Preparation and Comparative Properties of Antisera against Glyco-3β,12α-dihydroxy-5-cholen-24-oic Acid Linked to Bovine Serum Albumin at the C-3, C-15α, and C-24 Positions

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Anti glyco-3β,12α-dihydroxy-5-cholen-24-oic acid antisera were prepared by immunizing rabbits with hapten–bovine serum albumin (BSA) conjugates coupled at the C-3, C-15α, and C-24 positions on the bile acid molecule, and their properties were investigated by heterologous combination assay using ^{125}I-labeled tracer. The antiserum raised against the C-3 BSA conjugate showed poor titer and specificity, while the antisera from the other two conjugates showed satisfactorily high affinity constants ($K_a = 5.0 \times 10^8$ and 7.0×10^8 M^{-1}) and reasonable specificity, exhibiting negligible cross-reactivities with other major human bile acids and cholesterol. Among the unsaturated bile acids tested, high reactivity was observed with tauro-3β,12α-dihydroxy-5-cholen-24-oic acid, which suggested that bridge phenomena were significant in this assay system.

Keywords glyco-3β,12α-dihydroxy-5-cholen-24-oic acid; radioimmunoassay; bile acid; hapten synthesis; heterologous combination immunoassay; ^{125}I-label immunoassay

Oxidative cleavage of the cholesterol side chain is an important transformation in bile acid biosynthesis. Since this cleavage precedes the transformation of the ring structure initiated by 7α-hydroxylation of cholesterol, the key intermediate of this pathway is 3β-hydroxy-5-cholen-24-oic acid (Δ^5-3β-ol), which is converted in part to chenodeoxycholic acid.[1] Recently, another unsaturated bile acid, 3β,12α-dihydroxy-5-cholen-24-oic acid (Δ^5-3β,12α-diol), which may be a metabolite of Δ^5-3β-ol, has been found in urine of healthy subjects,[2] pregnant women,[3] and also patients with liver disease.[3-5] Bremmelgaard and his colleagues have reported a positive correlation in the urinary excretion of these compounds,[4] indicating a metabolic relationship. These unsaturated bile acids, therefore, are of interest as regards both their contents in biological fluids and their metabolic fate in the human body. Measurements of Δ^5-3β-ol content in body fluids in relation to hepatobiliary diseases have been extensively described in the literature,[6] but little is known about its metabolite, Δ^5-3β,12α-diol. Accordingly, the plasma concentration of Δ^5-3β,12α-diol in normal subjects and in patients with hepatobiliary disease became of considerable interest. Because this bile acid was present at a concentration of less than 10 ng/ml in plasma of healthy subjects,[5] a highly sensitive assay procedure such as radioimmunoassay (RIA) was considered to be the most feasible method of measurement, and a specific assay had to be developed.

A suitable hapten and a radiolabeled ligand with high specific activity were required for such an assay. In general, preparation of a bile acid immunogen is done by utilizing the C-24 carboxyl group on the side chain to form an amide bond with a protein. The strategy to obtain antibody specific to the glyco-Δ^5-3β,12α-diol is to bind the carrier protein to a point a long way from the characteristic functional groups of the molecule. Therefore, the C-3, C-15 and C-24 positions were selected as sites suitable for conjugation with the protein, since these positions leave functional groups at the A ring or the side chain available for recognition by antibody. It was also necessary to prepare ^{125}I-labeled tracer for the RIA because of the unavailability of a tritiated tracer. For these reasons, immunogens in which the haptenic derivatives are coupled with bovine serum albumin (BSA) at C-3, C-15α and C-24 were prepared, and the comparative properties of the antisera raised by administration of these immunogens were investigated by the ^{125}I-label assay.

Materials and Methods

Chemicals and Reagents Na[^{125}I] (17.4 Ci/mg) was purchased from New England Nuclear Co. (Boston, MA, U.S.A.). Bovine serum albumin (BSA, Fraction V) and complete Freund's adjuvant were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and Iatron Laboratories (Tokyo, Japan), respectively. The reference bile acids were either synthesized in our laboratory[5,7] or were obtained commercially and purified to homogenity by chromatography and/or recrystallization. All organic solvents and chemicals were of analytical reagent grade.

Apparatus All melting points were determined with a Mitamura micro hot-stage apparatus, and are uncorrected. Optical rotations were measured with a Union Giken-201 polarimeter. Ultraviolet (UV) spectra were recorded with a Shimadzu model UV-200 spectrometer. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded with Hitachi model R-40 spectrometer (90 MHz) in $CDCl_3$ unless otherwise stated. Chemical shifts are given as the δ value with tetramethylsilane as the internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). Infrared (IR) spectra were obtained using a Jasco IR A-102 spectrometer and are expressed in cm^{-1}. Column chromatography was performed with Kieselgel 60 (70—230 mesh, E. Merck). All organic solvent extracts were dried over anhydrous Na_2SO_4.

Methy *N*-(3β-Hemisuccinyloxy-12α-hydroxy-5-cholen-24-oyl)glycinate (1c) A solution of methyl 3β,12α-dihydroxy-5-cholen-24-oylglycinate[5] (**1b**, 200 mg) and succinic anhydride (200 mg) in pyridine (2 ml) was heated at 90°C for 1 h. After evaporation of pyridine *in vacuo*, the residue was dissolved in ether and the insoluble material was removed by filtration. The filtrate was washed with H_2O and dried. Evaporation of the solvent gave a crude product, which was chromatographed using benzene–acetone (3:1) as the eluent to give **1c** (130 mg, 53%). Colorless needles (MeOH), mp 191—192°C, $[\alpha]_D^{25}$ −13.6° (c=0.36, MeOH). IR (Nujol): 3380 (OH), 1720 (CO), 1700 (CO). ^{1}H-NMR (CD_3OD): 0.74 (3H, s, 18-CH_3), 1.03 (3H, s, 19-CH_3), 2.54 (4H, m, $COCH_2CH_2CO$), 3.67 (3H, s, $COOCH_3$), 3.88 (2H, s, $NHCH_2COO$), 3.98 (1H, m, 12β-H), 4.50 (1H, m, 3α-H), 5.35 (1H, m, 6-H). *Anal.* Calcd for $C_{31}H_{47}NO_8$: C, 65.23; H, 8.57. Found: C, 65.26; H, 8.59.

Methyl 3α,12α-Diacetoxy-15-oxo-5β-chol-8(14)-en-24-oate (3a) H_2O (20 ml), $CaCO_3$ (3 g), and *N*-bromosuccinimide (12 g) were added to a solution of methyl 3α,12α-diacetoxy-5β-chol-8(14)-en-24-oate[8] (**2**, 20 g) in tetrahydrofuran (THF) (30 ml), and the whole mixture was stirred at 0°C for 1 h under irradiation from a tungsten lamp (500 W). The reaction mixture was poured into ice-water, and the resulting solution was extracted with ether. The extract was washed with H_2O, dried, and evaporated *in vacuo*. Recrystallization of the product from MeOH gave **3a** (12.5 g, 63%). Colorless needles, mp 164—165°C, $[\alpha]_D^{25}$ +189.3° (c=0.81, $CHCl_3$). IR (Nujol): 1725 (CO), 1690 (CO), 1627 (C=C). ^{1}H-NMR: 0.84 (3H, s, 18-CH_3), 0.90 (3H, d, J=6 Hz, 21-CH_3), 1.03 (3H, s, 19-CH_3), 2.01 and 2.06 (each 3H, s, $OCOCH_3$) 3.64 (3H, s, $COOCH_3$), 4.06 (1H, m, 7-H), 4.75

(1H, m, 3β-H), 5.02 (1H, m, 12β-H). UV λ_{max}^{EtOH} nm (ε): 259 (16500). *Anal.* Calcd for $C_{29}H_{42}O_7$: C, 69.79; H, 8.42. Found: C, 69.92; H, 8.46. (lit.[8] mp 156—159°C).

3α,12α-Dihydroxy-15-oxo-5β-chol-8(14)-en-24-oic Acid (3b) A 2 N LiOH solution (70 ml) was added to a solution of **3a** (15 g) in MeOH (150 ml) and the whole mixture was refluxed for 3 h. The reaction mixture was acidified with 2 N HCl (70 ml) and methanol was evaporated off *in vacuo*. The resulting solution was extracted with ethyl acetate and the extract was washed with saturated NaCl, dried, and evaporated *in vacuo*. Recrystallization of the product from aqueous MeOH gave **3b** (11.6 g, 96%). Colorless plates, mp 210—211°C. IR (Nujol): 3300 (OH), 1680 (CO), 1635 (C=C). ^{1}H-NMR: 0.85 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 1.00 (3H, d, J=6 Hz, 21-CH_3), 3.50 (1H, m, 3β-H), 4.05 (1H, m, 12β-H). *Anal.* Calcd for $C_{24}H_{36}O_5$: C, 71.25; H, 8.79. Found: C, 71.25; H, 8.97. (lit.[8] mp 188—193°C).

Methyl 3α,12α,15α-Trihydroxy-5β-cholan-24-oate (4a) Li (1 g) was added to liquid ammonia (200 ml) at −50°C, and the mixture was stirred for 5 min. A solution of **3b** (5 g) in THF (10 ml) was added to this solution, and the reaction mixture was stirred for 1 h. After addition of ammonium chloride (5 g) to decompose the excess reagent, the ammonia was vaporized at room temperature. The resulting solution was diluted with MeOH (100 ml), and 30% MeOH–HCl (30 ml) was added. The whole mixture was refluxed for 10 min. After evaporation of MeOH, the residue was dissolved in ethyl acetate. This solution was washed with saturated NaCl, dried, and evaporated *in vacuo*. Recrystallization of the product from aqueous MeOH gave **4a** (3.2 g, 64%). Colorless needles, mp 265—267°C, $[\alpha]_D^{25}$ +80.6° (c=0.65, MeOH). IR (Nujol): 3300 (OH), 1720 (CO). ^{1}H-NMR (pyridine-d_5): 0.82 (3H, s, 18-CH_3), 0.97 (3H, s, 19-CH_3), 3.61 (3H, s, $COOCH_3$), 3.85 (1H, m, 3β-H), 4.00—4.30 (2H, m, 12β- and 15β-H). MS m/z: 404 (M^+-H_2O), 386 (M^+-2H_2O), 368 (M^+-3H_2O). (lit.[9] 256—259°C).

Methyl 3α,12α,15α-Triacetoxy-5β-cholan-24-oate (4b) Acetic anhydride (1 ml) and 4-dimethylaminopyridine (25 mg) were added to a solution of **4a** (100 mg) in pyridine (1 ml), and the whole was stirred at 0°C for 1 h. After addition of H_2O, the resulting solution was extracted with ether. The extract was washed with 2 N HCl, 5% $NaHCO_3$ and H_2O, dried, and evaporated *in vacuo* to give a crude product, which was then chromatographed. Elution with *n*-hexane–ethyl acetate (4 : 1) gave **4b** (98 mg, 76%). Colorless needles (MeOH), mp 126—127°C, $[\alpha]_D^{25}$ +110.4° (c=0.51, $CHCl_3$). ^{1}H-NMR: 0.81 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 2.02 (6H, s, 2×$OCOCH_3$), 2.13 (3H, s, $OCOCH_3$), 3.61 (3H, s, $COOCH_3$), 4.50—4.90 (2H, m, 3β- and 15β-H), 4.99 (1H, m, 12β-H). *Anal.* Calcd for $C_{31}H_{44}O_8$: C, 67.85; H, 8.82. Found: C, 67.94; H, 8.89.

Methyl 12α,15α-Diacetoxy-3-oxo-5β-cholan-24-oate (5a) A mixture of **4a** (2 g) and 50% Ag_2CO_3 on Celite (15 g) in toluene (500 ml) was refluxed for 24 h. After removal of the insoluble material by filtration, the filtrate was concentrated *in vacuo*. The residue was treated with acetic anhydride (1 ml) and 4-dimethylaminopyridine (500 mg) in pyridine (5 ml) at 0°C for 1 h. After addition of H_2O, the resulting mixture was extracted with ether. The extract was washed with 2 N HCl, 5% $NaHCO_3$, and H_2O, dried, and evaporated *in vacuo* to give a crude product, which was then chromatographed. Elution with *n*-hexane–ethyl acetate (4 : 1) gave **5a** (1.92 g, 78%). Colorless needles (MeOH), mp 168—170°C, $[\alpha]_D^{25}$ +101° (c=0.89, $CHCl_3$). IR (KBr): 1735 (CO), 1715 (CO). ^{1}H-NMR: 0.83 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.02 and 2.09 (each 3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.84 (1H, m, 15β-H), 5.05 (1H, m, 12β-H). *Anal.* Calcd for $C_{29}H_{44}O_7$: C, 69.02; H, 8.79. Found: C, 68.79; H, 8.75.

Methyl 12α,15α-Diacetoxy-4β-bromo-3-oxo-5β-cholan-24-oate (5b) A solution of 0.5 M Br_2 in AcOH (75 ml) was added to a solution of **5a** (1.8 g) in AcOH (25 ml) with stirring at room temperature, and the mixture was stirred for 30 min then poured into ice-water. The resulting solution was extracted with ethyl acetate. The extract was washed with 0.1 N NaOH and saturated NaCl, and dried. Evaporation of the solvent gave a crude product, which was then chromatographed. Elution with *n*-hexane–ethyl acetate (3 : 1) gave **5b** (1.9 g, 91%). Colorless needles (MeOH), mp 220—221 °C, $[\alpha]_D^{25}$ +108.4° (c=0.76, $CHCl_3$). IR (KBr): 1720 (C=O). ^{1}H-NMR: 0.77 (3H, d, J=6 Hz, 21-CH_3), 0.83 (3H, s, 18-CH_3), 1.06 (3H, s, 19-CH_3), 2.02 and 2.07 (each 3H, s, $OCOCH_3$), 3.62 (3H, s, $COOCH_3$), 4.84 (1H, m, 15β-H), 4.93 (1H, d, J=13 Hz, 4α-H), 5.03 (1H, m, 12β-H). *Anal.* Calcd for $C_{29}H_{43}BrO_7$: C, 59.68; H, 7.43. Found: C, 59.46; H, 7.38.

Methyl 12α,15α-Diacetoxy-3-oxo-4-cholen-24-oate (6) A solution of **5b** (1.9 g) and LiCl (1 g) in dimethylfomamide (DMF) (35 ml) was heated at 100 °C for 1 h. The reaction mixture was poured into water and the resulting solution was extracted with ethyl acetate. The extract was washed with saturated NaCl, dried, and evaporated *in vacuo* to give a crude product, which was chromatographed using *n*-hexane–ethyl acetate (5 : 2) as the eluent to give **6** (820 mg). Colorless needles (MeOH), mp 157—159 °C, $[\alpha]_D^{25}$ +144.2° (c=0.77, $CHCl_3$). IR (KBr): 1725 (CO) 1665 (CO), 1615 (C=C). ^{1}H-NMR: 0.85 (3H, s, 18-CH_3), 1.16 (3H, s, 19-CH_3), 2.03 and 2.10 (each 3H, s, $OCOCH_3$), 3.63 (3H, s, $COOCH_3$), 4.83 (1H, m, 15β-H), 5.02 (1H, m, 12β-H), 5.68 (1H, m, 4-H). *Anal.* Calcd for $C_{29}H_{42}O_7$: C, 69.29; H, 8.42. Found: C, 69.01; H, 8.44.

Methyl 12α,15α-Diacetoxy-3β-hydroxy-5-cholen-24-oate (7a) Bromotrimethylsilane (0.5 ml) and hexamethyldisilazane (2.5 ml) were added to a solution of **6** (102 mg) in pyridine (2 ml), and the mixture was allowed to stand at room temperature for 30 min. The reaction mixture was diluted with ether, washed with chilled 2 N HCl and H_2O, and dried. After evaporation of the solvent *in vacuo*, the residue was dissolved in isopropyl alcohol (20 ml), and $NaBH_4$ (300 mg) was added to this solution. The whole mixture was stirred at room temperature for 2 h. After addition of 10% AcOH to decompose the excess reagent, the isopropyl alcohol was removed *in vacuo* and the resulting solution was extracted with ethyl acetate. The extract was washed with 5% $NaHCO_3$ and saturated NaCl, and dried. Evaporation of the solvent gave a crude product, which was then chromatographed using *n*-hexane–ethyl acetate (2 : 1) as the eluent to give **7a** (72 mg, 71%) as colorless granules. $[\alpha]_D^{25}$ +70.0° (c=0.10, $CHCl_3$). IR (KBr): 3500 (OH), 1720 (CO). ^{1}H-NMR: 0.83 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 2.01 and 2.07 (each 3H, s, $OCOCH_3$), 3.42 (1H, m, 3α-H), 3.61 (3H, s, $COOCH_3$), 4.83 (1H, m, 15β-H), 5.03 (1H, m, 12β-H), 5.26 (1H, m, 6-H). *Anal.* Calcd for $C_{29}H_{44}O_7 \cdot 1/2H_2O$: C, 67.81; H, 8.83. Found: C, 67.87; H, 8.85.

Methyl 12α,15α-Diacetoxy-3β-*tert*-butyldimethylsilyloxy-5-cholen-24-oate (7b) Imidazole (37 mg) and *tert*-butyldimethylsilyl chloride (79 mg) were added to a solution of **7a** (150 mg) in DMF (1 ml), and the mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with ether, washed with H_2O, and dried. Evaporation of the solvent gave a crude product, which was then chromatographed using *n*-hexane–ethyl acetate (5 : 1) as the eluent to give **7b** (160 mg, 87%). Colorless needles (MeOH), mp 121—123 °C, $[\alpha]_D^{25}$ +52.9° (c=0.51, $CHCl_3$). IR (KBr): 1728 (CO). ^{1}H-NMR: 0.04 (6H, s, $Si(CH_3)_2$), 0.83 (3H, s, 18-CH_3), 0.87 (9H, s, *tert*-C_4H_9), 0.98 (3H, s, 19-CH_3), 1.97 and 2.04 (each 3H, s, $OCOCH_3$), 3.48 (1H, m, 3α-H), 3.59 (3H, s, $COOCH_3$), 4.80 (1H, m, 15β-H), 5.03 (1H, m, 12β-H) 5.25 (1H, m, 6-H). *Anal.* Calcd for $C_{34}H_{56}O_7Si$: C, 67.92; H, 9.45. Found: C, 67.94; H, 9.42.

12α,15α-Dihydroxy-3β-*tert*-butyldimethylsilyloxy-5-cholen-24-oic Acid (7c) A 2 N LiOH solution (3 ml) was added to a solution of **7b** (150 mg) in MeOH (10 ml), and the mixture was heated at 60 °C for 8 h. The reaction mixture was neutralized with AcOH, and MeOH was evaporated off *in vacuo*. The resulting solution was extracted with ethyl acetate. The extract was washed with H_2O, dried, and evaporated *in vacuo*. Recrystallization of the product from MeOH gave **7c** (130 mg, 87%) as colorless needles. mp 247—248 °C. $[\alpha]_D^{25}$ +41.3° (c=0.58, MeOH). IR (KBr): 3200 (OH), 1720 (COOH). ^{1}H-NMR (pyridine-d_5): 0.12 (6H, s, $Si(CH_3)_2$), 0.86 (3H, s, 18-CH_3), 0.97 (9H, s, *tert*-C_4H_9), 1.06 (3H, s, 19-CH_3), 3.55 (1H, m, 3α-H), 4.03—4.40 (2H, m, 12β- and 15β-H), 5.50 (1H, m, 6-H). *Anal.* Calcd for $C_{30}H_{52}O_5Si \cdot 1/2H_2O$: C, 68.01; H, 10.08. Found: C, 68.31; H, 10.06.

Methyl *N*-(12α,15α-Dihydroxy-3β-*tert*-butyldimethylsilyloxy-5-cholen-24-oyl)glycinate (8a) Diethyl cyanophosphonate (50 mg) and glycine methyl ester hydrochloride (53 mg) were added to a solution of **7c** (53 mg) in DMF (2 ml) which contained triethylamine (0.1 ml), and the mixture was stirred at room temperature overnight. After addition of water, the resulting solution was extracted with ethyl acetate. The extract was washed with 2 N HCl and saturated NaCl, dried, and evaporated *in vacuo*. Recrystallization of the product from MeOH gave **8a** (59 mg, 98%). Colorless needles, mp 185—187 °C, $[\alpha]_D^{25}$ +18.6° (c=0.65, $CHCl_3$). IR (KBr): 3350 (OH), 1720 (CO), 1650 (CONH). ^{1}H-NMR: 0.06 (6H, s, $Si(CH_3)_2$), 0.74 (3H, s, 18-CH_3), 0.87 (9H, s, *tert*-C_4H_9), 1.00 (3H, s, 19-CH_3), 3.45 (1H, m, 3α-H), 3.75 (3H, s, $COOCH_3$), 4.02 (2H, d, J=6 Hz, $NHCH_2COOH$), 3.82—4.10 (2H, m, 12β- and 15β-H), 5.31 (1H, m, 6-H), 6.35 (1H, m, NH). *Anal.* Calcd for $C_{33}H_{57}NO_6Si \cdot 1/2H_2O$: C, 65.96; H, 9.73. Found: C, 66.01; H, 9.83.

Methyl *N*-(15α-Hemisuccinyloxy-12α-hydroxy-3β-*tert*-butyldimethylsilyloxy-5-cholen-24-oyl)glycinate (8b) A solution of **8a** (80 mg) and succinic anhydride (160 mg) in pyridine (2 ml) was heated at 90 °C for 1 h. After evaporation of pyridine *in vacuo*, the residue was diluted with ether and the insoluble material was removed by filtration. The filtrate was washed with H_2O and dried. Evaporation of the solvent gave a crude product, which was then chromatographed using benzene–acetone (1 : 1) as the eluent to give **8b** (50 mg, 47%). Colorless granules, $[\alpha]_D^{25}$ +36.1° (c=0.55, MeOH). IR (KBr): 3350 (OH), 1715 (COOH), 1650 (CONH).

^{1}H-NMR: 0.07 (6H, s, Si $(CH_3)_2$), 0.79 (3H, s, 18-CH_3), 0.89 (9H, s, *tert*-C_4H_9), 1.01 (3H, s, 19-CH_3), 2.57 (4H, m, $COCH_2CH_2CO$), 3.48 (1H, m, 3α-H), 3.72 (3H, s, $COOCH_3$), 3.93 (2H, s, $NHCH_2COOH$), 3.95 (1H, m, 12β-H), 4.80 (1H, m, 15β-H), 5.30 (1H, m, 6-H). *Anal.* Calcd for $C_{37}H_{61}NO_9Si$: C, 62.59; H, 8.80. Found: C, 62.53; H, 8.61.

Methyl *N*-(3β,12α-Dihydroxy-15α-hemisuccinyloxy-5-cholen-24-oyl)-glycinate (8c) A 2 N HCl solution (0.5 ml) was added to a solution of **8b** (120 mg) in acetone (4 ml), and the mixture was stirred at room temperature for 1 h. After addition of H_2O, the resulting solution was extracted with ethyl acetate. The extract was washed with saturated NaCl, dried, and evaporated *in vacuo* to give a crude product, which was chromatographed. Elution with benzene–acetone (2 : 1) gave **8c** (80 mg, 80%). Colorless plates (isopropyl ether), mp 155—157 °C, $[\alpha]_D^{25}$ +47.2° (c=0.44, MeOH). IR (KBr): 3400 (OH), 1720 (CO), 1700 (CO), 1650 (CO). ^{1}H-NMR (CD_3OD): 0.78 (3H, s, 18-CH_3), 1.00 (3H, s, 19-CH_3), 2.61 (4H, m, $COCH_2CH_2CO$), 3.40 (1H, m, 3α-H), 3.72 (3H, s, $COOCH_3$), 3.98 (3H, m, 12β-H and $NHCH_2COOH$), 4.83 (1H, m, 15β-H), 5.27 (1H, m, 6-H). *Anal.* Calcd for $C_{31}H_{47}NO_9 \cdot 1/2H_2O$: C, 63.35; H, 8.24. Found: C, 63.39; H, 8.14.

Conjugation of Haptens with BSA A solution of a hapten (**1a**, **1c** or **8c**, 75 μmol) in dry dioxane (0.7 ml) was treated with tributylamine (0.02 ml) and isobutyl chlorocarbonate (0.01 ml) at 10 °C, and the whole mixture was stirred for 30 min. Then BSA (90 mg) in H_2O (2.2 ml)–dioxane (1.4 ml)–1 N NaOH (0.08 ml) was added under ice cooling and the mixture was stirred for 3 h. The resulting solution was dialyzed against cold running water for 2 d and the turbid protein solution was chromatographed on Sephadex G-25. The protein fractions (each 7 ml) that showed absorbance at 280 nm was combined and lyophilized to give the BSA conjugate as a fluffy powder. The molar ratio of the conjugated hapten to BSA was spectrophotometrically (392 nm) determined from the coloration with 83% H_2SO_4 to be 43 for **1a**, 30 for **1c**, and 36 for **8c**.

Immunization of Rabbits Two female domestic albino rabbits were used for immunization with each conjugate. The antigen (1 mg) was dissolved in sterile isotonic saline (0.5 ml) and emulsified with complete Freund's adjuvant (0.5 ml). The emulsion was injected into the rabbit subcutaneously at multiple sites over the back. This procedure was repeated at intervals of one week for a further one month and then once a month. The rabbits were bled one week after a booster injection. The sera were separated by centrifugation at 3000 rpm and stored at −20 °C. For use in RIA, the antisera were thawed and diluted with 0.1 M borate gelatin buffer (BGB, consisting of 0.1 M sodium borate at pH 8.0, 0.1% gelatin and 0.01% NaN_3).

***N*-(3β,12α-Dihydroxy-5-cholen-24-oyl)glycine–Histamine Conjugate (1d)** Diethyl cyanophosphonate (85 mg) and histamine dihydrochloride (82 mg) were added to a solution of *N*-(3β,12α-dihydroxy-5-cholen-24-oyl)glycine (**1a**, 104 mg) in DMF (6 ml) containing triethylamine (0.2 ml) and the mixture was stirred at room temperature for 18 h. The reaction mixture was diluted with ether and the insoluble material was removed by filtration. The filtrate was diluted with ethyl acetate, washed with 5% HCl, 5% $NaHCO_3$, and saturated NaCl, and dried. Evaporation of the solvent gave a crude product, which was then chromatographed using ethyl acetate–methanol–28% NH_4OH (10 : 1 : 0.5) and the eluent to give **1d** (50 mg, 52%) as colorless granules. ^{1}H-NMR ($CDCl_3$–CD_3OD=4 : 1): 0.73 (3H, s, 18-CH_3), 1.01 (3H, s, 19-CH_3), 1.04 (3H, d, J=3 Hz, 21-CH_3), 3.78 (2H, s, $NHCH_2CO$), 3.40 (1H, m, 3α-H), 4.00 (1H, m, 12β-H), 5.30 (1H, m, 6-H), 6.76 and 7.50 (each 1H, s, imidazole). *Anal.* Calcd for $C_{31}H_{48}N_4O_4 \cdot 2HCl$: C, 60.27; H, 8.21. Found: C, 60.25; H, 8.36.

^{125}I-Iodination of 1d Reaction was carried out in a vial containing Na[^{125}I] (2 mCi). The following solutions were added into the vial successively: **1d** (10 μl, 1 mg/ml in EtOH) and chloramine-T in 0.5 M phosphate buffer (pH 7.3) (10 μl, 6 mg/ml). The mixture was stirred vigorously for 50 s and $Na_2S_2O_5$ (240 μg) in 0.5 M phosphate buffer (pH 7.3) (20 μl) was added to stop the reaction. Separation of the product was performed by preparative thin layer chromatography (TLC) using ethyl acetate–MeOH–28% NH_4OH (10 : 2 : 1) as the developing solvent. The radioactive spot was scraped off and eluted with MeOH. After removal of silica gel particles by filtration, the filtrate was concentrated under N_2, and the resulting radioactive product was chromatographed on Sephadex LH-20 using benzene–MeOH (9 : 1) as the eluent. A major radioactive peak was collected and stored at 4 °C. The specific radioactivity of the ligand was defined to be 32.3 Ci/mmol by the method of Diekman *et al.*[10]

Quantitation of Antibody Titers Approximately 15000 dpm of the radioligand in BGB (100 μl) was added to each centrifuge tube (7 ml). An appropriately diluted antiserum (100 μl) was added, and the mixture was incubated at 37 °C for 90 min. After addition of dextran (0.05% w/v)–charcoal (1.5% w/v) suspension (500 μl) in BGB, the resulting solution was vortex-mixed and allowed to stand at 0 °C for 15 min, then centrifuged at 3000 rpm for 15 min at 4 °C. The radioactivities in the supernatants (0.5 ml) were counted.

Assay Procedure A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing a solution (100 μl) of *N*-(3β,12α-dihydroxy-5-cholen-24-oyl)glycine in 0.1—10 or 10—1000 ng/ml range. Aliquots (100 μl) of antiserum (1 : 100 or 1 : 9000 working dilution), and 100 μl of the radioligand (15000 dpm) were added. Incubation and separation were carried out as described above. The antibody bound fraction was expressed as B/B_0% (the amount bound relative to the amount bound for zero dose).

Cross-Reaction Study The specificities of antisera raised against *N*-(3β,12α-dihydroxy-5-cholen-24-oyl)glycine–BSA conjugate were tested by cross-reaction studies with 19 kinds of selected bile acids and cholesterol (Table II). The relative amounts required to reduce the initial binding of radioligand by half, where the mass of *N*-(3β,12α-dihydroxy-5-cholen-24-oyl)glycine was arbitrarily set at 100%, were calculated from the standard curves.

Measurement of Radioactivity Radioactivity counting was carried out with an Aloka ARC-300 auto well gamma system.

Results and Discussion

Initially, our efforts were focused on the synthesis of the haptenic derivatives. Of these compounds, glyco-Δ^5-3β,12α-diol[5] (**1a**), which can be directly reacted at the C-24 carboxyl with an amino group of BSA, was used at the C-24 hapten. On the other hand, the C-3 hapten, methyl *N*-(3β-hemisuccinyloxy-12α-hydroxy-5-cholen-24-oyl)glycinate (**1c**), was prepared by selective hemisuccinylation of glyco-Δ^5-3β,12α-diol methyl ester[5] (**1b**) at the C-3 hydroxyl group with succinic anhydride–pyridine. Fainally, the C-15 hapten possessing a half ester at the 15α-position was synthesized by the methods summarized in Chart 1.

In our synthetic route, 15α-hydroxydeoxycholic acid was required as a key intermediate leading to the desired hapten. Preparation of this compound by direct hydroxylation of deoxycholic acid by means of the ferro-ascorbate system[9] and microbial transformation[11] had the disadvantages of poor yield and difficulty in separation from by-products. We therefore developed a procedure in which a hydroxyl group can be unequivocally introduced at the C-15 position.

Allylic oxidation of methyl 3α,12α-diacetoxy-5β-chol-8(14)-en-24-oate (**2**), which can be obtained from cholic acid in three steps,[8] with *N*-bromosuccinimide under irradiation from a tungsten lamp (500 W) resulted in the regioselective formation of the 15-keto derivative (**3a**) in 75% yield. Hydrogenation of the $\Delta^{8,14}$-double bond to generate the 8β,14α-trans adduct was performed by Birch reduction[12] of the free acid (**3b**), which was readily obtained from **3a** by alkaline hydrolysis. Purification of the desired product was attained by transforming it to the methyl ester (**4a**) whose structure was confirmed by inspection of its mass and ^{1}H-NMR spectra compared with the reported values[9,11] and also by the analysis of its acetate (**4b**). Transformation of this intermediate into the desired hapten was then carried out.

Selective oxidation of the 3α hydroxyl in **4a** with silver carbonate on Celite[13] in refluxing toluene, following ordinary acetylation to protect the remaining hydroxyl group, afforded the 3-monoketone (**5a**) in a good yield. When **5a** was treated with an equimolar amount of bromine in acetic acid, monobromination occurred selectively at the 4β position to give the 4β-bromo-3-ketone (**5b**) as a sole product. A doublet signal (J=13 Hz) at 4.93 ppm in the ^{1}H-NMR

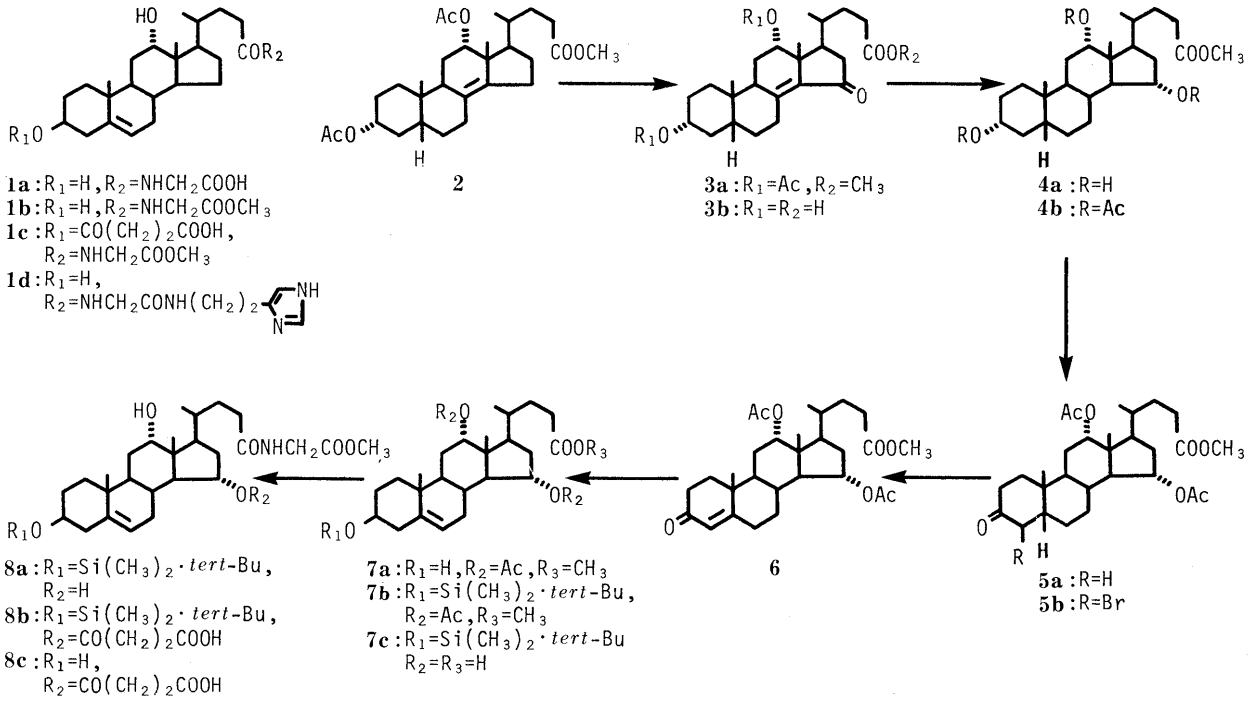

Chart 1

spectrum of **5b** revealed the 4β orientation of the bromine. Accordingly, **5b** was transformed into the Δ^4-3-ketone (**6**) by dehydrobromination with lithium chloride.

It is known that deconjugation of the Δ^4-3-ketone to the Δ^5-3β-ol can be accomplished by treatment with potassium *tert*-butoxide followed by reduction of the resulting $\Delta^{3,5}$-dienol.[14] However, retention of the 15α-acetyl group presented a difficulty because of the alkaline media. Therefore, an alternative method developed by Aringer and Eneroth[15] was employed. On treatment with bromotrimethylsilane and hexamethyldisilazane in pyridine, enol-silylation proceeded in the expected direction, yielding the $\Delta^{3,5}$-dienol silyl ether intermediate, which was immediately reduced with sodium borohydride in isopropanol to afford the deconjugated Δ^5-3β-ol (**7a**) in 80% yield. The structure of **7a** was confirmed by the ^{1}H-NMR spectrum with the characteristic multiplet signals due to the 3α- and 6-protons at 3.42 and 5.26 ppm, respectively, while the singlet signal due to the 4-proton (5.68 ppm) in **6** was absent. *tert*-Butyldimethylsilylation of **7a** was performed to protect the 3β-hydroxyl group in the usual manner. The resulting silyl ether (**7b**) was subjected to alkaline hydrolysis, yielding the free carboxylic acid (**7c**). When condensed with glycine methyl ester by the known method,[16] **7c** yielded the glycine amide (**8a**). Acylation of **8a** with succinic anhydride–pyridine selectively occurred at the 15α position to afford the 15-hemisuccinate (**8b**) in 60% yield. Finally, acid hydrolysis of **8b** on brief exposure to 5 N hydrochloric acid in acetone gave the desired hapten, methyl *N*-(3β,12α-dihydroxy-15α-hemisuccunyloxy-5-cholen-24-oyl)glycinate (**8c**).

These haptenic derivatives were covalently coupled to BSA by the mixed anhydride method.[17] A satisfactory number of hapten moieties was bound in each conjugate.

As a radioligand required for the RIA, ^{125}I-labeled tracer was prepared by radioiodination according to the method of Hunter and Greenwood[18] by utilizing the histamine conjugate (**1d**), which was obtained by condensation of **1a** with histamine hydrochloride.

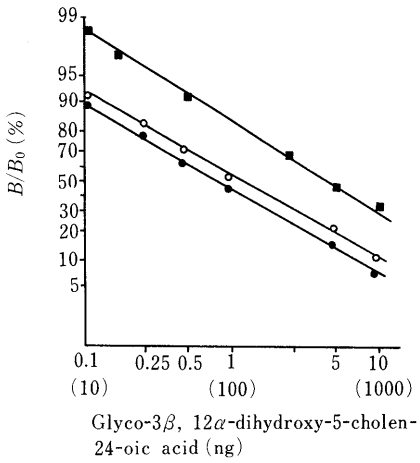

Fig. 1. Dose–Response Curves for Glyco-3β,12α-dihydroxy-5-cholen-24-oic Acid Using the Antisera Elicited against C-3 BSA Conjugate (■), C-15 BSA Conjugates (●), and C-24 BSA Conjugate (○)

The values in parenthesis refer to the C-3 BSA conjugate.

TABLE I. Affinity Constants and Sensitivities[a] of the Antisera

Antiserum (Final dilution)	K_a ($\times 10^8$ M^{-1})	50% displacement (ng)
C-3 (1:100)	1.7	470
C-15α (1:9000)	7.0	0.9
C-24 (1:9000)	5.0	1.7

a) Sensitivity is defined as the amount of unlabeled Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid required to displace 50% the radioligand from the antibody.

Antisera obtained from rabbits immunized with the C-15 and C-24 BSA conjugates exhibited satisfactory binding abilities at the dilution of 1:9000, at which 50% of added

TABLE II. Percent Cross-Reactivities of Anti Glyco-3β,12α-dihydroxy-5-cholen-24-oic Acid Antisera with Selected Compounds

Compound	% cross-reactivities (50%) C-3	C-15	C-24
Glyco-3β,12α-dihydroxy-5-cholen-24-oic acid	100	100	100
Glyco-3β-hydroxy-5-cholen-24-oic acid	47.0	3.54	15.0
Glyco-3β-sulfoxy-12α-hydroxy-5-cholen-24-oic acid	44.8	0.22	0.23
Glyco-3β-sulfoxy-5-cholen-24-oic acid	29.4	0.08	0.23
3β-Sulfoxy-5-cholen-24-oic acid	7.83	<0.01	<0.01
Glycolithocholic acid	6.20	<0.01	<0.01
Glycochenodeoxycholic acid	8.75	<0.01	<0.01
Glycodeoxycholic acid	9.66	<0.01	<0.01
Glycoursodeoxycholic acid	6.36	<0.01	<0.01
Glycocholic acid	7.00	<0.01	<0.01
Tauro-3β,12α-dihydroxy-5-cholen-24-oic acid	57.3	100	41.4
Tauro-3β-hydroxy-5-cholen-24-oic acid	8.39	14.4	7.50
Tauro-3β-sulfoxy-12α-hydroxy-5-cholen-24-oic acid	19.6	0.34	0.23
Tauro-3β-sulfoxy-5-cholen-24-oic acid	7.58	0.13	0.18
Taurolithocholic acid	3.40	<0.01	<0.01
Taurochenodeoxycholic acid	0.65	<0.01	<0.01
Taurodeoxycholic acid	0.80	<0.01	<0.01
Tauroursodeoxycholic acid	n.t.	<0.01	<0.01
Taurocholic acid	0.56	<0.01	<0.01
Cholesterol	4.70	<0.01	<0.01

n.t.: not tested.

tracer was bound, while that from the C-3 BSA conjugate was less satisfactory (50% binding at 1 : 100 dilution). No significant differences were observed among the antisera elicited in two rabbits for each conjugate.

Scatchard analysis[19] of these antisera disclosed high but variable affinity constants ($1.7—7.0 \times 10^8\ M^{-1}$). The affinities of these antisera are adequate, since the K_a values of anti-bile acid antibody are usually in the range $10^6—10^8\ M^{-1}$, allowing assay with a sensitivity ranging from 0.7 to 0.06 μmol.[20]

A log-logit plot of saturation curves constructed at the optimal dilution of these antisera is shown in Fig. 1. The antisera obtained from the C-15 and C-24 BSA conjugates provided standard curves which were linear in the range of 0.1 to 10 ng, while that of the C-3 conjugate was markedly shifted to the range of 10 to 1000 ng because of the poor reactivity.

The sensitivity of the saturation curve was represented by the amount required to displace 50% of the radioligand from the antibody (Table I). The C-3 antibody exhibited the lowest sensitivity. This may be ascribable to the reduced immunoreactivity of the antibody, and not to a difference in affinity or assay conditions, since the K_a value was almost identical to those of the C-15 and C-24 antibodies. The sensitivity with the C-24 antibody was poorer than that with the C-15 antibody at the same dilution. This result can be attributed to a difference of binding ability of the tracer to the antibody. Since the ^{125}I-radioligand is made by condensation of glyco-Δ^5-3β,12α-diol at the C-24 position with histamine, which closely resembles part of the lysine chain of BSA, the binding of ^{125}I-radioligand with the C-24 antibody is tighter than that with the C-15 antibody. Therefore, it is more difficult to displace the tracer bound to the antibody with glyco-Δ^5-3β,12α-diol.

The cross-reactivities of the antisera with 19 bile acids and cholesterol are listed in Table II. Comparison of the cross-reactivity indicated that both C-15 and C-24 BSA conjugates produced more specific antibodies than the C-3 conjugate. Relatively high cross-reaction of the C-3 antibody is not necessarily due to the site of conjugation and may be due to the low binding ability of the antibody, because the specificity pattern is not significantly influenced by the site of conjugation. With the C-15 and C-24 antibodies, all the major human bile acids exhibited negligible cross-reactivities of less than 0.01%, though the structurally similar glycine and taurine conjugates of Δ^5-3β-ol showed slight cross-reactivities with both antibodies. In contrast, the taurine conjugate of Δ^5-3β,12α-diol exhibited remarkably high cross-reactivities in the order of C-15 (100%) > C-24 (41.4%). In hapten immunoassay employing an iodinated tracer, heterologous assay, where different derivatives of the hapten are used for the preparation of immunogen and tracer, has shown good sensitivity, but specificity is often diminished in contrast to homologous assay, where the same hapten is used.[21] This does appear to be the case here. However, the lack of specificity for the taurine conjugates could be useful in allowing measurement of all the Δ^5-3β,12α-diol conjugates including taurine conjugate. Development of this immunoassay method to determine the bile acid contents in human body fluids will be reported in the near future.

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