Fluorescent derivatives of bile salts. I. Synthesis and properties of NBD-amino derivatives of bile salts

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Abstract In order to visualize bile salt transport, fluorescent bile salt derivatives were synthesized by introduction of the relatively small fluorescent 4-nitrobenzo-2-oxa-1,3-diazol (NBD)-amino group in either the 3-, 7-, or 12-position of the steroid structure, thus providing a complete set of diastereomeric derivatives, 3α-NBD-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid, 3β-NBD-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid, 7α-NBD-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid, 7β-NBD-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid, 12α-NBD-amino-3α,7α-dihydroxy-5β-cholan-24-oic acid, 12β-NBD-amino-3α,7α-dihydroxy-5β-cholan-24-oic acid, as well as their taurine conjugates. Their optical properties with absorption maxima at about 490 nm and emission maxima at 550 nm make them suitable for fluorescent microscopic studies. Fluorescence of the NBD-derivatives is strongly dependent on polarity of the solvent, on the concentration of the bile salt derivatives, and only slightly on temperature.—**Schneider, S., U. Schramm, A. Schreyer, H-P. Buscher, W. Gerok, and G. Kurz.** Fluorescent derivatives of bile salts. I. Synthesis and properties of NBD-amino derivatives of bile salts. *J. Lipid Res.* 1991. **32:** 1755–1767.

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Bile salts are synthesized in the liver, secreted into bile, and exert their physiological functions in the intestine. They are reabsorbed from the intestine, transported back to the liver via portal blood, and subjected in this way to an intensive recycling in the enterohepatic circulation. Effective reabsorption of bile salts for salvage also occurs in kidney.

Thus, transcellular transport of bile salts is of extreme importance in the organs of the enterohepatic circulation and in the kidney. These organs exhibit a complex morphological structure and in each organ only distinct cells are involved in bile salt transport. Organization of bile salt transport is not completely understood either on the cellular or on the organ level and information about alterations attending pathological conditions is even more lacking. This lack of knowledge is, to a large extent, caused by the chemical nature of the bile salts which as unobtrusive molecules "im schlichten Gewand der Farblosigkeit einherziehend, weder in ihrem Äusseren noch in ihren Eigenschaften irgendwie hervortreten" (1).[3]

Lack of appropriate chemical reactivity of bile salts has been overcome by the synthesis of photolabile derivatives (2–4), which allow the identification of structures that interact with bile salts at the molecular level by photoaffinity labeling (3–10). In order to compensate for the lack of color of bile salts we provided them with advantageous optical properties and synthesized fluorescent derivatives by introducing the relatively small NBD-amino function in different positions of the steroid structure of unconjugated and taurine-conjugated bile salts. The syntheses and physicochemical properties of different diastereomeric derivatives are described here and their suitability for the study of hepatobiliary transport is reported in the accompanying paper (11).

MATERIAL AND METHODS

Materials

Cholic acid, silica gel 60 (40–63 μm), and silica gel plates (Kieselgel 60 without fluorescence indicator) for TLC and HPTLC chromatography were purchased from

Abbreviations: DCI, direct chemical ionization; EI, electron impact; FAB, fast atom bombardment; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; LSC, liquid scintillation counting; NBD, 4-nitrobenzo-2-oxa-1,3-diazol; NBD-NC, N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-amino-dihydroxy-5β-cholan-24-oic acid; NBD-NCT, {N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-amino-dihydroxy-5β-cholan-24-oyl}-2'-aminoethanesulfonate; TLC, thin-layer chromatography.

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[3]". . . roaming about in their unadorned garment of colorlessness, are in no way conspicuous, neither in their outward appearance nor in their properties."

Merck (Merck, Darmstadt, Germany). NBD-Cl and taurine were from Sigma (Sigma GmbH, Taufkirchen, Germany). [2-^{3}H(N)]Taurine (750–1500 GBq/mmol) was obtained from NEN/Du Pont de Nemours GmbH Division (Dreieich, Germany). All other chemicals were of the highest quality available from commercial sources.

Detection of radioactivity

Radioactivity in organic solvents was determined after addition of 4 ml of Quickszint 501 (Zinsser Analytic GmbH, Frankfurt, Germany) by liquid scintillation counting (RackBeta 1217, Pharmacia LKB, Freiburg, Germany). Radioactivity on thin-layer plates was detected with a radioscanner (Linear Analyser, Berthold, Wildbad, Germany).

Analysis of organic compounds

Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer GmbH, Überlingen, Germany). Ultraviolet absorption spectra were measured with a Perkin-Elmer UV/VIS-Spectrophotometer Lambda 5 (Perkin-Elmer GmbH). Fluorescence spectra were recorded with a fluorescence spectrophotometer connected with a data unit DC-SU-2 (Perkin-Elmer GmbH). Quantum yields were determined by using the quantum counter Basic Blue 3 [2,7-bis-(diethylamino)-phenazoxonium chloride]. ^{1}H-NMR-spectra were measured on a Bruker-250-MHz-NMR spectrometer (Bruker GmbH, Karlsruhe, Germany). Values were in parts per million relative to tetramethylsilane as internal standard. Mass spectra of unconjugated bile salt derivatives were recorded with a Finnigan MAT 44S mass spectrometer connected with a data unit SS 2000 (Finnigan, Sunnyvale, CA). Unconjugated bile salts and their derivatives were ionized by EI (electron impact ionization) with an electron energy of 70 eV and by DCI (direct chemical ionization) with an electron energy of 170 eV using ammonia as reactant gas at a pressure of 30 Pa. In both cases positive ions were recorded (12–14). Mass spectra of taurine-conjugated bile salt derivatives were recorded with a VG 70-SE mass spectrometer (VG Instruments Inc., Stanford, CT). Taurine-conjugated bile salt derivatives were ionized by bombardment with xenon with an energy acceleration voltage of 8 kV using the FAB (fast atom bombardment) method.

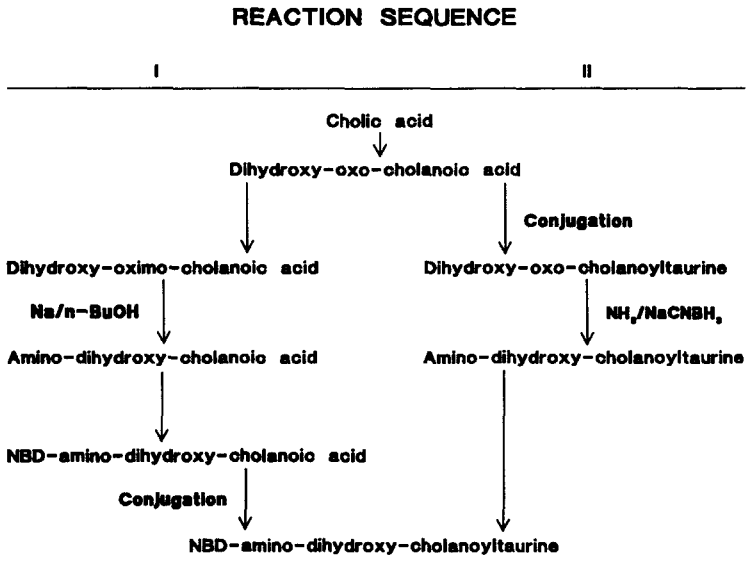
Syntheses

Column chromatography was performed on 100 × 5 cm-columns of silica gel 60 (63–200 μm), and flash chromatography (15) on either 25 × 5 cm or 25 × 3 cm-columns of silica gel 60 (40–63 μm). Solvent systems for chromatographic separations were: solvent system 1, ethyl acetate–cyclohexane–acetic acid 23:7:3 (v/v/v); solvent system 2, n-butanol–acetic acid–water 9:2:1 (v/v/v).

Bile acids and their derivatives were detected on TLC plates by spraying the dried plates with concentrated sulfuric acid and then heating at 120°C for 5 min.

7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oic acid, 3α,12α-dihydroxy-7-oxo-5β-cholan-24-oic acid, and 3α,7α-dihydroxy-12-oxo-5β-cholan-24-oic acid (**Scheme 1**) were synthesized exactly as described (2, 4). All analytical data and the elemental analyses were consistent with the reported values.

Dihydroxy-oximo-5β-cholan-24-oic acids (Scheme 1, reaction sequence I). A sample of 5 g (12.3 mmol) of the cor-



Scheme 1. Syntheses of NBD-amino derivatives of unconjugated and taurine-conjugated bile salts.

responding dihydroxy-oxo-5β-cholan-24-oic acid was dissolved in 50 ml of methanol. After addition of a solution of 3 g (36.6 mmol) sodium acetate and 1.5 g (21.6 mmol) hydroxylamine hydrochloride in 5 ml of distilled water, the reaction mixture was heated at reflux temperature for 3 h. The hot solution was filtered and the filtrate was concentrated by evaporation under reduced pressure to about half of its volume. This solution was slowly poured into 500 ml of acidified brine (pH 2.0) under vigorous stirring. The white precipitate was filtered off and dried. The crude product was purified by column chromatography using solvent system 1. Starting with each oxo derivative the yield of pure product was about 3.1 g (7.4 mmol, 60% yield). The crude products could be used without chromatographic purification for the synthesis of the amino-dihydroxy-5β-cholan-24-oic acids.

7α,12α-Dihydroxy-3-oximo-5β-cholan-24-oic acid. Yield: 3.6 g (8.5 mmol, 70%); mp, 158°C; TLC: R_f = 0.60 (solvent system 1); ^{1}H-NMR (d_4-MeOH): δ = 0.74 (s, C$\underline{H}_3$-18), 0.98 (s, C$\underline{H}_3$-19), 1.04 (d, J=6Hz, C$\underline{H}_3$-21), 3.85 (m, C$\underline{H}$-7), 4.0 (m, C$\underline{H}$-12); anal. calcd. for $C_{24}H_{39}O_5N$ (421.6): C, 68.37, H, 9.32, N, 3.32; found: C, 68.03, H, 9.12, N, 3.24.

3α,12α-Dihydroxy-7-oximo-5β-cholan-24-oic acid. Yield: 3.1 g (7.4 mmol, 60%); mp, 160°C; TLC: R_f = 0.56 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.6 (s, C$\underline{H}_3$-18), 0.98 (s, C$\underline{H}_3$-19), 0.93 (d, J=5Hz, C$\underline{H}_3$-21), 3.36 (m, C$\underline{H}$-3), 3.82 (m, C$\underline{H}$-12); anal. calcd. for $C_{24}H_{39}O_5N$ (421.6): C, 68.37, H, 9.32, N, 3.32; found: C, 67.82, H, 9.2, N, 3.38.

3α,7α-Dihydroxy-12-oximo-5β-cholan-24-oic acid. Yield: 3.1 g (7.4 mmol, 60%); mp, 160°C; TLC: R_f = 0.64 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.85 (s, C$\underline{H}_3$-18), 0.9 (s, C$\underline{H}_3$-19), 0.9 (d, J=5Hz, C$\underline{H}_3$-21), 3.2 (m, C$\underline{H}$-3), 3.69 (m, C$\underline{H}$-7); anal. calcd. for $C_{24}H_{39}O_5N$ (421.6): C, 68.37, H, 9.32, N, 3.32; found: C, 67.85, H, 8.97, N, 3.21.

Amino-dihydroxy-5β-cholan-24-oic acids (Scheme 1, reaction sequence I). A solution of 5 g (11.8 mmol) of the corresponding dihydroxy-oximo-5β-cholan-24-oic acid in 250 ml of dry n-butanol was heated at reflux temperature. Over a period of 1 h, 5 g (217 mmol) of sodium was added cautiously in small portions to the reaction mixture. To complete the reaction, the mixture was stirred at reflux temperature for an additional period, at least 1 h. The progress of the reaction was monitored by TLC using solvent system 2. After the reduction was shown to be finished, 250 ml of ice was added to the reaction mixture and the apparent pH value was adjusted to pH 2 with HCl. After separation of two layers, the organic phase was evaporated to dryness under reduced pressure. To achieve hydrolysis the partially esterified crude product was heated in 50 ml of 10% NaOH–methanol for about 1 h. After evaporation of the solvent under reduced pressure the crude product was purified by flash chromatography using the solvent system chloroform–methanol–acetic acid 7:2:0.1 (v/v/v). Starting with each oximo derivative, 2 g (5.0 mmol, 45% yield) of pure product was obtained as a mixture of α- and β-isomers. Whereas the mixture of the α- and β-isomers could be used for the syntheses of the NBD-derivatives without further separation, for analytical purposes the α- and β-isomers were separated by column chromatography again using chloroform–methanol–acetic acid 7:2:0.1 (v/v/v). The yields were quite different depending on the considered isomer; they are indicated below for each distinct isomer. All isomers were characterized by ^{1}H-NMR.

3α-Amino-7α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 1.4–1.6 g (3.5–4 mmol, 32–36%); mp, 219°C; TLC: R_f = 0.38 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.72 (s, C$\underline{H}_3$-18), 0.97 (s, C$\underline{H}_3$-19), 1.02 (d, J=6Hz, C$\underline{H}_3$-21), 2.93 (m, C$\underline{H}$-3), 3.82 (m, C$\underline{H}$-7), 3.98 (m, C$\underline{H}$-12).

3β-Amino-7α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 0.4–0.6 g (1–1.5 mmol; 9–13%); mp, 205°C; TLC: R_f = 0.33 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.7 (s, C$\underline{H}_3$-18), 0.94 (s, C$\underline{H}_3$-19), 1.01 (d, J=6Hz, C$\underline{H}_3$-21), 3.47 (m, C$\underline{H}$-3), 3.85 (m, C$\underline{H}$-7), 4.0 (m, C$\underline{H}$-12); anal. calcd. for $C_{24}H_{40}O_4NNa$ (429.6): C, 67.10, H, 9.39, N, 3.26; found: C, 67.36, H, 9.65, N, 3.22.

7α-Amino-3α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 0.4–0.6 g (1–1.5 mmol; 9–13%); mp, 209°C; TLC: R_f = 0.42 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.6 (s, C$\underline{H}_3$-18), 0.83 (s, C$\underline{H}_3$-19), 0.91 (d, J=5Hz, C$\underline{H}_3$-21), 3.22 (m, C$\underline{H}$-3), 3.07 (m, C$\underline{H}$-7), 3.77 (m, C$\underline{H}$-12).

7β-Amino-3α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 1.4–1.6 g (3.5–4 mmol, 32–36%); mp, 200°C; TLC: R_f = 0.40 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.6 (s, C$\underline{H}_3$-18), 0.83 (s, C$\underline{H}_3$-19), 0.91 (d, J=5Hz, C$\underline{H}_3$-21), 3.36 (m, C$\underline{H}$-3), 2.88 (m, C$\underline{H}$-7), 3.77 (m, C$\underline{H}$-12); anal. calcd. for $C_{24}H_{40}O_4N$ (406.6): C, 70,89, H, 9.92, N, 3.44; found: C, 71.16, H, 10.18, N, 3.48.

12α-Amino-3α,7α-dihydroxy-5β-cholan-24-oic acid. Yield: 0.4–0.6 g (1–1.5 mmol; 9–13%); mp, 210°C; TLC: R_f = 0.43 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.64 (s, C$\underline{H}_3$-18), 0.8 (s, C$\underline{H}_3$-19), 0.9 (d, J=5Hz, C$\underline{H}_3$-21), 3.18 (m, C$\underline{H}$-3), 3.6 (m, C$\underline{H}$-7), 3.02 (m, C$\underline{H}$-12).

12β-Amino-3α,7α-dihydroxy-5β-cholan-24-oic acid. Yield: 1.4–1.6 g (3.5–4 mmol, 32–36%); mp, 198°C; TLC: R_f = 0.45 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.69 (s, C$\underline{H}_3$-18), 0.84 (s, C$\underline{H}_3$-19), 0.94 (d, J=5Hz, C$\underline{H}_3$-21), 3.22 (m, C$\underline{H}$-3), 3.61 (m, C$\underline{H}$-7), 2.8 (m, C$\underline{H}$-12); anal. calcd. for $C_{24}H_{40}O_4N$ (406.6): C, 70,89, H, 9.92, N, 3.44; found: C, 70.2, H, 10.3, N, 3.48.

NBD-Amino-dihydroxy-5β-cholan-24-oic acids (Scheme 1, reaction sequence I). To a solution of 100 mg (245 μmol) of the corresponding amino-dihydroxy-5β-cholan-24-oic acid in 50 ml of dry ethanol enough solid $NaHCO_3$ was added so that a sediment remained. Dry ethanol was obtained by refluxing commercially available absolute ethanol over mag-

nesium turnings in presence of CCl_4 followed by distillation. A solution of 60 mg (300 μmol) of NBD-Cl in 5 ml dry dioxane was added dropwise and the reaction mixture was stirred at room temperature. The progress of the reaction was monitored by TLC using solvent system 1. After about 24 h the reaction mixture was filtered and the filtrate was evaporated under reduced pressure to dryness. The crude product was purified by flash chromatography using the solvent system ethyl acetate–cyclohexane–acetic acid 100:40:1 (v/v/v). Occasionally rechromatography proved to be necessary. The separation of the isomers had to be monitored by HPTLC using solvent system 1. Only fractions containing the pure product were combined and evaporated under reduced pressure to dryness. In order to obtain the isomer present in smaller amount, rechromatography was inevitable. The products obtained after chromatography were finally separated from silica gel by adsorption chromatography with Amberlite XAD-2. The total yield of the respective α- and β-isomers was about 50 mg (85 μmol, 35%). All isomers were characterized by ^{1}H-NMR and mass spectrometry.

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-3α-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (3α-NBD-NC). Yield: 35–40 mg (60–68 μmol, 25–28%); mp, 182°C; TLC: R_f = 0.45 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.67 (s, C$\underline{H}_3$–18), 0.97 (s, C$\underline{H}_3$–19), 1.0 (d, J = 5Hz, C$\underline{H}_3$–21), 3.56 (m, C$\underline{H}$–3), 3.66 (m, C$\underline{H}$–7), 3.85 (m, C$\underline{H}$–12), 6.44 (d, J = 10Hz, C$\underline{H}$=CNH), 8.43 (d, J = 10Hz, C$\underline{H}$=C–NO_2), 9.62 (m, –N$\underline{H}^+$).

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-3β-amino-7α,12α-dihydroxy-5β-cholan-24-oic acid (3β-NBD-NC). Yield: 10–15 mg (17–25 μmol, 7–11%); mp, 168°C; TLC: R_f = 0.38 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.61 (s, C$\underline{H}_3$–18), 0.9 (s, C$\underline{H}_3$–19), 0.94 (d, J = 6Hz, C$\underline{H}_3$–21), 4.14 (m, C$\underline{H}$–3), 3.64 (m, C$\underline{H}$–7), 3.82 (m, C$\underline{H}$–12), 6.46 (d, J = 9Hz, C$\underline{H}$=CNH), 8.48 (d, J = 9Hz, C$\underline{H}$=C–NO_2), 9.67 (m, –N$\underline{H}^+$); mass spectrum (EI); *m/z* = 355 M-(2 H_2O + amino-NBD), 269 M-(2 H_2O + NBD + side chain), 253 M-(2 H_2O + side chain + amino-NBD); mass spectrum (DCI): *m/z* = 588 $(M+NH_4)^+$, 424 $(M+NH_4\text{-NBD})^+$, 571 $(M)^+$, 371 $(M\text{-NBD})^+$.

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7α-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (7α-NBD-NC). Yield: 10–15 mg (17–25 μmol, 7–11%); mp, 180°C; TLC: R_f = 0.51 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.66 (s, C$\underline{H}_3$–18), 0.94 (s, C$\underline{H}_3$–19), 0.91 (d, J = 8Hz, C$\underline{H}_3$–21), 3.4 (m, C$\underline{H}$–3), 3.5 (m, C$\underline{H}$–7), 3.8 (m, C$\underline{H}$–12), 6.38 (d, J = 9Hz, C$\underline{H}$=CNH), 8.5 (d, J = 9Hz, C$\underline{H}$=C–NO_2).

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oic acid (7β-NBD-NC). Yield: 35–40 mg (60–68 μmol, 25–28%); mp, 172°C; TLC: R_f = 0.45 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.8 (s, C$\underline{H}_3$–18), 1.05 (s, C$\underline{H}_3$–19), 1.02 (d, J = 7Hz, C$\underline{H}_3$–21), 3.57 (m, C$\underline{H}$–3), 3.83 (m, C$\underline{H}$–7), 3.99 (m, C$\underline{H}$–12), 6.3 (d, J = 9Hz, C$\underline{H}$=CNH), 8.53 (d, J = 9Hz, C$\underline{H}$=C–NO_2); mass spectrum (EI); *m/z* = 355 M-(2 H_2O + amino-NBD), 269 M-(2 H_2O + NBD + side chain), 253 M-(2 H_2O + side chain + amino-NBD); mass spectrum (DCI): *m/z* = 588 $(M+NH_4)^+$, 424 $(M+NH_4\text{-NBD})^+$, 571 $(M)^+$, 371 $(M\text{-NBD})^+$.

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-12α-amino-3α,7α-dihydroxy-5β-cholan-24-oic acid (12α-NBD-NC). Yield: 10–15 mg (17–25 μmol, 7–11%); mp, 185°C; TLC: R_f = 0.39 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.85 (s, C$\underline{H}_3$–18), 0.85 (s, C$\underline{H}_3$–19), 0.53 and 0.68 (d, J = 5Hz, C$\underline{H}_3$–21), 3.18 (m, C$\underline{H}$–3), 3.7 (m, C$\underline{H}$–7), 4.35 (m, C$\underline{H}$–12), 6.58 and 7.02 (d, J = 9Hz, C$\underline{H}$=CNH), 8.41 and 8.44 (d, J = 9Hz, C$\underline{H}$=C–NO_2).

N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-12β-amino-3α,7α-dihydroxy-5β-cholan-24-oic acid (12β-NBD-NC). Yield: 35–40 mg (60–68 μmol, 25–28%); mp, 185°C; TLC: R_f = 0.46 (solvent system 1); ^{1}H-NMR (d_6-DMSO): δ = 0.83 (s, C$\underline{H}_3$–18), 0.89 (s, C$\underline{H}_3$–19), 0.41 and 0.6 (d, J = 5Hz, C$\underline{H}_3$–21), 3.15 (m, C$\underline{H}$–3), 3.71 (m, C$\underline{H}$–7), 4.3 (m, C$\underline{H}$–12), 6.59 and 6.63 (d, J = 9Hz, C$\underline{H}$=CNH), 8.32 and 8.34 (d, J = 9Hz, C$\underline{H}$=C–NO_2); mass spectrum (EI): *m/z* = 355 M-(2 H_2O + amino-NBD), 269 M-(2 H_2O + NBD + side chain), 253 M-(2 H_2O + side chain + amino-NBD); mass spectrum (DCI): *m/z* = 588 $(M+NH_4)^+$, 424 $(M+NH_4\text{-NBD})^+$, 571 $(M)^+$, 371 $(M\text{-NBD})^+$.

(Dihydroxy-oxo-5β-cholan-24-oyl)-2′-aminoethanesulfonates (Scheme 1, reaction sequence II). The conjugation of the dihydroxy-oxo-5β-cholan-24-oic acids with taurine was performed exactly as described (2). Starting with 2 g of the corresponding dihydroxy-oxo-5β-cholan-24-oic acid, the yield was about 65% (1.75 g, 3.2 mmol). The conjugated oxo derivatives were characterized by ^{1}H-NMR.

(7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oyl)-2′-aminoethanesulfonate. Yield: 1.9 g (3.5 mmol, 70%); mp, 225–226°C; TLC: R_f = 0.28 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.74 (s, C$\underline{H}_3$–18), 1.03 (s, C$\underline{H}_3$–19), 1.04 (d, J = 6Hz, C$\underline{H}_3$–21), 2.97 (t, J = 7Hz, C$\underline{H}_2$–NH), 3.6 (t, J = 7Hz, C$\underline{H}_2$–SO_3^-), 3.86 (d, J = 3Hz, C$\underline{H}$–7), 4.01 (m, C$\underline{H}$–12).

(3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oyl)-2′-aminoethanesulfonate. Yield: 1.75 g (3.2 mmol, 65%); mp, 216°C; TLC: R_f = 0.28 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.59 (s, C$\underline{H}_3$–18), 1.12 (s, C$\underline{H}_3$–19), 0.92 (d, J = 5Hz, C$\underline{H}_3$–21), 2.58 (t, J = 9Hz, C$\underline{H}_2$–NH), 3.28 (t, J = 9Hz, C$\underline{H}_2$–SO_3^-), 3.3 (m, C$\underline{H}$–3), 3.8 (m, C$\underline{H}$–12), 2.89 (dd, $J_{8,14\ od.\ 9}$ = 12Hz and 6Hz, C$\underline{H}$–8).

(3α,7α-Dihydroxy-12-oxo-5β-cholan-24-oyl)-2′-aminoethanesulfonate. Yield: 1.6 g (3 mmol, 60%); mp, 222°C; TLC: R_f = 0.29 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.94 (s, C$\underline{H}_3$–18), 0.94 (s, C$\underline{H}_3$–19), 0.74 (d, J = 5Hz,

CH_3-21), 2.58 (t, J=10Hz, CH_2-NH), 3.29 (t, J=10Hz, CH_2-SO_3^-), 3.19 (m, CH-3), 3.71 (m, CH-7), 7.75 (m, CO-NH).

(Amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonates (Scheme 1, reaction sequence II). Two g (3.7 mmol) of the corresponding (dihydroxy-oxo-5β-cholan-24-oyl)-2'-aminoethanesulfonate was dissolved in 100 ml of dry methanol and dry ammonia was bubbled through the solution for 15 min. Subsequently the solution was cooled to 0°C and the apparent pH value was adjusted with acetic acid to pH 6. After addition of 0.5 g (8 mmol) of sodium cyanoborohydride, the reaction mixture was stirred at room temperature for 24 h. The solution was then acidified with diluted HCl to remove HCN, which was absorbed in 20% aqueous KOH (caution!). After adjusting the apparent pH value with 1 N NaOH to pH 7, the solution was evaporated under reduced pressure to dryness. The crude product was purified by flash chromatography using the solvent system chloroform–methanol 3:1 (v/v). Starting with each oxo derivate 1 g (1.9 mmol, 50% yield) of pure product was obtained as a mixture of α- and β-isomers. Whereas the mixture of the α- and β-isomers could be used for the syntheses of the NBD-derivatives without further separation, for analytical purposes the α- and β-isomers were separated by column chromatography using chloroform–methanol 3:1 (v/v) as solvent system. The isomers obtained after chromatography were freed from silica gel by adsorption chromatography with Amberlite XAD-2. All isomers were characterized by ^{1}H-NMR.

(3α-Amino-7α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 0.5 g (0.9 mmol, 25%); mp, 250°C; TLC: R_f = 0.1 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.7 (s, CH_3-18), 0.95 (s, CH_3-19), 1.03 (d, J=6Hz, CH_3-21), 2.95 (t, J=7Hz, CH_2-NH), 3.59 (t, J=7Hz, CH_2-SO_3^-), 2.92 (m, CH-3), 3.8 (m, CH-7), 3.98 (m, CH-12), 2.49 (q, J=12Hz, CH_a-2 or CH_a-4).

(3β-Amino-7α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 0.5 g (0.9 mmol, 25%); mp, 245°C; TLC: R_f = 0.05 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.72 (s, CH_3-18), 0.93 (s, CH_3-19), 1.02 (d, J=7Hz, CH_3-21), 3.33 (m, CH-3), 2.97 (t, J=7Hz, CH_2-NH), 3.6 (t, J=7Hz, CH_2-SO_3^-), 3.8 (m, CH-7), 3.96 (m, CH-12).

(7α-Amino-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 0.95 g (1.8 mmol, 47%); mp, 250°C; TLC: R_f = 0.11 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.61 (s, CH_3-18), 0.86 (s, CH_3-19), 0.94 (d, J=5Hz, CH_3-21), 3.28 (m, CH_2-NH), 2.58 (m, CH_2-SO_3^-), 3.22 (m, CH-3), 3.18 (m, CH-7), 3.8 (m, CH-12).

(7β-Amino-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 50 mg (0.1 mmol, 3%); mp, 244°C; TLC: R_f = 0.07 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.6 (s, CH_3-18), 0.86 (s, CH_3-19), 0.93 (d, J=5Hz, CH_3-21), 2.58 (m, CH_2-NH), 3.29 (m, CH_2-SO_3^-), 3.3 (m, CH-3), 2.75 (m, CH-7), 3.79 (m, CH-12).

(12α-Amino-3α,7α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 0.9 g (1.7 mmol, 45%); mp, 244°C; TLC: R_f = 0.1 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.75 (s, CH_3-18), 0.84 (s, CH_3-19), 0.97 (d, J=5Hz, CH_3-21), 2.56 (t, J=10Hz, CH_2-NH), 3.29 (t, J=10Hz, CH_2-SO_3^-), 3.19 (m, CH-3), 3.62 (m, CH-7), 3.1 (m, CH-12), 7.8 (m, CO-NH).

(12β-Amino-3α,7α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate. Yield: 0.1 g (0.19 mmol, 10%); mp, 246°C; TLC: R_f = 0.15 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.78 (s, CH_3-18), 0.88 (s, CH_3-19), 0.99 (d, J=5Hz, CH_3-21), 2.56 (t, J=10Hz, CH_2-NH), 3.29 (t, J=10Hz, CH_2-SO_3^-), 3.22 (m, CH-3), 3.63 (m, CH-7), 2.8 (m, CH-12), 7.8 (m, CO-NH).

(NBD-Amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonates (Scheme 1). According to the required end product, the different stereoisomeric (NBD-amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonates were synthesized either by introduction of the NBD-residue into the corresponding (amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonates or by conjugation of the unconjugated NBD-derivatives with taurine.

3α-NBD-NCT, 7β-NBD-NCT, and 12β-NBD-NCT were synthesized starting with 100 mg (175 μmol) of the respective NBD-amino-dihydroxy-5β-cholan-24-oic acids (Scheme 1, reaction sequence I) as described.

3β-NBD-NCT, 7α-NBD-NCT, and 12α-NBD-NCT were synthesized following the procedure described for the synthesis of the NBD-amino-dihydroxy-5β-cholan-24-oic acids starting with 100 mg (186 μmol) of the mixture of α- and β-isomers of the corresponding (amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (Scheme 1, reaction sequence II). Because reductive amination of (7α,12α-dihydroxy-3-oxo-5β-cholan-24-oyl)-2'-aminoethanesulfonate results in equal amounts of the α- and β-amino compounds, this procedure may also be used for the synthesis of 3α-NBD-NCT.

The crude products were purified by flash chromatography using the solvent system chloroform–methanol–acetic acid 7:2:0.1 (v/v/v). Fractions containing the pure product were combined and evaporated under reduced pressure to dryness. The products obtained after flash chromatography were finally separated from silica gel by adsorption chromatography with Amberlite XAD-2.

Coupling of the amino derivative with the NBD-residue resulted in yields of about 35%; conjugation of the NBD-amino-acids gave yields of about 50%. All isomers were characterized by ^{1}H-NMR and mass spectrometry.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-3α-amino-7α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (3α-NBD-NCT). Yield: 45 mg (65 μmol, 35%), 75 mg (105 μmol, 60%); mp, 260–265°C, TLC: R_f = 0.37 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.73 (s, CH_3-18), 1.0 (s, CH_3-19), 1.03 (d, J=7Hz, CH_3-21), 3.0 (t, J=7Hz, CH_2-NH), 3.64 (t, J=7Hz, CH_2-SO_3^-), 3.56 (m, CH-3),

3.86 (m, CH-7), 4.01 (m, CH-12), 6.28 (d, J=9Hz, CH=CNH), 8.51 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-3β-amino-7α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (3β-NBD-NCT). Yield: 45 mg (65 μmol, 35%); mp, 255–260°C, TLC: R_f = 0.32 (solvent system 2); ^{1}H-NMR (d_4-MeOH): δ = 0.72 (s, CH_3-18), 1.0 (s, CH_3-19), 1.04 (d, J=7Hz, CH_3-21), 2.98 (t, J=7Hz, CH_2-NH), 3.61 (t, J=7Hz, CH_2-SO_3^-), 3.81 (m, CH-7), 3.94 (m, CH-3), 3.98 (m, CH-12), 6.39 (d, J=9Hz, CH=CNH), 8.5 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7α-amino-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (7α-NBD-NCT). Yield: 45 mg (65 μmol, 35%); mp, 265°C, TLC: R_f = 0.23 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.65 (s, CH_3-18), 0.92–0.96 (m, CH_3-19 and CH_3-21), 2.58 (t, J=7Hz, CH_2-NH), 3.28 (t, J=7Hz, CH_2-SO_3^-), 3.46 (m, CH-7), 3.8 (m, CH-12), 6.35 (d, J=9Hz, CH=CNH), 8.51 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-7β-amino-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (7β-NBD-NCT). Yield: 75 mg (105 μmol, 60%); mp, 260°C, TLC: R_f = 0.2 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.8 (s, CH_3-18), 1.01 (s, CH_3-19), 1.05 (s, CH_3-21), 2.58 (t, J=7Hz, CH_2-NH), 3.29 (t, J=7Hz, CH_2-SO_3^-), 3.83 (m, CH-3), 3.46 (m, CH-7), 3.98 (m, CH-12), 6.34 (d, J=9Hz, CH=CNH), 8.5 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-12α-amino-3α,7α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (12α-NBD-NCT). Yield: 45 mg (65 μmol, 35%); mp, 244°C, TLC: R_f = 0.37 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.81 (s, CH_3-18), 0.87 (s, CH_3-19), 0.5 and 0.62 (d, J=5Hz, CH_3-21), 2.79 (t, J=10Hz, CH_2-NH), 3.6 (t, J=7Hz, CH_2-SO_3^-), 3.2 (m, CH-3), 3.7 (m, CH-7), 4.21 (m, CH-12), 6.3 and 6.61 (d, J=9Hz, CH=CNH), 8.32 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

(N-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-12β-amino-3α,7α-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonate (12β-NBD-NCT). Yield: 75 mg (105 μmol, 60%); mp, 254°C, TLC: R_f = 0.35 (solvent system 2); ^{1}H-NMR (d_6-DMSO): δ = 0.78 (s, CH_3-18), 0.89 (s, CH_3-19), 0.5 and 0.62 (d, J=5Hz, CH_3-21), 2.79 (t, J=10Hz, CH_2-NH), 3.6 (t, J=10Hz, CH_2-SO_3^-), 3.17 (m, CH-3), 3.71 (m, CH-7), 4.19 (m, CH-12), 6.3 and 6.61 (d, J=9Hz, CH=CNH), 8.31 (d, J=9Hz, CH=C-NO_2); mass spectrum (FAB): *m/z*=700 M^+ (Na-salt), 722 $(M+Na)^+$, 542 (M+Na-amino-NBD)$^+$.

Synthesis of radioactively labeled bile salt derivatives

The radioactively labeled (NBD-amino-dihydroxy-5β-cholan-24-oyl)-2'-aminoethanesulfonates were synthesized exactly as described (2). The radiochemical yields of pure products were 4.6 MBq (50%) with specific activities of 750–1500 Bq/mmol. In order to reduce radiolytic decomposition, the products were dissolved in dry methanol (1 Bq/μl) and the solutions were kept in the dark at 20°C.

RESULTS

Syntheses

In order to have discriminative fluorescent derivatives of bile salts available for the study of different biological problems, a set of six diastereomers bearing the fluorescent NBD-amino group in positions 3, 7, and 12, respectively, was synthesized (**Fig. 1**).

The starting step in the synthesis of all amino derivatives (Scheme 1) was the selective oxidation of one of the hydroxyl groups of cholic acid, making use of their different reactivities towards oxidation and acylation reagents. Whereas the 7-hydroxyl group was selectively oxidized with N-bromosuccinimide, oxidation of the 3-hydroxyl group with the same reagent was started using the 7α,12α-diformoxy derivative easily obtained by selective hydrolysis of the triformylated cholic acid (4, 16–18). Oxidation of the 12-hydroxyl group was performed with the stronger oxidation reagent chromic acid after protection of the other hydroxyl groups of cholic acid methylester by acetylation (19, 20).

The three dihydroxy-oxo-cholanoic acids were used for the syntheses of the unconjugated amino-dihydroxy-cholanoic acids via oxime formation followed by reduction with sodium (Scheme 1, reaction sequence I) as well as for the syntheses of the conjugated amino derivatives where taurine conjugation preceeds direct reductive amination with sodium cyanoborohydride (Scheme 1, reaction sequence II).

Reduction of the oxime with sodium was performed principally according to Redel et al. (21), using n-butanol instead of amyl alcohol as a solvent. Acidification of the reaction mixture with 6 N HCl resulted in the separation of an aqueous NaCl-containing phase and an n-butanol phase containing the amino-dihydroxy-bile acids partially as n-butylesters. After mild hydrolyses with methanolic KOH, the amino-dihydroxy-cholanoic acids were isolated chromatographically as mixtures of α- and β-diastereomers. The ratio of the α- and β-diastereomers was different for the different positions as shown in **Table 1.** Separation of the diastereomers could be obtained by HPLC

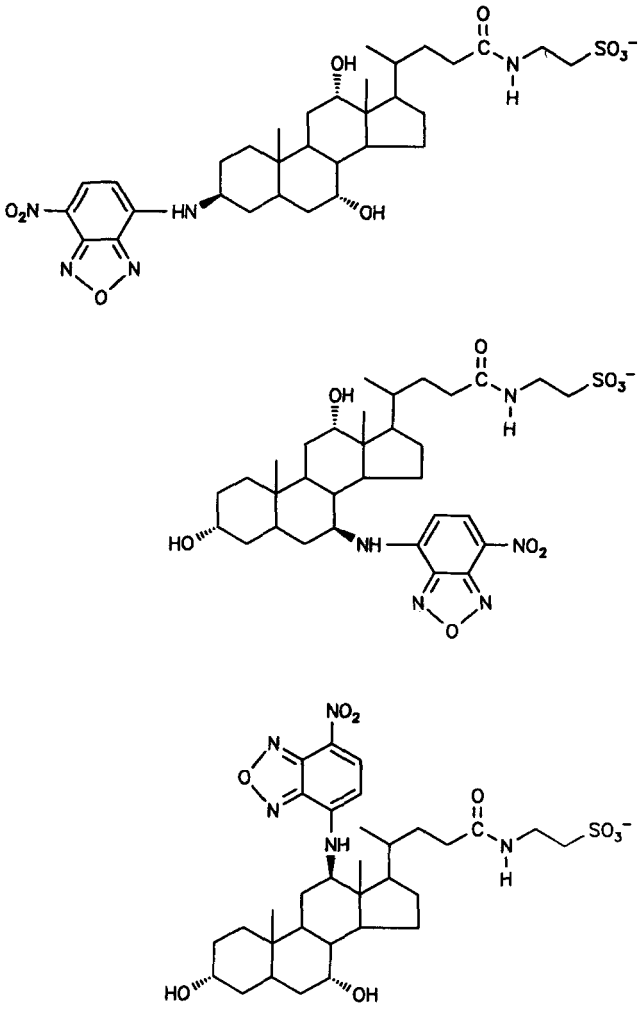


Fig. 1. Chemical structure of taurine-conjugated fluorescent bile salt derivatives (only β-orientations of the NBD-amino group are shown). Top: 3β-NBD-NCT; middle: 7β-NBD-NCT; bottom: 12β-NBD-NCT.

on a reversed-phase column; but this laborious isolation of stereochemically pure amino-dihydroxy-cholanoic acids proved not to be necessary for the preparation of the corresponding NBD-derivatives. After introduction of the NBD-group, the isomers could easily be separated by flash chromatography.

The conjugation of the stereochemically pure NBD-amino-dihydroxy-cholanoic acids with taurine was carried out using the mixed anhydride method (2) resulting in yields of about 50%. Synthesis of radioactively labeled conjugated bile salt derivatives was performed in the same way using [^{3}H]taurine.

Reductive amination of the taurine-conjugated dihydroxy-oxo-cholanoic acids using sodium cyanoborohydride as a complex hydride was performed by bubbling dry ammonia through the methanolic solutions and adjusting the apparent pH value to 6, instead of using ammonium acetate (22). Subsequent reduction with sodium cyanoborohydride resulted in mixtures of α- and β-isomers of the conjugated amino compound with a total yield of about 50–60%. The ratios of the α- and β-isomers formed, shown in **Table 2,** were different from those resulting from the reduction with sodium (Table 1).

The coupling of the amino function with NBD-Cl was performed in dry ethanol and the apparent pH value was adjusted with $NaHCO_3$ to 9.5. NBD-Cl was dissolved in dry dioxane and the solution was dropped slowly into the reaction mixture at a temperature of 25°C. With both the unconjugated and the conjugated bile salt derivatives only moderate yields of about 30% were obtained. The α- and β-NBD-substituted diastereomers of the amino-dihydroxy-cholanoic acids, whether unconjugated or conjugated, could be separated into the stereochemically pure products by flash chromatography.

Spectroscopic properties of the NBD-derivatives of bile salts

Absorption. The absorption spectra of the taurine-conjugated 7β-NBD-derivative of amino-dihydroxy-cholanoic acid in 150 mM phosphate buffer, pH 7.4, and ethanol are shown in **Fig. 2.** The spectra show four absorption maxima at 206, 230, 352, and 497 nm with a shoulder at 477 nm in phosphate buffer, pH 7.4, and four maxima at 201, 232, 336, and 477 nm in ethanol. The spectra of all the other taurine-conjugated NBD-amino-dihydroxy-cholanoic acids are very similar. The maxima at about 200 nm are assigned to the amide bond of the conjugated bile salt derivatives, whereas the other absorp-

TABLE 1. Ratio of α- and β-isomers formed by reduction of dihydroxy-oximo-cholanoic acids with elemental sodium in n-butanol

Starting Compound	Products	
	α-Amine	β-Amine
	%	
7α,12α-Dihydroxy-3-oximo-5β-cholan-24-oic acid	70–80	20–30
3α,12α-Dihydroxy-7-oximo-5β-cholan-24-oic acid	20–30	70–80
3α,7α-Dihydroxy-12-oximo-5β-cholan-24-oic acid	20–30	70–80

TABLE 2. Ratio of α- and β-isomers formed by reduction of taurine-conjugated dihydroxy-oxo-cholanoic acids with sodium cyanoborohydride

Starting Compound	Products	
	α-Amine	β-Amine
	%	
(7α,12α-Dihydroxy-3-oxo-5β-cholan-24-oyl)-taurine	45–55	45–55
(3α,12α-Dihydroxy-7-oxo-5β-cholan-24-oyl)-taurine	90–95	5–10
(3α,7α-Dihydroxy-12-oxo-5β-cholan-24-oyl)-taurine	90–95	5–10

tion maxima are caused by the NBD-amino function. The positions of these absorption maxima are in good agreement with those of other NBD-amino compounds (23–28). The most interesting absorption band is the intense longest wavelength absorption band between 460 and 500 nm, the position of which is strongly dependent on the solvent. The absorption of this band depends on the hydrophobicity of the solvent and shows a hypsochromic shift with increasing hydrophobicity of the solvent (27, 28). The longest wavelength absorption maxima of the different derivatives between 480 and 497 nm in aqueous solutions were shifted to absorption maxima between 463 and 473 nm in n-octanol, as summarized in **Table 3.** This characteristic blue shift is accompanied by a decrease in absorptivity (ϵ = 25000 $M^{-1}cm^{-1}$ in H_2O, ϵ = 21000 $M^{-1}cm^{-1}$ in ethanol) and by the disappearance of the shoulder beside the maximum.

The influence of the nature and pH value of buffer on absorption was investigated in a pH range relevant for in vivo studies. Variation of pH value in the range from 5 to 8 in different buffers (50–200 mM phosphate buffer, acetate buffer, and in standard incubation medium for hepatocytes (11)) had no effect on the absorption of all NBD-amino-dihydroxy-bile salt derivatives.

In order to examine whether the fluorescent bile salt derivatives form aggregates, absorption and absorption maxima were determined within the concentration range between 1 μM and 1 mM. The absorption maxima of all taurine-conjugated fluorescent derivatives did not change in this concentration range as demonstrated with 7β-NBD-NCT in **Fig. 3** for the range between 100 μM and 1 mM. The extinction increased linearily with increasing concentration of the fluorescent compound (Fig. 3, insert).

Fluorescence. The quantum yield of fluorescence of all fluorescent bile salt derivatives decreases with increasing temperature. Therefore, all measurements were performed at a constant temperature of 25°C.

The emission spectra of all taurine-conjugated fluorescent bile salt derivatives show maxima in the range between 540 nm and 570 nm depending on the polarity of the solvent, which is in good accordance with other NBD-amino compounds (27, 28). The emission spectra of 7β-NBD-NCT in solvents of different hydrophobicity are shown in **Fig. 4.** The emission maxima of 7β-NBD-NCT exhibit an increasing hypsochromic shift with increasing hydrophobicity of the solvent (**Table 4**) resulting in a shift from 567 nm in aqueous solution to 542 nm in n-octanol.

The hydrophobicity of the solvent had an even greater influence on the quantum yield (Table 4). The quantum yield increases with increasing hydrophobicity of the solvent, from Φ = 0.025 in water or phosphate buffer, pH 7.4, to Φ = 0.398 in n-octanol. pH variation of the buffer in the range between 5 and 8 had no influence on the wavelength of the emission maximum or on the quantum yield. The emission maxima and the quantum yields of the other fluorescent bile salt derivatives showed the same solvent dependency as 7β-NBD-NCT.

The absorption and emission spectra of the fluorescent bile salt derivatives exhibit spectral overlap, as shown in **Fig. 5** for 7β-NBD-NCT in ethanol, causing reabsorption of emitted light. Being dependent on the concentration of the fluorescent derivative, this reabsorption altered the shape of the emission spectrum (**Fig. 6**) and caused a loss of quantum yield with increasing concentration. In order to determine the influence of the concentration of the

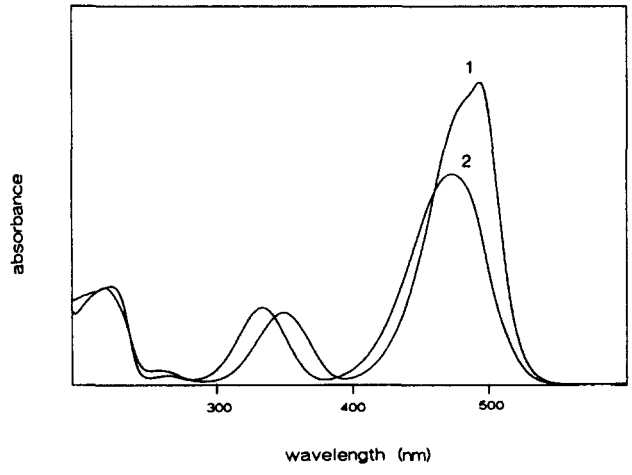


Fig. 2. Absorption spectra of 7β-NBD-NCT (5 μM). (1) Phosphate buffer, pH 7.4; (2) ethanol.

TABLE 3. Longest wavelength absorption maxima of taurine-conjugated NBD-amino derivatives of bile salts in different solvents; position of shoulder beside the maximum is given in parentheses

Compound	Absorption Maximum (nm) in Solvent				
	n-Octanol	n-Butanol	Ethanol	Methanol	Water
3α-NBD-NCT	463	465	466	468	494 (475)
3β-NBD-NCT	463	465	466	468	480 (494)
7α-NBD-NCT	473	475	477	478	497 (477)
7β-NBD-NCT	473	475	477	478	497 (477)
12α-NBD-NCT	460	461	463	464	482
12β-NBD-NCT	472	475	476	477	483

fluorescent bile salt derivatives on the quantum yield, the fluorescence was investigated in phosphate buffer, pH 7.4, in the concentration range from 1 μM to 1 mM. The data obtained with 7β-NBD-NCT demonstrate a clear decrease in quantum yield with increasing concentration of the fluorescent derivative (**Table 5**). At a concentration of 1 mM of the fluorescent bile salt derivative, the quantum yield amounted to only 0.5% of the quantum yield measured with a 1 μM solution. The quantum yields for all other taurine-conjugated NBD-amino-dihydroxy-cholanoic acids showed the same concentration dependency.

DISCUSSION

The study of transcellular transport of bile salts by fluorescence microscopy requires the introduction of a fluorophor (fluorescent group), emitting light in the visible range, into the otherwise non-fluorescent molecules. Such a fluorophor must have a relatively extended π-electron system and the spatial requirements of an appropriate substituent may have an influence on the physiological interactions of bile salt molecules.

Of many different fluorescent derivatives, the compounds bearing the NBD-group (4-nitrobenzo-2-oxa-1,3-diazol-group) seemed well suitable (25, 28) because, in comparison to other fluorophores, its introduction results in relatively small structural changes of the physiological bile salt molecules. In order to obtain light emission in the visible range, the NBD-group was linked to the steroid structure by an amino function. Due to the electron withdrawing effect of the NBD-group, the amino function is not protonated under physiological conditions, thus not changing the monoanionic character of the bile salts. Furthermore, NBD-amino derivatives, in contrast to NBD-thiol derivatives, are chemically stable under physiological conditions.

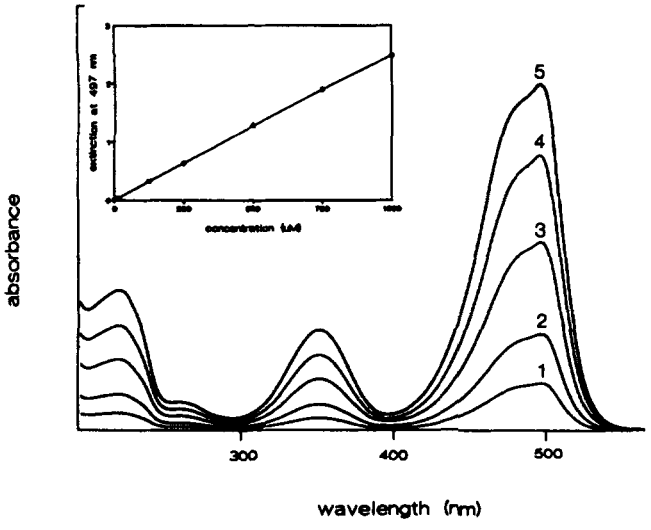


Fig. 3. Absorption spectra of different concentrations of 7β-NBD-NCT in phosphate buffer, pH 7.4. (1) 125 μM, (2) 250 μM, (3) 500 μM, (4) 750 μM, (5) 1000 μM 7β-NBD-NCT. Insert: Dependency of extinction (497 nm) on concentration of 7β-NBD-NCT.

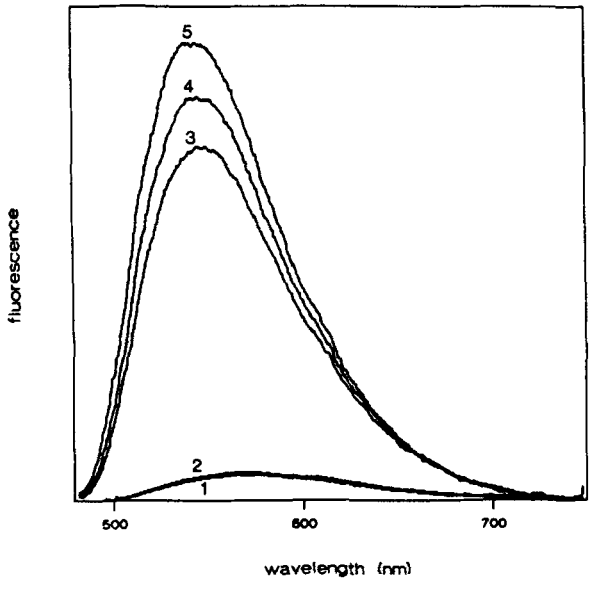


Fig. 4. Emission spectra of 7β-NBD-NCT (5 μM) in different media. (1) Water, (2) phosphate buffer, pH 7.4 (3) ethanol, (4) n-butanol, (5) n-octanol.

TABLE 4. Emission maxima and quantum yields of 7β-NBD-NCT (10 μM) in different media

Medium	Emission Maximum	Quantum Yield
	nm	
Water	567	0.025
Ethanol	545	0.312
n-Butanol	544	0.352
n-Octanol	542	0.398

The NBD-amino group, dependent on its position in the molecule, may change the structure of a bile salt to such an extent that physiological transport is affected. Because, with the exception of different uptake processes (29–32), the specificity of interactions of bile salts with binding proteins during their transcellular transport is not sufficiently known and because differences in functionally comparable processes in different cell types must be taken into consideration, a set of derivatives bearing the fluorescent group in different positions was synthesized (Fig. 1). A set of molecules bearing the fluorophor in different positions and with different orientations provides the chance to select an appropriate molecule not only for transport studies but also for physicochemical studies of micelle formation and of interaction of bile salts with biomembranes.

The syntheses of the NBD-derivatives of bile salts by coupling the fluorescent group with an amino function required first of all the synthesis of the monoamino derivatives of bile salts. Therefore, starting with cholic acid, each of the three hydroxyl groups had to be converted selectively into an amino function either in the equatorial or the axial position.

The introduction of the amino function can principally be achieved by two different reaction pathways both starting with the corresponding monooxo compounds, which

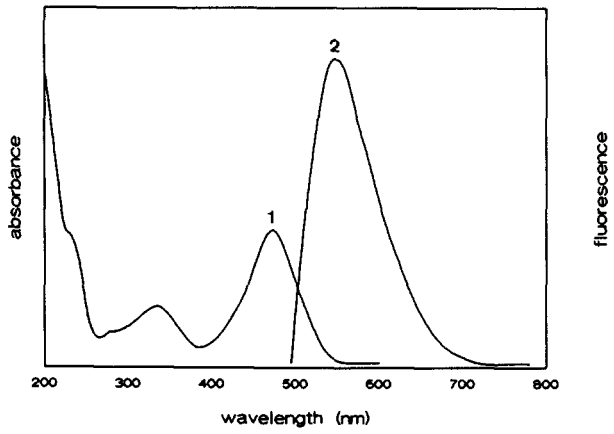


Fig. 5. Absorption and emission of 7β-NBD-NCT in ethanol. (1) Absorption spectrum, (2) emission spectrum.

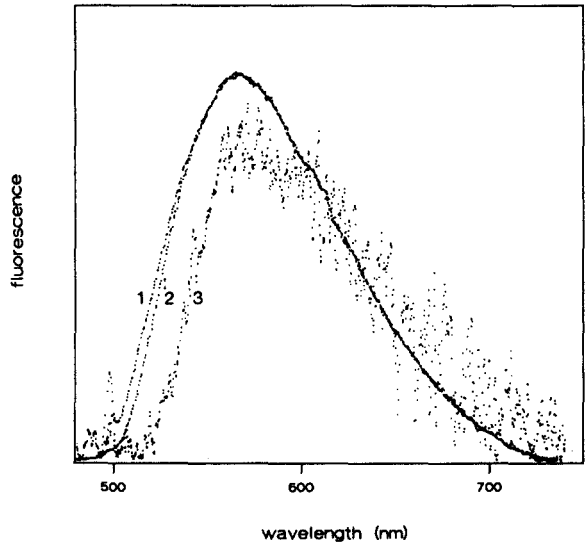


Fig. 6. Standardized emission spectra of 7β-NBD-NCT in phosphate buffer, pH 7.4. (1) 10 μM, (2) 100 μM, (3) 1 mM 7β-NBD-NCT.

can be converted into an amino function either via oxime formation followed by their reduction (Scheme 1) or by direct reductive amination. Whereas reduction of the corresponding oximes with elemental sodium could only be realized with the unconjugated compounds, the direct reductive amination of the carbonyl compounds, is useful for the synthesis of the unconjugated as well as for the taurine-conjugated derivatives. For the syntheses of taurine-conjugated amino-dihydroxy-cholanoic acids by reduction with the complex hydride, the conjugation step was performed prior to reductive amination, because the yields of taurine conjugation are higher with the oxo than with the NBD-amino derivatives.

Introduction of the amino function by both reaction sequences (Scheme 1) leads to a mixture of α- and β-diastereomers, but due to the different reaction mechanisms the α- and β-diastereomers are formed at different ratios (Table 1 and 2).

Reduction by electron transfer on the surface of elemental sodium resulted with all three dihydroxy-oximo-cholanoic acids in the preferential formation of the ther-

TABLE 5. Dependency of quantum yield on concentration of 7β-NBD-NCT in phosphate buffer, pH 7.4

Concentration	Quantum Yield
μM	
1	0.028
5	0.026
10	0.025
100	0.019
200	0.001
1000	0.00014

modynamically favored diastereomer bearing the amino function in the equatorial position. According to this, the amines predominantly formed are the 7β- and 12β-diastereomers as contrasted to the α-diastereomer in the 3-position (Table 1). Reduction of 3-oximo-coprostan by the same method resulted likewise in the preferential formation of the α-diastereomer (33).

Reductive amination using sodium cyanoborohydride as a complex hydride resulted in the predominant formation of the diastereomeric amino derivatives, the formation of which is favored by the steric requirements of the transition state. The accessibility of the 7- and 12-positions resulted in the preferential formation of the diastereomers bearing the amino function in the axial positions, whereas the 3-position is more open for an approach of the reagent from both sides. Consequently, in the 7- and 12-positions α-diastereomers are mainly formed, whereas in the 3-position α- and β-diastereomers are produced in similar amounts (Table 2).

Due to the different formation of diastereomers, reduction of the corresponding oxime with elemental sodium was chosen if the syntheses of 3α-NBD-NCT, 7β-NBD-NCT, and 12β-NBD-NCT (Table 1) were to be performed, and direct reductive amination if 3β-NBD-NCT, 7α-NBD-NCT, and 12α-NBD-NCT (Table 2) were to be synthesized.

The coupling of the amino function with NBD-Cl proceeded with the unconjugated as well as with the conjugated bile salt derivatives. For a synthesis without too many side products, the reaction conditions with regard to solvent, pH value, concentration of starting compounds, kind and concentration of the base, reaction temperature, and time were very important (33–41). It turned out that the coupling of NBD-Cl with the amino bile salt derivatives could best be performed at 25°C in dry ethanol, adjusting the apparent pH value with $NaHCO_3$ to 9.5. In order to minimize the amount of side products, a reaction time of 24 h was never exceeded, even if coupling was not complete. With these reaction conditions the formation of side products could be reduced but not completely prevented.

The NBD group acts as a protecting group for the amino function and therefore, in contrast to the unsubstituted amino-dihydroxy-cholanoic acids, their NBD derivatives can easily be conjugated with taurine. Taking advantage of this protecting function, conjugation with [^{3}H]taurine was performed resulting in radioactively labeled conjugated bile salt derivatives bearing the isotope in a metabolic stable position necessary for the investigation of biological transport processes.

A prerequisite for the investigation of bile salt transport by fluorescence microscopy with the aid of NBD-substituted bile salt derivatives is the knowledge of their optical properties. The dependency of absorption and emission on the concentration of the fluorescent compounds and on the environment of the fluorophor is of special interest.

Absorption of light by the NBD-amino bile salt derivatives in the longest wavelength absorption band with a maximum around 490 nm results in fluorescence emission with a maximum around 550 nm. As to be expected for NBD-derivatives, the positions of both the absorption and the emission maximum strongly depend on the hydrophobicity of the solvent, exhibiting an increasing hypsochromic shift with increasing hydrophobicity of the solvent.

Bile salts are amphipathic compounds and are expected to be involved in hydrophobic interactions during biological transport (2, 37–39). Therefore, the influence of the hydrophobicity of the solvent on the quantum yield of fluorescence was of prime interest. The quantum yield in aqueous solutions is very low, but increases clearly with increasing hydrophobicity of the solvent, being 16-fold higher in n-octanol than in water (Table 4). This increase in quantum yield in less polar solutions than water is a promising basis for the investigation of hydrophobic interactions of bile salts.

Quantum yield of fluorescence is not only dependent on the environment of the fluorophor but may also be influenced by its concentration. Fluorescence of solutions of NBD-amino bile salt derivatives at high concentrations is altered by a drastic loss of quantum yield (Table 5). Of the different mechanisms known to account for this loss of quantum yield, reabsorption of emitted light provides the only possible explanation. The formation of ground-state dimers can be excluded, because, even in high concentrations, the appearance of additional absorption maxima, characteristic for a formation of aggregates of the molecule, cannot be observed (Fig. 3). The formation of excited-state dimers can also be excluded, because no additional emission maximum can be observed in the fluorescence spectra (Fig. 6).

The study of transport processes needs not only variation of concentration but also variation of buffer and pH value. In a pH range that had to be taken into consideration for in vivo transport studies, no effects on the absorption and emission of all NBD-amino-dihydroxy-bile salt derivatives have been observed.

The synthesis of different diastereomeric unconjugated and taurine-conjugated fluorescent derivatives of bile salts was developed to provide new tools for the study of interactions and transport of bile salts. One of the major advantages of the synthesized NBD-amino bile salt derivatives, their environmental sensitivity, may be used for the determination of interactions of bile salts by changes either in quantum yield or in fluorescence polarization, allowing the study of binding of bile salts to distinct proteins and of their incorporation into micelles (Thom, H., Schramm, U. and Kurz, G., unpublished results). Above all, the fluorescent bile salt derivatives, provided they are

proved to be true bile salt analogues, give the chance for the visualization of biological bile salt transport by fluorescence microscopy (10, 40–42). The uncomplicated synthetic accessibility of the NBD-amino-dihydroxy-cholanoic acids and their taurine conjugates should promote their use for the investigation of a variety of biological problems.

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