3-Diazirine-derivatives of bile salts for photoaffinity labeling

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Abstract
New carbene-generating photolabile bile salt derivatives, 3,3-azo-7α,12α-dihydroxy-5β[3H]cholan-24-oic acid and (3,3-azo-7α,12α-dihydroxy-5β[3H]cholan-24-0yl)-2-aminoethanesulfonic acid were synthesized with high specific radioactivity. These 3-diazirine-derivatives could be activated to the corresponding carbenes by irradiation with ultraviolet light at 350 nm with a half-life time of 2 min. The 3-diazirine derivatives behaved in enterohepatic circulation like the natural bile salts. The uptake of [3H]taurocholate into isolated hepatocytes was competitively inhibited by (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-0yl)-2-aminoethanesulfonic acid indicating that the 3,3-azo-derivative of taurocholate shares the hepatic transport systems for natural bile salts. It was demonstrated that the radioactively labeled 3-diazirine bile salt derivatives are useful probes for photoaffinity labeling of bile salt binding proteins especially in intact cells and tissues.

EXPERIMENTAL

Materials
Cholic acid, taurine, silica gel N 60 (40-63 μm) and silica plates for analytic and preparative thin-layer chromatography were obtained from Merck (Darmstadt, E R. G.). Sodium borohydride (5-10 Ci/mmol) was obtained from Amersham Buchler GmbH (Braunschweig, E R. G.). All other chemicals were purchased in best quality from the usual commercial sources.

Methods
Elemental analyses were carried out with a Perkin-Elmer 240 analyzer (Perkin-Elmer, Friedrichshafen, F. R. G.). 1H-NMR-spectra were recorded on a Bruker 250 MHz spectrometer Spectrospin WM 250 (Bruker Physics, Karlsruhe, F. R. G.). Values are given in parts per million relative to tetramethylsilane as internal standard. Mass spectra were measured with a Finnigan MAT 445 (Finnigan MAT GmbH, Bremen, F. R. G.) mass spectrometer. Photolyses were carried out in a Rayonet photochemical reactor RPR 100 (The Southern Ultraviolet Company, Hamden, Conn., USA) equipped with 16 RPR 3500 A lamps using the cuvettes and techniques described (2, 4, 11).

Bile acid derivatives were detected on thin-layer chro-

Photolabile bile salt derivatives (1) have been successfully used for the identification of bile salt transport systems in blood (2), liver (3-7), small intestine (8,9), and kidney (10). The labeling pattern of the respective bile salt binding polypeptides in the different tissues is dependent upon the chemical structure of the utilized derivative. These differences are caused by the different reactivities of the photogenerated carbenes and nitrenes and the position of the photoactive group within the bile salt molecule. Therefore, a set of different carbene- and nitrene-generating photolabile derivatives with the photolabile groups at different positions of the bile salt molecule is necessary to ensure that no bile salt-binding protein will escape detection. The 7-diazirino-bile salt derivatives have short half-life times under irradiation at 350 nm of about 2 min, compared to 8.5-18 min for the 3- and 11-azido-derivatives upon irradiation at 300 nm (1). Due to their short half-life time upon photolysis the diazirine-derivatives are especially suited for photoaffinity labeling of intact cells and tissues (6,11,12), because short irradiation times are desirable to avoid photolytic damage of cells. In the present study we describe the synthesis of new diazirine-derivatives of bile salts with the photolabile diazirino-group attached at position 3 of the steroid nucleus.

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matograms by spraying the plates with conc. sulfuric acid and then heating at 80°C for 5 min.

Hepatocytes were isolated according to Berry and Friend (13). Uptake studies into isolated hepatocytes were performed by the rapid centrifugation method as described (14,15). For perfusion studies the respective bile salt derivatives (1-2 μCi dissolved in 200 μl of 0.9% sodium chloride solution) were injected into the upper mesenterial vein of anesthetized rats and bile was collected from the cannulated common bile duct. Radioactivity in bile samples was determined by liquid scintillation counting. The composition of bile salts in the bile samples was analyzed by thin-layer chromatography and subsequent radiochromatography scanning.

For photoaffinity labeling freshly prepared hepatocytes (1-2 × 10⁶ hepatocytes in 1-2 ml of 118 mM sodium/potassium phosphate buffer (pH 7.4), 4.74 mM KCl, 24.87 mM NaHCO₃, 1.185 mM MgCl₂, 1.25 mM CaCl₂, 5.5 mM D-glucose were incubated for 1 min with radioactive-labeled bile salt derivatives in the dark and subsequently photolyzed for 5 min at a wavelength of 350 nm in a Rayonet RPR-100 photochemical reactor. After irradiation the cells were diluted with 10 mM sodium phosphate buffer (pH 7.4), and after a further 10-min incubation the osmotically shocked cells were centrifuged at 15,000 g for 30 min. For analysis of incorporated radioactivity, the resulting pellets were dissolved in 100 μl of 62.5 mM Tris-HCl buffer (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.001% bromophenol blue, and polypeptides were separated by SDS gel electrophoresis on slab gels (180×350 mm) (9).

After fixing and staining, the gels were scanned and the distribution of radioactivity was determined either by fluorography (16) with sodium salicylate as scintillator or by liquid scintillation counting of the gels after slicing of the gel tracks into 2-mm pieces and digestion of proteins with Bio-lute (Zinsser Analytic GmbH, Frankfurt/F. R. G.).

**Syntheses**

The solvent systems used for chromatographic separations were: solvent system 1: ethylacetate-cyclohexane-acetic acid 23:7:3; solvent system 2: ethylacetate-cyclohexane-acetic acid 100:40:1; solvent system 3: ethylacetate-cyclohexane-acetic acid 200:20:1; solvent system 4: n-butanol-acetic acid-water 9:2:1.

**3α-Hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid**

Two g (4.05 mmol) of 3α,7α,12α-triformyloxy-5β-cholan-24-oic acid (17) was dissolved in 20 ml of dry methanol. Five ml of a 1 M methanolic solution of potassium hydroxide was added and after 8 min of vigorous stirring at room temperature the reaction mixture was poured into 400 ml of 1 M HCl. The precipitate was collected by filtration yielding 1.8 g (3.8 mmol) of 3α-hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid, which was recrystallized from ethanol/water. Yield: 93.8%; mp: 180-181°C. TLC: \( R_f = 0.66 \) (solvent system 1), 0.25 (solvent system 2), 0.24 (solvent system 3). \( ^1H-NMR \) (CDCl₃): \( \delta = 0.75 \) (s, \( CH_3 \)), 0.85 (d, \( CH_3 \)), 0.93 (s, \( CH_3 \)), 3.53 (m, \( CH = CH \)), 5.06 (s, \( CH-OCHO-7 \)), 5.28 (s, \( CH=OCHO-12 \)), 5.15 (s, \( CH-OCHO-12 \)), 8.15 (s, \( CH=OCHO-H \)). Anal. calcd. for \( C_{36}H_{48}O_8 \): C, 87.22, H, 8.68; found: C, 87.14, H, 8.75.

**3-Oxo-7α,12α-diformyloxy-5β-cholan-24-oic acid**

3α-Hydroxy-7α,12α-diformyloxy-5β-cholan-24-oic acid (3.72 g, 8 mmol) was dissolved in 400 ml of 80% aqueous acetone and subsequently 1.7 equivalents (13.6 mmol, 2.42 g) of solid N-bromosuccinimide were added. The solution immediately became orange and after about 2 h the orange color disappeared. Thin-layer chromatographic monitoring using solvent system 2 revealed that the reaction was complete. The solution was concentrated by evaporation to about 50 ml and after addition of 20 ml of dimethylsulfoxide the bile acid was precipitated from 500 ml of 1 M HCl. Yield: 3.6 g (7.8 mmol), 97.5%. The compound was recrystallized from ethanol/water. mp: 160-162°C. TLC: \( R_f = 0.73 \) (solvent system 1), 0.42 (solvent system 2), 0.32 (solvent system 3). \( ^1H-NMR \) (CDCl₃): \( \delta = 0.81 \) (s, \( CH_3 \)), 0.87 (d, \( CH = CH \)), 1.05 (s, \( CH_3 \)), 3.02 (CH=OCHO-7), 0.52 (s, \( CH-OCHO-12 \)), 1.56 (s, \( CH=OCHO-H \)), 5.32 (s, \( CH-OCHO-12 \)), 8.10 (s, \( CH=OCHO-H \)), 8.16 (s, \( CH-OCHO-12 \)). Anal. calcd. for \( C_{36}H_{48}O_8 \): C, 67.52, H, 8.28; found: C, 66.94, H, 8.32.

**3-Oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid**

3-Oxo-7α,12α-diformyloxy-5β-cholan-24-oic acid (2.3 g, 5 mmol) was dissolved in 70 ml of a 1 M methanolic solution of potassium hydroxide at 25°C. After 1-2 h the formyl groups had been removed as monitored by thin-layer chromatography in solvent system 1. The product was precipitated from 500 ml of 1 M HCl and recrystallized from ethanol/water yielding 1.78 g (4.41 mmol) of pure 3-oxo-7α,12α-dihydroxy-5β-cholan-24-oic acid. Yield: 88.2%; mp: 174-175°C. TLC: \( R_f = 0.50 \) (solvent system 1), 0.12 (solvent system 2), 0.13 (solvent system 3). \( ^1H-NMR \) (CDCl₃): \( \delta = 0.72 \) (s, \( CH_3 \)), 0.87 (d, \( CH = CH \)), 6.98 (s, \( CH_3 \)), 3.39 (CH=2 or CH=4, \( J = 15 Hz \)), 3.92 (s, \( CH = CH \)), 4.05 (s, \( CH-H \)). Anal. calcd. for \( C_{34}H_{48}O_8 \) (406.6): C, 70.89, H, 9.42. Found: C, 70.44, H, 9.82.
lute methanol and dry ammonia was bubbled through the solution at \(-10^\circ C\) for 10 h. Subsequently a solution of 2 g \((18 \text{ mmol})\) of hydroxylamine-O-sulfonic acid in 20 ml of absolute methanol was added during 30 min. The mixture was allowed to warm up and was subsequently stirred for 20 h. After filtration 1 ml of N-triethylamine was added and the solution was evaporated to dryness. The residue was redissolved in 30 ml of absolute methanol and after addition of 1 ml of N-triethylamine solid iodine was added in 50-mg portions until the brown color persisted. The reaction mixture was evaporated to dryness, the residue was dissolved in 20 ml of dimethylsulfoxide, and the crude diazirine was precipitated from 400 ml of 1 M HCl. The product was dried and dissolved in 25 ml of ethylacetate-cyclohexane-acetic acid 100:40:1 \((v/v/v)\) and purified by flash chromatography \((18)\) on a 30 cm column of silica gel N 60 using the aforementioned solvent as eluent. The fractions containing the pure 3-diazirine were kept at \(-20^\circ C\) for 10 h. Three hundred fifty mg of pure 3,3-azo-7a,12a-dihydroxy-5\(\beta\)-cholest-24-oic acid was obtained as white crystalline needles and another 260 mg was obtained from the mother liquor by evaporation. Yield: 54%; mp: 162–164\(^\circ\)C (decomposition). TLC: \(R_f\) = 0.68 \((\text{solvent system 1})\), 0.38 \((\text{solvent system 2})\), 0.37 \((\text{solvent system 3})\).

UV (methanol): \(\lambda_{max} = 370 \text{ nm} (\epsilon = 55)\), 352 nm \((\epsilon = 62)\). Mass spectrum: \(m/e 372 (M^+ - N_2)\), \(m/e 354 (M^+ - N_2 H_2 O)\), \(m/e 253 (M^+ - N_2 H_2 O - side chain with \ m/e 101, base peak)\). \(^1\)H-NMR (CDCl/CD_{2}OD = 3/1, \(\nu /\nu\)): \(\delta = 0.73 (s, \text{CH}_3 - 18)\), 1.00 \((d, \text{CH}_2 - 21, J = 6 \text{ Hz})\), 1.02 \((s, \text{CH}_3 - 19)\), 2.91 \((\text{CH}-2 or \text{CH}-4, J = 15 \text{ Hz})\), 3.84 \((s, \text{CH}-7)\), 3.97 \((s, \text{CH}-12)\). Anal. calcd. for \(C_{24}H_{38}N_2O_4\) \((418.56)\): C, 68.86, H, 9.15, N, 6.69 found: C, 68.25, H, 9.10, N, 6.31.

The synthesis of \(3,3\)-azo-7\(\alpha\)-12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oyl)-2-aminoethanesulfonic acid was performed via the mixed anhydride method as previously described \((1,19)\) starting from 3,3-azo-7\(\alpha\)-12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid.

**Synthesis of 3,3-azo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid and (3,3-azo-7\(\alpha\),
12\(\alpha\)-dihydroxy-5\(\beta\)[7\(\beta\)-\(^3\)H]cholestan-24-oic acid and (3,3-azo-7\(\alpha\),
12\(\alpha\)-dihydroxy-5\(\beta\)[7\(\beta\]-\(^3\)H]cholestan-24-oyl)-
2-aminoethanesulfonic acid**

Two hundred nine mg \((0.5 \text{ mmol})\) of 3,3-azo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid was dissolved in 20 ml of 80\% aqueous acetone and subsequently 140 mg \((0.75 \text{ mmol})\) of N-bromosuccinimide was added. After 2–4 h the solution was evaporated to dryness at 30\(^\circ\)C. The crude product was purified by flash chromatography \((18)\) on a 15 \(\times\) 3 cm column of silica gel N 60 using ethylacetate-cyclohexane-acetic acid 100:40:1 \((\nu /\nu /\nu)\) as solvent. One hundred fifty-five mg \((0.37 \text{ mmol})\) of 3,3-azo-7-oxo-12\(\alpha\)-hydroxy-5\(\beta\)-cholestan-24-oic acid \((R_f = 0.57, \text{ solvent system 2})\) and 49 mg \((0.12 \text{ mmol})\) of 3,3-azo-7-oxo-12-oxo-5\(\beta\)-cholestan-24-oic acid \((R_f = 0.68 \text{ in solvent system 2})\) were obtained. \((3,3\text{-Azo-7-oxo-12\(\alpha\)-hydroxy-5\(\beta\)-cholestan-24-oyl})-2-
aminoethanesulfonic acid was synthesized from 3,3-azo-7-oxo-12\(\alpha\)-hydroxy-5\(\beta\)-cholestan-24-oic acid as described \((1)\).

For the synthesis of radiolabeled 3-diazirine derivatives of bile salts in unconjugated and taurine-conjugated form, 0.02 mmol of 3,3-azo-7-oxo-12\(\alpha\)-hydroxy-5\(\beta\)-cholestan-24-oic acid or its taurine conjugate was dissolved in 300 \(\mu\)l of dioxane and 30 \(\mu\)l of 1 M aqueous sodium hydroxide. This solution was added to 100 mCi of sodium boron\(^3\)H]-hydride \((5.2 \text{ Ci/mmold})\). After 12 h at 20\(^\circ\)C in the dark, 50 \(\mu\)l of 2 M HCl was added and after a further 2 h the reaction mixture was put on preparative high performance thin-layer chromatography plates \((20 \text{ cm} \times 5 \text{ cm} \times 1 \text{ mm})\). The chromatograms were developed in solvent system 2 for the unconjugated and solvent system 4 for the taurine-conjugated derivatives. The compounds were extracted as described \((1)\) yielding 10–15 mCi of the corresponding photolabile salt derivatives with a specific radioactivity of 1.3 Ci/mmold.

**RESULTS**

**Synthesis of 3,3-azo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid**

For the synthesis of 3,3-azo-derivatives of bile salts, the corresponding 3-oxo-compounds are necessary as starting material. 3-Oxo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid has been synthesized from cholic acid methylester by Oppenauer-oxidation \((20)\) or by oxidation with silver carbonate on Celite \((21,22)\) and subsequent alkaline hydrolysis of the resulting 3-oxo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid methylester. However, during alkaline hydrolysis of the 3-oxo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid methylester obtained with these procedures, the 3-oxo-group was destroyed and only tiny amounts \((<5\%)\) of 3-oxo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid could be isolated after chromatography; this vulnerability of the 3-oxo group in bile acids during alkaline conditions was also found by others \((23)\). Therefore, 3-oxo-7\(\alpha\),12\(\alpha\)-dihydroxy-5\(\beta\)-cholestan-24-oic acid was synthesized by a route similar to that of Leppick \((24)\). Triformyloxycholic acid was selectively deformylated at position 3 to give 3-hydroxy-7\(\alpha\),12\(\alpha\)-dif-
lution and subsequent oxidation of the 3-diaziridine with 
iodine as described (1,25). In contrast to the synthesis of 
7,7-azo-derivatives of bile salts, this reaction step from the 
3-oxo to the 3-diazirine-derivatives (1) is extremely sensi-
tive to moisture. The formation of the 3-diazirine 
occurring only with absolutely dry compounds and solvents.
The 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was 
characterized by elemental analysis, UV, NMR, and 
mass spectrometry. 3,3-Azo-7α,12α-dihydroxy-5β-cholan-
24-oic acid shows the typical ultraviolet absorption of the 
diazirino-function with a doublet at 370 and 352 nm 
(1,25,26). The NMR-spectrum proved the structure of 
3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid; the typical 
signals of the H-atoms at position 7 (δ = 3.84) and position 
12(δ = 3.97) were present, whereas no proton signal 
at δ = 3.30 was observed, since the 3α-hydroxyl group and 
the H-atom at 3β-position are replaced by the 3,3-
azo-function. As with the 7,7-azo-isomere (1) no mole-
cular ion peak at m/e 418 could be detected in the mass 
spectrum of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid. 
The fragmentation pattern was very similar to that of the 
7-isomer with loss of Nα and H2O molecules (fragments 
m/e 372 and 354) and of the side chain (m/e 101) leading 
to the base peak m/e 253. Photolysis of 3,3-azo-7α,12α-di-
hydroxy-5β-cholan-24-oic acid at 350 nm in a Rayonet 
photochemical reactor revealed a half-life time about 2 
min under the conditions used (Fig. 1).

For the preparation of radioactively labeled 3,3-azo-
7α,12α-dihydroxy-5β-cholan-24-oic acid and its taurine 
conjugate, the 7α-hydroxy group was oxidized with N-bro-
mosuccinimide in 80% aqueous acetone to give the corre-
sponding 7-oxo-derivatives. Radioactivity was introduced 
by reduction of the 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-
24-oic acid and its taurine conjugate with sodium boro-
[3H]hydride in dioxane water (1). The reduction of 7-oxo-
derivatives of bile salts with sodium borohydride leads 
nearly completely to the 7α-hydroxy-derivative (27,28).
After reduction of 3,3-azo-7-oxo-12α-hydroxy-5β-cholan-
24-oic acid with sodium borohydride, only one radio-
actively labeled band comigrating with 3,3-azo-7α,12α-di-
hydroxy-5β-cholan-24-oic acid was detectable after thin-
layer chromatography of the reaction mixture in different 
solvent systems. A further confirmation of the structure of 
3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid was given 
by control experiments where 7-oxo-3α,12α-dihydroxy-
5β-cholan-24-oic acid and 3,3-azo-7-oxo-12α-hydroxy-
5β-cholan-24-oic acid were reduced with sodium borohy-
dride. After purification of the reaction products by prepa-
trative thin-layer chromatography, the signal of the 
H-atom at position 7 in the 1H-NMR-spectra was ob-
erved at δ = 3.84 indicating an equitorial orientation of 
the H-atom and an axial configuration of the 7-hydroxy-
group (i.e. α-position); a 7β-hydroxyl-derivative 
have given a signal of the 7-H atom at δ = 3.58 (28).
Therefore, reduction of 3,3-azo-7-oxo-derivatives of bile 
salts with Na[3H]BH4 yields photoreactive bile salt deriv-
atives with α-orientation of the hydroxyl groups at posi-
tions 7 and 12 as in the natural bile salts. Photoaffinity 
labeling experiments with hepatocyte membranes using 
the 12α-azo-derivatives and the 12β-azo-derivatives of 3β-
azido- and 7,7-azo-derivatives of cholate and taurocholate 
revealed no significant differences in the labeling pattern 
(W. Kramer, unpublished results).

**Photoaffinity labeling**

In order to determine whether the new photolabile 
3,3-azo-derivatives of bile salts behave like the natural bile 
salts, [14C]taurocholate and (3,3-azo-7α,12α-dihydroxy-
5β[7β,3H]cholan-24-oyl)-2-aminoethanesulfonic acid were 
simultaneously injected into the superior mesenteric vein 
of an anesthesized rat after cannulation of the common 
bile duct. Both bile acids appear in bile with a maximum 
of secretion of taurocholate at 4 min and at 6 min for 
(3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethane-
sulfonic acid (Fig. 2). During a bile collection period of 60 
min, 87% of (3,3-azo-7α,12α-dihydroxy-5β[7β,3H]cholan-
24-oyl)-2-aminoethanesulfonic acid and 93% of [14C]tau-
rocholate appeared in bile. The slightly retarded excre-
tion of bile salts without a 3-hydroxyl group was formerly 
observed with 3β-azo-derivatives of taurocholate (29,30).
After installation of (3,3-azo-7α,12α-dihydroxy-5β[7β,3H]
cholan-24-oyl)-2-aminoethanesulfonic acid into the lumen 
of the terminal ileum, the compound also appeared 
in bile, indicating that this substance is also absorbed 
from the small intestine similar to the natural bile salts.
Thin-layer chromatography of the respective bile samples 
revealed that the 3,3-azo-derivative of taurocholate 
underwent the enterohepatic circulation without metabolic

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**Fig. 1.** Photolysis of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid. A solution of 1.14 mg of 3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oic acid in 1 ml of methanol was photolyzed in a Rayonet RPR 100 photochemical reactor equipped with 16 RPR 3500 Å lamps for 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, and 8 min. The absorption of the diazirino group was recorded after the respective times of photolysis.
Fig. 2. Biliary excretion of (3,3-azo-7α,12α-dihydroxy-5β-[13H]cholan-24-oyl)-2-aminoethanesulfonic acid and [14C]taurocholate. Two μCi of (3,3-azo-7α,12α-dihydroxy-5β-[13H]cholan-24-oyl)-2-aminoethanesulfonic acid (O) and 1 μCi [14C]taurocholate (■) dissolved in 200 μl 0.9% sodium chloride solution was injected into the superior mesenteric vein of an anesthetized rat. Bile was collected at 2-min intervals and radioactivity was determined by liquid scintillation counting.

transformation like taurocholate. Fig. 3 shows that the Na⁺-dependent uptake of [3H]taurocholate into isolated hepatocytes was competitively inhibited by (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid, indicating that the photoreactive derivative shares common uptake systems with taurocholate.

With photolabile bile salt derivatives carrying nitrene-generating aliphatic azido groups at positions 3 or 11 and a carbene-generating diazirino group at position 7, bile salt-binding proteins in different tissues have been characterized (2-12,29,31). Due to their short half-life time of about 2 min compared to 8-18 min for the azido-derivatives (1,29) the 7,7-azo-derivatives are especially suited for the labeling of intact cells and living tissue (11,12). In sinusoidal surfaces of hepatocytes, two polypeptides of molecular weights 54,000 and 48,000 were identified as putative bile salt transport systems (3-6). The 54,000 molecular weight polypeptide was predominantly labeled with the (7,7-azo-3α-12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid, whereas the 3- and 11-azido derivatives led to highest labeling of the polypeptide of molecular weight 48,000. Similar differences in the labeling pattern of bile salt-binding proteins by 7-diazirine and 3- or 11-azido bile salt derivatives were observed in the small intestine (9) and in blood (2).

In order to determine whether the different chemical reactivities of carbenes and nitrenes or the position of the photolabile group within the bile salt molecule are predominantly responsible for these differences in the labeling pattern, the labeling of bile salt-binding polypeptides in isolated hepatocytes was chosen as an example to demonstrate that by use of only one photolabile substrate analogue some binding polypeptides may escape detection. For the identification of bile salt-binding polypeptides in hepatocyte membranes, freshly prepared isolated hepatocytes were submitted to photoaffinity labeling with 3,3-azo- and 7,7-azo-derivatives of taurocholate. After photoaffinity labeling the cells were washed with hypotonic medium, and after separation from soluble proteins by centrifugation the resulting pellets containing cell organelles were submitted to SDS gel electrophoresis.

Fig. 3. The effect of (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid on the Na⁺-dependent uptake of [3H]taurocholate into isolated hepatocytes. Freshly prepared isolated hepatocytes in 118 mM sodium/potassium phosphate buffer (pH 7.4), 4.74 mM KCl, 24.87 mM NaHCO₃, 1.185 mM MgCl₂, 1.25 mM CaCl₂, 5.5 mM D-glucose were incubated at 37°C with the indicated concentrations of [3H]-taurocholate in the absence (O) and in the presence (■) of 100 μM (3,3-azo-7α,12α-dihydroxy-5β-cholan-24-oyl)-2-aminoethanesulfonic acid. Under the conditions used the inhibition constant Kᵢ was 53 μM. [TC]: concentration of taurocholate.
Fig. 4A shows a fluorogram of these fractions after photoaffinity labeling of hepatocytes either with (3,3-azo-7α,12α-dihydroxy-5β[7β-3H]cholan-24-oyl)-2-aminoethanesulfonic acid (track a) or (7,7-azo-3α,12α-dihydroxy-5β[3β-3H]cholan-24-oyl)-2-aminoethanesulfonic acid (track b). With the 7,7-azo-derivative predominantly the bile salt-binding polypeptide of molecular weight 54,000 of the hepatocyte membrane was labeled, whereas the membrane polypeptide of molecular weight 48,000 was only slightly labeled; a predominant labeling of the mitochondrial polypeptide of molecular weight 33,000 was also found. Minor labeled bands with apparent molecular weights of 100,000, 95,000, 75,000, 67,000, and 24,000 were also detected. In contrast, labeling of the same hepatocyte preparation with the 3,3-azo-isomere resulted in a markedly different labeling pattern. (3,3-Azo-7α,12α-dihydroxy-5β[7β-3H]cholan-24-oyl)-2-aminoethanesulfonic acid led to a predominant labeling of the Na+-dependent bile salt carrier protein of molecular weight 48,000–49,000 (3,4,32); the bile salt-binding membrane protein of molecular weight 54,000—the predominantly labeled polypeptide by the 7,7-azo-derivative of taurocholate and assumed to be a component of the Na+-independent bile salt transport system (31)—was labeled to a much lesser extent than the polypeptide of molecular weight 48,000. The mitochondrial polypeptide of molecular weight 33,000 was hardly labeled whereas predominant labeling of a hitherto unidentified bile salt binding polypeptide of molecular weight 24,000 occurred. Labeled polypeptides with apparent molecular weights of 100,000, 95,000, 75,000, and 67,000 were also detected.

In order to evaluate the specificity of the labeled polypeptides for bile salts, competition photoaffinity labeling experiments of isolated hepatocytes in the presence of un-
labeled taurocholate were performed either with the 3,3-azo- or the 7,7-azo-derivatives of taurocholate (Fig. 4B). For detection of radioactivity in the labeled polypeptides both fluorography and liquid scintillation counting of the gels after slicing were used. The presence of unlabeled taurocholate predominantly reduced the labeling of the bile salt-binding proteins having molecular weights of 48,000 and 54,000 independently whether the 3,3-azo- or the 7,7-azo-derivative of taurocholate was used as photoprobe; the labeling of the polypeptides of molecular weight 67,000 (identified as membrane-bound albumin (4)) and 75,000 (presumably proalbumin as indicated from immunoprecipitation experiments) was also reduced by taurocholate. The labeling of the polypeptides of molecular weights 95,000 and 100,000, the latter possibly from immunoprecipitation experiments) was also reduced by taurocholate. The extent of labeling of the predominantly labeled polypeptide when the 7,7-azo-derivative was used, was also decreased by taurocholate, but to a lesser extent than the abovementioned polypeptides. The labeling of the hitherto unidentified polypeptide of molecular weight 24,000 which was very strongly labeled by (3,3-azo-7a,12a-dihydroxy-5β[7β-3H]-cholan-24-yl)-2-aminothanesulfonic acid, was decreased by the presence of taurocholate; the labeling of this polypeptide was less sensitive to the presence of taurocholate than the labeling of the other polypeptides and for a clear reduction in the extent of labeling, significant higher concentrations of taurocholate were necessary. In order to determine whether the hitherto unidentified bile salt-binding polypeptide of molecular weight 24,000 may be a further component of bile salt transport systems of the hepatocyte sinusoidal membrane, subfractions enriched with sinusoidal surfaces (4) were submitted to photoaffinity labeling with (3,3-azo-7a,12a-dihydroxy-5β[7β-3H]-cholan-24-yl)-2-aminothanesulfonic acid. The bile salt carrier protein of molecular weight 48,000 and membrane-bound albumin (M, 67,000) were the predominantly labeled polypeptides whereas the polypeptide of molecular weight 54,000 was only slightly labeled; no significant labeling of the polypeptides having molecular weights of 33,000 and 24,000 occurred. Therefore, from these experiments it is unlikely that the hitherto unidentified bile salt-binding polypeptide of molecular weight 24,000 is a component of the hepatocyte sinusoidal membrane.

The labeling experiments presented here clearly demonstrate that for photoaffinity labeling studies a set of different photolabile derivatives with the photolabile group attached at different positions of the molecule is necessary. Only the use of such a set of photoprobes minimizes the possibility that a bile salt-binding protein will escape detection. The labeling of hepatocytes has been chosen as an example for such differences in the labeling patterns. Similar effects on the labeling pattern between 3,3-azo- and 7,7-azo-derivatives of taurocholate were found in labeling experiments of human serum, brush border membrane vesicles from rat small intestine and kidney (34), and basolateral membranes from rat renal cortex (35,36). In conclusion, the carbene-generating 3-diazirine-derervatives of bile salts described here are useful new photoprobes for the identification of bile salt binding proteins.

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REFERENCES


