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Original article

Preparation of betulinic acid derivatives by chemical and biotransformation methods and determination of cytotoxicity against selected cancer cell lines

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ABSTRACT

Several novel 2,4-dinitrophenylhydrazone betulinic acid derivatives have been prepared by chemical and biotransformation methods using fungi and carrot cells. Some compounds showed significant cytotoxicity and selectivity against some tumor cell lines. The most active, 3-[(2,4-dinitrophenyl)hydrazono]lup-(20*R*)-29-oxolupan-28-oic acid, showed IC_{50} values between 1.76 and 2.51 μM against five human cancer cell lines. The most selective, 3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]-29-norlupan-28-oic acid, was five to seven times more selective for cancer cells when compared to fibroblasts. Cell cycle analysis and apoptosis induction were studied for the most active derivatives.

1. Introduction

Betulinic acid (**1**) is a bioactive pentacyclic triterpene. It is one of the most common triterpenes found in plants together with ursolic and oleanolic acids [1]. It has been isolated from many plants such as birch tree (*Betula* spp.), *Ziziphus* spp., *Syzygium* spp., *Diospyros* spp. and *Paeonia* spp. [2], sometimes in concentrations as high as 2–3% [3,4]. Important pharmacological properties have been described for betulinic acid including antitumor, anti-HIV [5], anti-inflammatory, antibacterial, antimalarial, antitrypanosomal and analgesic [6]. Other triterpenes, e.g. boswellic acid and glycyrrhetinic acids, have also shown antitumor activity [7,8].

The activity of betulinic acid against a large spectrum of tumor cell lines has attracted considerable attention. It is active in micromolar concentrations, with high selectivity [9–13]. It is highly selective against the melanoma cells and malignant tumors, inducing apoptosis. The mechanism of action involves mitochondrial membrane permeabilization with release of factors like cytochrome *c*, Smac or AIF in a permeability transition pore-dependent manner, activating caspases and nuclear fragmentation [14,15].

The antitumor properties of betulinic acid motivated studies of structure–activity relationship. Derivatives have been prepared by chemical methods and many exhibited increased cytotoxicity [16]. Biotransformations using microorganisms or plant cells have also been used for that purpose [17,18].

In this report we describe the preparation, characterization and *in vitro* anti-cancer activity of new derivatives of **1** and also the known compounds **2** [19], **8** [20], **11** and **13** [21] for comparison. They were obtained by chemical and biotransformation methods using fungi and carrot cells. The structure–activity relationships are proposed and could contribute to the understanding of the cytotoxic profile of this class of compounds.

2. Results and discussion

2.1. Chemistry

The known betulonic acid (**2**) (Scheme 1), and compounds **8** and **11**, **13** (Schemes 3 and 4) were prepared according to literature

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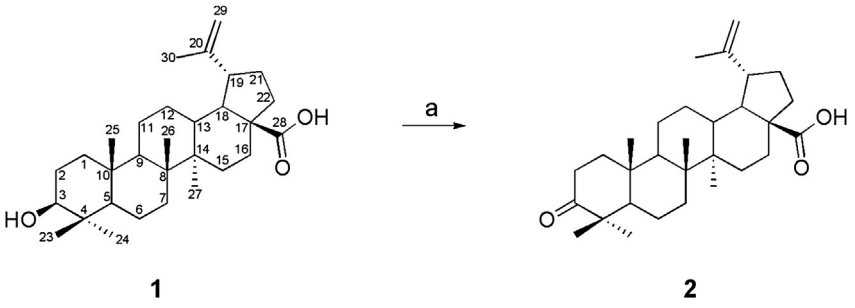


Scheme 1. a) CrO_3, H_2SO_4, 1.5 h, rt.

methods and their spectroscopic data were comparable with those previously reported [19–21].

Compounds containing the 2,4-dinitrophenylhydrazone (2,4-DNPH) moiety, e.g. 4,4′-dihydroxybenzophenone-2,4-dinitrophe nylhydrazone, have been shown to be active against cutaneous cancer metastasis [22,23]. Thus, we decided to prepare some 2,4-DNPH derivatives of betulinic acid.

The ^{13}C NMR spectra of compounds **3**–**10** and **12** (Schemes 2–4), containing a 2,4-DNPH group, showed the characteristic signals of C3 (~167.0 ppm) and C20 (161–162.5 ppm). The ^{1}H NMR spectra also showed the signal of –NH group at 11.00 ppm.

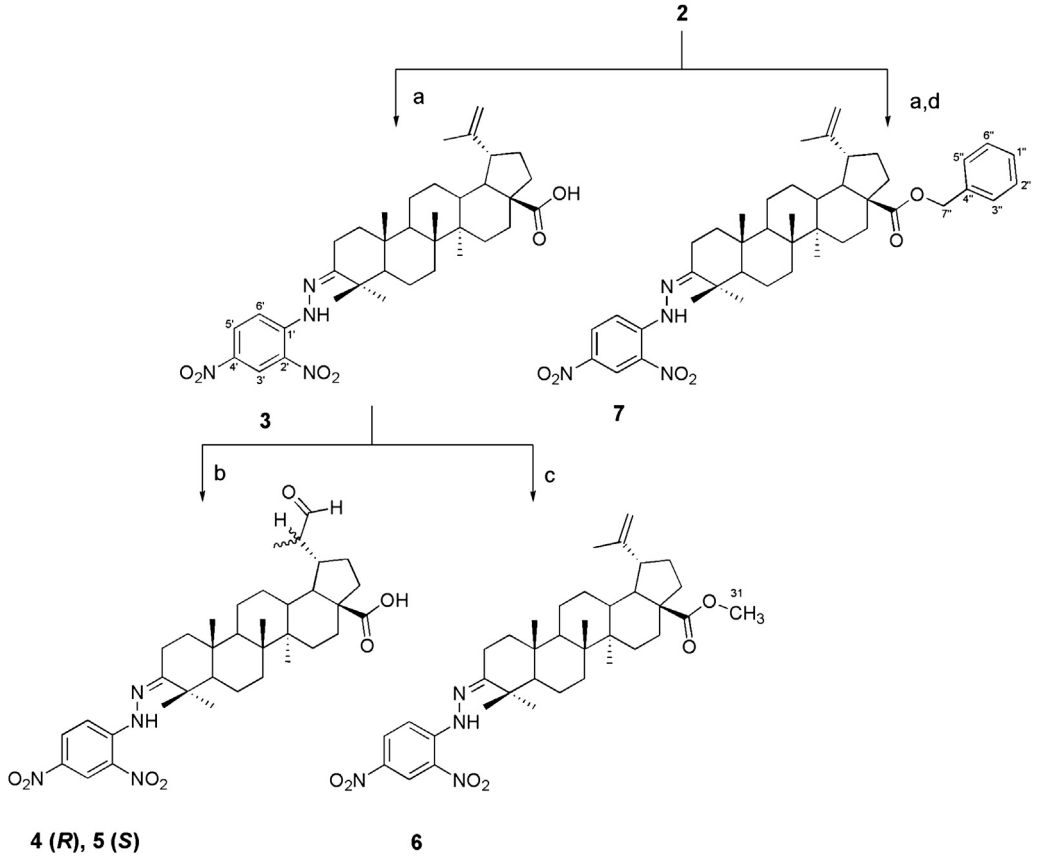
An attempt was made to prepare the epoxide of **3** which could not be isolated because it quickly rearranged into **4** and **5**. Their ^{1}H and ^{13}C NMR spectra showed the characteristic signals of aldehyde groups 9.87, 207.2 and 9.66, 204.8 ppm, respectively. This 1,2 hydrogen-shift rearrangement of terminal epoxides has been explored for the preparation of chiral aldehydes [24].

The biotransformation experiments produced compounds **2**, **14** and **15** (Scheme 5). They have been also obtained by biotransformations using *Bacillus megaterium* [25] and *Nocardia* sp. [26,27].

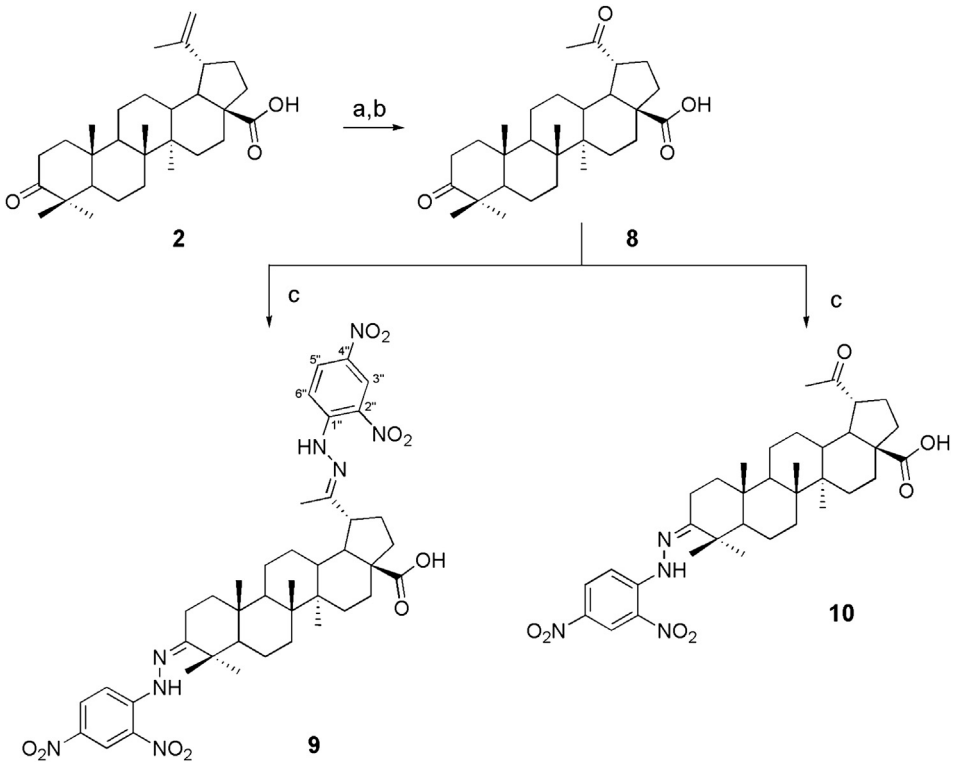
2.2. Cytotoxicity

The *in vitro* cytotoxic activity was evaluated against 518A2 (melanoma), 8505c (anaplastic thyroid tumor), A2780 (ovarian cancer), A549 (lung cancer) and MCF-7 (breast cancer) cell lines, by SRB colorimetric assay. The compounds were initially dissolved in DMSO and then with RPMI-1640 medium. The DMSO concentration was kept below 0.5% which was non-toxic to the cells. The compounds showed antitumor activity in a dose–response manner and the IC_{50} values are listed on Table 1.

Our results show that most derivatives have superior cytotoxic activity compared to the parent compound, **1** (IC_{50} = 8.75–14.8 μM).



Scheme 2. a) 2,4-DNPH, H_2SO_4, 15 h, rt; b) *m*-CPBA, 5 h, 0 °C; c) diazomethane, rt; d) benzyl chloride, K_2CO_3, 2 h, reflux.

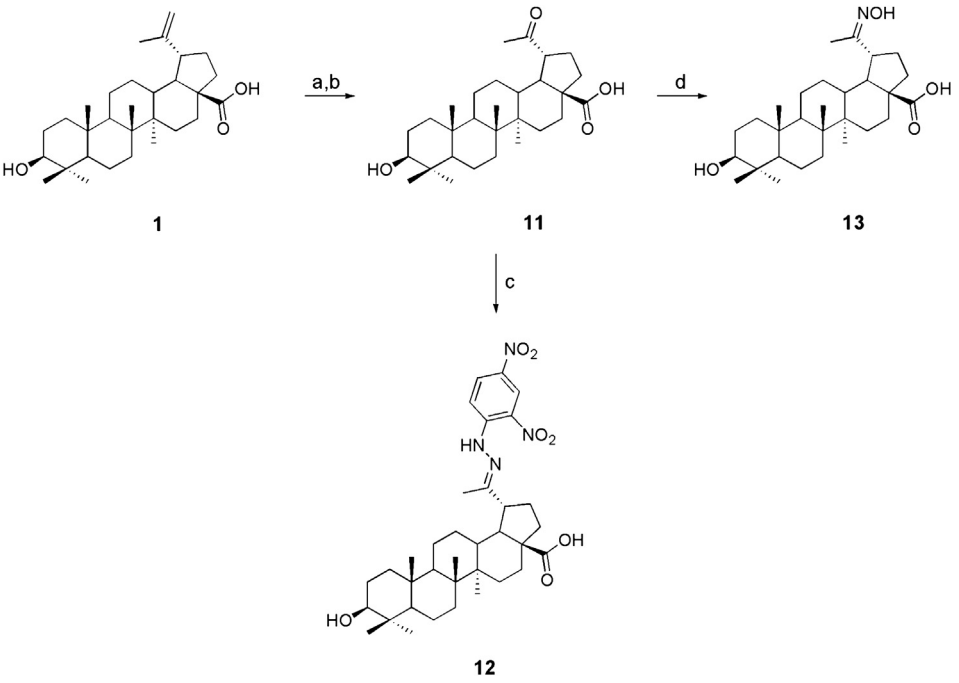


Scheme 3. a) OsO_4, dioxane:H_2O, 1 h, rt; b) $NaIO_4$, 3 days, rt; c) 2,4-DNPH, H_2SO_4, 15 h, rt.

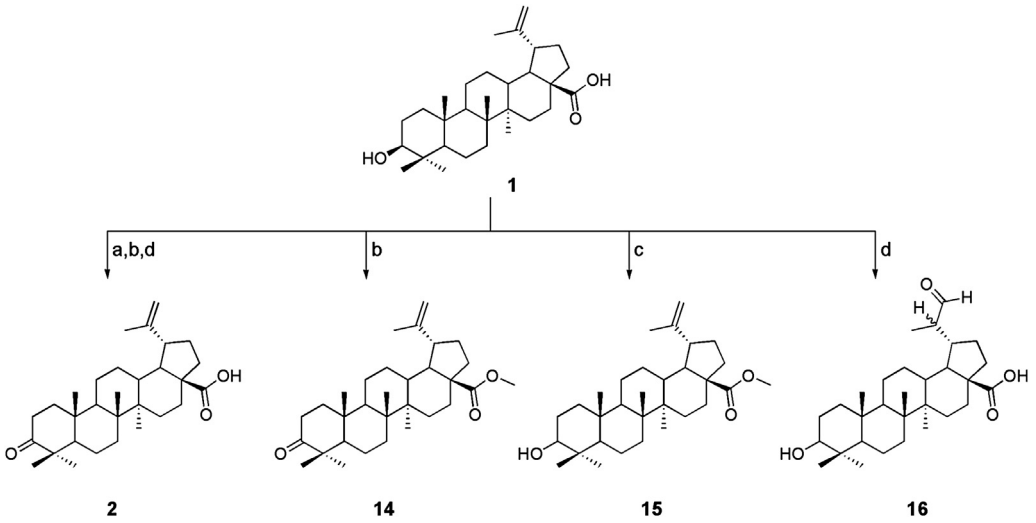
Those with $IC_{50} < 20$ μM are considered highly active, values between 20 and 75 μM are moderately active, and those between 75 and 165 μM are slightly active or inactive [28].

The highest activity was observed for isomers **4** and **5**, which were five to eight times more active than **1** against all tested cell lines (IC_{50} 1.76–2.51 μM). The stereochemistry at C20 does not have significant influence on activity since both isomers have comparable cytotoxicities.

Compound **2** (IC_{50} = 5.97–14.0 μM) was slightly more active than **1** against most cell lines. Compounds **10** and **12** showed higher cytotoxicity against MCF-7 cell line (IC_{50} = 7.52 and 6.84 μM; respectively), while compound **12** was also more active than **1**



Scheme 4. a) OsO_4, dioxane:H_2O, 1 h, rt; b) $NaIO_4$, 3 days, rt; c) 2,4-DNPH, H_2SO_4, 15 h, rt; d) hydroxylammonium chloride, sodium acetate, overnight, rt.



Scheme 5. a) *Mycelia sterilia*; b) *Penicillium* sp.; c) *Penicillium citreonigrum*; d) *Daucus carota* cells suspension.

against 8505c, 518A2 and A549 cell lines (IC_{50} = 8.72, 9.09 and 7.62 μM; respectively). Compound **15**, obtained by biotransformation, was active (IC_{50} = 16.3–17.2 μM), but less toxic than **1**. A drastic decrease in the cytotoxicity of compounds **7**–**9**, **11**, **13**, **14** and **16** was observed for most cell lines, with IC_{50} values between 36.2 and >100 μM. An exception was observed for compound **8** against A2780 ovarian cancer cell line, showing a small reduction in the activity (IC_{50} = 16.1 μM) compared to **1**. Compound **6** was inactive (IC_{50} = >100 μM). Compound **3** showed an improvement in the cytotoxic profile against A2780, 518A2 and MCF-7 cell lines (IC_{50} = 6.22, 13.50 and 8.17 μM; respectively), but with reduced activity against 8505c and A549 (IC_{50} = 25.9 and 21.2 μM; respectively).

The changes in activity according to structural modifications of **1** described above can be summarized as follows. The introduction of 2,4-DNPH moiety at C3 (compound **3**) changed slightly the cytotoxic activity. The absence of free carboxyl moieties led to a decrease or total loss of activity, as observed for compounds **6** and **7**. The same trend has been reported for other esters [29–31].

The addition of two 2,4-DNPH groups at C3 and C20 (compound **9**) led to a decrease of activity, despite the free carboxylic group. On the other hand, the presence of a 2,4-DNPH moiety, together with two oxygen functions, proved to be the most efficient modification, as observed for compounds **4**, **5**, **10** and **12**. In all of them the free carboxylic moiety was maintained at C28 and at C20 a C=O moiety was introduced (aldehyde in compounds **4** and **5**; ketone in compound **10**) or the free hydroxyl at C3 was kept (compound **12**). The simple modifications at the double bound at C20 of **1**, such as ketone (compound **8**), oxime (compound **13**) or aldehyde (compound **16**) led to a significant reduction of cytotoxicity, showing that modifications in this position are not favorable to get more potent derivatives when C3 hydroxyl is free. In this case, the presence of 2,4-DNPH moiety is essential when other oxygen functions are present, mainly at C20.

Compound **14**, obtained by biotransformation of **1**, was less active than **2**, showing once again the importance of free carboxylic group at C28. The biological esterification of **1** to compound **15** did not change significantly the antitumor properties demonstrating the importance of hydroxyl group at C3 for the cytotoxic activity.

These results suggest that the presence of moieties capable of making hydrogen bonds is important for the cytotoxic effect of the new 2,4-dinitrophenylhydrazone betulinic acid derivatives.

Table 1
IC_{50} [μM] of betulinic acid derivatives on tumor cell lines determined by SRB colorimetric assay, after 96 h.

Compound	A2780	8505c	518A2	MCF-7	A549
1	8.75 ± 0.96	12.63 ± 1.67	14.8 ± 0.60	14.03 ± 1.21	13.3 ± 0.82
2	5.97 ± 0.37	14.0 ± 0.69	12.93 ± 0.29	10.42 ± 0.49	10.42 ± 0.86
3	6.22 ± 0.14	25.9 ± 2.66	13.50 ± 1.21	8.17 ± 0.68	21.35 ± 1.20
4	1.76 ± 0.11	2.44 ± 0.06	1.91 ± 0.04	2.51 ± 0.26	1.79 ± 0.18
5	1.81 ± 0.05	2.17 ± 0.30	1.76 ± 0.11	2.41 ± 0.20	2.00 ± 0.25
6	>100	>100	>100	>100	>100
7	66.7 ± 3.82	>100	>100	>100	>100
8	16.1 ± 1.68	64.07 ± 2.60	43.50 ± 1.27	47.15 ± 1.34	59.13 ± 4.21
9	53.77 ± 4.65	>100	>100	60.25 ± 1.77	>100
10	9.1 ± 0.74	16.93 ± 0.40	14.60 ± 0.35	7.52 ± 0.47	12.1 ± 1.00
11	68.05 ± 1.91	93.6 ± 2.25	97.47 ± 2.19	63.23 ± 3.00	75.37 ± 3.10
12	11.3 ± 0.52	8.72 ± 0.35	9.09 ± 0.37	6.84 ± 0.47	7.62 ± 0.47
13	54.43 ± 2.25	55.93 ± 1.33	63.17 ± 1.44	52.23 ± 2.15	53.67 ± 1.27
14	36.25 ± 2.05	>100	68.05 ± 1.91	98.10 ± 2.69	>100
15	16.27 ± 0.75	17.17 ± 0.81	16.93 ± 0.40	16.93 ± 0.40	17.17 ± 0.40
16	50.3 ± 5.80	55.17 ± 1.33	52.93 ± 1.27	44.40 ± 1.80	46.87 ± 2.14

Values are derived from dose–response curves obtained by measuring the percentage of viable cells relative to untreated controls after 96 h exposure of the test compounds to A2780 (ovarian cancer), 8505c (anaplastic thyroid cancer), 518A2 (melanoma), MCF-7 (breast cancer) and A549 (lung carcinoma) cell lines using SRB-assay. Values are the average from at least three independent experiments.

Hydrazone derivatives of **1**, as well modifications at C20, have been reported. Hydrazones showed increased activity against ovarian and prostate tumor cell lines when compared to 3-hydroxyloxime, 3-acetyl, 3-(2-acetoxy)-propionyl and 5,3-*O*-trimethylacethyl derivatives [32]. When the olefin at C20 of **1** was oxidized to a ketone and then reduced to secondary alcohol or to oximes, led to a fall in the cytotoxic profile ($IC_{50} > 50$ μM). It is possible that the presence of highly electronegative oxygen atoms can change the electrostatic properties of **1** leading to less toxic derivatives. It was also observed that the double bound is not essential to the activity, since dihydrobetulinic acid kept the same IC_{50} values, indicating that this position, when modified alone, is not favorable to modifications to produce more potent derivatives [21]. Additionally, the free carboxylic group at C28 seems to have an importance in cytotoxicity and selectivity of these derivatives [28].

2.3. Selectivity

The active cytotoxic compounds **3**, **4**, **10** and **12** were chosen for selectivity test with CCD 18co normal colon human fibroblasts. The selectivity index was calculated by IC_{50} value in fibroblast divided by IC_{50} value in cancer cell lines. IC_{50} value of **1** was 43.6 ± 3.70 μM, while compounds **3**, **4**, **10** and **12** showed 18.1 ± 3.60, 8.30 ± 0.5, 43.5 ± 1.30 and 56.7 ± 3.30 μM, respectively. All compounds were less toxic in human fibroblasts in comparison with the cancer cell lines, except compound **3** for 8505c and A549 cell lines. The most active compound (**4**) showed three to five times more toxicity against tumor cells, as observed similarly to **1**. Compound **3** showed one to three times more selectivity to A2780, 518A2 and MCF-7 cell lines, while it was equally toxic to the others. Compound **10** showed 2.5 to five times more selectivity than **1**. Compound **12** was the most selective (5–7 times) against tumor cells. The test compounds were selective towards tumor cells and the human fibroblast showed better tolerance. However compound **3**, which has 2,4-DNPH moiety at C3 and the double bound at C20, was more toxic to fibroblasts and less selective to tumor cells. This was changed when the double bond was oxidized to aldehyde and ketone, or the addition of phenylhydrazone moiety at C20.

2.4. Cell cycle analysis

Cell cycle perturbations of A549 cells induced by **1** and **4** at their IC_{80} values were evaluated (Fig. 1, Table 2). The results showed that the compounds act in a time-dependent manner. Cell arrest was not induced by **1** in any cycle phase, but it induced directly cell death after 24 h. This was evidenced by a SubG1 peak (18.8%) and significant reduction of G0/G1. Compound **4** induced G0/G1 arrest (60.9%), reducing significantly G2/M peak after 24 h.

After 48 h almost all cells treated with **1** were not viable (SubG1 = 98.5%), with absence of G0/G1, S and G2/M phases. The percentage of arrested cells in G0/G1 by **4** decreased, while an increase of SubG1 peak was observed. Arrested cells in S and G2/M phases remained practically similar to after 24 h. Normally every cell that suffers apoptosis can be detected as a subdiploid peak (SubG1) by flow cytometry. SubG1 peak corresponds to cells with fragmented DNA, a feature of the apoptotic cell death.

The results show that **4** interfere in cell cycle of A549 cell line by the arrest of G0/G1 phase after 24 h treatment. However, when compared to **1**, apoptosis induction seems to be slower.

2.5. Apoptosis

Compounds **1**, **3**, **4**, **10** and **12** were investigated regarding their potential to induce apoptosis. The compounds were tested in acridine orange/ethidium bromide (AO/EB) assay. All compounds showed apoptosis features such as chromatin condensation, DNA

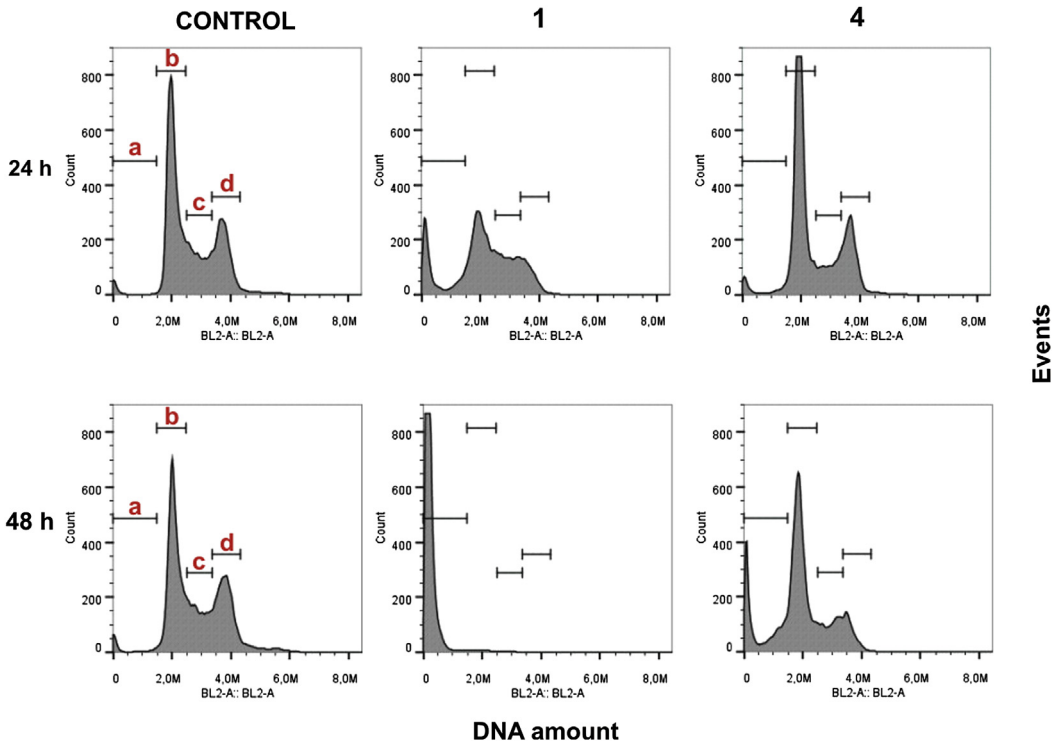


Fig. 1. Distribution of A549 cells in each cell cycle phase after treatment with IC_{80} concentration of **1** and **4** for 24 and 48 h. The results were obtained by the analysis of PI stained-DNA amount by flow cytometry, and were compared to an untreated control. Cell cycle phases: a. SubG1, b. G0/G1, c. S, d. G2/M. The histograms are representative of three independent experiments.

Table 2
Cell cycle analysis of A549 cells untreated (control) and treated with the IC_{80} concentration of **1** and **4** for 24 and 48 h.

Phase	Control	**1**	**4**
24 h			
subG1	2.73 ± 0.49	18.8 ± 1.01	8.77 ± 2.70
G0/G1	49.5 ± 2.51	36.7 ± 2.72	60.9 ± 1.71
S	20.2 ± 0.81	20.1 ± 0.78	14.8 ± 1.05
G2/M	24.9 ± 1.46	20.1 ± 2.46	13.9 ± 4.68
48 h			
subG1	3.23 ± 0.25	98.5 ± 0.38	18.3 ± 1.09
G0/G1	48.5 ± 1.82	0.96 ± 0.05	49.7 ± 3.38
S	19.1 ± 1.05	0.33 ± 0.23	14.6 ± 1.89
G2/M	26.0 ± 1.10	0.17 ± 0.11	15.9 ± 3.86

Values are the average from three independent experiments.

fragmentation, membrane blebbing and apoptotic bodies. Another evidence of the induction of apoptosis by these active compounds was given by DNA fragmentation assay. The occurrence of typical DNA ladders in the floating cells of A549 cell line after treatment with **4** was observed (Fig. 2).

Cell death induction by **1** and **4** was also assessed by annexin V-FITC staining assay. An increase of the number of apoptotic cells was observed in treated cells comparing to control after 24 h. It was observed an increase around 32% apoptosis (early apoptosis + secondary necrosis) for cells treated with **1**, while 12% apoptosis was verified to **4**. After 48 h, more than 50% of the cells treated with **1** were apoptotic, while the amount of 8% was observed to **4** (Fig. 3).

Despite **1** ($IC_{50} = 13.3\ \mu M$) was less active than **4** ($IC_{50} = 1.79\ \mu M$), the precursor induces a higher magnitude of apoptosis in a shorter time and **4** showed a slower mode of action. This observation is in agreement with the cell cycle results. Physical and pharmaceutical properties of the molecules, such as solubility in aqueous medium, intracellular penetration capability, stability, metabolic activation, etc., could explain the different rate of apoptosis between **1** and **4**.

3. Experimental

3.1. Materials and methods

Solvents were distilled before use. The progress of reactions were monitored by TLC on silica gel 60 F254 plates (Merck) using sulfuric-vanilin reagent for detection of the spots. Isolation of the products was carried out by column chromatography and circular chromatography (Chromatotron). All NMR spectra (^1H and ^{13}C NMR, bidimensional) were recorded on Bruker DPX 200 and DPX 400 instruments using $CDCl_3$, pyridin-d_5 or CD_3OD as solvent with TMS as internal standard. Chemical shifts are expressed in δ and coupling constant in Hertz. MS experiments were carried out on an Applied Biosystems MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer with electrospray ionization (ESI) source, in both positive and negative modes.

3.2. Isolation of betulinic acid (*1*)

Betulinic acid was isolated from the outer barks of *Platanus orientalis* L., from specimens located at Curitiba-PR, Brazil, using a published procedure [33]. A voucher was authenticated by a taxonomist and deposited at Department of Botany of the Universidade Federal do Paraná (UPCB 47812). The ground bark was extracted with *n*-hexane during 30 min and then exhaustively with $CHCl_3$ until the total removal of **1**. After evaporation of the solvent the residue was recrystallized from $CHCl_3$:MeOH (1:2). The pure white crystals of **1**, m.p. 298–300 °C, were characterized by spectroscopic analysis and were in agreement with published data [34].

3.3. Preparation of betulonic acid (*2*)

A solution of **1** (0.52 g, 1.14 mmol) in acetone (20 mL) was cooled, and 10 mL of Jones reagent (27 g CrO_3, 0.27 mol; 40 mL distilled H_2O; 23 mL H_2SO_4) was added dropwise during 1.5 h at 0 °C under stirring. Then MeOH (10 mL) was added, the mixture was stirred for 5 min, and the mixture was diluted with H_2O (30 mL). Organic solvents were evaporated under reduced pressure and the aqueous solution was extracted with ethyl acetate (5 × 20 mL). The crude extract was purified by flash chromatography using *n*-hexane:ethyl acetate as mobile phase. Compound **2** was obtained as a white-yellowish solid (0.3 g, yield 58%), m.p. 250–252 °C. MS $[M - H]^-$ m/z 453.3, $C_{30}H_{46}O_3$, and NMR spectra were compared with published data [35].

3.4. Preparation of 3-[(2,4-dinitrophenyl)hydrazono]lup-20(29)-en-28-oic acid (*3*)

A solution of **2** (0.5 g, 1.10 mmol) in ethanol (20 mL) was warmed, 10 mL of a solution of 2,4-DNPH (0.8 g 2,4-DNPH, 4.04 mmol; 4 mL H_2SO_4; 6 mL distilled H_2O) was added and the mixture was stirred at room temperature for 15 h. Water (40 mL) was added and the mixture was extracted with ethyl acetate (4 × 20 mL). The crude product was purified by column chromatography using silica gel 60 (CH_2Cl_2 100%) to give compound **3** (0.37 g, yield 73%) as a dark orange solid, m.p. 265–268 °C. UV–vis (MeOH–CH_3CN) λ_{max} 350, 388 nm. MS $[M + CH_3OH - H]^-$ m/z 665.5, $C_{36}H_{50}N_4O_6$. ^1H NMR (200 MHz, $CDCl_3$) δ 0.94 (3H, s, H25), 0.99 (3H, s, H27), 1.00 (3H, s, H26), 1.15 (3H, s, H24), 1.28 (3H, s, H23), 1.70 (3H, s, H30), 4.62 (1H, s, H29), 4.75 (1H, s, H29), 7.94 (1H,

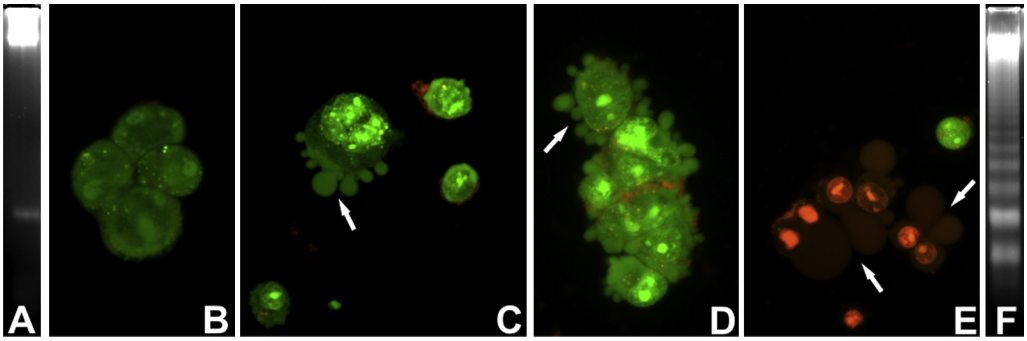


Fig. 2. DNA laddering assay (a,f) and acridine orange/ethidium bromide staining test (b–e) after treatment of A549 cells with IC_{80} concentration of **4** for 72 and 24 h, respectively. a, b. untreated controls; c, d. early apoptosis–chromatin condensation, DNA fragmentation and blebbings (arrows); e. late apoptosis–apoptotic bodies (arrows).

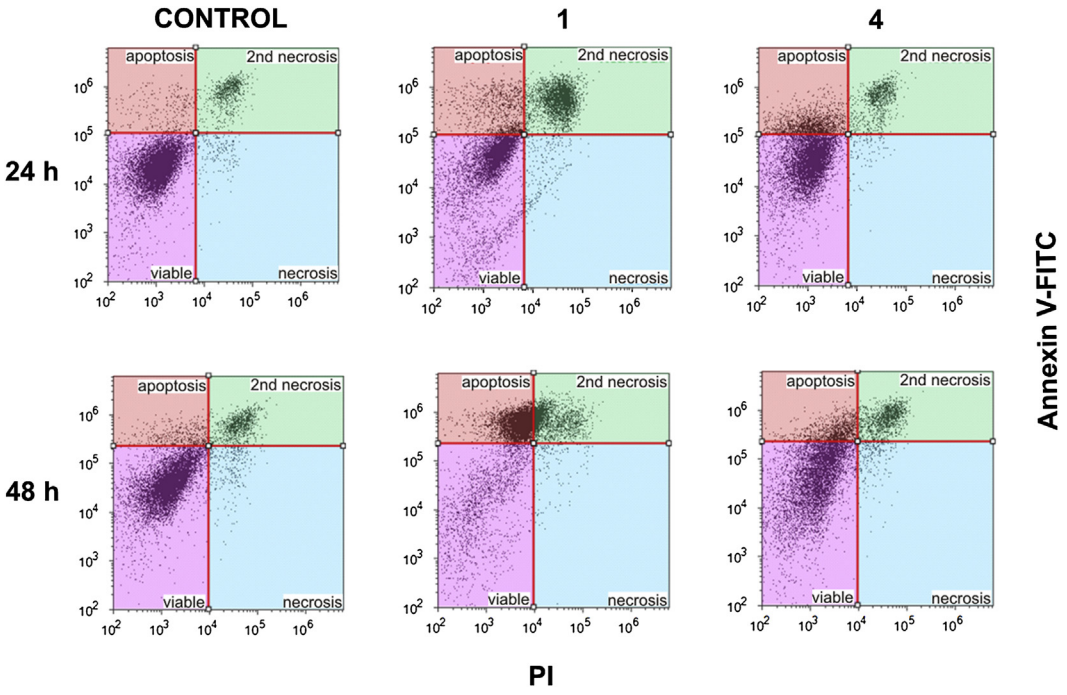


Fig. 3. Annexin V-FITC staining assay: A549 cells treated with IC_{80} doses of **1** and **4** after 24 and 48 h, in comparison to an untreated control. The histograms are representative of two independent experiments.

d, *J* 9.6 Hz, H6′), 8.29 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.12 (1H, d, *J* 2.5 Hz, H3′), 11.17 (1H, s, −NH). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.6 (C25), 16.0 (C26), 19.4 (C30), 19.7 (C6), 20.8 (C2), 21.4 (C11), 23.8 (C24), 25.5 (C12), 28.9 (C23), 29.7 (C15), 30.6 (C21), 32.2 (C16), 33.8 (C7), 37.1 (C10, C22), 38.4 (C1), 38.5 (C13), 40.8 (C8), 42.3 (C4), 42.5 (C14), 47.0 (C19), 49.2 (C18), 49.8 (C9), 55.2 (C5), 56.5 (C17), 109.8 (C29), 116.5 (C6′), 123.6 (C3′), 129.0 (C2′), 129.9 (C5′), 137.5 (C4′), 145.6 (C1′), 150.3 (C20), 167.4 (C3), 182.4 (C28).

3.5. Preparation of epimers 3-[(2,4-dinitrophenyl)hydrazono]-(20R,S)-29-oxolupan-28-oic acids (**4** and **5**)

A solution of **3** (0.25 g, 0.39 mmol) in CH_2Cl_2 (5 mL) was cooled on ice bath, *m*-chloroperbenzoic acid (0.41 g, 2.4 mmol) was added, and the mixture was stirred at 0 °C for 5 h. The solution was allowed to warm to room temperature, aqueous 10% (*w*/*v*) $Na_2S_2O_5$ (10 mL) was added, and the mixture was stirred for 10 min. The mixture was then extracted with CH_2Cl_2 (2 × 10 mL), the organic phase was washed with saturated aqueous $NaHCO_3$ (3 × 10 mL) and evaporated under reduced pressure. The crude extract was fractionated by circular chromatography (CH_2Cl_2 100%), yielding compounds **4** (dark orange solid, 123.3 mg, yield 48.4%, m.p. 226–229 °C) and **5** (orange solid, 28.7 mg, yield 11.2%, m.p. 220–222 °C).

Compound **4**. UV–vis (MeOH–CH_3CN), λ_{max} 361 nm. MS $[M - H]^-$ *m*/*z* 649.5, $C_{36}H_{50}N_4O_7$. ^{1}H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.93 (3H, s, H25), 0.98 (3H, s, H27), 1.02 (3H, s, H26), 1.11 (3H, s, H30), 1.15 (3H, s, H24), 1.29 (3H, s, H23), 7.95 (1H, d, *J* 9.6 Hz, H6′), 8.28 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.11 (1H, d, *J* 2.5 Hz, H3′), 9.87 (1H, d, *J* 1.6 Hz, H29), 11.19 (1H, s, −NH). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 14.4 (C27, C30), 15.6 (C25), 16.0 (C26), 19.7 (C6), 20.7 (C2), 21.3 (C11), 23.8 (C24), 25.8 (C12), 27.5(C21), 28.8 (C23), 29.7 (C15), 32.1 (C16), 33.8 (C7), 37.0 (C10, C22), 38.2 (C13), 38.3 (C1), 40.7 (C8), 42.2 (C4), 42.6 (C14, C19), 49.4 (C9, C20), 49.9 (C18), 55.0 (C5), 56.5 (C17), 116.5 (C6′), 123.5 (C3′), 128.9 (C2′), 129.9 (C5′), 137.4 (C4′), 145.5 (C1′), 167.4 (C3), 179.0 (C28), 207.2 (C29).

Compound **5**. UV–vis (MeOH–CH_3CN), λ_{max} 361 nm. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.94 (3H, s, H25), 1.00 (6H, s, H27, H30), 1.03 (3H, s, H26), 1.16 (3H, s, H24), 1.29 (3H, s, H23), 7.94 (1H, d, *J* 9.6 Hz, H6′), 8.28 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.11 (1H, d, *J* 2.5 Hz, H3′), 9.66 (1H, s, H29), 11.17 (1H, s, −NH). ^{13}C NMR (50 MHz, $CDCl_3$) δ 6.79 (C30), 14.4 (C27), 15.6 (C25), 16.0 (C26), 19.7 (C6), 20.7 (C2), 21.3 (C11), 23.8 (C24), 24.5 (C12), 26.7 (C21), 28.8 (C23), 29.7 (C15), 32.3 (C16), 33.8 (C7), 37.0 (C10, C19), 37.6 (C22), 38.2 (C1), 38.4 (C13), 40.8 (C8), 42.2 (C4), 42.7 (C14), 48.1 (C18), 49.5 (C9), 50.1 (C20), 55.1 (C5), 56.3 (C17), 116.5 (C6′), 123.5 (C3′), 128.9 (C2′), 129.9 (C5′), 137.4 (C4′), 145.6 (C1′), 167.3 (C3), 178.9 (C28), 204.8 (C29).

3.6. Preparation of methyl 3-[(2,4-dinitrophenyl)hydrazono]lup-20(29)-en-28-oate (**6**)

A solution of **3** (0.20 g, 0.31 mmol) in ethyl ether (2 mL) and an ethereal solution of diazomethane was added dropwise. The end of the reaction was detected by TLC. The crude product was purified by circular chromatography (*n*-hexane:CH_2Cl_2, 70:30), producing compound **6** (orange solid, 196 mg, yield 98%, m.p. 198–201 °C). UV–vis (MeOH–CH_3CN) λ_{max} 350, 380 nm. MS $[M - H]^-$ *m*/*z* 647.1, $C_{37}H_{52}N_4O_6$. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.92 (3H, s, H25), 0.97 (3H, s, H26, H27), 1.16 (3H, s, H24), 1.28 (3H, s, H23), 1.69 (3H, s, H30), 3.68 (3H, s, H31), 4.61 (1H, s, H29), 4.74 (1H, s, H29), 7.94 (1H, d, *J* 9.6 Hz, H6′), 8.29 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.12 (1H, d, *J* 2.5 Hz, H3′), 11.17 (1H, s, −NH). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.6 (C25), 15.9 (C26), 19.4 (C30), 19.7 (C6), 20.8 (C2), 21.4 (C11), 23.8 (C24), 25.5 (C12), 28.8 (C23), 29.6 (C15), 30.6 (C21), 32.1 (C16), 33.8 (C7), 36.9 (C22), 37.1(C10), 38.3 (C13), 38.4 (C1), 40.7 (C8), 42.2 (C4), 42.5 (C14), 46.9 (C19), 49.4 (C18), 49.8 (C9), 51.3 (C31), 55.2 (C5), 56.6 (C17), 109.7 (C29), 116.5 (C6′), 123.6 (C3′), 128.9 (C2′), 129.9 (C5′), 137.5 (C4′), 145.6 (C1′), 150.4 (C20), 167.4 (C3), 176.6 (C28).

3.7. Preparation of benzyl 3-[(2,4-dinitrophenyl)hydrazono]lup-20(29)-en-28-oate (**7**)

A solution of **2** (0.10 g, 0.22 mmol), benzyl chloride (0.5 mL) and K_2CO_3 (0.20 g, 1.45 mmol) in acetone (10 mL) was refluxed during 2 h. The mixture was filtered, the solvent evaporated, and the crude

product was purified by column chromatography (CH_2Cl_2 100%) to give the corresponding benzyl ester (94.5 mg, yield 90%).

A solution of the benzyl ester was prepared in ethanol (5 mL), a solution of 2,4-DNPH (0.10 g 2,4-DNPH, 0.5 mmol; 0.5 mL H_2SO_4; 0.75 mL distilled H_2O) was added and the mixture was stirred for 15 h at room temperature. Water (20 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The crude extract was fractionated by column chromatography (*n*-hexane:CH_2Cl_2, 70:30 and 50:50), producing compound **7** (colorless resin, 67.4 mg, yield 71.3%). UV–vis (MeOH–CH_3CN) λ_{max} 354, 371 nm. MS $[M - H]^-$ *m/z* 723.5, $C_{43}H_{56}N_4O_6$. ^1H NMR (200 MHz, $CDCl_3$) δ 0.81 (3H, s, H26), 0.90 (3H, s, H25), 0.94 (3H, s, H27), 1.15 (3H, s, H24), 1.27 (3H, s, H23), 1.68 (3H, s, H30), 4.60 (1H, s, H29), 4.73 (1H, s, H29), 5.12 (1H, d, *J* = 12.2 Hz, H7″, $-OCH_2-$Ph), 5.20 (1H, d, *J* = 12.2 Hz, H7″, $-OCH_2-$Ph), 7.36 (5H, m, H2″-6″), 7.94 (1H, d, *J* 9.6 Hz, H6′), 8.28 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.12 (1H, d, *J* 2.5 Hz, H3′), 11.17 (1H, s, –NH). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.6 (C25), 15.7 (C26), 19.4 (C30), 19.7 (C6), 20.7 (C2), 21.3 (C11), 23.8 (C24), 25.5 (C12), 28.8 (C23), 29.7 (C15), 30.6 (C21), 32.1 (C16), 33.7 (C7), 36.9 (C22), 37.0 (C10), 38.2 (C13), 38.4 (C1), 40.7 (C8), 42.2 (C4), 42.5 (C14), 46.9 (C19), 49.4 (C18), 49.8 (C9), 55.2 (C5), 56.5 (C17), 65.8 ($-OCH_2-$Ar, 7″), 109.7 (C29), 116.4 (C6′), 123.6 (C3′), 128.1 (C4″), 128.2 (C2″, C6″), 128.5 (C3″, C5″), 128.9 (C2′), 129.9 (C5′), 136.5 (C1″), 137.4 (C4′), 145.6 (C1′), 150.4 (C20), 167.4 (C3), 175.8 (C28).

3.8. Preparation of 3,20-dioxo-29-norlupan-28-oic acid (**8**)

Compound **2** (0.30 g, 0.66 mmol) was dissolved in dioxane:H_2O (10.5:1.5), OsO_4 (15 mg, 0.059 mmol) was added and the mixture was stirred for 1 h. $NaIO_4$ (1.5 g, 7.01 mmol) was added, in small portions, during 3 h and the mixture was stirred for 3 days at room temperature. Ethyl acetate was added and the mixture was washed with H_2O (3 × 50 mL). The organic phase was separated, the solvent evaporated and the residue purified by column chromatography (CH_2Cl_2 100%). Compound **8** (151 mg, yield 50.3%) was obtained as a white-grayish solid, m.p. 230–233 °C. MS $[2M + Na - 2H]^-$ *m/z* 933.3, $C_{29}H_{44}O_4$. ^1H NMR (200 MHz, $CDCl_3$) δ 0.91 (3H, s, H25), 0.95 (3H, s, H26), 1.01 (3H, s, H27), 1.02 (3H, s, H24), 1.07 (3H, s, H23), 2.19 (3H, s, H30). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.7 (C25), 16.0 (C26), 19.6 (C6), 21.0 (C24), 21.4 (C11), 26.8 (C23), 27.2 (C12), 28.2 (C21), 29.7 (C15), 30.1 (C30), 31.4 (C16), 33.5 (C7), 34.0 (C2), 36.7 (C22), 36.9 (C10), 37.6 (C13), 39.5 (C1), 40.5 (C8), 42.3 (C14), 47.3 (C4), 49.0 (C18), 49.7 (C9), 51.2 (C19), 54.7 (C5), 56.2 (C17), 181.9 (C28), 212.2 (C20), 218.3 (C3).

3.9. Preparation of 3,20-[(2,4-dinitrophenyl)hydrazono]lupan-28-oic acid (**9**) and 3-[(2,4-initrophenyl)hydrazono]-20-oxolupan-28-oic acid (**10**)

A solution of **8** (126 mg, 0.27 mmol) in ethanol (6.5 mL), a solution of 2,4-DNPH (0.13 g 2,4-DNPH, 0.65 mmol; 0.65 mL H_2SO_4, 1 mL distilled H_2O) was added and the mixture was stirred for 15 h at room temperature. Water (20 mL) was added and the mixture was extracted with CH_2Cl_2 (3 × 20 mL). The organic phase was separated, the solvent evaporated and the residue was fractionated by column chromatography (CH_2Cl_2 100%; CH_2Cl_2:MeOH, 99:1) producing compounds **9** (orange solid, 53.1 mg, yield 42.1%, m.p. 292–295 °C) and **10** (dark orange solid, 48.3 mg, yield 38.3%, m.p. 163–165 °C).

Compound **9**. UV–vis (MeOH–CH_3CN) λ_{max} 360 nm. MS $[M - H]^-$ *m/z* 815.0, $C_{41}H_{52}N_8O_{10}$. ^1H NMR (200 MHz, $CDCl_3$) δ 0.93 (3H, s, H25), 1.02 (3H, s, H26), 1.07 (3H, s, H27), 1.14 (3H, s, H24), 1.28 (3H, s, H23), 2.07 (3H, s, H30), 7.94 (2H, t, *J* 9.7 Hz, H6′,H6″), 8.26 (1H, dd, *J* 2.5 Hz, 5.5 Hz, H5′), 8.31 (1H, dd, *J* 2.5 Hz, 5.5 Hz, H5″), 9.09 (1H, d, 2.5 Hz, H3″), 9.12 (1H, d, 2.5 Hz, H3′), 11.05 (1H, s, –NH″), 11.13 (1H, s, –NH′). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.0 (C25), 15.7 (C26), 15.9 (C30), 19.6 (C6), 20.6 (C2), 21.3 (C11), 23.7 (C24), 26.9 (C12), 28.8 (C23), 29.2 (C15), 29.7 (C21), 31.9 (C16), 33.8 (C7), 36.8 (C22), 37.1 (C10), 38.1 (C13), 38.3 (C1), 40.7 (C8), 42.2 (C4), 42.5 (C14), 48.0 (C19), 49.3 (C18), 49.7 (C9), 55.1 (C5), 56.2 (C17), 116.3 (C6″), 116.4 (C6′), 123.6 (C3′, C3″), 128.9 (C2″), 129.1 (C2′), 129.9 (C5′), 130.0 (C5″),137.5 (C4″), 137.6 (C4′), 145.2 (C1″), 145.5 (C1′),161.4 (C20), 167.0 (C3), 181.3 (C28).

Compound **10**. UV–vis (MeOH–CH_3CN) λ_{max} 360 nm. MS $[M - H]^-$ *m/z* 635.5, $C_{35}H_{48}N_4O_7$. ^1H NMR (200 MHz, $CDCl_3$) δ 0.93 (3H, s, H25), 0.98 (3H, s, H26), 1.02 (3H, s, H27), 1.15 (3H, s, H24), 1.28 (3H, s, H23), 2.20 (3H, s, H30), 7.93 (1H, d, *J* 9.6 Hz, H6′), 8.29 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5′), 9.12 (1H, d, 2.5 Hz, H3′), 11.17 (1H, s, –NH′). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.6 (C25), 15.9 (C26), 19.6 (C6), 20.7 (C2), 21.3 (C11), 23.7 (C24), 27.2 (C12), 28.2 (C21), 28.8 (C23), 29.7 (C15), 30.1 (C30), 31.4 (C16), 33.6 (C7), 36.7 (C22), 37.0 (C10), 37.5 (C13), 38.3 (C1), 40.6 (C8), 42.2 (C4), 42.3 (C14), 49.0 (C18), 49.6 (C9), 51.1 (C19), 55.0 (C5), 56.2 (C17), 116.4 (C6′), 123.6 (C3′), 128.9 (C2′), 129.9 (C5′), 137.5 (C4′), 145.6 (C1′), 167.2 (C3), 181.7 (C28), 212.1 (C20).

3.10. Preparation of 3-hydroxy-20-oxo-29-norlupan-28-oic acid (**11**)

Compound **1** (1.03 g, 2.25 mmol) was dissolved in dioxane:H_2O (35:5), OsO_4 (85 mg, 0.33 mmol) was added and the mixture was stirred for 1 h. Then $NaIO_4$ (5 g, 23.4 mmol) was added, in small portions, during 3 h and the mixture was stirred for 3 days at room temperature. Ethyl acetate (100 mL) was added, and the mixture was washed with H_2O (3 × 100 mL). The organic phase was separated, dried and the solvent evaporated. The residue was then fractionated by flash chromatography using mixtures of CH_2Cl_2:MeOH and heptane:ethyl acetate as mobile phases. Compound **11** was isolated (840 mg, yield 81.3%) as a grayish solid, m.p. 275–277 °C. MS $[2M - H]^-$ *m/z* 914.4, $C_{29}H_{46}O_4$. ^1H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.78 (3H, s, H24), 0.81 (3H, s, H25), 0.95 (3H, s, H26), 0.99 (3H, s, H23), 1.02 (3H, s, H27), 2.18 (3H, s, H30). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 14.7 (C27), 15.5 (C24), 16.0 (C25), 16.1 (C26), 18.3 (C6), 20.9 (C11), 27.4 (C2, C12), 28.1 (C23), 28.5 (C21), 29.8 (C15), 30.1 (C30), 31.9 (C16), 34.3 (C7), 36.9 (C22), 37.2 (C10), 37.4 (C13), 38.8 (C1), 39.0 (C4), 40.6 (C8), 42.3 (C14), 49.4 (C18), 50.5 (C9), 51.5 (C19), 55.4 (C5), 56.2 (C17), 78.5 (C3), 179.0 (C28), 212.8 (C20).

3.11. Preparation of 3-hydroxy-20-[(2,4-dinitrophenyl)hydrazono]-29-norlupan-28-oic acid (**12**)

A solution of **11** (192 mg, 0.42 mmol) was prepared in ethanol (10 mL), a solution of 2,4-DNPH (0.25 g 2,4-DNPH, 1.26 mmol; 1 mL H_2SO_4; 1.5 mL distilled H_2O) was added and the mixture was stirred for 15 h, at room temperature. Water (20 mL) was added and the mixture was extracted with CH_2Cl_2 (4 × 25 mL). The organic phase was dried, the solvent evaporated and the residue was fractionated by column chromatography using CH_2Cl_2:MeOH as mobile phase. Compound **12** (140.8 mg, yield 73.3%) was obtained as an orange solid, m.p. 285–288 °C. UV–vis (MeOH–CH_3CN) λ_{max} 355, 372 nm. MS $[M - H]^-$ *m/z* 637.5, $C_{35}H_{50}N_4O_7$. ^1H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.79 (3H, s, H24), 0.80 (3H, s, H25), 0.99 (3H, s, H26), 1.00 (3H, s, H23), 1.07 (3H, s, H27), 2.04 (3H, s, H30), 7.99 (1H, d, *J* 9.6 Hz, H6″), 8.29 (1H, dd, *J* 2.5 Hz, 9.6 Hz, H5″), 9.09 (1H, d, 2.5 Hz, H3″), 11.04 (1H, s, –NH″). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 14.6 (C27, C30), 15.6 (C25), 16.1 (C24, C26),18.4 (C6), 21.0 (C11), 26.9 (C21), 27.4 (C2), 28.1 (C23), 29.3 (C12), 29.8 (C15), 32.4 (C16), 34.4 (C7), 37.0 (C22), 37.2 (C10), 37.9 (C13), 38.9 (C1), 39.0 (C4), 40.8

(C8), 42.4 (C14), 48.2 (C19), 49.7 (C18), 50.5 (C9), 55.5 (C5), 56.1 (C17), 78.3 (C3), 116.5 (C6″), 123.5 (C3″), 128.9 (C2″), 129.9 (C5″), 137.4 (C4″), 145.3 (C1″), 162.5 (C20), 178.8 (C28).

3.12. Preparation of 3-hydroxy-20-hydroxy-ethanimidoyl-lupan-28-oic acid (**13**)

A solution of **11** (217.2 mg, 0.47 mmol) was prepared in MeOH (4 mL), hydroxylammonium chloride (880 mg, 13.6 mmol) dissolved in 4 mL ethanol:H_2O (1:1) was added followed by CH_3COONa (880 mg, 10.7 mmol). The mixture was stirred for 2 h, and then left standing overnight at room temperature. Water (20 mL) was added and the mixture was extracted with ethyl acetate (3 × 15 mL). The extract was dried, the solvent evaporated ant the residue was fractionated by column chromatography (CH_2Cl_2:MeOH, 95:5). Compound **13** was isolated as a white solid, m.p. 268–270 °C (116.4 mg, yield 53.6%). MS $[M + H]^+$ *m/z* 474.3, $C_{29}H_{47}N_1O_4$. ^{1}H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.79 (3H, s, H24), 0.80 (3H, s, H25), 0.96 (3H, s, H26), 0.99 (3H, s, H23), 1.00 (3H, s, H27), 1.89 (3H, s, H30). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 11.2 (C30), 14.6 (C27), 15.6 (C24), 16.0 (C25), 16.1 (C26), 18.4 (C6), 20.9 (C11), 25.8 (C12), 27.4 (C2), 28.1 (C23), 28.6 (C21), 29.7 (C15), 32.5 (C16), 34.4 (C7), 37.2 (C10, C22), 37.8 (C13), 38.9 (C1), 39.0 (C4), 40.7 (C8), 42.4 (C14), 45.1 (C19), 49.4 (C18), 50.5 (C9), 55.5 (C5), 55.9 (C17), 78.4 (C3), 161.4 (C20), 179.0 (C28).

3.13. Biotransformation experiments

3.13.1. Biotransformations with fungi

The fungi strains, *Mycelia sterilia*, *Penicillium citreonigrum* and *Penicillium* sp., provided by Microbiology Section (Department of Pathology, UFPR), were kept in potato-dextrose-agar (PDA) at 4 °C until use. The fungi were cultivated in conical flasks containing 50 mL of a culture medium (20 g/L D-(−)-glucose, 8 g/L malt extract, 4 g/L yeast extract). The flasks were shaken (150 rpm) in the dark, at 28 °C, for 72 h. The mycelia were separated and transferred to conical flasks containing 250 mL of fresh culture medium, and cultured for additional 48 h. Compound **1** (65 mg per flask), dissolved in DMSO, was added to each flask except one (control) which received only DMSO. After seven days the broth was filtered and extracted with ethyl acetate (3 × 100 mL). The mycelia were sonicated with $CHCl_3$:MeOH (1:1) for 2 h. The organic fractions were combined, dried with anhydrous Na_2SO_4 and analyzed by TLC. The crude extracts were then fractionated using column, flash or circular chromatography [19].

M. sterilia converted **1** (585 mg, 9 flasks) to the known **2** (15.1 mg). *Penicillium* sp. biotransformed **1** (1.17 g, 18 flasks) to **2** (27.4 mg) and methyl 3-oxolup-20(29)-en-28-oate (**14**) (yellowish resin, 19.7 mg). *P. citreonigrum* transformed **1** (1.75 g, 27 flasks) to methyl 3-hydroxylup-20(29)-en-28-oate (**15**) (white-yellowish solid, 31.6 mg, m.p. 214–217 °C).

Compound **14**. ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.92 (3H, s, H25), 0.95 (3H, s, H26), 0.97 (3H, s, H27), 1.02 (3H, s, H24), 1.07 (3H, s, H23), 1.69 (3H, s, H30), 3.67 (3H, s, H31), 4.60 (1H, s, H29), 4.74 (1H, s, H29). ^{13}C NMR (50 MHz, $CDCl_3$) δ 14.6 (C27), 15.8 (C25), 15.9 (C26), 19.4 (C30), 19.6 (C6), 21.0 (C24), 21.4 (C11), 25.5 (C12), 26.6 (C23), 29.7 (C15), 30.6 (C21), 32.1 (C16), 33.6 (C7), 34.1 (C2), 36.9 (C10, C22), 38.3 (C13), 39.6 (C1), 40.6 (C8), 42.4 (C14), 46.9 (C19), 47.3 (C4), 49.4 (C18), 49.9 (C9), 51.3 (C31), 55.0 (C5), 56.5 (C17), 109.6 (C29), 150.5 (C20), 176.6 (C28), 218.1 (C3).

Compound **15**. ^{1}H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.79 (3H, s, H24), 0.82 (3H, s, H25), 0.92 (3H, s, H23), 0.97 (3H, s, H26), 1.00 (3H, s, H27), 1.69 (3H, s, H30), 3.66 (3H, s, H31), 4.61 (1H, s, H29), 4.75 (1H, s, H29). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 14.7 (C27), 15.6 (C25), 16.0 (C26), 16.1 (C24), 18.3 (C6), 19.4 (C30), 20.9 (C11), 25.5 (C12), 27.5 (C2), 28.1 (C23), 29.7 (C15), 30.6 (C21), 32.2 (C16), 34.4 (C7), 37.0 (C22), 37.2 (C10), 38.3 (C13), 38.9 (C1), 39.0 (C4), 40.7 (C8), 42.4 (C14), 47.0 (C18), 49.5 (C19), 50.6 (C9), 51.2 (C31), 55.5 (C5), 56.5 (C17), 78.5 (C3), 109.6 (C29), 150.5 (C20), 176.6 (C28).

3.13.2. Biotransformations with carrot cells

Commercial carrot seeds (*Daucus carota* var. Nantes) were sterilized with 20% sodium hypochlorite and 70% ethanol during 2 and 1 min, respectively. The seeds were rinsed three times with sterilized water and transferred to semisolid MS culture medium [36], without growth hormones, and kept under controlled temperature and light conditions. After a month the hypocotyls of the sprouts were cut in 2 cm pieces and transferred to a fresh semisolid MS culture medium containing 2.5 μM 2,4-dichlorphenoxyacetic acid (2,4-D) to induce the callus formation. The calli (2 g cells/flask) were transferred to conical flasks containing 50 mL of liquid MS culture medium with 2.5 μM 2,4-D at 25 °C stirring (120 rpm) in the dark. After a week, **1** (30 mg in acetone) was added into each cell suspension flask, except one which received only acetone (control). The flasks were shaken for 14 more days, the suspensions were diluted (1 part cells/1 part fresh MS medium) and more **1** (15 mg) was added. This procedure was repeated 6 times and then the suspensions were filtered. The filtrate was extracted with ethyl acetate (3 × 100 mL), and the cells where macerated and sonicated with CH_2Cl_2:MeOH (1:1) for 2.5 h. The organic extracts were dried over anhydrous Na_2SO_4 and analyzed by TLC. The crude extract of the cells was purified by flash chromatography using a gradient of heptane:CH_2Cl_2 and CH_2Cl_2:MeOH. Compound **2** (19.7 mg) was isolated and crystallized from *n*-hexane–MeOH. Further fractionation produced 3-hydroxy-(20*R*)-29-oxolupan-28-oic acid (**16**) (white-yellowish solid, 26.2 mg, m.p. 219–222 °C).

Compound **16**. MS $[M - H]^-$ *m/z* 471.3, $C_{30}H_{48}O_4$. ^{1}H NMR (200 MHz, $CDCl_3$ + Pyr-d_5) δ 0.82 (3H, s, H24), 0.83 (3H, s, H25), 0.98 (3H, s, H26), 1.04 (3H, s, H23, H27), 1.11 (3H, d, *J* 6.8 Hz, H30), 3.26 (1H, t, H3), 9.88 (1H, d, *J* 1.5 Hz, H29). ^{13}C NMR (50 MHz, $CDCl_3$ + Pyr-d_5) δ 14.5 (C27,C30), 15.7 (C24, C25), 16.1 (C26), 18.4 (C6), 20.9 (C11), 25.8 (C12), 27.5 (C2), 27.6 (C21), 28.2 (C23), 29.8 (C15), 32.2 (C16), 34.5 (C7), 37.1 (C22), 37.2 (C10), 38.2 (C13), 38.9 (C1), 39.0 (C4), 40.8 (C8), 42.6 (C14), 42.7 (C19), 49.4 (C20), 50.0 (C9), 50.3 (C18), 55.5 (C5), 56.5 (C17), 78.2 (C3), 178.9 (C28), 207.0 (C29).

3.14. Cytotoxicity assay

3.14.1. Preparation of solutions of compounds

Stock solutions of test compounds were prepared in DMSO (20 mM) and then diluted with nutrient medium (RPMI-1640 with L-glutamine (PAALabs, Pasching, Austria)) containing 10% heat inactivated fetal bovine serum (FBS) (Biochrom AG, Berlin, Germany) and 1% penicillin/streptomycin (100×) (PAA Labs, Pasching, Austria).

3.14.2. Cell culture

The cell lines 518A2 (melanoma), 8505c (anaplastic thyroid tumor), A2780 (ovarian cancer), A549 (lung cancer), MCF-7 (breast cancer) and CCD Co18 (normal human colon fibroblast) were included in this study. All these cell lines were kindly provided by Dr. Thomas Müller, Department of Hematology/Oncology, Martin-Luther-University of Halle-Wittenberg, Halle (Saale), Germany. Cultures were maintained as monolayers in RPMI-1640 supplemented with 10% heat inactivated FBS, and 1% penicillin/streptomycin, at 37 °C, in a humidified atmosphere with 5% CO_2.

3.14.3. SRB assay

The cytotoxic activities of all the compounds were evaluated using the sulforhodamine-B (SRB) microculture colorimetric assay

[37]. In short, exponentially growing cells were seeded into 96 well plates on day 0 (zero), at the appropriate cell densities, to prevent confluence of the cells during the period of experiment. After 24 h, the cells were treated with serial dilutions of the compounds (0–100 μM), for 96 h. The percentages of surviving cells relative to untreated controls were determined 96 h after the beginning of drug exposure. The supernatant medium from the 96 well plates was thrown away, and the cells were fixed with 10% TCA. For a thorough fixation plates were allowed to stand at 4 °C, for at least 2 h. After fixation the cells were washed in a strip washer. The washing was done five times with water using alternate dispensing and aspiration procedures. The plates were then dyed with 100 mL of 0.4% SRB, for about 45 min. After dying, the plates were again washed to remove the dye with 1% acetic acid, and allowed to air dry overnight. Next day Tris base solution (100 μL, 10 mM) was added to each well and absorbance was measured at 570 nm using a plate reader (Tecan Spectra, Crailsheim, Germany). The IC_{50} values were calculated from the semi-logarithmic dose–response curves.

3.14.4. Cell cycle analysis

Cell cycle was assessed by flow cytometry (Attune Acoustic Focusing Cytometer, Applied Biosystems, USA). 2.0×10^5 A549 cells were seeded in 25 cm^2 flasks with 10 mL of nutrient medium and kept at 37 °C, 5% CO_2, for 24 h. Next day, **1** and **4** at their IC_{80} values were added. Cells were treated during 24 and 48 h. The adherent cells and the supernatant were harvested, centrifuged (1500 rpm, 5 min, 4 °C) and the pellet was washed with PBS. The cells were fixed with 1 mL ice cold 70% ethanol and the samples were kept at 4 °C for 1 h prior to the analysis. Fixed cells were centrifuged and the pellet resuspended with 1 mL staining buffer (FBS, 2% NaN_3, PBS). After further centrifugation, the cells were dissolved in 100 μL RNAse A (100 μg/mL) and incubated at 37 °C for 30 min. 1 mL propidium iodide (PI) (20 μg/1 mL of staining buffer) was added and the samples were kept in the dark during 30 min before the analysis. Each analysis was done recording 2.0×10^4 events and the results were compared with untreated controls.

3.14.5. Apoptosis test

3.14.5.1. AO/EB staining assay. The cell culture flasks with 70–80% confluence were treated with IC_{80} doses of **1**, **3**, **4**, **10** and **12** for 24 h. The adherent and floating cells were collected after treatment and centrifuged (1500 rpm, 5 min, 4 °C). The cell pellet was resuspended in PBS. Equal amounts of cell suspension and AO/EB solution (10 μg/ml, 1:1) were mixed and analyzed under a fluorescence microscope (488 nm). Control cells were obtained harvesting untreated A549 cells by trypsinization.

3.14.5.2. DNA fragmentation assay. Determination of apoptotic cell death was performed by DNA gel electrophoresis. Briefly, A549 cells were treated with respective IC_{80} doses of **1** and **4** for 72 h. Floating cells induced by drug exposure were collected, washed with PBS and lysed with DNA lysis buffer (100 mM Tris–HCl pH 8.0; 20 mM EDTA; 0.8% SDS; all from Sigma Aldrich). Then, cells were treated with RNAse A at 37 °C for 2 h and proteinase K at 50 °C overnight (both from Roche Diagnostics Chemical Company, Mannheim, Germany). DNA laddering was observed by running the samples on 2% agarose gel followed by ethidium bromide (Sigma Aldrich) staining.

3.14.5.3. Annexin V-FITC staining assay. Apoptotic cells were detected using the Annexin V-FITC Apoptosis Detection Kit (BD Pharmingen, BD Biosciences, Heidelberg, Germany). 4.0×10^5 A549 cells were treated with **1** and **4** at their IC_{80} values during 24 and 48 h. Adherent and floating cells were harvested, centrifuged (1500 rpm, 5 min, 4 °C) and washed twice with 1 mL PBS (with Ca^{2+}/Mg^{2+}). The cells were resuspended in 400 μL binding buffer (1×) and the concentration of the samples was adjusted to 1.0×10^6 cell/mL. 100 μL of each sample was treated with 5 μL PI and 5 μL annexin V-FITC, and kept in the dark for 15 min. 400 μL binding buffer (1×) was added to each sample and they were analyzed by flow cytometry, using about 1×10^4 events.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.07.012.

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