New Lupane Derived Compounds with Pro-Apoptotic Activity in Cancer Cells: Synthesis and Structure−Activity Relationships

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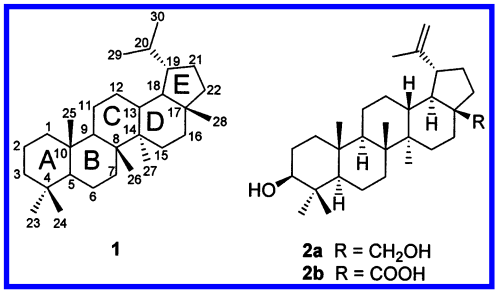
Cellular screening of various synthetic triterpenoid compounds formally derived from lupane has identified a number of analogues as potential anticancer drug candidates. Here we describe the synthesis and structure−activity relationships of betulin and betulinic acid derivatives containing an E-ring modified with different oxygen functions. Thus compounds containing the lup-18-en-21-one, lup-18-ene-21,22-dione, 18,19-secolupane, and the highly oxygenated 18,19-secolupane systems, as well as des-E-lupane derivatives, were prepared from the readily available natural pentacyclic triterpene betulin using oxidative procedures. These compounds were named betulinines. We demonstrate that only selected compounds, particularly those containing a lupane E-ring-derived unsaturated ketone or diketone function, possessed in vitro cytotoxic activity against tumor cell lines, suggesting a structure−activity relationship.

Introduction

The antitumor properties of lupane (**1**, Chart 1) derived triterpenoid compounds were first discovered over 20 years ago when extracts from the stem bark of various plants was tested for cytostatic activity using different in vivo cancer model systems.[1−5] This led to the isolation of the pentacyclic triterpene betulinic acid (BA; **2b**), the best-known representative of the lupane-derived compounds with antiproliferative properties.[6] Early data about both the in vitro and in vivo antitumor activity of BA remain controversial, however. For example, original observations[2] regarding the efficacy of BA in the Walker 256 murine carcinosarcoma model could not be reproduced using a similar system.[3] Antitumor activity of BA against P388D1 murine leukaemia tumors was also suggested.[4]

More recent studies have shown selective cytotoxicity of BA against human melanoblastoma cells.[7] Cell cycle analysis of melanoma cells exposed to BA revealed a G_0/G_1 block after 32 h exposure, and induction of apoptosis was observed after 56−72 h. In addition to this antimelanoma specificity, other studies demonstrated an antitumor effect against neuroectodermal tumors. Furthermore, there is also evidence that BA induces apoptosis via the activation of caspases and that the cytotoxic activity is independent of cellular p53 gene status and CD95 activation.[8] Interestingly, apoptosis is mediated via direct effects on mitochondria, since induction of mitochondrial permeability transition by BA alone was sufficient to trigger a full apoptosis progression.[9] It is also worth mentioning that BA itself, as well as some derivatives, have been described as potent anti-HIV agents.[10,11]

Chart 1



The screening of various lupane-derived triterpenoid compounds synthesized in our laboratories has now identified a number of novel potentially active anticancer drugs. In this report, we describe the synthesis and structure−activity relationships of derivatives of BA, in which the E-ring is modified with different oxygen functions, e.g., 18-lupen-21-one, 18-lup-21,22-dione, 18,19-secolupane, the highly oxygenated 18,19-secolupane, and des-E-lupane. We have named these compounds betulinines. It is known[12] from the literature that the cytotoxic activity of isoprenoid carboxylic acid derivatives is often related to the presence of a free carboxyl group in the molecule. The activity of the corresponding alkyl esters is often much less.[12] This fact is often explained by the comparatively poor hydrolytic lability of alkyl esters under physiological conditions. Pivaloyloxymethyl (Pom) and acetoxymethyl (Acm) esters, on the other hand, which are based on dihydroxymethane, are hydrolyzed readily by various nonspecific enzymes.[13] These esters can be used as suitable

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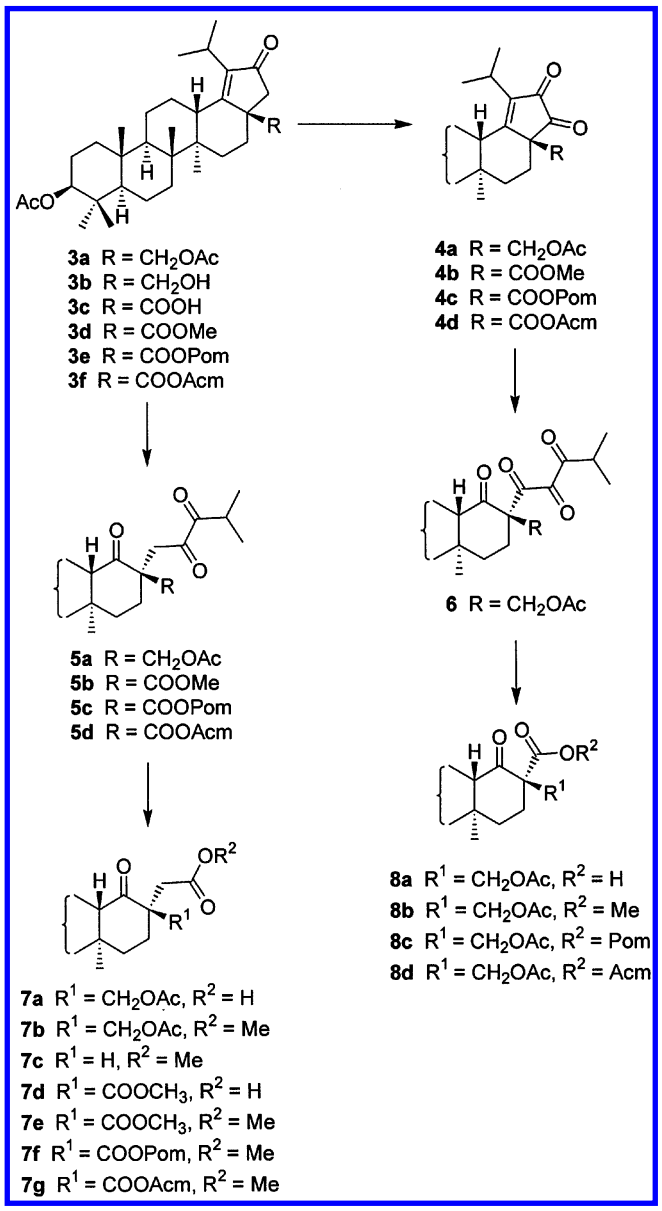
Scheme 1



prodrug groups for carboxylic acids. Because of their lipophilic character, they are membrane permeable and their purification is much easier than that of free carboxylic acids.

In this study, 3β-acetoxylupane derivatives with oxidative modifications of the E-ring and variously bearing acetoxymethyl, carboxyl, methoxycarbonyl, (pivaloyloxy)methoxycarbonyl, and acetoxymethoxycarbonyl groups at C17 were prepared and tested. The cytotoxic activity of this series of derivatives was assessed comparatively using human tumor cell lines.

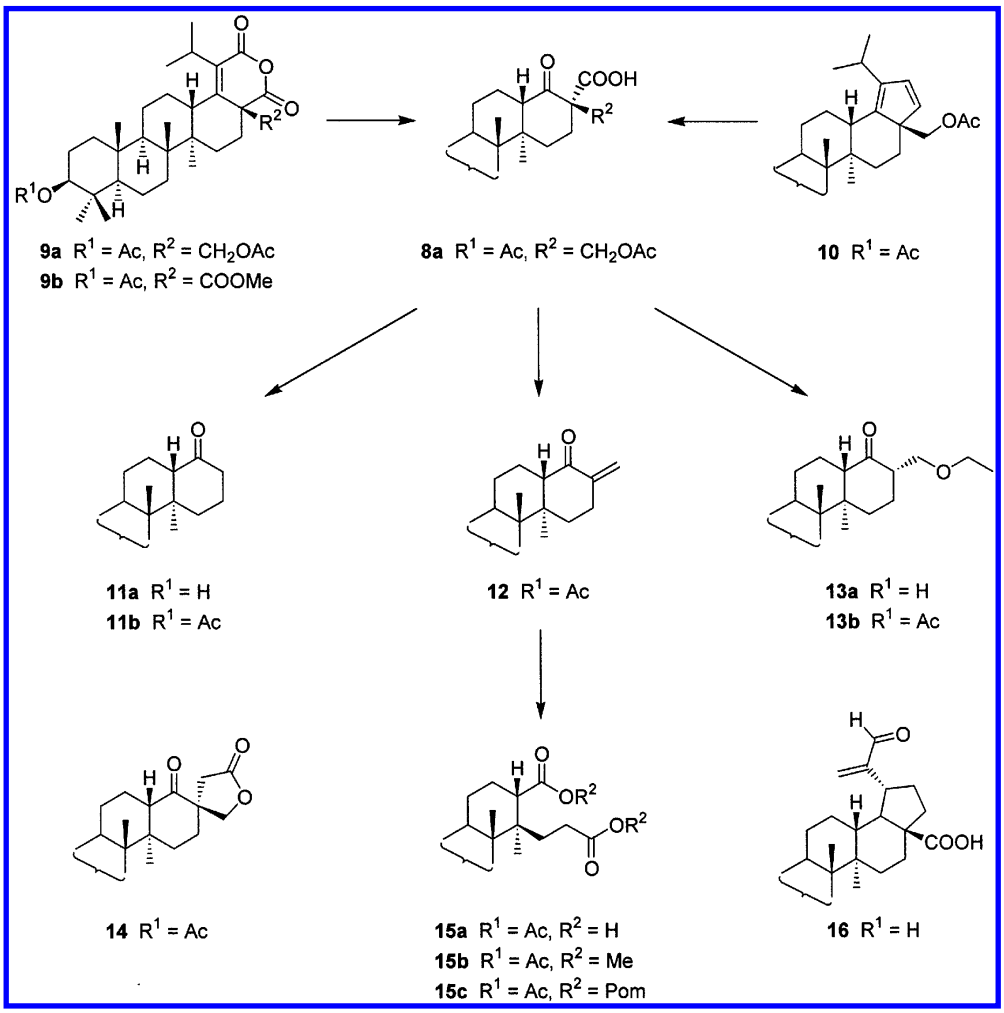
Chemistry

The starting material for the preparation of the compounds described here was 21-oxolup-18-ene-3β,28-diyl diacetate **3a** (Scheme 1). This unsaturated ketone is readily obtained from the natural triterpene betulin **2a** through successive acetylation, acid-catalyzed isomerization of the 20(29)-double bond,[14] and allylic oxidation of the resulting 18,19-unsaturated product.[15] Selective saponification of the 28-acetate ester then furnished hydroxy ketone **3b**,[16] whose carbinol function was oxidized to the carboxylic acid **3c** in high yield with RuO_4. Methyl ester **3d**, which has been described previously,[17] was also characterized. 1,8-Diazabicyclo-[5.4.0]undec-7-ene (DBU)-mediated reactions of keto acid **3c** with chloromethyl pivalate[13] or with bromomethyl acetate in dichloromethane−acetonitrile mixtures afforded the Pom and Acm esters **3e** and **3f**, respectively. Selenium dioxide oxidation of 21-ketones **3a** and **3d**−**f** in dioxane/acetic acid cleanly yielded the corresponding 21,22-diketones **4a**−**d**. Using ^{1}H NMR analysis it was found that α-diketones **4b**−**d** reacted very easily with simple alcohols, producing complicated mixtures of hemiketals and ketals; this finding ruled out the use of alcohols as recrystallization solvents.

The 21-ketones **3a** and **3d**−**f**, as well as 21,22-diketone **4a**, were used for the synthesis of the 18,19-secolupane derivatives **5** and **6**. For fission of the 18,19-double bond in the 21-oxo and 21,22-dioxo derivatives, ruthenium(IV) oxide was used in a catalytic mode (15 mol %). Ruthenium(VIII) oxide was generated in situ from ruthenium(IV) oxide with sodium periodate in a two-phase system of ethyl acetate and water. A similar approach was used by Russian authors[18] for the fission of the double bond in unsubstituted 18-lupenes. Regioselectivity and reaction yields were excellent with this system, although reaction times were often long (1−6 days). The resulting triketones **5a**−**d** and tetraketone **6** are stable crystalline compounds, existing, according to ^{13}C NMR, in exclusively the nonhydrated oxo-form. However, attempts to cleave the diketone functions in these compounds with lead tetraacetate or sodium periodate did not lead to nor-derivatives of tetranoracids of type **7**. Instead, the latter compounds were obtained through Baeyer−Villiger rearrangement with the aid of peroxyacetic acid and they were characterized as the free acids **7a** and **7d**, or in the form of methyl esters **7b** and **7e**−**g**. Tetranoracid **7a** was then lactonized easily to the previously known spirolactone **14**.[19] Through retro-aldol reaction of **7a**, followed by methylation and acetylation, pentanorlupane derivative **7c** was obtained. This compound had previously been prepared by a different approach.[19]

Tetraketone **6** was obtained by ruthenium-catalyzed oxidative degradation of diketone **4a**. Yields of **6** were improved by addition of acetonitrile to the reaction mixture[20] and an increase in the catalytic amount of ruthenium(IV) oxide to 17 mol %. Carboxylic acid **8a** was prepared by cleavage of tetraketone **6** with peroxyacetic acid as reported.[21] Compound **8a** could also be obtained by oxidative fission of the diene **10**, which in turn was obtained by a single-step reaction[22] from lup-18-ene-3β,28-diyl diacetate. However, despite the shorter synthetic route from betulin, the latter method is not as suitable for larger scale preparations of key compound **8a** as the former route via tetraketone **6**, or indeed an alternative route via anhydride **9a** we have reported on previously.[23] Methyl ester **8b** was obtained in the usual manner, whereas attempts to prepare Pom-ester **8c** were not successful. Similarly, application of the DBU-mediated alkylation method referred to above did not permit preparation of Acm-ester **8d** and only decomposition products were obtained. Nevertheless, **8d**

Scheme 2



could be prepared by alkylation of the silver carboxylate salt of acid **8a** in dimethylformamide.

The relative stability of keto acid **8a**, which on theoretical grounds one would expect to decarboxylate spontaneously, is probably due to steric congestion induced by the 17β-acetoxymethyl group, thus hindering formation of the requisite six-membered ring intermediates necessary in the cyclic mechanism of decarboxylation between the 18-oxo and 17α-carboxylate groups. It is known,[18] however, that if the carboxylic function is in position 17β, the corresponding 18-keto derivatives decarboxylate spontaneously.

β-Keto acid **8a** was used for the preparation of other highly degraded lupane and des-E lupane derivatives (Scheme 2). Heating of β-keto acid **8a** in boiling diglyme led to formation of a complicated product mixture through thermal decomposition. Two of the three main components could be identified, the known heptanorketoacetate **11b**[24] and methylene ketone **12**, an unstable compound that was isolated by lyophilization from benzene; the third compound was very unstable and could not be identified.

Very unusual is the reaction of β-keto acid **8a** with potassium hydroxide. If this reaction is performed in the presence of ethanol, the ether **13a** appears in the product mixture along with the normal product of the retroaldol reaction, which then undergoes decarboxylation to the heptanorketone **11a**. One of the possible explanations of the origin of the ethoxy derivative **13a** is Michael addition of ethanol to methylene ketone **12** under the basic conditions applied. If dioxane instead of ethanol is used as the solvent, high yields of pure heptanor ketone **11a** could be isolated.

Oxidative fission of the 17(28)-double bond of methylene ketone **12** produced heptanor-17,18-seco diacid **15a**.[25] This acid can also be prepared by fission of the double bond in hydroxy ketone **3b** or acid **3c**. Dimethyl ester **15b**[25] was prepared from the diacid **15a**. The new diPom-ester **15c** was also prepared, but it was impossible to obtain it in crystalline form. The structures of all the compounds were confirmed by spectral analysis. Furthermore, the structure and absolute configuration of key compound **8a** was confirmed through a single-crystal X-ray structure (see Figure 1; refer also to the Supporting Information).

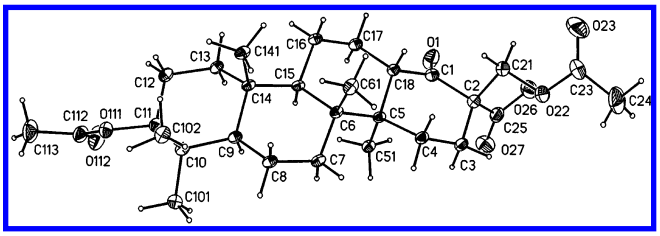


Figure 1. ORTEP-style plot of one of the two crystallographically independent molecules of **8a** in the single-crystal structure. Ellipsoids are drawn at the 30% level.

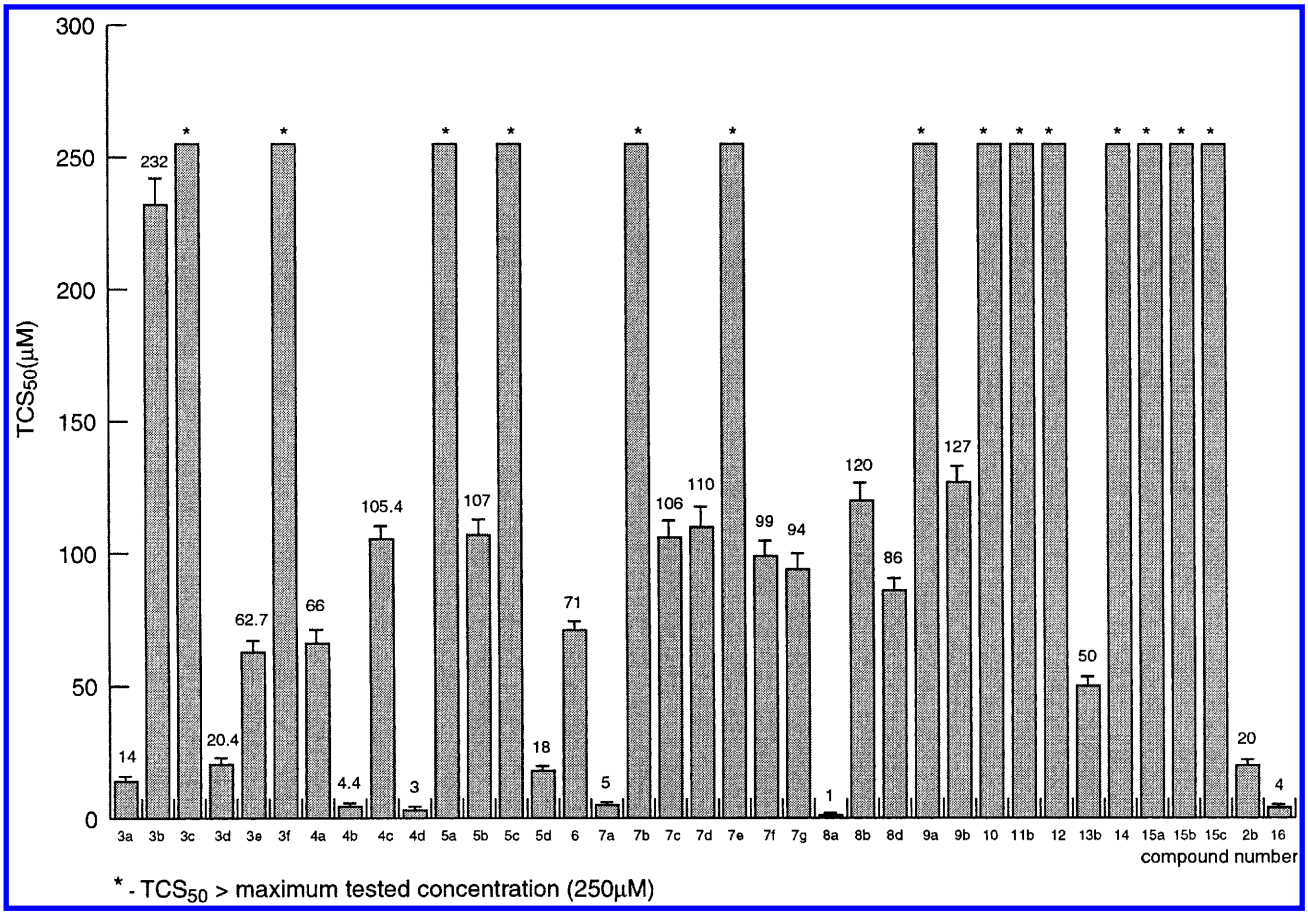


Figure 2. Screening of cytotoxic activity of synthesized lupane derivatives on the chemosensitive CEM T-lymphoblastic leukaemia cell line.

Synthesis of the compound **8a**, described here, was successfully scaled-up to 100 g, with the same yields and purity. Betulin, the starting material for the six-step synthesis of **8a**, is a waste product from the industrial production of paper from birch wood. Estimated production of betulin is thousands of tons per annum. Therefore, a large-scale availability of the compound **8a** is both cost-effective and ecological. Ecology of production is further supported by the fact that the six-step process was optimized with no industrial waste.

Results and Discussion

Since betulinines are complicated natural compounds with lipophilic and rigid carbon skeleton, they are soluble in chlorinated solvents (e.g. chloroform) or ethers (e.g. THF, dioxane) but poorly soluble in water and water-based solvents. However, some of these compounds, particularly those with a free carboxylic group, can be dissolved in a water−ethanol system using pharmaceutically acceptable vehicles.

To evaluate the anticancer potency of our betulinines, their cytotoxic activity against the drug-sensitive CEM cell line was initially examined in the screening assay (Figure 2). It was followed by expanded activity analysis against typical representatives of epithelial, neuroectodermal, and mesodermal tumor cell lines (refer to the Supporting Information). Potent compounds with TCS_{50} (concentration with 50% tumor cell survival) in the low micromolar concentration range were further tested on a panel of cell lines of different histogenetic and species origin (Table 1). It can be seen that nonmalignant cells, e.g. NIH3T3 fibroblasts and normal human lymphocytes, tolerated substantially higher doses of betulinines than the tumor cells, thus demonstrating a preferential cytotoxicity for malignant cells and a favorable therapeutic index.

We show here that only selected derivatives of BA (**2b**) were active against CEM cells under in vitro conditions, suggesting a structure−activity relationship. From the results of the in vitro MTT tests it follows that the cytotoxic activity of the betulinines is often due to the presence in the triterpenic molecule of a β-dicarbonyl group, e.g. as in compounds **4b**, **4d**, **5d**, **7a**, and **8a**, or of an α-unsaturated ketone, e.g. in compounds **3a** and **16**.

The cell cycle and apoptosis analyses clearly demonstrate that the most active BA (**2b**) derivatives (**4a, 8a, 16**) induce apoptosis in CEM cells, which was demonstrated by both the flow-cytometry and scanning electron microscopy techniques (Figures 4 and 5). However, the compounds differed mostly in time dependency of apoptosis induction. The most active was compound **8a**, where the first sub-G1 cells appeared as soon as 3 h after the treatment, although no specific cell cycle alterations were observed. On the other hand, in the CEM cells treated with compounds **4b** and **16** the first signs of apoptosis were between 24 and 48 h after treatment. Interestingly, compound **16** also induced an accumulation of cells in the G2/M and S regions. We hypothesize that the increase in the S phase fraction could be due to the induction of apoptosis at the G2/M

Table 1. Cytotoxicity of Selected Lupane Derivatives (**2b**, **4b**, **8a**, **16**) in a Panel of Cell Lines of Different Histogenetic Origin and Having Various Phenotype Alterations (mean ± SD)

		cytotoxicity (TCS_{50}, μM)			
cell line	description[a]	**2b**	**4b**	**8a**	**16**
B16	mouse melanoma	30.5 ± 3.72	35.4 ± 3.47	0.43 ± 0.25	9.32 ± 1.05
B16F	mouse melanoma, metastatic	4.6 ± 0.38	67.9 ± 5.14	4.66 ± 0.52	12.4 ± 0.92
SW620	human colon cancer, metastasis	>250	5.89 ± 0.47	1.22 ± 0.11	5.86 ± 0.61
U87MG	human glioblastoma	>228 ± 24.7	23.8 ± 2.43	1.85 ± 0.53	98.3 ± 3.95
HepG2	human hepatocellular carcinoma	3.61 ± 0.28	5.14 ± 0.55	1.67 ± 0.09	7.12 ± 0.82
A549	human lung adenocarcinoma	79.3 ± 6.3	6.7 ± 0.73	2.8 ± 0.34	43.27 ± 3.78
MCF-7	human breast cancer, estrogen dependent, $p53^{+/+}$, $RB^{+/+}$	194 ± 15.8	12.9 ± 1.17	2.3 ± 0.20	4.3 ± 0.52
U2OS	human osteosarcoma, $p53^{+/-}$, $RB^{+/-}$	>250	4.28 ± 0.44	1.53 ± 0.15	3.94 ± 0.43
Saos2	human rhabdomyosarcoma, $p53^{-/-}$, $RB^{-/-}$	>250	7.9 ± 0.83	1.78 ± 0.22	4.8 ± 0.51
BT549	human breast cancer, $p53^{mut}$/mut	>250	15.3 ± 1.31	2.03 ± 0.17	6.28 ± 0.49
MDA-MB-2 38	human breast cancer, estrogen independent, p53mut/mut	195 ± 15.3	3.28 ± 0.39	1.35 ± 0.1	2.37 ± 0.18
LNCaP	human prostate cancer, androgen dependent	244 ± 11.7	3.26 ± 0.29	1.12 ± 0.15	3.55 ± 0.36
DU145	human prostate cancer, androgen independent, $RB^{-/-}$	241 ± 22.9	1.98 ± 0.25	0.84 ± 0.11	2.75 ± 0.30
HT-29	human colon cancer	>250	5.23 ± 0.44	3.57 ± 0.24	12.31 ± 1.68
OVCAR-3	human ovarian cancer	164 ± 15.8	4.65 ± 0.38	0.9 ± 0.10	2.59 ± 0.19
Caco-2	human colon cancer	19.6 ± 2.04	8.13 ± 0.79	2.97 ± 0.28	7.72 ± 0.86
MEL-3	human melanoma	2.65 ± 0.35	5.86 ± 0.63	1.28 ± 0.21	3.68 ± 0.37
lymphocytes	human normal lymphocytes	>250	85.3 ± 9.15	12.5 ± 1.13	24.9 ± 2.11
NIH3T3	mouse immortalized fibroblasts	>250	96.8 ± 10.37	7.21 ± 0.83	15.7 ± 1.84
K562	human promyelocytic leukemia	53.9 ± 5.79	1.85 ± 0.22	0.19 ± 0.20	15.12 ± 1.93
K562-CdA	human promyelocytic leukemia, cladribin resistant	>250	5.17 ± 0.62	0.28 ± 0.03	2.13 ± 0.24
K562-GEM	human promyelocytic leukemia, gemcitabin resistant	101 ± 9.77	5.04 ± 0.61	0.94 ± 0.10	2.36 ± 0.25
K562-ARA-C	human promyelocytic leukemia, cytarabin resistant	>250	6.42 ± 0.49	0.63 ± 0.07	1.59 ± 0.18
K562-FLU D	human promyelocytic leukemia, fludarabin resistant	>250	4.85 ± 0.54	0.35 ± 0.41	1.27 ± 0.13
CEM	human T-lymphoblastic leukemia	>250	4.4 ± 0.23	1.0 ± 0.14	4.0 ± 0.32
CEM-DNR 1/C2	human T-lymphoblastic leukemia, daunorubicin resistant	>250	10.3 ± 0.11	0.61 ± 0.58	1.58 ± 0.17
CEM-DNR bulk	human T-lymphoblastic leukemia, daunorubicin resistant	>250	5.18 ± 0.49	1.05 ± 0.09	3.26 ± 0.41
CEM-VCR 1/F3	human T-lymphoblastic leukemia, vincristin resistant	19.1 ± 2.04	38.3 ± 4.15	3.27 ± 0.39	5.94 ± 0.47
CEM-VCR 3/D5	human T-lymphoblastic leukemia, vincristin resistant	24.1 ± 2.63	12.5 ± 1.08	2.93 ± 0.33	7.82 ± 0.86
CEM-VCR bulk	human T-lymphoblastic leukemia, vincristin resistant	68.5 ± 7.19	28.2 ± 2.95	2.51 ± 3.07	6.95 ± 0.58

[a] The majority of genetic alterations referred to here are described at http://www.atcc.org/.

transition and the appearance of the sub-G2 cells, i.e., the population analogous to sub-G1 cells but having a tetraploid DNA content. Although this explanation is further supported by the persistence of the S phase/sub-G2 cells for as long as 18 h after the disappearance of the G0/1 populations among the paclitaxel-treated CEM lymphoblasts, further studies should precisely specify the site and mechanism of action of these molecules.

Although BA has been reported to be preferentially effective against neuroectodermal tumors,[7−9] its derivatives described in this study have lost their specificity and demonstrated a broad anticancer potency (Table 1). Thus, the lack of significant activity correlation of BA with active betulinines of this study (Figure 3) is consistent with this observation. On the other hand, the effectiveness of most of the active betulinines described in Table 1 is significantly correlated (Figure 3), suggesting a common mechanism of action, although the different kinetics of apoptosis induction of **8a** versus **4b**/**16** compounds would suggest independent targets (Figure 4). However, we propose that this difference in timing of apoptosis is rather due to the dissimilar physical and/or pharmaceutical properties of these molecules, e.g., solubility, intracellular penetration, stability, metabolic activation.

Notably, a similar effectiveness of the betulinines was also found on cell lines bearing various mutations or deletions in the cell-cycle-associated proteins (Table 1). This fact indicates that betulinines such as compound **8a** should be equally effective on tumors with various alterations of tumor suppressor genes, such as p53 and the retinoblastoma protein pRb. We also note that the cytotoxic activity of the betulinines is independent of the hormonal status of the cancer cells, and therefore, compounds should be equally effective in the treatment of hormone-dependent and -independent cancers (Table 1). Finally, the betulinines were effective on both the drug-resistant cell lines and the drug-sensitive counterparts. These data demonstrate that the classical mechanisms of multidrug resistance will apparently not apply to this novel generation of compounds, and thus, they could provide significant therapeutic benefit to chemotherapy-resistant cancer patients.

Experimental Section

General. Melting points were determined using a Kofler block and are uncorrected. Optical rotations were measured using $CHCl_3$ solutions (unless otherwise stated) on an Autopol III (Rudolph Research, Flanders, NJ) polarimeter, with an accuracy of ±2°. NMR spectra were recorded on a Varian UNITY INOVA 400 instrument (^{1}H NMR spectra at 399.95 MHz) using $CDCl_3$ solutions (unless otherwise stated), with $SiMe_4$ as an internal standard. EIMS spectra were recorded on an INCOS 50 (Finigan MAT) spectrometer at 70 eV and an ion source temperature of 150 °C. The samples were introduced from a direct exposure probe at a heating rate of 10 mA/s. Relative abundances stated are related to the most abundant ion in the region of $m/z > 50$. TLC was carried out using silica gel 60 F_{254}, and detection was by spraying with 10% aqueous H_2SO_4 and heating to 150−200 °C. Column chromatography was performed using silica gel 60 (Merck 7734). For preparative HPLC, a normal phase silica gel column (250 × 25 mm, Biospher 7 μm), pump Gilson, and differential-refractometer detector (Laboratorní přístroje, Praha, Czech Republic) were used.

Acetate esters were prepared by overnight esterification at room temperature with a 1:1 mixture of pyridine and Ac_2O. Workup refers to pouring of the reaction mixture into H_2O; extraction of the product with Et_2O; and washing of the organic

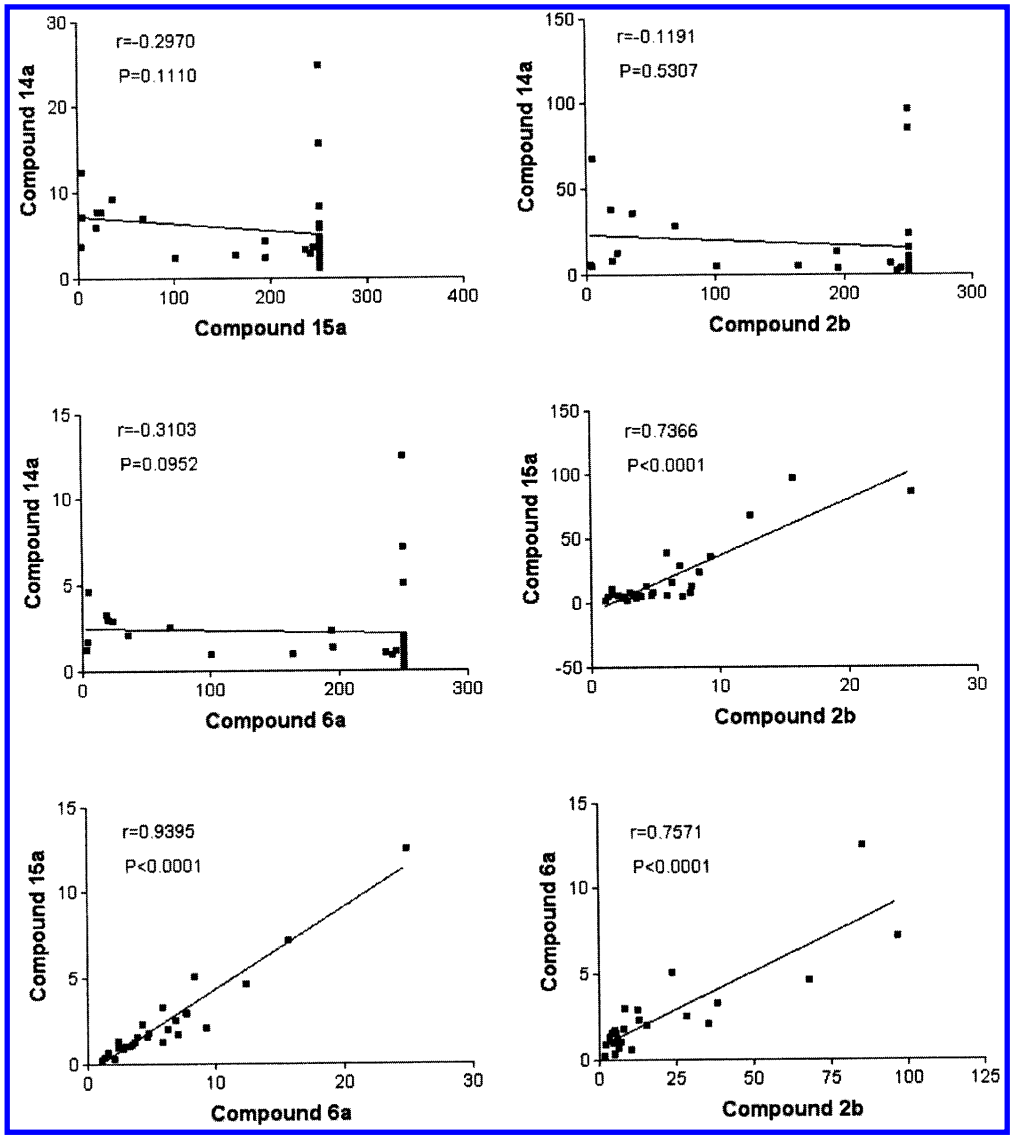


Figure 3. Spearmann correlation analysis of in vitro cytotoxic activity of betulinic acid (**2b**) and the most effective derivatives **16**, **4b**, and **8a** (*r*, correlation coefficient; *p*, statistical signicance).

layer successively with H_2O, dilute aqueous HCl, H_2O, saturated aqueous $NaHCO_3$, and again H_2O, followed by drying over $MgSO_4$, filtration, and evaporation of the filtrate under the reduced pressure. Analytical samples were dried over P_2O_5 under diminished pressure. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), bromomethyl acetate (Acm-Br), chloromethyl pivalate (Pom-Cl), silver carbonate, and ruthenium(IV) oxide hydrate were purchased from Aldrich. Ketone diacetate **3a**,[15] diene **10**,[22] and aldehyde **16**[26] were prepared according to literature procedures. Betulinic acid **2b** was obtained by extraction of the bark of plane trees (*Platanus x hybrida*) with MeOH and several crystallizations from the same solvent.

28-Hydroxy-21-oxolup-18-en-3*β*-yl Acetate (3b). A slightly modified literature procedure[16] was used: a solution of usaturated ketone diacetate **3a** (10 g, 18.5 mmol) and KOH (1.25 g, 22.5 mmol) in PhMe/EtOH (1:1, 0.6 L) was stirred vigorously for 6 h, when TLC (hexane/EtOAc 1:1) indicated complete reaction. After workup a white crystalline residue was obtained, which was recrystallized from EtOAc to afford **3b** (8.2 g, 90%, mp 310−313 °C dec): $[\alpha]_D$ −67° (*c* 0.48); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.95 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.19 (d, J = 6.9 Hz, 3H, CH_3), 1.21 (d, J = 6.9 Hz, 3H, CH_3), 1.92 (d, J = 18.6 Hz, 1H, C$^{22}H^{\alpha}$), 2.05 (s, 3H, OCOCH_3), 2.44 (d, J = 18.6 Hz, C$^{22}H^{\beta}$), 2.78 (dd, J = 12.5 Hz, J' = 3.4 Hz, 1H, C$^{13}H^{\beta}$), 3.19 (sept., J = 6.9 Hz, 1H, C^{20}H), 3.67 (d, J = 10.7 Hz, 1H, C$^{28}H^{\alpha}$), 3.72 (d, J = 10.7 Hz, 1H, C$^{28}H^{\beta}$), 4.49 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^3H^{\alpha}$). Ref.[16] mp 292−294 °C, $[\alpha]_D$ −69° (*c* 0.57).

3*β*-Acetoxy-21-oxolup-18-en-28-oic Acid (3c). A suspension of hydroxy ketone **3b** (1.59 g, 3.2 mmol) in EtOAc (135 mL) was added to a mixture of Ru(IV)O_2·H_2O (40 mg, 0.3 mmol), $NaIO_4$ (4 g, 19 mmol), H_2O (120 mL), and CF_3COOH (2 mL). The biphasic mixture was stirred vigorously for 6 h. EtOH was then added and the mixture was filtered. The organic phase was separated and filtered through a short column of SiO_2. The column was washed with EtOAc and the filtrate was evaporated under reduced pressure. The residue was washed with Et_2O and was crystallized from butanone to afford **3c** (1.06 g, 66%, mp 222−239 °C): $[\alpha]_D$ −40° (*c* 0.42 dioxane); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.21 (d, 3H (J = 7.0 Hz, CH_3), 1.22 (d, J = 7.0 Hz, CH_3, 3H), 2.03 (s, 3H, OCOCH_3), 2.20 (br. d, J = 18.6 Hz, 1H, C$^{22}H^{\alpha}$), 2.47 (m, 1H, C$^{16}H^{\beta}$), 2.59 (d, J = 18.6 Hz, 1H, C$^{22}H^{\beta}$), 2.76 (dd, J = 12.7 Hz, J' = 3.2 Hz, 1H, C$^{13}H^{\beta}$), 3.21 (sept., J = 7.0 Hz, 1H, C^{20}H), 4.49 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^3H^{\alpha}$); EIMS *m*/*z* 512 (M^+, 13), 468 (16), 452 (8), 437 (4), 426 (1), 409 (28), 393 (7), 365 (8), 317 (3), 264 (8), 249 (78), 229 (11), 218 (11), 205 (100), 189 (94). Anal. ($C_{32}H_{48}O_5$) C, H.

Methyl 3*β*-Acetoxy-21-oxolup-18-en-28-oate (3d). This compound was obtained from keto acid **3c** (250 mg, 0.49 mmol) by esterification with diazomethane. Crystallization from

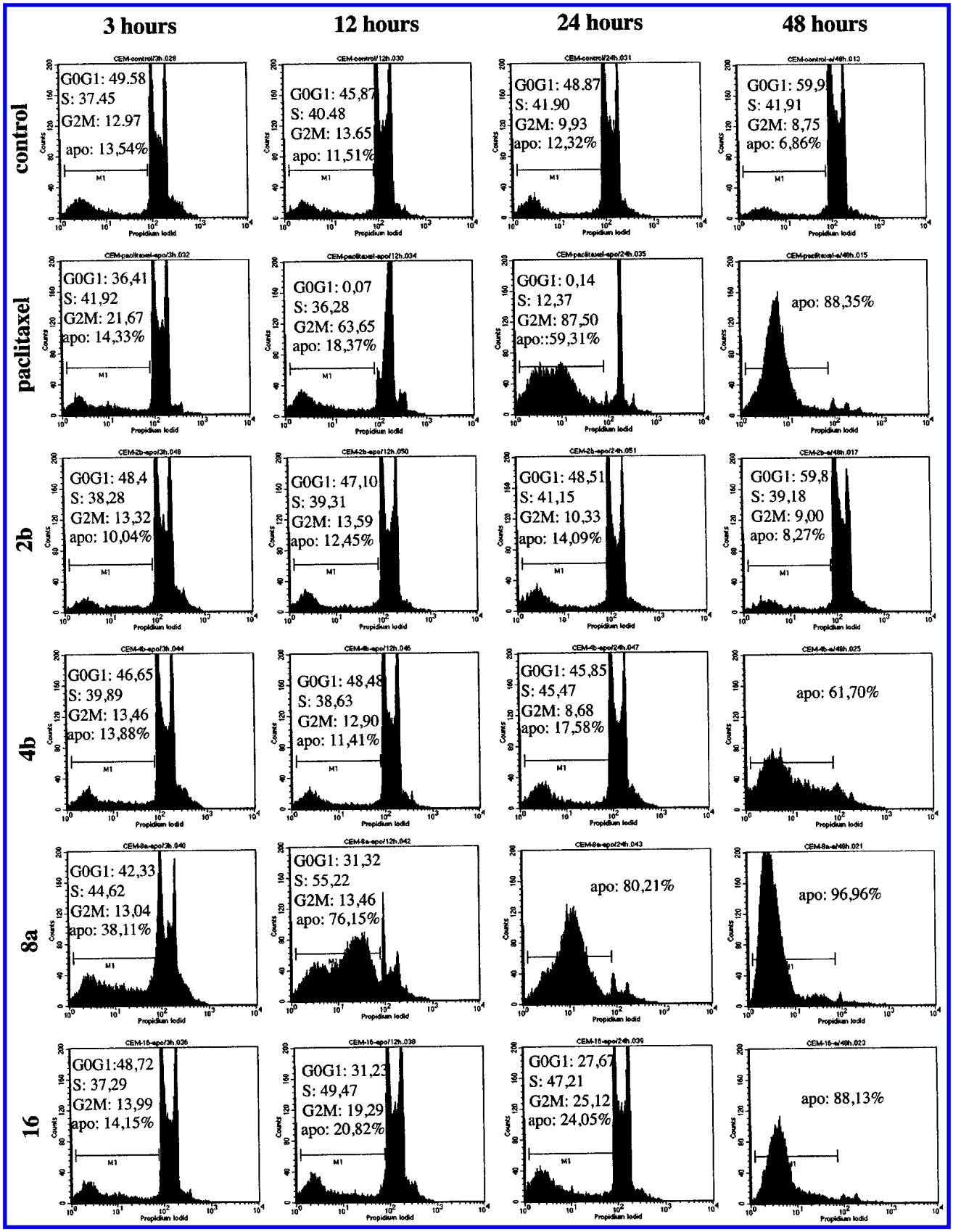
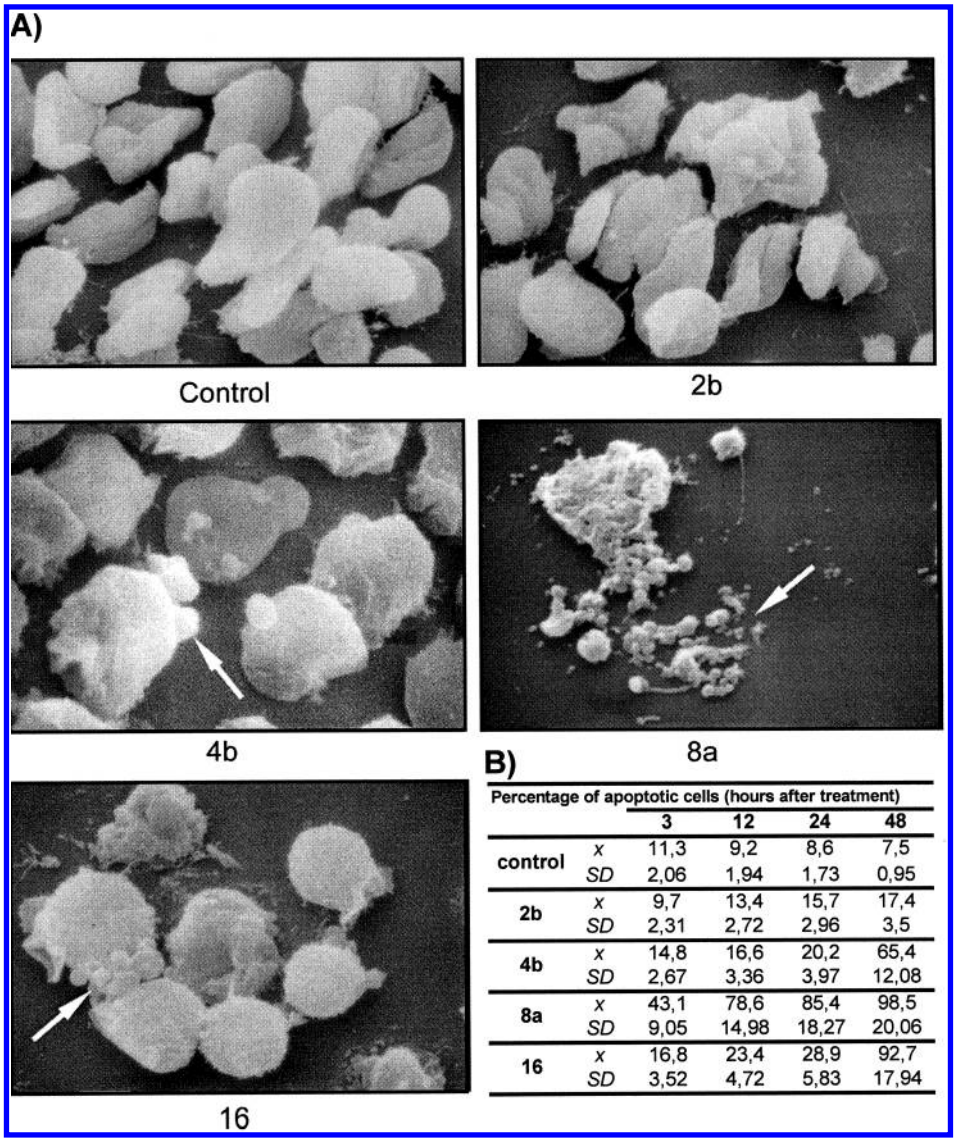


Figure 4. Apoptosis and cell cycle analysis of CEM cells untreated (control) or treated with betulinic acid (**2b**) and the most effective derivatives **16**, **4b**, and **8a** (10 μM). Paclitaxel-treated cells (1 μM) were used as a positive control for both apoptosis and specific cell cycle block on G2/M transition (typical example of several independnet experiments).

MeOH afforded **3d** (213 mg, 83%, mp 220−222 °C): $[\alpha]_D$ −33° (*c* 0.39). ^{1}H NMR δ 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.03 (s, 3H, CH_3), 1.21 (d, J = 7.0 Hz, 6H), 2.05 (s, 3H, OCOCH_3), 2.13 (d, J = 18.7 Hz, 1H, C$^{22}H^{\alpha}$), 2.46 (d, J = 18.7 Hz, 1H, C$^{22}H^{\beta}$), 2.48 (ddd, J = 13.5 Hz, J' = 3.9 Hz, J'' = 2.7 Hz, 1H, C$^{16}H^{\beta}$), 2.64 (dd, J = 12.7 Hz, J' = 3.2 Hz, 1H, C$^{13}H^{\beta}$), 3.20 (sept., J = 7.0 Hz, 1H, C^{20}H), 3.70 (s, 3H, (OCH_3), 4.49 (dd, J = 11.0 Hz, J' = 5.3 Hz, 1H,



Percentage of apoptotic cells (hours after treatment)

		3	12	24	48
control	*x*	11,3	9,2	8,6	7,5
	SD	2,06	1,94	1,73	0,95
2b	*x*	9,7	13,4	15,7	17,4
	SD	2,31	2,72	2,96	3,5
4b	*x*	14,8	16,6	20,2	65,4
	SD	2,67	3,36	3,97	12,08
8a	*x*	43,1	78,6	85,4	98,5
	SD	9,05	14,98	18,27	20,06
16	*x*	16,8	23,4	28,9	92,7
	SD	3,52	4,72	5,83	17,94

Figure 5. Scanning electron microscopy study of apoptosis induction in CEM cells untreated (control) or treated with betulinic acid (**2b**) and the most effective derivatives **16**, **4b**, and **8a** (10 μM). (A) Typical morphology of cells after 24-hour treatment. Arrows indicate morphological markers of apoptosis: cytoplasm membrane blabbing, cellular fragmentation, and formation of apoptotic bodies (magnification 3600×). (B) Dynamics of the process is expressed as percentages of apoptotic cells ($x \pm$ SD) at different time points.

C^3H^α); EIMS *m*/*z* 526 (M^+, 4), 483 (1), 466 (2), 441 (1), 435 (1), 423 (5), 369 (1), 331 (1), 315 (1), 287 (1), 276 (3), 263 (56), 203 (13), 189 (22). Ref.[17] mp 238−241 °C (CH_2Cl_2/MeOH), $[\alpha]_D$ −29° ($CHCl_3$). Anal. ($C_{33}H_{50}O_5$), C, H.

(Pivaloyloxy)methyl 3*β*-Acetoxy-21-oxolup-18-en-28-oate (3e). DBU (165 μL, 1.1 mmol) and Pom-Cl (160 μL; 1.1 mmol) were added to a solution of the keto acid **3c** (500 mg, 0.98 mmol) in CH_2Cl_2 (2.5 mL) and MeCN (1 mL). The mixture was stirred at room temperature for 3 h. It was then diluted with ice-cold H_2O and extracted with $CHCl_3$. The extract was washed with cold brine, dried, and evaporated. The residual viscous pale yellow oil (750 mg) was chromatographed on SiO_2 (PhMe). Crystallization from MeOH afforded the Pom-ester **3e** as colorless needles (453 mg, 72%, mp 179−180 °C): $[\alpha]_D$ −18° (*c* 0.36); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.19 (d, J = 7.0 Hz, 3H, CH_3), 1.21 (d, J = 7.0 Hz, 3H, CH_3), 1.20 (s, 9H, C(CH_3)$_3$), 2.05 (s, 3H OCOCH_3), 2.14 (dd, J = 18.7 Hz, J' = 0.8 Hz, 1H, $C^{22}H^\alpha$), 2.46 (d, J = 18.7 Hz, 1H, $C^{22}H^\beta$), 2.48 (ddd, J = 13.4 Hz, J' = 4.1 Hz, J'' = 2.6 Hz, 1H, $C^{16}H^\beta$), 2.67 (dd, J = 12.7 Hz, J' = 3.7 Hz, 1H, $C^{13}H^\beta$), 3.19 (sept., J = 7.0 Hz, 1H, $C^{20}H$), 4.49 (dd, J = 11.0 Hz, J' = 5.4 Hz, 1H, C^3H^α), 5.66 (d, J = 5.5 Hz, 1H, OCH_2O), 5.84 (d, J = 5.5 Hz, 1H, OCH_2O). EIMS *m*/*z* 626 (M^+, 6), 611 (1), 596 (28), 583 (4), 566 (4), 536 (5), 512 (4), 467 (22), 452 (12), 437 (2), 423 (2), 409 (20), 391 (4), 363 (17), 333 (76), 249 (48), 216 (7), 203 (100), 189 (38). Anal. ($C_{38}H_{58}O_7$) C, H.

Acetoxymethyl 3*β*-Acetoxy-21-oxolup-18-en-28-oate (3f). Keto acid **3c** (500 mg, 0.98 mmol) was treated with Acm-Br and DBU in CH_2Cl_2 (2.5 mL) and MeCN (1 mL) in the same manner as in the preparation of Pom-ester **3e**. Crystallization from butanone produced the Acm-ester **1f** as colorless needles (433 mg, 76%, mp 233−235 °C): $[\alpha]_D$ −26° (*c* 0.4); ^{1}H NMR δ 0.84 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 1.05 (s, 3H, CH_3), 1.20 (d, J = 7.0 Hz, 3H CH_3), 1.21 (d, J = 7.0 Hz, 3H CH_3), 2.05 (s, 3H, OCOCH_3), 2.10 (s, 3H, OCOCH_3), 2.15 (dd, J = 18.6 Hz, 1H, $C^{22}H^\alpha$), 2.47 (d, J = 18.6 Hz, 1H, $C^{22}H^\beta$), 2.48 (ddd, J = 13.4 Hz, J' = 4.1 Hz, J'' = 2.7 Hz, 1H, $C^{16}H^\beta$), 2.67 (dd, J = 12.6 Hz, J' = 3.3 Hz, 1H, $C^{13}H^\beta$), 3.19 (sept., J = 7.0 Hz, 1H, $C^{20}H$), 4.49 (dd, J = 11.0 Hz, J' = 5.3 Hz, 1H, C^3H^α), 5.70 (d, J = 5.5 Hz, 1H, OCH_2O), 5.79 (d, J = 5.5 Hz, 1H, OCH_2O). EIMS *m*/*z* 584 (M^+, 30), 554 (11), 541 (23), 512 (44), 481 (48), 467 (26), 452 (49), 409 (77), 391 (11), 321 (100), 249 (59), 203 (33), 189 (25). Anal. ($C_{35}H_{52}O_7$) C, H.

21,22-Dioxolup-18-ene-3*β*,28-diyl Diacetate (4a). A solution of ketone diacetate **3a** (2 g, 3.7 mmol) and SeO_2 (1.6 g,-14.4 mmol) in dioxane (40 mL), AcOH (20 mL), and Ac_2O (2

mL) was heated under reflux until reaction was complete by TLC (PhMe/Et_2O 10:1). After cooling, precipitated selenium was removed by filtration and the filtrate was poured slowly into an excess of vigorously stirred water. The red-orange precipitate was filtered under reduced pressure, washed carefully with H_2O, and air-dried. The crude product was dissolved in $CHCl_3$ and the solution was filtered through a column of alumina. The column was eluted with $CHCl_3$ and the filtrate was evaporated under reduced pressure. The residue was crystallized from MeOAc to afford **4a** as pale-orange crystals (1.45 g, 82%, mp 267−270 °C): $[\alpha]_D$ −127° (*c* 0.32); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 s, 3H, CH_3), 0.94 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.18 (s, 3H, CH_3), 1.24 (d, J = 7.0 Hz, 3H, CH_3), 1.26 (d, J = 7.0 Hz, 3H, CH_3), 1.91 (s, 3H, OCOCH_3), 2.06 (s, 3H, OCOCH_3), 3.12 (dd, J = 12.5 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.36 (sept., J = 7.0 Hz, 1H, C^{20}H), 4.02 (d, J = 11.0 Hz, 1H, C$^{28}H^{\alpha}$), 4.49 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$), 4.84 (d, J = 11.0 Hz, C$^{28}H^{\alpha}$). Ref.[16] mp 269−271 °C, $[\alpha]_D$ −136° (*c* 0.6).

Methyl 3*β*-Acetoxy-21,22-dioxolup-18-en-28-oate (4b). Ketomethyl ester **3d** (500 mg, 0.98 mmol) was oxidized with SeO_2 (400 mg, 3.6 mmol) in a mixture of dioxane (10 mL), AcOH (5 mL), and Ac_2O (0.5 mL) in the same manner as in the preparation of diketone diacetate **4a**. Crystallization from dimethyl carbonate afforded **4b** as pale-orange needles (387 mg, 73%, mp 228−234 °C dec): $[\alpha]_D$ −100° (*c* 0.43); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.26 (d, J = 7.0 Hz, 3H, CH_3), 1.29 (d, J = 7.0 Hz, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.54 (ddd, J = 13.5 Hz, J' = 4.4 Hz, J'' = 2.2 Hz, 1H, C$^{16}H^{\beta}$), 2.78 (dd, J = 12.7 Hz, J' = 3.3 Hz, 1H, C$^{13}H^{\beta}$), 3.36 (sept., J = 7.0 Hz, 1H, C^{20}H), 3.72 (s, 3H, OCH_3), 4.49 (dd, J = 11.2 Hz, J' = 5.3 Hz, 1H, C$^{3}H^{\alpha}$); EIMS m/z 540 (M$^+$, 1), 525 (1), 512 (3), 501 (1), 484 (100), 469 (37), 279 (3), 217 (4), 203 (7), 191 (17). Anal. ($C_{33}H_{48}O_6$) C, H.

Pivaloyloxymethyl 3*β*-Acetoxy-21,22-dioxolup-18-en-28-oate (4c). The keto-Pom ester **3e** (200 mg, 0.32 mmol) was oxidized with SeO_2 (160 mg, 1.4 mmol) in dioxane (4 mL), AcOH (2 mL), and Ac_2O (0.2 mL) in the same manner as in the preparation of diketone diacetate **4a**. Lyophilization from benzene afforded **4c** as a pale-orange powder (145 mg, 71%, mp 104−108 °C): $[\alpha]_D$ −135° (*c* 0.43); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.19 (s, 9H, C(CH_3)$_3$), 1.26 (d, J = 7.0 Hz, 3H, CH_3), 1.26 (d, J = 7.0 Hz, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.54 (ddd, J = 13.5 Hz, J' = 4.5 Hz, J'' = 2.2 Hz, 1H, C$^{16}H^{\beta}$), 2.80 (dd, J = 12.4 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.34 (sept., J = 7.0 Hz, 1H, C^{20}H), 5.55 (d, J = 5.4 Hz, 1H, OCH_2O), 5.91 (d, J = 5.4 Hz, 1H, OCH_2O); EIMS m/z 640 (M$^+$, 2), 610 (2), 582 (2), 565 (1), 550 (1), 542 (1), 506 (1), 498 (45), 481 (6), 470 (100), 455 (12), 439 (9), 217 (6), 203 (9), 189 (12). Anal. ($C_{38}H_{56}O_8$) C, H.

Acetoxymethyl 3*β*-Acetoxy-21,22-dioxolup-18-ene-28-oate (4d). Keto-Acm ester **3f** (250 mg, 0.43 mmol) was oxidized with SeO_2 (200 mg, 1.8 mmol) in dioxane (5 mL), AcOH (2.5 mL), and Ac_2O (0.25 mL) in the same manner as in the preparation of diketone diacetate **4a**. Crystallization from butanone afforded **4d** as pale-orange needles (176 mg, 69%, mp 227−228 °C): $[\alpha]_D$ −177° (c 0.43); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 0.97 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.26 (d, J = 7.0 Hz, 3H, CH_3), 1.27 (d, J = 7.0 Hz, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.09 (s, 3H, OCOCH_3), 2.55 (ddd, J = 13.7 Hz, J' = 4.5 Hz, J'' = 2.4 Hz, 1H, C$^{16}H^{\beta}$), 2.79 (dd, J = 12.6 Hz, J' = 3.4 Hz, 1H, C$^{13}H^{\beta}$), 3.35 (sept., J = 7.0 Hz, 1H, C^{20}H), 4.49 (dd, J = 11.1 Hz, J′ = 5.3 Hz, 1H, C$^{3}H^{\alpha}$), 5.62 (d, J = 5.5 Hz, 1H, OCH_2O), 5.82 (d, J = 5.5 Hz, 1H, OCH_2O); EIMS m/z 598 (M$^+$, 8), 568 (2), 540 (10), 498 (40), 470 (100), 455 (18), 439 (6), 285 (5), 259 (5), 243 (10), 217 (20), 203 (29), 190 (46). Anal. ($C_{35}H_{50}O_8$) C, H.

18,19,21-Trioxo-18,19-secolupan-3*β*,28-diyl Diacetate (5a). A solution of ketone diacetate **3a** (500 mg, 0.93 mmol) in EtOAc (21 mL) was added to a mixture of Ru(IV)O_2·H_2O (15 mg, 0.11 mmol), $NaIO_4$ (1.3 g, 6.1 mmol), H_2O (18 mL), and CF_3COOH (0.5 mL). The biphasic mixture was stirred vigorously at room temperature for 4 h, at which time reaction was complete by TLC (PhMe/Et_2O 6:1). EtOH was added, the mixture was filtered, and the organic layer was chromatographed on a short SiO_2 column (EtOAc). The eluate was evaporated under reduced pressure and the residue was crystallized from Me_2CO to afford **5a** as gold petals (339 mg, 64%, mp 226−228 °C): $[\alpha]_D$ +71° (*c* 0.39); ^{1}H NMR δ 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.13 (d, J = 6.9 Hz, 3H, CH_3), 1.16 (d, J = 6.9 Hz, 3H, CH_3), 1.84 (ddd, J = 13.9 Hz, J' = 4.4 Hz, J'' = 2.2 Hz, 1H, C$^{16}H^{\beta}$), 2.05 (s, 6H, 2 OCOCH_3), 2.31 (td, J = 13.9 Hz, J' = 13.9 Hz, J'' = 4.6 Hz, 1H, C$^{16}H^{\alpha}$), 2.68 (dd, J = 11.6 Hz; J' = 3.3 Hz, 1H, C$^{13}H^{\beta}$), 2.45 (d, J = 16.6 Hz, 1H, C$^{22}H^{\alpha}$), 3.25 (d, J = 16.6 Hz, 1H, C$^{22}H^{\beta}$), 3.38 (sept., J = 6.9 Hz, 1H, C^{20}H), 4.20 (d, J = 11.3 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (d, J = 11.3 Hz, 1H, C$^{28}H^{\beta}$), 4.48 (dd, $J \approx$ 10 Hz, $J' \approx$ 6 Hz, 1H, C$^{3}H^{\alpha}$); EIMS m/z 572 (M$^+$, 0.2), 557 (0.4), 501 (15), 459 (16), 441 (53), 399 (8), 381 (32), 371 (15), 353 (1), 339 (1), 215 (6), 201 (17), 189 (50), 71 (100). Anal. ($C_{34}H_{52}O_7$) C, H.

Methyl 3*β*-Acetoxy-18,19,21-trioxo-18,19-secolupan-28-oate (5b). Ketomethyl ester **3d** (400 mg; 0.76 mmol) was oxidized with Ru(VIII)O_4 (from Ru(IV)O_2·H_2O; 13 mg, 0.1 mmol) and $NaIO_4$ (1.1 g) in EtOAc (18 mL), H_2O (15 mL), and CF_3COOH (0.4 mL) in the same manner as in the preparation of triketone diacetate **5a**. Crystallization from MeOH afforded **5b** as yellow petals (259 mg, 61%, mp 173−175 °C): $[\alpha]_D$ +43° (*c* 0.45); ^{1}H NMR δ 0.82 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 1.12 (d, J = 7.0 Hz, 3H, CH_3), 1.14 (d, J = 7.0 Hz, 3H, CH_3), 2.04 (s, 3H, OCOCH_3), 2.87 (d, J = 16.5 Hz, 1H, C$^{22}H^{\alpha}$), 2.99 (dd, J = 11.9 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.15 (d, J = 16.5 Hz, 1H, C$^{22}H^{\beta}$), 3.36 (sept., J = 7.0 Hz, 1H, C^{20}H), 3.78 (s, 3H, OCH_3), 4.47 (dd, J = 11.0 Hz, J' = 5.4 Hz, 1H, C$^{3}H^{\alpha}$); EIMS m/z 558 (M$^+$, 4), 527 (1), 487 (17), 444 (4), 427 (98), 395 (6), 367 (6), 277 (5), 249 (8), 231 (8), 215 (11), 201 (32), 189 (100). Anal. ($C_{33}H_{50}O_7$) C, H.

(Pivaloyloxy)methyl 3*β*-Acetoxy-18,19,21-trioxo-18,19-secolupan-28-oate (5c). Keto-Pom ester **3e** (400 mg, 0.64 mmol) was oxidized with Ru(VIII)O_4 (from Ru(IV)O_2·H_2O; 13 mg, 0.1 mmol) and $NaIO_4$ (2.1 g) in EtOAc (16 mL), H_2O (14 mL), and CF_3COOH (0.4 mL) in the same manner as in the preparation of triketone diacetate **5a**. Crystallization from MeOH afforded **5c** as yellow petals (273 mg, 65%, mp 152−154 °C): $[\alpha]_D$ +40° (*c* 0.38); ^{1}H NMR δ 0.81 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.11 (d, J = 7.0 Hz, 3H, C$^{30}H_3$), 1.14 (d, J = 7.0 Hz, 3H, C$^{30}H_3$), 1.22 (s, 9H, C(CH_3)$_3$), 2.04 (s, 3H, OCOCH_3), 2.36 (br. m, 1H, C$^{16}H^{\alpha}$), 2.88 (d, J = 16.8 Hz, 1H, C$^{22}H^{\alpha}$), 3.06 (dd, J = 11.9 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.18 (d, J = 16.9 Hz, 1H, C$^{22}H^{\beta}$), 3.35 (sept., J = 7.0 Hz, 1H, C^{20}H), 4.47 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$); 5.75 (d, J = 5.5 Hz, 1H, OCH_2O), 5.85 (d, J = 5.5 Hz, 1H, OCH_2O); EIMS m/z 658 (M$^+$, 1), 612 (2), 598 (7), 584 (2), 544 (3), 498 (16), 484 (6), 470 (32), 438 (7), 413 (23), 349 (13), 249 (8), 235 (20), 217 (8), 203 (20), 189 (100). Anal. ($C_{38}H_{58}O_9$) C, H.

Acetoxymethyl 3*β*-Acetoxy-18,19,21-trioxo-18,19-secolupan-28-oate (5d). Keto-Acm ester **3f** (400 mg, 0.68 mmol) was oxidized with Ru(VIII)O_4 (from Ru(IV)O_2·H_2O; 13 mg, 0.1 mmol) and $NaIO_4$ (2.1 g) in EtOAc (16 mL), H_2O (14 mL), and CF_3COOH (0.4 mL) in the same manner as in the preparation of the triketone diacetate **5a**. Crystallization from MeOH afforded **5d** as yellow petals (273 mg, 65%, mp 215−235 °C dec): $[\alpha]_D$ +32° (*c* 0.44); ^{1}H NMR δ 0.81 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 1.11 (d, J = 7.0 Hz, 3H, CH_3), 1.14 (d, J = 7.0 Hz, 3H, CH_3), 2.04 (s, 3H, CH_3), 2.12 (s, 3H, 2 OCOCH_3), 2.37 (br. m, 1H, C$^{16}H^{\alpha}$), 2.85 (d, J = 16.8 Hz, 1H, C$^{22}H^{\alpha}$), 3.06 (dd, J = 11.9 Hz, J' = 3.4 Hz, 1H, C$^{13}H^{\beta}$), 3.21 (d, J = 16.8 Hz, 1H, C$^{22}H^{\beta}$), 3.35 (sept., J = 7.0 Hz, 1H, C^{20}H), 4.47 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$), 5.76 (d, J = 5.5 Hz, 1H, OCH_2O), 5.83 (d, J = 5.5 Hz, 1H, OCH_2O); EIMS m/z 616 (M$^+$, 1), 574 (1), 557 (1), 515 (4), 485 (5), 473 (9), 455 (8), 413 (25), 395 (18), 367 (15), 275 (3), 249 (11), 215 (8), 189 (100). Anal. ($C_{35}H_{52}O_9$) C, H.

18,19,21,22-Tetraoxo-18,19-secolupan-3*β*,28-diyl Diacetate (6). A solution of diketone diacetate **4a** (500 mg, 0.93 mmol) in EtOAc (20 mL) and MeCN (5 mL) was added to a mixture of $Ru(IV)O_2 \cdot H_2O$ (20 mg, 0.15 mmol), $NaIO_4$ (3.5 g, 16.4 mmol), H_2O (18 mL), and CF_3COOH (0.5 mL). The biphasic mixture was stirred vigorously at room temperature for 20 h, when TLC (PhMe/EtOAc 20:1) indicated complete reaction. EtOH was added, the mixture was filtered, the phases were separated, and the organic layer was dried and evaporated under a reduced pressure. The residue (643 mg), which according to ^{1}H NMR analysis contained tetraketone **6** and *β*-keto acid **8a** in a ratio of approximately 1:1, was chromatographed on SiO_2 (50 g) in PhMe/EtOAc (20:1). Crystallization from Me_2CO/isopentane afforded pure **6** as yellow-orange crystals (190 mg, 36%, mp 202−205 °C): $[\alpha]_D$ +37° (*c* 0.27); ^{1}H NMR *δ* 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 1.21 (d, J = 6.9 Hz, 3H, CH_3), 1.22 (d, J = 6.9 Hz, 3H, CH_3), 2.02 (s, 3H, OCOCH_3), 2.05 (s, 3H, OCOCH_3), 2.43 (br. m, 1H, C$^{16}H^{\beta}$), 2.84 (dd, J = 11.6 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.41 (sept. J = 7.0 Hz, 1H, C^{20}H), 4.48 (m, $\Sigma J \approx$ 15 Hz, 1H, C$^{3}H^{\alpha}$), 4.48 (d, J = 11.1 Hz, 1H, C$^{28}H^{\alpha}$), 4.77 (d, J = 11.1 Hz, 1H, C$^{28}H^{\beta}$); EIMS *m*/*z* 586 (M^+, 3), 544 (2), 526 (4), 514 (33), 513 (9), 498 (2), 487 (78), 474 (12), 459 (17), 444 (11), 427 (7), 417 (6), 400 (27), 383 (11), 367 (8), 357 (31), 339 (28), 322 (12), 297 (10), 276 (12), 249 (20), 229 (13), 216 (17), 203 (50), 189 (100). Anal. ($C_{34}H_{50}O_8$) C, H.

3*β*,28-Diacetoxy-18-oxo-19,20,29,30-tetranorlupan-21-oic Acid (7a). A solution of triketone **5a** (300 mg, 0.52 mmol) and AcOOH (5 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (40 mL) was stirred at room temperature for 24 h, when TLC (PhMe/Et_2O 6:1) indicated complete reaction. The colorless reaction mixture was diluted with cold H_2O and was extracted with $CHCl_3$. The combined organic layers were washed successively with 5% aqueous KI solution (400 mL), saturated aqueous sodium sulfite solution (200 mL), and brine (2 × 200 mL) and then dried and evaporated. According to ^{1}H NMR analysis the resulting pale-yellow oil (268 mg) was a mixture of the keto acid **7a** and spirolactone **14**[19] in a ratio of approximately 4:1. Preparative TLC ($CHCl_3$/EtOAc/AcOH 100:10:1), followed by crystallization from Et_2O/hexane, afforded **7a** as a white crystalline powder (165 mg, 61%, mp 152−155 °C and 185−189 °C dec): $[\alpha]_D$ +26° (*c* 0.30); ^{1}H NMR *δ* 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.90 (s, 3Hm CH_3), 1.14 (s, 3H, CH_3), 2.03 (s, 3H, OCOCH_3), 2.05 (s, 3H, OCOCH_3), 2.53 (d, J = 15.6 Hz, 1H, C$^{22}H^{\alpha}$), 2.60 (d, J = 15.6 Hz, 1H, C$^{22}H^{\beta}$), 2.81 (dd, J = 11.9 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 4.20 (d, J = 11.4 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (dd, J = 11.0 Hz, J' = 5.3 Hz, 1H, C$^{3}H^{\alpha}$), 4.60 (d, J = 11.4 Hz, 1H, C$^{28}H^{\beta}$); EIMS *m*/*z* 518 (M^+, 3), 503 (1), 486 (2), 458 (100), 443 (38), 430 (6), 415 (60), 398 (27), 383 (16), 355 (17), 323 (7), 255 (32), 241 (16), 204 (15), 189 (15). Anal. ($C_{30}H_{46}O_7$) C, H.

Methyl 3*β*,28-Diacetoxy-18-oxo-19,20,29,30-tetranorlupan-21-oate (7b). This compound was obtained by esterification of keto acid **7a** with diazomethane (mp 209−211 °C, MeOH): $[\alpha]_D$ +52° (*c* 0.46); ^{1}H NMR *δ* 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 2.03 (s, 3H, OCOCH_3), 2.05 (s, 3H, OCOCH_3), 2.43 (d, J = 15.9 Hz, 1H, C$^{22}H^{\alpha}$), 2.66 (d, J = 15.9 Hz, 1H, C$^{22}H^{\beta}$), 2.75 (dd, $J \approx$ 12 Hz, $J' \approx$ 4 Hz, 1H, C$^{13}H^{\beta}$), 3.66 (s, 3H, OCH_3), 4.19 (d, $J \approx$ 11 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (dd, $J \approx$ 10 Hz, $J' \approx$ 6 Hz, 1H, C$^{3}H^{\alpha}$), 4.56 (d, $J \approx$ 11 Hz, 1H, C$^{28}H^{\beta}$); EIMS *m*/*z* 532 (M^+, 3), 517 (1), 501 (1), 472 (40), 457 (11), 441 (4), 429 (17), 412 (3), 397 (4), 381 (3), 369 (2), 339 (3), 269 (91), 255 (23), 204 (14), 195 (30), 189 (100). Anal. ($C_{31}H_{48}O_7$) C, H.

Methyl 3*β*-Acetoxy-18-oxo-19,20,28,29,30-pentanorlupan-21-oate (7c). A suspension of methyl keto ester **7b** (140 m, 0.27 mmol) in a solution of KOH (140 mg, 2.43 mmol) in EtOH (7.5 mL) was refluxed for 3 h, at which time all starting material had dissolved. After cooling, the reaction mixture was poured into H_2O (50 mL). The resulting solution was acidified with 10% aqueous HCl (2 mL) and was extracted twice with Et_2O. The combined organic extracts were washed with H_2O, dried, and evaporated. The residue (102 mg) was treated with diazomethane and then acetylated with Ac_2O (2 mL) in pyridine (2 mL). The usual workup gave a white foamy residue. Purification by preparative TLC (PhMe/Et_2O 10:1) and crystallization from $CHCl_3$/MeOH afforded **7c** (76 mg, 58%, mp 192−194 °C): $[\alpha]_D$ +11° (*c* 0.50); ^{1}H NMR *δ* 0.79 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 2.04 (s, 3H, OCOCH_3), 2.13 (dd, J = 16.0 Hz, J' = 5.5 Hz, 1H, C$^{22}H^{\alpha}$), 2.56 (dd, J = 12.4 Hz, J' = 3.7 Hz, 1H, C$^{13}H^{\beta}$), 2.73 (dd, J = 16.0 Hz, J′ = 7.3 Hz, 1H, C$^{22}H^{\beta}$), 2.78 (br. m, $\Sigma J \approx$ 28 Hz, 1H, C^{17}H), 3.67 (s, 3H, OCH_3), 4.48 (dd, J = 11.2 Hz, J' = 5.3 Hz, 1H, C$^{3}H^{\alpha}$). Ref.[19] mp 193−195 °C, $[\alpha]_D$ +12°.

28-Methyl-21-hydrogen 3*β*-Acetoxy-18-oxo-19,20,29,30-tetranorlupane-21,28-dioate (7d). Triketomethyl ester **5b** (200 mg, 0.36 mmol) was oxidized with AcOOH (3 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (30 mL) in the same manner as in the preparation of the keto acid **7a**. Crystallization from Et_2O afforded **7d** as small white crystals (129 mg, 71%, mp 177−180 °C): $[\alpha]_D$,+64° (*c* 0.44); ^{1}H NMR *δ* 0.82 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.46 (ddd, J = 14.0 Hz, J' = 4.1 Hz, J'' = 2.7 Hz, 1H, C$^{16}H^{\beta}$), 2.62 (d, J = 16.3 Hz, 1H, C$^{22}H^{\alpha}$), 2.85 (dd, J = 12.0 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 2.85 (d, J = 16.3 Hz, 1H, C$^{22}H^{\beta}$), 3.73 (s, 3H, OCH_3); 4.47 (dd, J = 11.0 Hz, J' = 5.3 Hz, 1H, C$^{3}H^{\alpha}$); EIMS *m*/*z* 504 (M^+, 2), 461 (1), 444 (20), 429 (8), 401 (10), 385 (1), 335 (3), 309 (4), 264 (7), 254 (6), 241 (59), 228 (8), 223 (30), 216 (8), 204 (15), 197 (10), 189 (100). Anal. ($C_{29}H_{44}O_7$) C, H.

Dimethyl 3*β*-Acetoxy-18-oxo-19,20,29,30-tetranorlupane-21,28-dioate (7e). This compound was obtained by reaction of acid **7d** with diazomethane (mp 127−129 °C, MeOH): $[\alpha]_D$ +74° (*c* 0.40); ^{1}H NMR *δ* 0.83 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.07 (s, 3H, CH_3), 2.04 (s, 3H, OCOCH_3), 2.43 (ddd, J = 13.9 Hz, J' = 4.2 Hz, J'' = 2.7 Hz, 1H, C$^{16}H^{\beta}$), 2.60 (d, J = 16.0 Hz, 1H, C$^{22}H^{\alpha}$), 2.81 (d, J = 16.0 Hz, 1H, C$^{22}H^{\beta}$), 2.83 (dd, J = 12.0 Hz, J' = 3.6 Hz, 1H, C$^{13}H^{\beta}$), 3.66 (s, 3H, OCH_3), 3.75 (s, 3H, OCH_3), 4.47 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$); EIMS *m*/*z* 518 (M^+, 26), 458 (100), 443 (52), 427 (39), 415 (66), 353 (27), 255 (61), 223 (27), 189 (35). Anal. ($C_{30}H_{46}O_7$) C, H.

21-Methyl-28-(pivaloyloxy)methyl 3*β*-Acetoxy-18-oxo-19,20,29,30-tetranorlupane-21,28-dioate (7f). The triketo-Pom ester **5c** (200 mg, 0.30 mmol) was oxidized with AcOOH (10 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (30 mL) in the same manner as in the preparation of keto acid **7a**. The resulting colorless solid (157 mg) contained a polar compound as the major component and a less polar impurity according to TLC (PhMe/Et_2O 6:1). Crude methyl ester **7f** was obtained by treatment with diazomethane. Preparative HPLC (15% EtOAc in hexane) and crystallization from MeOH afforded pure **7f** (83 mg, 44%, mp 153−156 °C): $[\alpha]_D$ +63° (*c* 0.30); ^{1}H MNR *δ* 0.82 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.89 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 1.21 (s, 9H, (C(CH_3)$_3$), 2.04 (s, 3H, OCOCH_3), 2.42 (ddd, J = 13.9 Hz, J' = 4.2 Hz, J'' = 2.7 Hz, 1H, C$^{16}H^{\beta}$), 2.61 (d, J = 16.2 Hz, 1H, C$^{22}H^{\alpha}$), 2.82 (d, J = 16.2 Hz, 1H, C$^{22}H^{\beta}$), 2.90 (dd, J = 12.1 Hz, J' = 3.7 Hz, 1H, C$^{13}H^{\beta}$), 3.66 (s, 3H, OCH_3), 4.47 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$), 5.69 (d, J = 5.5 Hz, 1H, OCH_2O), 5.87 (d, J = 5.5 Hz, 1H, OCH_2O); EIMS *m*/*z* 618 (M^+, 4), 558 (69), 543 (16), 515 (21), 486 (26), 427 (65), 383 (29), 355 (100), 337 (21), 269 (29), 223 (27), 189 (29). Anal. ($C_{35}H_{54}O_9$) C, H.

28-Acetoxymethyl-21-methyl 3*β*-Acetoxy-18-oxo-19,20,29,30-tetranorlupane-21,28-dioate (7 g). Triketo-Acm ester **5d** (200 mg, 0.32 mmol) was oxidized with AcOOH (10 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (30 mL) in the same manner as in the preparation of the keto acid **7a**. The resulting colorless solid (143 mg) contained a polar compound as the major component and a less polar impurity according to TLC (PhMe/Et_2O 6:1). Crude methyl ester **7g** was obtained by treatment with diazomethane. Preparative HPLC (25% EtOAc in hexane) and crystallization from MeOH afforded pure **7g** (73 mg, 39%, mp 127−129 °C): $[\alpha]_D$ +66° (*c* 0.27); ^{1}H NMR *δ* 0.82 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.08 (s, 3H, CH_3), 2.04 (s, 3H,

OCOCH_3), 2.11 (s, 3H, OCOCH_3), 2.42 (ddd, J = 13.9 Hz, J' = 4.2 Hz, J'' = 2.7 Hz, 1H, C$^{16}H^{\beta}$), 2.61 (d, J = 16.2 Hz, 1H, C$^{22}H^{\alpha}$), 2.83 (d, J = 16.2 Hz, 1H, C$^{22}H^{\beta}$), 2.91 (dd, J = 12.1 Hz, J' = 3.5 Hz, 1H, C$^{13}H^{\beta}$), 3.66 (s, 3H, OCH_3), 4.47 (dd, J = 11.0 Hz, J' = 5.5 Hz, 1H, C$^{3}H^{\alpha}$), 5.76 (d, J = 5.6 Hz, 1H, OCH_2O), 5.83 (d, J = 5.6 Hz, 1H, OCH_2O); EIMS m/z 576 (M^+, 7), 552 (3), 528 (3), 516 (100), 501 (22), 473 (42), 427 (47), 383 (22), 353 (25), 313 (13), 223 (20), 189 (28). Anal. ($C_{32}H_{48}O_9$) C, H.

3β,28-Diacetoxy-18-oxo-19,20,21,29,30-pentanorlupan-22-oic Acid (8a). From Tetraketone 6. A solution of **6** (102 mg, 0.17 mmol) and AcOOH (6 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (15 mL) was stirred at room temperature for 17 h when TLC (PhMe/Et_2O 6:1) indicated complete reaction. The colorless reaction mixture was diluted with cold H_2O and was extracted with chloroform (3 × 20 mL). The combined extracts were washed successively with 5% aqueous KI, saturated aqueous Na_2SO_3, and brine, dried, and evaporated. The resulting colorless solid (96 mg) contained a polar compound as the major component according to TLC (PhMe/Et_2O 6:1). Preparative TLC ($CHCl_3$/EtOAc/AcOH 100:10:1) and crystallization from CH_2Cl_2/Et_2O afforded pure **8a** as small white needles (36 mg, 41%, mp 137−140 °C dec): $[\alpha]_D$ +40° (*c* 0.37); ^{1}H NMR δ 0.85 (s, 3 H, CH_3), 0.86 (s, 3 H, CH_3), 0.91 (s, 6 H, CH_3), 1.14 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.06 (s, 3H, OCOCH_3), 2.79 (dd, J = 11.7 Hz, J' = 3.6 Hz, 1H, C$^{13}H^{\beta}$), 4.46 (d, $J \approx$ 1 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (dd, $J \approx$ 11 Hz, $J' \approx$ 6 Hz, 1H, C$^{3}H^{\alpha}$), 4.64 (d, $J \approx$ 11 Hz, 1H, C$^{28}H^{\beta}$); EIMS m/z 444 (M^+ − 60, 1), 416 (3), 414 (3), 400 (22), 382 (2), 354 (6), 340 (15), 325 (10), 297 (9), 291 (7), 281 (7), 278 (9), 276 (31), 271 (7), 264 (27), 257 (7), 231 (7), 216 (33), 204 (40), 189 (100). Anal. ($C_{29}H_{44}O_7$) C, H.

From Anhydride 9a. A suspension of **9a** (500 mg, 0.88 mmol) in EtOAc (20 mL) and MeCN (5 mL) was added to a mixture of Ru(IV)O_2·H_2O (40 mg, 0.30 mmol), $NaIO_4$ (7.5 g, 35.1 mmol), H_2O (18 mL) and CF_3COOH (0.5 mL). The biphasic mixture was stirred vigorously at room temperature for 6 d, after which time TLC (PhMe/EtOAc 20:1) indicated complete reation. EtOH was added, the mixture was filtered, and the organic layer was dried and evaporated under reduced pressure. The white residue (643 mg) was crystallized from CH_2Cl_2/Et_2O to afford **8a** as small white needles (237 mg, 54%, mp 138−140 °C): $[\alpha]_D$ +40° (*c* 0.37). This material was identical to the sample obtained from the procedure described above.

Methyl 3β,28-Diacetoxy-18-oxo-19,20,21,29,30-pentanorlupan-22-oate (8b). A solution of diene diacetate **10** (500 mg, 0.95 mmol) in EtOAc (15 mL) and MeCN (5 mL) was added to a mixture of Ru(IV)O_2·H_2O (20 mg, 0.15 mmol), $NaIO_4$ (3.5 g, 16.4 mmol), H_2O (15 mL), and CF_3COOH (0.5 mL). The biphasic mixture was stirred vigorously at room temperature for 60 h, after which time TLC (PhMe/Et_2O 10:1) indicated complete reaction. EtOH ethanol was added, the mixture was filtered, and the organic layer was separated and filtered through a short column of silica gel. The column was eluted with EtOAc and the eluate was evaporated under reduced pressure. The resulting red-brown solid (437 mg) contained, according to TLC (PhMe/Et_2O 6:1), a polar compound as the major component and a less polar impurity. The crude β-ketomethyl ester **8b** was obtained by treatment with diazomethane. Preparative TLC (PhMe/Et_2O 10:1) and crystallization from $CHCl_3$/MeOH afforded pure **8b** (232 mg, 47%, mp 255−258 °C dec): $[\alpha]_D$ +68° (*c* 0.26); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.94 (s, 3H, CH_3), 1.13 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.06 (s, 3H, OCOCH_3), 2.45 (m, ΣJ = 34 Hz, 1H, C$^{16}H^{\alpha}$), 2.61 (dd, J = 11.7 Hz, J' = 3.6 Hz, 1H, C$^{13}H^{\beta}$), 3.77 (s, 3H, OCH_3), 4.44 (d, J = 11.2 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (dd, $J \approx$ 11 Hz, $J' \approx$ 6 Hz, 1H, C$^{3}H^{\alpha}$), 4.56 (d, J = 11.2 Hz, 1H, C$^{28}H^{\beta}$); EIMS m/z 487 (M^+ − 31, 3), 458 (45), 443 (15), 440 (3), 430 (9), 415 (21), 398 (6), 389 (5), 376 (6), 370 (7), 349 (4), 323 (4), 255 (95), 241 (23), 223 (23), 204 (18), 199 (18), 189 (100). Anal. ($C_{30}H_{46}O_7$) C, H.

Acetoxymethyl 3β,28-Diacetoxy-18-oxo-19,20,21,29,30-pentanorlupan-22-oate (8d). Well-powdered Ag_2CO_3 (83 mg, 0.30 mmol) was added to the solution of β-keto acid **8a** (100 mg, 0.20 mmol) and bromomethyl acetate (34 mg, 0.22 mmol) in dry DMF (5 mL) and this heterogeneous mixture was stirred at room temperature for 2 d, when TLC (PhMe/Et_2O 6:1) indicated complete reaction. The reaction mixture was filtered, poured into cold H_2O (50 mL), and extracted with $CHCl_3$. The combined extracts were washed twice with brine, dried, and evaporated. The resulting gray oil (131 mg) was purified by preparative TLC (PhMe/Et_2O 5:1) and the product was crystallized from butanone to afford pure **8d** as white needles (45 mg, 39%, mp 234−236 °C): $[\alpha]_D$ +69° (*c* 0.46); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.93 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.05 (s, 3H, OCOCH_3), 2.13 (s, 3H, OCOCH_3), 2.44 (m, 1H, C$^{16}H^{\alpha}$), 2.60 (dd, J = 11.8 Hz, J' = 3.6 Hz, 1H, C$^{13}H^{\beta}$), 4.43 (d, J = 11.2 Hz, 1H, C$^{28}H^{\alpha}$), 4.48 (dd, J = 11.1 Hz, J' = 5.2 Hz, 1H, C$^{3}H^{\alpha}$), 4.56 (d, J = 11.2 Hz, C$^{28}H^{\beta}$), 5.79 (d, J = 5.5 Hz, 1H, OCH_2O), 5.80 (d, J = 5.5 Hz, 1H, OCH_2O); EIMS m/z 576 (M^+, 2), 534 (2), 516 (100), 501 (25), 473 (32), 444 (16), 383 (14), 338 (13), 313 (30), 223 (27), 209 (13), 189 (52). Anal. ($C_{32}H_{48}O_9$) C, H.

Anhydride of 3β,28-Diacetoxy-21,22-secolup-18-ene-21,22-dioic Acid (9a). A solution of diketone diacetate **4a** (1 g, 1.81 mmol) and AcOOH (20 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (120 mL) was stirred at room temperature for 7 d when TLC (PhMe/Et_2O 6:1) indicated complete reaction. The colorless reaction mixture was diluted with cold H_2O and extracted with $CHCl_3$. The combined extracts were washed successively with 5% aqueous KI, saturated aqueous Na_2SO_3, and brine and then dried and evaporated. The resulting pale-yellow oil (1.2 g) was crystallized from $CHCl_3$/MeOH to afford **9a** as a white crystalline powder (875 mg, 85%, mp 306−309 °C): $[\alpha]_D$ +88° (*c* 0.45); ^{1}H NMR δ 0.86 (s, 6H, CH_3), 0.90 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 1.15 (d, J = 6.7 Hz, 3H, CH_3), 1.31 (d, J = 7.0 Hz, 3H, CH_3), 2.02 (s, 3H, OCOCH_3), 2.05 (s, 3H, OCOCH_3), 2.53 (dt, J = 14.4 Hz, J' = J'' = 3.5 Hz, 1H, C$^{16}H^{\beta}$), 2.72 (dd, J = 12.7 Hz, J' = 2.9 Hz, 1H, C$^{13}H^{\beta}$), 3.26 (sept., J = 6.9 Hz, 1H, C^{20}H), 3.89 (d, J = 11.0 Hz, 1H, C$^{28}H^{\alpha}$), 4.47 (dd, J = 11.3 Hz, J' = 5.0 Hz, 1H, C$^{3}H^{\alpha}$), 4.54 (d, J = 11.0 Hz, 1H, C$^{28}H^{\beta}$). Ref.[18] mp 308−310 °C, $[\alpha]_D$ +87° (*c* 0.60).

Anhydride of 3β-Acetoxy-17β-methoxycarbonyl-28-nor-21,22-secolup-18-ene-21,22-dioic Acid (9b). Diketomethyl ester **4b** (500 mg, 0.93 mmol) was oxidized by treatment with AcOOH (10 mL of a 32% solution in glacial acetic acid) in $CHCl_3$ (55 mL) in the same manner as in the preparation of the anhydride **9a**. Crystallization from $CHCl_3$/MeOH afforded **9b** as small white crystals (355 mg, 69%, mp 231−237 °C dec): $[\alpha]_D$ +116° (*c* 0.49); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 1.12 (s, 3H, CH_3), 1.16 (d, J = 6.7 Hz, 3H, CH_3), 1.34 (d, J = 7.0 Hz, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.38 (ddd, J = 14.5 Hz, J' = 12.1 Hz, J'' = 3.7 Hz, 1H, C$^{16}H^{\alpha}$), 2.49 (ddd, J = 14.5 Hz, J' = 5.3 Hz, J'' = 4.0 Hz, 1H, C$^{16}H^{\beta}$), 2.85 (dd, J = 12.6 Hz, J' = 3.0, 1H, C$^{13}H^{\beta}$), 3.32 (sept., J = 6.9 Hz, 1H, C^{20}H), 3.76 (s, 3H, OCH_3), 4.47 (dd, J = 11.1 Hz, J' = 5.2 Hz, 1H, C$^{3}H^{\alpha}$); EIMS m/z 556 (M^+,7), 541 (4), 510 (1), 496 (7), 484 (5), 481 (3), 469 (7), 453 (9), 437 (5), 409 (2), 315 (1), 293 (10), 279 (8), 249 (8), 236 (10), 221 (9), 203 (23), 189 (100). Anal. ($C_{33}H_{48}O_7$) C, H.

3β-Hydroxy-19,20,21,22,28,29,30-heptanorlupan-18-one (11a). β-Keto acid **8a** (150 mg, 0.30 mmol) was added to a solution of KOH (160 mg, 2.86 mmol) in dioxane (5.3 mL) and H_2O (3.7 mL) and the mixture was refluxed for 3 h under Ar. All starting material had dissolved during this time. After cooling, the reaction mixture was poured into H_2O (50 mL); the resulting solution was acidified with 10% aqueous HCl (2 mL) and was extracted twice with Et_2O. The combined extracts were washed with H_2O, dried, and evaporated. The resulting white crystalline solid (96 mg) contained a polar compound by TLC (PhMe/Et_2O 1:1). This was purified by preparative TLC (PhMe/Et_2O 1:1) and crystallization from $CHCl_3$/MeOH to afford pure **11a** as a white crystalline powder (67 mg, 61%, mp 191−193 °C sublim): $[\alpha]_D$ +10° (*c* 0.24); ^{1}H NMR δ 0.77 (s, 3H, CH_3), 0.83 (s, 3H, CH_3), 0.87 (d, J = 0.9 Hz, 3H, CH_3),

0.97 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 2.16−2.34 (m, 2H, C$^{17}H_2$), 2.49 (dd, $J \approx 12$ Hz, $J' \approx 4$ Hz, 1H, C$^{13}H^{\beta}$), 3.20 (dd, $J = 10.8$ Hz, $J' = 5.4$ Hz, 1H, C$^{3}H^{\alpha}$); EIMS *m/z* 346 (M^+, 17), 328 (15), 313 (5), 310 (2), 295 (1), 285 (2), 234 (14), 222 (8), 216 (7), 207 (78), 204 (17), 189 (43), 135 (37), 121 (33), 111 (100). Anal. ($C_{23}H_{38}O_2$) C, H, N.

18-Oxo-19,20,21,22,28,29,30-heptanorlupan-3*β*-yl Acetate (11b). This was obtained by acetylation of **11a** with Ac_2O in pyridine and the usual workup procedure (mp 215−218 °C, $CHCl_3$/MeOH): $[\alpha]_D$ +11° (*c* 0.48); ^{1}H NMR δ 0.83 (s, 3H, CH_3), 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 1.11 (s, 3H, CH_3), 2.04 (s, 3H, OCOCH_3), 2.16−2.35 (m, 2H, C$^{17}H_2$), 2.48 (dd, $J \approx 12$ Hz, $J' \approx 3$ Hz, 1H, C$^{13}H^{\beta}$), 4.48 (dd, $J \approx 11$ Hz, $J' \approx 6$ Hz, 1H, C$^{3}H^{\alpha}$); EIMS *m/z* 388 (M^+, 22), 373 (2), 370 (12), 328 (49), 313 (17), 310 (5), 295 (5), 285 (24), 276 (9), 264 (24), 259 (12), 246 (6), 216 (20), 204 (27), 189 (100), 111 (68). Anal. ($C_{25}H_{40}O_3$) C, H, N. Ref.[15] mp 200−203 °C, $[\alpha]_D$ +22°.

18-Oxo-19,20,21,22,29,30-hexanorlup-17(28)-en-3*β*-yl Acetate (12). A solution of *β*-keto acid **8a** (1 g 1.98 mmol) in diglyme (10 mL) was refluxed for 10 min, after which time TLC (PhMe/Et_2O 6:1) indicated complete reaction. After cooling, the reaction mixture was poured into excess H_2O and this was extracted with $CHCl_3$. The combined extracts were washed with H_2O, dried, and evaporated. According to TLC (PhMe/Et_2O 6:1) the resulting pale-yellow oil (1.5 g) was a mixture of three major components (heptanor ketone **11b**, methylene ketone **12,** and an unidentified compound) in the ratio of approximately 1:1:1. This mixture was separated by preparative HPLC (10% EtOAc in hexane).

Pure 12 (72 mg, 9%, mp 268−269 °C, lyophilisate from benzene): $[\alpha]_D$ +32° (*c* 0.37); ^{1}H NMR δ 0.85 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.90 (s, 3H, CH_3), 0.91 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.46 (dd, $J = 12$ Hz, $J' = 4$ Hz, 1H, C$^{13}H^{\beta}$), 2.49−2.65 (br. m, 2H, C$^{16}H_2$), 4.49 (m, $\Sigma J = 16.5$ Hz, 1H, C$^{3}H^{\alpha}$), 5.06 (dt, $J = 1.2$ Hz, $J' = J'' = 2.1$ Hz, 1H, C$^{28}H^{\alpha}$), 5.72 (dt, $J = 0.8$ Hz, $J' = J'' = 2.0$ Hz, 1H, C$^{28}H^{\beta}$); EIMS *m/z* 400 (M^+, 48), 337 (100), 320 (38), 297 (24), 283 (25), 276 (97), 264 (63), 249 (24), 189 (19). Anal. ($C_{26}H_{40}O_3$) C, H.

Heptanor ketone acetate 11b (131 mg, 17%) was identical with an authentic sample.

17α-Ethoxymethyl-18-oxo-19,20,21,22,28,29,30-heptanorlupan-3*β*-yl Acetate (13b). A suspension of *β*-keto acid **8a** (120 mg, 0.24 mmol) in a solution of KOH (120 mg, 2.14 mmol) in EtOH (6.3 mL) was refluxed for 3 h. All starting material had dissolved during this time. After cooling, the reaction mixture was poured into H_2O (50 mL) and the resulting solution was acidified with 10% aqueous HCl (2 mL). This was extracted twice with Et_2O. The combined extracts were washed with H_2O, dried, and evaporated. According to ^{1}H NMR analysis, the resulting pale-yellow oil (78 mg) was a mixture of heptanor ketone **11a** and ethoxyheptanor ketone **13a** in a ratio of approximately 1:1. The residue was acetylated with Ac_2O (1.5 mL) in pyridine (1.5 mL). Usual workup gave a white foamy residue, which was fractionated by preparative HPLC (10% EtOAc in hexane).

Heptanor ketone acetate 11b (14 mg, 15%, mp 216−219 °C, $CHCl_3$/MeOH): $[\alpha]_D$ +11° (*c* 0.48), was identical to an authentic sample.

Ethoxyheptanor ketone acetate 13b (18 mg, 17%. mp 139−144 °C, $CHCl_3$/MeOH): $[\alpha]_D$ +23° (*c* 0.32). ^{1}H NMR δ 0.80 (s, 3H, CH_3), 0.85 (s, 6H, CH_3), 0.90 (s, 3H, CH_3), 1.10 (s, 3H, CH_3), 1.18 (t, $J = 7.0$ Hz, 3H, OCH$_2$CH_3), 2.05 (s, 3H, OCOCH_3), 2.43−2.55 (m, 2H, C^{13}H & C^{17}H), 3.28 (dd, $J = 9.6$ Hz, $J' = 7.5$ Hz, 1H, C$^{28}H^{\alpha}$), 3.40−3.58 (m, 2H, OCH_2CH$_3$), 3.77 (dd, $J = 9.6$ Hz, $J' = 5.1$ Hz, 1H, C$^{28}H^{\beta}$), 4.48 (m, $\Sigma J = 16.5$ Hz, 1H, C$^{3}H^{\alpha}$); EIMS *m/z* 446 (M^+, 49), 431 (1), 428 (1), 417 (3), 402 (7), 387 (4), 386 (4), 371 (7), 343 (7), 276 (6), 264 (5), 216 (7), 204 (8), 189 (47), 183 (77), 169 (68), 114 (100). Anal. ($C_{28}H_{46}O_4$) C, H.

3*β*-Acetoxy-19,20,21,22,28,29,30-heptanor-17,18-secolupan-17,18-dioic Acid (15a). From Hydroxy Ketone 3b. A solution of **3b** (500 mg, 1.00 mmol) in EtOAc (10 mL), MeOAc (10 mL), and MeCN (5 mL) was added to a mixture of Ru(IV)-O_2·H_2O (20 mg, 0.15 mmol), $NaIO_4$ (3.5 g, 16.4 mmol), H_2O (15 mL), and CF_3COOH (0.5 mL). The reaction mixture was stirred vigorously at room temperature for 3 d, after which time TLC ($CHCl_3$/EtOAc/AcOH 100:10:1) indicated complete reaction. EtOH ethanol was added, the mixture was filtered, and the organic layer was separated and filtered through a short column of silica gel. The column eluted with EtOAc and the eluate was evaporated under reduced pressure. The resulting brown solid (379 mg) contained, according to TLC ($CHCl_3$/EtOAc/AcOH 100:10:1), a polar compound as the major component and a less polar impurity. Preparative TLC ($CHCl_3$/EtOAc/AcOH 100:10:1) and crystallization from Et_2O/hexane afforded **15a** (122 mg, 27%, mp 247−248 °C dec): $[\alpha]_D$ −4° (*c* 0.8, dioxane); ^{1}H NMR (in the mixture of $CDCl_3$ and CD_3OD) δ 0.84 (s, 3H, CH_3), 0.86 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 1.00 (s, 3H, CH_3), 1.09 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.29 (br. m, 2H, C$^{16}H_2$), 2.64 (dd, $J = 11.9$ Hz, $J' = 4.7$ Hz, 1H, C$^{13}H^{\beta}$), 4.48 (dd, $J = 11.2$ Hz, $J' = 5.1$ Hz, 1H, C$^{3}H^{\alpha}$). Ref.[25] mp 246−248 °C, hexane/Me_2CO); $[\alpha]_D$ −4.7° (*c* 0.42, $CHCl_3$).

From Acid 3c. A solution of **3c** (200 mg, 0.39 mmol) in EtOAc (5 mL), MeOAc (15 mL), and MeCN (5 mL) was added to a mixture of Ru(IV)O_2·H_2O (15 mg, 0.11 mmol), $NaIO_4$ (1.8 g, 8.4 mmol), H_2O (15 mL), and CF_3COOH (0.5 mL). The biphasic reaction mixture was stirred vigorously at room temperature for 60 h. Then the reaction mixture was worked up as in the previous example. Diacid **15a** (58 mg 33%, mp 245−247 °C dec): $[\alpha]_D$ −6° (*c* 0.42, dioxane), was identical to an authentic sample.

From Methylene Ketone 12. A solution of **12** (50 mg; 0.12 mmol) in EtOAc (2 mL) and MeCN (0.5 mL) was added to a mixture of Ru(IV)O_2·H_2O (1.5 mg, 0.01 mmol), $NaIO_4$ (150 mg, 0.7 mmol), H_2O (2 mL), and CF_3COOH (50 μL) was stirred vigorously at room temperature for 20 h. The reaction mixture was worked up as in the previous example. The diacid **15a** (22 mg, 39%, mp 244−246 °C dec): $[\alpha]_D$ −3° (*c* 0.3, dioxane), was identical to an authentic sample.

Dimethyl 3*β*-Acetoxy-19,20,21,22,28,29,30-heptanor-17,18-secolupan-17,18-dioate (15b). This compound was obtained by reaction of diacid **15a** with diazomethane (mp 143−145 °C, MeOH): $[\alpha]_D$ +10° (*c* 0.33); ^{1}H NMR δ 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.87 (d, $J = 0.8$ Hz, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.04 (s, 3H, CH_3), 2.05 (s, 3H, OCOCH_3), 2.62 (dd, $J = 12.7$ Hz, $J' = 4.0$ Hz, 1H, C$^{13}H^{\beta}$), 3.63 (s, 3H, OCH_3), 3.65 (s, 3H, OCH_3), 4.48 (dd, $J = 11.3$ Hz, $J' = 5.2$ Hz, 1H, C$^{3}H^{\alpha}$). Ref.[16] mp 140−144 °C, EtOH): $[\alpha]_D$ +12.2° (*c* 1, $CHCl_3$).

Bis[(pivaloyloxy)methyl] 3*β*-Acetoxy-19,20,21,22,28,29,30-heptanor-17,18-secolupane-17,18-dioate (15c). DBU (17 μL, 0.11 mmol) and Pom-Cl (16 μL, 0.11 mmol) were added to a solution of diacid **15a** (25 mg, 57 μmol) in CH_2Cl_2 (300 μL) and MeCN (120 μL). The mixture was stirred at room temperature for 3 h and was then diluted with ice-cold H_2O and extracted with $CHCl_3$. The combined extracts were washed with cold brine. After drying and evaporating, a viscous pale-brown oil (37 mg) was obtained. This was chromatographed on SiO_2 (PhMe/Et_2O 10:1). Pure **15c** (28 mg, 72%) was obtained as a colorless oil: $[\alpha]_D$ +19° (*c* 0.46); ^{1}H NMR δ 0.84 (s, 3H, CH_3), 0.85 (s, 3H, CH_3), 0.87 (s, 3H, CH_3), 0.99 (s, 3H, CH_3), 1.06 (s, 3H, CH_3), 1.21 (s, 18H, C(CH_3)$_3$), 2.05 (s, 3H, OCOCH_3), 2.12−2.30 (br m, 2H, C$^{16}H_2$), 2.65 (dd, $J = 12.0$ Hz, $J' = 4.6$ Hz, 1H, C$^{13}H^{\beta}$), 4.48 (dd, $J = 11.3$ Hz, $J' = 5.0$ Hz, 1H, C$^{3}H^{\alpha}$), 5.66 (d, $J = 5.5$ Hz, 1H, OCH_2O), 5.71 (d, $J = 5.5$ Hz, 1H, OCH_2O), 5.74 (d, $J = 5.5$ Hz, 1H, OCH_2O), 5.76 (d, $J = 5.5$ Hz, 1H, OCH_2O); EIMS *m/z* 678 (M^+, 0), 647 (6), 604 (2), 587 (63), 503 (13), 489 (16), 444 (18), 419 (11), 375 (19), 359 (13), 331 (7), 315 (21), 276 (21), 249 (9), 229 (16), 203 (8), 189 (100). Anal.($C_{37}H_{60}O_{10}$) C, H.

Crystallography. Single crystals of *β*-keto acid **8a** were grown from a $CHCl_3$/EtOAc/petroleum ether/MeOH solvent system. Crystal structure data: unit cell parameters, $a = 7.4459(9)$ Å, $\alpha = 90°$, $b = 11.0454(9)$ Å, $\beta = 94.002(11)°$, $c = 36.178(4)$ Å, $\gamma = 90°$; $C_{30.50}H_{50}O_{8.50}$ ($C_{29}H_{44}O_7$·1.5MeOH) = 552.70, two unit formulas per unit cell; calculated density 1.237 Mg m^3; space group P21; $\lambda = 1.54178$ Å, 10 322 independent reflections collected; the structure was solved by

direct methods and refined by full-matrix least-squares against F^2 (SHELXS-97); the program used for refinement was SHELXL-97; refinement with statistical weights [$w = 1/\sigma^2(F^2)$] gave a final Flack parameter of −0.05(9); the final R value was 3.63%. Full crystallographic data are included in the Supporting Information.

Cell Lines. All cells were purchased from the American Tissue Culture Collection (ATTC), unless otherwise indicated. Cladribine-, gemcitabine-, cytosinarabinoside-, and fludarabine-resistant sublines of K562 cells (K562-CdA, K562-GEM, K562-ARA-C, K562-FLUD) were kindly provided by Dr. J. Dummont (University of Lyon, Lyon, France). The drug-resistant sublines of CEM cells (CEM-DNR bulk, CEM-DNR 1/C2, CEM-VCR bulk, CEM-VCR 1/F3, CEM-VCR 3/D5) were selected in our laboratory by the cultivation of maternal cell lines in increasing concentrations of daunorubicine or vincristine, respectively.[27] The human T-lymphoblastic leukemia cell line, CEM, was used for routine screening of compounds. To prove a common mechanism of action, selected compounds that showed activity in the screening assay were tested in a panel of cell lines (Table 1). These lines were from different species and of various histogenetic origin, and they possess various alterations in their cell cycle-regulatory proteins and hormone receptor status (Table 1). The cells were maintained in Nunc/Corning 80 cm^2 plastic tissue culture flasks and cultured in cell culture medium (DMEM/RPMI 1640 with 5 g/L glucose, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10% fetal calf serum, and $NaHCO_3$).

Cytotoxic MTT Assay.[28] Cell suspensions were prepared and diluted according to the particular cell type and the expected target cell density (2500−30 000 cells/well based on cell growth characteristics). Cells were added by pipet (80 μL) into 96-well microtiter plates. Inoculates were allowed a preincubation period of 24 h at 37 °C and 5% CO_2 for stabilization. Four-fold dilutions, in 20-μL aliquots, of the intended test concentration were added at time zero to the microtiter plate wells. All test compound concentrations were examined in duplicate. Incubation of the cells with the test compounds lasted for 72 h at 37 °C, in a 5% CO_2 atmosphere at 100% humidity. At the end of the incubation period, the cells were assayed using MTT. Aliquots (10 μL) of the MTT stock solution were pipetted into each well and incubated for a further 1−4 h. After this incubation period formazan produced was dissolved by the addition of 100 μL/well of 10% aqueous SDS (pH = 5.5), followed by a further incubation at 37 °C overnight. The optical density (OD) was measured at 540 nm with a Labsystem iEMS Reader MF. Tumor cell survival (TCS) was calculated using the following equation: $TCS = (OD_{drug-exposed}/\text{mean } OD_{control}) \times 100\%$. The TCS_{50} value, the drug concentration lethal to 50% of the tumor cells, was calculated from appropriate dose−response curves.

Apoptosis and Cell Cycle Analysis by FACS.[29] CEM cells (1×10^6/mL) were cultured in 6-well plates, with or without 10 μM concentration of BA (**2b**), betulinines, **16**, **4b**, **8a**, and paclitaxel (1μM) as a reference drug, at 37 °C and 5% CO_2 for 3−24 h. Following the incubation, cells were pelleted, washed in Hank's buffered salt solution (HBSS), and fixed in 96% ethanol overnight at −20 °C. Low molecular weight apoptotic DNA was extracted in citrate buffer and RNA was cleaved by RNAse (50μg/mL). The DNA was stained by ethidium bromide, and the cells were analyzed by flow-cytometry using a 488 nm single beam laser (Becton Dickinson, San Jose, CA).

Morphological Analysis of Apoptosis by Scanning Electron Microscopy. CEM cells (1×10^6/mL) were cultured in 6-well plates, with or without 10 μM concentration of BA (**2b**), betulinines, **16**, **4b**, and **8a**, at 37 °C and 5% CO_2 for 3−24 h. Following incubation, cells were pelleted, washed in Hank's buffered salt solution, and fixed in 2% glutaraldehyde/HBSS at 4 °C overnight. Cellular suspensions were cytocentrifuged on microscopic slides, dried, and covered with gold under vacuum. The surfaces of (un)treated cells were examined by scanning electron microscope (Tesla BS340) for typical morphological markers of apoptosis: cytoplasmic membrane blabbing, cellular fragmentation, and formation of apoptotic bodies.

Statistics. The cytotoxic activities of BA (**2b**) and the most effective compounds, **16**, **4b**, and **6**, listed in Table 1 were mutually compared in order to investigate for a common mechanism of action. The nonparametric Spearman correlation analysis was performed using software Prisma, version 4.0. The correlation diagrams, correlation coefficients, and statistical significances are shown in Figure 3.

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Supporting Information Available: Full crystallographic information of β-keto acid **8a** and ^{13}C NMR data and expanded cytotoxic activity of all unpublished compounds. This material is available free of charge via Internet at http://pubs.acs.org.

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