Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling

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Abstract In an approach to the identification of bile salt-binding carriers, the photoactivable bile acid derivatives A) 3β-azido,7α,12α-dihydroxy-5β-cholan-24-oic acid, B) 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid, and C) 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid were synthesized in unconjugated and taurine-conjugated form. Photolysis of the 3β-azido derivatives was studied using a light source with a maximum emission at 300 nm and established a half-life time of 18.5 min. The photochemistry of the 7,7-azo derivatives was investigated using light with a maximum at 350 nm and had a half-life time of 2.2 min. The 11ξ-azido-12-oxo derivatives were photolyzed with light having a maximum at 300 nm resulting in a half-life time of 8.5 min. The suitability of the 7,7-azo derivatives for photoaffinity labeling was demonstrated by photolyses in ^{14}C-labeled methanol and acetonitrile. The generated carbene reacted with the solvents under covalent bond formation of 6 to 12%. The efficiency of all synthesized photolabile derivatives for photoaffinity labeling of bile salt binding proteins was demonstrated.—**Kramer, W., and G. Kurz.** Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling. *J. Lipid Res.* 1983. **24:** 910–923.

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Bile salts are subjected to biological recycling with the involvement of liver, terminal ileum, kidney, and blood (1–5). In liver they are taken up by a carrier-mediated active transport into the hepatocytes (6–11), in terminal ileum into distinct enterocytes (12, 13), and in kidney into the epithelial cells of the proximal tubules (14). Within the cells bile salts are subjected to intracellular transport and finally they are returned into the cycle by secretory membrane transport. In all these different transport processes as well as in their inter-organ transport by the blood, the bile salts are assumed to be carried in specific interactions with distinct proteins or carriers (15, 16). The identification of the proteins involved in such carrier-mediated transport processes takes advantage of specific ligand-receptor interactions being successful, in particular circumstances with affinity labeling and, most generally, with photoaffinity labeling.

In order to develop a means for the detection of virtually all bile salt-binding receptors, we have synthesized a series of different photolabile derivatives of bile salts and used them for the identification of carriers for bile salts in the enterohepatic circulation (16–22). In the present study the synthesis, their properties, and their suitability for photoaffinity labeling are reported. The behavior of these photolabile derivatives as analogues of the physiologically occurring bile salts in enterohepatic circulation will be described elsewhere.

EXPERIMENTAL

Materials

Cholic acid, taurine, silica gel N 60 (40–63 μm), and silica gel plates for thin-layer and high performance thin-layer chromatography were purchased from Merck (Merck Darmstadt, F.R.G.). [24-^{14}C]Cholic acid (40–60 mCi/mmol) and [2-^{3}H(N)]ethanesulfonic acid (23.15 Ci/mmol) were from New England Nuclear (Dreieich, F.R.G.). Sodium boro[^{3}H]hydride (5–10 Ci/mmol) was obtained from Amersham Radiochemical Center (Amersham, England). All other chemicals were of the best quality available from commercial sources.

Methods

Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer, Friedrichshafen, F.R.G.). Ultraviolet absorption spectra were measured with a spectrophotometer model 25 (Beckman Instruments, München, F.R.G.). Infrared spectra were recorded with a Perkin-Elmer 325 infrared spectrophotometer or an Infracord 137 (Perkin-Elmer, Friedrichshafen, F.R.G.).

^{1}H-NMR-spectra were measured on a Varian EM 390-A spectrometer (Varian GmbH, Darmstadt, F.R.G.); values are in parts per million relative to tetramethyl-

Abbreviation: TLC, thin-layer chromatography.

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silane as internal standard. Mass spectra were recorded with a Finnigan 3200 mass spectrometer connected with a data unit 6000 (Finnigan, Sunnyvale, CA, USA).

Photolyses were carried out at 30°C in a photoreactor 400 (Gräntzel, Karlsruhe, F.R.G.) equipped with quartz tubes coated with luminescent material having its maximum ultraviolet radiation at 320 nm or 350 nm, or in a Rayonet RPR 100 reactor (The Southern Ultraviolet Company, Hamden CT, USA) equipped with 16 RPR 3000 Å or 16 RPR 3500 Å lamps using the cuvettes and techniques described (17).

Bile acids and their derivatives were detected on thin-layer chromatograms by spraying the dried plates with concentrated sulfuric acid and then heating at 80°C for 5 min.

Syntheses

Solvent systems for chromatographic separations were: solvent system 1, ethyl acetate–cyclohexane–acetic acid 23:7:3 (v/v/v); solvent system 2, ethyl acetate–cyclohexane–acetic acid 100:40:1 (v/v/v); solvent system 3, ethyl acetate–cyclohexane–acetic acid 100:10:0.5 (v/v/v); and solvent system 4, n-butanol–acetic acid–water 9:2:1 (v/v/v).

3α-p-Toluenesulfonyl-7α,12α-dihydroxy-5β-cholan-24-oic acid. The 3α-tosylate of cholic acid was synthesized principally according to Barnett and Reichstein (23). In the course of 30 min, a concentrated solution of freshly recrystallized *p*-toluenesulfonyl chloride, 5 g (26 mmol), in pyridine was added with stirring at 0°C to a solution of 10 g (24.7 mmol) of cholic acid in 50 ml of absolute pyridine. The solution was allowed to warm up, stirred at room temperature for about 3 hr, and the progress of the reaction was monitored by TLC using solvent system 1. After the reaction had finished, the solution was slowly added to 2 liters of 1.5 M hydrochloric acid. The white precipitate was collected by filtration and dried in vacuo. Crude 3α-tosylate, 11.25 g (20 mmol, 80% yield), with a purity of more than 90% was obtained. The further purification of the crude product was achieved by chromatography on a 80 × 5-cm column of silica gel using solvent system 1 and yielded 10.1 g (17.9 mmol, 74.7% yield) of the pure product. MP, 131°C (decomposition); TLC: R_f = 0.86 (solvent system 1), 0.36 (solvent system 2); IR: 1730 cm^{-1} (C=0), 1165 cm^{-1}; ^{1}H-NMR ($CDCl_3$): δ = 0.66 (s, C$\underline{H}_3$-18), 0.84 (s, C$\underline{H}_3$-19), 0.96 (s, b, C$\underline{H}_3$-21), 2.25 (m, C$\underline{H}_2$-23), 2.40 (s,C$\underline{H}_3$-C_6H_4), 3.77 (s, C$\underline{H}$-7), 3.90 (s, C$\underline{H}$-12), 4.20 (m,b, C$\underline{H}$-3), 7.23 (d, J = 8 Hz, C$\underline{H}$-arom-CH_3), 7.70 (d, J = 8 Hz, C$\underline{H}$-arom-SO_2); anal. calcd. for $C_{31}H_{46}SO_7$ (562.68): C, 66.16, H, 8.24, S, 5.68; found: C, 65.68, H, 8.34, S, 5.87.

3α-p-Toluenesulfonyl-12-oxo-7α-hydroxy-5β-cholan-24-oic acid. Starting with 6.25 g (15.37 mmol) of 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid, synthesized according to Fieser and Rajagopalan (24), the aforementioned procedure yielded 6 g (10.7 mmol, 69.6% yield) of 3α-*p*-toluenesulfonyl-12-oxo-7α-hydroxy-5β-cholan-24-oic acid with a purity of about 85%. The dried crude product was dissolved in 15 ml of solvent system 1 and purified by chromatography on a 100 × 5-cm column of silica gel using the same solvent system. The fractions containing the pure product were combined and the solution was evaporated nearly to dryness. The residue was dissolved in 25 ml of dimethylformamide and this solution was slowly added to 1 liter of 1.5 M hydrochloric acid. The precipitate was collected by filtration and dried. Pure product, 3.8 g (6.76 mmol, 44% yield), was obtained as a white powder. MP, 161°C; TLC: R_f = 0.89 (solvent system 1), 0.40 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.88 (d, J = 4 Hz, C$\underline{H}_3$-21), 1.00 (s, C$\underline{H}_3$-19), 1.02 (s, C$\underline{H}_3$-18), 2.37 (m, C$\underline{H}_2$-23), 2.40 (s, C$\underline{H}_3$-C_6H_4), 3.89 (m, C$\underline{H}$-7), 4.30 (m, b, C$\underline{H}$-3), 7.17 (d, J = 8 Hz, C$\underline{H}$-arom-CH_3), 7.70 (d, J = 8 Hz, C$\underline{H}$-arom-SO_2); anal. calcd. for $C_{31}H_{44}SO_7$ (560.66): C, 66.41, H, 7.91, S, 5.70; found: C, 67.06, H, 8.32, S, 5.34.

3β-Azido-7α,12α-dihydroxy-5β-cholan-24-oic acid. Four grams (7.1 mmol) of 3α-*p*-toluenesulfonyl-7α,12α-dihydroxy-5β-cholan-24-oic acid was dissolved with stirring in a warm solution of 4 g (60.5 mmol) of sodium azide in 90 ml of dry dimethylsulfoxide and the resulting solution was kept at 100°C in the dark for 5 hr. All subsequent operations were performed in dim light. When all tosylate had disappeared as monitored by TLC using solvent system 1, the reaction mixture was poured with vigorous stirring into 2 liters of 1 M HCl. The precipitate was worked up and the dried product obtained was dissolved in solvent system 2 to yield a saturated solution. It was purified further by chromatography on a 100 × 5-cm column of silica gel using solvent system 2. The fractions were analyzed by TLC and the appropriate fractions containing the pure product were combined. The solution was concentrated by evaporation to about one-third of its original volume. After having kept the concentrated solution at −15°C for 24 hr, 300 mg of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid was obtained as white crystals. In order to gain a second crop of product, the mother liquor was evaporated to give a solid which in turn was redissolved in 20 ml of dimethylformamide. This solution was slowly added to 1 liter of 1 M HCl. The precipitate was worked up and an additional 1.5 g of pure product was obtained. Altogether 1.8 g (4.1 mmol, 58% yield) of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid was obtained. MP, 203–205°C; TLC: R_f = 0.83 (solvent sys-

tem 1), 0.29 (solvent system 2); IR: 2105 cm^{-1} (C-N_3), 1720 cm^{-1} (C=O); UV (methanol): λ_{max} = 286 nm (ϵ = 52), 223 nm (ϵ = 265); mass spectrum: m/e 405 (M^+-N_2), m/e 397 (M^+-2 × H_2O), m/e 390 (M^+-HN_3), m/e 372 (M^+-(HN_3 + H_2O)), m/e 354 (M^+-(HN_3 + 2 × H_2O)); ^{1}H-NMR (DMSO-d_6/$CDCl_3$ = 3:1): δ = 0.60 (s, CH_3-18), 0.88 (s, CH_3-19), 0.92 (d, J = 5 Hz, CH_3-21), 2.10 (m, CH_2-23), 3.60 (s,b, CH-7), 3.76 (s,b, CH-12), 3.85 (s,b, CH-3); anal. calcd. for $C_{24}H_{39}N_3O_4$ (433.58): C, 66.47, H, 9.06, N, 9.69; found: C, 66.40, H, 9.22, N, 9.20.

3β-Azido-12-oxo-7α-hydroxy-5β-cholan-24-oic acid. Starting with 6 g (10.7 mmol) of 3α-*p*-toluenesulfonyl-12-oxo-7α-hydroxy-5β-cholan-24-oic acid, the aforementioned procedure yielded, after chromatographic purification of the crude product using solvent system 2, 2.01 g (4.81 mmol, 45% yield) of 3β-azido-12-oxo-7α-hydroxy-5β-cholan-24-oic acid. MP, 125–130°C; TLC: R_f = 0.84 (solvent system 1), 0.28 (solvent system 2); IR: 2100 cm^{-1} (C-N_3), 1700 cm^{-1} (C=O); UV (methanol): λ_{max} = 285 nm (ϵ = 167), 221 nm (ϵ = 321); mass spectrum: m/e 431 (M^+), m/e 413 (M^+-H_2O), m/e 330 (M^+-side chain with m/e 101), m/e 312 (M^+-(side chain with m/e 101 + H_2O)), m/e 290 (base peak, M^+-$C_8H_{19}O_2$ with m/e 141 after rearrangement with loss of side chain and ring D); ^{1}H-NMR ($CDCl_3$): δ = 0.82 (s, CH_3-21), 1.00 (s, CH_3-18, CH_3-19), 2.27 (m, CH_2-23), 3.90 (m,b, CH-7, CH-3); anal. calcd. for $C_{24}H_{37}N_3O_4$ (431.56): C, 66.78, H, 8.64, N, 9.74; found: C, 66.68, H, 9.10, N, 9.22.

7-Oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid. 7-Oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid was synthesized according to Fieser and Rajagopalan (25) starting with 40 g (97.89 mmol) of cholic acid. After purification of the crude product in portions of 5 g by chromatography on a 100 × 5-cm column of silica gel using solvent system 1, 21.8 g (53.6 mmol, 54.7% yield) of 7-oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid was obtained. Despite the fact that the desired 7-oxo derivative and six to seven other products were in the crude product, it could be used without further chromatographic purification for the synthesis of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid. MP, 198°C (Lit.: 200°C); TLC: R_f = 0.53 (solvent system 1), 0.08 (solvent system 2); UV (methanol): λ_{max} = 267 nm (ϵ = 44), 220 nm (ϵ = 57); ^{1}H-NMR (DMSO-d_6): δ = 0.61 (s, CH_3-18), 0.93 (s,b, CH_3-19, CH_3-21), 2.11 (m, CH_2-23), 2.80 (dd, $J_{8/14 \text{ or } 9}$ = 12 Hz and 6 Hz, 1 proton), 3.30 (m,b, CH-3), 3.74 (m, CH-12); anal. calcd. for $C_{24}H_{38}O_5$ (406.58): C, 70.90, H, 9.42; found: C, 70.65, H, 9.70.

7,7-Azo-3α,12α-dihydroxy-5β-cholan-24-oic acid. One gram (2.46 mmol) of 7-oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid was dissolved in 20 ml of dry methanol and dry ammonia was bubbled through the solution at 0°C for 2 hr. Then a solution of 1 g (9 mmol) of hydroxylamine-O-sulfonic acid in 10 ml of dry methanol was added at 0°C during 20 min with stirring. All subsequent operations were performed in dim light. The mixture was allowed to warm up to room temperature, stirred for 6–12 hr and filtered. After addition of 1 ml of triethylamine, the solution was evaporated to dryness. The residue was dissolved in 20 ml of dry methanol and after addition of 1 ml of triethylamine a solution of 1 g (4 mmol) of iodine in 10 ml of methanol was added at room temperature in the dark in the course of 20 min. After the excess of iodine was reduced by the addition of solid sodium dithionite, the slightly yellow solution was slowly added to 300 ml of 1 M hydrochloric acid with concomitant stirring. The precipitate was worked up and the crude product was purified either by chromatography on a 100 × 5-cm column of silica gel using solvent system 1, or by flash chromatography (26) on a 30 × 3-cm column with solvent system 2 as eluant. The fractions containing the pure product were combined and evaporated to dryness. The residue was dissolved in 20 ml of dimethylsulfoxide and the solution was slowly added to 300 ml of 1 M HCl with stirring. The precipitate yielded 450 mg (1.07 mmol, 43% yield) of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid. MP, 135–140°C (decomposition); TLC: R_f = 0.71 (solvent system 1), 0.18 (solvent system 2); IR: 1710 cm^{-1} (C=O), 1570 cm^{-1} (C-N_2); UV (methanol): λ_{max} = 367 nm (ϵ = 52), 350 nm (ϵ = 57), 260 nm (ϵ = 146), 232 nm (ϵ = 239); mass spectrum: m/e 372 (M^+-(N_2 + H_2O)), m/e 354 (M^+-(N_2 + 2 × H_2O)), m/e 271 (M^+-(N_2 + H_2O + side chain with m/e 101)), m/e 253 (M^+-(N_2 + 2 × H_2O + side chain with m/e 101), base peak); ^{1}H-NMR (DMSO-d_6): δ = 0.55 (s, CH_3-18), 0.95 (s,b, CH_3-19, CH_3-21), 2.10 (m, CH_2-23), 3.30 (m,b, CH-3), 3.73 (m, CH-12); anal. calcd. for $C_{24}H_{38}N_2O_4$ (418.56): C, 68.86, H, 9.15, N, 6.69; found: C, 68.11, H, 8.73, N, 6.32.

3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid methylester. 3α,7α,12α-Trihydroxy-5β-cholan-24-oic acid methyl ester was prepared according to Grand and Reichstein (27) with a yield of 80% after recrystallization. MP, 150°C (Lit.: 142°C); TLC: R_f = 0.74 (solvent system 1), 0.06 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.68 (s, CH_3-18), 0.88 (s, CH_3-19), 1.00 (d, J = 4 Hz, CH_3-21), 2.28 (m, CH_2-23), 3.35 (m,b, CH-3), 3.62 (s, CH_3-OCO-), 3.79 (s, CH-7), 3.89 (s, CH-12); anal. calcd. for $C_{25}H_{42}O_5$ (422.58): C, 71.05, H, 10.01; found: C, 70.91, H, 10.32.

3α,7α-Diacetoxy-12α-hydroxy-5β-cholan-24-oic acid methylester. 3α,7α-Diacetoxy-12α-hydroxy-5β-cholan-24-oic acid methylester was prepared according to Fieser and

Rajagopalan (24) with a yield of 55% after recrystallization. MP, 176–178°C (Lit.: 181–183°C); TLC: R_f = 0.87 (solvent system 1), 0.60 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.68 (s, C$\underline{H}_3$-18), 0.96 (s, C$\underline{H}_3$-19), 1.00 (d, J = 4 Hz, C$\underline{H}_3$-21), 2.05, 2.10 (2 × C$\underline{H}_3$-CO at C-3 and C-7), 2.25 (m, C$\underline{H}_2$-23), 3.65 (s, C$\underline{H}_3$-OCO-), 3.95 (m, C$\underline{H}$-12), 4.59 (m,b, C$\underline{H}$-3), 4.85 (m, C$\underline{H}$-7); anal. calcd. for $C_{29}H_{46}O_7$ (506.66): C, 68.74, H, 9.15; found: C, 68.64, H, 9.35.

12-Oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid methylester. 12-Oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid methylester was prepared according to Fieser and Rajagopalan (24) with a yield of 99%. MP, 174–177°C (Lit.: 179–182°C); TLC: R_f = 0.90 (solvent system 1), 0.64 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.87 (d, J = 5 Hz, C$\underline{H}_3$-21), 1.05 (s, C$\underline{H}_3$-18, C$\underline{H}_3$-19), 2.01 (s, C$\underline{H}_3$CO- at C-3 and C-7), 2.30 (m, C$\underline{H}_2$-23), 3.60 (s, C$\underline{H}_3$-OCO-), 4.50 (m,b, C$\underline{H}$-3), 4.92 (m, C$\underline{H}$-7); anal. calcd. for $C_{29}H_{44}O_7$ (504.64): C, 69.02, H, 8.79; found: C, 68.81, H, 9.02.

12-Oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid. Fifteen grams (29.7 mmol) of 12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid methylester was added in portions of 1–2 g to 750 ml of boiling 2 M aqueous NaOH solution. Boiling was continued until all solid was dissolved. After cooling, the yellow solution was filtered and the filtrate was diluted with water to about 2 liters. The bile acid was precipitated by slow addition of 400 ml of 5 M HCl with concomitant stirring. The crystalline precipitate yielded 11 g (27.3 mmol, 92% yield) of 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid. MP, 217°C (Lit.: 219°C); TLC: R_f = 0.53 (solvent system 1), 0.08 (solvent system 2); UV (methanol): λ_{max} = 287 nm (ϵ = 41), 220 nm (ϵ = 42); ^{1}H-NMR (DMSO-d_6): δ = 0.76 (d, J = 5 Hz, C$\underline{H}_3$-21), 0.94, 0.96 (2 × s, C$\underline{H}_3$-18, C$\underline{H}_3$-19), 2.16 (m, C$\underline{H}_2$-23), 3.25 (m,b, C$\underline{H}$-3), 3.70 (m, C$\underline{H}$-7); anal. calcd. for $C_{24}H_{38}O_5$ (406.54): C, 70.90, H, 9.42; found: C, 69.88, H, 9.87.

11ξ-Bromo-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid. Five grams (12.3 mmol) of 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid was dissolved in 50 ml of acetic acid and after addition of 50 μl of 47% aqueous hydrobromic acid, the solution was heated to 60°C with stirring. After about 2 hr, the 3α,7α-diacetate was completely formed, as monitored by TLC using solvent system 3. Subsequently, 2.16 g (13.5 mmol, 700 μl) of bromine dissolved in 15 ml of acetic acid was added in the course of 30 min. Stirring at 60°C was continued for about 6 hr and then the red reaction mixture was carefully poured into 500 ml of water. The yellowish precipitate was worked up and the crude product was purified by chromatography on a 100 × 5-cm column of silica gel using solvent system 2. The fractions containing the desired product were pooled and evaporated to dryness. The resulting residue was dissolved in 20 ml of dimethylformamide and the solution was slowly added to 1 liter of 1 M HCl. The precipitate yielded 4.55 g (7.99 mmol, 65% yield) of 11ξ-bromo-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid. MP, 188–196°C; TLC: R_f = 0.87 (solvent system 1), 0.40 (solvent system 2); IR: 1720 cm^{-1} (C=O), 1240 cm^{-1}; UV (methanol): λ_{max} = 260 nm (ϵ = 606), 225 nm (ϵ = 896); ^{1}H-NMR (DMSO-d_6/$CDCl_3$ = 3:1): δ = 0.76 (d, J = 5 Hz, C$\underline{H}_3$-21), 0.97 (s, C$\underline{H}_3$-19), 1.20 (s, C$\underline{H}_3$-18), 1.91 (s, C$\underline{H}_3$CO- at C-3), 1.94 (s, C$\underline{H}_3$CO- at C-7), 2.15 (m, C$\underline{H}_2$-23), 4.45 (m,b, C$\underline{H}$-3), 4.79 (m, C$\underline{H}$-7), 5.29 (d, J = 10.5 Hz, C$\underline{H}$Br at C-11); anal. calcd. for $C_{28}H_{41}BrO_7$ (569.61): C, 59.03, H, 7.25, Br, 14.04; found: C, 58.86, H, 7.48, Br, 14.82.

11ξ-Azido-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid. Two and one-half grams (4.38 mmol) of 11ξ-bromo-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid was dissolved in 25 ml of dry dimethylsulfoxide containing 250 μl of acetic acid. All subsequent operations were performed in dim light. This solution was added to a solution of 3.5 g (46 mmol) of sodium azide in 40 ml of dry dimethylsulfoxide and the reaction mixture was kept at exactly 70°C for 8–10 hr. The progress of the reaction was monitored by TLC using solvent system 3. After completion, the brown reaction mixture was slowly poured into 2 liters of 1.5 M HCl with vigorous stirring. The precipitated and dried crude product was purified by chromatography on a 120 × 3-cm column of silica gel using solvent system 2. The appropriate fractions were combined and worked up as usual. Nine hundred and fifty milligrams (1.78 mmol, 40.6% yield) of 11ξ-azido-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid was obtained as a slightly yellowish powder. MP, 196–202°C; TLC: R_f = 0.87 (solvent system 1), 0.43 (solvent system 2); IR: 2105 cm^{-1} (C-N_3), 1705 cm^{-1} (C=O), 1245 cm^{-1}; UV (methanol): λ_{max} = 283 nm (ϵ = 948), 225 nm (ϵ = 1113); ^{1}H-NMR (DMSO-d_6/$CDCl_3$ = 3:1): δ = 0.78 (d, J = 5 Hz, C$\underline{H}_3$-21), 0.98 (s, C$\underline{H}_3$-19), 1.13 (s, C$\underline{H}_3$-18), 1.92 (s, C$\underline{H}_3$CO- at C-3), 1.95 (s, C$\underline{H}_3$CO- at C-7), 2.12. (m, C$\underline{H}_2$-23), 4.40 (m, 2 protons, C$\underline{H}$-3 and C$\underline{H}N_3$ at C-11), 4.79 (m, C$\underline{H}$-7); anal. calcd. for $C_{28}H_{41}N_3O_7$ (531.64): C, 63.25, H, 7.77, N, 7.90; found: C, 63.33, H, 8.15, N, 7.54.

11ξ-Azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid. Two grams (3.76 mmol) of 11ξ-azido-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid was dissolved in 20 ml of methanol and after addition of 4 ml of a 1 M methanolic solution of KOH the solution was kept at 20°C for about 100 hr. The product was precipitated by pouring the reaction mixture into 2 liters of 1.5 M HCl and finally purified by chromatography on a 120 × 3-cm column

of silica gel using solvent system 1. After the usual workup, 200 mg (0.45 mmol, 11.9% yield) of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid was obtained. MP, 135–138°C (decomposition); TLC: R_f = 0.57 (solvent system 1), 0.11 (solvent system 2); IR: 2105 cm^{-1} (C-N_3), 1710 cm^{-1} (C=O); UV (methanol): λ_{max} = 288 nm (ϵ = 1237), 226 nm (ϵ = 1134); mass spectrum: m/e 447 (M^+), m/e 419 (M^+-N_2), m/e 404 (M^+-HN_3), m/e 401 (M^+-(N_2 + H_2O)), m/e 386 (M^+-(HN_3 + H_2O)), m/e 368 (M^+-(HN_3 + 2 × H_2O)), m/e 318 (M^+-(N_2 + side chain with m/e 101)), m/e 300 (M^+-(N_2 + H_2O + side chain with m/e 101)); ^{1}H-NMR (DMSO-d_6/$CDCl_3$ = 3:1): δ = 0.80 (d, J = 5 Hz, CH_3-21), 1.00 (s, CH_3-19), 1.08 (s, CH_3-18), 2.13 (m, CH_2-23), 3.30 (m, CH-3), 3.69 (m, CH-7), 4.23 (m, C$H$$N_3$ at C-11); anal. calcd. for $C_{24}H_{37}N_3O_5$ (447.56): C, 64.64, H, 8.33, N, 9.39; found: C, 64.34, H, 8.37, N, 9.08.

Synthesis of taurine-conjugated bile salt derivatives

The respective unconjugated bile salt derivative (3.45 mmol) was dissolved in 5 ml of dry dioxane. After addition of 825 μl (3.45 mmol) of tri-n-butylamine, the solution was cooled to 15°C and 375 μl (3.45 mmol) of chloroethylcarbonate was added. The mixture was stirred at 15°C for 30 min and then a solution of 475 mg (3.8 mmol) of taurine in 3.8 ml of 1 M aqueous NaOH was added with vigorous stirring. Stirring was continued for 5–6 hr and the completion of the reaction was established by TLC using solvent system 4. Subsequently the reaction mixture was diluted with 30 ml of water. The pH was adjusted to pH 2 and unconjugated bile acids were extracted three times with 40 ml of ether each. From the organic phase the unconjugated bile acid derivative was recovered by evaporation. The aqueous phase was adjusted to pH 8 with 1 M NaOH and evaporated to dryness at 35°C. The residue was dissolved in 20 ml of warm dry ethanol and filtered to separate inorganic salt and taurine. The ethanolic solution of the taurine-conjugated bile acids was refluxed and ether was added until a faint turbidity occurred. After several days at 0°C a part of the taurine conjugate crystallized. The main portion, however, was obtained after evaporation of the solvent, redissolving the residue in 200 ml of water, and removal of the water by lyophilization. The total yields of the conjugated bile acid derivatives were between 60 and 65%.

Synthesis of radioactively labeled bile salt derivatives

General methods: all solvents were removed under a stream of nitrogen at 25°C bath temperature; all synthesized radioactive compounds were stored at −20°C dissolved in ethanol.

Synthesis of radioactively labeled bile acid derivatives

3β-Azido-7α,12α-dihydroxy-5β[12β-^{3}H]cholan-24-oic acid. 3β-Azido-12-oxo-7α-hydroxy-5β-cholan-24-oic acid (4.33 mg, 10 μmol) was dissolved in 150 μl of methanol and 30 μl of water. An ampoule with 100 mCi of sodium boro[^{3}H]hydride (5–10 Ci/mmol) was opened and the solution of the bile acid was added. After 6–8 hr, 50 μl of 2 M HCl was added to destroy excess borohydride. After 2–3 hr, the reaction mixture was put on three high performance TLC plates (20 × 10 cm) and the chromatograms were developed in solvent system 2 with a migration distance of the solvent of 16–18 cm. The plates were dried in vacuo and radioactivity distribution was determined with the aid of a radiochromatogram scanner (TLC-Analyzer LB 2820, Berthold, Wildbad, F.R.G.). Two main peaks could be detected: R_f = 0.21: 3β-azido-7α,12α-dihydroxy-5β[12β-^{3}H]cholan-24-oic acid and R_f = 0.33; 3β-azido-7α,12β-dihydroxy-5β[12α-^{3}H]cholan-24-oic acid. The ratio of 12α-hydroxy to 12β-hydroxy-compound was about 65:35. Two further compounds in low amounts could also be identified: R_f = 0.46: 3β-azido-7α,12α-dihydroxy-5β[12β-^{3}H]cholan-24-oic acid methylester and R_f = 0.63: 3β-azido-7α,12β-dihydroxy-5β[12α-^{3}H]cholan-24-oic acid methyl ester. The plates were cautiously sprayed with water to prevent dusting and the bands containing radioactivity were scraped off. The compounds were extracted from silica gel with methanol (10–20 times with 700 μl of methanol). More than 95% of the labeled bile acids could be extracted from the silica gel with this procedure. The yield of 3β-azido-7α,12α-dihydroxy-5β[12β-^{3}H]cholan-24-oic acid was 5–10 mCi (1.25–2.5 Ci/mmol). The compounds were stored in methanolic solution (0.5–1 μCi/μl) at −20°C in the dark. Prior to photoaffinity labeling experiments, the solution containing the desired amount of radioactivity was evaporated to dryness in a desiccator at 60 torr and the residue was dissolved in an appropriate buffer.

7,7-Azo-3α,12α-dihydroxy-5β[3β,12β-^{3}H]cholan-24-oic acid. 7,7-Azo-3α,12α-dihydroxy-5β-cholan-24-oic acid (1.46 g, 3.5 mmol) was dissolved in 40 ml of 80% aqueous acetone. After addition of 1.25 g (7 mmol) of N-bromosuccinimide, the yellow solution was stirred at room temperature overnight. The resulting solution was concentrated by evaporation to about 10 ml and worked up as usual. By flash chromatography (26) of the crude product on a 30 × 3-cm column of silica gel using solvent system 2, two pure diazirine derivatives could be isolated: 870 mg (2.1 mmol, 60.3% yield) of 7,7-azo-3-oxo-12-oxo-5β-cholan-24-oic acid and 218 mg (0.525 mmol, 15% yield) of 7,7-azo-3-oxo-12α-hydroxy-5β-cholan-24-oic acid. 7,7-Azo-3-oxo-12-oxo-5β-cho-

lan-24-oic acid (4.14 mg, 10 μmol) was dissolved in 150 μl of methanol and 30 μl of water and reduced with 100 mCi (7.1 Ci/mmol) of sodium boro[^{3}H]hydride as described for 3β-azido-7α,12α-dihydroxy-5β[12β-^{3}H]cholan-24-oic acid. After separation of the radioactive bile acid derivatives by TLC using high performance TLC plates with solvent system 2, two labeled bands could be detected: R_f = 0.30: 7,7-azo-3α,12α-dihydroxy-5β[3β,12β-^{3}H]cholan-24-oic acid and R_f = 0.38: 7,7-azo-3α,12β-dihydroxy-5β[3β,12α-^{3}H]-cholan-24-oic acid. The ratio of 12α-hydroxy to 12β-hydroxy-compound was 65:35. The isolation procedure was identical as described above, yielding 5–10 mCi of 7,7-azo-3α,12α-dihydroxy-5β[3β,12β-^{3}H]cholan-24-oic acid (3.55 Ci/mmol).

3α,7α,12α-Trihydroxy-5β[24-^{14}C]cholan-24-oic acid methyl ester. One hundred microcuries (50 mCi/mmol) of [24-^{14}C]cholic acid was dissolved in 200 μl of methanol that had been treated for 5 min with dry gaseous HCl. After 24 hr all cholic acid had been converted to the methyl ester as monitored by radiochromatogram scanning (about 100% yield).

3α,7α-Diacetoxy-12α-hydroxy-5β[24-^{14}C]cholan-24-oic acid methyl ester. One hundred microcuries (50 mCi/mmol) of [24-^{14}C]cholic acid methyl ester was dissolved in 100 μl of dry dioxane. After addition of 50 μl of pyridine–acetic acid 1:1 (v/v), the solution was kept at 20°C for 48 hr. Radiochromatography and cochromatography with unlabeled compounds showed complete reaction to the desired product (about 100% yield).

12-Oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid methyl ester. One hundred microcuries (50 mCi/mmol) of 3α,7α-diacetoxy-12α-hydroxy-5β[24-^{14}C]cholan-24-oic acid methyl ester was dissolved in 200 μl of acetic acid and then 5 μl of an aqueous solution of potassium chromate (2.6 mg K_2CrO_4) was added. The mixture was shaken for 2 hr and then kept at 25°C for 24 hr. After addition of 500 μl of water, the bile acid was extracted with four portions of 400 μl of ether and isolated (69% yield).

12-Oxo-3α,7α-dihydroxy-5β[24-^{14}C]cholan-24-oic acid. Seventy five microcuries (50 mCi/mmol) of 12-oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid methyl ester was dissolved in 50 μl of tetrahydrofuran and 200 μl of 2 M aqueous NaOH was added. The mixture was kept at 80°C for 90 min. After cooling, 0.85 ml of 1.5 M HCl was added and the bile acid was extracted with four portions of 400 μl of ether and isolated (85% yield).

11ξ-Bromo-12-oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid. Eighty microcuries (50 mCi/mmol) of 12-oxo-3α,7α-dihydroxy-5β[24-^{14}C]cholan-24-oic acid was dissolved in 50 μl of acetic acid. Five microliters of aqueous HBr (47%) and 65 μl of a solution containing 10 μl of bromine in 500 μl of acetic acid were added and the solution was kept at 50°C for 1 hr. After dilution with 600 μl of water, excess bromine was reduced by addition of 20 μl of a 1 M aqueous hyposulfite solution. Extraction of the bile acid with four portions of 400 μl of ether and evaporation of the ether yielded a crude product composed of four to six compounds. Purification was achieved by TLC on high performance TLC plates with solvent system 2 (68% yield).

11ξ-Azido-12-oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid. Fifty five microcuries (50 mCi/mmol) of 11ξ-bromo-12-oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid was dissolved in 50 μl of dry dimethylsulfoxide. After addition of 50 μl of a solution of 36.5 mg of sodium azide in 1 ml of dimethylsulfoxide, the mixture was kept in the dark at 70°C for 6 hr. After cooling, 250 μl of 1 M HCl was added and the bile acid was extracted with four portions of 400 μl of ether. After evaporation of the ether, the residue was redissolved in 50 μl of ethanol and the compound was purified by TLC using solvent system 2. The band with the desired azido-compound was scraped off and the bile acid was extracted from the silica gel with methanol (75% yield).

11ξ-Azido-12-oxo-3α,7α-dihydroxy-5β[24-^{14}C]cholan-24-oic acid. Forty one microcuries (50 mCi/mmol) of 11ξ-azido-12-oxo-3α,7α-diacetoxy-5β[24-^{14}C]cholan-24-oic acid was dissolved in 100 μl of a 0.5 M methanolic solution of KOH and kept at 25°C for about 100 hr with regular monitoring by TLC. After complete deacetylation, 250 μl of 1 M HCl was added and the bile acids were extracted with four portions of 400 μl of ether. After evaporation of the ether, the residue was redissolved in ethanol and the compound was purified by TLC in solvent system 1 (13–18% yield).

Synthesis of radioactively labeled taurine-conjugated bile acids. Two hundred fifty microcuries (23.15 Ci/mmol) of [2-^{3}H(N)]taurine was dissolved in 20 μl of 10 mM aqueous NaOH and this solution was shaken for 25 min. After centrifugation, 25 μl of dioxane was added. The corresponding unconjugated bile acid derivative (2.5 mg) was dissolved in 15 μl of freshly distilled dry dioxane. This solution was mixed with 5 μl of a solution of 10 μl of tri-n-butylamine in 20 μl of dioxane. After 30 min at 20°C, 5 μl of a solution of 10 μl of chloroethylcarbonate in 50 μl of dioxane was added and the solution was kept at 20°C for 2 hr. Subsequently, 0.6 μl of this solution was transferred to 5 μl of dry dioxane, and to this solution of the mixed anhydride the solution of the radioactive taurine was added. After 12 hr the reaction mixture was applied onto a high performance TLC plate and separated using solvent system 4. The labeled compound was isolated as described above (75–90% yield).

Alternatively the labeled taurine-conjugated bile ac-

ids were separated by electrophoresis at 1600 V and 10 mA and 10°C on Whatman 3 MM paper (40 × 4.5 cm) in the course of 2 hr using 15% acetic acid–5% formic acid as buffer (pH 1.9). The band containing the radioactive derivative was cut out and the bile acid was eluted with 1.5–5 ml of water (60–85% yield).

RESULTS

Syntheses

The synthesis of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid (**Fig. 1A**) takes advantage of the selective tosylation of cholic acid in pyridine (23). In order to avoid its partial hydrolysis, the tosylate was converted into the 3β-azido-derivative with sodium azide in absolute dimethylsulfoxide instead of dimethylformamide–water solutions.

For the synthesis of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid (Fig. 1B), the starting material 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid methylester was obtained according to Fieser and Rajagopalan (24). Hydrolysis of the 12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid methyl ester was carried out by addition of the solid compound in small portions to a boiling 2 M aqueous solution of NaOH. The saponification of the ester should not be performed in alcoholic solutions of NaOH or KOH because destruction of oxo-derivatives of bile salts may occur (28). In aqueous solutions the saponification of 12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid methyl ester leads to the wanted oxo-compound in a pure form, whereas use

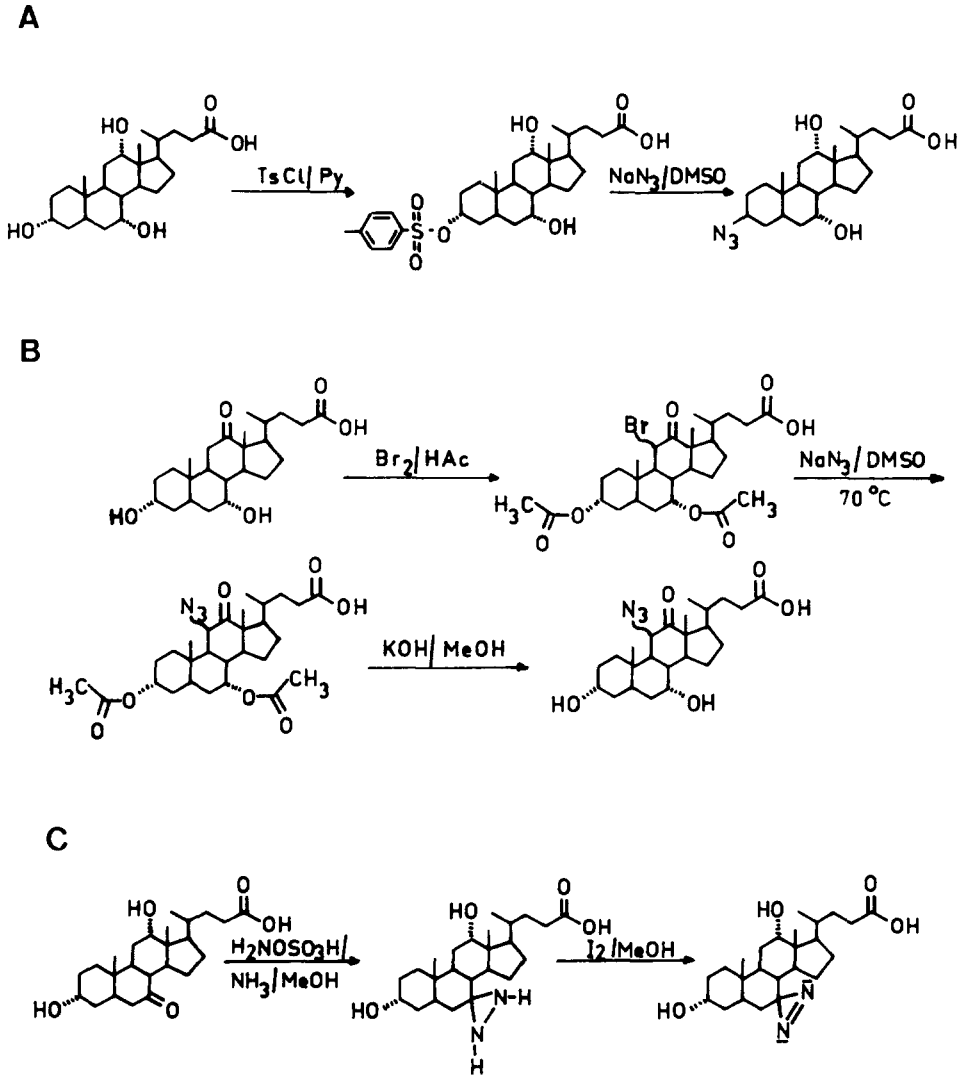

Fig. 1. Reaction scheme for the synthesis of photolabile derivatives of bile acids. A, 3β-Azido-7α,12α-dihydroxy-5β-cholan-24-oic acid; B, 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid; C, 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid.

of methanolic KOH resulted in the formation of about 30% unidentified nonketone products. Bromination of the 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid was performed using the conditions of Riegel and McIntosh (29), which resulted in the acetylation of the hydroxyl groups at positions 3 and 7. The bromination leads to a mixture of the 11α- and 11β-bromo-derivatives of about 2:1 (30). The subsequent formation of the 11ξ-azido-12-oxo-3α,7α-diacetoxy-5β-cholan-24-oic acid was found to be very sensitive to reaction temperature. High yields were obtained by performing the reaction in absolute dimethylsulfoxide at exactly 70°C for 4–6 hr. Higher temperature caused formation of the α,β-unsaturated ketone by elimination of hydrogen bromide, presumably from the 11β-isomer (30, 31). Subsequent deacetylation by KOH in methanolic solution yielded a crude product, from which the 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid must be isolated by column chromatography.

For the preparation of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid (Fig. 1C) selective oxidation of the hydroxyl group in position 7 of cholic acid was achieved by treatment of cholic acid in aqueous solution of $NaHCO_3$ with 1.25 equivalents of N-bromosuccinimide (25). Under these conditions about 50–65% of the desired 7-oxo-3α,12α-dihydroxy-5β-cholan-24-oic acid was formed. For the following synthesis of the 7-diazirine, the procedure of Church et al. was used (32, 33).

The synthesis of taurine conjugates of bile acid derivatives was performed using a modified procedure of the mixed anhydride method of Norman (34). It is worth mentioning that the mixed anhydride method, when applied for conjugation of bile acids at a μg-scale as necessary for the synthesis of radioactively labeled conjugates, leads to the formation of N-ethoxycarbonyl-2-aminoethanesulfonic acid (**Fig. 2**). Such side reactions were previously encountered in peptide synthesis (35).

Synthesis of radioactively labeled photolabile bile acids

For photoaffinity labeling, especially for experiments with isolated intact cells, relatively large amounts of photolabile derivatives with high specific radioactivities are desired. Bile acid derivatives with high specific radioactivity were obtained either by reduction of appropriate oxo-derivatives of unconjugated and conjugated bile acids by sodium boro[^{3}H]hydride or by introduction of labeled taurine into unconjugated compounds.

The synthesis of tritium-labeled 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid was started from 12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid, obtained according to Fieser and Rajagopalan (24). Introduction of the azido group in 3-position was performed by procedures paralleling those for the corresponding 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid (Fig. 1A). Reduction of the 3β-azido-12-oxo-7α-hydroxy-5β-cholan-24-oic acid with [^{3}H]$NaBH_4$ (5–10 Ci/mmol) in a methanol–water mixture resulted in the formation of about 60% 3β-azido-7α,12α-dihydroxy-5β[12β-^{3}H]-cholan-24-oic acid and about 40% of the diastereomeric 3β-azido-7α,12β-dihydroxy-5β[12α-^{3}H]cholan-24-oic acid, which were separated by TLC.

For the synthesis of the tritium-labeled 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid, the unlabeled compound was oxidized with N-bromosuccinimide, yielding the products 7,7-azo-3-oxo-12α-hydroxy-5β-cholan-24-oic acid and 7,7-azo-3-oxo-12-oxo-5β-cholan-24-oic acid, which were easily separable by column chromatography. The molar ratio of both products depends upon the molar excess of N-bromosuccinimide related

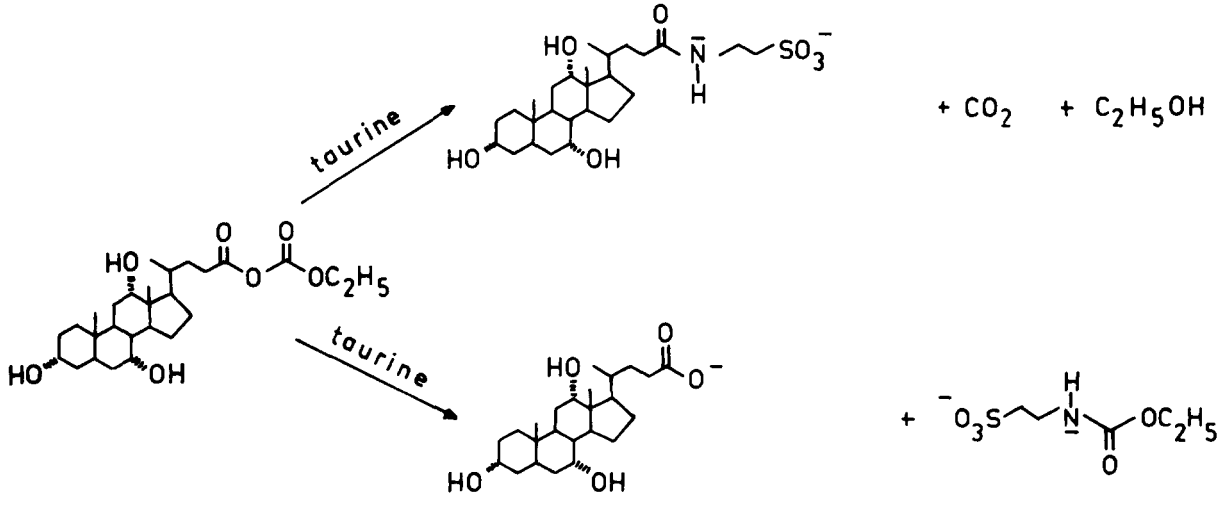

Fig. 2. Reaction scheme for reactions of bile acid mixed anhydrides with taurine.

to the bile acid.[2] At a molar ratio of 1:1.5, about 80% of the 3-oxo-compound and about 20% of the 3-oxo-12-oxo-compound were formed; whereas at a molar ratio of 1:2, about 80% of the 7,7-azo-3-oxo-12-oxo-5β-cholan-24-oic acid was formed. In order to achieve very high specific radioactivities in the desired 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid, we preferred the use of the dioxo-compound for reduction with [^{3}H]$NaBH_4$. The diastereomeric bile acid derivatives obtained by reduction of the corresponding oxo-compounds with [^{3}H]$NaBH_4$ were separated by TLC.

Because of the reductive selectivity and high specific radioactivity of [^{3}H]$NaBH_4$ (5–10 Ci/mmol), the labeling procedure described above was also applied to the synthesis of radioactively labeled taurine-conjugated photolabile bile acids. The azido- or azo-derivatives of a conjugated bile acid were usually oxidized by 1.5 equivalents of N-bromosuccinimide in 80% aqueous acetone and the resulting oxo-compounds separated by column chromatography were reduced with [^{3}H]$NaBH_4$.

For many investigations of bile salt transport and metabolism, the use of conjugated derivatives bearing the radioactive label in the conjugating amino acid is necessary. Therefore, the taurine conjugates of all photolabile derivatives of bile acids were synthesized using ^{3}H-labeled taurine (20–40 Ci/mmol). Complete separation of the labeled taurine conjugates was performed either by paper electrophoresis at pH 1.9 or by high performance TLC.

Mass spectroscopy

The synthesized photolabile bile acid derivatives were characterized by UV-, IR-, and NMR-spectroscopy and their identification was ascertained by mass spectroscopy. Without being transformed into more volatile derivatives, the compounds under consideration were subjected to mass spectroscopy. The mass spectrum of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid yielded the molecular ion at m/e 447. A characteristic peak at m/e 419 is explained by elimination of N_2 and the fragment m/e 404, presumably by loss of HN_3.

The mass spectra of both other photolabile derivatives exhibited no molecular ions. The fragmentation pattern of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid is interpreted by the elimination of H_2O resulting in the fragments m/e 415 and 397 and by loss of HN_3 leading to the fragment m/e 390. The fragmentation pattern of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid shows a small intensity peak at m/e 388, presumably resulting by loss of N_2H_2, whereas the loss of N_2 and H_2O molecules led to the fragmentation products at m/e 372 and 354.

Duration of photolysis

In order to evaluate the time necessary for photoaffinity labeling, the photolabile bile acid derivatives were irradiated and the time dependency of photolysis was followed spectrophotometrically. The photochemical conditions for photolysis were such that they could be applied to experiments with biological systems. Photoaffinity labeling of biological material should be carried out using light at wavelengths greater than 300 nm to minimize photolytical destruction. Therefore, the azido-derivatives were photolyzed with the aid of a Rayonet photochemical reactor RPR-100 equipped with 300-nm lamps (RPR-3000 Å) and the 7,7-azo-derivatives were photolyzed with a photoreactor 400 (Gräntzel, Karlsruhe, F.R.G.) using quartz tubes coated with luminescent material having its maximum of ultraviolet radiation at 350 nm, or with a Rayonet RPR-100 photochemical reactor equipped with 3500-Å lamps (17). With both photochemical reactors a filter tube 5 mm thick of Duran 50 glass was used to cut off the light emitted at wavelengths lower than 305 nm. Because further experiments with intact cells must be performed under aerobic conditions, no efforts were made to exclude the presence of oxygen.

The photolysis of the azido-derivatives of bile acids cannot be followed by ultraviolet spectroscopy because some of the predominant products absorb in the same wavelength range as the photolabile compounds. Therefore, photolysis of these azido-derivatives was monitored by infrared spectroscopy, taking advantage of the infrared absorption of the azido-group at about 2100 cm^{-1}. The time dependency of photolysis of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid under the stated conditions is demonstrated in **Fig. 3.** The disappearance of the absorption at 2100 cm^{-1} obeyed first order kinetics (insert in Fig. 3) and the half-life time of photolysis of the 11ξ-azido-12-oxo-derivative has been determined to be 8.5 min. The decrease of the absorption at 2100 cm^{-1} of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid caused by photolysis likewise followed first order kinetics (**Fig. 4**) with a half-life time of 18.5 min (insert of Fig. 4). As expected, the half-life time of the 3β-azido-derivatives is longer than that of the 11ξ-azido-12-oxo-derivatives.

Time dependency of photolysis of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid could be followed by ultraviolet absorption spectroscopy (**Fig. 5**). Photolysis of the 7,7-azo-derivative followed under the conditions used first order kinetics with a half-life time of 2.2 min (insert of Fig. 5).

[2] Giese, U., and G. Kurz. Unpublished results.

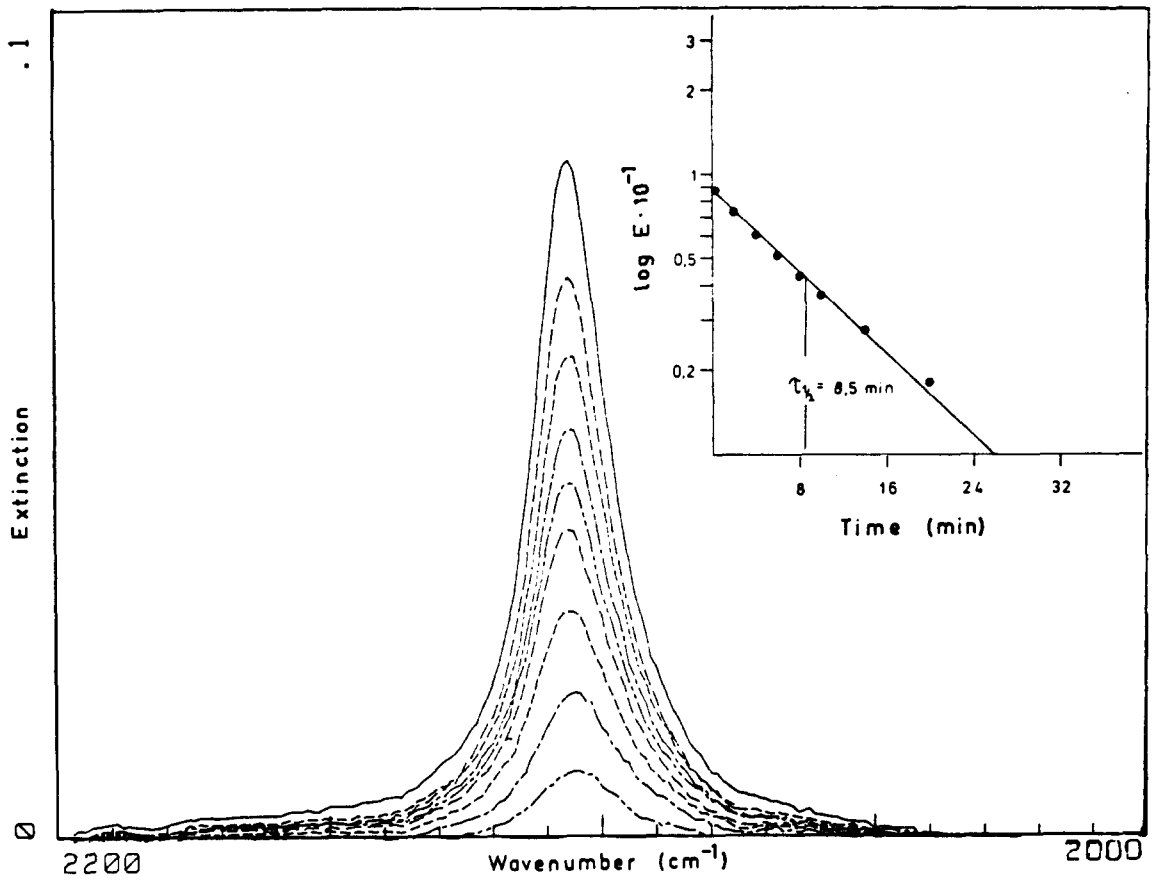

Fig. 3. Photolysis of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid monitored by infrared spectroscopy. A 24-mM solution of 11ξ-azido-12-oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid in dioxane was photolyzed in a Rayonet RPR-100 photochemical reactor equipped with 16 RPR-3000 Å lamps for 0, 2, 4, 6, 8, 10, 14, 20, and 30 min. The absorption of the azido group around 2100 cm^{-1} was recorded after different times of photolysis. The insert shows the determination of the half-life time of the azido group during photolysis.

Extent of photoaffinity labeling

Whereas azido-derivatives, mainly arylazides, as nitrene precursors have been used with success for photoaffinity labeling of molecules of biological interest (36, 37), the application of aliphatic diazirines as carbene precursor species has been limited (38–41). In order to evaluate the suitability of diazirine derivatives of bile acids as agents for photoaffinity labeling, we have determined the extent of covalent bond formation upon photolysis with solvents as well as with proteins known to interact specifically with taurine-conjugated bile salts.

For evaluation of the extent of covalent bond formation with solvents, photolysis was performed using [^{14}C]methanol and [^{14}C]acetonitrile. Subsequent to photolysis, which was performed in sealed ampoules, the radioactive solvent was separated by distillation. Because bile acids form solvates (42) that are only barely transformed into the unsolvated form, the residues obtained were redissolved in unlabeled solvent and again evaporated to dryness. This procedure had to be repeated many times until no radioactivity was detectable in the distillate. The final residue was dissolved in a definite volume of solvent and examined qualitatively by radio TLC as well as quantitatively for radioactivity. With methanol as solvent, 6–12% of the photolyzed 7,7-azo-derivative reacted forming one main product, as demonstrated by TLC (**Fig. 6A**). Photolysis of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid in acetonitrile resulted in 6–8% bond formation with the solvent. Two main products, whose identification was not intended, were found (Fig. 6B). Although a higher yield of insertion than about 10% would be desirable, it should be sufficient for the identification of bile salt-binding proteins in biological systems. This ability was tested with human serum albumin (17), which is involved in the transport of bile salts in blood (43–45) and with homogeneous hydroxycholanoyl-CoA:acceptor hydroxycholanoyl transferase (E.C. 2.3.1.?) isolated from rat liver. The hydroxycholanoyltransferase catalyzes the last step in the formation of conjugated bile salts and

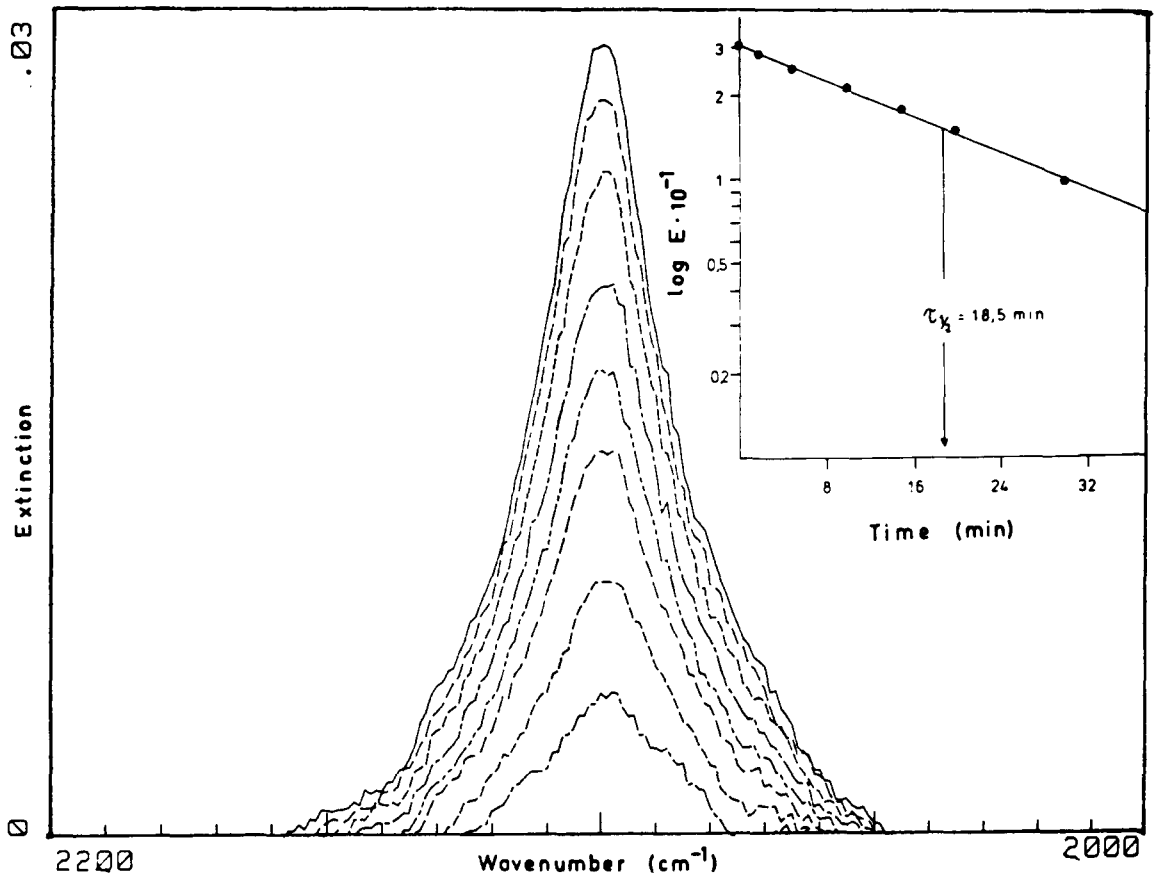

Fig. 4. Photolysis of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid monitored by infrared spectroscopy. A 14.2-mM solution of 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid in dioxane was photolyzed in a Rayonet RPR-100 photochemical reactor equipped with 16 RPR-3000 Å lamps for 0, 2, 5, 10, 15, 20, 30, and 45 min. For all other conditions, see legend of Fig. 3.

is subjected to product inhibition by conjugated bile salts.[3]

As with albumin (17), photoaffinity labeling of hydroxycholanoyl transferase (10 μM) resulted with the different photolabile derivatives (1–5 μM) in a different extent of incorporation of radioactivity, decreasing under the experimental conditions used from 0.7% with the 7,7-azo-derivative, to 0.3% with the 11ξ-azido-12-oxo-derivative and to 0.05% with the 3β-azido-derivative. The extent of photoaffinity labeling is low, but sufficient for the identification of bile salt-binding receptors.

DISCUSSION

The primary purpose of this work was to design derivatives of bile acids that may be useful for photoaffinity labeling and to show their suitability for the identification of bile salt-binding receptors in different biological systems. This objective, comprising research with cell organelles as well as with different cell types, must account for the specificity of these biological systems and implies distinct structural requirements for the photolabile derivatives. The introduction of bulky aromatic residues into the bile acid molecules in principle had to be left out of consideration and the specificity of distinct enzymes and transport systems in liver and terminal ileum[3] (46–48) made a modification of the side chain of the bile acid molecule (49) of little promise. Furthermore, to ensure that no bile salt-binding receptor may elude detection, the synthesis of more than one photolabile bile acid derivative had to be performed. Thus, starting from the cholic acid molecule, we introduced the relatively small azido- and diazirino-groups into different positions of the steroid nucleus, leaving two of the three hydroxyl groups unchanged. The synthesized photolabile bile acid derivatives 3β-azido-7α,12α-dihydroxy-5β-cholan-24-oic acid, 11ξ-azido-12-

[3] Abberger, H., and G. Kurz. Unpublished results.

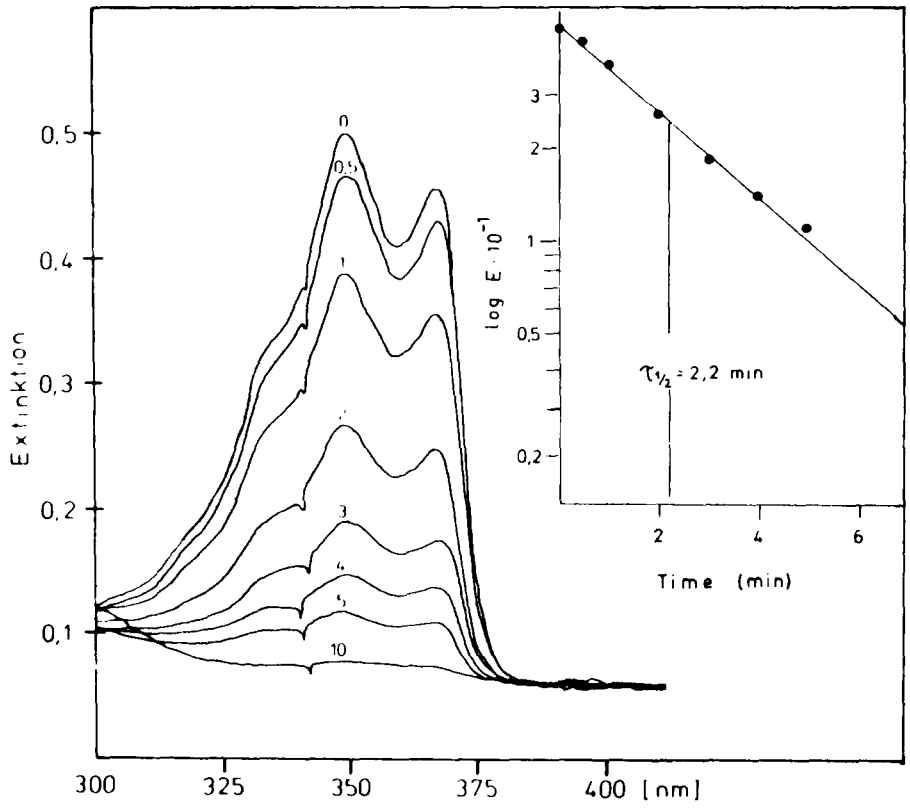

Fig. 5. Photolysis of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid monitored by ultraviolet spectroscopy. A 4.4-mM solution of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid in methanol was photolyzed in a Rayonet RPR-100 photochemical reactor equipped with 16 RPR-3500 Å lamps for 0, 0.5, 1, 2, 3, 4, 5, and 10 min. The absorption of the diazirino group was recorded after different times of photolysis. The insert shows the determination of the half-life time of the diazirino group during photolysis.

oxo-3α,7α-dihydroxy-5β-cholan-24-oic acid, 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid, and their corresponding taurine conjugates behave in enterohepatic circulation in all respects as analogues of the physiological bile salts (50).[4]

In order to have the possibility of performing photoaffinity labeling with intact whole cells using physiological bile salt concentrations, the radiolabeled photolabile derivatives had to be synthesized with specific radioactivities exceeding 1 Ci/mmol. This radiolabeling was achieved by reduction of the corresponding oxo-derivatives formed by oxidation with N-bromosuccinimide with [^{3}H]$NaBH_4$ and by conjugation with ^{3}H-labeled taurine. Because the tritium introduced into the steroid part of the photolabile molecule may be lost in some cases by oxidation and that of the conjugating taurine by hydrolysis, it might be advisable to use both types of radiolabeled derivatives.

[4] Buscher, H-P., J. Flad, W. Gerok, W. Kramer, and G. Kurz. Unpublished results.

All three photolabile bile salt derivatives are susceptible to photolysis at wavelengths longer than 305 nm, where biological systems are reasonably stable to irradiation. Furthermore, their relative short half-life times during irradiation make a duration of photolysis possible in which an impairment of whole cells can be disregarded. This applies especially to the 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid and its taurine conjugate, which need photolysis times shorter than 5 min for sufficient photoaffinity labeling. But even the azido-derivatives of the bile acids were successfully used in photoaffinity labeling experiments performed with isolated whole cells (51).

The photolabile bile acid derivatives, whose synthesis is described here, are, in principal, suitable for the identification of polypeptides and structures interacting physiologically with bile acids. However, the relative intensities of labeling of different polypeptides are dependent upon the specific polypeptide and the photolabile derivative used. The application of a set of different photolabile bile acid derivatives eliminates the

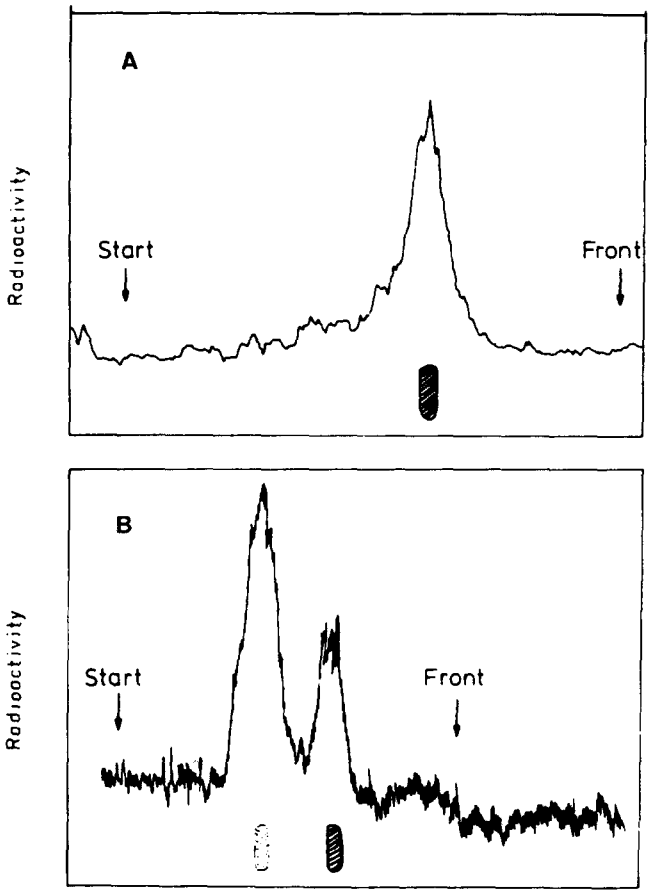

Fig. 6. TLC of the products obtained by photolysis of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid in radioactively labeled solvents. Samples (4.18 mg, 10 μM) of 7,7-Azo-3α,12α-dihydroxy-5β-cholan-24-oic acid in 100 μl (160 μCi) of [^{14}C]acetonitrile or 100 μl (126 μCi) of [^{14}C] methanol were photolyzed in sealed ampoules at 30°C in a Rayonet RPR-100 photochemical reactor equipped with 16 RPR-3500 Å lamps for 10 min. After complete evaporation of the radioactively labeled solvent, the residues were dissolved in definite volumes of the corresponding unlabeled solvents and subjected to thin-layer chromatography using solvent system 4. A, After photolysis in [^{14}C]methanol; B, after photolysis in [^{14}C]acetonitrile.

possibility that a distinct polypeptide eludes detection and allows the identification of virtually all polypeptides interacting with bile acids. This approach has been used for the identification of bile salt-binding proteins in different biological systems (16–22, 51–54).

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