Steroids 172 (2021) 108876

Contents lists available at ScienceDirect

Steroids

journal homepage: www.elsevier.com/locate/steroids

Type and position of linkage govern the cytotoxicity of oleanolic acid rhodamine B hybrids

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ARTICLE INFO

Keywords:
Oleanolic acid
Rhodamine B
Hybrids
Cytotoxicity

ABSTRACT

Oleanolic acid/rhodamine B hybrids exhibit different cytotoxicity depending on the way these two structural elements are linked. While a hybrid holding a piperazinyl spacer at C-28 proved to be cytotoxic in the nano-molar concentration range, hybrids with a direct linkage of the Rho B residue to C-3 of the triterpenoid skeleton are cytotoxic only in the low micro-molar concentration range without any selectivity. This once again underlines the importance of selecting the right spacer and the most appropriate position on the skeleton of the triterpene to achieve the most cytotoxic hybrids possible.

1. Introduction

Cancer affects the lives of numerous people every year; this disease ends fatally for many of them. Thus, despite many advances, state-of-the-art therapy and early diagnosis, the number of people suffering from cancer continues to rise. Whereas in 2008 there were around 12.7 million persons affected, by 2020 the figure had already risen to 19.3 million, and a further increase to 28.4 million persons is forecast for 2040. In 2020 alone, 10 million deaths were recorded. In addition to the personal and professional impact on the concerned persons and their families, the costs of treating the disease are also very high. It is estimated that the total cost of cancer worldwide will exceed in 2030 US$ 450 billion [1,2].

Drug targeting by definition refers to the targeted and selective accumulation or release of a drug at one or more desired sites of action. In cancer therapy, this ultimately reflects the ability to distinguish between malignant and normal cells. An insufficient selectivity is the cause of severe side effects. This inevitably leads to a poor compliance of patients because of facing a reduced quality of life due to the drugs. Asa result, an early discontinuation of therapy takes place.

Extending the original concept of drug-targeting directed at different tissues, a new line of research ("third level drug targeting") [3] has emerged in recent years that applies drug-targeting to subcellular entities and compartments ("organelle specific drug targeting") [3–6]. The focus here is on the endoplasmic reticulum, lysosomes and especially mitochondria.

In recent years, derivatives of pentacyclic triterpenoic acids have been identified as promising and, in some cases, highly cytotoxic compounds, starting with studies on the cytotoxic activity of betulinic acid and melanoma [7]. While betulinic acid causes parallel damage in both mitochondrial and lysosomal compartments thereby inducing autophagy [8], we were able to show for triterpene-derived saphirinium derivatives [9] that the endoplasmic reticulum is the target. Rhodamine B derivatives [10–17], as well as several phosphonium salts [18–20] or F-16 conjugates [21] on the other hand, act as mitocans; their targets are the mitochondria [22–26].

Hereby we could show that EC_{50} values in the low micro or even in the nano-molar concentration range for cytotoxicity on human cancer cell lines were observed depending very strongly on the triterpene scaffold, the spacer and the cationic residue. Thus, derivatives of maslinic acid were found to be more active than those derived from betulinic acid [10,14,17]. Rhodamine B derivatives were more cytotoxic than comparable malachite green derivatives [27], and compounds holding a (homo)-piperazinyl spacer [16] were significantly more effective than those with an ethylenediamine spacer [14,28]. The latter derivatives showed EC_{50} values > 30 μM and thus are usually considered non-cytotoxic.

Thus, the question of the spacer and its linkage is of decisive

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https://doi.org/10.1016/j.steroids.2021.108876
Received 1 March 2021; Received in revised form 17 May 2021; Accepted 25 May 2021
Available online 12 June 2021

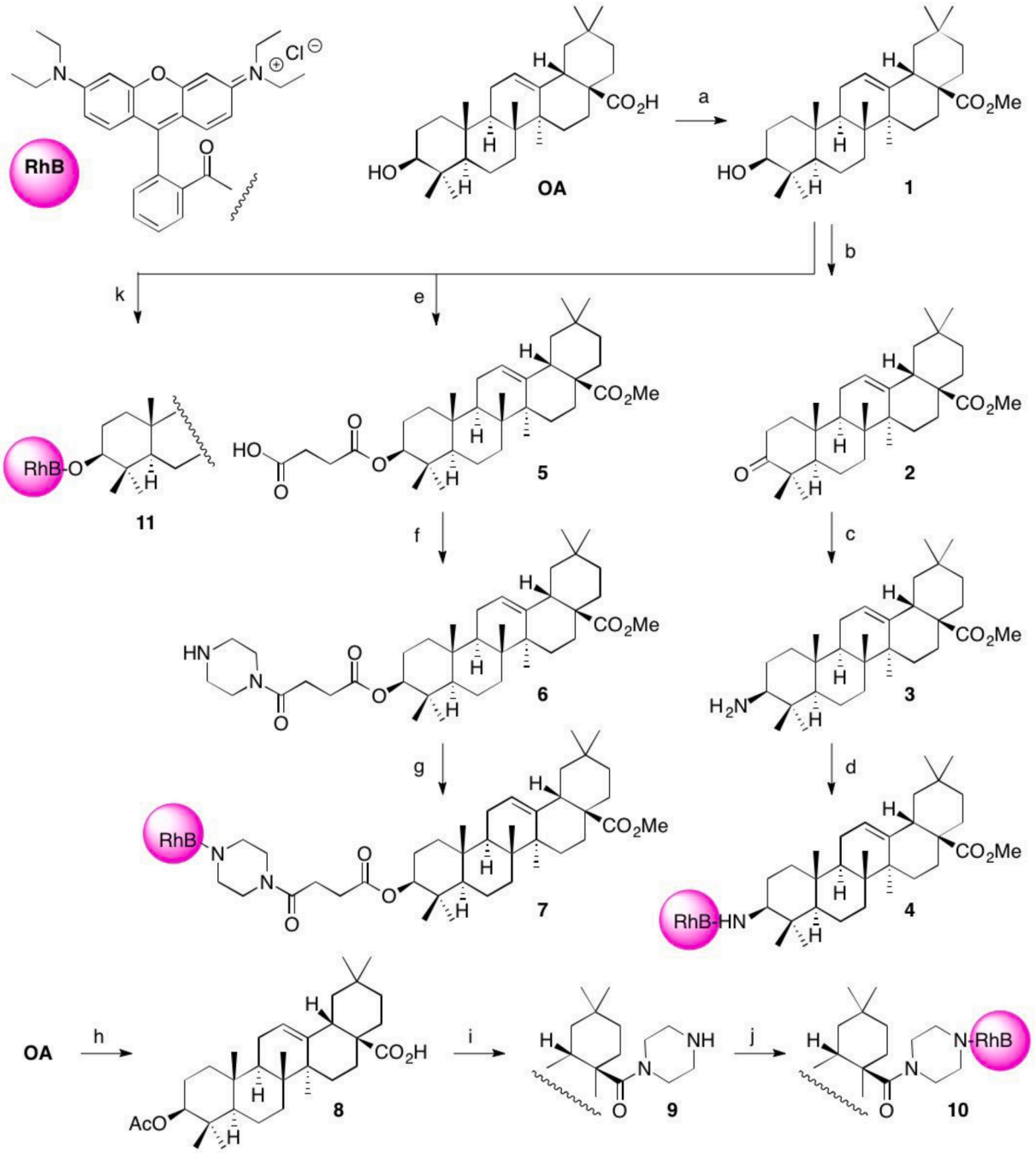
importance. For this comparative study, oleanolic acid (OA) was chosen as the basic structure to access a small library of compounds. OA derivatives had previously been shown to hold good cytotoxicity [29–31]. Furthermore, OA is also readily and commercially available in larger quantities.

2. Results and discussion

Thus, OA was converted into methyl ester 1 (Scheme 1) [32–35], the oxidation of which with freshly prepared Jones reagent afforded ketone 2 in 71% isolated yield [32]. Reductive amination of 2 with ammonium acetate and sodium cyanoborohydride gave the 3β-configured amine 3 [36–40]; the corresponding 3α epimer [36–41] could be determined in trace amounts on TLC and detected by mass spectrometry using HPTLC-ASAP MS but could not be isolated.

Amide 4 was formed in 65% yield from the reaction of rhodamine B (Rho B) in acetonitrile in the presence of EDC and TEA showing an UV absorption λ_{max} = 550 nm being characteristic for the presence of an intact Rho B moiety.

Reaction of 1 with succinic anhydride in the presence of TEA gave chain elongated 5 whose reaction with oxalyl chloride followed by the addition of piperazine furnished 6. The use of a succinyl spacer has been



Scheme 1. Reactions and conditions: a) K_2CO_3, DMF, MeI, 23 °C, 24 h, 77.5%; b) Jones oxidation, 71.3%; c) $(NH_4)_2CO_3$, $NaBH_3CN$, MeOH, 23 °C, 24 h, 65%; d) Rho B, EDC, TEA, 23 °C, 3 d, 65%; e) succinyl anhydride, pyridine, DMAP (cat.), 23 °C, 8 h, 79%; f) $(CO)_2Cl_2$, DMF, then piperazine, DCM, TEA, DMAP, 23 °C, 1d, 94%; g) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, then 6, TEA, DMAP (cat), 23 °C, 1 d, 75%; h) Ac_2O, DCM, TREA, DMAP (cat), 23 °C, 1 d, 75%; i) $(CO)_2Cl_2$, DMF, then piperazine, DCM, REA, DMAP (cat.), 23 °C, 1 d, 84%; j) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, the 9, TEA, DMAP (cat.), 23 °C, 1 d, 79%; k) Rho B, DCM, $(CO)_2Cl_2$, DMF (cat.), 23 °C, 1 h, then 1, TEA, DMAP (cat.), 23 °C, 1 d, 41%.

applied very successfully in the past for the synthesis of biologically active triterpene carboxylic acid derivatives. Rho B was activated with oxalyl chloride and allowed to react with 6 to afford 7 as a purple solid showing $\lambda_{max} = 559$ nm.

Acetylation of OA gave well known acetate 3 whose reaction – as described above – yielded piperazinyl amide 9. This compound was coupled with Rho B to afford 10, again as a pink colored solid.

To get an insight onto the influence of an oxygen substituent at position C-3 of the triterpenoid skeleton (as compared to a nitrogen substituent as in 4), compound 1 was coupled with Rho B to yield 11. To evaluate the cytotoxic activity of these compounds, photometric sulforhodamine B assays (SRB) were performed employing several human tumor cell lines as well as non-malignant fibroblasts (NIH 3 T3). The results from these assays are compiled in Table 1.

The results from the SRB assays showed no significant difference between compounds 4 and 11; the cytotoxic effect was independent of whether the RhoB moiety was bound to C-3 via an ester linkage or as an amide. Their EC_{50} values for all tumor cells were low, but these compounds also lacked selectivity. In this series of compounds, 10 performed best. Compounds 7 and 10 showed EC_{50} values in the nano-molar or low micro-molar concentration range. This highlights the importance of the piperazinyl moiety as well as the nature of the attachment of the Rho B residue to the triterpenoid backbone. Compound 10 is thus 25 times more cytotoxic (to A2780 cells) than compound 7 and >1000 times more cytotoxic than parent compound OA.

Molecular modeling calculations, as they have been performed in the past, are of limited value, only. These calculations had shown, using similar compounds as examples, that some mitochondrial enzymes (e.g. NAD(P)H-quinone oxidoreductase or succinate dehydrogenase) could possibly be inhibited; however, the experimental evidence for this is still pending [42]. The cytotoxicity of the compounds could also be caused by changes in the potential of mitochondrial membranes or their ability to increase the concentration of reactive oxygen species. To get a deeper insight, most cytotoxic compound 10 was subjected to flow cytometric measurements (Annexin V/PI assay). Thereby, A375 cells were treated with 2 × EC_{50} concentrations of 10 for 48 h, and the results from these experiments are depicted in Fig. 1.

In Fig. 1, the BL1-A signal corresponds to the FITC signal for annexin V (x-axis); PI was detected at BL3-A (y-axis). As a vonsequence, cells found in R1 areo necrotic cells, those in R2 are late apoptotic, while cells in *R*3 are viable cells, and cells in R4 have died from apoptosis. Thus, from the 48 h incubation of 10, 44.9% of the A375 cells have died by apoptosis and 19.6% by late apoptosis. The number of necrotic cells remained small (0.9%).

Table 1
Cytotoxicity of selected compounds; 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 3 T3 (non-malignant fibroblasts); cut-off 30 μM, n.s. not soluble, n.d. not determined. Betulinic acid (BA), oleanolic acid (OA) and doxorubicin (DX) have been used as positive standards.

#	A375	HT29	MCF-7	A2780	NIH 3 T3
OA	>30	>30	>30	>30	>30
Rho B	>30	>30	>30	>30	>30
1	>30	>30	>30	>30	>30
2	3.8 ± 1.0	4.7 ± 0.5	4.5 ± 0.5	3.8 ± 0.2	6.3 ± 0.4
3	2.9 ± 0.3	4.0 ± 0.4	2.9 ± 0.2	2.9 ± 0.5	2.8 ± 0.5
4	1.8 ± 0.1	2.4 ± 0.3	1.5 ± 0.2	1.5 ± 0.1	5.5 ± 0.4
5	n.s.	n.s.	n.s.	n.s.	n.s.
6	9.2 ± 0.6	11.3 ± 0.6	10.7 ± 0.4	12.7 ± 1.3	7.9 ± 0.3
7	1.1 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	1.4 ± 0.1
8	13.0 ± 1.1	20.5 ± 1.7	12.9 ± 1.9	9.4 ± 0.5	17.5 ± 1.5
9	1.9 ± 0.2	1.3 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	2.1 ± 0.1
10	0.06 ± 0.004	0.09 ± 0.01	0.06 ± 0.004	0.032 ± 0.001	0.137 ± 0.006
11	1.6 ± 0.2	2.8 ± 0.4	2.3 ± 0.1	1.1 ± 0.2	2.6 ± 0.2
BA	n.d.	12.7 ± 1.8	18.4 ± 2.0	12.0 ± 1.7	16.1 ± 1.4
DX	n.d.	0.9 ± 0.2	1.1 ± 0.3	0.02 ± 0.01	0.06 ± 0.03

3. Conclusion

In this small study, using OA as the starting material, it was shown that OA Rho B hybrids exhibit different cytotoxicity depending on the way these two structural elements are linked. Hybrids with a direct linkage of the Rho B residue to C–3 of the triterpenoid skeleton (whether as ester or as amide) are cytotoxic in the low micro-molar concentration range but also not selective. In contrast, a hybrid of OA, Rho B and a piperazinyl spacer at C–28 proved to be cytotoxic in the nano-molar concentration range, whereas no increase in cytotoxicity can be observed when binding to C–3 via a succinyl spacer. This once again underlines the importance of selecting the right spacer and the most appropriate position on the skeleton of the triterpene to achieve the most cytotoxic hybrids possible. Compound 10 is significantly more cytotoxic than parent compound OA and acts mainly by apoptosis while the number of necrotic cells remains small.

4. Experimental part

4.1. General

NMR spectra were recorded using the Varian spectrometers DD2 and VNMRS (400 and 500 MHz, respectively, δgiven in ppm, J in Hz; typical experiments: APT ^{13}C, HMBC, HSQC); MS spectra were taken on a Finnigan MAT LCQ 7000 (electrospray, voltage 4.1 kV, sheath gas nitrogen) or an Advion expression^{L} CMS. TLC was performed ion silica gel (Merck 5554, detection with cerium molybdate reagent); melting points are uncorrected (Leica hot stage microscope). IR spectra were recorded on a Perkin Elmer FT-IR spectrometer 1000 or on a Perkin Elmer Spectrum Two (UATR Two unit). The solvents were dried according to usual procedures. Chemicals were obtained from local suppliers; oleanolic acid was bought "Betulinines" (Stříbrná Skalice, Czech Republic) and used as received. SRB assays were performed as previously described [14,34,43].

4.2. Biology

4.2.1. Cell lines and culture conditions

Following human cancer cell lines A375 (malignant melanoma), HT29 (colon adenocarcinoma), MCF-7 (breast cancer), A2780 (ovarian carcinoma), and non-malignant mouse fibroblasts NIH 3 T3 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.

4.2.2. Cytotoxicity assay (SRB assay)

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

4.2.3. Annexin V/PI assay

Approx. 600,000 cells (A375) were seeded in cell culture flasks; they were allowed to grow for 1 day. The medium was removed, and the substance loaded medium was added; incubation lasted for for 48 h. All

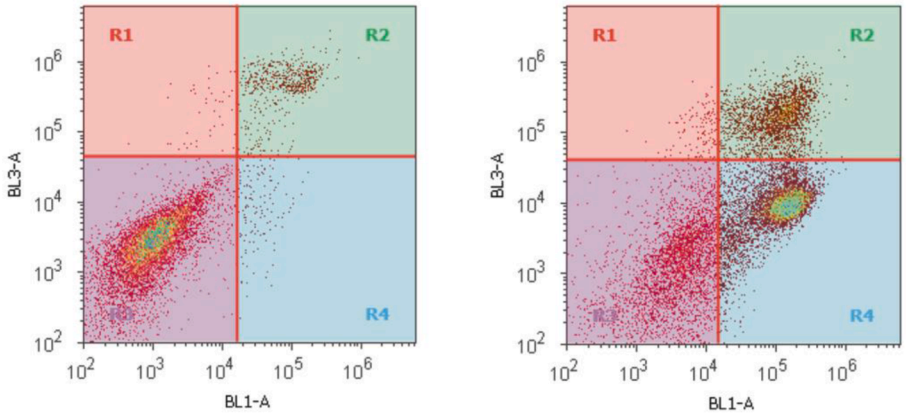


Fig. 1. Annexin V/PI flow cytometry of 10 employing A375 cells (48 h of incubation, 2 × EC_{50} concentration); control experiment (left), incubation with 10 (right).

cells were harvested, centrifuged (1200 rpm, 5 min) and washed twice (PBS (w/w)). Approx. 100,000 cells were washed with annexin V bounding buffer (BD Biosciences®) and treated with a propidium iodide solution (3 μL, 1 mg/mL) and annexin V (5 μL, BD Biosciences®) for 15 min at room temperature in the dark. After adding annexin V bounding buffer (400 μL) the suspension was submitted to a FACS measurement. Calculation was performed as suggested from the supplier (BD Biosciences®).

4.2.4. Synthesess

4.2.4.1. Methyl 3 β -hydroxyolean-12-en-28-oate (1). Oleanolic acid (15.0 g, 32.8 mmol) was dissolved in DMF (200 mL) and potassium carbonate (4.5 g, 32.8 mmol) was added. The reaction mixture was stirred at 23 °C for 30 min. Iodomethane (2.5 mL, 39.7 mmol) was added dropwise, and the mixture was stirred at 23 °C for 24 h. HCl (0.1 m, 65 mL) and water (0.5 L) were added, the precipitate was collected, washed with water (2 × 250 mL) and dried. Recrystallization from ethanol gave 1 as a white solid (11.63 g, 77.5%); m.p. 203 °C (lit.:[44] 200–202 °C); $[\alpha]_D = +66.5°$ (*c* 0.34, $CHCl_3$) [lit.:[44] $[\alpha]_D = 70°$ (*c* 1.0, $CHCl_3$)]; $R_F =$ 0.78 (SiO_2, hexanes/EtOAc, 7:3); UV–Vis ($CHCl_3$): λ_{max} (logε) = 257 nm (3.74) IR (ATR): ν = 3441 s, 2947 m, 1728 m, 1636*w*, 1464*w*, 1386*w*, 1163*w*, 1032*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.26 (*m*,1H, 12-H) 3.60 (s, 3H, OMe), 3.19 (dd, 1H, *J* = 11.0, 4.4 Hz, 3-H), 2.84 (dd, 1H, *J* = 13.9, 4.2 Hz, 18-H), 1.95 (ddd, 1H, *J* = 14.5, 14.4, 4.1 Hz, 16-H_a), 1.88–1.82 (*m*, 2H,11-$H_{a,b}$), 1.67 (ddd, 1H, *J* = 13.9, 13.9, 4.4 Hz, 22-H_a), 1.63–1.47 (*m*, 9H, 9-H, 1-H_a, 19-H_a, 6-H_a, 15-H_a, 22-H_b, 16-H_b, 2-H), 1.43–1.22 (*m*, 4H, 21-H_a, 7-H, 6-H_b), 1.19–1.12 (*m*, 2H, 19-H_b, 21-H_b), 1.10 (s, 3H, 27-H), 1.03(dd, 1H, *J* = 14.9, 4.0 Hz, 15-H_b) 0.97–0.92 (*m*, 1H, 1-H_b), 0.96 (s, 3H, 23-H), 0.90 (s, 3H, 30-H), 0.88 (s, 3H, 25-H), 0.87 (s, 3H, 29-H), 0.76 (s, 3H, 24-H), 0.73–0.68 (*m*, 1H, 5-H), 0.70 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.2 (C-28), 143.7 (C-13), 122.3 (C-12), 79.0 (C-3), 55.2 (C-5), 51.5 (OMe), 47.6 (C-9), 46.7 (C-17), 45.8 (C-19), 41.6 (C-14), 41.3 (C-18), 39.2 (C-8), 38.7 (C-4.), 38.4 (C-1), 37.0 (C-10), 33.8 (C-21), 33.1 (C-29), 32.6 (C-7), 32.3 (C-22), 30.6 (C-20), 28.1 (C-23), 27.7 (C-15), 27.1 (C-2), 25.9 (C-27), 23.6 (C-30), 23.4 (C-11), 23.0 (C-16), 18.3 (C-6), 16.8 (C-26), 15.5 (C-24), 15.3 (C-25) ppm; MS (ESI, MeOH): *m/z* 453.1 (68%, $[M + H\text{-}H_2O]^+$), 471.3 (16%, $[M + H]^+$) 493.2 (100%, $[M + H + H_2O]^+$); analysis calcd for $C_{31}H_{50}O_3$ (470.73): C 79.10, H 10.71; found: C 78.85, H 10.97.

4.2.4.2. Methyl 3-oxoolean-12-en-28-oate (2). A solution of 1 (5.61 g, 12.28 mmol) in acetone (300 mL) was heated under reflux for 30 min. After cooling to 0 °C, Jones reagent [prepared from CrO_3 (6.27 g, 62.7 mmol), water (26 mL), and conc. H_2SO_4 (6.23 mL)] was slowly added, and the mixture was stirred for 1 h at 0 °C. Then MeOH (10 mL) was added, and stirring was continued for another 30 min. The solvents were removed under diminished pressure, water was added, and the aqueous phase was extracted with DCM (3 × 25 mL). After evaporation of the DCM, column chromatography (silica gel, hexane/EtOAc, 9:1) furnished 2 as a white solid (5.18 g, 71.3%); m.p. 186 °C (lit.:[45] 184 °C) ; $[\alpha]_D =$ +93.2° (*c* 0.320, $CHCl_3$) [lit.:[46] $[\alpha]_D = 90°$ (*c* 1.2, $CHCl_3$); $R_F = 0.66$ (SiO2, hexanes/EtOAc, 8:2)]; UV–Vis ($CHCl_3$): λ_{max} (log ε) = 310 nm (3.09); IR (ATR): ν = 3432 s, 2941 s, 1726*vs*, 1703 s, 1458 m, 1382*w*, 1364*w*, 1264*w*, 1205 m, 1163 m, 1125*w*, 1040*w*, 1016 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.29 (*m*, 1H, 12-H), 3.61 (s, 3H, OMe), 2.86 (dd, 1H, *J* = 13.7, 4.3 Hz, 18-H), 2.53 (ddd, 1H, *J* = 16.0, 11.2, 7.3 Hz, 2-H_a), 2.34 (ddd, 1H, *J* = 15.9, 6.7, 3.6 Hz, 2-H_b), 1.99–1.-83 (*m*, 4H, 1-H_a, 16-H_a, 11-H), 1.67 (ddd, 1H, *J* = 14.0, 13.9, 4.6 Hz, 22-H_a), 1.64–1.56 (*m*, 4H, 9-H, 15-H_a, 19-H_a, 16-H_b), 1.53–1.44 (*m*, 4H, 22-H_b, 7-H_a, 6-H), 1.42–1.26 (*m*, 4H, 1-H_b, 21-H_a, 7-H_b, 5-H), 1.20–1.11 (*m*, 2H, 19-H_b, 21-H_b), 1.12 (s, 3H, 27-H), 1.09–1.04 (*m*, 1H, 15-H_b), 1.06 (s, 3H, 23-H), 1.02 (s, 3H, 24-H), 1.02 (s, 3H, 25-H), 0.91 (s, 3H, 30-H), 0.87 (s, 3H, 29-H), 0.76 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, CDCl3): δ = 217.6 (C-3), 178.2 (C-28), 143.8 (C-13), 122.1 (C-12), 55.3 (C-5), 51.5 (OMe), 47.4 (C-9), 46.9 (C-4), 46.7 (C-17), 45.8 (C-19), 41.7 (C-14), 41.4 (C-18), 39.2 (C-8), 39.1 (C-1), 36.7 (C-10), 34.1 (C-2), 33.8 (C-21), 33.1 (C-29), 32.3 (C-7), 32.2 (C-22), 30.7 (C-20), 27.7 (C-15), 26.4 (C-23), 25.8 (C-27), 23.6 (C-30), 23.5 (C-11), 23.0 (C-16), 21.4 (C-24), 19.6 (C-6), 16.7 (C-26), 15.0 (C-25) ppm; MS (ESI, MeOH): *m/z* 469.2 (100%, $[M + H]^+$), 491.0 (32%, $[M + Na]^+$), 408.4 (12%, $[M + Na + H_2O]^+$); analysis calcd for $C_{30}H_{46}O_3$ (454.69): C 79.25, H 10.20; found: C 78.98, H 10.32.

4.2.4.3. Methyl 3 β -aminoolean-12-en-28-oate (3). A suspension of 2 (580 mg, 1.24 mmol)) and ammonium acetate (950 mg, 12.4 mmol) in MeOH (50 mL) stirred at 23 °C for 10 min. A 1 m solution of sodium cyanoborohydride in THF (0.54 mL) was added, and the reaction mixture was stirred at 23 °C for 24 h. Usual aqueous workup and purification by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 3 as a white solid (196 mg, 71.4%); m.p. 216–220 °C; $[\alpha]_D$ = +39.0° (*c* 0.134, $CHCl_3$); $R_F = 0.36$ (SiO_2, $CHCl_3$/MeOH, 9:1); IR (ATR): ν = 3413 m, 2946*w*, 1726*w*, 1635 m, 1328 s, 1191*w*, 1040*w*, 824 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.33–5.23 (*m*, 1H, 12-H), 3.62 (s, 3H, 31-H), 3.20–3.08 (*m*, 1H, 3-H), 2.86 (dd, *J* = 13.7, 4.6 Hz, 1H, 18-H), 2.20–1.17 (*m*, 19H, 16-H, 11-H_a, 7-H, 22-H, 2-H, 11-H_b, 19-H_a, 15-H_a, 6-H, 1-H, 21-H, 9-H), 1.13 (s, 3H, 30-H), 1.09 (s, 3H, 23-H), 1.08–1.03 (*m*, 2H, 15-H_b, 19-H_b), 0.96 (s, 3H, 24-H), 0.93 (s, 6H, 25-H, 26-H), 0.88 (s, 3H, 29-H), 0.81–0.76 (*m*, 1H, 5-H), 0.72 (s, 3H; 27-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$) δ = 178.5 (C-28), 144.1 (C-13), 122.3 (C-12), 58.5 (C-3), 56.3 (C-5), 48.3 (C-9), 46.9 (C-19), 46.7 (C-17), 42.0 (C-14), 41.6 (C-18), 39.5 (C-8), 37.0 (C-4), 35.5 (C-10), 34.1 (C-1), 34.1 (C-21), 33.2 (C-29), 32.6 (C-2), 32.5 (C-7), 32.4 (C-22), 30.9 (C-20), 27.9 (C-15), 27.7 (C-23), 25.9 (C-30), 23.8 (C-26), 23.3 (C-16), 22.9 (C-24), 17.0 (C-27), 15.4 (C-25) ppm; MS (ESI, MeOH): *m/z* 470.1 (100%, $[M + H]^+$); analysis calcd for $C_{30}H_{49}NO_2$ (455.72): C 79.07, H 10.84, N 3.07; found:

C 78.83, H 11.03, N 2.86.

4.2.4.4. 6-(Diethylamino)-N,N-diethyl-9-(2-{[(3 β)-28-methoxy-28-oxoolean-12-en-3-yl]carbamoyl}phenyl)3H-xanthen-3-iminium chloride (4). To a solution of rhodamine B (184.9 mg, 0.386 mmol) in acetonitrile (60 mL), EDC (74.0 mg, 0.386 mmol) and TEA (0.06 mL, 0.386 mmol) were added, and the mixture was stirred at 23 °C for 90 min. Compound 3 (165 mg, 0.351 mmol) was added, and stirring at 23 °C was continued for 3 days. Usual aqueous workup followed by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 4 as a purple solid (160 mg, 65%); m.p. 247–250 °C; $R_F = 0.48$ (SiO_2, $CHCl_3$/MeOH, 9:1); UV–Vis ($CHCl_3$): λ_{max} (log ε) = 550 nm (4.97); IR (ATR): ν = 2938*w*, 1645 m, 1587 s, 1409 m, 1332 s, 1178 s, 1130 m, 1073 m, 823 m, 683 m cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 7.93$ (d, $J = 7.7$ Hz, 1H, 37-H), 7.67–7.62 (*m*, 1H, 35-H), 7.61–7.51 (*m*, 2H, 38-H, 41-H), 7.47 (d, $J = 9.4$ Hz, 1H, 41′-H), 7.22 (dd, $J = 7.5$, 1.1 Hz, 1H, 36-H), 6.99 (d, $J = 9.4$ Hz, 1H, 42-H), 6.91 (d, $J = 9.4$ Hz, 1H, 42′-H), 6.70–6.65 (*m*, 2H, 44-H, 44′-H), 5.23 (t, $J = 3.7$ Hz, 1H, 12-H), 3.60 (s, 3H, 31-H), 3.64–3.51 (*m*, 8H, 46-H, 46-H'), 3.47–3.37 (*m*, 1H, 18-H), 2.82 (dd, $J = 13.7$, 4.6 Hz, 1H), 1.97–0.93 (*m*, 20H, 16-H, 2-H_a, 22-H_a, 15-H_a, 19-H_a, 22-H_b, 1-H_a, 6-H, 9-H, 7-H_a, 21-H_a, 7-H_b, 21-H_b, 2-H_b, 19-H_b, 15-H_b), 1.35–1.24 (*m*, 12H, 47-H, 47′-H), 1.03 (s, 3H, 30-H), 0.90 (s, 3H, 26-H), 0.86 (d, $J = 1.6$ Hz, 6H, 25-H, 29-H), 0.82 (s, 1H, 1-H_b), 0.81 (s, 3H, 27-H), 0.70 (*m*, 1H, 5-H), 0.67 (s, 3H, 24-H), 0.58 (s, 3H, 23-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.4 (C-28), 168.0 (C-32), 160.2 (C-39), 158.0 (C-45′), 157.9 (C-45), 155.7 (C-43′), 155.4 (C-43), 143.8 (C-13), 138.3 (C-34), 133.2 (C-41′), 132.9 (C-41), 130.8 (C-33), 130.5 (C-35), 130.1 (C-38), 129.3 (C-36), 128.6 (C-37), 122.6 (C-12), 114.6 (C-40′), 114.3 (C-42′), 114.2 (C-40), 114.2 (C-42), 96.0 (C-44′), 96.0 (C-44), 57.8 (C-3), 56.3 (C-5), 51.6 (C-31), 47.7 (C-9), 46.9 (C-17), 46.1 (C-46, C-46′), 46.1 (C-19), 41.7 (C-14), 41.5 (C-18), 39.4 (C-1), 39.3 (C-8), 38.3 (C-4), 37.0 (C-10), 34.0 (C-21), 33.2 (C-29), 32.8 (C-7), 32.5 (C-22), 30.8 (C-20), 28.6 (C-23), 27.8 (C-15), 25.9 (C-30), 24.6 (C-2), 23.8 (C-26), 23.5 (C-11), 23.2 (C-16), 18.6 (C-6), 16.9 (C-24), 16.7 (C-27), 15.3 (C-25), 12.8 (C-47, C-47′) ppm; MS (ESI, MeOH): *m/z* 895.2 (100%, $[M + H]^+$); analysis calcd for $C_{59}H_{80}N_3O_4Cl$ (930.74): C 76.14, H 8.66, N 4.52; found: C 75.95, H 8.90, N 4.31.

4.2.4.5. 4-{[(3 β)-28-Methoxy-28-oxoolean-12-en-3-yl]oxy}-4-oxobutanoic acid (5). Compound 1 (2 g, 4.23 mmol) and catalytic amounts of DMAP were added to a solution of succinic anhydride (2.1 g, 21.15 mmol) in dry pyridine (50 mL), and the mixture was stirred under reflux for 8 h. Usual aqueous workup followed by column chromatography (SiO_2, hexanes/EtOAc, 7:3) gave 5 as a white solid (1.9 g, 79%); m.p. 210–212 °C; $[\alpha]_D = +60.8°$ (*c* 0.201, $CHCl_3$); $R_F = 0.29$ (SiO_2, hexanes/EtOAc, 7:3); IR (ATR): ν = 2935 m, 1728 s, 1710 s, 1440*w*, 1381*w*, 1317 m, 1175 s, 1148 m, 1036*w*, 1013*w*, 985 m, 801*w*, 645 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.27$ (t, $J = 3.7$ Hz, 1H, 12-H), 4.52 (dd, $J = 9.0$, 6.9 Hz, 1H, 3-H), 3.62 (s, 3H, 31-H), 2.86 (dd, $J = 13.9$, 4.5 Hz, 1H, 18-H), 2.73–2.56 (*m*, 4H, 33-H, 34-H), 2.02–1.91 (*m*, 1H, 16-H_a), 1.90–1.83 (*m*, 2H, 11-H), 1.74–1.14 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.12 (s, 3H, 27-H), 1.09–0.99 (*m*, 2H, 15-H_b, 1-H_b), 0.92 (s, 3H, 25-H), 0.92 (s, 3H, 30-H), 0.89 (s, 3H, 29-H), 0.85 (s, 3H, 23-H), 0.85 (s, 3H, 24-H), 0.82–0.76 (*m*, 1H, 5-H), 0.72 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 178.5 (C-28), 177.8 (C-32), 171.9 (C-35), 144.0 (C-13), 122.4 (C-12), 81.7 (C-3), 55.5 (C-5), 51.7 (C-31), 47.7 (C-9), 46.9 (C-17), 46.0 (C-19), 41.8 (C-14), 41.5 (C-18), 39.4 (C-8), 38.2 (C-1), 37.9 (C-4), 37.1 (C-10), 34.0 (C-21), 33.2 (C-29), 32.8 (C-7), 32.5 (C-22), 30.8 (C-20), 29.5 (C-33), 29.1 (C-34), 28.1 (C-23), 27.8 (C-15), 26.1 (C-27), 23.8 (C-30), 23.6 (C-16), 23.2 (C-2), 18.4 (C-6), 17.0 (C-26), 16.8 (C-24), 15.5 (C-25) ppm; MS (ESI, MeOH): *m/z* 493.1 (38%, $[M + Na]^+$), 1163.3 (100%, $[2\ M + Na]^+$); analysis calcd for $C_{35}H_{54}O_6$ (570.80): C 73.65, H 9.54.

4.2.4.6. Methyl (3 β)-3-{[(4-oxo-4-(piperazin-1-yl)butanoyl]oxy}olean-12-en-28-oate (6). Compound 5 (300 mg, 0.526 mmol) was dissolved in dry DCM (20 mL), and oxalyl chloride (4 eq.) and catalytic quantities of DMF were added. The reaction mixture was stirred at 23 °C for one hour. The volatiles were removed under diminished pressure, the residue was dissolved in dry DCM (15 mL), and piperazine (4 eq.), TEA (1 eq.) and DMAP (cat.) were added. The reaction mixture was stirred at 23 °C for one day. Usual aqueous workup followed column chromatography (SiO_2, hexanes/EtOAc, 7:3) gave 6 as a white solid (295 mg, 94%); m.p. 78–82 °C; $[\alpha]_D = +11.4°$ (*c* 0.163, $CHCl_3$); $R_F = 0.21$ (SiO_2, hexanes/EtOAc, 7:3); IR (ATR): ν = 3442 m, 2946 s, 1725 s, 1628 m, 1328 s, 1173 m, 1036*w*, 822 m cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.27$ (t, $J = 3.6$ Hz, 1H, 12-H), 4.50 (dd, $J = 8.8$, 7.1 Hz, 1H, 3-H), 3.68–3.44 (*m*, 8H, 36-H, 37-H, 38-H, 39-H), 3.62 (s, 3H, 31-H), 2.85 (dd, $J = 13.8$, 4.5 Hz, 1H, 18-H), 2.71–2.59 (*m*, 4H, 33-H, 34-H), 2.02–1.91 (*m*, 1H, 16-H_a), 1.91–1.84 (*m*, 2H, 11-H), 1.73–1.14 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.12 (s, 3H, 27-H), 1.08–0.98 (*m*, 2H, 1-H_b, 15-H_b), 0.92 (d, $J = 1.3$ Hz, 6H, 25-H, 30-H), 0.89 (s, 3H, 29-H), 0.86 (s, 3H, 23-H), 0.85 (s, 3H, 24-H), 0.82–0.79 (*m*, 1H, 5-H), 0.72 (s, 3H, 26-H) ppm; ^{13}C NMR (101 MHz, CDCl3): δ = 178.7 (C-28), 173.1 (C-32), 170.6 (C-35), 144.3 (C-13), 122.7 (C-12), 81.7 (C-3), 55.8 (C-5), 52.0 (C-31), 48.0 (C-9) 47.2 (C-17), 46.3 (C-19), 45.5 (C-37, C-38), 42.1 (C-14), 42.1 (C-36, C-39), 41.8 (C-18), 39.8 (C-8), 38.6 (C-1), 38.2 (C-4), 37.4 (C-10), 34.3 (C-21), 33.6 (C-29), 33.1 (C-7), 32.8 (C-22), 31.2 (C-20), 30.0 (C-34), 28.5 (C-23), 28.4 (C-33), 28.2 (C-15), 26.4 (C-27), 24.1 (C-30), 24.0 (C-16), 23.9 (C-11), 23.5 (C-2), 18.7 (C-6), 17.3 (C-26), 17.2 (C-24), 15.8 (C-25) ppm; MS (ESI, MeOH): *m/z* 639.3 (100%, $[M + H]^+$), 1277.2 (12%, $[2\ M + H]^+$), 1299.3 (100%, $[2\ M + Na]^+$); analysis calcd for $C_{39}H_{62}N_2O_5$ (638.92): C 73.31, H 9.78, N 4.38; found: C 73.05, H 9.98, N 4.18.

4.2.4.7. 6-(Diethylamino)-N,N-diethyl-9-(2-{[(3 β)-2-methoxy-28-oxoolean-12-en3-yl]carbamoyl}phenyl)–3H-xanthen-3-iminium chloride (7). Rhodamine B (277 mg, 0.6 mmol) was dissolved in dry DCM (15 mL) and oxalyl chloride (4 eq.) and catalytic quantities of DMF were added. The reaction mixture was stirred at 23 °C for one hour. The volatiles were removed under diminished pressure, the residue was dissolved in dry DCM (15 mL), and compound 6 (200 mg, 0.469 mmol), TEA (1 equiv.) and DMAP (cat.) were added. The reaction mixture was stirred at 23 °C for one day. Usual aqueous workup followed column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1) gave 7 as a purple solid (151 mg, 75.5%); m.p. 165–175 °C; $R_F = 0.44$ (SiO_2, $CHCl_3$/MeOH, 9:1); UV–Vis ($CHCl_3$): λ_{max} (log ε) = 559 nm (4.92); IR (ATR): ν = 3411 m, 2932*w*, 1722*w*, 1633 m, 1588 m, 1411 m, 1334 s, 1245 m, 1179 m, 1074*w*, 1007*w*, 979*w*, 823*w*, 683*w* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 7.78–6.64 (*m*, 10*H*, 45-H, 43-H, 46-H, 49-H, 49′-H, 44-H, 50-H, 50′-H, 44-H, 44′-H), 5.29–5.22 (*m*, 1H, 12-H), 4.45 (t, $J = 7.9$ Hz, 1H, 3-H), 3.61 (s, 3H, 31-H), 3.76–3.26 (*m*, 16H, 54-H, 54′-H, 36-H, 37-H, 38-H, 39-H), 2.85 (dd, $J = 14.4$, 4.8 Hz, 1H, 18-H), 2.73–2.52 (*m*, 4H, 33-H, 34-H), 2.01–1.90 (*m*, 1H, 16-H_a), 1.86 (dd, $J = 9.5$, 4.0 Hz, 2H, 11-H), 1.74–1.12 (*m*, 16H, 22-H_a, 16-H_b, 2-H, 19-H_a, 1-H_a, 15-H_a, 9-H, 6-H_a, 7-H, 6-H_b, 21-H_a, 22-H_b, 21-H_b, 19-H_b), 1.31 (s, 12H, 55-H, 55′-H), 1.11 (s, 3H, 27-H), 1.08–0.95 (*m*, 2H, 15-H_b, 1-H_b), 0.91 (s, 3H, 30-H), 0.90 (s, 3H, 25-H), 0.89 (s, 3H, 29-H), 0.83 (s, 3H, 23-H), 0.81 (s, 3H, 24-H), 0.78–0.76 (*m*, 1H, 5-H), 0.71 (s, 3H, 26-H) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 178.4 (C-28), 173.1 (C-32), 170.5 (C-35), 168.0 (C-40), 163.1 (C-47), 157.9 (C-53, C-53′), 155.9 (C-51, C-51′), 143.9 (C-13), 137.2 (C-42), 132.6 (C-49, C-49′), 131.0 (C-41), 130.1, 127.8 (C-45), 122.4 (C-12), 115.3 (C-48, C-48′, C-50, C-50′), 96.1 (C-52, C-52′), 81.2 (C-3), 55.5 (C-5), 51.6 (C-31), 47.7 (C-9), 46.9 (C-17), 46.2 (C-54, C-54′), 46.0 (C-19), 45.5 (C-37, C-38), 42.4 (C-36′, C-39), 41.8 (C-14), 41.4 (C-18), 39.4 (C-8), 38.2 (C-1), 37.9 (C-4), 37.0 (C-10), 34.0 (C-21), 33.2 (C-29), 32.7 (C-7), 32.5 (C-22), 32.3 (C-20), 30.8 (C-34), 28.2 (C-23), 28.0 (C-33), 27.8 (C-15), 26.0 (C-27), 23.8 (C-30), 23.6 (C-16), 23.5 (C-11), 23.2 (C-2), 18.3 (C-6), 17.0 (C-26), 16.9 (C-24), 15.5 (C-25), 12.7

(C-55, C-55′) ppm; MS (ESI, MeOH): *m/z* 1064.1 (100%, $[M + H]^+$); analysis calcd for $C_{67}H_{91}N_4O_7Cl$ (1099.92): C 73.16, H 8.34, N 5.09; found: C 72.96, H 8.51, N 4.87.

4.2.4.8. 3 β -Acetyloxy-olean-12-en-28-oic acid (8). This compound was prepared by acetylation of OA as previously reported, and 8 (10 g, 75%) was obtained as a colorless solid; m.p. 265–267 °C (lit.:[47] 264–265 °C); MS (ESI, MeOH): *m/z* 499.2 (13%, $[M + H]^+$), 521.2 (35%, $[M + Na]^+$, 1019.4 $[2 M + Na]^+$).

4.2.4.9. (3 β) 28-Oxo-28-(piperazine-1-yl)-olean-12-en-3-yl acetate (9). As described for the synthesis of 6, from 8 and piperazine, compound 9 (0.42 g, 84%) was obtained as a colorless solid; m.p. 171–175 °C; (lit.: [48,49] m.p. 170–176 °C); MS (ESI, MeOH): *m/z* 567.2 (52%, $[M + H]^+$).

4.2.4.10. 9-(2-{4[(3 β)-3-Acetyloxy-28-oxoolean-12-en-28-yl]-piperazine-1-carbonyl}phenyl)-6-(diethylamino)-N,N-diethyl-3H-xanthen-3-iminium chloride (10). As described for the synthesis of 7, from 9 and piperazine, compound 10 (0.67 g, 79%) was obtained as a colorless solid; m.p. 244–247 °C, (lit.:[14] 245–248 °C); MS (ESI, MeOH): *m/z* 991.7 (100%, $[M-Cl]^+$).

4.2.4.11. 6-(Diethylamino)-N,N-diethyl-9-[2-({[(3 β)-28-methoxy-28-oxoolean-12-en-3-yl]oxy}carbonyl)phenyl]–3H-xanthen-3-iminium chloride (11). This compound was prepared as previously described; 11 was obtained as a pink solid (0.68 g, 41%); m.p. 237–240 °C (lit.:[17] 235–240 °C); MS (ESI, MeOH): *m/z* 896.1 (100%, $[M-Cl]^+$).

CRediT authorship contribution statement

Niels Heise: Investigation, Writing - review & editing. **Sophie Hoenke:** Investigation, Writing - review & editing. **Vivienne Simon:** Investigation, Writing - review & editing. **Hans-Peter Deigner:** Conceptualization, Writing - original draft, Writing - review & editing. **Ahmed Al-Harrasi:** Conceptualization, Writing - original draft, Writing - review & editing. **René Csuk:** Conceptualization, Writing - original draft, Writing - review & editing.

Acknowledgments

We like to thank Th. Schmidt for the MS spectra, and Dr. D. Ströhl, Y. Schiller and S. Ludwig for numerous NMR spectra. IR and UV/vis spectra, optical rotations and micro-analyses were performed by M. Schneider. The cell lines were provided by Dr. Th. Müller (Dep. of Oncology, MLU).

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