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Converting bile acids into mitocans

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ABSTRACT

Cholic acid (**1**, CD), deoxycholic (**3**, DCA), chenodeoxycholic acid (**5**, CDCA), ursodeoxycholic acid (**7**, UDCA), and lithocholic acid (**9**, LCA) were acetylated and converted into their piperazinyl spacered rhodamine B conjugates **16**–**20**. While the parent bile acids showed almost no cytotoxic effects for several human tumor cell lines, the piperazinyl amides were cytostatic but an even superior effect was observed for the rhodamine B conjugates. Extra staining experiments showed these compounds as mitocans; they led to a cell arrest in the G1 phase.

1. Introduction

Recently rhodamine B conjugates of substituted, spacered of pentacyclic triterpenes attracted increased scientific interest, since some of them held most promising cytotoxic properties [1–8]. Cancer is the leading death cause with million deaths worldwide [9], resulting in both an additional burden on health institutions especially in poorer counties but also a social burden for individuals and their family. Since lipophilic derived cations are accumulated in the mitochondria, they have been used for the synthesis of various drug conjugates [4,6,10–15]. Hereby, we report the synthesis of novel tetracyclic triterpene, bile acid derived rhodamine B conjugates and their biological evaluation, thereby employing derivatives of bile acids litho-, deoxy-, chenodeoxy-, and ursodeoxycholic acid (Fig. 1), respectively.

Bile acids are natural detergents. They facilitate the absorption of fat in the intestine but they are also essential in the maintenance of the intestine epithelium homeostasis. Depending on type and concentration they show a dual behavior both as pro-survival or pro-death molecules [16–18]. Recently, cytotoxic activity [19] was established for bile acid-paclitaxel hybrids [20], a camptothecin conjugate [21] as well as for a dihydroartemisinine [22] analog. Furthermore, some bile carboxylamides have been shown to exert pro-apoptotic effects in human colon adenocarcinoma cells DLD-1, HCT-116 and HT29. Pro-apoptotic activity has also been observed on multiple myeloma as well as on glioblastoma multiforme [23]. We have previously demonstrated the cytotoxic activity of triterpenoid-rhodamine B conjugates [4]. It was therefore reasonable to extend our investigations to bile acids, especially since these compounds show a priori a better solubility in biological systems than triterpenoids by comparison due to their amphiphilic nature. In addition, bile acids have emerged as important starting materials for a variety of different bioactive conjugates [24,25].

2. Results and discussion

2.1. Chemistry

Cholic acid (**1**, CA), deoxycholic (**3**, DCA), chenodeoxycholic acid (**5**, CDCA), ursodeoxycholic acid (**7**, UDCA), and lithocholic acid (**9**, LCA) were bought from commercial vendors and acetylated to their respective acetates **2**, **4**, **6**, **8**, and **10** with acceptable yields (Scheme 1). Subsequently, these acetylated bile acids (**ABA**) were allowed to react with oxalyl chloride in the presence of catalytic amounts of DMF followed by adding piperazine to yield piperazinyl amides **11**–**15** (55–66 %, Scheme 2).

Finally, the desired rhodamine B conjugates were easily synthesized employing the well-established EDC·HCl / HOBt method to afford the conjugates in **16**–**20** in 47 %–66 % isolated yields, respectively.

2.2. Biology

The cytotoxicity of the synthesized compounds was evaluated using photometric SRB assays employing several human tumor cell lines (cut-off at 30 μM; Table 1), and the results of which are summarized in Table 1. While the parent bile acids **2**, **4**, **6** and **8** as well as rhodamine B (**Rhd B**) showed no activity towards the cell lines used in the SRB-assay, their acetates held increased biological activity towards the cell lines A375, MCF7, and A2780. Compound **10** proved to be insoluble under the conditions of the assay. At first glance, this seems surprising since

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compounds **2**, **4**, **6** and **8** were readily soluble. However, the poor solubility of **10** also follows the solubility behavior reported for the unsubstituted **GA**s. Here, too, LCA is the most poorly soluble with 0.38 mg/L while CA, for example, has a solubility of 175 mg/L in water. [26] For the piperazinyl amides **12** and **13**, however, even lower EC_{50} values were observed. The observation that piperazinyl amides hold lower EC_{50} values than their corresponding carboxylic acids parallels earlier results having been observed also in the field of terpenoid, in particular of triterpenoid carboxylic acids [1,2,27]. Finally, for the rhodamine B conjugates **16**–**20** and the malignant cell lines very low EC_{50}-values in the range of 0.2–1.2 μM were measured. These conjugates, however, showed only low selectivity for the non-malignant cell line NIH 3 T3 with **16** being the best overall compound holding a tumor cell/non-tumor cell selectivity ranging from S = 1.33 – 3.27. The best selectivity for this series of compounds was measured for the breast adenocarcinoma cell line MCF-7; this again, parallels previous finding for similar steroid conjugates [28].

To further investigate the mode of action of the piperazinyl amides **12** and **13**, as well as of the rhodamine B conjugate **16**, annexin-V-FITC/PI staining assays were used to assess the triggered mode of cell death onto the tumor cell line A2780 at double the EC_{50} concentrations (Fig. 2). Thereby, the samples (sixfold sample repetitions) were incubated for 24 h and 48 h with and without added compounds. Two technical repetitions in reference to the control group were used for calculation. Interestingly, compound **16** showed no significant difference from the control group (Table 2). In contrast to this compound, compounds **12** and **13** showed a significantly lower number of necrotic and vital cells and, in addition, they also held significantly more apoptotic cells at 24 h incubation. These trends continued after 48 h of incubation with compound **12** being the best with an average of 34 % more apoptotic and −26 % vital cells in comparison to the control cell line.

Furthermore, cell cycles were analyzed by FACS employing the ovarian cancer cell line A2780 applying an incubation time of 24 h and 48 h, respectively. Analysis of the results from these experiments showed compounds **12** and **16** to lock treated cells in the G1-phase and a decreased number of cells was found in the S-phase at 24 h incubation (Fig. 3; Fig. 4).

This parallels most recent findings for **12**; R. Yang et al. have previously shown that this compound arrests HepG2 hepatoma cells in G0/G1 and induces apoptosis by the PI3K/AKT/mTor pathway [29]. Compound **12** also induced nearly 39 % of apoptosis as compared to the control cell line (Fig. 3). After an incubation time of 48 h, cells treated with **12** showed 51.38 % apoptotic cells, while the number of cells in the G1 phase had decreased (Fig. 4). The increased cytostatic effect of compound **16** seems to be the result of fewer cells being able to enter the S and G2/M phase.

To assess whether **16** acts as a mitocan (an acrynom describing compounds exerting their anti-cancer activity via their molecular targets within mitochondria), some extra staining experiments were performed, the results of which are depicted in Fig. 5. The dye rhodamine 123 is known to stain mitochondria specifically; this dye emits green light after excitation; compound **16** emits red light.

Consequently, a merged image of the two excitations would cause an observed orange color, and gives evidence whether **16** is accumulated in the mitochondria of A2780 cells (Fig. 5). A microscopic investigation indeed showed a good match of the rhodamine 123 dye with **16**, and an orange color was observed. Moreover, staining with Hoechst 33,342, a blue-emitting nucleus targeting dye, showed **16** not to enter the nucleus of the cancer cells.

3. Conclusion

Several bile acids, i.e. cholic acid (**1**), deoxycholic (**3**), chenodeoxycholic acid (**5**), ursodeoxycholic acid (**7**), and lithocholic acid (**9**) were converted into their acetylated piperazinyl amides. The latter were coupled with rhodamine B to yield conjugates. These conjugates were cytostatic for a panel of human tumor cell lines; they led to a cell arrest in G1, and are accumulated in the mitochondria of the tumor cells. The conjugates do not enter the nucleus. Albeit their cytostatic effect is lower than that of pentacyclic triterpenoid analogs, they represent interesting starting materials for the development of analogs of even higher cytotoxicity and improved tumor cell/non-malignant cell selectivity while still retaining their good solubility properties.

4. Experimental part

4.1. Methods and equipment

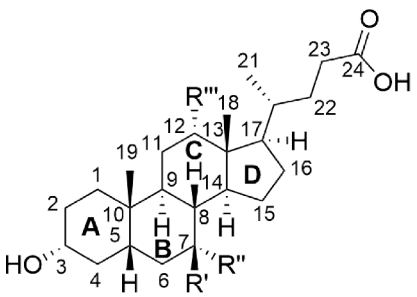
Cholic (**1**), deoxycholic (**2**), chenodeoxycholic (**3**), ursodeoxycholic (**4**), and lithocholic acid (**5**) were obtained from Carl Roth and abcr GmbH and were used as received. Equipment and lab equipment was used as previously described. Details can be found in the Supplementary materials file. For the Annexin V-FITC/PI assay as well as for the cell cycle analysis the Attune® Cytometric Software (1.2.5) and MSExcel were used for calculations.

4.2. General procedure (GP1) for the acetylation of bile acids

The bile acid (**1**–**5**, 1 eq) and cat. amounts of DMAP were dissolved in a minimal amount of dry pyridine (20 mL), and acetic anhydride (3–7 eq, depending on the number of hydroxyl groups) was added. After stirring at 25 °C for 24 h, the solution was diluted with DCM (100 mL), washed with HCl (0.1 M, 50 mL), and water (2 × 100 mL). The organic phase was dried ($MgSO_4$), the solvent evaporated under reduced pressure, and the residue was purified by column chromatography (SiO_2, *n*-hexane/ethyl acetate).

4.3. General procedure (GP2) for the synthesis of acetylated piperazinyl amides

The acetylated bile acid (**2**, **4**, **6**, **8**, **10**, 1 eq) was dissolved in a minimal amount of dry DCM (10 mL) under argon and cooled to 0 °C. Cat. amount of dry DMF and oxalyl chloride (4 eq) were added, and the



Cholic acid:	R'=OH,	R''=OH,	R'''=OH
Lithocholic acid:	R'=H,	R''=H,	R'''=H
Deoxycholic acid:	R'=H,	R''=H,	R'''=OH
Chenodeoxycholic acid:	R'=H,	R''=OH,	R'''=H
Ursodeoxycholic acid:	R'=OH,	R''=H,	R'''=H

Fig. 1. Structure and numbering scheme of bile acids.

reaction was allowed to warm to 25 °C. After stirring for an additional 2 h, the volatiles were removed under reduced pressure. To a solution containing piperazine (3.8 eq) and triethylamine (1.1 eq) in a minimal amount of dry DCM at –21 °C under argon was added dropwise the acid chloride, dissolved in dry DCM (20 mL). The reaction mixture was stirred at 25 °C for 24 h, quenched with water (100 mL), extracted with DCM (3 × 100 mL), the combined organic phases were dried ($MgSO_4$), and the solvent was evaporated under reduced pressure to obtain a solid which was purified by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1).

4.4. General procedure (GP3) for the amidation with rhodamine B

Rhodamine B (1 eq), 1-hydroxybenztriazole hydrate (1.1 eq), and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (1.1 eq) were dissolved in minimal amounts of DMF under argon. After stirring for 24 h, compound **11**–**15**, (1 eq) dissolved in a minimal amount of DMF, was added. Stirring was continued for 24 h, and for work-up aqueous HCl (1 M) and $CHCl_3$ were added; evaporation of the organic layer under reduced pressure afforded the crude product, which was purified by column chromatography (SiO_2, $CHCl_3$/MeOH, 9:1).

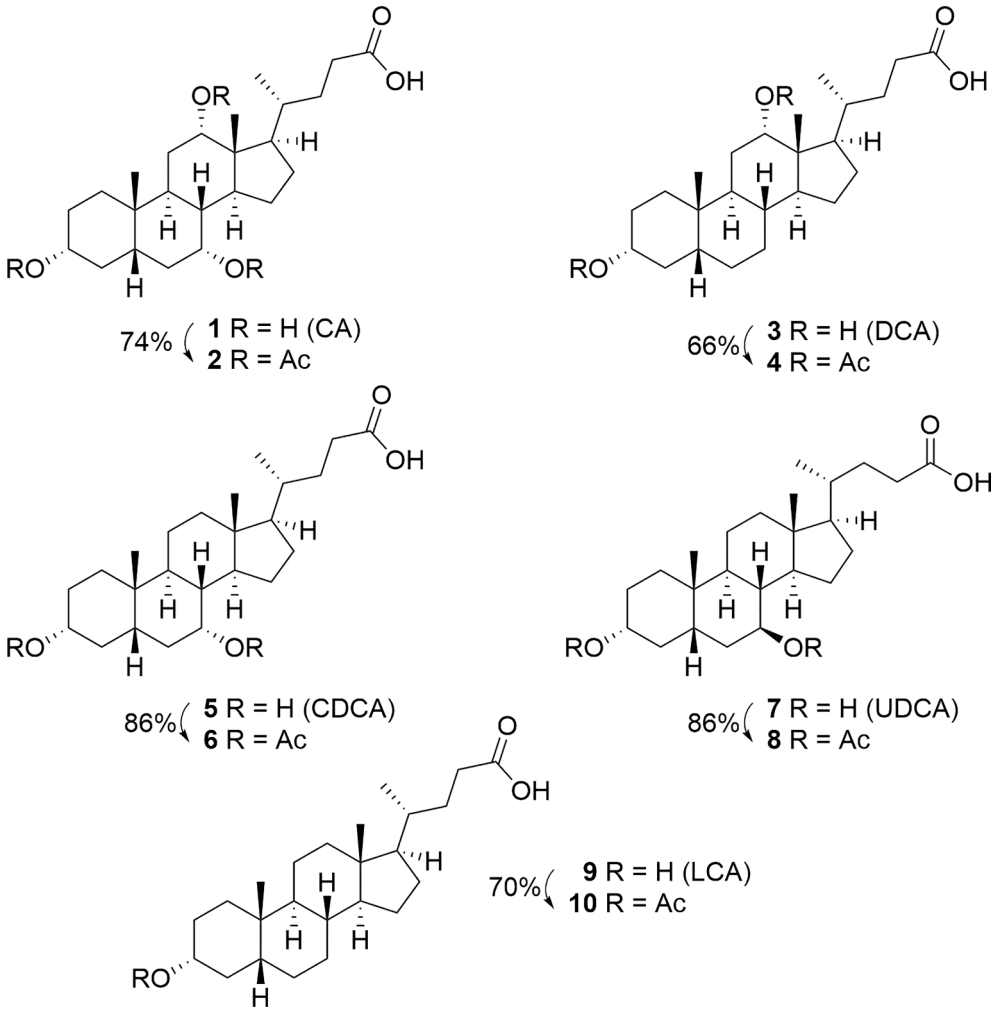
4.5. Syntheses

4.5.1. 3α,7α,12α-Tris(acetyloxy)-5β-cholic acid (2)

Following GP1, from cholic acid (**1**, 3.0 g, 7.3 mmol) **2** (2.2 g, 74 %) was obtained as a white solid; m.p. 92 °C (lit.: [30] 69–70 °C); R_F = 0.3 (*n*-hexane/ethyl acetate, 2:3); $[\alpha]_D$ = +69.6° (*c* 0.249, $CHCl_3$) [lit.: [31] $[\alpha]_D$ = +22.9° (*c* 0.34, $CHCl_3$)]; IR (ATR): $\tilde{\nu}$ = 498*vw*, 584*vw*, 608*w*, 660*vw*, 638*vw*, 722*vw*, 800*w*, 890*w*, 938*w*, 965*w*, 1023 *m*, 1062*w*, 1128*vw*, 1152*w*, 1233 *s*, 1365 *m*, 1377 *m*, 1439*w*, 1731 *s*, 2872*w*, 2941 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.08 (t, *J* = 2.9 Hz, 1H, H-12), 4.90 (q, *J* = 3.2 Hz, 1H, 7-H), 4.57 (tt, *J* = 11.4, 4.3 Hz, 1H, 3-H), 2.31 (dddd, *J* = 70.3, 15.8, 9.6, 5.8 Hz, 2H, 23-H_2), 2.13 (s, 3H, 28-H_3), 2.08 (s, 3H, 30-H_3), 2.04 (s, 3H, 26-H_3), 2.04 – 1.75 (m, 7H, 1-H_a + 4-H_a + 6-H_a + 9-H + 14-H + 16-H_a + 22-H_a), 1.75 – 1.49 (m, 7H, 1-H_b + 2-H_a + 6-H_b + 8-H + 11-H_2 + 17-H), 1.49 – 0.96 (m, 8H, 2-H_b + 4-H_b + 5-H + 15-H_2 + 16-H_b + 20-H + 22-H_b), 0.91 (s, 3H, 19-H_3), 0.82 (d, *J* = 6.5 Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 179.5 (C-24), 170.7 (C-25), 170.7 (C-27), 170.6 (C-29), 75.5 (C-12), 74.3 (C-3), 70.9 (C-7), 47.5 (C-17), 45.2 (C-13), 43.6 (C-14), 41.1 (C-5), 37.9 (C-8), 34.9 (C-1), 34.8 (C-4), 34.7 (C-20), 34.5 (C-10), 34.5 (C-6), 31.4 (C-23), 30.9 (C-22), 30.7 (C-22), 29.0 (C-9), 27.3 (C-16), 27.0 (C-2), 25.7 (C-11), 23.0 (C-15), 22.7 (C-19), 21.7 (C-30), 21.6 (C-28), 21.6 (C-26), 17.6 (C-21), 12.4 (C-18) ppm; MS (ESI, MeOH): *m/z* = 552.13 (20 %, $[M + NH_4]^+$), 557.27 (100 %, $[M + Na]^+$), 573.27 (7 %, $[M + K]^+$).

4.5.2. 3α,12α-Bis(acetyloxy)-5β-cholan-24-oic acid (4)

Following GP1 from deoxycholic acid (**3**, 3.0 g, 7.6 mmol) **4** (1.96 g, 66 %) was obtained as a white solid; m.p. 89 °C (lit.: [30] 92–93 °C); R_F = 0.47 (toluene/ethyl acetate/heptane/HCOOH, 80:26:10:5); $[\alpha]_D$ = +90.6° (*c* 0.263, $CHCl_3$) [lit.: [32] $[\alpha]_D$ = +80.5° (*c* 1.00, $CHCl_3$)]; IR (ATR): $\tilde{\nu}$ = 579*w*, 617*w*, 665*w*, 681*w*, 754*w*, 800*w*, 851*w*, 888*w*, 915*w*,



Scheme 1. Synthesis of bile acid derived acetates 2, 4, 6, 8 and 10; conditions: Ac_2O, cat. DMAP, pyridine, 25 °C, 1 day.

954*w*, 971 *m*, 1025 *m*, 1070*w*, 1091*w*, 1160 *m*, 1193 *m*, 1240 *s*, 1363 *m*, 1378 *m*, 1449*w*, 1467*w*, 1707 *m*, 1733 *s*, 2868*w*, 2941 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 5.08$ (s, 1H, 12-H), 4.70 (dt, $J = 11.3$, 6.6 Hz, 1H, 3-H), 2.49 – 2.18 (m, 2H, 23-H_2), 2.10 (s, 3H, 28-H_3), 2.03 (s, 3H, 26-H_3), 1.96 – 1.75 (m, 4H, 4-H_a + 6-H_a + 16-H_a + 22-H_a), 1.75 – 1.51 (m, 9H, 1-H_a + 2-H_a + 4-H_b + 11-H_2 + 14-H + 15-H_a + 17-H + 20-H), 1.50 – 0.94 (m, 10*H*, 1-H_b + 2-H_b + 6-H_b + 7-H_2 + 8-H + 9-H + 15-H_b + 16-H_b + 22-H_b), 0.90 (s, 3H, 19-H_3), 0.81 (d, $J = 6.4$ Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 179.6$ (C-24), 170.8 (C-25), 170.7 (C-27), 76.1 (C-12), 74.4 (C-3), 49.6 (C-14), 47.7 (C-17), 45.2 (C-13), 42.0 (C-5), 35.8 (C-9), 34.9 (C-1), 34.8 (C-8), 34.6 (C-20), 34.2 (C-10), 32.4 (C-4), 31.0 (C-23), 30.8 (C-22), 27.5 (C-16), 27.0 (C-6), 26.8 (C-2), 26.0 (C-7), 25.8 (C-11), 23.6 (C-15), 23.2 (C-19), 21.6 (C-26), 21.5 (C-28), 17.7 (C-21), 12.6 (C-18) ppm; MS (ESI, MeOH): $m/z =$ 357.20 (18 %, $[M + H–2HOAc]^+$), 494.20 (38 %, $[M + NH_4]^+$), 499.27 (100 %, $[M + Na]^+$), 915.53 (23 %, $[2M\text{-}HOAc + Na]^+$).

4.5.3. 3α,7α-Bis(acetyloxy)-5β-cholan-24-oic acid (6)

Following GP1 from chenodeoxycholic acid (**5**, 3.0 g, 7.6 mmol) **6** (2.6 g, 86 %) was obtained as a white solid; m.p. 100 °C (lit.: [33] 99 °C); $R_F = 0.28$ (*n*-hexane/ethyl acetate, 2:1); $[\alpha]_D = +15.6°$ (*c* 0.309, $CHCl_3$) [lit.: [34] $[\alpha]_D = +13.2°$ (*c* 1.16, $CHCl_3$)]; IR (ATR): $\tilde{\nu} =$ 450*w*, 609*w*, 888*vw*, 938*w*, 967*w*, 1023 *m*, 1067*w*, 1140*w*, 1233 *s*, 1245 *s*, 1363 *m*, 1376 *m*, 1439*w*, 1732 *s*, 2870*w*, 2938 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.97 – 4.78$ (m, 1H, 7-H), 4.59 (dt, $J = 11.4$, 6.9 Hz, 1H, 3-H), 2.33 (dddd, $J = 54.0$, 15.8, 9.8, 5.8 Hz, 2H, 23-H_2), 2.05 (s, 3H, 26-H_3), 2.04 – 2.01 (m, 4H, 28-H_3 + 6-H_a), 2.01 – 1.65 (m, 6H, 2-H_a + 4-H_a + 8-H + 12-H_a + 16-H_a + 22-H_a), 1.65 – 1.40 (m, 5H, 2-H_b + 5-H + 9-H + 11-H_a + 20-H), 1.40 – 0.98 (m, 8H, 11-H_b + 12-H_b + 14-H + 15-H_2 + 16-H_b + 17-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.93 (s, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 179.6$ (C-24), 170.8 (C-25), 170.6 (C-27), 74.3 (C-3), 71.4 (C-7), 55.9 (C-14), 50.6 (C-17), 42.9 (C-13), 41.1 (C-5), 39.7 (C-12), 38.1 (C-9), 35.4 (C-20), 35.1 (C-1), 34.9 (C-6), 34.8 (C-10), 34.2 (C-8), 31.5 (C-4), 31.0 (C-23), 30.9 (C-22), 28.2 (C-16), 26.9 (C-2), 23.7 (C-15), 22.8 (C-19), 21.7 (C-26), 21.6 (C-28), 20.8 (C-11), 18.5 (C-21), 11.9 (C-18) ppm; MS (ESI, MeOH): $m/z =$ 552.13 (20 %, $[M + NH_4]^+$), 557.27 (100 %, $[M + Na]^+$), 573.27 (7 %, $[M + K]^+$).

4.5.4. 3α,7β-Bis(acetyloxy)-5β-cholan-24-oic acid (8)

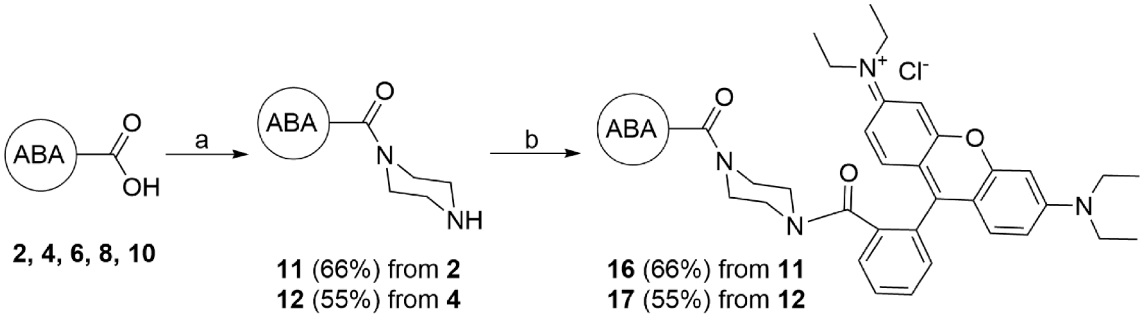
Following GP1 from ursodeoxycholic acid (**7**, 3.0 g, 7.6 mmol) **8** (2.4 g, 86 %) was obtained as a white solid; m.p. 110 °C (lit.: [35] 98–102 °C); $R_F = 0.31$ (hexane/ethyl acetate, 2:1); $[\alpha]_D = +53.8°$ (*c* 0.231, $CHCl_3$); IR (ATR): $\tilde{\nu} =$ 2946 *m*, 2872*w*, 1732 *s*, 1707 *m*, 1451*w*, 1381*w*, 1364 *m*, 1236*vs*, 1167*w*, 1123*w*, 1094*w*, 1075*w*, 1043 *m*, 1021 *m*, 987*w*, 977*w*, 956*w*, 932*w*, 925*w*, 908*w*, 892*w*, 864*w*, 853*w*, 800*vw*, 774*w*, 693*vw*, 666*vw*, 620*w*, 608*w*, 593*vw*, 568*vw*, 544*vw*, 530*vw*, 515*vw*, 504*vw*, 471*w*, 453*vw*, 416*vw* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): $\delta = 4.76$ (td, $J = 11.1$, 5.3 Hz, 1H, 7-H), 4.66 (tt, $J = 10.8$, 5.1 Hz, 1H, 3-H), 2.38 (ddd, $J = 15.5$, 10.0, 5.2 Hz, 1H, 23-H_a), 2.25 (ddd, $J = 15.9$, 9.5, 6.7 Hz, 1H, 23-H_b), 2.01 (s, 3H, 26-H_3), 2.01 – 1.98 (m, 1H,12-H_a), 1.97 (s, 3H, 28-H_3), 1.84 – 1.66 (m, 8H, 1-H_2 + 2-H_a + 4-H_a + 6-H_a + 8-H + 16-H_a + 22-H_a), 1.66 – 1.59 (m, 1H, 6-H_b), 1.58 – 1.49 (m, 2H, 5-H + 9-H), 1.48 – 1.12 (m, 10*H*, 2-H_b + 11-H_2 + 12-H_2 + 14-H + 15-H_2 + 16-H_b + 22-H_b), 1.10 – 0.99 (m, 2H, 4-H_b + 17-H), 0.96 (s, 3H, 19-H_3), 0.92 (d, $J = 6.5$ Hz, 3H, 21-H_3), 0.67 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): $\delta = 179.7$ (C-24), 170.6 (C-27), 170.6 (C-25), 73.6 (C-7), 73.6 (C-3), 55.2 (C-14), 55.0 (C-17), 43.6 (C-13), 42.0 (C-5), 40.0 (C-8), 39.9 (C-12), 39.4 (C-9), 35.2 (C-20), 34.5 (C-4), 34.0 (C-10), 32.9 (C-1 + C6), 30.9 (C-23), 30.7 (C-22), 28.4 (C-16), 26.4 (C-2), 25.6 (C-15), 23.2 (C-19), 21.8 (C-28), 21.4 (C-26), 21.2 (C-11), 18.3 (C-21), 12.1 (C-18) ppm; MS (ESI, $MeOH/CHCl_3$, 4:1, positive): $m/z = 499.1$ (100 %, $[M + Na]^+$); MS (ESI, $MeOH/CHCl_3$, 4:1): $m/z = 475.2$ (80 %, $[M–H]^-$), 951.3 (100 %, $[2\ M–H]^-$).

4.5.5. 3α-Acetyloxy-5β-cholan-24-oic acid (10)

Following GP1 from lithocholic acid (**9**, 3.0 g, 8.0 mmol) **10** (2.1 g, 70 %) was obtained as a white solid; m.p. 172 °C (lit.: [36] 170–171 °C); $R_F = 0.32$ (*n*-hexane/ethyl acetate, 2:1); $[\alpha]_D = +44.0°$ (*c* 0.310, $CHCl_3$) [lit.: [37] $[\alpha]_D = +41.6°$ (*c* 0.01, $CHCl_3$)]; IR (ATR): $\tilde{\nu} =$ 419*vw*, 449*vw*, 483*vw*, 616 *m*, 661*vw*, 887*vw*, 906*vw*, 931*vw*, 949*vw*, 981*vw*, 1026 *m*, 1065*vw*, 1096*vw*, 1164*w*, 1240 *s*, 1362 *m*, 1379 *m*, 1448 *m*, 1706 *s*, 1735 *s*, 2866 *m*, 2931 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): $\delta = 4.71$ (td, $J =$ 11.3, 5.6 Hz, 1H, 3-H), 2.32 (dddd, $J = 56.0$, 15.8, 9.9, 5.8 Hz, 2H, 23-H_2), 2.03 (s, 3H, 26-H_3), 2.00 – 1.73 (m, 6H, 1-H_a + 4-H_a + 6-H_a + 12-H_a + 16-H_a + 22-H_a), 1.73 – 1.49 (m, 3H, 2-H_a + 4-H_b + 15-H_a), 1.48 – 0.96 (m, 17H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_b + 16-H_b + 17-H + 20-H + 22-H_b), 0.93 (d, $J =$ 2.0 Hz, 3H, 19-H_3), 0.91 (s, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): $\delta = 180.0$ (C-24), 170.9 (C-25), 74.6 (C-3), 56.7 (C-14), 56.2 (C-17), 42.9 (C-13), 42.1 (C-5), 40.6 (C-9), 40.3 (C-20), 36.0 (C-12), 35.5 (C-8), 35.2 (C-1), 34.7 (C-10), 32.4 (C-4), 31.1 (C-23), 30.9 (C-22), 28.3 (C-16), 27.2 (C-6), 26.8 (C-2), 26.5 (C-7), 24.3 (C-15), 23.5 (C-19), 21.6 (C-26), 21.0 (C-11), 18.4 (C-21), 12.2 (C-18) ppm; MS (ESI, MeOH): $m/z = 359.20$ (18 %, $[M\text{-}HOAc + H]^+$), 436.20 (17 %, $[M + NH_4]^+$), 441.27 (100 %, $[M + Na]^+$), 799.53 (32 %, $[2\ M\text{-}HOAc + Na]^+$), 859.33 (15 %, $[2\ M + Na]^+$), 875.40 (40 %, $[2\ M + Na]^+$).

4.5.6. 3α,7α,12α-Tris(acetyloxy)-24-(1-piperazinyl)-5β-cholan-24-one (11)

Following GP2 from **2** (500 mg, 1.0 mmol) **11** (330 mg, 66 %) was obtained as a white solid; m.p. 136 °C; $R_F = 0.10$ ($CHCl_3$/MeOH, 95:5); $[\alpha]_D = +21.1°$ (*c* 0.131, $CHCl_3$); IR (ATR): $\tilde{\nu} =$ 450*w*, 608*w*, 751*w*, 965*w*, 1023 *m*, 1151*vw*, 1233 *s*, 1365 *m*, 1376 *m*, 1435 *m*, 1645 *m*, 1730 *m*,



2, 4, 6, 8, 10

11 (66%) from **2**
12 (55%) from **4**
13 (62%) from **6**
14 (59%) from **8**
15 (64%) from **10**

16 (66%) from **11**
17 (55%) from **12**
18 (52%) from **13**
19 (65%) from **14**
20 (47%) from **15**

Scheme 2. Synthesis of the acetylated bile acid (ABA) conjugates 16–20: a) cat. DMF, $(COCl)_2$, DCM, 0 °C → 25 °C, 2 h → piperazine, TEA, DCM, −21 °C → 25 °C, 24 h; b) rhodamine B, HOBt, EDC·HCl, DMF, 25 °C, 24 h.

Table 1
Cytotoxicity of synthesized compounds and rhodamine B (Rhd B) assessed from SRB-assays (EC_{50} values [μM] after 72 h of treatment). Human cancer cell lines: A375 (epithelial melanoma), HT29 (colorectal adenocarcinoma), MCF-7 (breast adenocarcinoma), A2780 (ovarian carcinoma), FaDu (hypopharyngeal carcinoma); non-malignant: NIH 3 T3 (fibroblasts); n.s. not soluble; n.d. not determined; S (selectivity) calculated S = $EC_{50\ of\ NIH\ 3T3}$ / $EC_{50\ of\ tumor\ cell\ line}$. Positive control: doxorubicin (DX).

	A375	HT29	MCF-7	A2780	FaDu	NIH 3T3
Rhd B	>30	>30	>30	>30	>30	>30
2	23.7 ± 3.3	>30	28 ± 4	23.8 ± 2.1	>30	>30
4	>30	>30	>30	24.8 ± 3.1	>30	>30
6	>30	>30	>30	>30	>30	>30
8	>30	>30	>30	>30	>30	>30
10	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
11	23.7 ± 2.6 (S = 0.94)	14.2 ± 1.5 (S = 1.57)	21.3 ± 2.5 (S = 1.04)	17.9 ± 2.2 (S = 1.25)	23.3 ± 3.6 (S = 0.96)	22.3 ± 2.7
12	4.1 ± 0.3 (S = 0.85)	3.2 ± 0.3 (S = 1.09)	4.1 ± 0.2 (S = 0.85)	3.4 ± 0.2 (S = 1.03)	3.0 ± 0.4 (S = 1.17)	3.5 ± 0.3
13	4.5 ± 0.4 (S = 0.87)	4.8 ± 0.4 (S = 0.81)	4.6 ± 0.3 (S = 0.85)	4.2 ± 0.5 (S = 0.93)	3.8 ± 0.6 (S = 1.03)	3.9 ± 0.4
14	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
15	6.61 ± 0.3 (S = 0.55)	3.59 ± 0.1 (S = 1.01)	4.12 ± 0.3 (S = 0.88)	5.50 ± 0.4 (S = 0.66)	6.67 ± 0.2 (S = 0.54)	3.62 ± 0.4
16	1.0 ± 0.1 (S = 1.6)	1.0 ± 0.1 (S = 1.6)	0.49 ± 0.05 (S = 3.27)	0.6 ± 0.1 (S = 2.67)	1.2 ± 0.3 (S = 1.33)	1.6 ± 0.1
17	0.66 ± 0.05 (S = 1.11)	0.68 ± 0.04 (S = 1.07)	0.32 ± 0.03 (S = 2.28)	0.36 ± 0.04 (S = 2.03)	0.4 ± 0.2 (S = 1.33)	0.73 ± 0.02
18	0.7 ± 0.1 (S = 0.93)	0.6 ± 0.1 (S = 1.08)	0.207 ± 0.004 (S = 3.14)	0.24 ± 0.03 (S = 2.71)	0.5 ± 0.1 (S = 1.3)	0.65 ± 0.09
19	0.42 ± 0.04 (S = 1.05)	0.39 ± 0.07 (S = 1.13)	0.20 ± 0.01 (S = 2.2)	0.214 ± 0.002 (S = 2.06)	0.5 ± 0.2 (S = 0.88)	0.44 ± 0.08
20	0.73 ± 0.05 (S = 1.18)	0.71 ± 0.03 (S = 1.21)	0.32 ± 0.03 (S = 2.69)	0.41 ± 0.06 (S = 2.1)	0.8 ± 0.1 (S = 1.08)	0.86 ± 0.03
DX	n.d.	0.91 ± 0.01 (S = 0.45)	1.10 ± 0.3 (S = 0.37)	0.01 ± 0.01 (S = 41.0)	n.d.	0.41 ± 0.07

2872*w*, 2939 *m* cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$): δ = 5.09 (s, 1H, 12-H), 4.90 (d, *J* = 2.7 Hz, 1H, 7-H), 4.61 – 4.51 (m, 1H, 3-H), 3.64 – 3.36 (m, 4H, 32-H_2 + 32'–H_2), 2.87 – 2.78 (m, 4H, 31-H_2 + 31'–H_2), 2.40 – 2.15 (m, 2H, 23-H_2), 2.12 (s, 3H, 26-H_3), 2.07 (s, 3H, 30-H_3), 2.03 (s, 4H, 28-H_3 + 9-H), 2.01 – 1.81 (m, 4H, 1-H_a + 6-H_a + 14-H + 16-H_a), 1.81 – 1.55 (m, 8H, 1-H_a + 2-H_a + 4-H_a + 6-H_b + 8-H + 11-H_a + 17-H + 22-H_a), 1.54 – 0.96 (m, 8H, 2-H_b + 4-H_b + 5-H + 11-H_b + 15-H_2 + 16-H_b + 20-H + 22-H_b), 0.90 (s, 3H, 19-H_3), 0.82 (d, *J* = 6.6 Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, $CDCl_3$): δ = 172.0 (C-24), 170.7 (C-25), 170.7 (C-27), 170.5 (C-29), 75.6 (C-12), 74.2 (C-3), 70.8 (C-7), 47.8 (C-17), 47.0 (C-31), 46.5 (C-31'), 46.0 (C-32), 45.3 (C-13), 43.5 (C-14), 42.7 (C-32'), 41.1 (C-5), 37.9 (C-8), 35.2 (C-20), 34.8 (C-1), 34.8 (C-4), 34.5 (C-10), 31.4 (C-6), 31.4 (C-22), 30.5 (C-23), 29.0 (C-9), 27.4 (C-16), 27.0 (C-2), 25.7 (C-11), 23.0 (C-15), 22.7 (C-19), 21.7 (C-30), 21.6 (C-28), 21.6 (C-26), 17.9 (C-21), 12.4 (C-18) ppm; MS (ESI, MeOH): *m/z* = 423.40 (8 %, $[M\text{-}3HOAc + H]^+$), 483.33 (12 %, $[M\text{-}2HOAc + H]^+$), 543.27 (12 %, $[M\text{-}HOAc + H]^+$), 603.27 (100 %, $[M + H]^+$), 615.40 (32 %, $[M + C + H]^+$), 633.20 (35 %, $[M + HCHO + H]^+$); analysis calcd for $C_{34}H_{54}N_2O_7$ (602.81): C 67.74, H 9.03, N 4.64; found: C 67.51, H 9.30, N 4.42.

4.5.7. 3α,12α-Bis(acetyloxy)-24-(1-piperazinyl)-5β-cholan24-one (12)

Following GP2 from **4** (500 mg, 1.0 mmol) **12** (273 mg, 55 %) was obtained as a white solid [29]; m.p. 87 °C; R_F = 0.38 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = +91.1° (*c* 0.297, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 497*vw*, 608*w*, 617*w*, 661*vw*, 755*vw*, 887*w*, 971*w*, 1025 *s*, 1090*w*, 1161*w*, 1195*w*, 1241*vs*, 1319*w*, 1363 *m*, 1377 *m*, 1434 *m*, 1446 *m*, 1644 *m*, 1732 *m*, 2867*w*, 2938*w* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 5.09 (t, *J* = 2.9 Hz, 1H, 12-H), 4.70 (ddt, *J* = 16.0, 10.8, 4.6 Hz, 1H, 3-H), 3.80 – 3.57 (m, 4H, 29-H_2 + 29'–H_2), 3.13 – 3.01 (m, 4H, 30-H_2 + 30'–H_2), 2.28 (dddd, *J* = 64.6, 15.4, 10.3, 5.3 Hz, 2H, 23-H_2), 2.10 (s, 3H, 28-H_3), 2.03 (s, 3H, 26-H_3), 1.94 – 1.74 (m, 4H, 6-H_a + 16-H_a + 22-H_2), 1.74 – 1.51 (m, 8H, 1-H_a + 2-H_a + 4-H_2 + 8-H + 14-H + 15-H_a + 17-H), 1.50 – 0.94 (m, 12H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 9-H + 11-H_2 + 15-H_b + 16-H_b + 20-H), 0.90 (s, 3H, 19-H_3), 0.82 (d, *J* = 6.4 Hz, 3H, 21-H_3), 0.72 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.3 (C-24), 170.7 (C-25), 170.6 (C-27), 76.0 (C-12), 74.3 (C-3), 49.6 (C-14), 48.0 (C-17), 45.3 (C-29), 45.2 (C-13), 45.0 (C-29'), 44.7 (C-30), 42.0 (C-5), 40.7 (C-30'), 35.8 (C-9), 35.2 (C-20), 34.9 (C-1), 34.6 (C-8), 34.2 (C-10), 32.4 (C-4), 31.3 (C-22), 30.5 (C-23), 27.6 (C-16), 27.0 (C-6), 26.8 (C-2), 26.0 (C-7), 25.8 (C-11), 23.6 (C-15), 23.2 (C-19), 21.6 (C-26), 21.6 (C-28), 17.9 (C-21), 12.6 (C-18) ppm; MS (ESI, MeOH): *m/z* = 425.33 (15 %, $[M\text{-}2HOAc + H]^+$), 485.27 (38 %, $[M\text{-}HOAc + H]^+$), 545.27 (100 %, $[M + H]^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.55, H 9.62, N 5.14; found: C 70.39, H 9.95, N 4.97.

4.5.8. 3α,7α-Bis(acetyloxy) 24-(1-piperazinyl)-5β-cholan-24-one (13)

Following GP2 from **6** (500 mg, 1.1 mmol) **13** (310 mg, 62 %) was obtained as a white solid [29]; m.p. 85 °C; R_F = 0.33 ($CHCl_3$/MeOH, 9:1); $[\alpha]_D$ = +16.2° (*c* 0.324, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 419*vw*, 449*w*, 553*vw*, 609*w*, 794*vw*, 888*w*, 938*w*, 968*w*, 1022 *m*, 1067*w*, 1140*w*, 1233 *s*, 1245 *s*, 1319 *m*, 1363 *m*, 1375 *m*, 1435 *m*, 1636 *m*, 1729 *s*, 2867*w*, 2938 *m* cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ = 4.86 (s, 1H, 7-H), 4.58 (dt, *J* = 11.3, 6.8 Hz, 1H, 3-H), 3.56 (d, *J* = 60.6 Hz, 4H, 29-H_2 + 29'–H_2), 2.93 (d, *J* = 14.6 Hz, 4H, 30-H_2 + 30'–H_2), 2.43 – 2.12 (m, 2H, 23-H_2), 2.04 (s, 3H, 28-H_3), 2.02 (s, 4H, 26-H_3 + 6-H_a), 2.01 – 1.90 (m, 2H, 12-H_a + 4-H_a), 1.90 – 1.66 (m, 5H, 1-H_a + 2-H_a + 16-H_a + 20-H + 22-H_a), 1.65 – 0.99 (m, 16H, 1-H_b + 2-H_b + 4-H_b + 5-H + 6-H_b + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_2 + 16-H_b + 17-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.92 (s, 3H, 21-H_3), 0.64 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.2 (C-24), 170.8 (C-25), 170.6 (C-27), 74.3 (C-3), 71.4 (C-7), 56.0 (C-17), 50.6 (C-14), 46.0 (C-30), 45.8 (C-30'), 45.4 (C-29), 42.9 (C-13), 41.8 (C-29'), 41.1 (C-5), 39.7 (C-12), 38.1 (C-8), 35.8 (C-9), 35.0 (C-1), 34.9 (C-6), 34.8 (C-10), 34.2 (C-20), 31.5 (C-22), 31.5 (C-4), 30.4 (C-23), 28.3 (C-16), 26.9 (C-2), 23.7 (C-15), 22.8 (C-19), 21.7 (C-28), 21.6 (C-26), 20.8 (C-11), 18.6 (C-21), 11.9 (C-18) ppm; MS (ESI, MeOH): *m/z* = 425.33 (17 %, $[M\text{-}2HOAc + H]^+$), 485.27 (27 %, $[M\text{-}HOAc + H]^+$), 545.27 (100 %, $[M + H]^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.55, H 9.62, N 5.14; found: C 70.19, H 9.98, N 4.94.

4.5.9. 3α-Acetyloxy-24-(1-piperazinyl)-5β-cholan-24-one (14)

Following GP2 from **8** (500 mg, 1.2 mmol) **14** (330 mg, 59 %) was obtained as a white solid; m.p. 79 °C; R_F = 0.10 ($CHCl_3$/MeOH, 95:5); $[\alpha]_D$ = +39.5° (*c* 0.324, $CHCl_3$) [lit.: [38] $[\alpha]_D$ = +20.5° (*c* 0.98, $CHCl_3$)]; IR (ATR): $\tilde{\nu}$ = 483*vw*, 557*w*, 614*w*, 792*w*, 887*w*, 1026 *m*, 1240 *s*, 1362 *m*, 1379 *m*, 1446 *m*, 1640 *m*, 1733 *m*, 2864 *m*, 2929 *m* cm^{-1}; ^1H

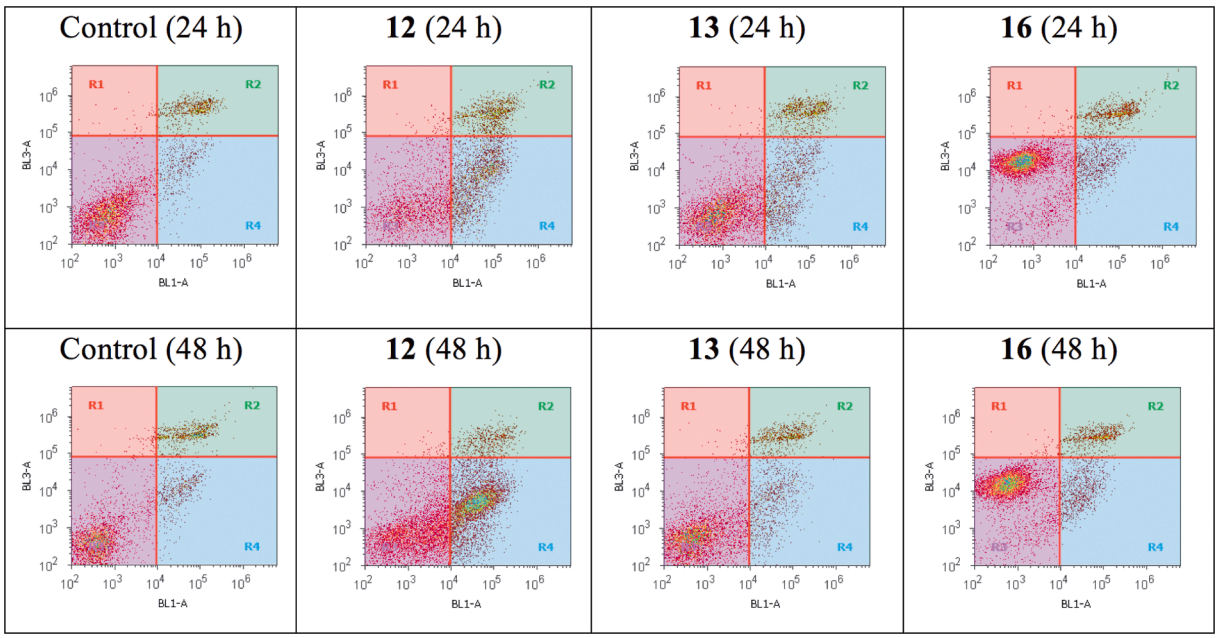
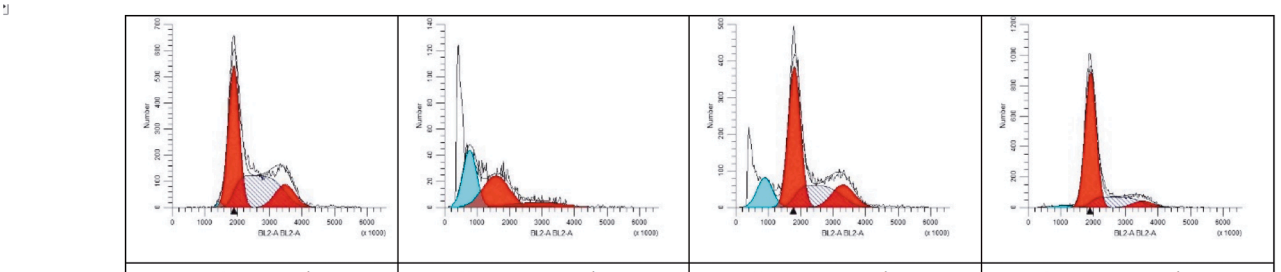


Fig. 2. Annexin V-FITC/PI assay plots. Representative examples of density plots determined by flow cytometry after 24 h and 48 h, respectively; R1: necrotic, R2: secondary necrotic/late-stage apoptotic, *R*3: vital, R4: apoptotic.

Table 2
Annexin V-FITC/PI assay average difference to control results tested for significance with T-test (red = P > 0.05; orange = P < 0.05; green = P < 0.01).

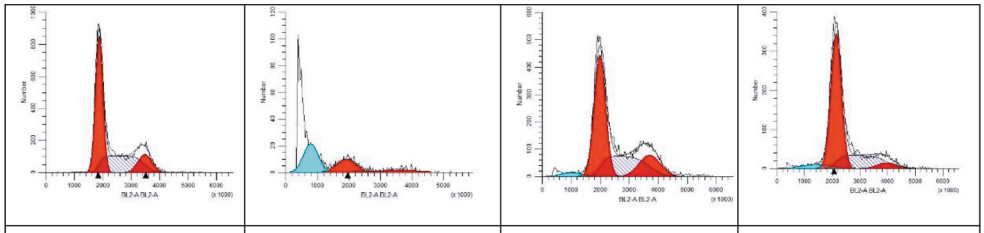
Incubation time	Necrotic			Secondary necrotic Late-stage apoptotic			vital			apoptotic		
	12	**13**	**16**	**12**	**13**	**16**	**12**	**13**	**16**	**12**	**13**	**16**
24 h	−3%	−3%	−1%	1 %	1 %	1 %	−24 %	−11 %	0 %	27 %	13 %	0 %
48 h	−3%	−2%	−1%	−5%	−3%	1 %	−26 %	−4%	−4%	34 %	9 %	4 %



	Control	Compound **12**	Compound **13**	Compound **16**
G1	47.12%	70.84% (23.72%)	55.86% (8.74%)	72.31% (25.19%)
S	38.81%	6.98% (-31.83%)	27.54% (-11.27%)	21.12% (-17.69%)
G2/M	14.07%	22.18% (8.11%)	16.60% (2.54%)	6.57% (-7.5%)
Apop.	0.93%	39.89% (38.96%)	12.93% (13.7%)	2.30% (1.37%)

Fig. 3. Cell cycle analysis of cell line A2780 with compounds 12, 13, and 16 in comparison with a control group after an incubation of 24 h. Left red = G1; right red = G2/M; blue striped = S phase; light blue subG1/apoptosis. In brackets the difference to control is shown.

NMR (400 MHz, $CDCl_3$): δ = 4.71 (d, *J* = 4.9 Hz, 1H, 3-H), 3.50 (d, *J* = 60.9 Hz, 4H, 27-H_2 + 27'–H_2), 2.93 – 2.76 (m, 4H, 28-H_2 + 28'–H_2), 2.44 – 2.11 (m, 2H, 23-H_2), 2.02 (s, 3H, 26-H_3), 2.00 – 1.48 (m, 9H, 1-H_a + 2-H_a + 4-H_2 + 6-H_a + 12-H_a + 15-H_a + 16-H_a + 22-H_a), 1.48 – 0.97 (m, 17H, 1-H_b + 2-H_b + 5-H + 6-H_b + 7-H_2 + 8-H + 9-H + 11-H_2 + 12-H_b + 14-H + 15-H_b + 16-H_b + 17-H + 20-H + 22-H_b), 0.94 (s, 3H, 19-H_3), 0.92 (s, 3H, 21-H_3), 0.64 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.3 (C-24), 170.8 (C-25), 74.5 (C-3), 56.7 (C-14), 56.3 (C-17), 47.1 (C-27), 46.6 (C-27'), 46.1 (C-28), 42.9 (C-28'), 42.8 (C-13), 42.1 (C-5), 40.6 (C-9), 40.3 (C-12), 36.0 (C-8), 35.9 (C-20), 35.2 (C-1),



	Control	Compound **12**	Compound **13**	Compound **16**
G1	54.81%	64.26% (9.45%)	52.73% (-2.08%)	69.01% (14.2%)
S	31.59%	9.02% (-22.21%)	29.89% (-1.7%)	25.33% (-6.26%)
G2/M	13.60%	26.72% (13.12%)	17.38% (3.78%)	5.66% (-7.94%)
Apop.	0.30%	51.38% (51.08%)	3.00% (2.7%)	5.63% (5.33%)

Fig. 4. Cell cycle analysis of cell line A2780 with compounds 12, 13, and 16 compared to a control group after an incubation of 48 h. Left red = G1; right red = G2/M; blue striped = S phase; light blue subG1/apoptosis. In brackets the difference to control is shown.

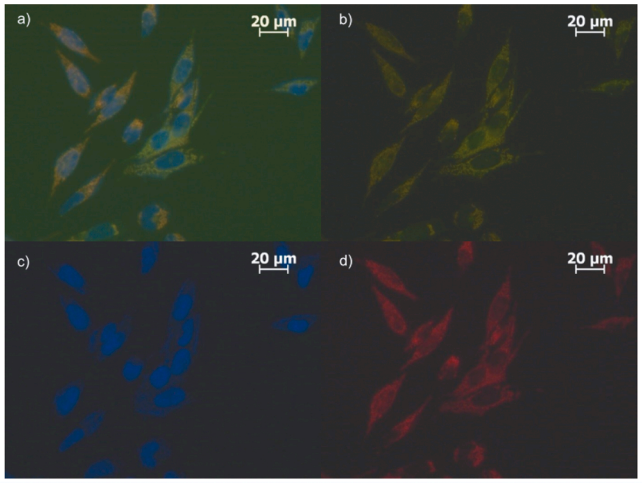


Fig. 5. Fluorescence images of A2780 tumor cells treated with 16, rhodamine 123 and Hoechst 33,342, respectively: a) merged imaged, b) rhodamine 123, c) Hoechst 33,342, d) compound 16.

34.7 (C-10), 32.4 (C-4), 31.6 (C-22), 30.5 (C-23), 28.4 (C-6), 27.2 (C-16), 26.8 (C-2), 26.5 (C-7), 24.4 (C-15), 23.5 (C-19), 21.6 (C-26), 21.0 (C-11), 18.7 (C-21), 12.2 (C-18) ppm; MS (ESI, MeOH): m/z = 427.37 (3 %, [M-HOAc + H]$^+$), 487.33 (100 %, [M + H]$^+$), 509.27 (2 %, [M + Na]$^+$), 973.33 (2 %, [2 M + H]$^+$); analysis calcd for $C_{30}H_{50}N_2O_3$ (486.73): C 74.03, H 10.35, N 5.76; found: C 73.76, H 10.59, N 5.47.

4.5.10. 3α,7β-Bis(acetyloxy)-24-(1-piperazinyl)- 5β-cholan-24-one (15)

Following GP2 from **10** (500 mg, 1.0 mmol) **15** (365 mg, 64 %) was obtained as a white solid; m.p. 94.3 °C; R_F = 0.20 ($CHCl_3$/MeOH/NH_4OH, 98:1.8:0.2); $[\alpha]_D$ = +48.2° (*c* 0.188, $CHCl_3$); IR (ATR): $\tilde{\nu}$ = 468*w*, 477*w*, 559*w*, 608 *m*, 693*w*, 799*w*, 891*w*, 956*w*, 1021 *s*, 1042 *m*, 1122 *w*, 1237*vs*, 1319*w*, 1364 *m*, 1380*w*, 1433 *m*, 1636 *m*, 1729 *s*, 2871*w*, 2944*w* cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): 4.75 (tt, J = 9.0, 4.1 Hz, 1H, 7-H), 3.71 – 3.45 (m, 5H, 3-H, 29-H_2 + 29′-H_2), 3.17 – 2.84 (m, 4H, 30-H_2 + 30′-H_2), 2.35 (ddt, J = 15.5, 10.6, 5.4 Hz, 1H, 23-H_a), 2.19 (ddt, J = 15.3, 10.5, 6.2 Hz, 1H, 23-H_b), 2.03 – 1.96 (m, 4H, 12-H_a + 26-H_3), 1.96 (s, 3H, 28-H_3), 1.86 – 1.57 (m, 9H, 1-H_a + 2-H_a + 4-H_2 + 6-H_2 + 8-H + 16-H_a + 22-H_a), 1.57 – 1.36 (m, 4H, 5-H + 9-H + 11-H_a + 20-H), 1.37 – 1.09 (m, 8H, 2-H_b + 11-H_b + 15-H_2 + 16-H_b + 17-H + 22-H_b), 1.09 – 0.99 (m, 2H, 1-H_b + 14-H), 0.99 – 0.89 (m, 6H, 19-H_3 + 21-H_3), 0.66 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, $CDCl_3$): δ = 172.2 (C-24), 170.7 (C-27), 170.6 (C-25), 73.8 (C-7), 71.2 (C-3), 55.2 (C-17), 54.9 (C-14), 45.5 (C-30), 45.1 (C-30′), 43.6 (C-13), 42.2 (C-5), 41.4 (C-29 + C-29′), 40.0 (C-8), 39.9 (C-12), 39.4 (C-9), 37.1 (C-4), 35.4 (C-20), 34.8 (C-1), 33.9 (C-10), 33.0 (C-6), 31.3 (C-22), 30.1 (C-23), 28.5 (C-16), 26.4 (C-2), 25.6 (C-15), 23.2 (C-19), 21.8 (C-28), 21.4 (C-26), 21.2 (C-11), 18.6 (C-21), 12.1 (C-18) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): m/z = 545.1 (74 %, [M + H]$^+$), 567.1 (100 %, [M + Na]$^+$); analysis calcd for $C_{32}H_{52}N_2O_5$ (544.78): C 70.65, H 9.62, N 5.14; found: C 70.39, H 9.95, N 4.97.

4.5.11. 9-[2-[[4-(3α,7α,12α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl]carbonyl] phenyl]-3,6-bis(diethylamino]-xanthylium chloride (16)

Following GP3, from **11** (100 mg, 166 µmol), rhodamine B (88 mg, 182 µmol), 1-hydroxybenztriazole hydrate (30.5 mg, 199 µmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (39 mg, 199 µmol) **16** (112 mg, 66 %) was obtained as a purple solid; m.p. 118 °C; R_F = 0.55 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 448*w*, 498*w*, 583*w*, 607*w*, 666*w*, 683 *m*, 772 *m*, 798 *m*, 922*w*, 938*w*, 966*w*, 1022 *s*, 1073 *s*, 1094 *m*, 1132 *m*, 1160 *m*, 1180 *s*, 1197 *m*, 1245*vs*, 1338 *s*, 1363 *m*, 1378 *m*, 1394 *m*, 1414 *m*,1466 *m*, 1529*w*, 1588 *s*, 1634 *m*, 1644 *m*, 1728 *s*, 2869*w*, 2925 *m*, 2959 *m* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.79), 355 (3.61), 309 (3.93), 282 (4.00), 262 (4.29) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.77 – 7.64 (m, 3H, 35-H + 37-H + 38-H), 7.58 – 7.46 (m, 1H, 36-H), 7.18 – 7.03 (m, 4H, 42-H + 42′-H), 7.03 – 6.89 (m, 2H, 45-H + 45′+H), 4.95 (d, J = 4.3, 1H, 12-H), 4.77 (s, 1H, 3-H), 4.44 (tt, J = 11.1, 4.2, 1H, 7-H), 3.70 – 3.57 (m, 8H, 47-H_2 + 47′-H_2 + 47″–H_2 + 47‴–H_2), 3.45 – 3.16 (m, 8H, 31-H_2 + 31′-H_2 + 32-H_2 + 32′-H_2), 2.33 – 2.08 (m, 2H, 23-H_2), 2.07 – 2.02 (m, 3H, 26-H_3), 1.98 (s, 3H, 30-H_3), 1.97 – 1.95 (m, 4H, 6-H_a + 28-H_3), 2.01 – 1.86 (m, 2H, 1-H_a + 9-H), 1.85 – 1.71 (m, 2H, 14-H + 16-H_a), 1.72 – 1.53 (m, 6H, 2-H_a + 4-H_a + 8-H + 11-H_a + 17-H + 22-H_a), 1.53 – 1.39 (m, 4H, 1-H_b + 5-H + 6-H_b + 11-H_b), 1.38 – 1.24 (m, 3H, 2-H_b + 15-H_a + 20-H), 1.24 – 1.14 (m, 14H, 16-H_b + 22-H_b + 48-H_3 + 48′-H_3 + 48″–H_3 + 48‴–H_3), 1.13 – 0.93 (m, 2H, 4-H_b + 15-H_b), 0.93 – 0.82 (m, 3H, 19-H_3), 0.80 – 0.69 (m, 3H, 21-H_3), 0.70 – 0.64 (m, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 175.3 (C-24), 170.3 (C-27), 170.2 (C-25), 170.1 (C-29), 166.8 (C-33), 157.5 (C-46 + C-46′), 156.1 (C-40), 155.6 (C-44 + C-44′), 135.7 (C-39), 132.2 (C-42 + C-42′), 131.2 (C-34), 130.9 (C-36), 130.5 (C-35 + C-38), 128.0 (C-37), 114.7 (C-43 + C-32′), 113.5 (C-41 + C-41′), 96.4 (C-45 + C-45′), 75.0 (C-12), 73.8 (C-7), 70.6 (C-3), 47.6 (C-17), 45.8 (C-47 + C-47′+C-47″+C-47‴), 45.1 (C-13 + C-31 + C-31′), 43.5 (C-14), 41.3 (C-32 + C-32′), 40.6 (C-5), 37.3 (C-8), 34.8 (C-1), 34.7 (C-20), 34.4 (C-10), 34.4 (C-4), 31.2 (C-6), 31.1 (C-22), 29.9 (C-23), 28.8 (C-9), 27.1 (C-

16), 26.9 (C-2), 25.5 (C-11), 22.7 (C-15), 22.6 (C-19), 21.7 (C-30), 21.6 (C-28), 21.5 (C-26), 17.9 (C-21), 12.9 (C-48 + C-48′+C-48″+C-48‴), 12.4 (C-18) ppm; MS (ESI, MeOH/$CHCl_3$, 4:1): *m/z* = 1027.9 (50 %, [M-Cl]$^+$), 1028.9 (40 %, [M-Cl + H]$^+$); analysis calcd for $C_{62}H_{83}N_4O_9Cl$ (1063.82): C 70.00, H 7.86, N 5.27; found: C 69.73, H 8.01, N 4.98.

4.5.12. 9-[2-[[4-(3α,12α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl]carbonyl] phenyl]-3,6-bis(diethylamino]-xanthylium chloride (17)

Following GP3, from **12** (100 mg, 184 μmol), rhodamine B (97 mg, 202 μmol), 1-hydroxybenztriazole hydrate (34 mg, 220 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (43 mg, 220 μmol) **17** (102 mg, 55 %) was obtained as a purple solid; m.p. 156 °C; R_F = 0.56 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 498*w*, 543*w*, 578*w*, 619*w*, 666*w*, 683 *m*, 747*w*, 784*w*, 823*w*, 922*w*, 972*w*, 1007 *m*, 1026 *m*, 1074 *m*, 1132 *m*, 1160 *m*, 1179*vs*, 1195 *m*, 1244*vs*, 1273 *m*, 1336 *s*, 1380 *m*, 1394 *m*, 1412 *s*, 1448 *m*, 1466 *m*, 1504 *m*, 1507 *m*, 1528*w*, 1556*w*, 1587*vs*, 1632 *m*, 1730 *m*, 2868*w*, 2934*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.91), 357 (3.76), 308 (4.10), 281 (4.14), 261 (4.44) nm; ^{1}H NMR (400 MHz, DMSO-d_6): δ = 7.78 – 7.65 (m, 3H, 33-H + 35-H + 36-H), 7.51 (ddd, *J* = 9.3, 5.6, 1.8, 1H, 34-H), 7.19 – 7.04 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.93 (d, *J* = 2.3, 2H, 43-H + 43′-H), 4.94 (d, *J* = 4.1, 1H, 12-H), 4.56 (td, *J* = 11.1, 5.7, 1H, 3-H), 3.73 – 3.59 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.49 – 3.18 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.32 – 2.08 (m, 2H, 23-H_2), 2.01 (s, 3H, 28-H_3), 1.95 (s, 3H, 26-H_3), 1.84 – 1.64 (m, 3H, 4-H_a + 6-H_a + 16-H_a), 1.65 – 1.49 (m, 8H, 1-H_a + 2-H_a + 8-H + 11-H_a + 14-H + 15-H_a + 17-H + 22-H_a), 1.49 – 1.32 (m, 5H, 4-H_b + 5-H + 7-H_a + 9-H + 11-H_b), 1.31 – 1.23 (m, 1H, 20-H), 1.24 – 1.15 (m, 15H, 2-H_b + 6-H_b-16-H_b + 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.15 – 0.93 (m, 4H, 1-H_b + 7-H_b + 15-H_b + 22-H_b), 0.86 (s, 3H, 19-H_3), 0.76 – 0.63 (m, 3H, 21-H_3), 0.65 (s, 3H, 18-H_3) ppm; ^{13}C NMR (101 MHz, DMSO-d_6): δ = 171.5 (C-24), 170.2 (C-25), 170.1 (C-27), 167.0 (C-31), 157.5 (C-44 + C-44′), 156.1 (C-38), 155.6 (C-42 + C-42′), 135.7 (C-37), 132.2 (C-40 + C40′), 131.2 (C-32), 130.9 (C-34), 130.3 (C-33), 130.2 (C-36), 128.0 (C-35), 114.7 (C-41 + C-41′), 113.5 (C-39 + C-39′), 96.4 (C-43 + C-43′), 75.4 (C-12), 73.8 (C-3), 49.5 (C-14), 47.7 (C-17), 47.3 (C-29 + C29′), 45.8 (C-45 + C-45′+C-45″+C-45‴), 45.0 (C-13), 41.8 (C-30 + C-30′), 41.5 (C-5), 35.5 (C-9), 34.8 (C-20), 34.7 (C-1), 34.3 (C-8), 34.0 (C-10), 32.3 (C-4), 31.1 (C-22), 29.8 (C-23), 27.2 (C-6), 26.9 (C-16), 26.6 (C-2), 26.0 (C-7), 25.6 (C-11), 23.5 (C-15), 23.2 (C-19), 21.6 (C-26), 21.4 (C-28), 17.9 (C-21), 12.9 (C-46 + C-46′+C-46″+C-46‴), 12.6 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 970.2 (100 %, [M-Cl]$^+$), 971.3 (70 %, [M-Cl + H]$^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.43, H 8.39, N 5.26.

4.5.13. 9-[2-[[4-(3α,7α-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (18)

Following GP3, from **13** (100 mg, 184 μmol), rhodamine B (97 mg, 202 μmol), 1-hydroxybenztriazole hydrate (34 mg, 220 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)carbodiimide hydrochloride (43 mg, 220 μmol) **18** (92 mg, 52 %) was obtained as a purple solid; m.p. 144 °C; R_F = 0.53 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 484*w*. 490w, 498*w*, 546*w*, 580*w*, 611*w*, 666*w*, 683 *m*, 760*w*, 822*w*, 922*w*, 970 *m*, 979 *m*, 1006 *s*, 1024 *s*, 1049 *m*, 1072 *m*, 1133 *m*, 1160 *m*, 1179*vs*, 1245 *s*, 1273 *s*, 1337 *s*, 1378 *m*, 1394 *m*, 1413 *s*, 1448 *m*, 1467 *m*, 1481 *m*, 1529 *m*, 1558*w*, 1587*vs*, 1630 *m*, 1726 *m*, 2870*w*, 2936*w*, 3402*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 560 (4.87), 355 (3.71), 309 (4.04), 280 (4.06), 261 (4.39) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.77 – 7.64 (m, 3H, 33-H + 35-H + 36-H), 7.54 – 7.46 (m, 1H, 34-H), 7.16 – 7.05 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.92 (d, J = 2.3, 2H, 43-H + 43′-H), 4.77 – 4.72 (m, 1H, 7-H), 4.50 – 4.40 (m, 1H, 3-H), 3.68 – 3.58 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.44 – 3.17 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.26 (ddd, J = 15.2, 10.0, 5.3, 1H, 23-H_a), 2.20 – 2.10 (m, 1H, 23-H_b), 1.96 (s, 3H, 28-H_3), 1.95 (s, 3H, 26-H_3), 2.00 – 1.86 (m, 3H, 4-H_a + 6-H_a + 12-H_a), 1.82 – 1.65 (m, 3H, 1-H_a + 16-H_a + 20-H), 1.64 – 1.53 (m, 3H, 2-H_a + 8-H + 22-H_a), 1.49 (d, J = 12.2, 1H, 6-H), 1.45 – 1.38 (m, 3H, 4-H_b + 5-H + 11-H_a), 1.38 – 1.21 (m, 4H, 2-H_b + 9-H + 14-H + 15-H_a), 1.18 (t, J = 7.1, 12H, 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.21 – 1.14 (m, 2H, 11-H_b + 16-H_b), 1.15 – 1.06 (m, 3H, 12-H_b + 17-H + 22-H_b), 1.06 – 0.97 (m, 2H, 1-H_b + 15-H_b), 0.88 (s, 3H, 19-H_3), 0.87 – 0.83 (m, 3H, 21-H_3), 0.58 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.6 (C-24), 170.3 (C-25), 170.1 (C-27), 167.0 (C-31), 157.5 (C-44 + C44′), 156.1 (C-38), 155.6 (C-42 + C42′), 135.7 (C-37), 132.2 (C-40 + C-40′), 131.1 (C-32), 130.8 (C-34), 130.3 (C-33), 130.2 (C-36), 128.0 (C-35), 114.7 (C-41 + C-41′), 113.5 (C-35 + C-35′), 96.4 (C-43 + C-43′), 73.8 (C-3), 71.0 (C-7), 55.8 (C-17), 50.5 (C-14), 47.3 (C-29 + C-29′), 45.9 (C-45 + C-45′+C45″+C-45‴), 42.7 (C-13), 41.5 (C-30 + C-30′), 40.7 (C-5), 40.5 (C-10), 39.5 (C-12), 37.5 (C-8), 35.4 (C-9), 34.8 (C-1), 34.7 (C-6), 34.1 (C-20), 31.3 (C-4), 31.2 (C-22), 29.8 (C-23), 28.0 (C-16), 26.9 (C-2), 23.5 (C-15), 22.8 (C-19), 21.7 (C-28), 21.6 (C-26), 20.7 (C-11), 18.8 (C-21), 12.9 (C-46 + C-46′+C-46″+C-46‴), 12.0 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 969.3 (100 %, [M-Cl]$^+$), 970.3 (80 %, [M-Cl + H]$^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.42, H 8.40, N 5.39.

4.5.14. 9-[2-[[4-(3α-Acetyloxy-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (19)

Following GP3, from **14** (100 mg, 205 μmol), rhodamine B (108 mg, 202 μmol), 1-hydroxybenztriazole hydrate (38 mg, 247 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (48 mg, 247 μmol) **19** (121 mg, 65 %) was obtained as a purple solid; m.p. 147 °C; R_F = 0.58 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 498*w*, 666*w*, 683 *m*, 822*w*, 921*w*, 977*w*, 1007 *m*, 1026 *m*, 1073 *m*, 1095*w*, 1132 *m*, 1160 *m*, 1179*vs*, 1196 *m*, 1243 *s*, 1272 *m*, 1336 *s*, 1381 *m*, 1394 *m*, 1412 *s*, 1449 *m*, 1466 *m*, 1480 *m*, 1508*w*, 1528*w*, 1587*vs*, 1632 *m*, 1731*w*, 2864*w*, 2928*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 562 (4.90), 356 (3.71), 309 (4.03), 282 (4.06), 262 (4.37) nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.77 – 7.71 (m, 2H, 31-H + 34-H), 7.72 – 7.66 (m, 1H, 33-H), 7.54 – 7.47 (m, 1H, 32-H), 7.17 – 7.05 (m, 4H, 38-H + 38′-H + 39-H + 39′-H), 6.93 (d, *J* = 2.3, 2H, 41-H + 41′-H), 4.58 (tt, *J* = 11.2, 4.7, 1H, 3-H), 3.70 – 3.59 (m, 8H, 43-H_2 + 43′-H_2 + 43″–H_2 + 43‴–H_2), 3.47 – 3.17 (m, 9H, 12-H_a + 27-H_2 + 27′-H_2 + 28-H_2 + 28′-H_2), 3.11 – 2.95 (m, 1H, 12-H_b), 2.39 – 2.08 (m, 2H, 23-H_2), 1.95 (s, 3H, 26-H_3), 1.93 – 1.87 (m, 1H, 15-H_a), 1.84 – 1.68 (m, 4H, 1-H_a + 4-H_a + 11-H_a + 16-H_a), 1.66 – 1.55 (m, 1H, 22-H_a), 1.55 – 1.39 (m, 3H, 4-H_b + 5-H + 6-H_a), 1.38 – 1.29 (m, 5H, 2-H_a + 7-H_a + 8-H + 9-H + 20-H), 1.27 – 1.11 (m, 17H, 7-H_b + 11-H_b + 15-H_b + 16-H_b + 22-H_b + 44-H_3 + 44′-H_3 + 44″–H_3 + 44‴–H_3), 1.13 – 0.93 (m, 5H, 1-H_b + 2-H_b + 6-H_b + 14-H + 17-H), 0.92 – 0.82 (m, 6H, 19-H_3 + 21-H_3), 0.61 – 0.57 (m, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.7 (C-24), 171.6 (C-29), 170.2 (C-25), 157.5 (C-42 + C-42′), 156.1 (C-36), 155.6 (C-40 + C-40′), 135.7 (C-35), 132.2 (C-38 + C-38′), 131.2 (C-30), 130.9 (C-32), 130.3 (C-31), 130.2 (C-34), 128.0 (C-33), 114.7 (C-39 + C-39′), 113.5 (C-37 + C-37′), 96.4 (C-41 + C-41′), 73.9 (C-3), 56.3 (C-14), 56.0 (C-17), 45.9 (C-43 + C-43′+C-43″+C-43‴), 45.1 (C-27 + C-27′), 42.7 (C-12), 41.6 (C-5), 40.6 (C-28 + C-28′), 40.3 (C-9), 40.0 (C-13 + C-15), 35.8 (C-8), 35.4 (C-20), 35.0 (C-1), 34.6 (C-10), 32.3 (C-4), 31.3 (C-22), 29.9 (C-23), 28.2 (C-16), 27.0 (C-11), 26.4 (C-2), 24.3 (C-6), 23.5 (C-19), 21.5 (C-26), 20.9 (C-7), 18.8 (C-21), 12.9 (C-44 + C-44′+C-44″+C-44‴), 12.3 (C-18) ppm; MS (ESI, propan-2-ol): *m/z* = 911.7 (100 %, [M-Cl]$^+$), 912.7 (65 %, [M-Cl + H]$^+$); analysis calcd for $C_{57}H_{91}N_4O_5Cl$ (946.67): C 72.25, H 9.69, N 5.92; found: C 71.96, H 9.95, N 5.66.

4.5.15. 9-[2-[[4-(3α,7β-Bis(acetyloxy)-5β-cholan-24-oyl)-1-piperazinyl] carbonyl]phenyl]-3,6-bis(diethylamino]-xanthylium chloride (20)

Following GP3, from **15** (100 mg, 188 μmol), rhodamine B (99 mg, 207 μmol), 1-hydroxybenztriazole hydrate (35 mg, 226 μmol) and *N*-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (44 mg, 226 μmol) **20** (88 mg, 47 %) was obtained as a purple solid; m.p. 169 °C; R_F = 0.59 ($CHCl_3$/MeOH, 9:1); IR (ATR): $\tilde{\nu}$ = 683 *m*, 822*w*, 922*w*, 957*w*, 977 *m*, 1007 *s*, 1023 *s*, 1043 *m*, 1074 *m*, 1095*w*, 1132 *m*, 1160 *m*, 1179*vs*,

1197 *m*, 1241 *s*, 1273 *s*, 1336 *s,* 1383 *m,* 1395 *m,* 1412 *s,* 1466 *m*, 1481 *m*, 1508 *m*, 1529 *m*, 1587*vs*, 1633 *m*, 1727 *m*, 2871*w*, 2938*w* cm^{-1}; UV/vis (MeOH): λ^{max} (log ε) = 558 (4.94), 355 (3.85), 306 (4.14), 257 (4.48), nm; ^{1}H NMR (500 MHz, DMSO-d_6): δ = 7.76 – 7.71 (m, 2H, 33-H + 36-H), 7.70 – 7.66 (m, 1H, 35-H), 7.55 – 7.47 (m, 1H, 34-H), 7.16 – 7.06 (m, 4H, 40-H + 40′-H + 41-H + 41′-H), 6.93 (d, J = 2.3, 2H, 43-H + 43′-H), 4.69 – 4.59 (m, 1H, 7-H), 4.53 (ddd, J = 15.6, 10.3, 4.8, 1H, 3-H), 3.68 – 3.60 (m, 8H, 45-H_2 + 45′-H_2 + 45″–H_2 + 45‴–H_2), 3.46 – 3.13 (m, 8H, 29-H_2 + 29′-H_2 + 30-H_2 + 30′-H_2), 2.30 – 2.09 (m, 2H, 23-H_2), 1.95 (s, 3H, 26-H_3), 1.91 (s, 3H, 28-H_3), 1.93 – 1.87 (m, 1H, 12-H_a), 1.79 – 1.45 (m, 11*H*, 1-H_a + 2-H_a + 4-H_a + 5-H + 6-H_2 + 8-H + 9-H + 16-H_a + 22-H_2), 1.45 – 1.25 (m, 4H, 2-H_b + 11-H_a + 15-H_a + 20-H), 1.26 – 1.12 (m, 5H, 11-H_b + 12-H_b + 14-H + 16-H_b + 15-H_b), 1.18 (t, J = 7.1, 12H, 46-H_3 + 46′-H_3 + 46″–H_3 + 46‴–H_3), 1.16 – 0.96 (m, 3H, 1-H_b + 4-H_b + 17-H), 0.90 (s, 3H, 19-H_3), 0.90 – 0.81 (m, 3H, 21-H_3), 0.60 (s, 3H, 18-H_3) ppm; ^{13}C NMR (126 MHz, DMSO-d_6): δ = 171.1 (C-24), 169.8 (C-27), 169.8 (C-25), 166.5 (C-31), 157.0 (C-44 + C-44′), 155.6 (C-38), 155.1 (C-42 + C-42′), 135.3 (C-37), 131.8 (C-40 + C-40′), 130.7 (C-32), 130.4 (C-34), 129.8 (C-36), 129.7 (C-33), 127.5 (C-35), 114.3 (C-41 + C-41′), 113.0 (C-39 + C-39′), 95.9 (C-43 + C-43′), 72.9 (C-3), 72.8 (C-7), 54.4 (C-17), 54.4 (C-14), 46.8 (C-29 + C-29′), 45.4 (C-45 + C-45′+C-45″+C-45‴), 43.1 (C-13), 41.2 (C-5 + C-30 + C-30′), 39.7 (C-8), 39.5 (C-10), 39.2 (C-12), 38.6 (C-9), 34.8 (C-20), 33.9 (C-1), 32.6 (C-6), 32.5 (C-22), 30.8 (C-4), 29.3 (C-23), 28.0 (C-16), 26.0 (C-2), 25.3 (C-15), 22.8 (C-19), 21.5 (C-28), 21.0 (C-26), 20.8 (C-11), 18.4 (C-21), 12.4 (C-46 + C-46′+C-46″+C-46‴), 11.8 (C-18) ppm; MS (ESI, MeOH): m/z = 969.5 (100 %, $[M\text{-}Cl]^+$), 970.5 (78 %, $[M\text{-}Cl + H]^+$); analysis calcd for $C_{60}H_{81}N_4O_7Cl$ (1005.78): C 71.65, H 8.12, N 5.57; found: C 71.43, H 8.39, N 5.37.

CRediT authorship contribution statement

Benjamin Brandes: Investigation, Writing – review & editing. **Sophie Hoenke:** Investigation, Writing – original draft. **Christian Schultz:** 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.steroids.2022.109148.

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