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Synthesis and Biological Activity of 22-Deoxo-23-oxa Analogues of Saponin OSW-1

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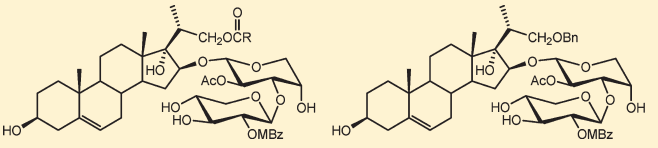
Supporting Information

ABSTRACT: Analogues of the potent cytotoxic saponin OSW-1 were prepared from the readily available steroidal 16β,17α,22-triol. The new 22-deoxo-23-oxa analogues of OSW-1 were screened against eight cancer cell lines and normal human fibroblasts. The analogues proved to be slightly less active than OSW-1 but also less toxic to normal cells. They induce concentration- and time-dependent apoptosis of mammalian cancer cells with caspase-3 activation.



INTRODUCTION

Natural products have proven to be rich sources of leads for compounds with anticancer properties. According to a recent report, 63% of anticancer agents approved between 1981 and 2008 were natural products, natural-product-derived, or natural-product-inspired.[1] Notably, Sashida's group have isolated a small family of cholestane glycoside saponins (Figure 1) from bulbs of *Ornithogalum saundersiae* that exhibit cytotoxic activity at nanomolar concentrations.[2,3] These saponins contain a novel 3β,16β,17α-trihydroxycholest-5-en-22-one aglycone with an acylated disaccharide at C-16. OSW-1, the major and most active of the saponins, is about 10−100 times more cytotoxic than clinically applied anticancer agents such as mitomycin C, adriamycin, cisplatin, camptothecin, and paclitaxel. This potency, in combination with a unique mechanism of action and selectivity toward malignant tumor cells, gives OSW-1 and its analogues great potential as anticancer agents.[4]

A number of synthesis pathways have been described for OSW-1.[5] However, this natural saponin has a relatively complex structure, and all of the methods reported to date are multistage and inefficient. Thus, there is a need for OSW-1 analogues with simpler structures that retain high and selective activity.[6] Here we describe the synthesis of new 22-deoxo-23-oxa analogues of OSW-1 and tests of their ability to inhibit the proliferation of cancer cells and induce apoptosis in them.

RESULTS AND DISCUSSION

Chemistry. Previous studies have shown that replacing the C-23 atom in the OSW-1 side chain by a heteroatom increases its antitumor activity.[6c] Therefore, we prepared a series of further 22-deoxo-23-oxa analogues of saponin OSW-1 with ether or ester moieties in the side chain in order to explore their biological activities and identify leads with closer to optimal selective cytoxicity. The synthesis of OSW-1 analogues **5** and **8a**−**k** is illustrated in Schemes 1 and 2. As a substrate for synthesizing analogues, the known 16β,17α,22-triol **2**,[7] which is readily available from commercial 3β-hydroxyandrost-5-en-17-one, was used. Compound **5** was obtained by three-step synthesis as shown in Scheme 1. The regioselective Williamson etherification of the 22-hydroxyl group of triol **2**[8] with 1.1 equiv of benzyl chloride in the presence of NaH was followed by glycosylation of the obtained compound **3** with OSW-1 disaccharide trichloroacetimidate under standard conditions.[5a,e] The desired 16β-glycoside **4** was obtained in 66% yield. After removal of the protective groups with *p*-TsOH/H_2O, the required ether analogue **5** was obtained in 43% overall yield (from triol **2**, three steps).

In contrast to etherification, regioselective esterification of the primary 22-hydroxyl group of triol **2** failed. For synthesis of 22-deoxo-22-acyloxy analogues, glycoside **4** described above was used as a substrate (Scheme 2). Hydrogenolysis of **4** allowed selective deprotection of the 22-hydroxyl group. Esterification of the obtained alcohol **6** with an acid in the presence of DCC and DMAP gave the desired protected 22-deoxo-22-acyloxy analogues **7a**−**k** in high yields. Routine removal of the protective groups (*p*-TsOH, dioxane/H_2O) afforded the OSW-1 analogues **8a**−**k**. In this manner, three series of ester analogues with saturated (**8a**−**f**), unsaturated (**8g**−**i**), and aromatic (**8j**,**k**) side chains were obtained.

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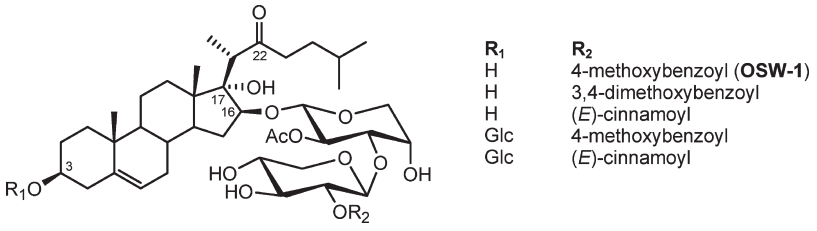
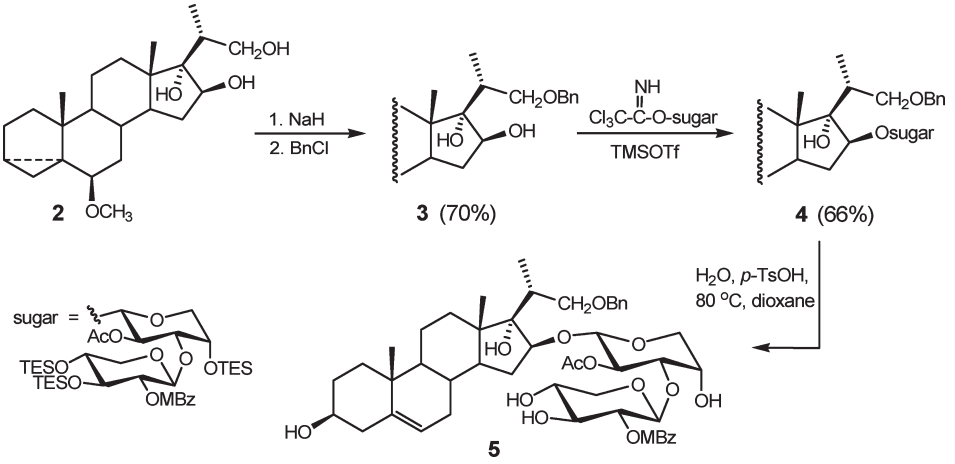
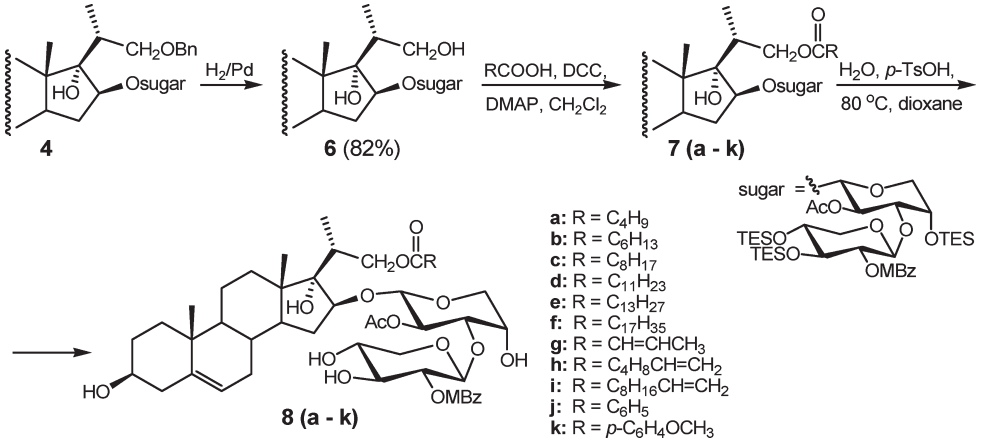


Figure 1

Scheme 1



Scheme 2



Cytotoxic Activities. The synthetic OSW-1 analogues were screened against various tumor cells. The suspension cell line CEM proved to be the most sensitive to the OSW-1 derivatives (Table 1); all of the tested compounds were effective at nanomolar concentrations against it. The highest antiproliferative activity was displayed by compounds containing 8−11 carbon atoms in the side chain (**8c**, **8d**, **8i**). Both shortening [**8a** (C_4), **8b** (C_6)] and extension [**8e** (C_{13}), **8f** (C_{17})] of the alkyl side chain led to slight loss of activity. These results are consistent with a recent report that OSW-1 forms a hydrophobic cluster involving 4-methoxybenzoate of disaccharide and the cholestane side chain (terminal methyl groups).[9] The van der Waals type interactions between these hydrophobic moieties were found both in solution and in the solid state. The hydrophobic cluster may have a biological importance as a recognition motif for binding proteins. Alkenyl side chains of corresponding lengths (**8h**, **8i**) had similar effects on the analogues' anticancer activity. It seems that small variations in the structure and length of the alkyl side chain do not affect the compounds' antiproliferative activity significantly.[6g] The cytotoxicity (IC_{50}) values of **8j** and **8k**, bearing a benzyl substituent, varied between 57 and 70 nM and were about 100-fold lower than that of OSW-1. The

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Table 1. IC_{50} (μM) Obtained from the Calcein AM Assays with the Tested Cancer and Normal Cell Lines[a]

OSW-1 derivative	cell line, IC_{50} (nM)								
	CEM	MCF7	G 361	HeLa	HOS	A 549	T98	HCT116	BJ
OSW-1 aglycone	>50000	>50000	>50000	>50000	>50000	>50000	>50000	>50000	>50000
OSW-1	0.3 ± 0.03	2.4 ± 0.2	1000 ± 100	3.4 ± 0.3	8200 ± 400	27 ± 3	70 ± 6	8400 ± 500	0.2 ± 0.01
5	20 ± 1	600 ± 100	750 ± 80	200 ± 10		100 ± 50			300 ± 40
8a	60 ± 10	500 ± 70	390 ± 60	170 ± 10		160 ± 40			500 ± 60
8b	10 ± 2	400 ± 40	1280 ± 200	30 ± 2		20 ± 5			600 ± 70
8c	7.2 ± 0.4	26 ± 8	15 ± 1	47 ± 4	2800 ± 900	590 ± 60	11 ± 3	4900 ± 800	7 ± 1
8d	6.3 ± 0.5	21 ± 1	8 ± 2	42 ± 18	1900 ± 700	930 ± 300	7 ± 1	2800 ± 600	5 ± 1
8e	16 ± 5	48 ± 22	30 ± 10	67 ± 2	2700 ± 600	720 ± 70	28 ± 1	2800 ± 400	83 ± 5
8f	58 ± 1	195 ± 21	300 ± 100	435 ± 64	>50000	550 ± 180	210 ± 50	>50000	80 ± 4
8g	340 ± 40	840 ± 51	890 ± 90	1940 ± 50	>50000	550 ± 200	940 ± 20	>50000	230 ± 80
8h	21 ± 3	54 ± 9	44 ± 4	61 ± 6	6500 ± 1400	840 ± 300	43 ± 9	11000 ± 700	20 ± 10
8i	6.1 ± 1.2	29 ± 12	6.7 ± 0.5	103 ± 88	1940 ± 50	600 ± 100	7 ± 1	2600 ± 700	3 ± 0.8
8j	70 ± 10	700 ± 100	1660 ± 90	240 ± 60		170 ± 40			100 ± 20
8k	57 ± 19	410 ± 120	170 ± 60	340 ± 10	5400 ± 1500	570 ± 190	55 ± 4	5000 ± 400	39 ± 6

[a] The cells were treated for 72 h with serial concentrations of the compounds. Mean ± SD values were obtained from three independent experiments performed in triplicate. OSW-1 was used as a positive control.

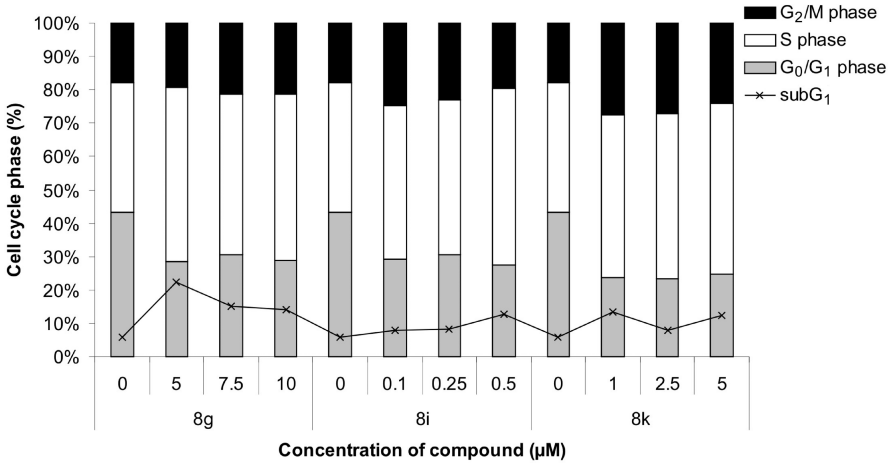

Figure 2. Histograms, obtained by flow cytometric analysis, showing the distributions of CEM cells in G_0/G1, S, and G_2/M cell cycle phases and the $subG_1$ fraction of cells after 24 h of treatment with **8g**, **8i**, and **8k** relative to untreated controls. Data indicate the percentage (%) of the number of cells in respective phases.

compounds were also tested for cytotoxicity to normal BJ human fibroblasts and proved to be (except compounds **8c**, **8d**, **8g**, **8h**, and **8i**) substantially (3- to 360-fold) less toxic toward them than toward malignant cell lines (Table 1). OSW-1 was more cytotoxic toward normal human fibroblasts than its derivatives, and the ester analogue **8b** exhibited the highest selectivity toward cancer cells versus normal cells. OSW-1 analogues also showed stronger effects than OSW-1 on some cancer cell lines (e.g., G-361, HOS, T98, and HCT 116). Several authors have found OSW-1 analogues to be cytotoxic at nanomolar concentrations toward various cancer cell lines.[6e,10,11] However, only Zhou et al.[4a] have previously assessed their cytotoxicity to both cancer cell lines and human nonmalignant cells. These authors found cancer cells to be about 40- to 150-fold more sensitive than normal human cells to the compounds. Our study confirms and extends these findings, since our tested analogues were 15- to 3000-fold less cytotoxic than OSW-1 to nonmalignant human cells, indicating that our OSW-1 analogues are likely to have wider therapeutic windows than OSW-1.

Cell Cycle and Apoptosis. Flow cytometric analysis was used to quantify the distribution of CEM cells in cell cycle phases, and the $subG_1$ fraction as a marker of the proportion of apoptotic cells, following incubation with OSW-1 and our prepared analogues. The results show that treatment with **8g**, **8i**, and **8k** increased the proportions of S-phase and G_2/M cells, with concomitant reductions in proportions of G_0/G_1 cells, in a dose-dependent manner (Figure 2). In addition, the proportion of cells with $subG_1$ amounts of DNA (apoptotic cells) increased following treatment with saponin derivative **8g** or **8k**, and the CEM cells yielded 3 times more cellular debris following treatment with **8g** than untreated controls after 24 h (Figure 2). Thus, the tested OSW-1 derivatives effectively induced cell cycle arrest

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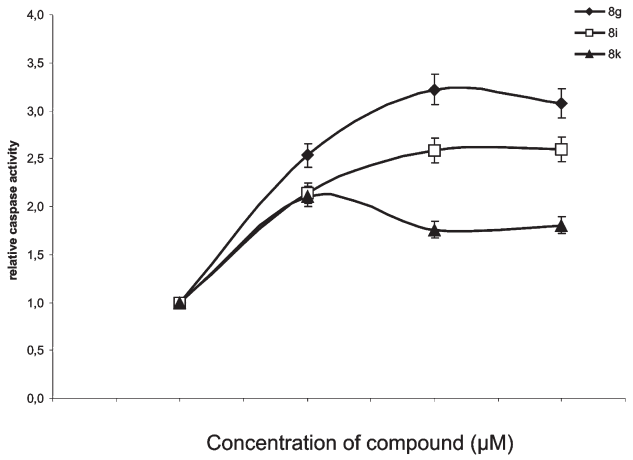

8g	0	5	7.5	10
8i	0	0.1	0.25	0.5
8k	0	1	2.5	5

Figure 3. Activity of caspases 3 and 7. Acute T-lymphoblastic leukemia cells CEM were treated for 24 h with the OSW-1 derivatives **8g**, **8i**, and **8k** and compared with untreated control cells. The data indicate the relative increase in activity of caspases 3 and 7. Experiments were repeated three times.

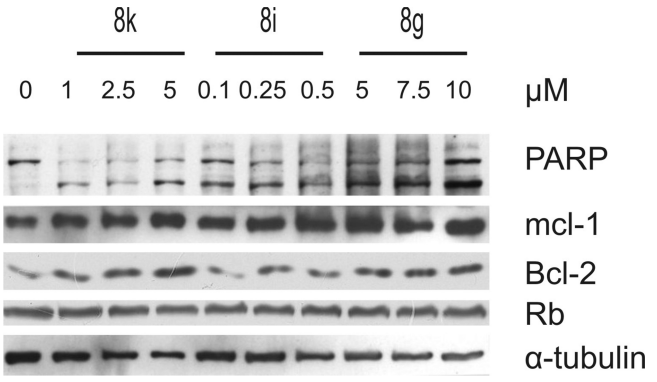

Figure 4. Western blot analysis of apoptosis-related proteins (Rb, PARP, Bcl-2, mcl-1, casapase-3) in leukemia cells (CEM) treated with OSW-1 derivatives **8g**, **8i**, and **8k** compared to their expression in untreated control cells, and expression of α-tubulin as a protein loading marker.

and apoptosis. Mitochondria and calcium regulation have been shown to be perturbed in cancer cells and following saponin treatment.[4a] Thus, the OSW-1 derivatives may induce increases in cytosolic calcium levels that are more toxic to cancer cells than to normal cells, explaining their selectivity.[4a]

Activities of Caspase 3/7. We determined the activities of effector caspases 3 and 7 (3/7) in CEM cells exposed to **8g**, **8i**, or **8k** using a fluorogenic substrate, Ac-DEVD-AMC, and/or the caspase 3/7 inhibitor Ac-DEVD-CHO. Cells were treated with a series of concentrations of the compounds exceeding their IC_{50} values, **8g** (5, 7.5, and 10 μM), **8i** (0.1, 0.25, and 0.5 μM), and **8k** (1, 2.5, and 5 μM), for 24 h. Compound **8g** induced ~3-fold increases in the activity of caspases 3 and 7 after 24 h at 7.5 and 10 μM compared with untreated controls (Figure 3). Compound **8i** induced ~2.5-fold increases after 24 h at the two highest tested concentrations, while **8k** induced about 2.1-, 1.7- and 1.8-fold increases at 1, 2, and 5 μM, respectively.

Western Blot Analysis. Western blot analyses were used to detect changes in expression of apoptosis-related proteins in the CEM leukemia cancer cell line 24 h after treatment with OSW-1 derivatives **8g** (5, 7.5, and 10 μM), **8i** (0.1, 0.25, and 0.5 μM), and **8k** (1, 2.5, and 5 μM). As shown in Figure 4, these treatments induced cleavage of PARP after 24 h, and levels of the anti-apoptotic Bcl-2 protein increased after **8g** and **8k** treatment in a dose-dependent manner. However, expression of the antiapoptotic protein mcl-1 and tumor suppressor retinoblastoma (Rb) protein was not changed after these treatments with OSW-1 analogues.

It is known that apoptosis is mediated by caspase cascade activation[12] and that caspase-3 is an executioner protease that cleaves PARP, resulting in DNA degradation and apoptotic death.[13] Our Western blot analysis demonstrated the dose-dependent accumulation of caspase-3 and cleavage of PARP after 24 h of treatment with OSW-1 derivatives **8g**, **8i**, and **8k** in the leukemia cancer cell line CEM (Figure 4). Thus, the results confirm that those OSW-1 derivatives can induce caspase-3 activated apoptosis (Figure 3).

CONCLUSIONS

OSW-1 is a saponin that is strongly cytotoxic to several types of malignant cells. However, although it has high selectivity toward cancer cells according to some reports, it is also very toxic to normal human cells. We report here the synthesis of a series of OSW-1 analogues that are easier to obtain by chemical synthesis than the natural product. The new analogues are slightly less active than OSW-1 but have a much wider therapeutic window, since they are less toxic to normal cells. The analogues (like OSW-1) induce apoptosis of mammalian cancer cells in a concentration-dependent manner.

EXPERIMENTAL SECTION

Chemical Synthesis. *General Remarks.* Melting points presented here were determined using Boetius-type Kofler apparatus. NMR spectra were recorded with Bruker Avance II 400 or Avance DPX 200 spectrometers operating at 400 and 200 MHz, respectively, using $CDCl_3$ solutions with TMS as the internal standard (only selected signals in the ^{1}H NMR spectra are reported). Infrared spectra (in chloroform solution) were recorded using a Nicolet series II Magna-IR 550 FTIR spectrometer. Mass spectra were obtained at 70 eV with an AMD-604 spectrometer. The reaction products were isolated by column chromatography, performed using 70−230 mesh silica gel (J. T. Baker).

Compound **2** and glycosyl trichloroacetimidates ($CCl_3C(NH)O$ sugar) were obtained according to procedures described respectively in refs 7, 5a, and 5e. The details of synthesis of compounds **3**− **8** and their spectroscopic date are described in the Supporting Information.

Purity of final compounds was found to be ≥95% as determined by HPLC analysis. HPLC was performed with LabAlliance apparatus comprising pumps (III Pump series), a UV−vis detector (525 dual-wavelength), and injection valve (Rheodyne model 7725i). Analytical HPLC was carried out with a normal phase Supelco-Si column (5 μm), 0.46 cm × 25 cm. The eluting solvent was dichloromethane/methanol (94:6), isocratic. The flow rate was 1.0 mL/min. The elution pattern of products was monitored continuously by UV at 260 nm. The retention times of final products were as follows: **5** (5.96 min), **8a** (8.93 min), **8b**

(8.53 min), **8c** (8.28 min), **8d** (8.06), **8e** (7.46 min), **8f** (7.45 min), **8g** (9.10 min), **8h** (8.67 min), **8i** (7.75 min), **8j** (9.01 min), **8k** (8.75 min).

(20R)-21-Benzyloxy-6β-methoxy-20-methyl-3α,5α-cyclopregnane-16β,17α-diol (***3***). To a solution of (20*R*)-6β-methoxy-20-methyl-3α,5α-cyclopregnane-16β,17α,21-triol (triol **2**, 0.5 g, 1.3 mmol) in dry THF (15 mL), NaH (1.5 equiv, 0.048 g) was added at 0 °C. The reaction mixture was stirred for 15 min at 0 °C. Then a solution of benzyl bromide (1.1 equiv, 0.17 mL) in THF (2 mL) was added dropwise. The reaction mixture was stirred for 1 h under reflux. The reaction was carefully quenched with water, and products were extracted with ether. The extract was dried over $MgSO_4$, and solvent was evaporated in vacuo. The crude product was purified by silica gel column chromatography with hexane−ethyl acetate (8:2 v/v). Yield: 70% (0.43 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.34 (m, 5H), 4.54 (s, 2H), 3.92 (bs, 1H), 3.90 (m, 1H), 3.70 (t, J = 9.0 Hz, 1H), 3.43 (dd, J_1 = 3.2 Hz, J_2 = 9.2 Hz, 1H), 3.33 (s, 3H), 2.78 (m, 1H), 1.04 (s, 3H), 1.01 (s, 3H), 0.95 (d, J = 7.1 Hz, 3H), 0.66 (m, 1H), 0.44 (dd, J_1 = 5.1 Hz, J_2 = 8.0 Hz, 1H).

(20R)-21-Benzyloxy-6β-methoxy-20-methyl-3α,5α-cyclopregnane-16β17α-diol 16-O-{2-O-(4-Methoxybenzoyl)-3,4-di-O-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-4′-O-triethylsilyl-α-L-arabinopyranoside} (***4***). A solution of compound **3** (0.19 g, 0.41 mmol) and 2-*O*-(4-methoxybenzoyl)-3,4-di-*O*-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-*O*-acetyl-4′-*O*-triethylosilyl-α-L-arabinopyranosyl trichloroacetimidate (1.2 equiv, 0.46 g) in dry dichloromethane (10 mL) was stirred with 4 Å molecular sieves (MS, 1.28 g) for 15 min at room temperature. Then the reaction mixture was cooled to −68 °C (dry ice−ethanol bath) and a 0.14 M solution of TMSOTf in dry dichloromethane (1.1 mL) was slowly added. The reaction mixture was stirred for an additional 30 min at −40 °C (dry ice−acetonitrile bath), and the reaction was quenched with triethylamine (0.5 mL). Then molecular sieves were filtered off and the solvent was evaporated in vacuo. The crude product was purified by silica gel column chromatography with hexane−ethyl acetate (95:5 v/v). Yield: 66% (0.34 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.98 (d, J = 9.0 Hz, 2H), 7.32 (m, 5H), 6.91 (d, J = 9.0 Hz, 2H), 4.90 (m, 2H), 4.72 (d, J = 5.3 Hz, 1H), 4.57 (d, J = 12.0 Hz, 1H), 4.37 (d, J = 12.0 Hz, 1H), 4.18 (s, 1H), 4.15 (m, 1H), 3.99 (m, 1H), 3.87 (s, 3H), 3.87−3.59 (m, 6H), 3.47 (dd, J_1 = 4.0 Hz, J_2 = 7.8 Hz, 1H), 3.32−3.25 (m, 3H), 3.30 (s, 3H), 2.74 (m, 1H), 1.85 (s, 3H), 1.10 (d, J = 7.2 Hz, 3H), 1.01−0.87 (m, 34H), 0.67−0.54 (m, 19H), 0.40 (dd, J_1 = 5.1 Hz, J_2 = 8.0 Hz, 1H).

(20R)-21-Benzyloxy-20-methylpregn-5-ene-3β,16β,17α-triol 16-O-{2-O-(4-Methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-α-L-arabinopyranoside} (***5***). To a solution of the glycoside **4** (0.018 g, 0.014 mmol) in dioxane−water (7:1 v/v, 3.2 mL) was added *p*-TsOH·H_2O (0.002 g). The reaction mixture was stirred for 1.5 h at 75 °C. Then the reaction mixture was poured into the water and product was extracted with ethyl acetate. The extract was dried over $MgSO_4$, and the solvent was evaporated in vacuo. The saponin **5** was purified by silica gel column chromatography (elution with dichloromethane−methanol, 97:3 v/v). Yield: 93% (0.015 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.02 (d, J = 8.7 Hz, 2H), 7.28 (m, 5H), 6.96 (d, J = 8.7 Hz, 2H), 5.31 (bs, 1H) 4.90 (dd, J_1 = 4.9 Hz, J_2 = 6.7 Hz, 1H), 4.84 (dd, J_1 = 6.8 Hz, J_2 = 7.3 Hz, 1H), 4.70 (d, J = 6.5 Hz, 1H), 4.51 (d, J = 12.0 Hz, 1H), 4.19 (d, J = 12.0 Hz, 1H), 4.16 (dd, J_1 = 4.5 Hz, J_2 = 11.6 Hz, 1H), 4.02 (s, 1H), 3.94 (m, 1H), 3.88 (s, 3H), 3.87 (m, 3H), 3.72 (m, 2H), 3.52 (m, 4H), 3.45−3.38 (m, 4H), 2.85 (m, 1H), 1.81 (s, 3H), 1.01 (s, 3H), 0.99 (d, J = 9.0 Hz, 3H), 0.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 169.3 (C), 166.3 (C), 164.1 (C), 140.6 (C), 137.6 (CH), 132.2 (2CH), 128.4 (2CH), 128.2 (2CH), 127.9 (CH), 121.6 (CH), 121.3 (C), 113.9 (2CH), 101.9 (CH), 101.8 (CH), 90.5 (CH), 87.2 (C), 80.4 (CH), 75.4 (CH_2), 74.5 (CH), 74.0 (CH_2), 73.6 (CH), 71.8 (CH), 70.7 (CH), 69.7 (CH), 66.4 (CH), 64.6 (CH_2), 63.3 (CH_2), 55.5 (CH_3), 49.7 (CH), 48.3 (CH), 46.2 (C), 42.3 (CH_2, C), 37.2 (CH_2), 36.4 (C), 35.1 (CH_2), 33.8 (CH), 32.4 (CH_2), 31.9 (CH), 31.6 (CH_2), 29.7 (CH_2), 20.6 (CH_2, CH_3), 19.4 (CH_3), 13.7 (CH_3), 12.9 (CH_3). ESI-MS m/z 917.4 (MNa^+). HRMS-ESI (m/z): $[M + Na]^+$ calcd for $C_{49}H_{66}O_{15}Na$, 917.4299; found, 917.4312.

(20R)-6β-Methoxy-20-methyl-3α,5α-cyclopregnane-16β,17α,21-triol 16-O-{2-O-(4-Methoxybenzoyl)-3,4-di-O-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-4′-O-triethylsilyl-α-L-arabinopyranoside} (***6***). To a stirred solution of glycoside **4** (0.5 g, 0.4 mmol) in ethyl acetate and anhydrous ethanol (1:1 v/v, 20 mL), 10% Pd/C (0.53 g) and triethylamine (0.18 mL) were added. The reaction was carried out under hydrogen atmosphere (5 MPa) at 50 °C for 20 h. Then the catalyst was filtered off and the solvent was evaporated in vacuo. The desired alcohol was purified by silica gel column chromatography with hexane−ethyl acetate (8:2 v/v) elution. Yield: 82% (0.38 g). ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.99 (d, J = 9.0 Hz, 2H), 6.91 (d, J = 9.0 Hz, 2H), 4.96 (dd, J_1 = 4.9 Hz, J_2 = 7.1 Hz, 1H), 4.91 (dd, J_1 = 5.5 Hz, J_2 = 6.9 Hz, 1H), 4.39 (d, J = 5.3 Hz, 1H), 4.36 (d, J = 4.9 Hz, 1H), 4.14 (m, 1H), 4.01 (m, 1H), 3.87 (s, 3H), 3.68−3.78 (m, 6H), 3.54 (dd, J_1 = 3.1 Hz, J_2 = 11.0 Hz, 1H), 3.36 (m, 1H), 3.32 (s, 3H), 3.24 (dd, J_1 = 7.7 Hz, J_2 = 11.4 Hz, 1H), 2.76 (m, 1H), 1.90 (s, 3H), 0.86−1.07 (m, 34H), 0.53−0.66 (m, 19H), 0.42 (dd, J_1 = 5.1 Hz, J_2 = 7.9 Hz, 1H).

General Procedure for Synthesis of Compounds ***7a*****−*****k***. A solution of compound **6** (1 equiv), carboxylic acid (1.2 equiv), DCC (1.2 equiv), and DMAP (0.1 equiv) in dichloromethane was stirred for 16 h at room temperature. Then *N*,*N*-dicyclohexylurea was filtered off, and the filtrate was washed with 5% acetic acid, water and dried over $MgSO_4$. The solvent was evaporated. Silica gel column chromatography (elution with hexane−ethyl acetate) afforded the ester **7**, which was subsequently subjected to deprotection of functional groups.

(20R)-21-O-[(E)-But-2-enoyl]-6β-methoxy-20-methyl-3α,5α-cyclopregnane-16β,17α,21-triol 16-O-{2-O-(4-Methoxybenzoyl)-3,4-di-O-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-4′-O-triethylsilyl-α-L-arabinopyranoside} (***7g***). Silica gel column chromatography (elution with hexane−ethyl acetate, 82:18 v/v) afforded the desired ester **7g** in 95% yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 7.98 (d, J = 8.7 Hz, 2H), 7.08 (dd J_1 = 6.9 Hz, J_2 = 15.5 Hz, 1H), 6.90 (d, J = 8.7 Hz, 2H), 5.77 (dq, J_1 = 1.7 Hz, J_2 = 15.5 Hz, 1H), 5.03 (m, 1H), 4.94 (m, 1H), 4.77 (m, 1H), 4.31 (m, 1H), 4.00−4.18 (m, 4H), 3.87 (s, 3H), 3.63−3.74 (m, 4H), 3.36 (m, 2H), 3.31 (s, 3H), 3.24 (m, 1H), 2.75 (m, 1H), 1.89 (s, 3H), 1.84 (d, J = 1.7 Hz, 3H), 0.87−1.02 (m, 34H), 0.52−0.66 (m, 19H), 0.42 (m, 1H).

(20R)-21-O-(Undec-10-enoyl)-20-methyl-6β-methoxy-3α,5α-cyclopregnan-16β,17α,21-triol 16-O-{2-O-(4-Methoxybenzoyl)-3,4-di-O-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-4′-O-triethylsilyl-α-L-arabinopyranoside} (***7i***). Silica gel column chromatography (elution with hexane−ethyl acetate, 7:1 v/v) afforded the desired ester **7i** in 63% yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ) 7.99 (d, J = 8.7 Hz, 2H), 6.90 (d, J = 8.7 Hz, 2H), 5.82 (m, 1H), 4.93−5.02 (m, 4H), 4.75 (m, 1H), 4.31 (m, 1H), 4.12 (m, 2H), 4.01 (m, 2H), 3.87 (s, 3H), 3.62−3.87 (m, 4H), 3.36 (m, 2H), 3.31 (s, 3H), 3.25 (m, 1H), 2,.5 (m, 1H), 2.23 (m, 2H), 1.91 (s, 3H), 0.89−1.02 (m, 40H), 0.56−0.64 (m, 19H), 0.42 (m, 1H).

(20R)-21-O-(4-Methoxybenzoyl)-6β-methoxy-20-methyl-3α,5α-cyclopregnane-16β,17α,21-triol 16-O-{2-O-(4-Methoxybenzoyl)-3,4-di-O-triethylsilyl-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-4′-O-triethylsilyl-α-L-arabinopyranoside} (***7k***). Silica gel column (hexane−ethyl acetate, 84:16 v/v) afforded the desired ester **7k** in 65% yield. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 8.07 (d, J = 8.8 Hz, 2H), 7.95 (d, J = 8.8 Hz, 2H), 6.96 (d, J = 8.8 Hz, 2H), 6.86 (d, J = 8.8 Hz, 2H), 5.06 (m, 1H), 4.95 (t, J = 6.1 Hz, 1H), 4.74 (m, 1H), 4.36 (m, 2H), 4.25 (dd, J_1 = 3.4 Hz, J_2 = 11.1 Hz, 1H), 4.12 (m, 1H), 4.01 (m, 1H), 3.89 (s, 3H), 3.85 (s, 4H), 3.60−3.75 (m, 4H), 3.37 (m, 1H), 3.31 (s, 3H), 3.24 (m, 1H), 2.75 (m, 1H), 1.85 (s, 3H), 0.86−1.27 (m, 34H), 0.53−0.66 (m, 19H), 0.42 (m, 1H).

Compounds **7a**−**f**, **7h**, and **7j** were also prepared by this general method, details of which are provided in Supporting Information.

General Procedure for Synthesis of Compounds ***8a*****−*****k***. To a solution of compound **7** in dioxane−water (7:1 v/v), a catalytic amount of *p*-TsOH·H_2O was added. The reaction mixture was stirred for 1.5 h at 75 °C. Then the reaction mixture was poured into the water and product was extracted with ethyl acetate. The extract was dried over $MgSO_4$, and the solvent was evaporated in vacuo. The saponin **8** was purified by silica gel column chromatography (elution with dichloromethane−methanol).

(20R)-21-O-[(E)-But-2-enoyl]-20-methylpregn-5-ene-3β,16β,17α,21-tetraol 16-O-{2-O-(4-Methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-α-L-arabinopyranoside} (***8g***). Silica gel column chromatography (dichloromethane−methanol, 94:6 v/v) afforded the desired saponin **8g** in 94% yield. $[\alpha]_D^{25}$ −29.3° (*c* 0.75, MeOH). IR (KBr) $\tilde{\nu}_{max}$: 3445, 1748, 1715, 1606, 1513, 1259 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$/MeOD, δ): 7.94 (d, *J* = 8.9 Hz, 2H), 6.88 (d, *J* = 8.9 Hz, 2H), 6.86 (dd, J_1 = 6.9 Hz, J_2 = 15.5 Hz, 1H), 5.70 (dq, J_1 = 1.7 Hz, J_2 = 15.5 Hz, 1H), 5.26 (m, 1H), 5.01 (dd, J_1 = 6.8 Hz, J_2 = 8.8 Hz, 1H), 4.87 (dd, J_1 = 6.98 Hz, J_2 = 8.1 Hz, 1H), 4.59 (d, *J* = 6.8 Hz, 1H), 4.25 (d, *J* = 6.8 Hz, 1H), 4.07 (dd, J_1 = 6.4 Hz, J_2 = 11.0 Hz, 1H), 4.00 (dd, J_1 = 4.6 Hz, J_2 = 11.7 Hz, 1H), 3.86−3.95 (m, 3H), 3.85 (s, 3H), 3.57−3.67 (m, 4H), 3.41 (m, 2H), 3.33 (t, *J* = 1.6 Hz, 1H), 3.28 (dd, J_1 = 8.8 Hz, J_2 = 11.7 Hz, 1H), 1.81 (dd, J_1 = 1.7 Hz, J_2 = 6.9 Hz, 1H), 1.63 (s, 3H), 0.94 (s, 3H), 0.90 (d, *J* = 7.0 Hz, 3H), 0.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, δ): 169.4 (C), 166.7 (C), 165.6 (C), 163.7 (C), 145.1 (CH), 140.5 (C), 131.9 (2CH), 122.2 (CH), 121.7 (C), 121.3 (CH), 113.6 (2CH), 102.4 (CH), 101.8 (CH), 88.1 (CH), 86.5 (C), 79.6 (CH), 74.0 (CH), 73.1 (CH), 71.3 (CH), 70.5 (CH), 69.3 (CH), 68.0 (CH_2), 67.5 (CH), 64.8 (CH_2), 64.5 (CH_2), 55.3 (CH_3), 49.6 (CH), 48.4 (CH), 46.5 (C), 41.8 (CH_2), 37.1 (CH_2), 36.3 (C), 35.1 (CH_2), 33.8 (CH), 32.3 (CH_2), 31.63 (CH), 31.59 (CH_2), 31.1 (CH_2), 20.4 (CH_2), 20.3 (CH_3), 19.1 (CH_3), 17.8 (CH_3), 12.7 (CH_3), 11.8 (CH_3). ESI-MS *m*/*z* (%) 895.4 (MNa^+). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calcd for $C_{46}H_{64}O_{16}Na$, 895.4092; found, 895.4117.

(20R)-21-O-(Undec-10-enoyl)-20-methylpregn-5-ene-3β,16β,17α,21-tetraol 16-O-{2-O-(4-Methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-α-L-arabinopyranoside} (***8i***). Silica gel column chromatography (dichloromethane−methanol, 95:5 v/v) afforded the desired saponin **8i** in 95% yield. $[\alpha]_D^{25}$ −17.8° (*c* 1.00, MeOH). IR (KBr) $\tilde{\nu}_{max}$: 3448, 1735, 1719, 1606, 1512, 1257, 1047 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$/MeOD, δ): 7.95 (d, *J* = 8.9 Hz, 2H), 6.89 (d, *J* = 8.9 Hz, 2H), 5.77 (m, 1H), 5.27 (d, *J* = 4.6 Hz, 1H), 5.01 (dd, J_1 = 6.7 Hz, J_2 = 8.6 Hz, 1H), 4.95 (dd, J_1 = 1.9 Hz, J_2 = 17.1 Hz, 1H), 4.88 (m, 2H), 4.61 (d, *J* = 6.6 Hz, 1H), 4.25 (d, *J* = 6.6 Hz, 1H), 4.02 (m, 2H), 3.93 (m, 2H), 3.83 (s, 4H), 3.59−3.67 (m, 4H), 3.42 (m, 2H), 3.30 (m, 1H), 2.00 (m, 2H), 1.68 (s, 3H), 0.95 (s, 3H), 0.89 (d, *J* = 7.0 Hz, 3H), 0.77 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD, δ): 174.0 (C), 169.4 (C), 165.6 (C), 163.7 (C), 140.5 (C), 139.0 (CH), 131.9 (2CH), 121.6 (C), 121.3 (CH), 114.0 (CH_2), 113.6 (2CH), 102.3 (CH), 101.7 (CH), 88.3 (CH), 86.6 (C), 79.6 (CH), 73.9 (CH), 73.1 (CH), 71.3 (CH), 70.4 (CH), 69.3 (CH), 68.3 (CH_2), 67.3 (CH), 64.6 (CH_2), 64.4 (CH_2), 55.3 (CH_3), 49.6 (CH), 48.4 (CH), 46.5 (C), 41.9 (CH_2), 37.1 (CH_2), 36.3 (C), 35.0 (CH_2), 34.2 (CH_2), 33.6 (CH_2, CH), 32.3 (CH_2), 31.64 (CH), 31.60 (CH_2), 31.2 (CH_2), 29.2 (CH_2), 29.1, (CH_2), 29.0 (CH_2), 28.9 (CH_2), 28.8 (CH_2), 24.8 (CH_2), 20.4 (CH_2), 20.3 (CH_3), 19.2 (CH_3), 12.7 (CH_3), 11.9 (CH_3). ESI-MS *m*/*z* (%) 993.5 (MNa^+). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calcd for $C_{53}H_{78}O_{16}Na$, 993.5188; found, 993.5186.

(20R)-21-O-(4-Methoxybenzoyl)-20-metylopregn-5-ene-3β,16β,17α,21-tetraol 16-O-{2-O-(4-Methoxybenzoyl)-β-D-xylopyranosyl-(1→3)-2′-O-acetyl-α-L-arabinopyranoside} (***8k***). Silica gel column chromatography (dichloromethane−methanol, 94:6 v/v) afforded the desired saponin **8k** in 90% yield. $[\alpha]_D^{25}$ −10.2° (*c* 1.00, MeOH). IR ($CHCl_3$) $\tilde{\nu}_{max}$: 3588, 3384, 1738, 1724, 1715, 1607, 1512, 1259, 1196 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$/MeOD, δ): 7.91 (d, *J* = 8.8 Hz, 2H), 7.87 (d, *J* = 8.8 Hz, 2H), 6.88 (d, *J* = 8.9 Hz, 2H), 6.85 (d, *J* = 8.9 Hz, 2H), 5.30 (m, 1H), 5.06 (dd, J_1 = 6.7 Hz, J_2 = 8.4 Hz, 1H), 4.89 (J_1 = 7.0 Hz, J_2 = 7.6 Hz, 1H), 4.63 (d, *J* = 6.5 Hz, 1H), 4.35 (d, *J* = 6.6 Hz, 1H), 4.26 (m, 1H), 4.11 (dd, J_1 = 3.5 Hz, J_2 = 11.0 Hz, 1H), 4.05 (dd, J_1 = 4.5 Hz, J_2 = 11.7 Hz, 1H), 3.95 (m, 2H), 3.84 (s, 3H), 3.82 (s, 3H), 3.61−3.71 (m, 4H), 3.45 (m, 2H), 3.34 (dd, J_1 = 8.6 Hz, J_2 = 11.6 Hz, 1H), 1.64 (s, 3H), 1.01 (d, *J* = 7.0 Hz, 3H), 0.98 (s, 3H), 0.83 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$/MeOD, δ): 169.5 (C), 166.6 (C), 165.7 (C), 163.8 (C), 163.5 (C), 140.6 (C), 131.9 (2CH), 131.4 (2CH), 122.4 (C),121.6 (C), 121.5 (CH), 113.71 (2CH), 113.69 (2CH), 102.4 (CH), 101.7 (CH), 88.4 (CH), 86.7 (CH), 79.7 (CH), 74.0 (CH), 73.1 (CH), 71.5 (CH), 70.5 (CH), 69.4 (CH), 68.5 (CH_2), 67.4 (CH), 64.53 (CH_2), 64.45 (CH_2), 55.40 (CH_3), 55.39 (CH_3), 53.38 (C), 48.5 (CH), 46.6 (C), 42.0 (CH_2), 37.2 (CH_2), 36.4 (C), 35.1 (CH_2), 34.0 (CH), 32.5 (CH_2), 31.74 (CH), 31.69 (CH_2), 31.3 (CH_2), 20.5 (CH_2), 20.4 (CH_3), 19.3 (CH_3), 12.9 (CH_3), 12.1 (CH_3). ESI-MS *m*/*z* (%) 961.4 (MNa^+). HRMS-ESI (*m*/*z*): $[M + Na]^+$ calcd for $C_{50}H_{66}O_{17}Na$, 961.4198; found, 961.4209.

Compounds **8a**−**f**, **8h**, and **8j** were also prepared by this general method, details of which are provided in Supporting Information.

Biological Tests. *Cell Culture.* Stock solutions (10 mmol/L) of OSW-1 derivatives were prepared by dissolving an appropriate quantity of each substance in DMSO. Dulbecco's modified Eagle's medium (DMEM, RPMI 1640 medium), fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin were purchased from Sigma (MO, U.S.). Calcein AM was obtained from Molecular Probes (Invitrogen Corporation, CA, U.S.).

The screening cell lines (T-lymphoblastic leukemia cell line CEM, breast carcinoma cell line MCF-7, cervical carcinoma cell line HeLa, human glioblastoma cell line T98, human malignant melanoma G-361, human osteogenic sarcoma cell line HOS, carcinomic human alveolar basal epithelial cells A549, human colon carcinoma cells HCT 116, and human fibroblasts BJ) were obtained from the American Type Culture Collection (Manassas, VA, U.S.). All cell lines were cultured in DMEM medium (Sigma, MO, U.S.) supplemented with 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, 10 000 U penicillin, and 10 mg/mL streptomycin. The cell lines were maintained under standard cell culture conditions at 37 °C and 5% CO_2 in a humid environment. Cells were subcultured 2 or 3 times a week using the standard trypsinization procedure.

Calcein AM Assay. Suspensions of tested cell lines (~1.0×10^5 cells/mL) were placed in 96-well microtiter plates, and after 3 h of stabilization (time zero) the tested compounds were added (in four 20 μL aliquots) in serially diluted concentrations in dimethylsulfoxide (DMSO). Control cultures were treated with DMSO alone, and the final concentration of DMSO in the incubation mixtures never exceeded 0.6%. The test compounds were typically evaluated at six 3-fold dilutions, and the highest final concentration was generally 50 μM (these conditions were varied in a few cases, depending on the compound). After 72 h of incubation, 100 μL calcein AM solution (Molecular Probes, Invitrogen, CA, U.S.) was added, and incubation was continued for another hour. The fluorescence of viable cells was then quantified using a Fluoroskan Ascent instrument (Labsystems, Finland). The percentage of surviving cells in each well was calculated by dividing the intensity of the fluorescence signals from the exposed wells by the intensity of signals from control wells and multiplying by 100. These ratios were then used to construct dose-response curves from which IC_{50} values, the concentrations of the respective compounds that were lethal to 50% of the tumor cells, were calculated. The results obtained for selected compounds are shown in Table 1.

Flow Cytometric Analysis of the Cell Cycle and Apoptosis. CEM leukemia cancer cells were seeded in 100 mm culture dishes, and immediately incubated with the test compounds. After 24 h, the cells were washed, fixed, and stained in 0.1% [m/v] sodium citrate, 0.1% [v/v] Triton X-100, 0.2 mg/mL RNase A, and 10 μg/mL propidium

iodide in PBS. Their DNA content was then assessed with a flow cytometer (Cell Lab Quanta SC-MPL, Beckman Coulter, CA, U.S.), and the distribution of cells in subG$_1$ ("apoptotic cells"), G$_0$/G$_1$, S, and G$_2$/M phases were quantified by histogram analysis using MultiCycle AV software (Phoenix Flow Systems, CA, U.S.).

Activities of Caspases 3/7. Treated CEM cells were harvested by centrifugation and homogenized in an extraction buffer (10 mM KCl, 5 mM HEPES, 1 mM EDTA, 1 mM EGTA, 0.2% CHAPS, plus protease inhibitors aprotinin, leupeptin, PMSF; pH 7.4) on ice for 20 min. The resulting homogenates were clarified by centrifugation at 10000*g* for 20 min at 4 °C. The protein contents of the samples were quantified by the Bradford method, and they were diluted to equivalent protein concentrations. Lysates were then incubated for 1 h with 100 mM Ac-DEVD-AMC as a substrate (Sigma-Aldrich) in an assay buffer (25 mM PIPES, 2 mM EGTA, 2 mM $MgCl_2$, 5 mM DTT, pH 7.3). For negative controls, the lysates were supplemented with 100 mM Ac-DEVD-CHO (Sigma-Aldrich) as a caspase 3/7 inhibitor. The fluorescence of the product was measured using a Fluoroskan Ascent microplate reader (Labsystems, Finland) at 346 nm/442 nm (excitation/emission).

Western Blotting. The cells were seeded into culture medium in 100 mm culture dishes at a density of 1.5×10^6 cells/mL and treated immediately with OSW-1 derivatives. DMSO was used as a vehicle for controls. After 24 h of treatment, the cells were washed three times with cold PBS (10 mM, pH 7.4) and lysed in ice-cold RIPA protein extraction buffer (20 mM Tris-HCl, pH 7.4, 5 mM EDTA, 2 mM EGTA, 100 mM NaCl, 2 mM NaF, 0.2% Nonidet P-40, 30 mM PMSF, 1 mM DTT, 10 mg/mL of aprotinin and leupeptin). The lysate was collected into a microfuge tube and incubated on ice for 1 h. It was then cleared by centrifugation at 10000*g* for 30 min at 4 °C, and the supernatant was collected. Proteins in lysates were quantified by the Bradford method and diluted with Laemmli electrophoresis buffer. The proteins were then separated on 10% or 12% SDS−polyacrylamide gels, transferred to nitrocellulose membranes (Bio-Rad Laboratories, CA, U.S.) and stained with Ponceau S to check equal protein loading. The membranes were blocked with 5% (w/v) nonfat dry milk and 0.1% Tween-20 in PBS for 2 h and probed with specific primary antibodies (Santa Cruz Biotechnology, CA, U.S.) overnight. After being washed in PBS and PBS with 0.1% Tween-20, the membranes were probed with horseradish peroxidase conjugated secondary antibodies and visualized with West Pico Supersignal chemiluminescent detection reagent (Thermo Fisher Scientific, Rockford, U.S.). To confirm equal protein loading, immunodetection was performed with anti-α-tubulin monoclonal antibody (Sigma, MO, U.S.). The experiments were repeated three times. The protein expression in treated cells was compared to untreated controls.

■ ASSOCIATED CONTENT

Supporting Information. Experimental procedures and analytical data for compounds **3**−**8**; ^{1}H NMR, ^{13}C NMR, IR, MS spectra of products. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

Ac-DEVD-AMC, *N*-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin; Ac-DEVD-CHO, *N*-acetyl-Asp-Glu-Val-Asp-CHO; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; EGTA, ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FBS, fetal bovine serum; Tf, trifluoromethanesulfonate; IC_{50}, the concentration of 50% cell inhibition; MBz, *p*-methoxybenzoyl; PARP, poly ADP-ribose polymerase-1; PIPES, 1,4-piperazinediethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TES, triethylsilyl

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