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MEDICINAL CHEMISTRY RESEARCH

ORIGINAL RESEARCH

Platanic acid derived amides are more cytotoxic than their corresponding oximes

Marie Kozubek[1] · Sophie Hoenke[1] · Theresa Schmidt[1] · Dieter Ströhl[1] · René Csuk[1]

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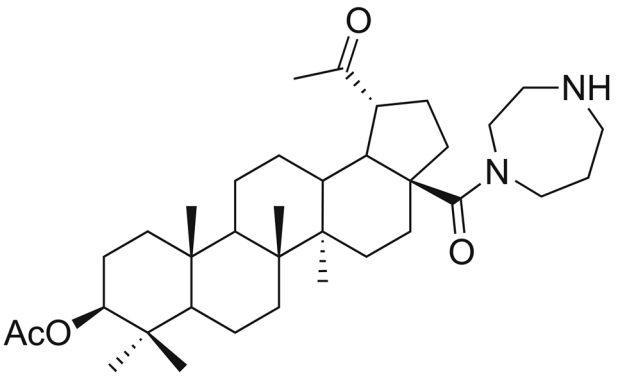
Abstract

Albeit platanic acid has been known since 1956, its potential to act as a valuable starting material for the synthesis of cytotoxic agents has been neglected for many years. Hereby we describe the synthesis of a small library of amides and oximes derived from 3-*O*-acetyl-platanic acid, and the results of their screening as cytotoxic agents for several human tumor cell lines. As a result, while the cytotoxicity of the oximes was diminished as compared to the parent amides, the homopiperazinyl amide **5** held the highest cytoxicity ($EC_{50} = 0.9$ μM for A375 human melanoma cells). Extra FACS and cell cycle measurements showed compound **5** to act onto A375 cells rather by apoptosis than by necrosis.

Clinical trial registration

No clinical trials are associated with this study

Graphical abstract



EC_{50} = 0.9 μM (for A375 human melanoma cells; from SRB assay)

Keywords Platanic acid · cytotoxicity · SRB assay

Introduction

Platanic acid (**PA**, Fig. 1) is a 20-oxo-30-norlupane analog to betulinic acid. The compound was first isolated by C. Djerassi [1] in 1956; it occurs in several plants, including *Platanus sp*., *Melaleucas sp*. or *Melilotus sp*. [2] This pentacyclic triterpenoic acid, however, can also be readily obtained in good yields by oxidative cleavage of the *exo*-

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✉ René Csuk
rene.csuk@chemie.uni-halle.de

[1] Martin–Luther–University Halle–Wittenberg, Organic Chemistry, Kurt–Mothes–Str. 2, D–06120 Halle (Saale), Germany

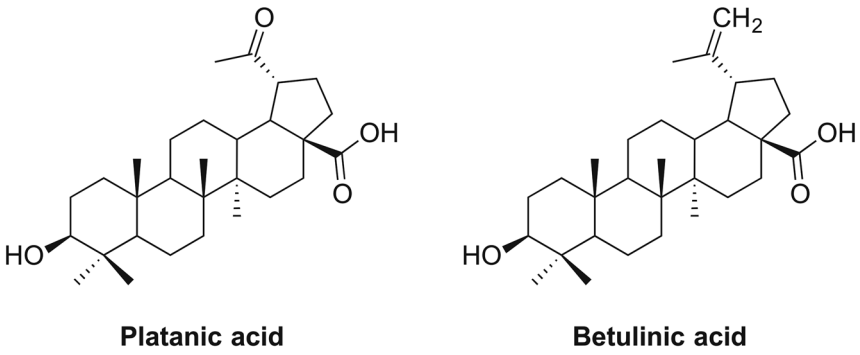


Fig. 1 Structures of platanic acid (**PA**) and betulinic acid (**BA**)

cyclic double bond of betulinic acid (**BA**) or derivatives thereof. Typically, OsO_4 [3], RuO_4 [4], or ozone [5] have been utilized for this reaction. Its reconversion can be performed by means of a Wittig reaction; [6] this reaction has been used to access labeled **PA** and derivatives thereof [7].

Despite the relatively good accessibility of platanic acid, the number of studies and structural modifications has remained relatively small over all these years. This is all the more astonishing since the number of publications on betulinic acid is almost unmanageably large, while less than 100 publications can be found under the keyword "platanic acid" in SciFinder (as of February 2022). This is surprising as several derivatives derived from platanic acid have proven to be strongly cytotoxic or enzyme inhibitors [8]. This included the synthesis of cytotoxic amides [9] but also an access to rhodamine B conjugates that acted as mitocans in several human tumor cell lines even in low nano-molar concentration [10–13]. Several derivatives were potent inhibitors of the enzymes acetyl and butyrylcholinesterase (AChE, BChE) [8] but also of xanthine oxidase [14, 15]. The development of inhibitors for AChE and/or BChE as therapeutics to alleviate the symptoms of neurological disorders such as Alzheimer's disease [16–19], Parkinson [20–26] or Lewy body dementias [27–32] has been in the focus of scientific interest now for many years. Inhibitors of xanthine oxidase are drugs intended for the therapy of hyperuricemia and gout but also for the management of reperfusion injury [33–45]. However, the focus of our own investigations was based on the cytotoxic potential of these compounds.

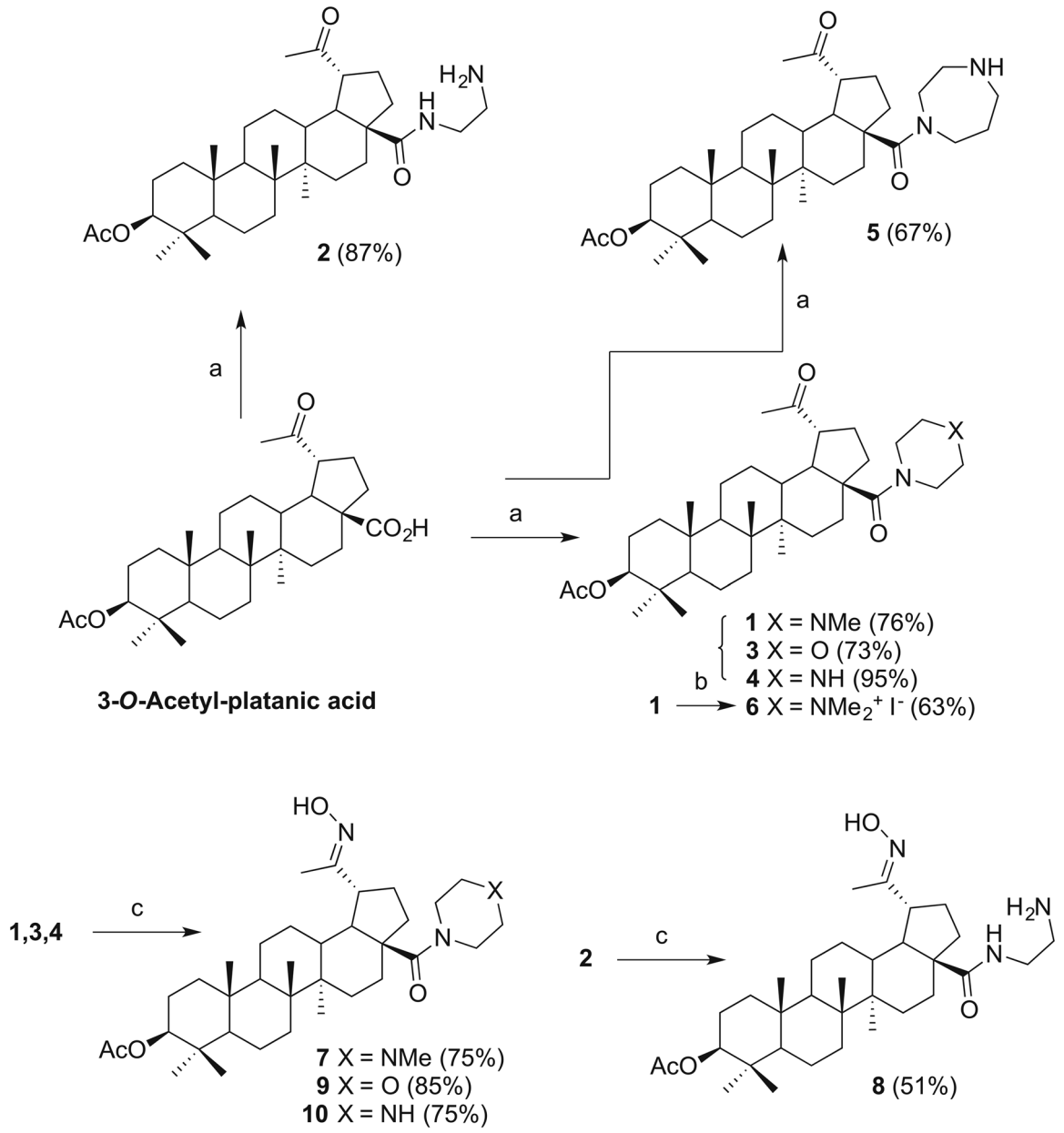
Results and discussion

3-*O*-Acetyl-platanic acid [8] (Scheme 1) was chosen as a starting material for the synthesis of the derivatives. Thus, this very valuable starting material was allowed to react with oxalyl chloride in the presence of catal. amounts of dimethylformamide (DMF) followed by the addition of *N*-methylpiperazine to yield amide **1** in 76% isolated yield. The same procedure was applied for the synthesis of analogs **2** (from ethylenediamine, 87%), **3** (from morpholine, 73%), and **4** (from piperazine, 95%). Activation of **1** with oxalyl chloride followed by the reaction with homopiperazine furnished **5** while from the reaction of **1** with an excess of iodomethane in dry DCM compound **6** was obtained. Reaction of amides **1**–**4** with hydroxylammonium chloride in dry pyridine at 60 °C for 3 h furnished (*E*) configurated oximes **7**–**10**, respectively. A 2D ROESY NMR spectrum (Fig. 2) of compound **9** was recorded to determine the absolute configuration of the oximes **7**-**10**. This spectrum revealed evidence of ROE interactions between the protons from the methyl group (H-29 at δ = 1.66 ppm) with the proton from the hydroxyl group (at δ = 10.04 ppm) indicated by the presence of a ROE cross peak. Based on these results, the oximes hold an (*E*) configuration. This result is in full agreement with previously obtained results. Careful examination of the synthesis of **9** revealed that very small amounts (as indicated via HPTLC-ESI-MS) of (*Z*) configured product were also formed during the reaction, but this material could not be isolated.

To assess their cytotoxicity, photometric sulforhodamine B (SRB) assays were performed employing a set of human malignant cell lines (A375, HT29, MCF-7, A2780, HeLa) and non–malignant fibroblasts (NIH 3T3). The results from these assays are compiled in Table 1.

Amides **2**–**5** were highly cytotoxic for all human tumor cell lines but also for non-malignant NIH 3T3 and HEK293 cells. The highest cytotoxicity was observed for amide **5** holding a homopiperazinyl moiety. This follows previous findings [12, 13] for homopiperazinyl holding derivatives of triterpenoids and their cytotoxicity. This compound showed an EC_{50} value for A375 human melanoma cells as low as 0.9 µM. Compounds **1**, **3** and **6** were not soluble under the conditions of the assay. The cytotoxicity of the oximes was diminished as compared to the cytotoxicity determined for the corresponding amides. Thereby piperazine derived compound **10** showed the lowest EC_{50} value (2.2 ± 0.4 µM) again for A375 tumor cells followed by EC_{50} = 2.7 ± 0.3 µM for human ovarian carcinoma cells A2780.

Extra FACS based investigation (Fig. 3) of compounds **2** and **5** (A375 cells, incubation time 48 h) showed 0.4%



Scheme 1 Reactions and conditions: (**a**) $(COCl)_2$, DCM, DMF (catal.), then N-methylpiperazine (→ **1**) or ethylenediamine (→ **2**) or morpholine (→ **3**) or piperazine (→ **4**) or homopiperazine (→ **5**), 3 h, 21 °C; (**b**) MeI, DCM, 24 h, 21 °C; (**c**) hydroxylammonium chloride, pyridine, **1** (→ **7**) or **2** (→ **8**) or **3** (→ **9**) or **4** (→ **10**), 3 h 60 °C

of the cells necrotic (R1), 15.5% late-apoptotic (R2) and 3.6% apoptotic (R3), while for **5** the percentage of necrotic cells was also low (0.7%) but the number of apoptotic cells ca. five-times larger (20.8%, R4) than for compound **2**.

Investigation of the cell cycle (48 h, A375 cells, Fig. 4) showed for **2** 73.8% of the cells in G1 phase, 15.9% in S, 10.4% in G2/M and 24.5% as apoptotic while for **5** under the same conditions the percentage of cells in G1 was slightly lowered (65.3%) while those in the S phase were higher (24.4%).

Conclusion

Long time neglected platanic acid was acetylated and subsequently converted into a variety of amides (**1**–**6**) and their respective oximes (**7**–**10**). Their cytotoxic potential was evaluated in SRB assays employing several human tumor cell lines as well as non-malignant NIH 3T3 and HEK293 cells. As a result, the amides held a higher cytotoxicity than the oximes. The highest cytotoxicity was observed for a homopiperazinyl amide **5** with an $EC_{50} = 0.9$ µM for human melanoma cells. These results as well as extra FACS and

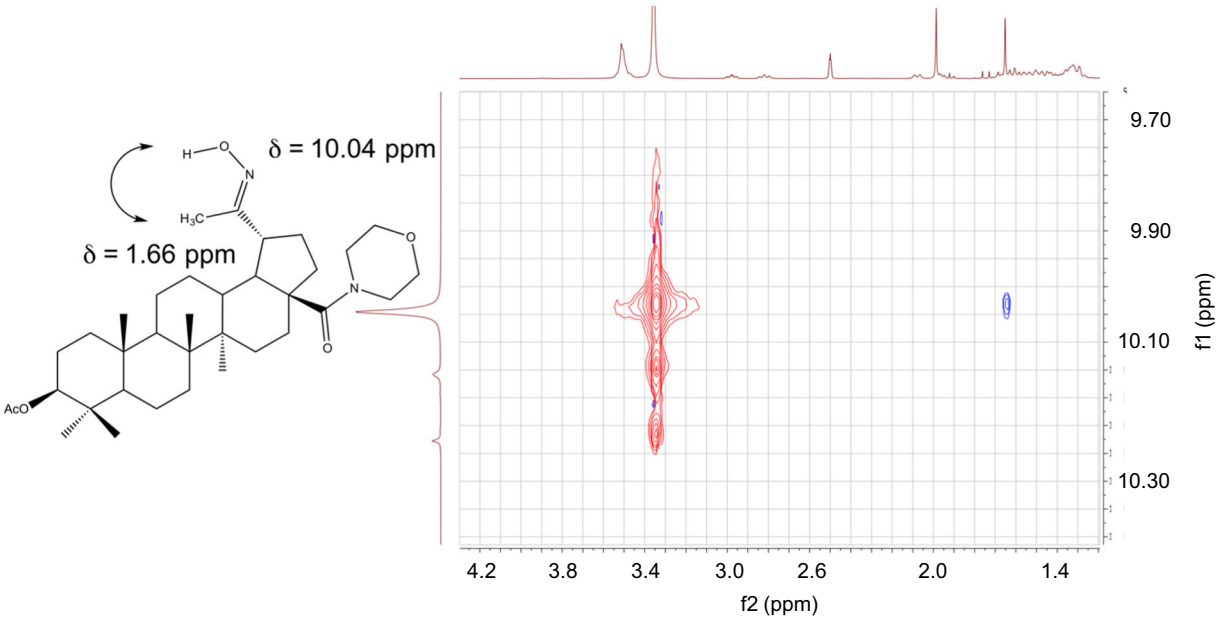


Fig. 2 Determination of the absolute configuration of oxime **9** by 2D ROESY NMR

Table 1 SRB assay EC_{50} values [μM] after 72 h of treatment; averaged from three independent experiments performed each in triplicate; confidence interval CI = 95%. Human cancer cell lines: A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), HeLa (cervical cancer), NIH 3T3 (non-malignant fibroblasts), HEK293 (human embryonic kidney cells); cut-off 30 μM, n.s. not soluble, n.d. not determined. Doxorubicin (**DX**) has been used as a positive standard

Compound	A375	HT29	MCF7	A2780	HeLa	NIH 3T3	HEK293
1	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
2	1.7 ± 0.7	3.8 ± 0.2	3.4 ± 0.2	3.0 ± 0.3	3.7 ± 0.2	1.6 ± 0.6	3.1 ± 0.2
3	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
4	1.9 ± 0.4	3.9 ± 0.2	2.7 ± 0.3	2.6 ± 0.4	2.9 ± 0.4	1.3 ± 0.1	2.7 ± 0.6
5	0.9 ± 0.1	2.3 ± 0.2	1.8 ± 0.2	1.6 ± 0.1	n.d.	0.6 ± 0.1	n.d.
6	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
7	7.9 ± 0.6	11.1 ± 0.3	6.3 ± 0.6	8.1 ± 0.8	9.8 ± 0.6	6.9 ± 0.9	6.1 ± 1.1
8	7.8 ± 1.2	10.1 ± 0.4	8.2 ± 0.4	10.0 ± 0.7	10.0 ± 0.5	5.5 ± 0.6	11.8 ± 1.2
9	9.0 ± 1.4	20.3 ± 1.0	6.8 ± 0.4	10.4 ± 0.8	13.6 ± 0.7	15.4 ± 1.8	7.4 ± 0.4
10	2.2 ± 0.4	4.8 ± 0.4	2.7 ± 0.3	3.5 ± 0.4	4.0 ± 0.2	1.8 ± 0.1	3.8 ± 0.3
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	n.d.	0.06 ± 0.03	n.d.

cell cycle measurements reflect that even small changes in the substitution pattern might lead to a significant change in activity and probably in their respective mode of action of these compounds.

Experimental

General

NMR spectra were recorded using the Varian spectrometers DD2 and VNMRS (400 and 500 MHz, respectively). MS spectra were taken on a Advion expression^{L} CMS mass spectrometer (positive ion polarity mode, solvent: methanol, solvent flow: 0.2 mL/min, spray voltage: 5.17 kV, source voltage: 77 V, APCI corona discharge: 4.2 μA, capillary temperature: 250 °C, capillary voltage: 180 V, sheath gas: N_2). Thin-layer chromatography was performed on pre-coated silica gel plates supplied by Macherey-Nagel. IR spectra were recorded on a Spectrum 1000 FT-IR-spectrometer from Perkin-Elmer. The UV/Vis-spectra were recorded on a Lambda 14 spectrometer from Perkin-Elmer. The optical rotations were measured either on a JASCO P-2000 or a Perkin-Elmer polarimeter at 20 °C. The melting points were determined using the Leica hot stage microscope Galen III and are uncorrected. Elemental analyses were performed on a Foss-Heraeus Vario EL (CHNS) unit. The solvents were dried according to usual procedures.

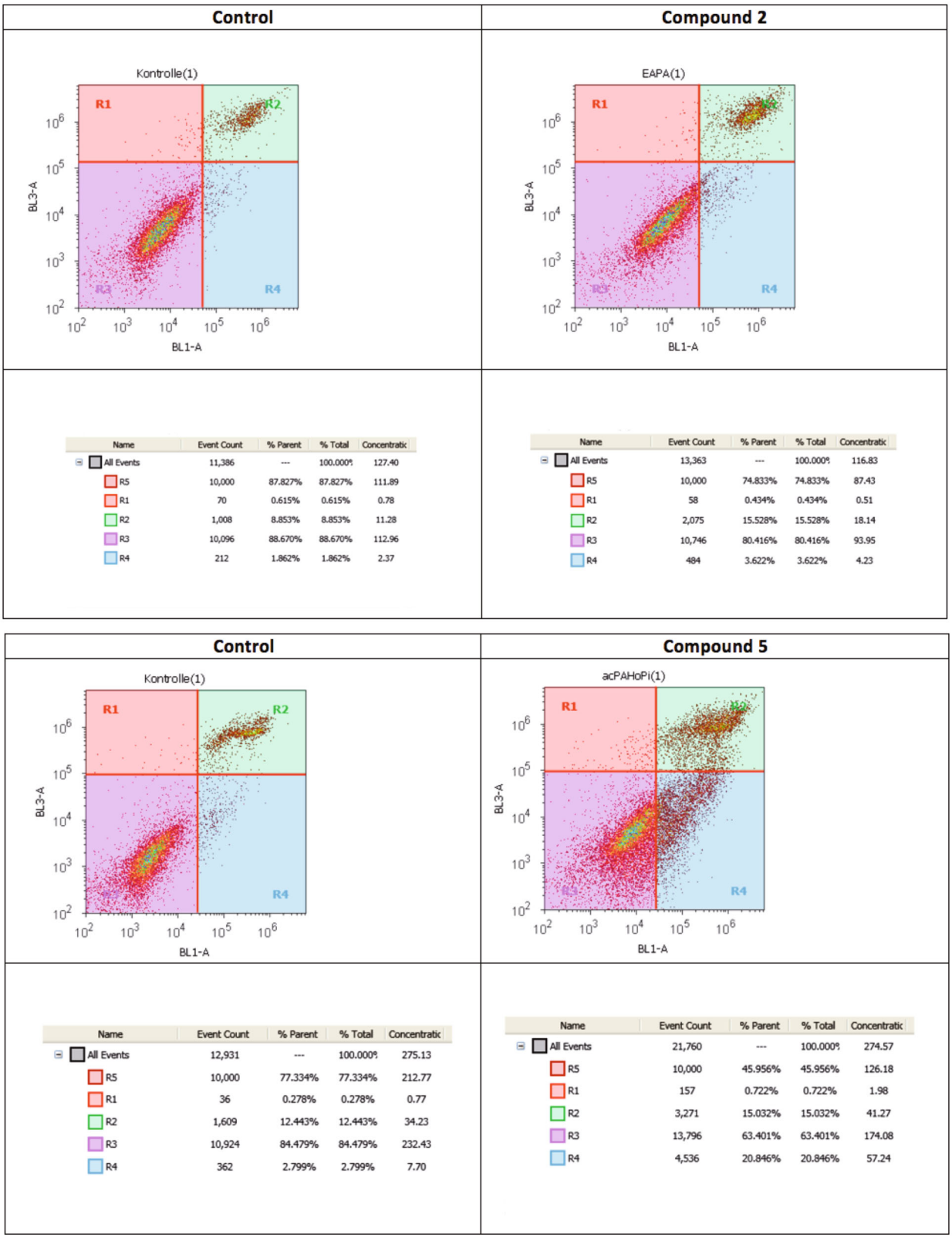
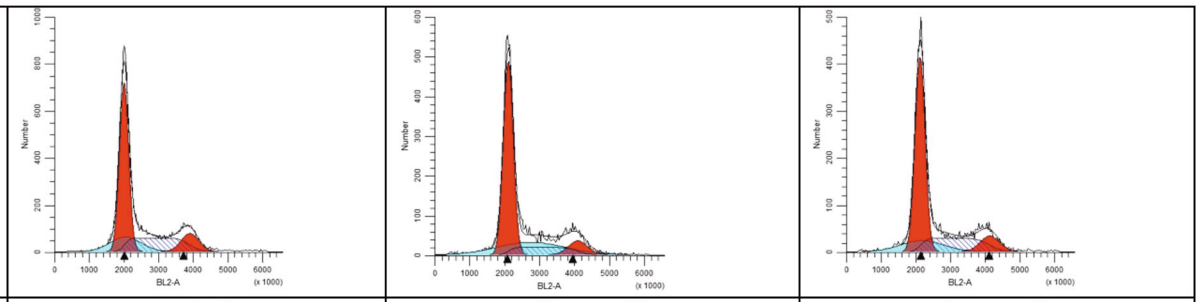


Fig. 3 FACS investigation of compounds **2** and **5** (48 h incubation, A375 cells)



	control	Compound 2	Compound 5
G1	59.9 %	73.8 %	65.3 %
S	27.5 %	15.9 %	24.4 %
G2/M	12.6 %	10.4 %	10.3 %
Apo	15.0 %	24.5 %	13.8 %

Fig. 4 Cell cycle investigation for compounds **2** and **5** (incubation 48 h, A375 cells), red G1 G2/M, striped S phase, blue apoptosis

Platanic acid was bought from "Betulinines" (Stříbrná Skalice, Czech Republic) and used as received.

Cell lines and culture conditions

Following human cancer cell lines A375 (malignant melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast cancer), A2780 (ovarian carcinoma), HeLa (cervical cancer), NIH 3T3 (non-malignant mouse fibroblasts) and HEK293 (human embryonic kidney cells) were used. All cell lines were obtained from the Department of Oncology (Martin-Luther-University Halle Wittenberg). Cultures were maintained as monolayers in RPMI 1640 medium with L-glutamine (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) supplemented with 10% heat-inactivated fetal bovine serum (Sigma-Aldrich GmbH, Steinheim, Germany) and penicillin/streptomycin (Capricorn Scientific GmbH, Ebsdorfergrund, Germany) at 37 °C in a humidified atmosphere with 5% CO_2.

Cytotoxicity assay (SRB assay)

For the evaluation of the cytotoxicity of the compounds the sulforhodamine-B (Kiton-Red S, ABCR GmbH, Karlsruhe, Germany) micro-culture colorimetric assay was used. The EC_{50} values were averaged from three independent experiments performed each in triplicate and calculated from semi-logarithmic dose-response curves applying a non-linear 4 P Hills-slope equation [10].

General procedure for the synthesis of amides (GPA)

To an ice-cold solution of 3-*O*-acetyl-platanic acid (1.8 g, 3.5 mmol) in dry DCM (20 mL), oxalyl chloride (0.6 mL, 7.0 mmol) and DMF (cat.) were added. After stirring for 3 h at 21 °C, the volatiles were removed under reduced pressure. The residue was dissolved in DCM (25 mL), and at 0 °C 4 equivalents of the corresponding amine were added; stirring at 21 °C was continued for 12 h. Usual aqueous workup followed by chromatographic purification of the crude product yielded the amides.

General procedure for the synthesis of oximes (GPB)

To a solution of the carboxamide in dry pyridine (19 mL), hydroxylammonium chloride was added, and the mixture was stirred at 60 °C for 3 h. The solvent was removed under reduced pressure by co-evaporating with toluene (3 × 20 mL). The residue was fractionated by column chromatography.

3β-Acetyloxy-N-(1-methylpiperazinyl) 20-oxo-30-norlupan-28-amide (1)

According to GPA with methylpiperazine (1.7 mL, 15.6 mmol) followed by column chromatography (silica gel, *n*-hexane/chloroform/methanol, 5:4.75:0.25) gave **1** (1.8 g, 76%) as a white solid; m. p. > 300 °C; $R_F = 0.22$ (*n*-hexane/chloroform/methanol, 5:4.75:0.25); $[\alpha]_D = -21.8°$ (*c* 0.12, $CHCl_3$); IR (ATR): $\nu = 2988$w, 2970 m, 2940 m, 2867 m, 2793 m, 1733s, 1716m, 1622s, 1458 m, 1413 m, 1369 m, 1346 m, 1300 m, 1289 m, 1248 s, 1222 m, 1195 m, 1167 m, 1143 m, 1133 m, 1111 m, 1077 m, 1036 m, 1027 m, 1009 m, 977 m, 900 m, 782 m, 751w, 695 m, 656w, 598 m, 549w, 509 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) $\delta = 4.45$ (dd, $J = 10.8$, 5.4 Hz, 1H), 3.62 (s, 4H, 32-H + 32'-H), 3.22 (dt, $J = 11.4$, 6.0 Hz, 1H), 2.67 (td, $J = 12.5$, 3.8 Hz, 1H), 2.39 (s, 4H, 33-H + 33'-H), 2.32 (s, 3H, 34-H), 2.14 (s, 3H, 29-H), 2.10–2.03 (m, 2H, 16-H_a + 18-H), 2.01 (s, 3H, 31-H), 1.99–1.85 (m, 2H, 22-H_a + 21-H_a), 1.66–1.55 (m, 4H, 1-H_a + 16-H_b + 2-H), 1.50–1.43 (m, 3H, 22-H_b + 21-H_b + 6-H_a), 1.43–1.32 (m, 5H, 11-H_a + 6-H_b + 7-H + 15-H_a), 1.31–1.25 (m, 2H, 11-H_b + 9-H), 1.18–1.13 (m, 1H, 15-

H_b), 1.06–1.00 (m, 1H, 12-H_a), 0.98–0.92 (m, 5H, 1-H_b + 27-H + 12-H_b), 0.90 (s, 3H, 26-H), 0.83 (s, 3H, 25-H), 0.82 (s, 3H, 23-H), 0.81 (s, 3H, 24-H), 0.79–0.75 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 212.9 (C-20), 173.2 (C-28), 170.8 (C-30), 80.8 (C-3), 55.4 (C-5), 55.0 (C-33 + C-33'), 54.3 (C-17), 52.4 (C-18), 50.5 (C-9), 49.9 (C-19), 45.7 (C-34), 41.6 (C-32 + C-32'), 40.5 (C-14), 38.2 (C-1), 37.7 (C-8), 37.0 (C-4 + C-10), 35.8 (C-13), 35.5 (C-22), 34.1 (C-7), 31.9 (C-16), 30.2 (C-29), CH3, 29.7 (C-15), 28.6 (C-21), 27.8 (C-23), 27.4 (C-12), 23.6 (C-2), 21.2 (C-31), 21.0 (C-11), 18.0 (C-6), 16.4 (C-24), 16.1 (C-25), 15.9 (C-26), 14.5 (C-27) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 605.4 ($[M + H + Na]^+$, 100%); analysis calcd for $C_{36}H_{58}N_2O_4$ (582.87): C 74.18, H 10.03, N 4.81; found: 73.86, H 10.29, N 4.57.

3β-Acetyloxy-N-(2-aminoethyl) 20-oxo-30-norlupan-28-amide (2)

According to GPA with ethylenediamine (0.7 mL, 11.0 mmol) followed by chromatography (silica gel, chloroform/methanol/ammonium hydroxide, 9:1:0.1) gave **2** (2.4 g, 87%) as a white solid; m.p. 230–233 °C (lit.: [46] 231–234 °C); R_F = 0.17 (chloroform/methanol/ammonium hydroxide, 9:1:0.1); $[\alpha]_D = -13.9°$ (*c* 0.13, $CHCl_3$) [lit].: [47] $[\alpha]_D = -8.5°$ (*c* 0.16, $CHCl_3$); IR (ATR): ν = 2941 m, 2866 m, 1732m, 1703m, 1633m, 1524 m, 1467 m, 1449 m, 1391 m, 1367 m, 1317 m, 1247 s, 1196 m, 1163 m, 1108w, 1073w, 1028 m, 979 m, 945 m, 901 m, 866 m, 820w, 753 m, 657 m, 610 m, 556 m, 506 m cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.44 (dd, *J* = 10.9, 5.3 Hz, 1H, 3-H), 3.42–3.24 (m, 3H, 19-H + 32-H), 2.86 (t, *J* = 5.9 Hz, 2H, 33-H), 2.21 (td, *J* = 12.0, 4.2 Hz, 1H, 13-H), 2.14 (s, 3H, 29-H), 2.12–2.02 (m, 2H, 18-H + 21-H_a), 2.01 (s, 3H, 31-H), 1.99–1.95 (m, 1H, 16-H_a), 1.81–1.76 (m, 1H, 22-H_a), 1.66–1.56 (m, 4H, 1-H_a + 16-H_b + 2-H), 1.52–1.43 (m, 3H, 22-H_b + 21-H_b + 6-H_a), 1.43–1.34 (m, 4H, 15-H_a + 11-H_a + 6-H_b + 7-H_a), 1.33–1.29 (m, 1H, 7-H_b), 1.28–1.20 (m, 2H, 11-H_b + 9-H), 1.18–1.13 (m, 1H, 15-H_b), 1.10–0.99 (m, 2H, 12-H), 0.97 (s, 3H, 27-H), 0.95–0.91 (m, 1H, 1-H_b), 0.89 (s, 3H, 26-H), 0.83–0.81 (m, 6H, 25-H + 23-H), 0.80 (s, 3H, 24-H), 0.78–0.74 (m, 1H, 5-H); ^{13}C NMR (126 MHz, $CDCl_3$) δ = 213.0 (C-20), 176.8 (C-28), 171.1 (C-30), 81.0 (C-3), 55.7 (C-17), 55.5 (C-5), 51.3 (C-19), 50.5 (C-9), 50.2 (C-18), 42.4 (C-14), 41.5 (C-33), 41.1 (C-32), 40.8 (C-8), 38.5 (C-1), 38.1 (C-22), 37.9 (C-4), 37.2 (C-10), 36.9 (C-13), 34.4 (C-7), 33.0 (C-16), 30.2 (C-29), 29.6 (C-15), 28.7 (C-21), 28.0 (C-23), 27.3 (C-12), 23.8 (C-2), 21.4 (C-31), 21.0 (C-11), 18.3 (C-6), 16.6 (C-24), 16.3 (C-25), 16.2 (C-26), 14.8 (C-27) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 541.3 ($[M]^-$, 100%); analysis calcd for $C_{33}H_{54}N_2O_4$ (542.79): C 73.02, H 10.03, N 5.16; found: 72.86, H 10.29, N 46.

3β-Acetyloxy-N-(4-morpholinyl) 20-oxo-30-norlupan-28-amide (3)

According to GPA with morpholine (1.4 mL, 16.4 mmol) followed by chromatography (silica gel, *n*-hexane/chloroform/acetone, 5:4.75:0.25) gave **3** (1.7 g, 73%) as a white solid; m.p. 255–258 °C; R_F = 0.17 (*n*-hexane/chloroform/acetone, 5:4.75:0.25); $[\alpha]_D = -20.5°$ (*c* 0.14, $CHCl_3$); IR (ATR): ν = 2943 m, 2864 m, 1732m, 1709m, 1634s, 1452 m, 1409 m, 1367 m, 1314w, 1263 m, 1244 s, 1188 s, 1173w, 1116 s, 1066w, 1031 s, 979 m, 901w, 844w, 751w, 609w, 598w, 577w, 556w, 507w cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.45 (dd, *J* = 10.5, 5.5 Hz, 1H, 3-H), 3.67–3.53 (m, 8H, 33-H + 33'-H + 32-H + 32'-H), 3.26 – 3.18 (m, 1H, 19-H), 2.68 (td, *J* = 12.2, 4.0 Hz, 1H, 13-H), 2.16 (s, 3H, 29-H), 2.11–2.03 (m, 2H, 16-H_a + 18-H), 2.02 (s, 3H, 31-H), 1.97–1.86 (m, 2H, 22-H_a + 21-H_a), 1.65–1.58 (m, 3H, 1-H_a + 16-H_b + 2-H_a), 1.58–1.45 (m, 4H, 2-H_b + 22-H_b + 21-H_b + 6-H_a), 1.44–1.42 (m, 1H, 11-H_a), 1.41–1.34 (m, 4H, 6-H_b + 7-H + 15-H_a), 1.31–1.23 (m, 2H, 9-H + 11-H_b), 1.21–1.14 (m, 1H, 15-H_b), 1.07–1.01 (m, 1H, 12-H_a), 0.97 (s, 3H, 27-H), 0.96–0.92 (m, 2H, 1-H_b + 12-H_b), 0.91 (s, 3H, 26-H), 0.83 (s, 3H, 25-H), 0.82 (s, 3H, 23-H), 0.82 (s, 3H, 24-H), 0.80–0.76 (m, 1H, 5-H) ppm;^{13}C NMR (101 MHz, $CDCl_3$) δ = 213.0 (C-20), 173.7 (C-28), 171.1 (C-30), 81.0 (C-3), 67.1 (C-33 + C-33' + C-32 + C-32'), 55.6 (C-5), 54.6 (C-17), 52.6 (C-18), 50.8 (C-9), 50.1 (C-19), 41.9 (C-14), 40.7 (C-8), 38.5 (C-1), 37.9 (C-4), 37.3 (C-10), 36.1 (C-13), 35.7 (C-22), 34.4 (C-7), 32.1 (C-16), 30.5 (C-29), 30.0 (C-15), 28.9 (C-21), 28.1 (C-23), 27.6 (C-12), 23.8 (C-2), 21.4 (C-31), 21.3 (C-11), 18.3 (C-6), 16.6 (C-24), 16.4 (C-25), 16.2 (C-26), 14.8 (C-27) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 592.5 ($[M + H + Na]^+$, 100%); analysis calcd for $C_{35}H_{55}NO_5$ (569.81): C 73.77, H 9.73, N 2.46; found: C 73.55, H 9.97, N 2.11.

3β-Acetyloxy-N-(1-piperazinyl) 20-oxo-30-norlupan-28-amide (4)

According to GPA with piperazine (0.7 g, 8.0 mmol) followed chromatography (silica gel, $CHCl_3$/MeOH, 9:1) gave compound **4** (1.1 g, 95%) as a white solid; m.p. 220–223 °C (lit.: [10]115–125 °C); R_F = 0.15 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D = -20.3°$ (*c* 0.13, $CHCl_3$); IR (ATR): ν = 2941 m, 2866 m, 1731m, 1709m, 1628s, 1450 m, 1413 m, 1393 m, 1367 m, 1318w, 1244 s, 1193 s, 1163 m, 1133 m, 1110w, 1102w, 1027 s, 979 m, 900w, 858w, 803w, 752 m, 679w, 659w, 608w, 572w, 552w 507w, 453w cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.45 (dd, *J* = 10.7, 5.4 Hz, 1H, 3-H), 3.78–3.63 (m, 4H, 32-H + 32'-H), 3.20 (t, *J* = 11.4 Hz, 1H, 19-H), 3.00–2.89 (m, 4H, 32-H + 32'-H), 2.64 (t, *J* = 10.3 Hz, 1H, 13-H), 2.15 (s, 3H, 29-H), 2.10–2.03 (m, 2H, 18-H + 16-H_a), 2.02 (s, 3H, 31-H), 1.94–1.84 (m, 2H, 22-H_a + 21-H_a),

1.67-1.55 (m, 4H, 1-H_a + 16-H_b + 2-H), 1.53-1.45 (m, 3 H, 22-H_b + 6-H_a + 21-H_b), 1.42-1.30 (m, 5H, 11-H_a + 6-H_b + 7-H + 15-H_a), 1-29-1.28 (m, 1H, 9-H), 1.25-1.21 (m, 1H, 11-H_b), 1.20-1.15 (m, 1H, 15-H_b), 1.07-1.00 (m, 1H, 12-H_a), 0.99-0.94 (m, 5H, 1-H_b + 27-H + 12-H_b), 0.90 (s, 3H, 26-H), 0.83 (s, 3H, 25-H), 0.82 (s, 3H, 23-H), 0.81 (s, 3H, 24-H), 0.80-0.75 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 212.9 (C-20), 173.7 (C-28), 171.1 (C-30), 81.0 (C-3), 58.5 (C-33 + C-33'), 55.6 (C-5), 54.6 (C-17), 52.6 (C-18), 50.7 (C-9), 50.1 (C-19), 45.4 (C-32 + C-32') 41.9 (C-14), 40.7 (C-8), 38.5 (C-1), 37.9 (C-4), 37.3 (C-10), 36.1 (C-13), 35.7 (C-22), 34.3 (C-7), 32.1 (C-16), 30.5 (C-29), 30.0 (C-15), 28.9 (C-21), 28.0 (C-23), 27.6 (C-12), 23.8 (C-2), 21.4 (C-31), 21.3 (C-11), 18.3 (C-6), 16.6 (C-24), 16.4 (C-25), 16.2 (C-26), 14.8 (C-27) ppm; MS (ESI, MeOH: $CHCl_3$, 4:1): m/z 569.6 ($[M]^+$, 100%); analysis calcd for $C_{35}H_{56}N_2O_4$ (568.83): C 73.90, H 9.92, N 4.92; found: C 73.65, H 10.13, N 4.75.

3β-Acetyloxy-N-(1-homopiperazinyl) 20-oxo-30-norlupan-28-amide (5)

According to GPA with homopiperazine (0.4 g, 4.0 mmol) followed by column chromatography (silica gel, $CHCl_3$/MeOH, 95:5) gave **5** (387 mg, 67%) as a white solid; m. p. 160-165 °C; R_F = 0.14 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = -29.2° (*c* 0.16, $CHCl_3$); IR (ATR): ν = 2940 *m*, 1732*s*, 1622*s*, 1367 *m*, 1244*vs*, 979 *m*, 750 *s* cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$): δ = 4.45 (*dd*, *J* = 10.7, 5.4 Hz, 1H, 3-H), 3.24 (*td*, *J* = 11.4, 3.6 Hz, 1H, 19-H), 3.89 – 2.55 (*m*, 8H, 32-H, 33-H, 34-H, 36-H), 2.73 (*t*, *J* = 12.4 Hz, 1H, 13-H), 2.15 (*s*, 3H, 29-H), 2.14 – 2.02 (*m*, 3H, 16-H_a, 18-H, 22-H_a), 2.02 (*s*, 3H, 31-H), 1.98 – 1.87 (*m*, 1H, 21-H_a), 1.68 – 1.12 (*m*, 17H, 35-H, 1-H_a, 2-H, 16-H_b, 22-H_b, 21-H_b, 6-H, 11-H_a, 7-H, 15-H_a, 9-H, 11-H_b, 15-H_b), 1.06 – 0.99 (*m*, 2H, 12-H), 0.97 (*s*, 3H, 27-H), 0.96 – 0.94 (*m*, 1H, 1-H_b), 0.91 (*s*, 3H, 26-H), 0.82 (*s*, 3H, 25-H), 0.82 (*s*, 3H, 23-H), 0.81 (*s*, 3H, 24-H), 0.79 – 0.74 (*m*, 1H, 5-H); ^{13}C NMR (126 MHz, $CDCl_3$): δ = 213.4 (C-20), 174.7 (C-28), 171.3 (C-30), 81.3 (C-3), 55.9 (C-5), 55.3 (C-17), 53.2 (C-18), 51.0 (C-9), 50.5 (C-19), 42.2 (C-14), 41.0 (C-8), 38.8 (C-1), 38.2 (C-4), 37.5 (C-10), 36.3 (C-13), 36.3 (C-22), 34.6 (C-7), 32.2 (C-16), 30.7 (C-29), 30.3 (C-15), 29.2 (C-21), 28.3 (C-23), 27.8 (C-12), 24.1 (C-2), 21.7 (C-31), 21.6 (C-11), 18.5 (C-6), 16.9 (C-24), 16.6 (C-25), 16.4 (C-26), 15.1 (C-27) ppm; MS (ESI, MeOH): m/z 583.3 ($[M]^+$, 100%); analysis calcd. for $C_{36}H_{58}N_2O_4$ (582.86): C 74.18, H 10.03, N 4.81; found: C 73.82, H 10.31, N 4.56.

3β-Acetyloxy-N-(1,1-dimethylpiperazin-1-ium-4-yl) 20-oxo-30-norlupan-28-amide iodide (6)

A solution of **1** (1.8 g, 3.0 mmol) and iodomethane (3.2 mL, 51 mmol) in dry DCM (60 mL) was stirred for 1 day at 21 °C. The volatiles were removed under reduced pressure followed by chromatography (silica gel, chloroform/methanol/formic acid, 4.5:0.4:0.1) to afford **6** (1.2 g, 63%) as a yellowish solid; m.p. 130-231 °C; R_F = 0.27 (chloroform/methanol/formic acid, 4.5:0.4:0.1); $[\alpha]_D$ = −1.3° (*c* 0.13, $CHCl_3$); IR (ATR): ν = 2825 m, 2776w, 2740w, 2696w, 1646m, 1578 s, 1382 m, 1351 s, 1227 m, 1066 m, 790 m, 764 m, 726 m cm^{-1}; ^{1}H NMR (500 MHz, CD_3OD): δ = 4.44-4.39 (m, 1H, 3-H), 4.05-3.89 (m, 4H, 32-H + 32'-H), 3.45 (s, 4H, 33-H + 33'-H), 3.25 (s, 6H, 34-H + 35-H), 3.19-3.11 (m, 1H, 19-H), 2.70-2.61 (m, 1H, 13-H), 2.15 (s, 3H, 29-H), 2.14-2.01 (m, 3H, 21-H_a + 18-H + 22-H_a), 1.99 (s, 3H, 31-H), 1.97-1.88 (m, 1H, 16-H_a), 1.71-1.55 (m, 4H, 1-H_a + 21-H_b + 2-H), 1.55-1.33 (m, 9H, 16-H_b + 22-H_b + 6-H_a + 11-H_a + 6-H_b + 7-H + 15-H_a + 9-H), 1.32-1.23 (m, 2H, 11-H_b + 15-H_b), 1.11-1.05 (m, 1H, 12-H_a), 0.99 (s, 3H, 27-H), 0.98-0.94 (m, 2H, 1-H_b + 12-H_b), 0.92 (s, 3H, 26-H), 0.87 (s, 3H, 25-H), 0.83 (s, 3H, 23-H), 0.83 (s, 3H, 24-H), 0.81-0.78 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, CD_3OD) δ = 215.6 (C-20), 175.6 (C-28), 172.9 (C-30), 82.5 (C-3), 62.4 (C-33 + C-33'), 56.9 (C-5), 55.8 (C-17), 53.9 (C-18), 52.0 (C-34 + C-35), 52.0 (C-9), 51.6 (C-19), 42.9 (C-14), 41.9 (C-8), 39.6 (C-1), 39.5 (C-32 + C-32'), 38.8 (C-4), 38.3 (C-10), 37.4 (C-13), 36.5 (C-22), 35.4 (C-7), 32.8 (C-21), 31.1 (C-15), 29.8 (C-29), 29.7 (C-16), 28.5 (C-12), 28.4 (C-23), 24.6 (C-2), 22.4 (C-11), 21.1 (C-31), 19.2 (C-6), 16.9 (C-24), 16.8 (C-25), 16.6 (C-26), 15.0 (C-27) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 597.0 ($[M-I]^+$, 100%); analysis calcd for $C_{37}H_{61}IN_2O_4$ (724.79): C 61.31, H 8.48, N 3.87; found: C 61.03, H 8.67, N 3.58.

(3β, 20E) 3-Acetyloxy-20-hydroxyimino-N-(1-methylpiperazinyl)-30-norlupan-28-amide (7)

According to GPB from **1** (1.9 g, 3.3 mmol) and hydroxylammonium chloride (1.5 g, 22 mmol) followed by chromatography (silica gel, *n*-hexane/chloroform/methanol, 5:4.5:0.5) **7** (1.5 g, 75%) was obtained as a white solid; m.p. 295-300 °C; R_F = 0.27 (*n*-hexane/chloroform/methanol, 5:4.5:0.5); $[\alpha]_D$ = +0.21° (*c* 0.12, MeOH); IR (ATR): ν = 2944 m, 2872 m, 2686w, 2583w, 2513w, 2452w, 1712m, 1627s, 1456 m, 1449 m, 1488 m, 1373 s, 1315w, 1300w, 1249 s, 1206 m, 1153 m, 1130w, 1086w, 1049 m, 1025 m, 975 s, 947 m, 911w, 850w, 763 m, 746 m, 690 m, 667 m, 606 m, 545 m, 511 m, 476 m cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ 4.36 (dd, *J* = 11.6, 4.6 Hz, 1H, 3-H), 3.70-3.30 (m, 6H, 33-H + 33'-H + 32-H), 3.00 – 2.93 (m, 1H, 19-H), 2.93-2.82 (m, 2H, 32'-H), 2.80-2.75 (m, 1H, 13-H), 2.74 (s, 3H, 34-H), 2.10-2.01 (m, 1H, 16-H_a), 1.98 (s, 3H, 31-H), 1.95-1.90 (m, 1H, 22-H_a), 1.77-1.70 (m, 1H, 21-H_a), 1.66 (s, 3H, 29-H), 1.64-1.37 (m, 9H, 18-H + 1-H_a + 2-H_a + 12-H_a + 16-H_b + 2-H_b + 22-H_b + 6-H_a + 21-H_b), 1.37-1.24 (m, 6H, 11-H_a + 6-H_b + 7-H + 9-H + 15-H_b), 1.16-

1.08 (m, 2H, 11-H_b + 15-H_b), 0.97-0.92 (m, 1H, 1-H_b), 0.91 (s, 3H, 27-H), 0.85 (s, 3H, 26-H), 0.83-0.81 (m, 1H, 12-H_b), 0.80 (s, 3H, 25-H), 0.79-0.74 (m, 7H, 23-H + 24-H + 5-H) ppm; ^{13}C NMR (126 MHz, DMSO-d_6) δ = 172.8 (C-28), 170.1 (C-30), 159.9 (C-20), 79.9 (C-3), 54.7 (C-5), 53.6 (C-17), 52.1 (C-33 + C-33‘ + C-32 + C-32‘), 51.5 (C-18), 49.8 (C-9), 43.3 (C-19), 41.8 (C-34), 41.4 (C-14), 40.2 (C-8), 37.8 (C-1), 37.4 (C-4), 36.6 (C-10), 35.0 (C-22), 35.8 (C-13), 33.7 (C-7), 31.5 (C-16), 29.3 (C-15), 28.5 (C-21), 27.6 (C-23), 25.1 (C-12), 23.4 (C-2), 21.0 (C-31), 20.6 (C-11), 17.7 (C-6), 16.4 (C-24), 15.9 (C-25), 15.7 (C-26), 14.3 (C-27), 10.7 (C-29) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 598.6 ($[M + CH_3OH + H]^+$, 100%) 1197.3 ($[2\,M + 2CH_3OH + H]^+$, 10%); analysis calcd for $C_{36}H_{59}N_3O_4$ (597.87): C 72.32, H 9.95, N 7.03; found: 72.08, H 10.15, N 6.80.

(3β, 20E) 3-Acetyloxy-20-hydroxyimino-N-(2-aminoethyl)-30-norlupan-28-amide (8)

According to GPB from **2** (1.3 g, 2.5 mmol) and hydroxylammonium chloride (1.2 g, 17 mmol) followed by chromatography (silica gel, *n*-hexane/chloroform/methanol, 4:3.5:1.5) **8** (0.7 g, 51%) was obtained as a white solid; m.p. > 300 °C; $R_F = 0.22$ (*n*-hexane/chloroform/methanol, 4:3.5:1.5); $[\alpha]_D = +3.12°$ (*c* 0.10, MeOH); IR (ATR): ν = 2940 s, 2872 m, 1733m, 1709m, 1657m, 1639m, 1516 m, 1467 m, 1451 m, 1367 s, 1317w, 1245 s, 1195 m, 1172w, 1156w, 1131w, 1108w, 1024 s, 978 s, 946 m, 902 m, 878w, 850 m, 803w, 774w, 691 m, 611 m, 560 m, 548 m, 500 m, 471 m cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ = 4.36 (dd, *J* = 11.5, 4.6 Hz, 1H, 3-H), 3.32-3.28 (m, 2H, 32-H), 3.10 (dt, *J* = 11.0, 5.6 Hz, 1H, 19-H), 2.82-2.73 (m, 2H, 33-H), 2.54-2.46 (m, 1H, 13-H), 2.24-2.17 (m, 1H, 16-H_a), 1.99 (s, 3H, 31-H), 1.90-1.84 (m, 1H, 22-H_a), 1.74-1.66 (m, 1H, 21-H_a), 1.64 (s, 3H, 29-H), 1.60-1.46 (m, 5H, 1-H_a + 2-H_a + 18-H + 12-H_a + 2-H_b), 1.46-1.37 (m, 3H, 6-H_a + 16-H_b + 22-H_b), 1.37-1.22 (m, 7H, 11-H_a + 21-H_b + 6-H_b + 7-H + 9-H + 15-H_a), 1.19-1.08 (m, 1H, 11-H_b), 1.06-1.01 (m, 1H, 15-H_b), 0.97-0.91 (m, 1H, 1-H_b), 0.90 (s, 3H, 27-H), 0.84 (s, 3H, 26-H), 0.83-0.81 (m, 1H, 12-H_b), 0.80 (s, 3H, 25-H), 0.79-0.74 (m, 7H, 23-H + 24-H + 5-H) ppm; ^{13}C NMR (126 MHz, DMSO-d_6) δ = 176.1 (C-28), 170.1 (C-30), 159.7 (C-20), 79.9 (C-3), 54.7 (C-5), 54.6 (C-17), 49.7 (C-9), 49.4 (C-18), 44.0 (C-19), 41.8 (C-14), 40.2 (C-8), 38.6 (C-33), 37.7 (C-1), 37.4 (C-4), 37.3 (C-22), 36.6 (C-10), 36.5 (C-32), 36.3 (C-13), 33.7 (C-7), 32.0 (C-16), 28.9 (C-15), 28.2 (C-21), 27.6 (C-23), 25.1 (C-12), 23.4 (C-2), 21.0 (C-31), 20.4 (C-11), 17.7 (C-6), 16.4 (C-24), 15.9 (C-25), 15.8 (C-26), 14.3 (C-27), 10.6 (C-29) ppm;; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 558.1 ($[M + H]^+$, 100%) 1170.5 ($[2\,M + H]^+$, 50%); analysis calcd for $C_{33}H_{55}N_3O_4$ (557.81): C 71.06, H 9.94, N 7.53; found: C 70.78, H 10.12, N 7.35.

(3β, 20E) 3-Acetyloxy-20-hydroxyimino-N-(4-morpholinyl)-30-norlupan-28-amide (9)

According to GPB from **3** (1.5 g, 2.6 mmol) and hydroxylammonium chloride (1.2 g, 17 mmol) followed by chromatography (silica gel, *n*-hexane/chloroform/methanol, 5:4.75:0.25) compound **9** (1.3 g, 85%) was obtained as a white solid; m.p. 283-286 °C; $R_F = 0.24$ (*n*-hexane/chloroform/methanol, 5:4.75:0.25); $[\alpha]_D = +0.76°$ (*c* 0.10, MeOH); IR (ATR): ν = 3400 m, 2967 m, 2940 m, 2927 m, 2860 m, 1735w, 1706s, 1636s, 1467w, 1445 m, 1393 m, 1385 m, 1363 m, 1313w, 1299w, 1268 s, 1224w, 1185 m, 1119 s, 1065w, 1046 m, 1031 m, 978 m, 948w, 915w, 904w, 881w, 854 m, 751w, 662 m, 646 m, 596 m, 550w, 510w cm^{-1}; ^{1}H NMR (500 MHz, DMSO-d_6) δ = 4.37 (dd, *J* = 11.6, 4.7 Hz, 1H, 3-H), 3.56-3.46 (m, 8H, 33-H + 33'-H + 32-H + 32'-H), 2.98 (q, *J* = 6.5 Hz, 1H, 19-H), 2.86-2.79 (m, 1H, 13-H), 2.11-2.05 (m, 1H, 16-H_a), 1.99 (s, 3H, 31-H), 1.97-1.92 (m, 1H, 22-H_a), 1.74-1.68 (m, 1H, 21-H_a), 1.65 (s, 3H; 29-H), 1.64-1.43 (m, 8H, 18-H + 1-H_a + 12-H_a + 2-H_a + 2-H_b + 16-H_b + 22-H_b + 6-H_a), 1.42-1.29 (m, 7H, 21-H_b + 6-H_b + 11-H_a + 7-H + 9-H + 15-H_a), 1.19-1.08 (m, 2H, 11-H_b + 15-H_b), 0.98-0.92 (m, 1H, 1-H_b), 0.91 (s, 3H, 27-H), 0.85 (s, 3H, 26-H), 0.84-0.82 (m, 1H, 12-H_b), 0.81 (s, 3H, 25-H), 0.79 (m, 7H, 23-H + 24-H + 5-H) ppm; ^{13}C NMR (126 MHz, DMSO-d_6) δ = 172.5 (C-28), 170.1 (C-30), 159.8 (C-20), 79.9 (C-3), 66.3 (C-33 + C-33‘ + C-32 + C-32‘), 54.7 (C-5), 53.5 (C-17), 51.6 (C-18), 49.8 (C-9), 43.4 (C-19), 41.4 (C-14), 40.2 (C-8), 37.8 (C-1), 37.4 (C-4), 36.6 (C-10), 35.8 (C-13), 35.0 (C-22), 33.7 (C-7), 31.4 (C-16), 29.2 (C-15), 28.5 (C-21), 27.6 (C-23), 25.1 (C-12), 23.4 (C-2), 20.9 (C-31), 20.6 (C-11), 17.7 (C-6), 16.4 (C-24), 15.9 (C-25), 15.7 (C-26), 14.3 (C-27), 10.6 (C-29) ppm; MS (ESI, MeOH:$CHCl_3$, 4:1): m/z 585.7 ($[M + H]^+$, 86%) 1115.2 ($[2\,M + H]^+$, 20%); analysis calcd for $C_{35}H_{56}N_2O_5$ (584.83): C 71.88, H 9.65, N 4.79; found: 71.64, H 9.87, N 4.51.

(3β, 20E) 3-Acetyloxy-20-hydroxyimino-N-(1-piperazinyl)-30-norlupan-28-amide (10)

According to GPB from **4** (1.5 g, 2.6 mmol) and hydroxylammonium chloride (1.2 g, 17 mmol) followed by chromatography (silica gel, *n*-hexane/chloroform/methanol, 4:3.5:1.5) compound **10** (1.1 g, 75%) was obtained as white solid; m.p. 211-215 °C; $R_F = 0.17$ (*n*-hexane/chloroform/methanol, 4:3.5:1.5); $[\alpha]_D = -14.4°$ (*c* 0.11, $CHCl_3$); IR (ATR): ν = 2943 s, 2713 s, 2667 s, 2163w, 1983w, 1894w, 1732m, 1623m, 1577 m, 1506 m, 1468 m, 1393 m, 1372 m, 1315 m, 1246 m, 1193 m, 1160 m, 1146 m, 1027 m, 999 s, 981 m, 947 m, 901 m, 850 m, 575 s, 540 s, 510 s, 471 s, 419 s cm^{-1}; ^{1}H NMR (500 MHz, $CDCl_3$) δ = 4.48 – 4.43 (m, 1H, 3-H), 3.78-3.67 (m, 4H, 33-H + 33'-H), 3.07 (m, 1H, 19-H),

3.04-2.90 (m, 4H, 32-H + 32'-H), 2.81 (m,1H, 13-H), 2.11-2.04 (m, 1H, 16-H_a), 2.03 (s, 3H, 31-H), 2.00-1.82 (m, 2H, 22-H_a + 21-H_a), 1.80 (s, 3H, 29-H), 1.75-1.46 (m, 9H, 18-H + 1-H_a + 2-H + 16-H_b + 12-H_a + 21-H_b + 6-H_a + 22-H_b), 1.43-1.39 (m, 1H, 11-H_a), 1.39-1.31 (m, 4H, 6-H_b + 7-H + 15-H_a), 1.30-1.24 (m, 2H, 9-H + 11-H_b), 1.19-1.13 (m, 1H, 15-H_b), 0.98-0.93 (m, 2H, 1-H_b + 12-H_b), 0.92 (s, 3H, 27-H), 0.90 (s, 3H, 26-H), 0.83 (s, 3H, 25-H), 0.82 (s, 3H, 23-H), 0.82 (s, 3H, 24-H), 0.79-0.75 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 173.4 (C-28), 171.2 (C-30), 162.7 (C-20), 81.1 (C-3), 58.5 (C-33 + C-33'), 55.7 (C-5), 54.4 (C-17), 52.7 (C-18), 50.8 (C-9), 45.0 (C-32 + C-32'), 44.0 (C-19), 41.9 (C-14), 40.8 (C-8), 38.6 (C-1), 37.9 (C-4), 37.3 (C-10), 36.5 (C-13), 35.8 (C-22), 34.4 (C-7), 32.5 (C-16), 29.9 (C-15), 29.3 (C-21), 28.1 (C-23), 26.0 (C-12), 23.8 (C-2), 21.4 (C-31), 21.3 (C-11), 18.3 (C-6), 16.6 (C-24), 16.4 (C-25), 16.2 (C-26), 14.7 (C-27), 11.8 (C-29) ppm; MS (ESI, MeOH: $CHCl_3$, 4:1): m/z 584.0 ($[M + H]^+$, 50%) 1167.0 ($[2\,M + H]^+$, 100%); analysis calcd for $C_{35}H_{57}N_3O_4$ (583.86): C 72.00, H 9.84, N 7.20; found: 71.86, H 10.03, N 6.97.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Compliance with ethical standards

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