Results in Chemistry 4 (2022) 100429

Contents lists available at ScienceDirect

Results in Chemistry

journal homepage: www.sciencedirect.com/journal/results-in-chemistry

Betulinic acid and glycyrrhetinic acid derived piperazinyl spacered rhodamine B conjugates are highly cytotoxic and necrotic

Marie Kozubek [a], Sophie Hoenke [a], Hans-Peter Deigner [b], René Csuk [a,*]

[a] Martin–Luther–University Halle–Wittenberg, Organic Chemistry, Kurt–Mothes–Str. 2, D–06120 Halle (Saale), Germany
[b] Furtwangen University, Institute of Precision Medicine, Medical and Life Science Faculty, Jakob–Kienzle–Str. 17, D-78054 Villingen–Schwenningen, Germany

ARTICLE INFO

Keywords:
Betulin
Betulinic acid
Glycyrrhetinic acid
Cytotoxicity
Rhodamine B conjugates

ABSTRACT

Pentacyclic triterpene-piperazine-rhodamine B conjugates with ursane or oleanane backbones have been shown in the past to be highly cytotoxic thereby acting as mitocans. Starting from betulinic acid or glycyrrhetinic acid, new analogues were now made available, and their cytotoxic activity was investigated employing several human tumor cell lines [A375 (melanoma), HT29 (colorectal carcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), and for comparison NIH 3T3 (non-malignant fibroblasts)]. For these conjugates it has been established that the linking position at ring E governs the magnitude of cytotoxicity. These conjugates were still highly cytotoxic but significantly less cytotoxic than those holding a oleanane skeleton. Staining experiments showed the rhodamine B conjugates as necrotic compounds and to act as mitocans. The most active compound (**8**) held an EC_{50} = 0.04 μM for A2780 ovarian carcinoma cells.

Introduction

For a long time, the potential of pentacyclic triterpene carboxylic acids was underestimated. Although betulin (**BN**, Fig. 1) was first isolated and described by J. T. Lowitz [1] as early as 1788, it was not until 1995 that the cytotoxic effect of the BN-derived betulinic acid (**BA**) against melanoma was recognized by E. Pisha et al. [2] For decades since then, a large number of pentacyclic triterpene carboxylic acids have been isolated from a wide variety of different plant sources and also studied for their cytotoxic potential. [3–11] However, many of these compounds were only weakly cytotoxic or not cytotoxic at all. Even **BA**-derived platanic acid (**PA**) did not live up to the expectations placed in it, since **PA** itself is also practically not cytotoxic and, in addition, it is even more poorly soluble than betulinic acid in biological fluids. [12–21].

More recently, a renaissance of this class of compounds has been achieved, as acylated amides have been shown to have good cytotoxicity, in particular a diacetylated benzylamide ("**EM2**") [22–27] derived from maslinic acid or (iso)-quinolinyl amides ("**IQAA**") [28] derived from augustic acid.

Triterpenoid piperazine amides also stand out as cytotoxic, but are far surpassed [29] by derivatives that have a general structure as an acetylated triterpene carboxylic acid-piperazine-rhodamine B conjugate. [30–36] Thereby, a triterpene carboxylic acid – acetylated one or more times in ring A – is linked to rhodamine B via a piperazine residue (attached to the triterpene carboxylic acid as an amide) at its distal nitrogen to form a cationic, lipophilic conjugate. These compounds are supposed to interact with mitochondrial membranes and therefore act as mitocans (mitochondria targeted drugs) even in the low nanomolar concentration range. [29].

Thereby, compounds of the ursane or oleanane type were mainly investigated. Little is known about derivatives of this type with a lupane or with a β-amyrin backbone, such as in compounds derived from betulin (**BN**), betulinic acid (**BA**), platanic acid (**PA**) or glycyrrhetinic acid (**GA**). This will be the subject of this study.

Results and discussion

BN, **BA**, **PA** and **GA** were selected as starting materials. They are available in large quantities and very good purity from local suppliers. Their acetylation (Scheme 1) gave the known acetates **1**–**3**. Reaction of **1**–**3** with oxalyl chloride followed by reaction with piperazine gave the amides **4**–**6**, and their reaction with rhodamine B (which was previously converted into the corresponding acid chloride with oxalyl chloride) led to the formation of the acetylated piperazinyl-spacered triterpene-rhodamine B conjugates **7**–**9**. These compounds are pink colored thus indicating the presence of an intact cationic rhodamine B moiety. This is

* Corresponding author.
E-mail address: rene.csuk@chemie.uni-halle.de (R. Csuk).

https://doi.org/10.1016/j.rechem.2022.100429
Received 23 May 2022; Accepted 30 June 2022
Available online 2 July 2022

usually regarded as a pre-requisite for obtaining compounds of mitocanic activity. [29].

Especially for a comparison with the corresponding glycyrrhetinic acid derivatives, two additional compounds were prepared (Scheme 2). **BN** was acetylated to form known diacetate **10** whose reduction with BH_3 in THF at 0 °C [37] gave **11**. Jones oxidation of the latter afforded **12** whose reaction with oxalyl chloride followed by the addition of piperazine yielded **13**. Reaction of **13** with rhodamine B (activated with oxalyl chloride) gave **14**. **BA** acetate **1** was converted into its corresponding benzylamide **15** whose reduction with BH_3 yielded **16**. The latter compound was oxidized, and acid **17** was obtained in 52% yield. Coupling of this compound with **18** (having been accessed from the reaction of rhodamine B with oxalyl chloride followed by the addition of piperazine) finally gave **19**.

To assess their cytotoxicity these compounds were subjected to sulforhodamine B (SRB) assays employing several human tumor cell lines. The results of these assays have been compiled in Table 1.

The results from extra staining experiments employing A375 cells (having been incubated with either **14** or **19** at 2 × EC_{50} concentration for 24 h and 48 h, respectively) are depicted in Fig. 2 and Fig. 3. These FITC/annexin V/propidium iodide staining experiments allowed a quantification of the apoptosis/necrosis inducing activity of compounds **14** and **19**, respectively. Thereby, cells found in R1 (upper left) are regarded necrotic, those in R2 (upper right) late apoptotic, in R3 (bottom left) viable cells can be found and in R4 (bottom right) apoptotic cells are registered. Treatment of A375 cells with **14** for 24 h showed 42% of the cells being necrotic; after 2 days 50% of the cells were necrotic, and only 1.6% of the cells had died by apoptosis.

As shown in Figs. 2 and 3, the number of necrotic cells after treatment with **19** for 2 days amounted to 32.8% and 5.7% of the cells having died by apoptosis. This clearly indicates that A375 cells die rather by necrosis than by apoptosis.

Although generalizations are always difficult, betulin derived **14** is more cytotoxic than betulinic acid derived **19**. Compounds **7**–**9** are approximately equally cytotoxic but significantly better than **14**. This might be caused by a higher bioavailability of the former compounds due to an increased solubility. Tumor/non-tumor cell selectivity is approximately the same in all cases but significantly worse than that previously measured for **EM2**. However, the results also show that betulin, betulinic acid and glycyrrhetinic acid derived conjugates are slightly less cytotoxic than the previously reported corresponding oleanolic and ursolic-piperazinyl-rhodamine-B hybrids. All compounds together, however, are significantly worse than those derivatives previously accessed from maslinic acid [38], tormentic acid [29] or madecassic acid [31]. This proves the original assumption that both the type of spacer (piperazine being better than ethylenediamine) is crucial, but also that the presence of a second acetoxy group in ring A improves cytotoxicity, and that the mode of attachment of the rhodamine residue (spacered better than directly bound) and the corresponding triterpene skeleton are also of crucial importance. Worthwhile to mention that **15** (albeit being not a rhodamine derivative) seems perhaps to be a more valuable compound over both, **14** and **19**, because **15** is much less cytotoxic to the non-malignant cells than **14** and **19**. Moreover, it shows certain selectivity in its effect.

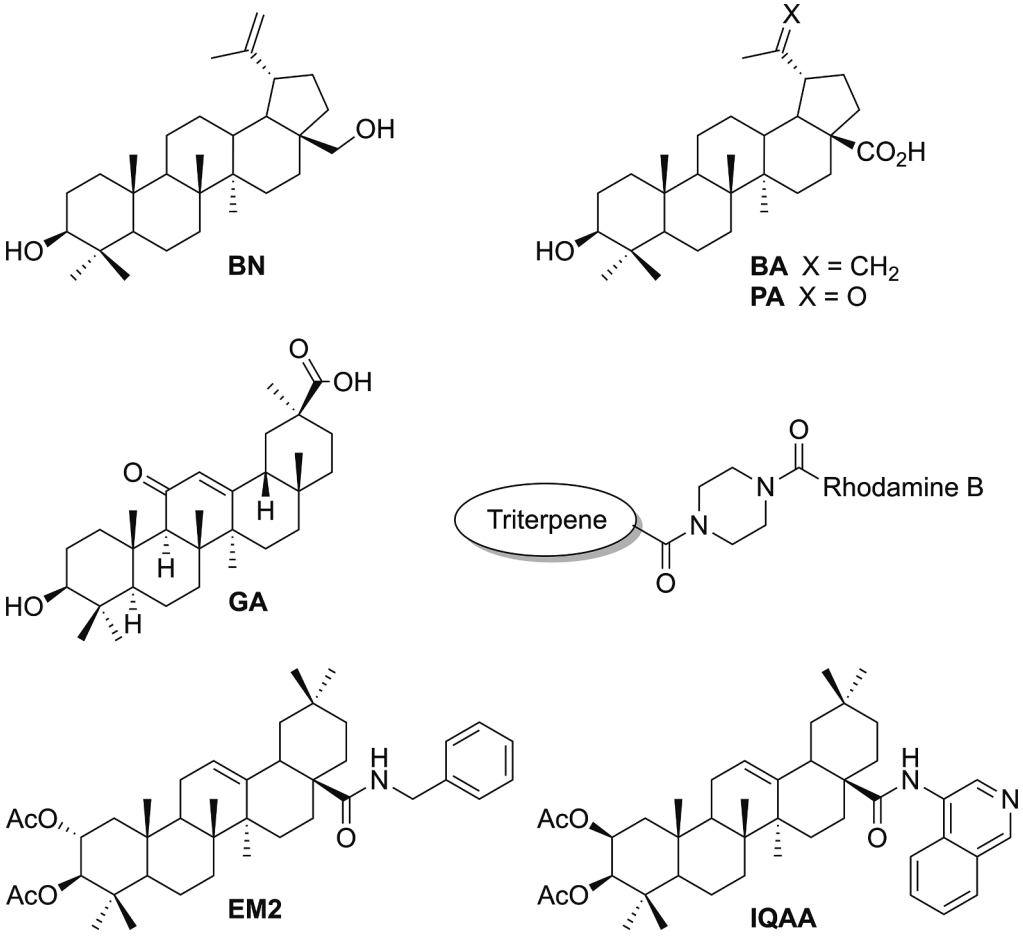
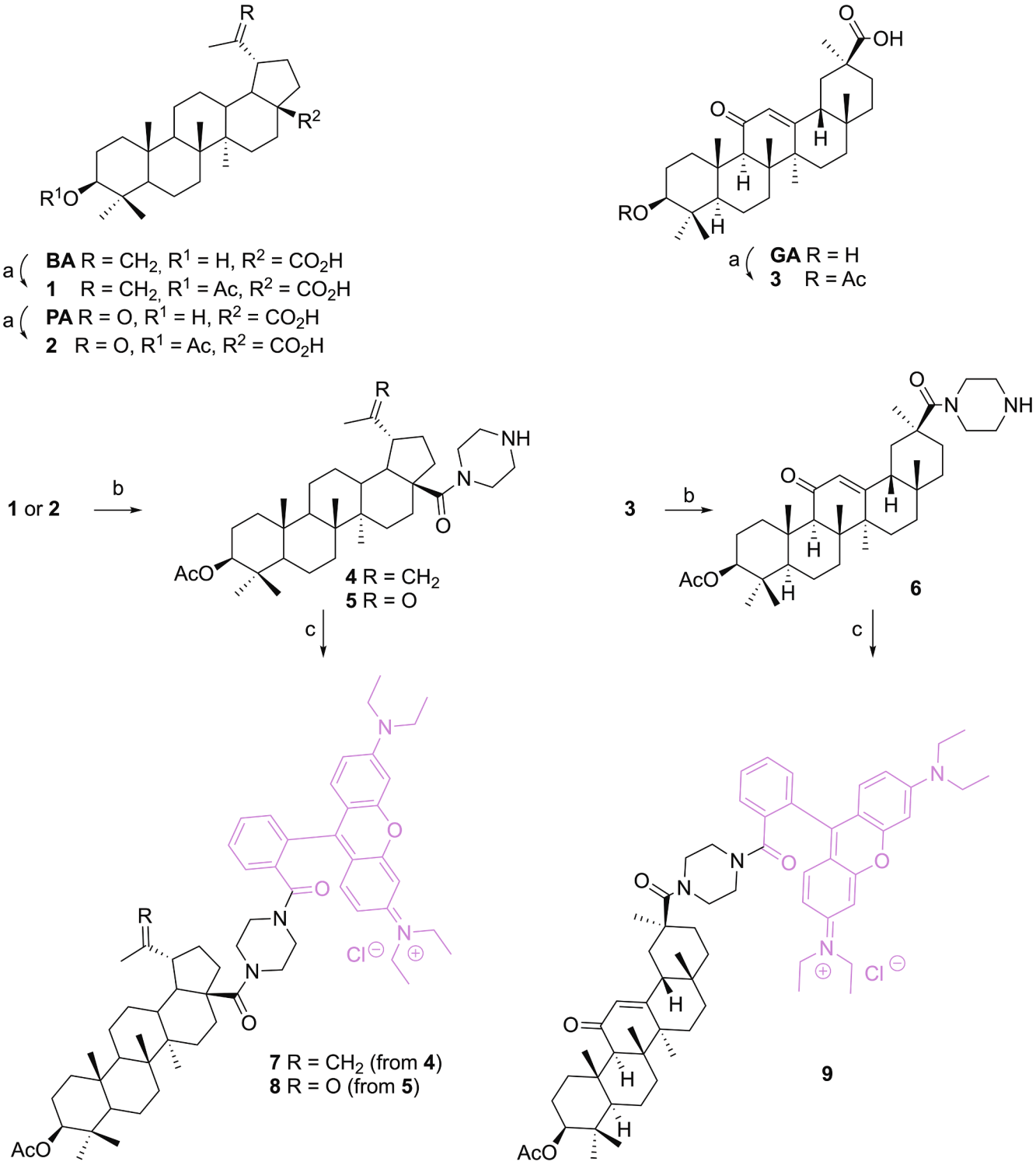


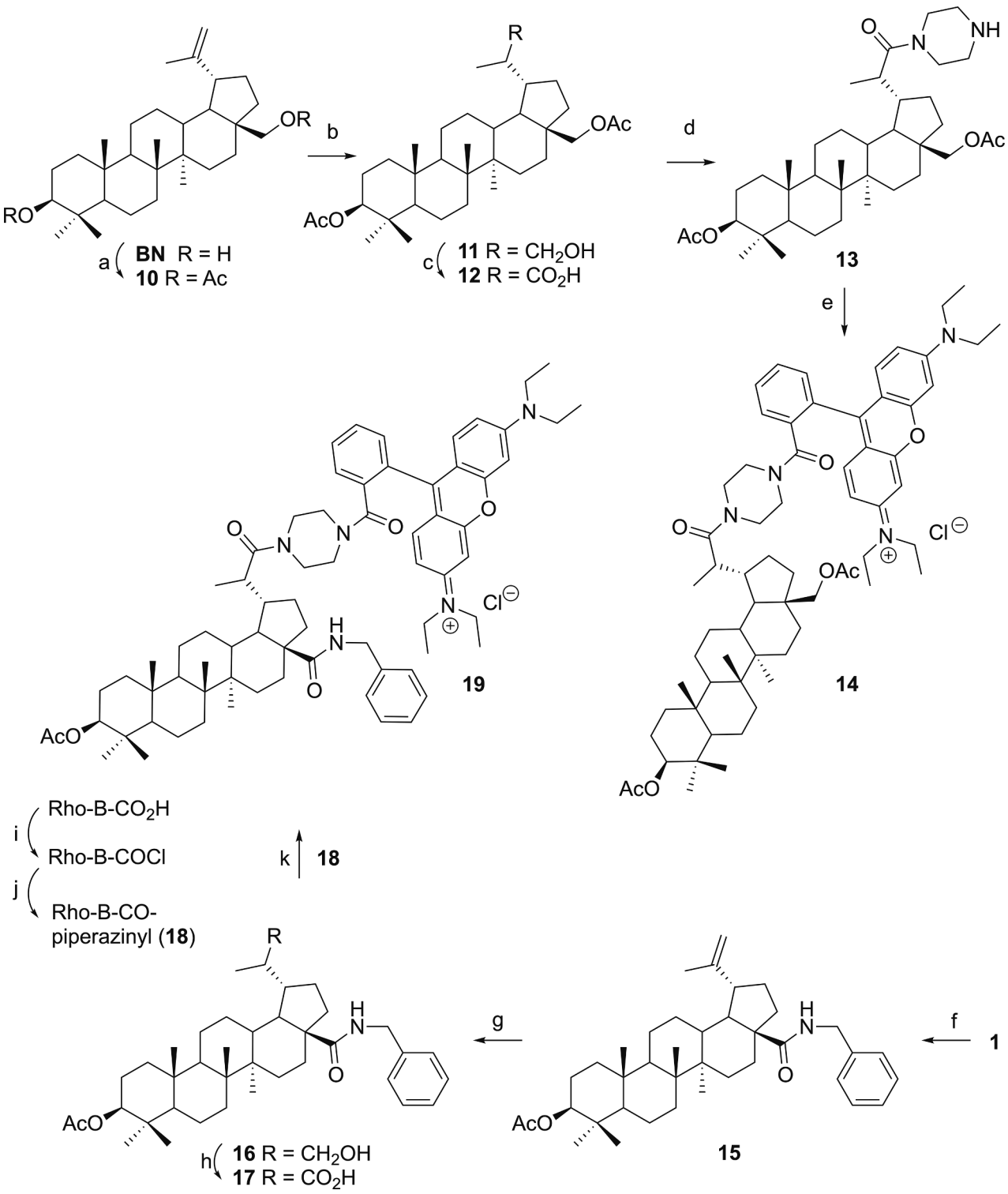
Fig. 1. Structure of betulin (**BN**), betulinic acid (**BA**), platanic acid (**PA**) and the generalized depiction of a piperazinyl spacered triterpene-rhodamine B conjugate as well as of most cytotoxic derivatives **EM2** and **IQAA**.

Conclusion

Pentacyclic triterpene-piperazine-rhodamine B conjugates derived from betulin, betulinic acid or glycyrrhetinic acid were synthesized and screened for their cytotoxic activity. This study is based on previous investigations showing similar compounds holding an ursane or oleanane backbone of high cytotoxicity thereby acting as mitocans. For these new conjugates it was shown, however, that the linking position at ring E governs the magnitude of cytotoxicity. As a result, these conjugates were still highly cytotoxic but significantly less cytotoxic than those holding a oleanane skeleton. Staining experiments showed the rhodamine B conjugates as necrotic compounds and to act as mitocans. The most active compound (**8**) held an EC_{50} = 0.04 μM for A2780 ovarian carcinoma cells.



Scheme 1. Synthesis of compounds **1**–**9**: a) Ac_2O, py, DMAP (cat.), 21 °C, 12 h, →**1** (75%), →**2** (79%), →**3** (91%); b) DCM, $(COCl)_2$, DMF (cat.) then piperazine, DCM, 21 °C, 12 h, →**4** (73%), →**5** (68%), →**6** (67%); c) rhodamine B, DCM, $(COCl)_2$, DMF (cat.), then **4**, **5** or **6**, DCM, 21 °C, 24 h, **4** → **7** (67%), **5** → **8** (70%), **6** → **9** (64%); the rhodamine B part has been colored in pink.



Scheme 2. Synthesis of conjugates **14** and **19**: a) Ac_2O, py, DMAP (cat.), 21 °C, 12 h, 83%; b) THF, BH_3·THF, 1 h, 0 °C then NaOAc, H_2O_2, 1 h, 0 °C, 67%; c) Jones oxidation (CrO_3/H_2SO_4), 0 °C, 1 h, 81%; d) $(COCl)_2$, DCM, DMF (cat.), then piperazine, 12 h, 21 °C, 91%; e) rhodamine B, $(COCl)_2$, DCM, DMF (cat.), then **13**, 24 h, 21 °C, 24%; f) DCM, $(COCl)_2$, DMF (cat.) then Bn-NH_2, 12 h, 21 °C, 67%; g) THF, BH_3·THF, 1 h, 0 °C then NaOAc, H_2O_2, 1 h, 0 °C, 70%; h) Jones oxidation (CrO_3/H_2SO_4), 0 °C, 1 h, 52%; i) $(COCl)_2$, DCM, DMF (cat.); j) piperazine, 12 h, 21 °C, 67%; k) DCM, $(COCl)_2$, DMF (cat), then **18**, DCM, 21 °C, 24 h, 9%.

Experimental

Equipment and general methods are described in the supplementary materials file.

3β-Acetyloxy-lup-20(29)-en-28-oic acid (1)

Following GPA, from betulinic acid (**BA**, 5.0 g, 0.011 mol) **1** (4.1 g, 75%) was obtained as a colorless solid; $R_f = 0.71$ (toluene/ethyl acetate/*n*-heptane/formic acid, 80:26:10:5); m.p. 274–277 °C (lit.: [39] 277–278 °C); $[\alpha]_D = +21.5°$ ($c = 0.40$, $CHCl_3$), lit.: [40] $[\alpha]_D = +26.4°$

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), NIH 3T3 (non-malignant fibroblasts); cut-off 30 μM, n.d. not determined; n.s. not soluble under the conditions of the assay; doxorubicin (**DX**) has been used as a positive standard.

Compound	A375	HT29	MCF-7	A2780	NIH 3T3
1	19.2 ± 1.7	21.3 ± 2.0	11.0 ± 0.5	18.3 ± 0.5	>30
2	>30	>30	>30	>30	>30
3	>30	>30	>30	>30	>30
4	1.5 ± 0.3	1.0 ± 0.1	1.4 ± 0.1	1.9 ± 0.1	0.9 ± 0.06
5	1.9 ± 0.4	3.9 ± 0.2	2.7 ± 0.3	2.6 ± 0.4	1.3 ± 0.1
6	3.7 ± 0.4	4.5 ± 0.6	8.4 ± 0.8	8.2 ± 0.5	8.7 ± 0.7
7	0.1 ± 0.04	0.2 ± 0.04	0.1 ± 0.05	0.05 ± 0.002	0.2 ± 0.05
8	0.1 ± 0.03	0.1 ± 0.04	0.1 ± 0.03	0.04 ± 0.006	0.2 ± 0.06
9	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.05	0.1 ± 0.03
10	18.7 ± 0.9	15.9 ± 1.3	20.4 ± 2.6	11.1 ± 1.4	>30
11	10.0 ± 0.3	18.2 ± 1.5	11.5 ± 1.7	9.9 ± 0.8	15.9 ± 0.8
12	21.5 ± 1.1	27.7 ± 0.8	16.1 ± 1.3	12.5 ± 1.8	25.3 ± 0.6
13	n.s.	n.s.	n.s.	n.s.	n.s.
14	0.5 ± 0.05	0.3 ± 0.04	0.3 ± 0.05	0.2 ± 0.06	0.5 ± 0.07
15	4.1 ± 0.2	>30	26.8 ± 6.8	6.3 ± 0.8	>30
16	>30	>30	25.1 ± 5.7	16.8 ± 2.6	>30
17	16.2 ± 1.4	26.1 ± 1.2	13.6 ± 0.9	14.1 ± 1.1	21.2 ± 1.5
18	>30	>30	17.8 ± 3.9	26.4 ± 2.1	>30
19	1.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	1.6 ± 0.1
DX	n.d.	0.9 ± 0.01	1.1 ± 0.3	0.01 ± 0.006	0.4 ± 0.07

(c = 0.54, $CHCl_3$); MS (ESI): m/z (%) = 497.1 ($[M-H]^-$, 25), 995.3 ([2 M−H]$^-$, 100).

3β-Acetyloxy-20-oxo-30-norlupan-28-oic acid (2)

Following GPA, from platanic acid (**PA**, 5.0 g, 0.011 mol) **2** (4.3 g, 79%) was obtained as a colorless solid; R_f = 0.52 (toluene/ethyl acetate/*n*-heptane/formic acid, 80:26:10:5); m.p. 259–261 °C (lit.: [41] 252–255 °C); $[\alpha]_D$ = –9.0° (c = 0.35, $CHCl_3$), [lit.: [41] $[\alpha]_D$ = –9.5° (c = 0.80, $CHCl_3$)]; MS (ESI): m/z (%) = 499.0 ([M−H]$^-$, 14), 999.2 ([2 M−H]$^-$, 100).

3β-Acetyloxy-11-oxo-olean-12-en-29-oic acid (3)

Following GPA, from glycyrrhetinic acid (**GA**, 5.0 g, 0.011 mol) **3** (5.0 g, 91%) was obtained as a colorless solid; R_f = 0.50 (*n*-hexane/ethyl acetate, 7:3); m.p. 316–318 °C (decomp.) (lit.: [42] 310–313 °C); $[\alpha]_D$ = +165.7° (c = 0.5, $CHCl_3$) [lit.: [42] $[\alpha]_D$ = +163.2° (c = 1.0, $CHCl_3$)]; MS (ESI): m/z (%) = 513.5 ($[M + H]^+$, 100), 535.5 ($[M + Na]^+$, 60), 567.0 ($[M + MeOH + Na]^+$,69).

3β-Acetyloxy-28-(1-piperazinyl)-lup-20(29)-en-28-one (4)

Following GPD, from **1** (2.5 g, 5 mmol) and piperazine (1.6 g, 20.0 mmol), compound **4** (2.1 g, 73%) was obtained as a colorless solid; R_f = 0.38 (chloroform/methanol, 9:1); m.p. = 166–173 °C (lit.: [38] 162–167 °C); $[\alpha]_D$ = − 1.4° (c = 0.21, MeOH), [lit.: [38] $[\alpha]_D$ = − 1.8° (c = 0.32, MeOH); MS (ESI): m/z (%) = 567.3 ($[M + H]^+$, 100).

3β-Acetyloxy-28-(1-piperazinyl)- 30-norlupane −20,28- dione (5)

Following GPD, from **2** (2.5 g, 5.0 mmol) and piperazine (1.6 g, 20.0 mmol), **5** (1.93 g, 68%) was obtained as a colorless solid; R_f = 0.40 (chloroform/methanol, 9:1); m.p. = 126–129 °C (lit: [38] 115–125 °C); $[\alpha]_D$ = − 20.3° (c = 0.13, $CHCl_3$); MS (ESI): m/z (%) = 569.3 ($[M + H]^+$, 100).

3β-Acetyloxy-30-(1-piperazinyl)-olean-12-ene-11,30-dione (6)

Following GPD, from **3** (0.5 g, 1.0 mmol) and piperazine (0.3 g, 4.0 mmol), **6** (0.4 g, 67%) was obtained as a colorless solid; R_f = 0.30 (chloroform/methanol, 9:1); m.p. 162–164 °C [lit.: [38] 160 °C (decomp.]; MS (ESI): m/z (%) = 581.4 ($[M + H]^+$, 42).

9- [2- [[4-(3β-Acetyloxy-28-oxo-lup-20(29)en-28-yl)-1-piperazinyl] carbonyl] phenyl] -3,6-bis(diethylamino] -xanthylium chloride (7)

Following GPE, from **4** (360 mg, 0.64 mmol) and rhodamine B, **7** (440 mg, 67%) was obtained as a dark purple solid; R_f = 0.37 (chloroform/methanol, 9:1); m.p. 246–251 °C (lit.: [38] m.p. 246–250 ∘C); MS (ESI, MeOH): m/z (%) = 991.6 ($[M-Cl]^+$, 100).

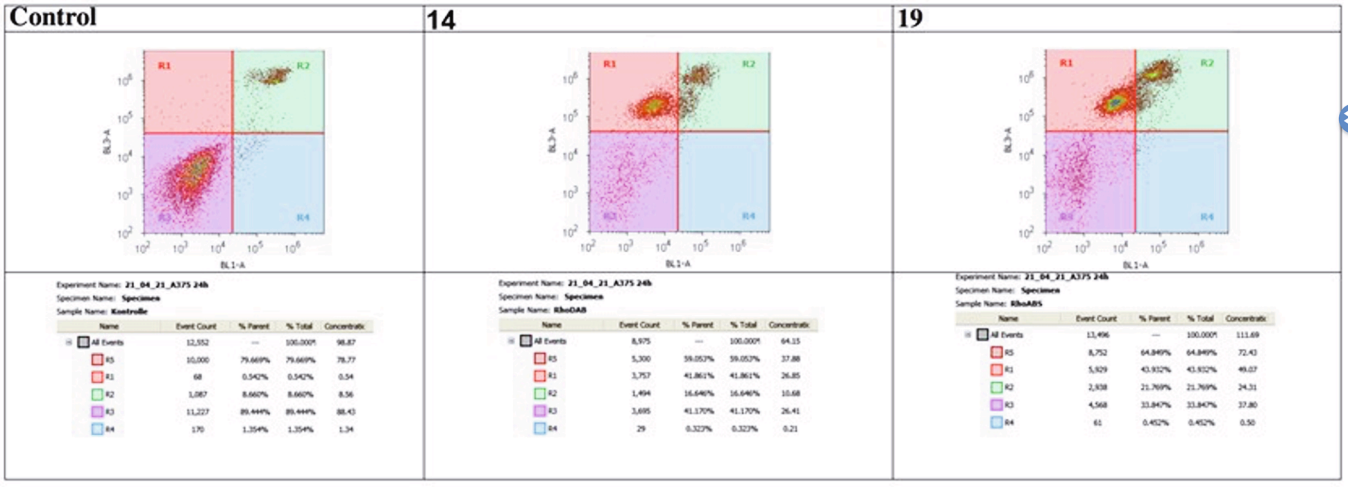


Fig. 2. FITC/Annexin V/Propidium iodide assay utilizing compounds **14** and **19** (A375 cells, 24 h, 2 × EC_{50} concentration).

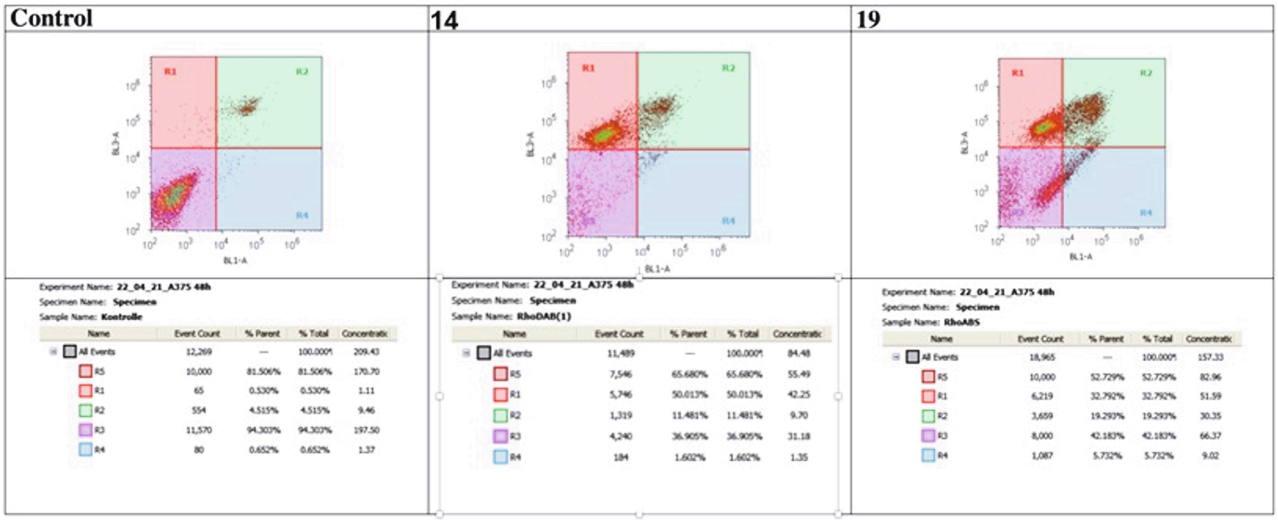


Fig. 3. FITC/Annexin V/propidium iodide assay utilizing compounds **14** and **19** (A375 cells, 48 h, 2 × EC_{50} concentration).

9-[2-[[4-(3β, 20*R*) 3-Acetyloxy-20,28-dioxo-30-norlupan-28-yl)-1-piperazinyl] carbonyl] phenyl] -3,6-bis(diethylamino)-xanthylium chloride (8)

Following GPE from **5** (360 mg, 0.64 mmol) and rhodamine B, **8** (460 mg, 70%) was obtained as a dark purple solid; $R_f = 0.35$ (chloroform/methanol, 9:1); m.p. 246–252 °C (lit.: [38] 235–243 ∘C); MS (ESI, MeOH): *m/z* (%) = 993.7 ($[M-Cl]^+$, 100).

9-[2-[[4-(3β, 20β)-3-Acetyloxy-11,29-dioxo-olean-12-en-29-yl) piperazinyl] carbonyl] phenyl] -3,6-bis(diethylamino] -xanthylium chloride (9)

As previously described, **9** (214 mg, 64%) was obtained as a violet solid; $R_f = 0.32$ (chloroform/methanol, 9:1); m.p. 237–240 °C (lit.: [38] 235–238 °C); MS (ESI): *m/z* (%) = 1005.8 ($[M-Cl]^+$, 100).

3β, 28-Diacetyloxylup-20(29)-ene (10)

Following GPA, betulin (20.0 g, 39.0 mmol) was acetylated, and **10** (16.6 g, 83%) was obtained as a colorless, crystalline solid; $R_f = 0.66$ (*n*-hexane/ethyl acetate, 7:1); m.p. 216–219 °C (lit.: [43] 223–224 °C); $[\alpha]_D$ = +21.0° (*c* = 0.20, $CHCl_3$) [lit.: [44] $[\alpha]_D$ = +23° (*c* = 0.46, $CHCl_3$)]; MS (ESI, MeCN): *m/z* (%) = 549.7 ($[M-Na]^+$, 34), 1075.7 ($[2M + Na]^+$, 100).

(3β, 20*R*) 3,28,29-lupanetriol-3,28-diacetate (11)

Following GPB from **10** (5.7 g, 10.8 mmol) and chromatographic purification (silica gel, chloroform/*n*-hexane/ethyl acetate, 8:7:1) **11** (2.6 g, 67%) was obtained as white solid; $R_f = 0.30$ (silica gel, chloroform/*n*-hexane/ethyl acetate, 8:7:1); m.p. 231–233 °C (lit.: [45,46] 235–236 °C); $[\alpha]_D$ = -13.4° (*c* = 0.15, $CHCl_3$) (lit.: [45,46] $[\alpha]_D$ = -14.0° (c = 0.65, $CHCl_3$)); MS (ESI, MeOH): *m/z* (%) = 567.6 ($[M + Na]^+$, 85), 1111.3 ($[2M + Na]^+$, 100).

(3β, 20*R*) 3, 28-Bis(acetyloxy)-lupan-39-oic acid (12)

Following GPC from **11** (3.5 g, 6.4 mmol) **12** (3.2 g, 81%) was obtained as a white solid; $R_f = 0.25$ (chloroform/*n*-hexane/ethyl acetate, 8:7:1); m.p. 231–234 °C (lit.: [45,46] 239–241 °C; 238–240 °C); $[\alpha]_D$ = -43.2° (*c* = 0.2, $CHCl_3$) (lit.: [45,46] $[\alpha]_D$ = -44.0° (c = 0.68, $CHCl_3$), $[\alpha]_D$ = -56° (c = 1, $CHCl_3$)[29,38]); MS (ESI, MeOH): *m/z* (%) = 557.4 ($[M-H]^-$, 75), 1115.3 ($[2M-H]^-$, 100).

(3β, 20*R*) 3, 28-Bis(acetyloxy)-(1-piperazinyl)-lupan-29-amide (13)

Following GPD from **12** (1.4 g, 2.5 mmol) and piperazine (0.86 g, 10 mmol), compound **13** (1.4 g, 91%) was obtained as white solid; R_f = 0.24 (*n*-heptane/chloroform/isopropanol, 6:2:2); m.p. 125–128 °C (lit.: [37] m.p. 127–130 °C); $[\alpha]_D$ = -19.4° (c = 0.20, $CHCl_3$) (lit.: [37] $[\alpha]_D$ = -18.3° (*c* = 0.16, $CHCl_3$); MS (ESI, MeOH/DCM (4:1)): *m/z* (%) = 627.5 ($[M-H]^+$, 100%).

9-[2-[[4-(3β-Diacetyloxy-(29-piperazinyl)-lupan-30-amid-37-oyl)-1-piperazinyl] carbonyl] phenyl] -3,6-bis(diethylamino] -xanthylium chloride (14)

Following GPE from **13** (0.5 g, 0.8 mmol) and rhodamine B (0.5 g, 1.0 mmol), **14** (0.2 g, 24 %) was obtained as a dark purple solid; R_f = 0.34 (chloroform/methanol, 9:1); IR (ATR): ν = 2970w, 2935w, 2870w, 1720 m, 1645 m, 1584 s, 1556 m, 1529 m, 1480 m, 1466 m, 1433 m, 1410 s, 1394 m, 1334 s, 1272 s, 1245 s, 1196 m, 1177 s, 1160 m, 1130 s, 1072 s, 1006 m, 976 m, 921 m, 868w, 822 m,758 m,709 m, 681 m, 666w, 619w, 608w, 580w, 547w, 520w, 496w, 486w, 465w, 456w cm^{-1}; UV–Vis (MeOH): λ_{max} (log ε) = 223 (4.6), 257 (4.6), 556 (5.1) nm; ^1H NMR (500 MHz, $CDCl_3$) δ = 8.28 (d, *J* = 7.8 Hz, 1H, 47-H), 7.83–7.66 (m, 3H, 46-H + 39-H + 42-H), 7.40–7.29 (m, 2H, 40-H + 41-H), 7.08–7.04 (m, 1H, 47-H), 6.89–6.86 (m, 1H, 47-H'), 6.81(m, 1H, 49′-H), 4.46 (m, 1H, 3-H), 4.20 (m, 1H, 28-H_a), 3.76–3.26 (m, 21H, 28-H_b + 35-H + 35′-H + 36-H + 36′-H + 51-H + 51′-H + 51′′-H + 51′′′-H), 2.24–2.17 (m, 1H, 2-H_a), 2.11 (m, 1H, 19-H), 2.05–2.01(m, 6H, 32-H + 34-H), 1.84–1.71 (m, 3H, 20-H + 16-H_a + 21-H_a), 1.67–1.62 (m, 4H, 1-H_a + 13-H + 15-H_a + 2-H_b), 1.55–1.40 (m, 5H, 12-H_a + 6-H_a + 12-H_b + 9-H + 11-H_b), 1.37–1.20 (m, 20H, 6-H_b + 7-H + 52-H + 52-H'+52′'-H + 52′′'-H + 11-H_b + 22-H_b + 18-H + 16-H_b + 21-H_b), 1.01 (s, 3H, 26-H), 0.99–0.92 (m, 2H, 1-H_b + 15-H_b), 0.90–0.77 (m, 15H, 27-H + 25-H + 24-H), 0.77–0.73 (m, 1H, 5-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$) δ = 175.3 (C-29), 171.7 (C-33), 171.2 (C-31), 167.9 (C-37), 165.6 (C-48), 159.1 (C-50), 159.1 (C-50′), 157.9 (C-48‘), 155.7 (C-45), 133.8 (C-50‘) 133.3 (C-46), 133.1 (C-39), 131.4 (C-42), 131.4 (C-47), 130.9 (C-46′), 130.6 (C-42), 130.4 (C-49), 130.3 (C-41), 129.9 (C-38), 114.3 (C-47′), 113.7 (C-43), 113.7 (C-45′), 96.6 (C-49‘), 81.1 (C-3), 62.9 (C-28), 55.6 (C-5), 50.2 (C-18), 48.8 (C-9), 46.9 (C-17), 46.4 (C-35 + C-35′+C-36 + C-36′), 46.3 (C-51 + C-51′+C-51′'+C-51′''), 43.9 (C-20), 43.0 (C-14), 41.0 (C-8), 38.7 (C-1), 37.9 (C-4), 37.2 (C-10), 36.8 (C-13), 34.3 (C-7), 33.8 (C-22), 29.8 (C-16), 29.8 (C-21), 28.1 (C-23), 27.7 (C-12), 27.0 (C-15), 23.8 (C-2), 21.7 (C-19), 21.4 (C-34), 21.1 (C-32), 20.9 (C-11), 18.3

(C-6), 16.6 (C-24 + C-25 + C-27), 16.2 (C-26), 16.1 (C-30), 12.8 (C-52 + C-52′+C-52′'+C-52′''') ppm; MS (ESI, MeOH): *m/z* (%) = 1051.6 ([M−Cl]$^+$, 100).

3β-Acetyloxylup-*N*-benzyl-lup-20(29)-en-28-amide (15)

Following GPD from **1** (3. g, 6.6 mmol) and benzylamine (1.9 mL, 17.6 mmol) followed by usual work-up and chromatographic purification (silica gel, *n*-hexane/ethyl acetate, 9:1) **15** (2.6 g, 67%) was obtained as a white solid; R_f = 0.26 (silica gel, *n*-hexane/ethyl acetate, 7:1); m.p. 124–126 °C (lit.: [37] 124–127 °C); $[\alpha]_D$ = +22.3° (*c* = 0.51, $CHCl_3$) [lit.: [37] $[\alpha]_D$ = +23.2° (*c* = 0.35, $CHCl_3$); MS (ESI, MeOH): *m/z* (%) = 588.4 ([M−H]$^+$, 100).

(20*R*) 3β-Acetyloxy-30-hydroxy-*N*-benzyl-lupan-17-carboxamide (16)

Following GPB, from **15** (7.0 g, 11.9 mmol) and chromatographic purification (silica gel, chloroform/*n*-hexane/ethyl acetate, 8:5:3) **16** (5.0 g, 70%) was obtained as a white solid; R_f = 0.35 (chloroform/*n*-hexane/ethyl acetate, 8:5:3); m.p. 142–144 °C (lit.: [37] m.p. 143–145 °C; $[\alpha]_D$ = -0.25° (c = 0.11, $CHCl_3$) (lit.: [37] $[\alpha]_D$ = -0.2° (*c* = 0.18, $CHCl_3$); MS (ESI, MeOH): *m/z* (%) = 604.0 ([M−H] $^-$, 100%).

(20*R*) 3β-Acetoxy-17-benzyl-carbamoyl-lupan-30-oic acid (17)

Following GPC from **16** (2.5 g, 4.1 mmol) **17** (1.3 g, 52%) was obtained as a white solid; R_F = 0.35 (chloroform/*n*-hexane/ethyl acetate, 8:7:1); m.p. 163–167 °C (lit.: [37] m.p. 162–165 °C; $[\alpha]_D$ = -26.5° (c = 0.15, $CHCl_3$) lit.: [37] $[\alpha]_D$ = -27.0° (*c* = 0.12, $CHCl_3$); MS (ESI, MeOH/$CHCl_3$ (4:1)): *m/z* (%) = 618.1 ([M−H]$^-$, 100%).

3,6-Bis(diethylamino)-9 [2-(1-piperazinyl)carbonyl] -xanthylium chloride (18)

Reaction of rhodamine B (10.0 g, 22.3 mmol) in dry DCM (250 mL) with oxalyl chloride (8.84 mL) at 0 °C followed by the addition of piperazine (10.0 g) as described above gave after 24 h and chromatographic purification (silica gel, chloroform/methanol, 9:1) **18** (7.2 g, 67%) as a dark purple amorphous solid; R_f = 0.12 (chloroform/methanol, 8:2); m.p. > 250 °C; MS (ESI, MeOH): *m/z* = 256.2 (26%, [M + H-Cl]$^{2+}$), 511.6 (100%, [M−Cl]$^+$); analysis calcd for $C_{32}H_{39}ClN_4O_2$ (547.14): C 70.25, H 7.18, N 10.24; found: C 69,98, H 7.29, N 9.97.

9- [2- [[4-(3β-Acetyloxy-17β-benzyl-carbamoyl-lupan-29-amid-40-oyl)-piperazinyl] carbonyl] phenyl] -3,6-bis(diethylamino] -xanthylium chloride (19)

Compound **17** (0.4 g, 0.6 mmol) was dissolved in dry DCM (20 mL), oxalyl chloride (0.3 mL) and DMF were added at 0 °C. After 2 h, the volatiles were removed under reduced pressure. The residue was dissolved in dry DCM (10 mL), and the solution was concentrated again to remove excess oxalyl chloride. The acyl chloride of **17** was diluted with dry DCM (15 mL) and added dropwise to a solution of **18** in dry DCM (20 mL). After completion of the reaction (as indicated by TLC), the solvent was removed under diminished pressure, and the residue was subjected to column chromatography (silica gel, chloroform/methanol, 9:1) to yield **19** (51 mg, 9 %) as a dark purple amorphous solid; R_f = 0.30 (chloroform/methanol, 9:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 8.16–8.10 (m, 1H), 8.09–8.02 (m, 1H), 7.95–7.53 (m, 4H), 7.48–7.14 (m, 8H), 7.08–6.64 (m, 3H), 4.52–4.43 (m, 1H), 3.96–3.95 (m, 1H), 3.76–3.69 (m, 5H), 3.69–3.53 (m, 6H), 3.50–3.48 (m, 2H), 3.44–3.42 (m, 1H), 3.41–3.23 (m, 4H), 3.19–3.08 (m, 1H), 3.04–2.86 (m, 2H), 2.08–2.01 (m, 6H), 1.99–1.89 (m, 8H), 1.88–1.79 (m, 3H), 1.75–1.57 (m, 6H), 1.55–1.38 (m, 9H), 1.37–1.30 (m, 9H), 1.28–1.22 (m, 8H), 1.19–1.11 (m, 3H), 1.10–1.06 (m, 3H), 1.05–0.96 (m, 2H), 0.93–0.79 (m, 12H), 0.79–0.71 (m, 1H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 175.6, 175.3, 170.1, 168.4, 157.2, 155.8, 155.3, 139.7, 137.2, 136.0, 132.2, 130.6, 129.4, 129.4, 128.3, 127.6, 114.7, 114.1, 113.4, 113.2, 98.5, 96.5, 80.2, 55.8, 53.5, 51.3, 50.7, 46.1, 45.7, 45.6, 45.0, 42.6, 42.4, 41.5, 40.0, 39.6, 38.7, 37.8, 37.0, 33.7, 32.6, 31.5, 29.4, 28.2, 27.4, 25.7, 24.2, 21.6, 19.0, 17.8, 16.6, 16.2, 15.7, 14.6, 12.4, 12.0 ppm; MS (ESI, MeOH): *m/z* (%) = 1113.9 ([M−Cl]$^+$, 12%) ppm; analysis calcd for $C_{71}H_{94}N_5O_6Cl$ (1148.99): C 74.22, H 8.25, N 6.10; found: C 73.87, H 8.51, N 5.86.

CRediT authorship contribution statement

Marie Kozubek: Investigation, Writing – review & editing. **Sophie Hoenke:** Investigation, Writing – original draft. **Hans-Peter Deigner:** Conceptualization, Writing – original draft, Writing – review & editing. **René Csuk:** Conceptualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

We like to thank Dr. D. Ströhl, Y. Schiller and S. Ludwig for the NMR spectra, and M. Schneider for measuring IR and UV/vis spectra as well as optical rotations and micro-analyses. The cell lines were provided by Dr. Th. Müller (Dept. Oncology, Martin-Luther-University Halle-Wittenberg). Experimental help in the lab was provided by V. Karsten (assays) and Dr. R. K. Wolfram (synthesis). We like to thank T. Schmidt for numerous MS spectra.

References

[1] J.T. Lowitz, Über eine neue fast benzoesäureartige Substanz der Birken, Crell's Ann. Chem. 2 (1788,) 312–317.
[2] E. Pisha, H. Chai, I.S. Lee, T.E. Chagwedera, N.R. Farnsworth, G.A. Cordell, C.W. W. Beecher, H.H.S. Fong, A.D. Kinghorn, D.M. Brown, M.C. Wani, M.E. Wall, T. J. Hieken, T.K. Dasgupta, J.M. Pezzuto, Discovery of betulinic acid as a selective inhibitor of human-melanoma that functions by induction of apoptosis, Nat. Med. 1 (10) (1995) 1046–1051.
[3] U. Bildziukevich, Z. Ozdemir, Z. Wimmer, Recent achievements in medicinal and supramolecular chemistry of betulinic acid and its derivatives, Molecules 24, (19) (2019) 3546.
[4] L. Borkova, J. Hodon, M. Urban, Synthesis of betulinic acid derivatives with modified a-rings and their application as potential drug candidates, Asian J. Org. Chem. 7 (8) (2018) 1542–1560.
[5] N. Gupta, A Review on recent developments in the anticancer potential of oleanolic acid and its analogs (2017–2020), Mini Rev. Med. Chem. 22 (4) (2022) 600–616.
[6] J. Hodon, L. Borkova, J. Pokorny, A. Kazakova, M. Urban, Design and synthesis of pentacyclic triterpene conjugates and their use in medicinal research, Eur. J. Med. Chem. 182 (2019), 111653.
[7] H. Hussain, I. Ali, D. Wang, F.L. Hakkim, B. Westermann, I. Ahmed, A.M. Ashour, A. Khan, A. Hussain, I.R. Green, S.T.A. Shah, Glycyrrhetinic acid: a promising scaffold for the discovery of anticancer agents, Expert Opin. Drug Discovery 16 (12) (2021) 1497–1516.
[8] Islam, M. T.; Ali, E. S.; Uddin, S. J.; Khan, I. N.; Shill, M. C.; de Castro e Sousa, J. M.; Barros de Alencar, M. V. O.; Melo-Cavalcante, A. A. C.; Mubarak, M. S., Anti-Cancer Effects of Asiatic Acid, a Triterpene from Centilla asiatica L: A Review. *Anti-Cancer Agents Med. Chem.* **2020,** 20, (5), 536-547.
[9] M. Olech, W. Ziemichod, N. Nowacka-Jechalke, The occurrence and biological activity of tormentic acid-a review, Molecules 26, (13) (2021) 3797.
[10] A. Sureda, M. Martorell, X. Capo, M. Monserrat-Mesquida, M.M. Quetglas-Llabres, M. Rasekhian, S.M. Nabavi, S. Tejada, Antitumor effects of triterpenes in hepatocellular carcinoma, Curr. Med. Chem. 28 (13) (2021) 2465–2484.
[11] A.K. Surowiak, L. Balcerzak, S. Lochynski, D.J. Strub, Biological activity of selected natural and synthetic terpenoid lactones, Int. J. Mol. Sci. 22, (9) (2021) 5036.
[12] L.C. Baratto, M.V. Porsani, I.C. Pimentel, A.B. Pereira Netto, R. Paschke, B. H. Oliveira, Preparation of betulinic acid derivatives by chemical and biotransformation methods and determination of cytotoxicity against selected cancer cell lines, Eur. J. Med. Chem. 68 (2013) 121–131.
[13] U. Bildziukevich, M. Malík, Z. Özdemir, L. Rárová, L. Janovská, M. Šlouf, D. Šaman, J. Šarek, N. Nonappa, Z. Wimmer, Spermine amides of selected

triterpenoid acids: dynamic supramolecular system formation influences the cytotoxicity of the drugs, J. Mater. Chem. B 8 (3) (2020) 484–491.
[14] B.A. Ganaie, M. Shahid, A. Rashid, T. Ara, J. Ahmad Banday, F. Malik, B.A. Bhat, Platanic acid-aryl enones as potential anticancer compounds: synthesis and biological profiling against breast, prostate and lung cancer cell lines, Chem. Biodiversity 18, (10) (2021) e2100292.
[15] S. Hoenke, M.A. Christoph, S. Friedrich, N. Heise, B. Brandes, H.-P. Deigner, A. Al-Harrasi, R. Csuk, The presence of a cyclohexyldiamine moiety confers cytotoxicity to pentacyclic triterpenoids, Molecules 26, (7) (2021) 2102.
[16] S. Hoenke, N.V. Heise, M. Kahnt, H.-P. Deigner, R. Csuk, Betulinic acid derived amides are highly cytotoxic, apoptotic and selective, Eur. J. Med. Chem. 207 (2020), 112815.
[17] M. Kahnt, L. Fischer, A. Al-Harrasi, R. Csuk, Ethylenediamine derived carboxamides of betulinic and ursolic acid as potential cytotoxic agents, Molecules 23 (10) (2018) 1–19.
[18] M. Kahnt, L. Heller, P. Grabandt, A. Al-Harrasi, R. Csuk, Platanic acid: A new scaffold for the synthesis of cytotoxic agents, Eur. J. Med. Chem. 143 (2018) 259–265.
[19] J.Y. Kim, H.M. Koo, D.S.H.L. Kim, Development of C-20 modified betulinic acid derivatives as antitumor agents, Bioorg. Med. Chem. Lett. 11 (17) (2001) 2405–2408.
[20] O. Kraft, M. Kozubek, S. Hoenke, I. Serbian, D. Major, R. Csuk, Cytotoxic triterpenoid-safirinium conjugates target the endoplasmic reticulum, Eur. J. Med. Chem. 209 (2021), 112920.
[21] S. Sommerwerk, L. Heller, R. Csuk, Synthesis and cytotoxic activity of pentacyclic triterpenoid sulfamates, Arch. Pharm. (Weinheim, Ger.) 348 (1) (2015) 46–54.
[22] S. Schwarz, A. Loesche, S.D. Lucas, S. Sommerwerk, I. Serbian, B. Siewert, E. Pianowski, R. Csuk, Converting maslinic acid into an effective inhibitor of acylcholinesterases, Eur. J. Med. Chem. 103 (2015) 438–445.
[23] B. Siewert, E. Pianowski, A. Obernauer, R. Csuk, Towards cytotoxic and selective derivatives of maslinic acid, Bioorg. Med. Chem. 22 (1) (2014) 594–615.
[24] S. Sommerwerk, L. Heller, J. Kuhfs, R. Csuk, Urea derivates of ursolic, oleanolic and maslinic acid induce apoptosis and are selective cytotoxic for several human tumor cell lines, Eur. J. Med. Chem. 119 (2016) 1–16.
[25] I.Z. Pavel, R. Csuk, C. Danciu, S. Avram, F. Baderca, A. Cioca, E.-A. Moaca, C.-V. Mihali, I. Pinzaru, D.M. Muntean, C.A. Dehelean, Assessment of the antiangiogenic and anti-inflammatory properties of a maslinic acid derivative and its potentiation using zinc chloride, Int. J. Mol. Sci. 20, (11) (2019) 2828.
[26] I.Z. Pavel, C. Danciu, C. Oprean, C.A. Dehelean, D. Muntean, R. Csuk, D. M. Muntean, In vitro evaluation of the antimicrobial ability and cytotoxicity on two melanoma cell lines of a benzylamide derivative of maslinic acid, Anal. Cell. Pathol. 2016 (2016) 1–6.
[27] I.Z. Pavel, C.A. Dehelean, L. Farczadi, D.M. Muntean, L. Vlase, C. Danciu, R. Csuk, F. Birsasteanu, D.M. Muntean, Assessment of a maslinic acid derivative and its metabolite in rat blood by liquid chromatography coupled with mass spectrometry, Rev. Chim. (Bucharest, Rom.) 68 (5) (2017) 1089–1094.
[28] S. Sommerwerk, L. Heller, J. Kuhfs, R. Csuk, Selective killing of cancer cells with triterpenoic acid amides - The substantial role of an aromatic moiety alignment, Eur. J. Med. Chem. 122 (2016) 452–464.
[29] S. Hoenke, I. Serbian, H.-P. Deigner, R. Csuk, Mitocanic Di- and triterpenoid rhodamine B conjugates, Molecules 25, (22) (2020) 5443.
[30] M. Kahnt, J. Wiemann, L. Fischer, S. Sommerwerk, R. Csuk, Transformation of asiatic acid into a mitocanic, bimodal-acting rhodamine B conjugate of nanomolar cytotoxicity, Eur. J. Med. Chem. 159 (2018) 143–148.
[31] O. Kraft, A.-K. Hartmann, S. Hoenke, I. Serbian, R. Csuk, Madecassic acid-a new scaffold for highly cytotoxic agents, Int J Mol Sci 23 (2022) (8).
[32] I. Macasoi, I.Z. Pavel, A.E. Moaca, S. Avram, V.L. David, D. Coricovac, A. Mioc, D. A. Spandidos, A. Tsatsakis, C. Soica, V. Dumitrascu, C. Dehelean, Mechanistic investigations of antitumor activity of a rhodamine B-oleanolic acid derivative bioconjugate, Oncol. Rep. 44 (3) (2020) 1169–1183.
[33] I. Serbian, S. Hoenke, R. Csuk, Synthesis of some steroidal mitocans of nanomolar cytotoxicity acting by apoptosis, Eur. J. Med. Chem. 199 (2020), 112425.
[34] J. Wiemann, L. Fischer, J. Kessler, D. Stroehl, R. Csuk, Ugi multicomponent-reaction: Syntheses of cytotoxic dehydroabietylamine derivatives, Bioorg. Chem. 81 (2018) 567–576.
[35] R.K. Wolfram, L. Fischer, R. Kluge, D. Ströhl, A. Al-Harrasi, R. Csuk, Homopiperazine-rhodamine B adducts of triterpenoic acids are strong mitocans, Eur. J. Med. Chem. 155 (2018) 869–879.
[36] R.K. Wolfram, L. Heller, R. Csuk, Targeting mitochondria: Esters of rhodamine B with triterpenoids are mitocanic triggers of apoptosis, Eur. J. Med. Chem. 152 (2018) 21–30.
[37] M. Kozubek, S. Hoenke, T. Schmidt, H.-P. Deigner, A. Al-Harrasi, R. Csuk, Synthesis and cytotoxicity of betulin and betulinic acid derived 30-oxo-amides, Steroids 182 (2022) 109014.
[38] S. Sommerwerk, L. Heller, C. Kerzig, A.E. Kramell, R. Csuk, Rhodamine B conjugates of triterpenoic acids are cytotoxic mitocans even at nanomolar concentrations, Eur. J. Med. Chem. 127 (2017) 1–9.
[39] M. Urban, J. Sarek, J. Klinot, G. Korinkova, M. Hajduch, Synthesis of a-seco derivatives of betulinic acid with cytotoxic activity, J. Nat. Prod. 67 (7) (2004) 1100–1105.
[40] D. Thibeault, C. Gauthier, J. Legault, J. Bouchard, P. Dufour, A. Pichette, Synthesis and structure-activity relationship study of cytotoxic germanicane- and lupane-type 3 beta-O-monodesmosidic saponins starting from betulin, Bioorg. Med. Chem. 15 (18) (2007) 6144–6157.
[41] A. Vystrcil, Budesins.M, Triterpenes., 16. Unusual epimerisation of C(19)-acetyl group of 20-Oxo-30-norlupane derivatives, Collect. Czech. Chem. Comm. 35 (1) (1970) 295–311.
[42] I. Beseda, L. Czollner, P.S. Shah, R. Khunt, R. Gaware, P. Kosma, C. Stanetty, M. C. del Ruiz-Ruiz, H. Amer, K. Mereiter, T. Da Cunha, A. Odermatt, D. Classen-Houben, U. Jordis, Synthesis of glycyrrhetinic acid derivatives for the treatment of metabolic diseases, Bioorg. Med. Chem. 18 (1) (2010) 433–454.
[43] L. Ruzicka, O. Isler, Polyterpene und polyterpenoide, CVI, Oxydation des dihydro-betulins and der dihydro-betulonsäure mit salpetersäure, Helv. Chim. Acta 19 (1936) 506–519.
[44] I.C. Sun, H.K. Wang, Y. Kashiwada, J.K. Shen, L.M. Cosentino, C.H. Chen, L. M. Yang, K.H. Lee, Anti-AIDS agents. 34. Synthesis and structure-activity relationships of betulin derivatives as anti-HIV agents, J. Med. Chem. 41 (23) (1998) 4648–4657.
[45] A. Vystrcil, V. Krecek, M. Budesinsky, Triterpenes., 42. Photooxidation of (20R)- and (20S)-29-Lupanol Derivatives, Collect. Czech. Chem. Commun. 40 (5) (1975) 1593–1603.
[46] A. Vystrcil, V. Pouzar, V. Krecek, Triterpenes., 32. absolute-configuration at C(20) in 29-substituted lupane derivatives, Collect. Czech. Chem. Commun. 38 (12) (1973) 3902–3911.