose) polymerase-1 enzyme.

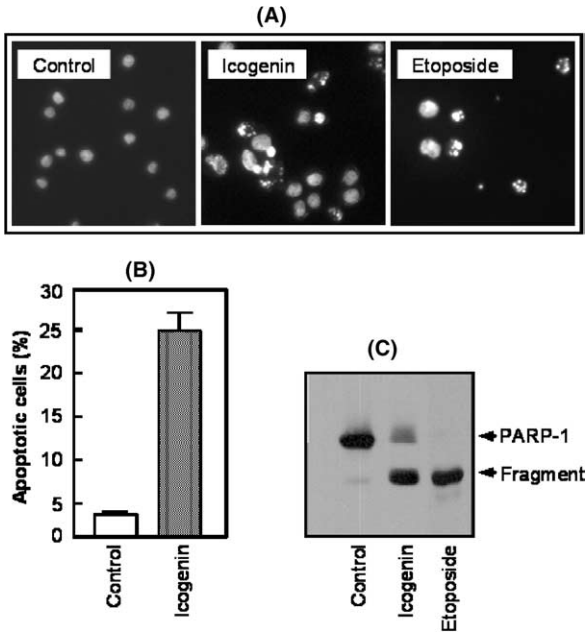

Figure 3. Induction of apoptosis in HL-60 cells by icogenin. (A) Cells were cultured in absence (control) or presence of 1 μM icogenin for 2 h, then they were stained with Hoechst 33258 and nuclei were visualized using fluorescence microscopy; etoposide (10 μM) was used as positive control. (B) HL-60 cells were counted and scored for apoptotic chromatin condensation by quantitative fluorescent microscopy; the results of a representative experiment are shown and each point represents the average ± SE of triplicate determinations. (C) Western blot analysis of poly(ADP-ribose)polymerase-1 (PARP-1) cleavage in HL-60 cells. Cells were incubated in absence or presence of icogenin for 4 h. Total cell lysates were analyzed by immunoblotting with anti-PARP-1 antibody; etoposide (10 μM), as positive control, was also included.

4. Experimental section

4.1. General methods

Optical rotations were measured using a Perkin–Elmer model 343 polarimeter. IR spectra were recorded using a Bruker model IFS-55 spectrophotometer. ^{1}H and ^{13}C NMR spectra were obtained on a Bruker model AMX-400 spectrometer and on a Bruker model AMX-300 with standard pulse sequences, operating at 400 MHz in ^{1}H and 75 MHz in ^{13}C NMR. $CDCl_3$, C_5D_5N, and $CO(CD_3)_2$ were used as solvents and TMS as internal standard. FAB and EIMS were taken on a Micromass model Autospec (70 eV) spectrometer. Column chromatography (CC) was carried out on silica gel (70–230 mesh, Merck), ODS silica gel (Aldrich Chemical Company), Sephadex LH-20 (Aldrich Chemical Company), and Diaion HP-20 (Supelco). Fractions obtained from CC were monitored by TLC (silica gel 60 F_{254}), and PTLC was carried out on silica gel 60 $PF_{254+366}$ plates (20 × 20 cm, 1 mm thick).

4.2. Plant material

The roots of *D. draco* were collected in Parque del Drago, Icod de los Vinos, Tenerife, Canary Islands, in winter 2001. The plant material was identified by JM and a voucher specimen PDED01 is deposited in the Herbarium of the Parque del Drago, Icod de los Vinos, Tenerife, Canary Islands.

4.3. Extraction and isolation

The root (895 g) of *D. draco* was extracted with 95% EtOH in a Soxhlet apparatus for 72 h. Removal of the solvent gave a residue (25 g), which was subjected to column chromatography on Si gel using *n*-hexane–AcOEt in increasing polarity and then with increasing percentages of MeOH to afford five fractions according to their TLC behavior. Twenty-three compounds were isolated, 22 of which have been identified as known compounds. Fraction I was eluted with *n*-hexane and *n*-hexane–AcOEt (20:1) and rechromatographed on Si gel with *n*-hexane–AcOEt (20:1), Sephadex LH-20 eluting with *n*-hexane–CH_2Cl_2–MeOH (2:1:1), and preparative TLC with *n*-hexane–AcOEt (9:1) to give (15*Z*)-phytoene[10] (2.5 mg), 8′-apo-β-carotin-8′-al[11] (3.9 mg), β-sitosterol[12] (4.0 mg), diosgenone (**2**)[13] (1.5 mg), and diosgenin (**3**)[14] (16.6 mg). Fraction II was eluted with *n*-hexane–AcOEt (8:2) and rechromatographed on Si gel with *n*-hexane–AcOEt (7:3), Sephadex LH-20 eluting with *n*-hexane–CH_2Cl_2–MeOH (2:1:1), and preparative TLC with CH_2Cl_2–acetone (20:1), toluene–^{i}PrOH (20:1) to give 4-allyl-catechol[15] (7.7 mg), 3-(4-hydroxybenzyl)-7,8-methylendioxychroman[16] (1.5 mg), (2*S*)-4′,7-dihydroxy-8-methylflavan[17] (2 mg), 7-hydroxy-3-(4-hydroxybenzyl)-8-methoxychroman[16] (4 mg), loureirin A[18] (4.8 mg), (−)-3′-hydroxy-4′-methoxy-7-hydroxy-8-methylflavan[19] (2 mg), 7-hydroxy-3-(4-hydroxybenzyl)chroman[20] (6 mg). Fraction III was eluted with *n*-hexane–AcOEt in increasing polarity and rechromatographed on Si gel with CH_2Cl_2–acetone (20:1), Sephadex LH-20 eluting with *n*-hexane–CH_2Cl_2–MeOH (1:1:1), and preparative TLC with CH_2Cl_2–acetone (20:1) and toluene–^{i}PrOH (20:1) to give (2*S*)-4′,5-dihydroxy-7-methoxy-8-methylflavan[20] (1.2 mg), (2*S*)-4′,7-dihydroxy-3′-methoxyflavan[21] (10 mg), loureirin C[18] (1.2 mg), isoliquiritigenin[22] (1.0 mg), shonanin[23] (4.0 mg), syringaresinol[24] (6 mg), and sitoindoside I[25] (2.5 mg). Fraction IV was eluted with CH_2Cl_2–MeOH in increasing polarity and rechromatographed on Si gel with CH_2Cl_2–MeOH (10:1), Sephadex LH-20 eluting with *n*-hexane–$CHCl_3$–MeOH (1:1:1), and preparative TLC with toluene–^{i}PrOH (7:3) to give 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)-β-D-glucopyranosyl]-diosgenin (**4**)[26] (73 mg). Fraction V was passed through a Diaion HP-20 column using gradients of MeOH in H_2O and rechromatographed on Si gel with CH_2Cl_2–MeOH (8:2), Sephadex LH-20 eluting with MeOH and preparative TLC with $CHCl_3$–MeOH (8:2) and on an ODS Si gel column eluting with MeOH–H_2O (2:1) and MeCN–H_2O (1:2) to give (23*S*,24*S*)-spirost-5,25(27)-diene-1β,3β,23,24-tetrol 1-*O*-{*O*-α-L-rhamnopyranosyl-(1 → 2)-α-L-arabinopyranoside} (**5**)[27] (7 mg), dioscin (**6**)[28] (75 mg), and icogenin (**1**) (40 mg).

4.3.1. Icogenin (1). Amorphous solid. $[\alpha]_D^{20}$ −61.2 (*c* 0.04, EtOH); IR (KBr) ν_{max} 3420, 2920, 1040 869, 837, 815 cm^{-1}; FABMS (positive ion) *m*/*z*: 907 $[M+Na-OMe]^+$; Anal. C 60.35%, H 8.25%, calcd for $C_{46}H_{76}O_{18}$, C 60.24%, H 8.35%. ^{1}H and ^{13}C NMR data: see Table 1.

4.3.2. Acid hydrolysis of icogenin (1). **1** (5 mg) was dissolved in 1 M HCl (dioxane–H_2O, 1:1.5, 8 mL) and then heated to 80 °C in a water bath for 1 h. After extraction with $CHCl_3$, the aqueous residue was evaporated to dryness and rechromatographed on Si gel with $CHCl_3$–MeOH (8:2) to give **1a**, as colorless amorphous powder (1.7 mg): $[\alpha]_D^{20}$ −88.6 (*c* 0.1, pyridine); IR (KBr) ν_{max} 3419, 981, 963, 920, 900, 867, 838, 813 cm^{-1}; ^{1}H NMR (pyridine-d_5) δ 0.65 (3H, d, *J* = 5.4 Hz, H-27), 0.79 (3H, s, H-18), 1.03 (3H, s, H-19), 1.09 (3H, d, *J* = 6.8 Hz, H-21), 1.70 (3H, d, *J* = 6.0 Hz, H-6″), 3.46 (1H, m, H-26), 3.53 (1H, m, H-26), 4.37 (1H, d, *J* = 10.8 Hz, H-6′), 4.55 (1H, m, H-6‴), 4.83 (1H, d, *J* = 5.95 Hz, H-1′), 5.29 (1H, d, *J* = 4.6 Hz, H-6), 5.04 (1H, d, *J* = 7.7 Hz, H-1‴), 6.31 (1H, br s, H-1″); ^{13}C NMR (pyridine-d_5) δ 15.9 (C-21), 17.2 (C-18), 18.2 (C-27), 19.5 (C-6″), 20.6 (C-19), 22.0 (C-11), 30.1 (C-24), 30.9 (C-2), 31.5 (C-25), 32.6 (C-8), 32.7 (C-23), 33.1 (C-7), 33.2 (C-15), 38.0 (C-10), 38.3 (C-1), 39.6 (C-4), 40.7 (C-12), 41.3 (C-13), 42.8 (C-20), 51.1 (C-9), 57.5 (C-14), 63.3 (C-6′), 63.3 (C-6‴), 63.7 (C-17), 67.7 (C-26), 70.4 (C-5″), 70.4 (C-4‴), 72.3 (C-4′), 73.3 (C-2″), 73.6 (C-3″), 74.9 (C-4″), 75.8 (C-2‴), 77.9 (C-3‴), 78.6 (C-5‴), 78.7 (C-5′), 79.3 (C-3), 79.5 (C-2′), 82.0 (C-16), 90.3 (C-3′), 100.8 (C-1′), 103.1 (C-1″), 105.4 (C-1‴), 110.1 (C-22), 122.7 (C-6), 141.6 (C-5); HRFABMS *m*/*z* 907.4310 $[M+Na]^+$ (calcd for $C_{45}H_{72}O_{17}Na$, 907.4667).

4.3.3. Acetylation of (1). To a solution of 26 mg of **1** in pyridine, an excess of Ac_2O was added at 25 °C. After 10 h the reaction was quenched with a diluted $NaHCO_3$ solution. Excess pyridine was eliminated by washing with aqueous $CuSO_4$. After extraction with $CHCl_3$ and purification on silica gel, **1b** was obtained as a colorless amorphous powder (36 mg): $[\alpha]_D^{20}$ −73.8 (*c* 0.17, $CHCl_3$); IR (KBr) ν_{max} 2950, 1749, 1368, 1225, 1048, 981, 900, 756 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.75 (3H, br s, *J* = 3.9 Hz, H-27), 0.75 (3H, s, H-18), 0.93 (3H, d, *J* = 6.8 Hz, H-21), 0.99 (3H, s, H-19), 1.14 (3H, d, *J* = 6.2 Hz, H-6″), 1.9–2.2 (27H, s, OAc), 3.34 (1H, t, *J* = 12.0 Hz, H-26), 3.43 (1H, m, H-26), 3.59 (1H, m, H-3), 3.53 (1H, d, *J* = 3.7 Hz, H-5′), 3.59 (1H, m, H-3), 3.71 (1H, d, *J* = 9.0 Hz, H-2′), 4.01 (1H, dd, *J* = 2.0, 12.2 Hz, H-6′), 4.04 (1H, dd, *J* = 2.0, 12.2 Hz, H-6′), 4.07 (1H, dd, *J* = 1.5, 12.2 Hz, H-6‴), 4.15 (1H, dd, *J* = 4.8, 12.2 Hz, H-6‴), 4.43 (1H, d, *J* = 7.9 Hz, H-1′), 4.69 (1H, d, *J* = 8.0 Hz, H-1‴), 5.22 (1H, br s, H-1″), 5.35 (1H, d, *J* = 4.8 Hz, H-6); ^{13}C NMR ($CDCl_3$) δ 14.4 (C-21), 16.2 (C-18), 17.0 (C-27), 17.1 (C-6‴), 19.2 (C-19), 20.3–20.7 (OAc), 20.6 (C-11), 28.7 (C-24), 29.4 (C-2), 30.2 (C-25), 31.4 (C-8), 31.4 (C-23), 31.8 (C-15), 32.0 (C-7), 37.1 (C-1), 36.8 (C-10), 38.3 (C-4), 39.7 (C-12), 40.2 (C-13), 41.5 (C-20), 50.0 (C-9), 56.4 (C-14), 61.8 (C-6′), 62.1 (C-17), 62.4 (C-6″), 66.6 (C-5‴), 66.8 (C-26),

68.3 (C-4′), 69.4 (C-2″), 70.8 (C-2‴), 71.1 (C-4‴), 71.3 (C-3‴), 71.7 (C-3″), 72.2 (C-5″), 72.7 (C-5′), 74.9 (C-4″), 76.1 (C-2′), 78.8 (C-3), 80.7 (C-3′), 80.8 (C-16), 96.8 (C-1″), 99.1 (C-1‴), 99.2 (C-1′), 109.2 (C-22), 122.0 (C-6), 140.1 (C-5), 168.8–170.7 (OAc); HRFABMS m/z 1285.6778 $[M+Na]^+$ (calcd for $C_{63}H_{90}O_{26}Na$, 1285.5618).

4.4. Cell culture

Human HL-60 myeloid leukemia cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Invitrogen) and 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The cultures were passed twice weekly exhibiting characteristic doubling times of ~24 h. 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 10 mM icogenin were made in dimethylsulfoxide (DMSO), and aliquots were frozen at −20 °C. Further dilutions of stock solutions of icogenin were made in culture media just before use. In all experiments, the final concentration of DMSO did not exceed 0.2% (v/v), a concentration, which is nontoxic to the cells.

4.5. Assay for growth inhibition and cell viability

The cytotoxicity of icogenin was assessed using the 3[4,5-dimethylthiazol-2-yl-]2,5-diphenyl tetrazolium bromide assay.[29] Briefly, 2×10^4 exponentially growing cells were seeded in 96-well microculture plates with various icogenin concentrations (0.3–20 μM) in a volume of 100 μL for 72 h. Then, surviving cells were detected based on their ability to metabolize 3[4,5-dimethylthiazol-2-yl-] 2,5-diphenyl tetrazolium bromide (Applichem) into formazan crystals. Optical density at 570 nm was used as a measure of cell viability. 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 De Lean et al.[30]

4.6. Quantitative fluorescent microscopy

HL-60 cells (1×10^6) were treated with 1 μM icogenin in RPMI 1640 medium for 2 h. Then cells were harvested and fixed in 3% paraformaldehyde and incubated at room temperature for 10 min. The fixative was removed and the cells were washed with phosphate-buffered saline (PBS), resuspended in 30–50 μL of PBS containing 16 μg/mL bis-benzimide trihydrochloride (Hoescht-33258),[31] and incubated at room temperature for 15 min. Ten microliter aliquots of the cells were placed on glass slides, and triplicate samples of 500 cells each were counted and scored for the incidence of apoptotic chromatin condensation using Zeiss fluorescent microscopy. Stained nuclei with condensed chromatin (supercondensed chromatin at the nuclear periphery), or nuclei that were fragmented into multiple smaller dense bodies were considered as apoptotic. Nuclei with uncondensed and dispersed chromatin were considered as not apoptotic.

4.7. Immunoblot analysis of poly(ADP-ribose) polymerase-1 degradation

Induction of apoptosis was also examined by proteolytic cleavage of poly(ADP-ribose) polymerase-1. Briefly, 7×10^5 exponentially growing HL-60 cells were treated with icogenin (1 μM, 4 h) at 37 °C. Cells were pelleted by centrifugation, and resuspended in lysis buffer containing 25 mM PBS, 0.1 mM phenylmethylsulfonylfluoride, and protease inhibitors leupeptin, aprotinin, and pepstatin A (5 μg/mL each). After centrifugation, the pellet was resuspended in the loading buffer containing 125 mM Tris–HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 5% glycerol, and 1% β-mercaptoethanol. The mixture was sonicated for 30 s at 4 °C and then boiled at 100 °C for 3 min. For western blotting, the cell lysates were fractionated on a 7.5% polyacrylamide gel containing 0.1% SDS. The gel and the nitrocellulose membrane (Amersham Pharmacia Biotech) were equilibrated for 20 min in transfer buffer (25 mM Tris, 192 mM glycine, 3.5 mM SDS, 20% methanol) and transferred to nitrocellulose by the semi-dry electrophoretic transfer system (Bio-Rad). The loading and transfer of equal amounts of protein were confirmed by staining the nitrocellulose membrane with Ponceau S. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h, followed by incubation with anti-poly(ADP-ribose) polymerase-1 monoclonal antibody (BD PharMingen; 1:1000 dilution in TBST supplemented with 3% nonfat milk) overnight. After washing and incubation with horseradish peroxidase-conjugated anti-mouse (Amersham Pharmacia Biotech), the antigen–antibody complexes were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) using the manufacturer's protocol. The appearance of an 85 kDa cleavage product was used as a measure of apoptosis.

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