Synthesis and applicability of photolabile 7,7-azo analogues of natural bile salt precursors

T. Gengenbacher,* W. Gerok,† U. Giese, [1,2,*] and G. Kurz[2,*]

Institut für Organische Chemie and Biochemie der Universität Freiburg,* D-7800 Freiburg, F. R. G. and Medizinische Universitätsklinik Freiburg,† D-7800 Freiburg, F. R. G.

Abstract In an approach to the identification of proteins involved in the side chain degradation of bile salt biosynthesis, the photolabile 7,7-azo derivatives of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,26-tetrol and 3α,7α,12α-trihydroxy-5β-cholestan-26-oate were synthesized. All 7,7-azo derivatives were metabolized by intact rat liver and freshly isolated rat hepatocytes in the same manner as the nonphotolabile physiological intermediates, resulting in the formation of the 7,7-azo analogues of cholyltaurine and cholylglycine. Photolysis of all three photolabile derivatives, using a light source with a maximum emission at 350 nm, occurred with a half-life of 2.1 min; their efficacy for photoaffinity labeling was demonstrated by incorporation into rat serum albumin. —**Gengenbacher, T., W. Gerok, U. Giese, and G. Kurz.** Synthesis and applicability of photolabile 7,7-azo analogues of natural bile salt precursors. *J. Lipid Res.* 1990. **31:** 315–327.

Supplementary key words bile salt biosynthesis • photoaffinity labeling

Photolabile derivatives of bile salts have been synthesized (1, 2) and used to identify polypeptides that bind these derivatives in the course of enterohepatic bile salt transport (2–11). The introduction of the small photolabile diazirine group in the 7-position of the steroid nucleus has proven to be of advantage. For this reason, the same position has been chosen to introduce the diazirine group into appropriate metabolites formed by side chain degradation of cholesterol during bile salt biosynthesis.

Side chain degradation starts with hydroxylation of 5β-cholestane-3α,7α,12α-triol to 5β-cholestane-3α,7α,12α,26-tetrol by mitochondria or endoplasmic reticulum (12, 13). The subsequent oxidation to 3α,7α,12α-trihydroxy-5β-cholestan-26-oate by two subsequent dehydrogenations occurs in the cytoplasm. The conversion of trihydroxycholestanoate to cholate takes place in peroxisomes (14) in a process analogous to the β-oxidation of fatty acids. Cholic acid is excreted in bile after conjugation with taurine and glycine.

In order to identify the proteins involved in side chain degradation, we have synthesized 7,7-azo-5β-cholestane-3α,12α-diol, 7,7-azo-5β-cholestane-3α,12α,26-triol, and 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26-oate, and have demonstrated their suitability for the investigation of bile salt biosynthesis and protein binding.

MATERIALS AND METHODS

Materials

Cholic acid, silica gel 60 (40–63 μm and 63–200 μm), and silica gel plates (Kieselgel 60, 10 × 20 cm) for thin-layer and high performance thin-layer chromatography were purchased from Merck (Merck Darmstadt, F. R. G.). Sodium boro[^{3}H]hydride (9.7–10.0 Ci/mmol and 80 Ci/mmol) was obtained from Amersham Buchler GmbH & Co. KG (Braunschweig, F. R. G)). All other chemicals were of the highest quality available from commercial sources. 7,7-Azo-3α,12α-dihydroxy-5β-cholan-24-oic acid, its taurine and glycine conjugates, and their tritium-labeled derivatives were synthesized as described previously (1).

Animals

Male Wistar rats (Tierzuchtanstalt Jautz, Hannover, F. R. G.) weighing 150–200 g were used. The animals had free access to food (standard rat diet Altromin 300 R, Altromin GmbH, Lage, F. R. G.) and tap water.

Liver infusion experiments

Five to 20 nmol of the tritium-labeled metabolites and their photolabile derivatives, dissolved either in 500 μl of 0.15 M NaCl, pH 7.0, or rat serum, were injected into a

Abbreviations: TLC, thin-layer chromatography; HPTLC, high performance thin-layer chromatography; HPLC, high performance liquid chromatography; EI, electron impact ionization; DCI, direct chemical ionization, THF, tetrahydrofuran.

[1]Present address: NATEC Institut für Naturwissenschaftliche-Technische Dienste GmbH, Behringstrasse 154, D-2000 Hamburg 50, F. R. G.

[2]To whom correspondence should be addressed.

peripheral mesenterial vein of rats anesthetized with pentobarbital (3 mg of sodium pentobarbital/100 g body weight, i.p.). Considering a portal blood flow of about 10 ml/min (15), the compounds were infused over a 30-sec period so that their final concentration in blood did not exceed 100 μM. Bile was collected at different times beginning 10 min before start of injection. Radioactivity in bile was determined by liquid scintillation counting, and the nature of the secreted compounds was determined by TLC using n-butanol–acetic acid–water 5:2:3 (v/v/v) as solvent system. Radioactivity on thin-layer plates was detected with a radioscanner (Linear Analyser, Berthold, Wildbad, F. R. G.).

Isolation and incubation of hepatocytes

The standard isolation and incubation medium in all experiments with hepatocytes contained 118 mM NaCl, 4.74 mM KCl, 1.2 mM $MgCl_2$, 0.59 mM KH_2PO_4, 0.59 mM Na_2HPO_4, 24 mM $NaHCO_3$, 1.25 mM $CaCl_2$, and 5.5 mM D-glucose, and was saturated with carbogen (95% O_2/5% CO_2) and adjusted to pH 7.4. Isolated hepatocytes from livers of pentobarbital-anesthetized rats (3 mg of sodium pentobarbital/100 g of body weight, i.p.) were prepared by collagenase perfusion (16). The yield of hepatocytes was about 2×10^8 cells/liver. Cell viability was estimated by determining Trypan blue exclusion. Only cell suspensions with a viability of >90% were used.

In some experiments the isolated hepatocytes (3×10^6 cells/ml) were preincubated with 100 μM taurine at 37°C for 5 min. Then 5–15 μM of the tritium-labeled bile salt metabolites and their photolabile derivatives were added and the whole cell suspension was gently shaken for another 10 min at 37°C. After this time the cell suspension was centrifuged and the bile salts were extracted from the precipitate twice with 200 μl methanol. Aliquots of the organic extract were analyzed by TLC.

Photolysis and photoaffinity labeling

Photolysis and photoaffinity labeling were carried out at 30°C in a Rayonet RPR 100 reactor (The Southern Ultraviolet Company, Hamden, CT) equipped with 16 RPR 3500 Å lamps (1). Ultraviolet absorption spectra were measured with a Perkin-Elmer UV/VIS-Spectrometer Lambda 5 (Perkin-Elmer, Überlingen, F. R. G.).

Polyacrylamide gel electrophoresis and detection of radioactivity

Discontinuous sodium dodecylsulfate/polyacrylamide gel electrophoresis (17) using vertical slab gels (200 × 180 × 2.8 mm) and detection of radioactivity were performed as described (18).

HPLC

Isolation of tritiated bile salt derivatives by HPLC was carried out with an LKB 2150 liquid chromatograph (Pharmacia LKB, Freiburg, F. R. G.) equipped with a Rheodyne injector (Cotati, CA). Zorbax ODS columns (4.6 × 250 mm or 9.4 × 250 mm, 5-μm particles, DuPont Instruments, Bad Nauheim, F. R. G.) were used with solvent system A, methanol–10 mM acetate buffer (pH 4.37) 82:18 (v/v) (19) or with solvent system B, a methanol–phosphate buffer mixture prepared as described (20). A flow rate of 0.7 ml/min was used with the analytical column and a flow rate of 2.5 ml/min with the semi-preparative column. Fractions of 0.7 ml were collected and assayed for radioactivity (19).

Analysis of organic compounds

Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer, Friedrichshafen, F. R. G.). Melting points were determined with a Büchi hotstage apparatus (Büchi, Flawil, Switzerland) and are uncorrected. ^{1}H-NMR-spectra were measured on a Bruker 250-MHz-NMR spectrometer (Bruker GmbH, Karlsruhe, F. R. G.); values are in parts per million relative to tetramethylsilane as internal standard. Mass spectra were recorded with a Finnigan 44S mass spectrometer connected with a data unit SS 2000 (Finnigan, Sunnyvale, CA). 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 (21–23).

Syntheses

Column chromatography was performed on 100 × 5 cm-columns of silica gel 60 (63–200 μm), and flash chromatography on 25 × 5 cm-columns of silica gel 60 (40–63 μm) (24). Solvent systems for chromatographic separations were: solvent system 1, ethyl acetate–cyclohexane–acetic acid 100:40:1 (v/v/v); solvent system 2, ethyl acetate–cyclohexane–acetic acid 23:7:3 (v/v/v); solvent system 3, ethyl acetate–cyclohexane–benzene 1:1:1 (v/v/v); solvent system 4, ethyl acetate; solvent system 5, ethyl acetate–cyclohexane–benzene 1:7:7 (v/v/v); and solvent system 6, chloroform–cyclohexane–ethyl acetate 5:5:1 (v/v/v).

5β-Cholestane-3α,7α,12α-triol (II) (**Fig. 1**). Cholestanetriol was synthesized according to Bergström and Krabisch (25) using 10 g (24.5 mmol) of cholic acid and 70 ml (644 mmol) of isovaleric acid as starting materials. The reaction was stopped immediately after the solution had become faintly yellow. The mixture was then evaporated to dryness in vacuo. The residue was dissolved in 100 ml of chloroform and this solution was extracted four times with 50 ml of 10% Na_2CO_3-solution. The organic phase was evaporated to dryness and the crude product was purified by flash chromatography using solvent system 4 and yielded 10.1 g (24 mmol, 97% yield) of the pure pro-

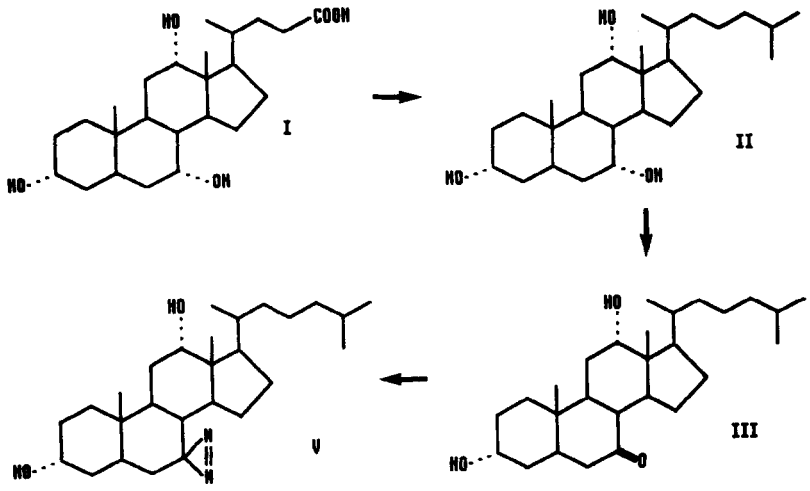

Fig. 1. Synthesis of 7,7-azo-5β-cholestane-3α,12α-diol; I, cholic acid; II, 5β-cholestane-3α,7α,12α-triol; III, 3α,12α-dihydroxy-5β-cholestan-7-one; V, 7,7-azo-5β-cholestane-3α,12α-diol.

duct. MP, 184°C (Lit.: 185°–186°C); TLC: R_f = 0.15 (solvent system 1), 0.46 (solvent system 2), 0.2 (solvent system 4); ^{1}H-NMR ($CDCl_3$): δ = 0.67 (s,C$\underline{H}_3$-18), 0.88 (s, C$\underline{H}_3$-19), 0.89 (d,J = 6 Hz,C$\underline{H}_3$-26/27), 0.97 (d,J = 6 Hz, C$\underline{H}_3$-21), 3.43 (m,b,C$\underline{H}$-3), 3.83 (m,b,C$\underline{H}$-7), 3.97 (m,b,C$\underline{H}$-12); mass spectrum (EI): *m/z* = 420, M^+; 402, M-H_2O; 384, M-2H_2O; 366, M-3H_2O; 271, M-(2H_2O + side chain); 253, M-(3H_2O + side chain); anal. calcd. for $C_{27}H_{48}O_3$ (420.7): C, 77.09, H, 11.50; found: C, 77.10, H, 11.66.

3α,12α-Dihydroxy-5β-cholestan-7-one (III) (Fig. 1). The 7-oxo derivative of cholestanetriol was synthesized principally according to Fieser and Rajagopalan (26), starting with 2 g (4.8 mmol) of 5β-cholestane-3α,7α,12α-triol. The crude product was purified by column chromatography using solvent system 1 and yielded 0.96 g (2.3 mmol, 49% yield) of the pure product. MP, 140°C; TLC: R_f = 0.25 (solvent system 1), 0.53 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.68 (s,C$\underline{H}_3$-18), 0.86 (d,J = 6 Hz, C$\underline{H}_3$-26/27), 0.96 (d,J = 6 Hz,C$\underline{H}_3$-21), 1.17 (s,C$\underline{H}_3$-19), 3.54 (m,b,C$\underline{H}$-3), 4.02 (m,b,C$\underline{H}$-12); anal. calcd. for $C_{27}H_{46}O_3$ (418.7): C, 77.46, H, 11.07; found: C, 76.34, H, 11.08.

7,7-Azo-5β-cholestane-3α,12α-diol (V) (Fig. 1). The 7,7-azo analogue of cholestanetriol was synthesized principally according to Kramer and Kurz (1), starting with 1.6 g (3.8 mmol) of 3α,12α-dihydroxy-5β-cholestan-7-one. The crude product was purified by column chromatgraphy using solvent system 3 and yielded 1.3 g (3 mmol, 79% yield) of the pure product. MP, 151°C; TLC: R_f = 0.43 (solvent system 1), 0.66 (solvent system 2), 0.18 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.61 (s,C$\underline{H}_3$-18), 0.85 (d,J = 6 Hz,C$\underline{H}_3$-26/27), 0.92 (d,J = 6 Hz,C$\underline{H}_3$-21), 1.00 (s,C$\underline{H}_3$-19), 3.61 (m,b,C$\underline{H}$-3), 4.00 (m,b,C$\underline{H}$-12); mass spectrum (EI): *m/z* = 384, M-(H_2O + N_2); 366, M-(2H_2O + N_2); 271, M-(H_2O + N_2 + side chain); 253, M-(2H_2O + N_2 + side chain); anal. calcd. for $C_{27}H_{46}O_2N_2$ (430.7):C, 75.30, H, 10.77, N, 6.50; found: C, 74.81, H, 10.88, N, 5.81.

7,7-Azo-12α-hydroxy-5β-cholestan-3-one (VI) (**Fig. 2**). The 3-oxo derivative of 7,7-azo-5β-cholestane-3α,12α-diol was synthesized principally according to Fieser and Rajagopalan (26), starting with 120 mg (0.28 mmol) of 7,7-azo-5β-cholestane-3α,12α-diol. The crude product was purified by column chromatography using solvent system 3 and yielded 100 mg (0.23 mmol, 83% yield) of the pure product. MP, 163°C; TLC: R_f = 0.45 (solvent system 1), 0.67 (solvent system 2), 0.41 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.62 (s,C$\underline{H}_3$-18), 0.85 (d,J = 6 Hz,C$\underline{H}_3$-26/27), 0.92 (d,J = 7 Hz,C$\underline{H}_3$-21), 1.05 (s,C$\underline{H}_3$-19), 4.00 (m,C$\underline{H}$-2); anal. calcd. for $C_{27}H_{44}O_2N_2$ (428.7): C, 75.65, H, 10.35, N, 6.54; found: C, 75.31, H, 10.48, N, 5.99.

7,7-Azo-5β-cholestane-3α,12α-diol 3-ethylcarbonate (VIII) (Fig. 2). 7,7-Azo-5β-cholestane-3α,12α-diol 3-ethylcarbonate was synthesized principally according to Fieser and Rajagopalan (26), with 1 g (2.6 mmol) of 7,7-azo-5β-cholestane-3α,12α-diol and 1.5 ml (15.7 mmol) of ethyl chloroformate as starting materials. The crude product was purified by column chromatography using solvent system 1 and yielded 1.1 g (2.2 mmol, 85% yield) of the pure product. MP, 210°C; TLC: R_f = 0.70 (solvent system 1), 0.84 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.61 (s,C$\underline{H}_3$-18), 0.84 (d,J = 6 Hz,C$\underline{H}_3$-26/27), 0.92 (d,J = 6 Hz, C$\underline{H}_3$-21), 1.01 (s,C$\underline{H}_3$-19), 1.31 (t,J = 7 Hz,OCOOCH$_2$C$\underline{H}_3$), 3.97 (m,b,C$\underline{H}$-12), 4.17 (q,J = 7 Hz, OCOOC$\underline{H}_2$CH$_3$), 4.57 (m,b,C$\underline{H}$-3); anal. calcd. for $C_{30}H_{50}O_4N_2$ (502.7): C, 71.61, H, 9.95, N, 5.57; found: C, 71.31, H, 9.74, N, 5.22.

7,7-Azo-3α-carboethoxy-5β-cholestan-12-one (IX) (Fig. 2). The 12-oxo derivative was synthesized principally accord-

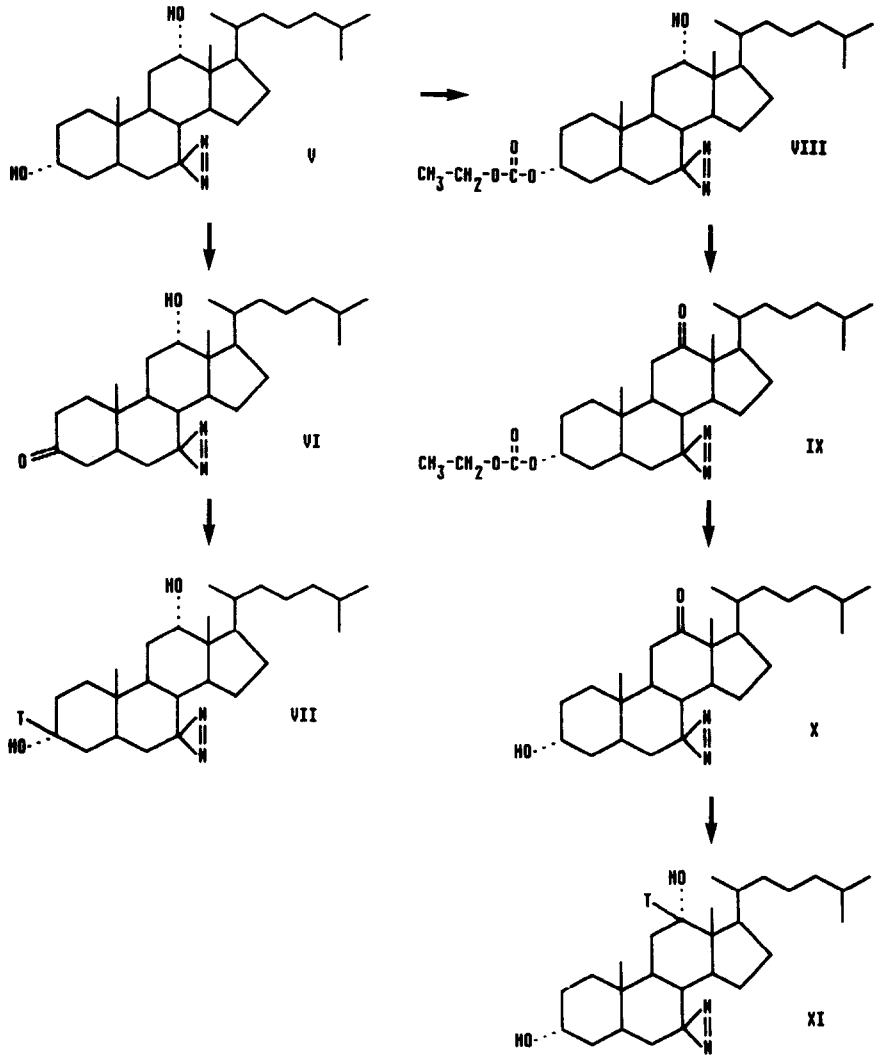

Fig. 2. Synthesis of tritium-labeled derivatives of 7,7-azo-5β-cholestane-3α,12α-diol. V, 7,7-azo-5β-cholestane-3α,12α-diol; VI, 7,7-azo-12α-hydroxy-5β-cholestan-3-one; VII, 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α-diol; VIII, 7,7-azo-5β-cholestane-3α,12α-diol 3-ethylcarbonate; IX, 7,7-azo-3α-carboethoxy-5β-cholestan-12-one; X, 7,7-azo-3α-hydroxy-5β-cholestan-12-one; XI, 7,7-azo-5β-[12β-^{3}H]cholestane-3α,12α-diol.

ing to Fieser and Rajagopalan (27), starting with 900 mg (1.8 mmol) of 7,7-azo-5β-cholestane-3α,12α-diol 3-ethylcarbonate. The crude product was purified by column chromatography using solvent system 5, and yielded 850 mg (1.7 mmol, 95% yield) of the pure product. MP, 220°C; TLC: R_f = 0.71 (solvent system 1), 0.85 (solvent system 2), 0.24 (solvent system 5); ^{1}H-NMR ($CDCl_3$): δ = 0.78 (d,J = 6 Hz,C$\underline{H}_3$-21), 0.85 (d,J = 6 Hz,C$\underline{H}_3$-26/27), 0.97 (s,C$\underline{H}_3$-18), 1.13 (s,C$\underline{H}_3$-19), 1.31 (t,J = 7 Hz,OCOOCH$_2$C$\underline{H}_3$), 4.18 (q,J = 7 Hz,OCOOC$\underline{H}_2$CH$_3$), 4.55 (m,b,C$\underline{H}$-3); anal. calcd. for $C_{30}H_{48}O_4N_2$ (500.7): C, 71.96, H, 9.66, N, 5.59; found: C, 71.21, H, 9.44, N, 5.02.

7,7-Azo-3α-hydroxy-5β-cholestan-12-one (X) (Fig. 2). Seven hundred mg (1.4 mmol) of 7,7-azo-3α-carboethoxy-5β-cholestan-12-one were dissolved in 20 ml of 5% KOH-methanol. The reaction mixture was stirred at room temperature overnight, and then slowly added to 300 ml of water. The precipitate was collected by filtration and dried in vacuo. The crude product was purified by column chromatography using solvent system 3 and yielded 600 mg (1.27 mmol, 91% yield) of the pure product. MP, 161°C; TLC: R_f = 0.44 (solvent system 1), 0.65 (solvent system 2), 0.21 (solvent system 3); ^{1}H-NMR ($CDCl_3$): δ = 0.78 (d,J = 6 Hz,C$\underline{H}_3$-21), 0.85 (d,J = 6 Hz, C$\underline{H}_3$-26/27), 0.97 (s,C$\underline{H}_3$-18), 1.12 (s,C$\underline{H}_3$-19), 3.60 (m,b,C$\underline{H}$-3); anal. calcd. for $C_{27}H_{44}O_2N_2$ (428.7): C, 75.65, H, 10.35, N, 6.54; found: C, 75.11, H, 10.52, N, 5.94.

3α,7α,12α-Triformoxy-5β-cholan-24-oic acid (XII) (**Fig. 3**). Triformylated cholic acid was prepared according to Ruzicka, Plattner, and Heusser (28) with a yield of 90%.

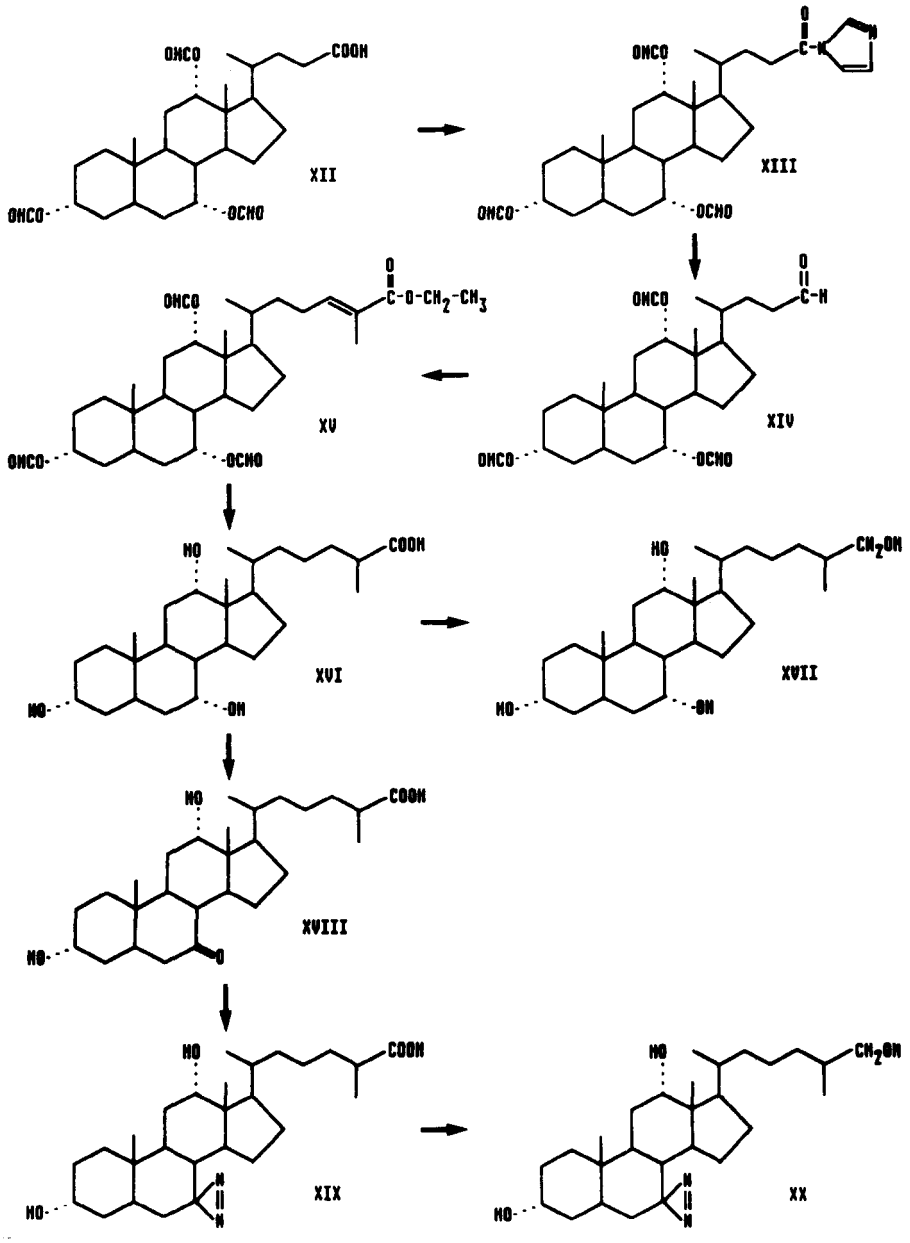

Fig. 3. Synthesis of 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid and 7,7-azo-5β-cholestane-3α,12α, 26(27)-triol. XII, 3α,7α,12α-triformoxy-5β-cholan-24-oic acid; XIII, 3α,7α,12α-triformoxy-5β-cholan-24-oic acid imidazolide; XIV, 3α,7α,12α-triformoxy-5β-cholan-24-aldehyde; XV, 3α,7α,12α-triformoxy-5β-cholest-24-en-26(27)-oic acid ethylester; XVI, 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid; XVII, 5β-cholestane-3α,7α,12α, 26(27)-tetrol; XVIII, 3α,12α-dihydroxy-7-oxo-5β-cholestan-26(27)-oic acid; XIX, 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid, XX, 7,7-azo-5β-cholestane-3α,12α,26(27)-triol.

MP, 200°C (Lit.: 198°–200°C); TLC: R_f = 0.53 (solvent system 1), 0.75 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.76 (s,C$\underline{H}_3$–18), 0.86 (d,J = 7 Hz,C$\underline{H}_3$–21), 0.95 (s,C$\underline{H}_3$–19), 4.71 m,b,C$\underline{H}$–3), 5.06 (m,C$\underline{H}$–7), 5.27 (m, C$\underline{H}$–12), 8.02 (s,C$\underline{H}$OO at C-3), 8.11 (s,C$\underline{H}$OO at C-7), 8.16 (s,C$\underline{H}$OO at C-12). Elemental analyses were consistent with the reported values.

3α,7α,12α-Triformoxy-5β-cholan-24-oic acid imidazolide (XIII) (Fig. 3). The imidazolide of the triformylated cholic acid was synthesized principally according to Staab and Bräunling (29). Thirty g (60 mmol) of 3α,7α,12α-triformoxy-5β-cholan-24-oic acid and 18 g (110 mmol) of carbonyldiimidazole were dissolved in 500 ml of dry THF. This solution was stirred for at least 20 h at room temperature and then evaporated to dryness. The residue was cautiously treated with a small volume of water. The insoluble product was filtered, washed with a small volume of ether, and dried in vacuo. This procedure yielded

27 g (54 mmol, 90% yield) of the pure product. MP, 145°C; TLC: R_f = 0.24 (solvent system 1), 0.38 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.78 (s,C$\underline{H}_3$-18), 0.93 (d,J = 7 Hz,C$\underline{H}_3$-21), 0.96 (s,C$\underline{H}_3$-19), 2.85 (m,b,C$\underline{H}_2$-23), 4.74 (m,b,C$\underline{H}$-3), 5.09 (m,C$\underline{H}$-7), 5.29 (m,C$\underline{H}$-12), 8.05 (s,C$\underline{H}$OO at C-3), 8.11 (s,C$\underline{H}$OO at C-7), 8.18 (s,C$\underline{H}$OO at C-12); anal. calcd. for $C_{30}H_{42}O_7N_2$ (542.8): C, 66.40, H, 7.80, N, 5.16; found: C, 65.81, H, 7.94, N, 5.44.

3α,7α,12α-Triformoxy-5β-cholan-24-aldehyde (XIV) (Fig. 3). The aldehyde was synthesized principally according to a method of Kerb et al. (30). Four g (7 mmol) of 3α,7α,12α-triformoxy-5β-cholan-24-oic acid imidazolide was dissolved in 30 ml of dry THF and a solution of 2.9 g (15 mmol) of LiAlH(t-BuO)$_3$ in 30 ml of dry THF was added at 0°C. The solution was stirred and allowed to warm up. Stirring at room temperature was continued for about 20 h. The progress of the reaction was monitored by TLC using solvent system 1. After the reaction had finished, the solution was slowly added to 500 ml of saturated aqueous NaCl, and acidified with 5 ml of concentrated hydrochloric acid. This mixture was extracted three times with 100 ml of ether. The combined ether extracts were washed with saturated aqueous NaCl and evaporated to dryness. The crude product was purified by flash chromatography using solvent system 6 and yielded 1.6 g (3.2 mmol, 46% yield) of the pure product. MP, 150°C; TLC: R_f = 0.65 (solvent system 1), 0.78 (solvent system 2), 0.18 (solvent system 6); ^{1}H-NMR ($CDCl_3$): δ = 0.76 (s,C$\underline{H}_3$-18), 0.85 (d,J = 7 Hz,C$\underline{H}_3$-21), 0.96 (s,C$\underline{H}_3$-19), 2.4 (m,b,C$\underline{H}_2$-23), 4.73 (m,b,C$\underline{H}$-3), 5.08 (m,C$\underline{H}$-7), 5.27 (m,C$\underline{H}$-12), 8.04 (s,C$\underline{H}$OO at C-3), 8.11 (s,C$\underline{H}$OO at C-7), 8.17 (s,C$\underline{H}$OO at C-12), 9.76 (s,C$\underline{H}$O-24); anal. calcd. for $C_{27}H_{40}O_7$ (476.6): C, 68.04, H, 8.46; found: C, 67.90, H, 8.73.

3α,7α,12α-Triformoxy-5β-cholest-24-en-26(27)-oic acid ethylester (XV) (Fig. 3). The ethylester was synthesized principally according to Boutagy and Thomas (31). Three hundred mg (10 mmol) of a 80% dispersion of sodium hydride in paraffin was suspended in 20 ml of ether. Triethyl 2-phosphonopropionate (2.4 g, 10 mmol) was added to this suspension and the mixture was stirred at room temperature until hydrogen evolution had ceased. This solution was added dropwise to a solution of 2.63 g (5.5 mmol) 3α,7α,12α-triformoxy-5β-cholan-24-aldehyde in 150 ml of ether. The reaction mixture was stirred at room temperature until the reaction was complete, as indicated by TLC using solvent system 6. After the reaction had finished, the solution was slowly poured into 500 ml of 1.5 M hydrochloric acid and this mixture was extracted five times with 100 ml of ether. The combined ether extracts were evaporated to dryness, and the crude product was purified by flash chromatography using solvent system 6. This procedure yielded 2.8 g (4.95 mmol, 90% yield) of the pure product. MP, 123°C; TLC: R_f = 0.69 (solvent system 1), 0.79 (solvent system 2), 0.23 (solvent system 6); ^{1}H-NMR ($CDCl_3$): δ = 0.75 (s, C$\underline{H}_3$-18), 0.89 (d,J = 7 Hz,C$\underline{H}_3$-21), 0.95 (s,C$\underline{H}_3$-19), 1.31 (t,J = 7 Hz,OCH$_2$C$\underline{H}_3$), 1.84 (s,C$\underline{H}_3$-26(27)), 4.19 (q,J = 7 Hz,OC$\underline{H}_2$CH$_3$), 4.74 (m,b,C$\underline{H}$-3), 5.1 (m,C$\underline{H}$-7), 5.31 (m,C$\underline{H}$-12), 5.96 (m,b,C$\underline{H}$-24,Z-form), 6.73 (m,b,C$\underline{H}$-24, E-form), 8.02 (s,C$\underline{H}$OO at C-3), 8.12 (s,C$\underline{H}$OO at C-7), 8.19 (s,C$\underline{H}$OO at C-12); anal. calcd. for $C_{32}H_{48}O_8$(560.7): C, 68.55, H, 8.63; found: C, 68.37, H, 8.89.

3α,7α,12α-Trihydroxy-5β-cholestan-26(27)-oic acid (XVI) (Fig. 3). Three g (5.3 mmol) of 3α,7α,12α-triformoxy-5β-cholest-24-en-26(27)-oic acid ethylester was dissolved in 150 ml of ethyl acetate. This solution was hydrogenated at 30 atm with Pd on activated carbon (5% Pd) as catalyst for 20 h at room temperature, filtered, and the filtrate was evaporated to dryness in vacuo. The residue was dissolved in 200 ml of 2 N KOH–methanol and this solution was stirred at room temperature for 20 h. The solution was then concentrated to 20 ml by evaporation and slowly poured into 500 ml of 1.5 M hydrochloric acid. The precipitate was collected by filtration, dried in vacuo, and purified by column chromatography using solvent system 2. The yield of the pure product was 2 g (4.4 mmol, 83% yield). MP, 178°–181°C (Lit.: 183°C (32)); TLC: R_f = 0.1 (solvent system 1), 0.41 (solvent system 2); ^{1}H-NMR (CD_3OD): δ = 0.71 (s,C$\underline{H}_3$-18), 0.91 (s,C$\underline{H}_3$-19), 1.00 (d,J = 7 Hz,C$\underline{H}_3$-21), 1.14 (d,J = 7 Hz,C$\underline{H}_3$-26(27)), 3.41 (m,b,C$\underline{H}$-3), 3.81 (m,C$\underline{H}$-7), 3.96 (m,C$\underline{H}$-12); mass spectrum (EI): *m/z* = 414, M–2H_2O; 396, M–3H_2O; 271, M–(2H_2O + side chain); 253, M– (3H_2O + side chain); mass spectrum (DCI): *m/z* = 468, $(M + NH_4)^+$; anal. calcd. for $C_{27}H_{46}O_5$ (450.6): C, 71.96, H, 10.28; found: C, 71.17, H, 10.72.

3α,12α-Dihydroxy-7-oxo-5β-cholestan-26(27)-oic acid (XVIII) (Fig. 3).The 7-oxo derivative of trihydroxycholestanoic acid was synthesized principally according to Fieser and Rajagopalan (26), starting with 1.5 g (3.3 mmol) of 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid. The crude product was purified by column chromatography using solvent system 1. The yield of the pure product was 1 g (2.2 mmol, 66% yield). MP, 128°–133°C; TLC: R_f = 0.19 (solvent system 1), 0.52 (solvent system 2); ^{1}H-NMR (CD_3OD): δ = 0.70 (s,C$\underline{H}_3$-18), 1.00 (d,J = 7 Hz,C$\underline{H}_3$-21), 1.13 (d,J = 7 Hz,C$\underline{H}_3$26(27)), 1.23 (s,C$\underline{H}_3$-19), 3.45 (m,b,C$\underline{H}$-3), 4.00 (m,C$\underline{H}$-12); anal. calcd. for $C_{27}H_{44}O_5$(448.6): C, 72.28, H, 9.89; found: C, 71.95, H, 9.92.

7,7,-Azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid (XIX) (Fig. 3). The 7,7,-azo analogue of trihydroxycholestanoic acid was synthesized principally according to Kramer and Kurz (1) starting with 500 mg (1.1 mmol) of

3α,12α-dihydroxy-7-oxo-5β-cholestane-26(27)-oic acid. Column chromatography, using solvent system 2, was performed to purify the crude product. The yield of the pure product was 300 mg (0.7 mmol, 60% yield). MP, 135°C; TLC: R_f = 0.36 (solvent system 1), 0.65 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.61 (s,C$\underline{H}_3$-18), 0.92 (d,J = 7 Hz,C$\underline{H}_3$-21), 1.00 (s,C$\underline{H}_3$-19), 1.16 (d,J = 7 Hz,C$\underline{H}_3$-26(27)), 3.63 (m,b,C$\underline{H}$-3), 4.01 (m,C$\underline{H}$-12); mass spectrum (EI): *m/z* = 414, M-(H_2O + N_2); 396, M-(2H_2O + N_2); 271, M-(H_2O + N_2 + side chain); 253, (M-2H_2O + N_2 + side chain); mass spectrum (DCI): *m/z* = 478, $(M + NH_4)^+$; 450, $(M + NH_4-N_2)^+$; anal. calcd. for $C_{27}H_{44}O_4N_2$ (460.6): C, 70.44, H, 9.57, N, 6.09; found: C, 70.19, H, 9.58, N, 5.98.

7,7-Azo-12α-hydroxy-3-oxo-5β-cholestan-26(27)-oic acid (XXIII) (**Fig. 4**). The 3-oxo derivative of 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid was synthesized principally according to Fieser and Rajagopalan (26), starting with 250 mg (0.54 mmol) of 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid. The crude product was purified by column chromatography using solvent system 1 and yielded 140 mg (0.31 mmol, 57% yield) of the pure product. MP, 150°C; TLC: R_f = 0.43 (solvent system 1), 0.71 (solvent system 2); ^{1}H-NMR ($CDCl_3$): δ = 0.64 (s,C$\underline{H}_3$-18), 0.94 (d,J = 7 Hz, C$\underline{H}_3$-21), 1.10 (s,C$\underline{H}_3$-19), 1.17 (d,J = 7 Hz,C$\underline{H}_3$-26(27)), 4.05 (m,C$\underline{H}$-12); anal. calcd. for $C_{27}H_{42}O_4N_2$ (458.6): C, 70.74, H, 9.17, N, 6.11; found: C, 70.59, H, 9.11, N, 5.99.

5β-Cholestane-3α,7α,12α,26(27)-tetrol (XVII) (Fig. 3). Two g (4.5 mmol) of 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid was dissolved in 50 ml of dry THF. After addition of 630 μl (4.5 mmol) of triethylamine the reaction mixture was stirred for 5 min. Then 440 μl (4.5 mmol) of ethyl chloroformate were added and after 30 min the mixture was filtered. One g (26 mmol) of sodium borohydride and 2 ml of water were added to the clear solution. After 1 h the solution was poured into 200 ml of 1.5 M hydrochloric acid. The crude product was purified by column chromatography using solvent system 2 and yielded 1.3 g (2.9 mmol, 60% yield) of the pure product. MP, 172°-175°C; TLC: R_f = 0.06 (solvent system 1), 0.34 (solvent system 2); ^{1}H-NMR (d_6-acetone): δ = 0.71 (s,C$\underline{H}_3$-18), 0.90 (s,C$\underline{H}_3$-19), 0.94 (d,J = 7 Hz,C$\underline{H}_3$-26(27)), 1.04 (d,J = 7 Hz,C$\underline{H}_3$-21), 3.11 (m,C$\underline{H}_2$OH-26(27)),

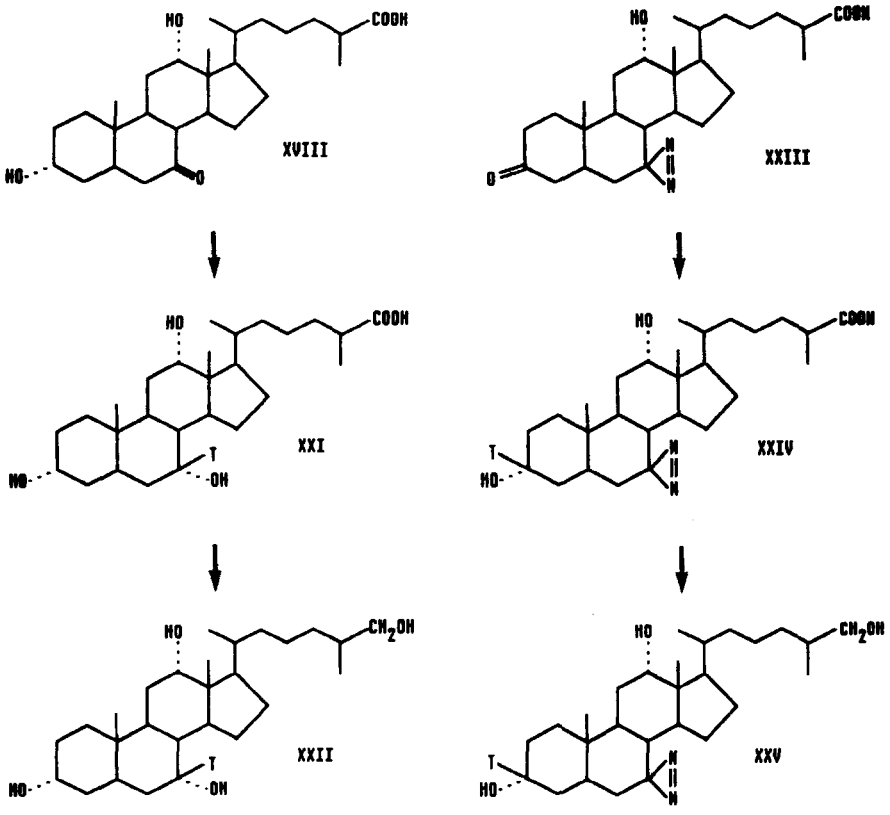

Fig. 4. Synthesis of tritium-labeled derivatives of 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid and 5β-cholestane-3α,7α,12α,26(27)-tetrol. XVIII, 3α,12α-dihydroxy-7-oxo-5β-cholestan-26(27)-oic acid; XXI, 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid; XXII, 5β-[7β-^{3}H]cholestane-3α,7α,12α,26(27)-tetrol; XXIII, 7,7-azo-12α-hydroxy-3-oxo-5β-cholestan-26(27)-oic acid; XXIV, 7,7-azo-3α,12α-dihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid; XXV, 7,7-azo-5β-[3β-^{3}H]cholestane-3α-12α,26(27)-triol.

3.79 (m,CH-3), 3.89 (m,CH-7), 3.94 (m,CH-12); mass spectrum (DCI): *m/z* = 454, $(M + NH_4)^+$; 436, M^+; 418, M- H_2O; 400, M-$2H_2O$; 382, M-$3H_2O$; 271, M-($2H_2O$ + side chain); 253, M-($3H_2O$ + side chain); anal. calcd. for $C_{27}H_{48}O_4$ (436.6): C, 74.27, H, 11.08; found: C, 74.01, H, 10.91.

7,7-Azo-5β-cholestane-3α,12α,26(27)-triol (XX) (Fig. 3). The 7,7-azo analogue of 5β-cholestane-3α,7α,12α,26(27)-tetrol was synthesized according to the aforementioned procedure. Starting with 4 mg (8.7 μmol) of 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid, the synthesis yielded 2 mg (4.5 μmol, 51% yield) of the pure product. MP, 148°-151°C; TLC: R_f = 0.34 (solvent system 1), 0.62 (solvent system 2); ^{1}H-NMR (d_6-acetone): δ = 0.66 (s,CH$_3$-18), 0.87 (d,J = 7 Hz,CH$_3$-26(27)), 0.98 (d,J = 7 Hz,CH$_3$-21), 1.03 (s,CH$_3$-19), 3.13 (m,CH$_2$OH-26(27), 3.78 (m,b,CH-3), 3.98 (m,CH-12); mass spectrum (EI): *m/z* = 400, M-(H_2O + N_2); 271, M-(H_2O + N_2 + side chain); 253, M-($2H_2O$ + N_2 + side chain); mass spectrum (DCI): *m/z* = 436, $(M + NH_4 - N_2)^+$; anal. calcd. for $C_{27}H_{46}O_3N_2$ (446.7): C, 72.61, H, 10.38, N, 6.27; found: C, 72.68, H, 10.14, N, 5.97.

Synthesis of tritium-labeled derivatives

Tritium-labeled compounds were synthesized by reduction of the appropriate oxo-derivatives with sodium boro[^{3}H]hydride. The 26(27)-hydroxy derivatives were obtained by reduction of the corresponding tritium-labeled acids after formation of the mixed anhydrides, using sodium borohydride. Usually, about 25 μmol of the respective oxo-derivative was dissolved in a mixture of 250 μl of THF and 25 μl of water or, in the case of the acids, of 25 μl of 1 N NaOH. In an ampoule, 100 mCi of sodium boro[^{3}H]hydride (9.7-10.0 Ci/mmol) was dissolved in a mixture of 360 μl of THF and 40 μl of water, adjusted to pH 7.5. Two hundred μl of this solution was added to the solution of the oxo-derivative and the reaction mixture was allowed to stand at room temperature overnight. In order to remove residual sodium boro[^{3}H]hydride, which possibly may have not reacted, 25 μl of an aqueous solution of 1 M D-glucose, pH 7.5, was added. After 2 h the tritium-labeled derivatives were purified either by HPTLC and/or HPLC.

5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (IV). Ten mg of 3α,12α-dihydroxy-5β-cholestan-7-one were reduced with 50 mCi of sodium boro[^{3}H]hydride (9.7 Ci/mmol). The crude product was purified in amounts of 1 mg by HPTLC using solvent system 2 and yielded 40 mCi (2.4 Ci/mmol) of the pure product. Alternatively, analytical amounts of about 10 μCi of the crude product were separated by HPLC using solvent system A.

7,7-Azo-5β-[3β-^{3}H]cholestane-3α,12α-diol (VII) (Fig. 2). Ten mg of 7,7-azo-12α-hydroxy-5β-cholestan-3-one was reduced with 50 mCi of sodium boro[^{3}H]hydride (9.7 Ci/mmol). The crude product was purified in amounts of 1 mg by HPTLC using solvent system 1 and yielded 38 mCi (2.4 Ci/mmol) of the purified stereochemically non-homogeneous product. Rechromatography by HPLC in amounts of about 1 mCi, using solvent system A, yielded 880 μCi of the stereochemically pure [3β-^{3}H]-labeled product per run.

7,7-Azo-5β-[12β-^{3}H]cholestane-3α,12α-diol (XI) (Fig. 2). Ten mg of 7,7-azo-3α-hydroxy-5β-cholestan-12-one was reduced with 50 mCi of sodium boro[^{3}H]hydride (9.9 Ci/mmol). Isolation of the mixture of isomers was achieved by HPTLC using solvent system 1. Separation of the 12α- and 12β-isomers was carried out by HPLC in amounts of about 1 mCi using solvent system A and yielded 500 μCi (2.5 Ci/mmol) of each isomer per run.

3α,7α,12α-Trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid (XXI) (Fig. 4). Ten mg of 3α,12α-dihydroxy-7-oxo-5β-cholestan-26(27)-oate was reduced with 50 mCi of sodium boro[^{3}H]hydride (10.0 Ci/mmol). The crude product was purified in amounts of 1 mg by HPTLC using solvent system 2 and yielded 39 mCi (2.5 Ci/mmol) of the pure product. Alternatively, analytical amounts of about 10 μCi of the crude product were separated by HPLC using solvent system B.

7,7-Azo-3α,12α-dihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid (XXIV) (Fig. 4). Ten mg of 7,7-azo-12α-hydroxy-3-oxo-5β-cholestan-26(27)-oate was reduced with 50 mCi of sodium boro[^{3}H]hydride (10.0 Ci/mmol). The crude product was purified in amounts of 1 mg by HPTLC using solvent system 1 and yielded 36 mCi (2.5 Ci/mmol) of the purified stereochemically nonhomogeneous product. Rechromatography in amounts of about 1 mCi by HPLC, using solvent system B, yielded 850 μCi of the stereochemically pure [3β-^{3}H]-labeled product per run. In order to get a product with a very high specific radioactivity, 500 mCi of sodium boro[^{3}H]hydride (80 Ci/mmol) was used for reduction. The total yield of the purified product, having a specific radioactivity of 20 Ci/mmol, was only 40 mCi.

5β-[7β-^{3}H]Cholestane-3α, 7α, 12α, 26(27)-tetrol (XXII) (Fig. 4). One μmol (2.5 mCi) of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid was transformed to the mixed anhydride and immediately reduced with sodium borohydride as described above. Purification of the crude product was achieved by HPTLC using solvent system 2 and yielded 1.2 mCi (2.5 Ci/mmol) of the pure product. Alternatively, analytical amounts of about 10 μCi of the crude product were separated by HPLC using solvent system A.

7,7-Azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol (XXV) (Fig. 4.). One μmol (2.5 mCi) or 50 nmol (2.0 mCi) of 7,7-azo-3α,12α-trihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid were transformed to the mixed anhydride and

immediately reduced with sodium borohydride as described above. Purification of the crude product was achieved by HPTLC using solvent system 2 and yielded 1.3 mCi (2.5 Ci/mmol) or 1.1 mCi (20 Ci/mmol) of the pure product. Alternatively, analytical amounts of about 10 μCi of the crude product were separated by HPLC using solvent system A.

RESULTS AND DISCUSSION

Syntheses

Synthesis of the photolabile trihydroxycholestane analogue 7,7-azo-5β-cholestane-3α,12α-diol (V) (Fig. 1) comprises elongation of the side chain of C_{24}-bile acid by electrolysis of cholic acid with isovaleric acid (25), followed by the introduction of the diazirine group in the 7-position (1). The only crucial point in the complete synthesis is to stop electrolysis in time, which should be done immediately after the solution becomes faintly yellow. In this case the main product 5β-cholestane-3α,7α,12α-triol could easily be separated from the side-products by flash chromatography.

Synthesis of radioactively labeled cholestane derivatives could be performed easily for 5β-cholestane-3α,7α,12α-triol (II) by introduction of tritium in the metabolically stable 7β-position. Reduction of the 7-oxo compound, 3α,12α-dihydroxy-5β-cholestan-7-one (III), with sodium boro[^{3}H]hydride went smoothly with a total yield of about 90% related to sodium boro[^{3}H]hydride and resulted in formation of 89% of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (IV, formula not shown) and 11% of an isomer probably with the opposite configuration at C-7. The ratio of the diastereomers formed by reduction of 3α,12α-dihydroxy-5β-cholestan-7-one with sodium borohydride is comparable to that obtained with other 7-oxo steroids (33, 34). Isolation of the desired 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol was achieved on a preparative scale by HPTLC on silica gel or on a semi-preparative scale by means of HPLC on a reversed-phase column. The stereochemical purity of the isolated [7β-^{3}H]-labeled compound was controlled by analytical HPLC and guaranteed by cochromatography with the authentic unlabeled 5β-cholestane-3α,7α,12α-triol. The diastereomeric [7α-^{3}H]-labeled compound could no more be detected.

For the photolabile 7,7-azo analogue, 7,7-azo-5β-cholestane-3α,12α-diol (V), tritium can be introduced either in position 3 or 12 (Fig. 2), the first being chemically more simple and convenient. Starting with 7,7-azo-5β-cholestane-3α,12α-diol (V), oxidation of the 3α-OH group was carried out with N-bromosuccinimide yielding 7,7-azo-12α-hydroxy-5β-cholestan-3-one (VI) as the main product and 7,7-azo-5β-cholestane-3,12-dione as minor product. The 3-oxo compound, which could easily be purified by column chromatography, was reduced with sodium boro[^{3}H]hydride and resulted in the formation of the 3-hydroxy-[3-^{3}H]-labeled derivatives with a yield of about 85% related to sodium boro[^{3}H]hydride. The major reduction product, the desired 7,7-azo-5β-[3β-^{3}H]-cholestane-3α,12α-diol (VII) and presumably and [3α-^{3}H]-labeled derivative were formed in a product ratio of 77:23, as judged by HPLC. This ratio is near to those obtained by reduction of other 3-oxo steroids with sodium borohydride (35, 36). Separation of the diastereomeric forms of the crude product by HPTLC resulted only in an enrichment of the [3β-^{3}H]-labeled derivative, the extent of which varied and was dependent upon the amount of substance applied to a plate. In order to obtain the stereochemically pure product, HPLC on a reversed-phase column proved necessary. After HPLC no [3α-^{3}H]-labeled derivative could be detected in the desired [3β-^{3}H]-labeled product.

Introduction of tritium in the 12-position by reduction of the corresponding 12-oxo derivative requires protection of the 3α-OH group in the course of the synthesis. Ethyl chloroformate was used as a convenient protecting reagent. The resulting 7,7-azo-5β-cholestane-3α,12α-diol 3-ethylcarbonate (VIII) was oxidized with potassium chromate (27) to 7,7-azo-3α-carboethoxy-5β-cholestan-12-one (IX), which could be easily hydrolyzed to 7,7-azo-3α-hydroxy-5β-cholestan-12-one (X). Reduction of the 12-oxo compound with sodium boro[^{3}H]hydride resulted in the formation of the diastereomeric mixture of 7,7-azo-5β-[12α-^{3}H]cholestane-3α,12β-diol and 7,7-azo-5β-[12β-^{3}H]cholestane-3α,12α-diol (XI) with a yield of about 80%. The product ratio of [12β-^{3}H]- to [12α-^{3}H]-labeled derivative of 50:50 was somewhat lower than those described for the reduction of other 12-oxo steroids by sodium borohydride (37–39). Separation of the diastereomers could be obtained only by HPLC.

Syntheses of the photolabile derivatives 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid (XIX) and 7,7-azo-5β-cholestane-3α,12α,26(27)-triol (XX) use a Wittig-Horner reaction for side chain elongation (Fig. 3). The required 3α,7α,12α-triformoxy-5β-cholan-24-aldehyde (XIV) was obtained by reduction of the activated cholic acid derivative 3α,7α,12α-triformoxy-5β-cholan-24-oic acid imidazolide (XIII), formed by reaction of 3α,7α,12α-triformoxy-5β-cholan-24-oic acid (XII) with carbonyldiimidazole. This reaction sequence proved to be more convenient than the synthesis of the aldehyde from the corresponding alcohol (40, 41). The subsequent Wittig-Horner reaction, using triethyl 2-phosphonopropionate resulted in higher yields of 3α,7α,12α-triformoxy-5β-cholest-24-en-26(27)-oic acid ethylester (XV), than those ob-

tained with (2-carboethoxy-ethyl)-triphenylphosphorane (32, 41). Subsequent catalytic hydrogenation and saponification gave excellent yields of 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid (XVI), which was oxidized to the 7-oxo derivative 3α,12α-dihydroxy-7-oxo-5β-cholestan-26(27)-oic acid (XVIII) and further transferred to 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid (XIX) as described (1).

5β-Cholestane-3α,7α,12α,26(27)-tetrol (XVII) and its photolabile analogue, 7,7-azo-5β-cholestane-3α,12α,26(27)-triol (XX), were synthesized by reduction of the corresponding activated acids with sodium borohydride in order to avoid reduction of the diazirine group.

The syntheses of the required tritium-labeled derivatives were started from the most simply accessibly oxo-derivatives (Fig. 4), which could be obtained in good yields by oxidation of 3α,7α,12α-trihydroxy-5β-cholestan-26(27)-oic acid and 7,7-azo-3α,12α-dihydroxy-5β-cholestan-26(27)-oic acid, respectively, with N-bromosuccinimide. Both oxo-derivatives, 3α,12α-dihydroxy-7-oxo-5β-cholestan-26(27)-oic acid XVIII) and 7,7-azo-12α-hydroxy-3-oxo-5β-cholestan-26(27)-oic acid (XXIII), were reduced with sodium boro[^{3}H]hydride resulting in the formation of mixtures of the corresponding diastereomeric compounds. The mixture of the 7-hydroxy-[7-^{3}H]diastereomers was obtained in yields of about 88%. The product ratio of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid (XXI) to the presumed [7α-^{3}H]-labeled derivative was 90:10. The desired [7β-^{3}H]-labeled derivative could be obtained as stereochemically pure product by HPTLC or by HPLC. The 3-hydroxy-[3-^{3}H]diastereomers were obtained in yields of about 80%. The 7,7-azo-3α,12α-dihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid XXIV), formed in a product ratio to the presumed [3α-^{3}H]-labeled derivative of 75:25, could be isolated by HPLC.

Starting from 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid and 7,7-azo-3α,12α-dihydroxy-5β-[3β-^{3}H]-cholestan-26(27)-oic acid, the corresponding bile alcohols, 5β-[7β-^{3}H]cholestane-3α,7α,12α,26(27)-tetrol (XXII) and 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol (XXV), could be synthesized by activation with ethyl chloroformate and subsequent reduction with sodium borohydride.

Biotransformation of the photolabile analogues

In order to be suitable for the investigation of side chain degradation by photoaffinity labeling, the photolabile analogues must pass through the same enzymatic sequences as the physiological metabolites. Thus, metabolism of the synthesized photolabile derivatives was compared with that of the unsubstituted metabolites in the intact rat liver in vivo and in freshly isolated rat hepatocytes.

For studying the comparative metabolism of photolabile derivatives by intact rat liver, infusion experiments were performed with aliquots of each of the different compounds as stereochemically pure substrates. After infusion of 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol (IV), only labeled cholyltaurine and cholylglycine were secreted into bile in the ratio of about 3.5:1. Maximum secretion was reached in about 10 min and 95% of the applied radioactivity was found in bile after 80 min (**Table 1**). Because position 7 of the steroid nucleus is occupied by the azo-group, tritium was introduced in position 3 as well as in position 12, respectively, of the photolabile analogue. Both compounds, 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α-diol (VII) and 7,7-azo-5β-[12β-^{3}H]cholestane-3α,12α-diol (XI), were completely and identically metabolized to the corresponding conjugated derivatives of 7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oic acid (Table 1). The times taken to their peak appearance in bile and the secretion of 95% of the infused radioactivity were only insignificantly slower than for the natural compound 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol. Under the experimental conditions used, no differences could be detected between the photolabile derivatives bearing the tritium either in 3β- or in 12β-position. Thus, for the photolabile analogues of intermediates following in side chain degradation, only the easily accessibly [3β-^{3}H]-labeled derivatives were synthesized.

Infusion of both the physiological intermediates 5β-[7β-^{3}H]cholestane-3α,7α,12α26(27)-tetrol (XXII) and the sodium salt of 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid (XXI) resulted in the secretion of taurine- and glycine-conjugated tritium-labeled cholic acid into

TABLE 1. Biotransformation of natural bile salt precursors and their 7,7-azo analogues by rat liver

Injected Compound	Secretion		Secreted Substances
	Maximum	95%	
	min		
IV	10	80	[7β-^{3}H]CT, [7β-^{3}H]CG
VII	12	85	[3β-^{3}H]AT, [3β-^{3}H]AG
XI	12	85	[12β-^{3}H]AT, [12β-^{3}H]AG
XXII	8	60	[7β-^{3}H]CT, [7β-^{3}H]CG
XXV	10	65	[3β-^{3}H]AT, [3β-^{3}H]AG
XXI	5	20	[7β-^{3}H]CT, [7β-^{3}H]CG
XXIV	7	22	[3β-^{3}H]AT, [3β-^{3}H]AG

Tritium-labeled compounds (5–20 nmol) dissolved in 500 μl of 0.15 M NaCl, pH 7.0, or rat serum were infused into a peripheral mesenteric vein over a 30-sec period. Bile was collected and its radioactivity was determined by liquid scintillation counting. The nature of the secreted metabolites was ascertained by TLC. IV, 5β-[7β-^{3}H]cholestane-3α,7α,12α-triol; VII, 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α-diol; XI, 7,7-azo-5β-[12β-^{3}H]cholestane-3α,12α-diol; XXI, 3α,7α,12α-trihydroxy-5β-[7β-^{3}H]cholestan-26(27)-oic acid; XXII, 5β-[7β-^{3}H]cholestane-3α,7α,12α,26(27)-tetrol; XXIV, 7,7-azo-3α,12α-dihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid; XXV, 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol; CT, cholyltaurine; CG, cholylglycine; AT, (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-taurine; AG, (7,7-azo-3α,12α-dihydroxy-5β-cholan-24-oyl)-2'-glycine.

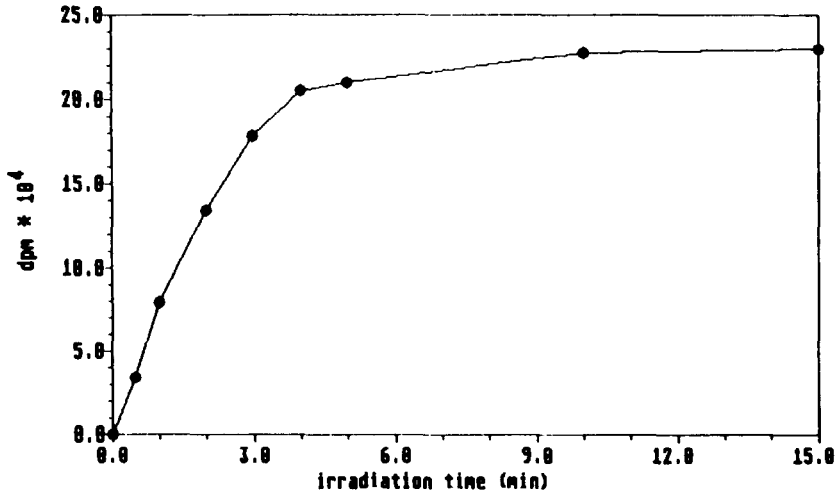

Fig. 5. Photoaffinity labeling of rat serum albumin by 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol. A solution of 1 μM 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol and of 1.8 μM rat serum albumin in 0.15 M NaCl, adjusted to pH 7.0, was irradiated at 350 nm for the times indicated. Incorporation of radioactivity into albumin was determined after sodium dodecylsulfate polyacrylamide gel electrophoresis by liquid scintillation counting (18).

bile in a ratio of about 3.5:1 (Table 1). The photolabile analogues 7,7-azo-5β-[3β-^{3}H]cholestane-3α,12α,26(27)-triol (XXV) and the sodium salt of 7,7-azo-3α,12α-dihydroxy-5β-[3β-^{3}H]cholestan-26(27)-oic acid (XXIV) were completely biotransformed and the conjugates of their transformation products were secreted by rat liver nearly as rapidly as those derived from the physiological intermediates. As expected, the maximum of secretion as well as the secretion of 95% of the applied radioactivity were attained more rapidly with the sodium salts of the C_{27}-acids than with the corresponding bile alcohols. The sodium salts of the C_{27}-acids as well as the C_{27}-bile alcohols obtained by reduction of the corresponding acids were applied as diastereomeric mixtures of (25R)- and (25S)-derivatives. This proved to be practical because not only (25R)- but also (25S)-3α,7α,12α-trihydroxy-5β-cholestanoate are completely biotransformed in liver (42–45).

Photoaffinity labeling studies may be performed at different levels of biological organization. Isolated hepatocytes are suitable for the study of bile salt biosynthesis (46–48) and are easily amenable to photoaffinity labeling studies (6, 49). Since it is desired to use the photolabile analogues for the identification of proteins involved in their metabolism and transport by intact cells, it is noteworthy that all these compounds were metabolized by isolated hepatocytes, identically with their physiological counterparts, only to taurine- and glycine- conjugated C_{24}-bile acids. Preincubation of hepatocytes with taurine resulted in the formation only of taurine- conjugated bile acids.

Suitability for photoaffinity labeling

Aliphatic diazirines exhibit an absorption maximum at about 350 nm with a second maximum or a shoulder at about 366 nm. As expected (1), the half-life of all 7,7-azo analogues, irradiated at 350 nm, was 2.1 min. The suitability of the tritium-labeled 7,7-azo analogues for photoaffinity labeling studies was demonstrated by photolytic incorporation into rat serum albumin with a half-life time of about 1.9 min (**Fig. 5**); about 0.9% of the protein was labeled.

The uncomplicated synthetic accessibility of the photolabile 7,7-azo analogues of 5β-cholestane-3α,7α,12α-triol, 5β-cholestane-3α,7α,12α,26-tetrol, and 3α,7α,12α-trihydroxy-5β-cholestan-26-oate, their behavior in metabolism, and their favorable photolytic properties indicate that they may be suitable for identification and localization of proteins involved in their metabolism and transport.

The authors express their gratitude to Dr. D. Hunkler and Dr. J. Wörth from the Institut für Organische Chemie and Biochemie der Universität Freiburg for the ^{1}H-NMR and mass spectra. This investigation was supported by the Deutsche Forschungsgemeinschaft and the Fritz-Thyssen-Stiftung. One of us (U. Giese) is indebted to the Fritz-Thyssen-Stiftung for a scholarship. The authors thank Prof. Dr. J. D. Ostrow for critically reading and correcting the manuscript.

Manuscript received 30 June 1989 and in revised form 13 October 1989.

REFERENCES

1. Kramer, W., and G. Kurz. 1983. Photolabile derivatives of bile salts. Synthesis and suitability for photoaffinity labeling. *J. Lipid Res.* **24:** 910–923.
2. von Dippe, P., and D. Levy. 1983. Characterization of the bile acid transport system in normal and transformed hepatocytes. Photoaffinity labeling of the taurocholate carrier protein. *J. Biol. Chem.* **258:** 8896–8901.
3. Kramer, W., H-P. Buscher, W. Gerok, and G. Kurz. 1979.

Bile salt binding to serum components. Taurocholate incorporation into high-density lipoproteins revealed by photoaffinity labeling. *Eur. J. Biochem.* **102:** 1-9.
4. Kramer, W., U. Bickel, H-P. Buscher, W. Gerok, and G. Kurz. 1982. Bile-salt-binding polypeptides in plasma membranes of hepatocytes revealed by photoaffinity labeling. *Eur. J. Biochem.* **129:** 13-24.
5. Fricker, G., S. Schneider, W. Gerok, and G. Kurz. 1987. Identification of different transport systems for bile salts in sinusoidal and canalicular membranes of hepatocytes. *Biol. Chem. Hoppe-Seyler.* **368:** 1143-1150.
6. Ruetz, S., G. Fricker, G. Hugentobler, K. Winterhalter, G. Kurz, and P. J. Meier. 1987. Isolation and characterization of the putative canalicular bile salt transport system of rat liver. *J. Biol. Chem.* **262:** 11324-11330.
7. Fricker, G., G. Hugentobler, P. J. Meier, G. Kurz, and J. L. Boyer. 1987. Identification of a single sinusoidal bile salt uptake system in skate liver. *Am. J. Physiol.* **252:** G816-G822.
8. Buscher, H-P., W. Gerok, M. Köllinger, G. Kurz, M. Müller, A. Nolte, and S. Schneider. 1988. Transport systems for amphipathic compounds in normal and neoplastic hepatocytes. *Adv. Enzyme Regul.* **27:** 173-192.
9. Kurz, G., M. Müller, U. Schramm, and W. Gerok. 1989. Identification and function of bile salt binding polypeptides of hepatocyte membrane. *In* Hepatic Transport in Organic Substances. E. Petzinger, R. K-H. Kinne, and H. Sies, editors. Springer-Verlag, Berlin, Heidelberg. 267-278.
10. Kramer, W., G. Burckhardt, F. A. Wilson, and G. Kurz. 1983. Bile salt-binding polypeptides in brush-border membrane vesicles from rat small intestine revealed by photoaffinity labeling. *J. Biol. Chem.* **258:** 3623-3627.
11. Burckhardt, G., W. Kramer, G. Kurz, and F. A. Wilson. 1987. Photoaffinity labeling studies of the rat renal sodium/bile salt cotransport system. *Biochem. Biophys. Res. Commun.* **143:** 1018-1023.
12. Cronholm, T., T. Curstedt, and J. Sjövall. 1982. Formation of bile acids and glycerophosphatides in liver. *In* Metabolic Compartmentation. H. Sies, editor. Academic Press, London, New York. 331-359.
13. Björkhem, I. 1985. Mechanism of bile acid biosynthesis in mammalian liver. *In* Sterols and Bile Acids. H. Danielsson, and J. Sjövall, editors. Elsevier Science Publishers B. V., Amsterdam, New York, Oxford. 231-278.
14. Pedersen, J. I., B. F. Kase, K. Prydz, and I. Björkhem. 1987. Liver peroxisomes and bile acid formation. *In* Peroxisomes in Biology and Medicine. H. D. Fahimi, and H. Sies, editors. Springer-Verlag, Berlin, Heidelberg. 67-77.
15. Ross, G., F. N. White, A. W. Brown, and A. Kolin. 1966. Regional blood flow in the rat. *J. Appl. Physiol.* **21:** 1273-1275.
16. Berry, M. N., and D. S. Friend. 1969. High-yield preparation of isolated rat liver parenchymal cells. A biochemical and fine structural study. *J. Cell Biol.* **43:** 506-520.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227:** 680-685.
18. Wieland, T., M. Nassal, W. Kramer, G. Fricker, U. Bickel, and G. Kurz. 1984. Identity of hepatic membrane transport systems for bile salts, phalloidin, and antamanide by photoaffinity labeling. *Proc. Natl. Acad. Sci. USA.* **81:** 5232-5236.
19. Prydz, K., B. F. Kase, and J. I. Pedersen. 1988. High pressure liquid chromatography solvent systems for studies of bile acid biosynthesis. *J. Lipid Res.* **29:** 532-537.
20. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28:** 589-595.
21. Lawson, A. M., and K. D. R. Setchell. 1988. Mass spectrometry of bile acids. *In* The Bile Acids. Chemistry, Physiology, and Metabolism. Volume 4. K. D. R. Setchell, D. Kritchevsky, and P. P. Nair, editors. Plenum Press, New York, London. 167-267.
22. Lawson, A. M. 1989. Mass spectrometry—the fundamental principles. *In* Mass Spectrometry. A. M. Lawson, editor. Walter de Gruyter, Berlin, New York. 1-52.
23. Setchell, K. D. R., and A. M. Lawson. 1989. Bile acids. *In* Mass Spectrometry. A. M. Lawson, editor. Walter de Gruyter, Berlin, New York. 53-125.
24. Still, W. C., M. Kahn, and A. Mitra. 1978. Rapid chromatographic technique for preparative separations with moderate resolution. *J. Org. Chem.* **43:** 2923-2925.
25. Bergström, S., and L. Krabisch. 1957. Preparation of some hydroxy-coprostanes; 3α,7α- and 3α,12α-dihydroxycoprostane. *Acta Chem. Scand.* **11:** 1067.
26. Fieser, L. F., and S. Rajagopalan. 1949. Selective oxidation with N-bromosuccinimide. I. Cholic acid. *J. Am. Chem. Soc.* **71:** 3935-3938.
27. Fieser, L. F., and S. Rajagopalan. 1950. Oxidation of steroids. III. Selective oxidations and acylations in the bile acid series. *J. Am. Chem. Soc.* **72:** 5530-5536.
28. Ruzicka, L., Pl. A. Plattner, and H. Heusser. 1944. Über β'-[3α,7α,12β-Trioxy-nor-cholanyl-(23)]-$\Delta^{\alpha',\beta'}$-butenolid, ein Homologes der digitaloiden Aglucone. *Helv. Chim. Acta.* **27:** 186-194.
29. Staab, H. A., and H. Bräunling. 1962. Reduktion von Carbonsäuren zu Aldehyden über Imidazolide. *Liebigs Ann. Chem.* **654:** 119-130.
30. Kerb, U., G. Schulz, P. Hocks, R. Wiechert, A. Furlenmeier, A. Fürst, A. Langemann, and G. Waldvogel. 1966. Zur Synthese des Ecdysons. Die Synthese des natürlichen Häutungshormons. *Helv. Chim. Acta.* **49:** 1601-1606.
31. Boutagy, J. S., and R. E. Thomas. 1971. Cardenolide analogues. I. Route for preparing semi-synthetic analogues of digitoxigenin. *Aust. J. Chem.* **24:** 2723-2728.
32. Okuda, K., and H. Danielsson. 1965. Synthesis and metabolism of 5β-cholestane-3α,7α,12α-triol-26-al. *Acta. Chem. Scand.* **19:** 2160-2165.
33. Mosbach, E. H., W. Meyer, and F. E. Kendall. 1954. Preparation and $NaBH_4$ reduction of 7-ketocholanic acid. *J. Am. Chem. Soc.* **76:** 5799-5801.
34. Maruyama, S., N. Ogihara, I. Adachi, J. Ohotawa, and M. Morisaki. 1987. Stereoselectivity in reduction of steroidal 7-ketones. *Chem. Pharm. Bull.* **35:** 1847-1852.
35. Heymann, H., and L. F. Fieser. 1951. A new route to 11-ketosteroids by fission of a $\Delta^{9(11)}$-ethylene oxide. *J. Am. Chem. Soc.* **73:** 5252-5265.
36. Dauben, W. G., R. A. Micheli, and J. F. Eastham. 1952. The reduction of steroidal enol acetates with lithium aluminium hydride and sodium borohydride. *J. Am. Chem. Soc.* **74:** 3852-3855.
37. Huffman, J. W., D. M. Alabran, T. W. Bethea, and A. C. Ruggles. 1964. The reduction of 12-keto steroids. *J. Org. Chem.* **29:** 2963-2968.
38. Huffman, J. W., and D. J. Copley. 1977. Reduction of 12-keto steroids. *J. Org. Chem.* **42:** 3811-3814.
39. Iida, T., and F. C. Chang. 1983. Potential bile acid metabolites. 8. 7,12-Dihydroxy- and 7β-hydroxy-5β-cholanic acids. *J. Org. Chem.* **48:** 1194-1197.
40. Yashima, H. 1963. Stero-bile acids and bile sterols. LIII. Synthesis and metabolism of cholic aldehyde. *J. Biochem.* **54:** 47-50.

41. Somanathan, R., and S. Krisans. 1984. Improved synthesis of 5β-cholestane-3α,7α,12α,26(27)-tetrol isomers from cholic acid. *Steroids.* **43:** 651–655.
42. Gustafsson, J. 1980. Biosynthesis of cholic acid in rat liver: formation of cholic acid from 3α,7α,12α-trihydroxy- and 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acids. *Lipids.* **15:** 113–121.
43. Swell, L., J. Gustafsson, H. Danielsson, C. C. Schwartz, and Z. R. Vlahcevic. 1981. Biosynthesis of bile acids in man. An in vivo evaluation of the conversion of R and S 3α,7α,12α-trihydroxy-5β-cholestanoic and 3α,7α,12α,24ξ-tetrahydroxy-5β-cholestanoic acids to cholic acid. *J. Biol. Chem.* **256:** 912–916.
44. Une, M., I. Morigami, K. Kihira, and T. Hoshita. 1984. Stereospecific formation of (24E)-3α,7α,12α-trihydroxy-5β-cholest-24-en-26-oic acid and (24R,25S)-3α,7α,12α,24-tetrahydroxy-5β-cholestan-26-oic acid from either (25R)- or (25S)-3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid by rat liver homogenate. *J. Biochem.* **96:** 1103–1107.
45. Une, M., Y. Tazawa, K. Tada, and T. Hoshita. 1987. Occurrence of both (25R)- and (25S)-3α,7α,12α-trihydroxy-5β-cholestanoic acids in urine from an infant with Zellweger's syndrome. *J. Biochem.* **102:** 1525–1530.
46. Yousef, I. M., J. Ho, and K. N. Jeejeebhoy. 1978. Bile acid synthesis in isolated rat hepatocytes. *Can. J. Biochem.* **56:** 780–783.
47. Whiting, M. J., and A. M. Edwards. 1979. Measurement of cholic acid synthesis and secretion by isolated hepatocytes. *J. Lipid Res.* **20:** 914–918.
48. Botham, K. M., G. J. Beckett, I. W. Percy-Robb, and G. S. Boyd. 1980. Bile acid synthesis in isolated rat liver cells. The effect of 7α-hydroxycholesterol. *Eur. J. Biochem.* **103:** 299–305.
49. Buscher, H-P., W. Gerok, G. Kurz, U. Schramm, and H. Thom. 1989. Hepatocyte primary culture and bile salt transport. *In* Trends in Bile Acid Research. G. Paumgartner, A. Stiehl, and W. Gerok, editors. Kluwer Academic Publishers, Dordrecht, Boston, London. 133–142.