Synthesis of new, UV-photoactive dansyl derivatives for flow cytometric studies on bile acid uptake†

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Four new fluorescent derivatives of cholic acid have been synthesized; they incorporate a dansyl moiety at 3α-, 3β-, 7α- or 7β- positions. These cholic acid analogs are UV photoactive and also exhibit green fluorescence. In addition, they have been demonstrated to be suitable for studying the kinetics of bile acid transport by flow cytometry.

Introduction

Uptake of bile acids into hepatocytes and excretion into the bile canaliculi are fulfilled by a panel of transporters located at the apical or at the sinusoidal pole of the plasma membrane.[1-3] Inhibition of the uptake can disrupt homeostasis and alter bile composition, resulting in a cascade of events that predisposes the liver to cellular injury.[4] We have recently developed flow cytometry assays for the study of bile acid transport in freshly isolated rat hepatocytes.[5,6] They are based on the use of fluorescent derivatives of cholic acid (ChA) such as cholylamidofluorescein (CamF) and 4-nitrobenzo-2-oxa-1,3-diazole (NBD) amino conjugates.

As a result of the recent availability of UV-light sources in standard flow cytometers and bioimage analyzers, suitable UV-absorbing probes are required to examine bile acid transport in multiparametric or high-content studies. Fluorescent derivatives meeting such a requirement would have the advantage of exploiting this (less frequent) excitation region. In addition to be UV-photoactivated, the selected fluorophores should also be low molecular weight moieties, in order to introduce only small structural changes for maintaining transport inside cells.

For this purpose, we have designed four new regio- and stereoisomers of cholic acid incorporating a dansyl fluorophore (Dns-ChA). Here, we report on their synthesis and photophysical characterization as well as on their capability to be transported inside cells. By using troglitazone, a well-known inhibitor of bile acid uptake,[7] the specificity of the new UV-absorbing Dns-ChA derivatives has been demonstrated.

Results and discussion

The synthetic strategy used for the preparation of **3α-**, **3β-**, **7α-** and **7β-Dns-ChA** is illustrated in Scheme 1. It is based on the regioselective transformation of the desired hydroxyl group into the corresponding amino function to give **3α-**, **3β-**, **7α-** and **7β-NH_2-ChMe**. These intermediates were conjugated with the fluorophore, and subsequent deprotection gave rise to the desired products.

The sequence started with protection of the carboxylic acid as the corresponding methyl ester (**ChMe**).[8] Then, regioselective oxidation of hydroxyl group at C-3 was achieved using Ag_2CO_3 supported on celite.[8] The resulting carbonyl group was subjected to stereoselective reductive amination using $NaBH_3CN/AcONH_4$, providing the 3α-epimer. The 3β-amino derivative was prepared in three steps from ChMe. Thus, the hydroxyl group at C-3 was converted into the mesylate and then subjected to a nucleophilic substitution using NaN_3.[9] Reduction of the resulting azide using $Pd\text{-}C/HCOONH_4$ gave **3β-NH_2-ChA**.

In the case of the C-7 amino derivatives, oxidation of the hydroxyl group at C-7 was performed regioselectively starting from ChA, using NBS.[10] Reductive amination of **7[O]ChA** followed by esterification in $MeOH/H^+$ led to **7α-NH_2-ChMe**. To obtain the 7β-diastereoisomer, **7[O]ChA** was converted into the oxime; then, subsequent reduction[11] using Na/1-BuOH followed by esterification gave a mixture (30:70) of **7α-** and **7β-NH_2-ChMe**, which was not resolved at this stage.

Conjugation of **3α-**, **3β-**, **7α-** and **7β-NH_2-ChMe** with dansyl chloride led to **3α-**, **3β-**, **7α-** and **7β-Dns-ChMe**, which after final deprotection afforded the desired compounds **3α-**, **3β-**, **7α-** and **7β-Dns-ChA**.

Photophysical characterization

The UV-absorption spectra of the four Dns-ChAs in ethanol exhibited maxima at 250 and 335 nm (see Fig. 1 for a representative example). The fluorescence spectra showed maxima in the range 504-514 nm (Table 1). From the intersection of the corresponding normalized excitation and emission spectra, singlet energy ($E_{0\text{-}0}$) values of *ca.* 286 kJ/mol were estimated. Emission quenching by oxygen was observed in the four cases. It was dynamic in nature as reflected by the shorter lifetimes of the singlet excited states

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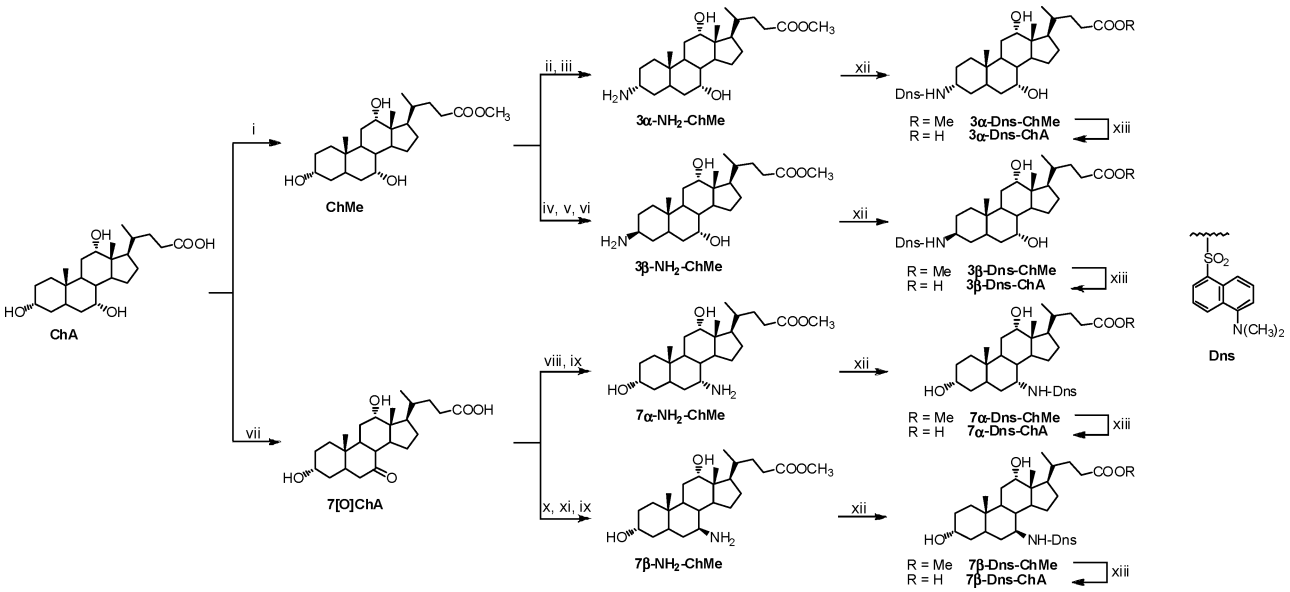

Scheme 1 Reagents and conditions: (i) MeOH, HCl, DMP; (ii) Ag_2CO_3, toluene; (iii) $NaBH_3CN$, $AcONH_4$, MeOH; (iv) MsCl, pyridine; (v) NaN_3, DMF; (vi) Pd-C 10%, $HCOONH_4$, AcOEt:MeOH; (vii) NBS, $NaHCO_3$ (aq); (viii) $NaBH_3CN$, $AcONH_4$, MeOH; (ix) MeOH-HCl; (x) $NH_2OH{\cdot}HCl$, AcONa, MeOH; (xi) Na, 1-BuOH; (xii) Dns-Cl, Et_3N, DMF; (xiii) KOH, MeOH.

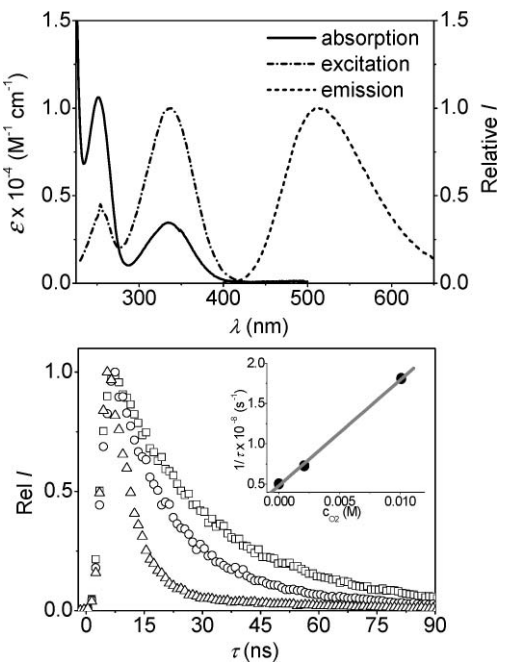

Fig. 1 Top: Normalized absorption, excitation and emission spectra of compound **3α-Dns-ChA** in ethanol as an example. Bottom: Fluorescence decay traces of **3α-Dns-ChA** at different O_2 concentrations (□ N_2, ○ air, Δ O_2); Inset: Stern–Volmer plot.

(see Fig. 1 bottom and Table 1). In aqueous media, the fluorescence maxima shifted to *ca.* 550 nm, and the emission quantum yields were markedly lower. Accordingly, the fluorescence lifetimes were considerably shorter (around 4-5 ns). In addition, all Dns-ChA derivatives exhibited aggregation behaviour in aqueous medium, in the mM range, as expected for bile acid derivatives.

Table 1 Photophysical properties of the Dns-ChA derivatives

Dns-ChA	λ_{em} (nm)	$\phi_F{}^a$ (τ_S in ns) N_2	air	O_2	$10^{-10} \times k_{O2}$ (s^{-1} M^{-1})
3α	514	0.38 (19.9)	0.26 (13.7)	0.11 (5.5)	1.31
3β	514	0.40 (20.2)	0.27 (12.7)	0.12 (4.1)	1.98
7α	504	0.42 (20.9)	0.28 (13.3)	0.13 (5.4)	1.38
7β	508	0.41 (19.5)	0.27 (12.8)	0.12 (4.4)	1.78

[a] Determined using an air-saturated solution of Coumarine30 in CH_3CN ($\phi_F = 0.67$) as a standard ($\lambda_{exc} = 370$ nm).

Flow cytometry

The suitability of the four dansyl conjugates for bile acid uptake was investigated on freshly isolated rat hepatocytes, using multiparametric flow cytometry. This technique allows examination of multiple simultaneous fluorescences of individual live cells in suspension. Initially, a kinetic assay of the uptake was performed on **3α-Dns-ChA**. Cell suspensions were stained with propidium iodide for 5 minutes prior to flow cytometric analysis, to identify and exclude dead cells from data acquisition (Fig. 2A).

Specific uptake by live cells was followed by a plot of the variation of green fluorescence intensity *versus* time (Fig. 2B). The results showed that living hepatocytes accumulated slowly but constantly **3α-Dns-ChA** along the experimental period. When endpoint fluorescence was measured after 30 minutes incubation in the presence of the dansyl derivative, a marked increase of intracellular accumulation was observed, more than 10-fold over the cellular autofluorescence of unstained cells (Fig. 2C).

In a second series of experiments the concentration-dependence of intracellular accumulation of the four Dns-ChA was examined in single end-point measurements after 30 min incubation. As

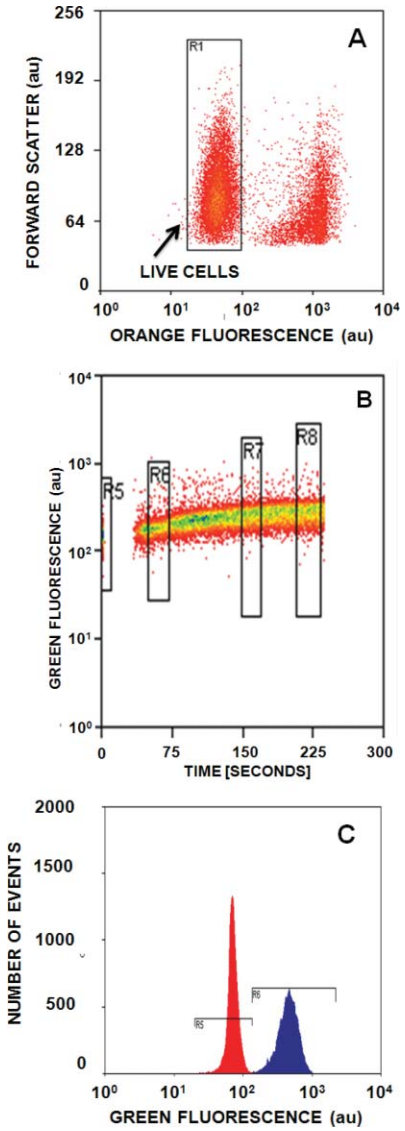

Fig. 2 Flow cytometric analysis of **3α-Dns-ChA** uptake by rat hepatocytes. **(A)** Live cells are delimited by the rectangular gate. Their selection is based on exclusion of propidium iodide. **(B)** Kinetics of **3α-Dns-ChA** uptake. Transport inside the live cell was detected and quantified by measuring the increase of green fluorescence with time. Marked rectangles indicate analytical regions for calculations from raw cytometric data. **(C)** End-point measurement of **3α-Dns-ChA** accumulation. Overlay of the green autofluorescence of unstained cells (red) and the green fluorescence of cells incubated with **3α-Dns-ChA** (blue).

shown in Fig. 3, all derivatives accumulated in a concentration-dependent fashion, the most efficient being **3α-Dns-ChA**.

The dependence of this effect on the operation of bile-acid transporters in the plasma membrane of liver cells was also addressed. For this purpose, troglitazone, an *in vivo* cholestatic compound,[12] that has been shown to be a strong inhibitor *in vitro* of bile acid uptake through sodium taurocholate cotransporting polypeptide (NTCP) and organic anion transporting polypeptide family (OATP) in hepatocytes,[13,14] was employed. In fact, preincubation of fresh hepatocyte suspensions with troglitazone provoked a strong and dose-dependent reduction in the uptake of every Dns-ChA derivative (Fig. 4).

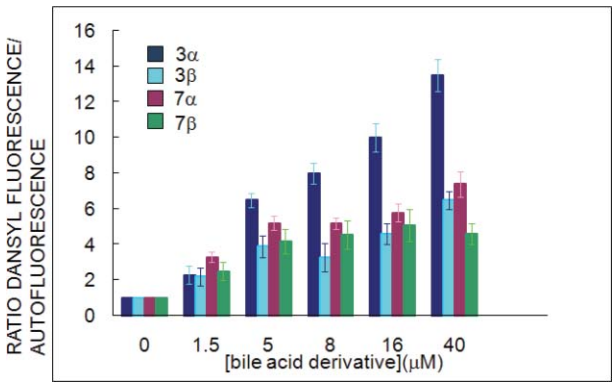

Fig. 3 Flow cytometric comparison of Dns-ChAs uptake by rat hepatocytes: Concentration-dependence of dansyl fluorescence after 30 min incubation. Intracellular accumulation of green fluorescence over the intrinsic basal autofluorescence.

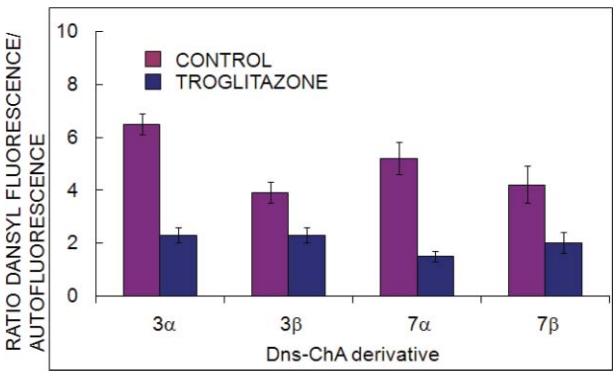

Fig. 4 Flow cytometric comparison of Dns-ChAs uptake by rat hepatocytes: Effect of bile-acid transport inhibitor troglitazone on the uptake of the fluorescent Dns-ChA derivatives. The bars show the intracellular accumulation of 5 μM Dns-ChA in the absence [violet bars] or in the presence [blue bars] of troglitazone [50 μM] after 30 min, prior to bile acid addition.

Conclusions

In summary, four new dansyl derivatives of cholic acid have been synthesized by selective conjugation of the Dns fluorophore at positions 3- and 7- of the ChA skeleton with well defined stereochemistries.

The obtained Dns-ChA probes can be photoactivated in the UV-region and exhibit green fluorescence; thus, they are suitable to follow the kinetics of bile acid transport by flow cytometry. Combination of these dansyl derivatives with the already existing blue-light absorbing analogs provides a valuable tool to increase the complexity of bile acid studies, as multiple transporters or interactions can, in principle, be assessed simultaneously.

Experimental

General

Cholic acid, dansyl choride (Dns-Cl), Coumarine30, anhydrous solvents and other reagents used for the synthesis of the fluorescent derivatives were purchased from Sigma Chemical Co. (Madrid, Spain) and used as received. Ethanol (99.9%) was from Merck

(Darmstadt, Germany). The TLC spots were visualized by spraying the plate with a 10% EtOH solution of phosphomolybdic acid followed by heating.

The ^{1}H and ^{13}C NMR spectra were measured by means of a Bruker (Rheinstetten, Germany) 300 MHz instrument; $CDCl_3$ and CD_3OD were used as solvents, and the signal corresponding to the solvent in each case was taken as the reference: $CDCl_3$ (δ = 7.26 for ^{1}H NMR, δ = 77.2 for ^{13}C NMR) and CD_3OD (δ = 3.31 for ^{1}H NMR, δ = 49.0 for ^{13}C NMR); coupling constants are given in Hz. Exact mass spectra are included for all final compounds.

Synthesis of N-dansyl-3α-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (3α-Dns-ChA)

To a stirred solution of cholic acid (2 g, 4.9 mmol) in MeOH (10 mL) containing 0.3 mL of conc. HCl, dimethoxypropanone (5 mL) was added. The reaction mixture was stirred overnight and then the solvent was evaporated. The crude solid was redissolved in AcOEt, washed with sat. $NaHCO_3$, brine, dried over $MgSO_4$ and concentrated to give methyl 3α,7α,12α-trihydroxy-5β-cholan-24-oate (**ChMe**)[8] as a white crystalline solid (1.95 g, 94%) that was used in the following step without any further purification. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.64 (s, 3H, Me-18), 0.85 (s, 3H, Me-19), 0.95 (d, J = 5.7, 3H, Me-21), 3.29 (br s, 3H, 3xOH), 3.39 (m, 1 H, CH_{ax}-3β), 3.63 (s, 3H, MeO), 3.80 (br s, 1H, CH_{eq}-7β), 3.92 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.6 (CH_3), 17.4 (CH_3), 22.6 (CH_3), 23.4 (CH_2), 26.4 (CH), 27.7 (CH_2), 28.3 (CH_2), 30.5 (CH_2), 31.1 (CH_2), 31.2 (CH_2), 34.8 (CH_2), 34.9 (CH), 35.4 (CH), 39.5 (CH_2), 39.6 (CH), 41.6 (CH), 41.7 (CH), 46.5 (CH), 47.1 (C), 51.6 (CH_3O), 68.5 (7-CH), 72.0 (3-CH), 73.2 (12-CH), 175.0 (COO).

To a stirred solution of **ChMe** (0.97 g, 2.3 mmol) in anhydrous toluene (30 mL), Ag_2CO_3@celite‡ (2.38 g, 3.45 mmol) was added. The mixture was refluxed in a Dean–Stark apparatus under N_2 for 7 hours. Then, it was filtered, washed with warm toluene and concentrated. After column chromatography (SiO_2, AcOEt:n-hexane 5:1), methyl 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oate (**3[O]ChMe**)[8] (0.81 g, 84%) was obtained as a white solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.72 (s, 3H, Me-18), 0.98 (m, 6H, Me-19 + Me-21), 3.40 (dd, J = 13.5, 15.0, 1H, CH_{ax}-4α), 3.66 (s, 3H, MeO), 3.92 (s, 1H, CH_{eq}-7β), 4.02 (s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.7 (CH_3), 17.5 (CH_3), 21.9 (CH_3), 23.3 (CH_2), 27.4 (CH), 27.6 (CH_2), 28.8 (CH_2), 31.0 (CH_2), 31.2 (CH_2), 34.0 (CH_2), 35.0 (C), 35.3 (CH), 36.8 (CH_2), 37.0 (CH_2), 39.7 (CH), 42.0 (CH), 43.2 (CH), 45.7 (CH_2), 46.8 (C), 47.5 (CH), 51.7 (CH_3O), 68.5 (7-CH), 73.0 (12-CH), 174.8 (COO), 213.2 (C=O).

A mixture of **3[O]ChMe** (0.81 g, 1.92 mmol), ammonium acetate (1.48 g, 19.2 mmol) and $NaBH_3CN$ (0.12 g, 1.92 mmol) in anhydrous MeOH (40 mL) was stirred at rt, under N_2, for 24 hours. Afterwards, the mixture was carefully acidified with conc. HCl to pH 3 and the solvent was removed under vacuum. The solid was washed with Et_2O, redissolved in 1-butanol (25 mL), filtered off, washed with brine and evaporated. After purification by recrystallization from MeOH:CH_2Cl_2, methyl 3α-amino-7α,12α-dihydroxy-5β-cholan-24-oate (**3α-NH_2-ChMe**)[15] (hydrochloride salt) was obtained as a white solid (0.49 g, 60%). ^{1}H-NMR (300 MHz, CD_3OD): δ 0.72 (s, 3H, Me-18), 0.97 (s, 3H, Me-19), 1.01 (d, J = 6.3, 3H, Me-21), 2.93 (m, 1H, CH_{ax}-3β), 3.65 (s, 3H, MeO), 3.81 (br s, 1H, CH_{eq}-7β), 3.99 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.0 (CH_3), 17.6 (CH_3), 23.0 (CH_3), 24.1 (CH_2), 26.7 (CH_2), 28.0 (CH), 28.7 (CH_2), 29.6 (CH_2), 31.9 (CH_2), 32.2 (CH_2), 35.4 (CH_2), 35.6 (CH_2), 35.8 (C), 36.0 (CH_2), 36.8 (CH), 41.0 (CH), 43.0 (CH), 43.1 (CH), 47.5 (C), 48.0 (CH), 52.0 (CH_3O), 52.7 (3-CH), 68.6 (7-CH), 73.7 (12-CH), 176.5 (COO).

To a solution of **3α-NH_2-ChMe** (0.1 g, 0.25 mmol) in anhydrous DMF (2.5 mL), Et_3N (0.1 mL, 0.75 mmol) was added, and the reaction mixture was cooled to 0 °C. Then, a solution of Dns-Cl (95 mg, 0.35 mmol) in anhydrous CH_3CN (1 mL) was added dropwise, under inert atmosphere, and the reaction mixture was stirred overnight at rt. Afterwards, the solvent was removed and the crude purified by column chromatography (AcOEt:n-hexane 2:1) to give methyl N-dansyl-3α-amino-7α,12α-dihydroxy-5β-cholan-24-oate (**3α-Dns-ChMe**)[15] as a light green crystalline solid (80 mg, 49%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.62 (s, 3H, Me-18), 0.79 (s, 3H, Me-19), 0.94 (d, J = 5.7, 3H, Me-21), 2.87 (s, 6H, Me_2N), 2.96 (m, 1 H, CH_{ax}-3β), 3.65 (s, 3H, MeO), 3.77 (br s, 1H, CH_{eq}-7β), 3.91 (br s, 1H, CH_{eq}-12β), 5.26 (d, J = 7.8, 1H, NH), 7.15 (d, J = 7.2, 1H, CH), 7.50 (m, 2H, CH), 8.26 (m, 2H, CH), 8.51 (d, J = 8.4, 1H, CH); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.6 (CH_3), 17.4 (CH_3), 22.6 (CH_3), 23.2 (CH_2), 26.7 (CH), 27.6 (CH_2), 28.3 (CH_2), 28.9 (CH_2), 31.0 (CH_2), 31.2 (CH_2), 34.6 (CH_2), 35.3 (CH), 35.9 (CH_2), 37.7 (CH_2), 39.5 (CH), 42.0 (CH), 42.1 (CH), 45.6 (CH_3), 46.5 (C), 47.2 (CH), 51.7 (CH_3), 54.4 (3-CH), 68.3 (7-CH), 73.0 (12-CH), 115.3 (CH), 119.5 (CH), 123.3 (CH), 128.2 (CH), 129.2 (CH), 129.8 (C), 129.9 (C), 130.1 (CH), 136.3 (C), 151.7 (C), 174.9 (COO). HRMS m/z 654.3710 (calc. for $C_{37}H_{54}N_2O_6S$ 654.3703).

To a solution of **3α-NH_2-ChMe** (80 mg, 0.12 mmol) in 2 mL of MeOH, a solution of KOH in MeOH (1 M, 1.2 mL) was added, and the resulting mixture was stirred overnight at rt. The solvent was evaporated and the mixture was redissolved in H_2O (2 mL), acidified with 1 M HCl, extracted twice with AcOEt and purified by column chromatography (SiO_2, AcOEt:n-hexane:AcOH, 70:30:1). N-Dansyl-3α-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (**3α-Dns-ChA**) was obtained as a light green crystalline solid (68 mg, 89%).^{1}H-NMR (300 MHz, CD_3OD): δ 0.65 (s, 3H, Me-18), 0.81 (s, 3H, Me-19), 0.98 (d, J = 6.0, 3H, Me-21), 2.87 (s, 7H, Me_2N + CH_{ax}-3β), 3.71 (br s, 1H, CH_{eq}-7β), 3.89 (br s, 1H, CH_{eq}-12β), 7.25 (d, J = 7.5, 1H, CH), 7.55 (m, 2H, CH), 8.20 (d, J = 7.2, 1H, CH), 8.34 (d, J = 8.7, 1H, CH), 8.53 (d, J = 8.4, 1H, CH); ^{13}C-NMR (75 MHz, CD_3OD): δ 12.9 (CH_3), 17.6 (CH_3), 23.1 (CH_3), 24.1 (CH_2), 27.8 (CH), 28.6 (CH_2), 29.5 (CH_2), 29.6 (CH_2), 32.1 (CH_2), 32.3 (CH_2), 35.6 (CH_2), 36.8 (CH), 37.1 (CH_2), 38.7 (CH_2), 40.9 (CH), 43.0 (CH), 43.9 (CH), 45.8 (CH_3), 47.4 (CH), 48.0 (C), 55.5 (3-CH), 68.8 (7-CH), 73.8 (12-CH), 116.3 (CH), 120.9 (CH), 124.2 (CH), 128.8 (CH), 129.8 (CH), 130.9 (CH), 131.0 (C), 131.2 (C), 138.3 (C), 153.1 (C), 178.3 (COO). HRMS m/z 640.3538 (calc. for $C_{36}H_{52}N_2O_6S$ 640.3546).

‡ Silver carbonate on celite (Ag_2CO_3@celite): To a stirred solution of Ag_2NO_3 (1.5 g, 8.75 mmol) in 10 mL of H_2O containing 1.25 g of Celite, a solution of Na_2CO_3 in 15 mL of H_2O (1.25 g, 11.75 mmol) was slowly added. The green precipitate was filtered off, washed with water and dried. Ag_2CO_3 supported on celite was obtained as a greenish solid (2.38 g, 93%); concentration: 1.72 mmol of the reactive/g of the solid.

Synthesis of N-dansyl-3β-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (3β-Dns-ChA)

To a cooled (0 °C) solution of **ChMe** (0.5 g, 1.2 mmol) in anhydrous pyridine (5 mL), mesyl chloride (0.19 mL, 2.4 mmol) was added dropwise. The reaction mixture was stirred at rt under N_2 atmosphere for 7 h. Afterwards, the mixture was poured into 100 mL of HCl (6 M) saturated with NaCl and extracted with CH_2Cl_2 (3x). The combined organic layers were washed with brine, dried over $MgSO_4$ and concentrated. Crude methyl 3α-methanesulfonyl-7α,12α-dihydroxy-5β-cholan-24-oate (**3α-Ms-ChMe**)[9] was purified on short column chromatography (AcOEt:n-hexane, 1:1) and obtained as a white solid (0.55 g, 92%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.68 (s, 3H, Me-18), 0.90 (s, 3H, Me-19), 0.97 (d, J = 6.3, 3H, Me-21), 2.98 (s, 3H, CH_3SO_2), 3.66 (s, 3H, MeO), 3.86 (br s, 1H, CH_{eq}-7β), 3.98 (br s, 1H, CH_{eq}-12β), 4.50 (m, 1 H, CH_{ax}-3β); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.7 (CH_3), 17.5 (CH_3), 22.5 (CH_3), 23.3 (CH_2), 26.7 (CH), 27.6 (CH_2), 28.0 (CH_2), 28.4 (CH_2), 31.0 (CH_2), 31.2 (CH_2), 34.3 (CH_2), 34.6 (C), 34.9 (CH_2), 35.3 (CH), 36.2 (CH_2), 39.0 (CH_3S), 39.6 (CH), 41.6 (CH), 42.0 (CH), 46.7 (C), 47.3 (CH), 51.7 (CH_3O), 68.2 (7-CH), 72.9 (12-CH), 82.9 (3-CH), 174.9 (COO).

A solution of **3α-Ms-ChMe** (0.55 g, 1.1 mmol) and NaN_3 (0.13 g, 1.98 mmol) in DMF (15 mL) was heated at 100 °C for 5 h in absence of light. Then, the solvent was evaporated, the crude was redissolved in CH_2Cl_2, washed with brine, dried over $MgSO_4$ and concentrated. After column chromatography (SiO_2, AcOEt:n-hexane, 1:1), methyl 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oate (**3β-N_3-ChMe**)[9] was obtained as a white solid (0.29 g, 59%): ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.69 (s, 3H, Me-18), 0.93 (s, 3H, Me-19), 0.97 (d, J = 6.0, 3H, Me-21), 3.66 (s, 3H, MeO), 3.86 (br s, 1H, CH_{eq}-7β), 3.90 (br s, 1 H, CH_{eq}-3α), 3.98 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.7 (CH_3), 17.5 (CH_3), 23.1 (CH_3), 23.3 (CH_2), 24.7 (CH_2), 26.4 (CH), 27.6 (CH_2), 28.7 (CH_2), 30.6 (CH_2), 31.0 (CH_2), 31.2 (CH_2), 33.2 (CH_2), 34.2 (CH_2), 35.2 (C), 35.3 (CH), 36.9 (CH), 39.6 (CH), 42.2 (CH), 46.7 (C), 47.4 (CH), 51.7 (CH_3O), 58.8 (3-CH), 68.5 (7-CH), 73.1 (12-CH), 174.8 (COO).

0.29 g (0.65 mmol) of **3β-N_3-ChMe** were dissolved in a mixture of AcOEt:MeOH 1:2 (9 mL). After addition of 0.41 g of ammonium formate (6.5 mmol) and 0.14 g of Pd-C 10% (20 mol%), the reaction mixture was refluxed for 6 h. The solution was then filtered, washed with 10% Et_3N/MeOH and concentrated. The crude was redissolved in CH_2Cl_2, washed with brine and concentrated. The amine was redissolved in MeOH containing conc. HCl (5%) to obtain the hydrochloride salt. The solvent was then evaporated and the resulting white solid was washed with Et_2O and dried. Methyl 3β-amino-7α,12α-dihydroxy-5β-cholan-24-oate (**3β-NH_2-ChMe**)[16] (hydrochloride salt) was obtained as a white powder (0.17 g, 63%). ^{1}H-NMR (300 MHz, CD_3OD): δ 0.72 (s, 3H, Me-18), 1.01 (m, 6H, Me-19 + Me-21), 3.50 (br s, 1H, CH_{eq}-3α), 3.65 (s, 3H, MeO), 3.81 (br s, 1H, CH_{eq}-7β), 3.97 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.0 (CH_3), 17.6 (CH_3), 23.0 (CH_3), 24.1 (CH_2), 24.2 (CH_2), 27.5 (CH), 28.7 (CH_2), 29.6 (CH_2), 30.5 (CH_2), 31.9 (CH_2), 32.2 (CH_2), 32.8 (CH_2), 34.9 (CH_2), 36.3 (C), 36.8 (CH), 37.3 (CH), 40.9 (CH), 43.0 (CH), 47.6 (C), 48.0 (CH), 49.4 (3-CH), 52.0 (CH_3O), 68.7 (7-CH), 73.8 (12-CH), 176.5 (COO).

Compound **3β-Dns-ChMe** was prepared from **3β-NH_2-ChMe** following the procedure described above for **3α-Dns-ChMe**. Thus, starting from **3β-NH_2-ChMe** (0.1 g, 0.24 mmol), methyl N-dansyl-3β-amino-7α,12α-dihydroxy-5β-cholan-24-oate (**3β-Dns-ChMe**) (85 mg, 55%) was obtained as a light green crystalline solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.60 (s, 3H, Me-18), 0.76 (s, 3H, Me-19), 0.90 (d, J = 6.3, 3H, Me-21), 2.87 (s, 6H, Me_2N), 3.41 (br s, 1H, CH_{eq}-3α), 3.63 (s, 3H, MeO), 3.69 (br s, 1H, CH_{eq}-7β), 3.87 (br s, 1H, CH_{eq}-12β), 5.02 (d, J = 6.3, 1H, NH), 7.15 (d, J = 7.5, 1H, CH_{Ar}), 7.50 (m, 2H, CH), 8.23 (d, J = 7.2, 1H, CH), 8.28 (d, J = 8.4, 1H, CH), 8.50 (d, J = 8.4, 1H, CH); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.6 (CH_3), 17.4 (CH_3), 23.0 (CH_3), 23.2 (CH_2), 25.7 (CH_2), 26.1 (CH), 27.5 (CH_2), 28.5 (CH_2), 30.3 (CH_2), 30.9 (CH_2), 31.2 (CH_2), 34.0 (CH_2), 34.2 (CH_2), 35.0 (C), 35.2 (CH), 36.7 (CH), 39.5 (CH), 41.9 (CH), 45.6 (CH_3), 46.5 (C), 47.3 (CH), 50.1 (3-CH), 51.6 (CH_3), 68.3 (7-CH), 72.9 (12-CH), 115.1 (CH), 118.7 (CH), 123.3 (CH), 128.4 (CH), 129.7 (CH), 129.8 (CH), 129.9 (C), 130.4 (CH), 135.5 (C), 152.1 (C), 174.8 (COO). HRMS m/z 654.3701 (calc. for $C_{37}H_{54}N_2O_6S$ 654.3703).

Compound **3β-Dns-ChA** was prepared from **3β-Dns-ChMe** following the procedure described above for **3α-Dns-ChA**. Thus, starting from **3β-Dns-ChMe** (85 mg), N-dansyl-3β-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (**3β-Dns-ChA**) (60 mg, 83%) was obtained as a pale green crystalline solid. ^{1}H-NMR (300 MHz, CD_3OD): δ 0.65 (s, 3H, Me-18), 0.76 (s, 3H, Me-19), 0.97 (d, J = 6.3, 3H, Me-21), 2.88 (s, 6H, Me_2N), 3.39 (br s, 1H, CH_{eq}-3α), 3.63 (br s, 1H, CH_{eq}-7β), 3.87 (br s, 1H, CH_{eq}-12β), 7.26 (d, J = 7.2, 1H, CH_{Ar}), 7.57 (m, 2H, CH), 8.20 (d, J = 7.2, 1.2, 1H, CH), 8.41 (d, J = 8.7, 1H, CH), 8.54 (d, J = 8.4, 1H, CH); ^{13}C-NMR (75 MHz, CD_3OD): δ 12.9 (CH_3), 17.6 (CH_3), 23.2 (CH_3), 24.1 (CH_2), 26.5 (CH_2), 27.3 (CH), 28.6 (CH_2), 29.6 (CH_2), 31.2 (CH_2), 32.1 (CH_2), 32.3 (CH_2), 34.9 (CH_2), 35.2 (CH_2), 36.0 (C), 36.8 (CH), 37.7 (CH), 40.8 (CH), 42.9 (CH), 45.8 (CH_3), 47.5 (C), 48.0 (CH), 51.4 (3-CH), 68.9 (7-CH), 73.9 (12-CH), 116.3 (CH), 120.7 (CH), 124.3 (CH), 128.9 (CH), 130.2 (CH), 131.0 (C), 131.1 (C), 137.9 (C), 153.2 (C), 178.3 (COO). HRMS m/z 640.3552 (calc. for $C_{36}H_{52}N_2O_6S$ 640.3546).

Synthesis of N-dansyl-7α-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (7α-Dns-ChA)

To a stirred warm solution (70 °C) of cholic acid (1 g, 2.45 mmol) in aqueous $NaHCO_3$ (3%, 40 mL), N-bromosuccinimide (1 g, 6.12 mmol) was added in small portions. The reaction mixture was stirred overnight, at rt, in absence of light and then it was warmed at 80 °C for further 2 hours. After cooling down it was acidified with HCl 6 M (40 mL) and the resulting precipitate was filtered and washed with water. The crude product was redissolved in AcOEt, washed with saturated aqueous NaCl and dried over $MgSO_4$. Purification by column chromatography (SiO_2, AcOEt:MeOH, 20:1) gave 7-oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid (**7[O]ChA**)[10] (0.58 g, 58%) as a white solid. ^{1}H-NMR (300 MHz, CD_3OD): δ 0.72 (s, 3H, Me-18), 1.02 (d, J = 6.3, 3H, Me-21), 1.22 (s, 3H, Me-19), 2.56 (dd, J = 11.4 both, 1H, CH-8), 2.98 (dd, J = 12.3, 6.0, 1 H, CH-6), 3.52 (m, 1H, CH_{ax}-3β), 3.99 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.2 (CH_3), 17.7 (CH_3), 23.3 (CH_3), 25.4 (CH_2), 28.7 (CH_2), 30.5 (CH_2), 30.6 (CH_2), 32.0 (CH_2), 32.3 (CH_2), 35.2 (CH_2), 35.9 (C), 36.6 (CH), 37.5 (CH), 38.3 (CH_2), 41.9 (CH), 46.3 (CH_2), 47.3

(CH), 47.5 (CH), 47.6 (C), 50.8 (CH), 71.6 (3-CH), 72.9 (12-CH), 178.2 (COOH), 214.8 (C=O).

A mixture of **7[O]ChA** (0.58 g, 1.4 mmol), $NaBH_3CN$ (0.09 g, 1.4 mmol) and ammonium acetate (1 g, 14 mmol) was dissolved in anhydrous MeOH (40 mL) and stirred at room temperature, under inert atmosphere, for 48 hours. Afterwards, the mixture was cautiously acidified with HCl to pH 2 and stirred for further 24 h to complete the esterification (the acid moiety was partially esterified during the reductive amination). Afterwards, the volume of the solvent was reduced until a white precipitate appeared; the solid was filtered off, washed with CH_2Cl_2, redissolved in 1-BuOH, washed with water and evaporated. The solid was once more washed with Et_2O and dried. 7α-Amino-3α,12α-dihydroxy-5β-cholan-24-oic acid methyl ester (**7α-NH_2-ChMe**) (hydrochloride salt) was obtained as a white powder (0.25 g, 42%): ^{1}H-NMR (300 MHz, CD_3OD): δ 0.73 (s, 3H, Me-18), 0.95 (s, 3H, Me-19), 1.01 (d, J = 5.7, 3H, Me-21), 2.98 (br s, 1H, CH_{eq}-7β), 3.39 (m, 1 H, CH_{ax}-3β), 3.65 (s, 3H, MeO), 3.97 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.0 (CH_3), 17.6 (CH_3), 23.1 (CH_3), 24.4 (CH_2), 27.4 (CH), 28.6 (CH_2), 29.3 (CH_2), 31.1 (CH_2), 31.8 (CH_2), 32.2 (CH_2), 34.9 (CH_2), 36.0 (C), 36.4 (CH_2), 36.7 (CH), 40.1 (CH), 40.9 (CH_2), 43.1 (CH), 43.2 (CH), 47.6 (C), 47.9 (CH), 49.6 (7-CH), 52.0 (CH_3O), 72.6 (3-CH), 73.8 (12-CH), 176.5 (COO). HRMS m/z 421.3193 (calc. for $C_{25}H_{43}NO_4$ 421.3192).

7α-Dns-ChMe was prepared from **7α-Dns-ChMe** following the procedure described above for **3α-Dns-ChMe**. Thus, starting from **7α-NH_2-ChMe** (42 mg), methyl N-dansyl-7α-amino-3α,12α-dihydroxy-5β-cholan-24-oate (**7α-Dns-ChMe**) was obtained as an orange crystalline solid (46 mg, 70%). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.45 (s, 3H, Me-18), 0.82 (s, 3H, Me-19), 0.85 (d, J = 5.1, 3H, Me-21), 2.86 (s, 6H, Me_2N), 3.26 (br s, 1H, CH_{eq}-7β), 3.42 (m, 1H, CH_{ax}-3β), 3.67 (s, 3H, MeO), 3.87 (br s, 1H, CH_{eq}-12β), 6.25 (d, J = 6.6, 1H, NH), 7.18 (d, J = 7.5, 1H, CH), 7.50 (t, 1H, CH), 7.63 (t, 1H, CH), 8.28 (d, J = 7.2, 1H, CH), 8.51 (d, J = 8.4, 2H, CH); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.8 (CH_3), 17.6 (CH_3), 23.1 (CH_3), 26.8 (CH_2), 28.0 (CH_2), 28.9 (CH_2), 30.3 (CH_2), 31.0 (CH_2), 31.2 (CH_2), 33.2 (CH), 33.4 (C), 34.9 (CH_2), 35.0 (CH), 35.4 (CH_2), 36.3 (CH_2), 41.8 (CH), 42.6 (CH), 45.5 (CH_3), 45.9 (CH), 46.6 (CH), 48.0 (C), 51.7 (CH_3), 54.3 (7-CH), 71.3 (3-CH), 72.2 (12-CH), 115.2 (CH), 118.8 (CH), 123.6 (CH), 128.4 (CH), 129.1 (CH), 129.7 (C), 129.9 (C), 130.3 (CH), 137.3 (C), 152.1 (C), 174.8 (COO). HRMS m/z 654.3682 (calc. for $C_{37}H_{54}N_2O_6S$ 654.3703).

7α-Dns-ChA was prepared from **7α-Dns-ChMe** following the procedure described above for **3α-Dns-ChMe**. Thus, starting from **7α-Dns-ChMe** (46 mg), N-dansyl-7α-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (**7α-Dns-ChA**) was obtained as a yellow crystalline solid (40 mg, 90%). ^{1}H-NMR (300 MHz, CD_3OD): δ 0.43 (s, 3H, Me-18), 0.87 (s, 3H, Me-19), 0.90 (d, J = 5.7, 3H, Me-21), 2.88 (s, 6H, Me_2N), 3.02 (br s, 1H, CH_{eq}-7β), 3.42 (m, 1H, CH_{ax}-3β), 3.86 (br s, 1H, CH_{eq}-12β), 7.31 (d, J = 7.5, 1H, CH), 7.54 (t, 1H, CH), 7.62 (t, 1H, CH), 8.19 (d, J = 7.2, 1H, CH), 8.55 (d, J = 8.4, 1H, CH), 8.60 (d, J = 8.7, 1H, CH); ^{13}C-NMR (75 MHz, CD_3OD): δ 12.7 (CH_3), 17.7 (CH_3), 22.9 (CH_3), 23.5 (CH_2), 27.6 (CH_2), 27.9 (CH), 29.2 (CH_2), 31.0 (CH_2), 31.1 (CH_2), 32.1 (CH_2), 34.6 (CH_2), 35.9 (CH), 36.0 (C), 36.4 (CH_2), 39.1 (CH), 40.2 (CH_2), 41.8 (CH), 43.2 (CH), 45.9 (CH_3N), 46.9 (CH), 47.0 (C), 52.8 (7-CH), 72.8 (3-CH), 73.5 (12-CH), 116.6 (CH), 121.6 (CH), 124.1 (CH), 128.9 (CH), 130.5 (CH), 131.0 (CH), 131.3 (C), 131.4 (C), 137.4 (C), 153.3 (C), 178.2 (COOH). HRMS m/z 640.3539 (calc. for $C_{36}H_{52}N_2O_6S$ 640.3546).

Synthesis of N-dansyl-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (7β-Dns-ChA)

To a stirred solution of **7[O]ChA** (0.7 g, 1.72 mmol) in MeOH (10 mL), a solution of hydroxylamine-hydrochloride (0.21 g, 3.1 mmol) and sodium acetate (0.42 g, 5.16 mmol) in water (1 mL) was added. After 4 h under reflux, the hot reaction mixture was filtered and concentrated to half its volume and poured into acidified (pH 2) brine (70 mL). The resulting precipitate was filtered, redissolved in AcOEt, washed with brine and evaporated. Purification on short column chromatography (SiO_2, AcOEt:MeOH, 20:1) gave 7-oximo-3α,12α-dihydroxy-5β-cholan-24-oic acid (**7[NOH]ChA**)[11] (0.49 g, 68%) as a white solid. ^{1}H-NMR (300 MHz, CD_3OD): δ 0.73 (s, 3H, Me-18), 1.02 (d, J = 6.3, 3H, Me-21), 1.08 (s, 3H, Me-19), 3.07 (dd, J = 12.9, 1.8, 1 H, CH-6), 3.52 (m, 1H, CH_{ax}-3β), 3.99 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.3 (CH_3), 17.7 (CH_3), 23.5 (CH_3), 26.0 (CH_2), 28.3 (CH_2), 28.6 (CH_2), 30.0 (CH_2), 30.7 (CH_2), 32.0 (CH_2), 32.3 (CH_2), 35.6 (C), 36.1 (CH), 36.6 (CH), 37.6 (CH_2), 37.8 (CH), 42.4 (CH), 43.5 (CH), 46.0 (CH_2), 47.4 (CH), 47.6 (C), 71.8 (3-CH), 73.3 (12-CH), 160.5 (C=NOH), 178.3 (COOH).

To a refluxing solution of **7[NOH]ChA** (0.49 g, 1.2 mmol) in 1-butanol (25 mL), Na (0.48 g, 21.6 mmol) was added over period of 1 h. The resulting mixture was refluxed for further 3 h and then poured into cold water (25 mL). After acidification with HCl (1M) to pH 2, the organic phase was separated and concentrated. Saponification of the partially formed butyl ester was performed by heating of the crude in 10% NaOH/MeOH for 1 h. Afterwards, the solvent was removed, the crude redissolved in slightly acidified H_2O and evaporated. To obtain the methyl ester, the crude was redissolved in MeOH (10 mL) containing a few drops of conc. HCl and stirred at rt overnight. Purification on column chromatography (SiO_2, CH_2Cl_2:MeOH:NH_3, 90:10:1) yielded 0.25 g (49%) of a white solid as a mixture of isomers **7α-NH_2-ChMe**:**7β-NH_2-ChMe**[17] (30:70) that was used into the following step and resolved after conjugation with the dansyl moiety. ^{1}H NMR (300 MHz, CD_3OD): δ 0.74 (m, 3H, Me-18(7α-NH_2-ChMe) + Me-18(7β-NH_2-ChMe)), 0.94 (s, 3H, Me-19), 1.02 (d, J = 6.3, 3H, Me-21), 2.82 (m, 0.7H, CH_{ax}-7β), 2.97 (br s, 0.3H, CH_{eq}-7α), 3.40 (m, 0.3H, CH_{ax}-3β(7α-NH_2-ChMe)), 3.51 (m, 0.7H, CH_{ax}-3β(7β-NH_2-ChMe)), 3.65 (s, 3H, MeO), 3.94 (br s, 1H, CH_{eq}-12β); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.0 (CH_3(7α-NH_2-ChMe)), 13.3 (CH_3(7β-NH_2-ChMe)), 17.6 (CH_3), 23.1 (CH_3(7α-NH_2-ChMe)), 23.7 (CH_3(7β-NH_2-ChMe)), 24.4 (CH_2), 27.4 (CH(7α-NH_2-ChMe)), 27.5 (CH(7β-NH_2-ChMe)), 28.6 (CH_2(7α-NH_2-ChMe)), 29.0 (CH_2(7β-NH_2-ChMe)), 29.4 (CH_2(7α-NH_2-ChMe)), 30.3 (CH_2(7β-NH_2-ChMe)), 30.9 (CH_2(7β-NH_2-ChMe)), 31.2 (CH_2(7α-NH_2-ChMe)), 31.8 (CH_2), 32.2 (CH_2(7α-NH_2-ChMe)), 33.5 (CH_2(7β-NH_2-ChMe)), 34.8 (CH_2(7β-NH_2-ChMe)), 34.9 (CH_2(7α-NH_2-ChMe)), 36.0 (C), 36.3 (CH_2(7β-NH_2-ChMe)), 36.4 (CH_2(7α-NH_2-ChMe)), 36.5 (CH(7β-NH_2-ChMe)), 36.7 (CH(7α-NH_2-ChMe)), 37.4 (CH_2(7β-NH_2-ChMe)), 37.9 (CH(7β-NH_2-ChMe)), 40.2 (CH(7α-NH_2-ChMe)), 41.0 (CH_2(7α-NH_2-ChMe)), 43.1 (CH(7α-NH_2-ChMe)), 43.2 (CH(7α-NH_2-ChMe)), 43.6 (CH(7β-NH_2-ChMe)), 45.0 (CH(7β-NH_2-ChMe)), 46.5 (CH(7β-NH_2-ChMe)), 47.6 (CH(7α-NH_2-ChMe)),

48.0 (C), 48.9 (7-CH(7β-NH_2-ChMe)), 49.5 (7-CH(7α-NH_2-ChMe)), 51.4 (CH_3O(7α-NH_2-ChMe)), 52.0 (CH_3O(7β-NH_2-ChMe)), 72.2 (3-CH(7β-NH_2-ChMe)), 72.6 (3-CH(7α-NH_2-ChMe)), 73.2 (12-CH(7β-NH_2-ChMe)), 73.8 (12-CH(7α-NH_2-ChMe)), 176.5 (COO).

7β-Dns-ChMe was prepared from a mixture of **7α-NH_2-ChMe**:**7β-NH_2-ChMe** following the procedure described above for **3α-Dns-ChMe**. Thus, starting from 0.15 g of the mixture of amines, methyl N-dansyl-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oate (**7β-Dns-ChMe**) (95 mg) was obtained as a pale green crystalline solid (41%): ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.66 (s, 3H, Me-18), 0.73 (s, 3H, Me-19), 0.96 (d, J = 6.0, 3H, Me-21), 2.89 (s, 6H, Me_2N), 3.36 (m, 2H, CH_{ax}-3β + CH_{ax}-7α), 3.67 (s, 3H, MeO), 3.94 (br s, 1H, CH_{eq}-12β), 4.22 (d, J = 9.3, 1H, NH), 7.17 (d, J = 7.5, 1H, CH), 7.54 (m, 2H, CH), 8.24 (d, J = 7.5, 2H, CH), 8.51 (d, J = 8.4, 1H, CH); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 12.4 (CH_3), 17.3 (CH_3), 22.3 (CH_2), 22.4 (CH_3), 26.6 (CH_2), 27.0 (CH), 27.8 (CH_2), 29.7 (CH_2), 30.1 (CH_2), 30.7 (CH_2), 32.2 (CH_2), 34.6 (C), 34.8 (CH), 35.2 (CH_2), 37.9 (CH), 39.3 (CH_2), 41.0 (CH), 41.3 (CH), 45.4 (CH_3), 46.2 (C), 46.5 (CH), 51.4 (7-CH), 51.5 (CH_3), 71.7 (3-CH), 72.6 (12-CH), 115.0 (CH), 119.6 (CH), 123.3 (CH), 128.1 (CH), 129.4 (CH), 129.8 (C), 130.0 (C), 130.1 (CH), 136.1 (C), 152.0 (C), 174.7 (COO). HRMS m/z 654.3739 (calc. for $C_{37}H_{54}N_2O_6S$ 654.3703).

7β-Dns-ChA was prepared from **7β-Dns-ChMe** following the procedure described above for **3α-Dns-ChA**. Thus, starting from **7β-Dns-ChMe** (95 mg), N-dansyl-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (**7β-Dns-ChA**) was obtained as a pale green crystalline solid (80 mg, 85%). ^{1}H-NMR (300 MHz, CD_3OD): δ 0.43 (s, 3H, Me-18), 0.87 (s, 3H, Me-19), 0.90 (d, J = 5.7, 3H, Me-21), 2.88 (s, 6H, Me_2N), 3.02 (br s, 1H, CH_{eq}-7β), 3.42 (m, 1H, CH_{ax}-3β), 3.86 (br s, 1H, CH_{eq}-12β), 7.31 (d, J = 7.5, 1H, CH), 7.54 (t, 1H, CH), 7.62 (t, 1H, CH), 8.19 (d, J = 7.2, 1H, CH), 8.55 (d, J = 8.4, 1H, CH), 8.60 (d, J = 8.7, 1H, CH); ^{13}C-NMR (75 MHz, CD_3OD): δ 13.4 (CH_3), 17.7 (CH_3), 23.5 (CH_3), 27.6 (CH_2), 29.1 (CH_2), 30.2 (CH_2), 30.8 (CH_2), 32.4 (CH_2), 32.5 (CH_2), 34.3 (CH), 34.6 (C), 36.0 (CH_2), 36.6 (CH), 37.2 (CH_2), 43.0 (CH), 43.3 (CH), 45.8 (CH_3N), 46.7 (CH), 48.0 (CH), 55.1 (7-CH), 72.0 (3-CH), 73.2 (12-CH), 116.3 (CH), 120.9 (CH), 124.4 (CH), 129.0 (CH), 129.7 (CH), 130.8 (CH), 131.1 (C), 131.2 (C), 139.6 (C), 153.1 (C), 178.7 (COOH). HRMS m/z 640.3549 (calc. for $C_{36}H_{52}N_2O_6S$ 640.3546).

Photophysical measurements

Absorption measurements (UV/Vis) were performed on a JASCO V-530 spectrometer (Japan). Fluorescence spectra were recorded on a FS900 fluorometer, and lifetimes were measured with a FL900 setup, both from Edinburgh Instruments (Reading, UK). Lifetime measurements were based on single-photon-counting using a hydrogen flashlamp (1.5 ns pulse width) as excitation source (λ_{exc} = 337 nm). The kinetic traces were fitted by monoexponential decay functions using a re-convolution procedure to separate from the lamp pulse profile. When required, the solutions were purged with nitrogen or oxygen for 15 minutes before the measurements. The absorbance of the solutions at the excitation wavelength was kept below 0.1. Cuvettes of 1 cm optical path length were employed, and experiments were performed in ethanol at room temperature.

The singlet excited state energy was calculated using the following formula:

$$E_S = N_A \frac{hc}{\lambda_{cr}} [Jmol^{-1}]$$

where N_A is Avogadro constant, h Planck constant, c the speed of light in a vacuum and λ_{cr} is the corresponding crossing point between the normalized excitation and emission spectra.

Fluorescence quantum yields were determined using the following formula:[18]

$$\phi_i = \phi_s \frac{n^2}{n_S{}^2} \frac{I_i}{I_S} \frac{1-10^{-A_S(\lambda_{exe})}}{1-10^{-A_i(\lambda_{exe})}}$$

where ϕ is fluorescence quantum yield, A the absorbance at the excitation wavelength, I the area under the corrected fluorescence spectra, and n is the refractive index of the solvent in which the sample fluorescence was collected. The subscripts "i" and "S" refer to the sample of interest and the standard respectively. Coumarine30 in CH_3CN (ϕ_F = 0.67, λ_{exc} = 370 nm) was used as a standard.[19]

Flow cytometry

Hepatocytes were obtained from 200-300 g Sprague Dawley male rats by perfusion of the liver with collagenase as described elsewhere.[20] Cell viability of suspensions, assessed by the trypan blue exclusion test, was higher than 85%.

All the flow cytometric measurements were performed in triplicate using a MoFlo Cell Sorter (Beckman-Coulter, Brea, CA) equipped with a water-cooled argon-ion laser emitting at both 350 nm (UV laser for excitation of dansyl derivatives) and 488 nm (blue laser for excitation of propidium iodide). Laser power was set up at 50 mW. The fluorescence emissions were collected at 530 nm (dansyl green fluorescence) and 625 nm (propidium iodide orange fluorescence). Measurements of forward angle laser light scatter (FS), an estimation of cell size, were used for gross morphological assessment of hepatocytes and the exclusion of debris. Data analysis was performed using the Summit V4.0 software (Beckman-Coulter, Brea, CA) interfaced to the cell sorter.

The uptake kinetics of the different Dns-ChA derivatives were evaluated by flow cytometry, as previously described.[6] Suspensions of freshly isolated rat hepatocytes were diluted at 5×10^5 cells/mL in Ham's F-12/Lebovitz L-15 (1:1) medium supplemented with 2% calf serum plus 0.2% bovine serum albumin and kept at 37 °C in a 5% CO_2 humidified atmosphere until analysis. Flow cytometric experiments were always performed within two hours after cell isolation. Hepatocyte suspensions were dispensed in standard polypropylene tubes and stained with appropriate concentrations of each dansyl derivative for 30 min at 37 °C in the dark, propidium iodide was added at 5 μg/mL final concentration for 5 minutes to identify and exclude dead cells from the analysis. Detector settings were adjusted to display live cells as the events with largest forward scatter and lowest orange fluorescence (corresponding to autofluorescence). Live cells were thus delimited by and selected according to the rectangular gate shown in Fig. 2A. Dead and dying cells appear as intense propidium-fluorescent events, while bare nuclei released by necrotic cells appear as small but fluorescent events.

At the starting time, each tube was loaded in the flow cytometer, and data acquisition was maintained for about 10 seconds, in

order to detect the green autofluorescence of cells. Then, data acquisition was paused, and an appropriate volume of stock solution (1 mg/mL in ethanol) of the corresponding dansyl derivative was added quickly to the tube for a final concentration of 5 μM. From this moment data acquisition was continued until 300 seconds. Transport of the cholic acid derivative inside the cell can be detected and quantified by measuring the increase of dansyl green fluorescence in cells along time. For this purpose, rectangular analytical regions are implemented by the cytometer-interfaced computer along the graph X-axis to obtain mathematical values from the raw cytometric data, as shown in Fig. 2B.

Single end-point flow cytometric measurements of cellular fluorescence were performed to determine the stability of intracellular fluorescence accumulation. Thus, following the initial kinetic measurement, each treated sample was run again in the flow cytometer at 30 min after addition of the dansyl derivative. In these end-point measurements fluorescence data from 10 000 live cells were acquired, as shown in Fig. 2C.

In order to check for specificity, hepatocyte suspensions were treated with troglitazone, a well known cholestatic drug, which inhibits bile salt uptake and efflux.[12] Dilute hepatocyte suspensions were incubated for 15 minutes at 37 °C with troglitazone (50 μM final concentration from a stock solution at 1 mg/mL in DMSO) or an appropriate volume of DMSO. Then, uptake of dansyl derivatives was determined by flow cytometry as described above.

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