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Preparation and Antigenic Properties of Methyl 3β-Hydroxy-19-oxo-5-cholen-24-oyl-glycinate 19-(*O*-Carboxymethyl)oxime–Bovine Serum Albumin Conjugate

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The preparation and antigenic properties of 3β-hydroxy-5-cholen-24-oyl-glycine–bovine serum albumin (BSA) conjugate in which the hapten is linked to the carrier protein through an (*O*-carboxymethyl)oxime bridge at the C-19 position on the steroid portion are described. Antibody raised against the antigen in rabbits possessed high titer and specificity to 3β-hydroxy-5-cholen-24-oyl-glycine, exhibiting no significant cross-reactions with various bile acids.

Keywords——radioimmunoassay; bile acid; hapten; 3β-hydroxy-5-cholen-24-oyl-glycine

Many studies on radioimmunoassay for bile acids[1-7] have been reported since the first report of Simmonds and his co-workers in 1973.[8] In these studies, the preparations of antisera used antigens in which the steroid conjugates were coupled to carrier proteins through the C-24 position. However, antisera elicited with these antigens did not have sufficient specificities to the functional groups on the side chain. In order to improve the speficicity, we synthesized new haptens which were coupled to bovine serum albumin (BSA) through the C-3 position for lithocholylglycine[9] and the C-19 position for 3β-hydroxy-5-cholen-24-oic acid.[10] These antigens were satisfactory in respect to specificity. 3β-Hydroxy-5-cholen-24-oic acid is an intermediate metabolite of a minor pathway of bile acid biosynthesis which begins with oxidation of the cholesterol side chain. In patients with cholestasis, 3β-hydroxy-5-cholen-24-oic acid has been detected,[11-14] and there is some evidence that synthesis of 3β-hydroxy-5-cholen-24-oic acid continues at a lower rate in healthy adults.[15] Minder *et al.* revealed the existence of 3β-hydroxy-5-cholen-24-oic acid in the sera of healthy subjects by radioimmunoassay.[16] We have synthesized a new hapten for radioimmunoassay for glycine conjugated form of 3β-hydroxy-5-cholen-24-oic acid. This paper describes the synthesis of the hapten, the 19-(*O*-carboxymethyl)oxime derivative of methyl 3β-hydroxy-19-oxo-5-cholen-24-oyl-glycinate, and its BSA-conjugate, the synthesis of [^{125}I]-iodinated ligand and the specificity of antiserum.

Results and Discussion

Our initial effort was directed to the preparation of a new type of hapten-carrier protein conjugate for obtaining specific antiserum. We attemped to prepare 3β-hydroxy-5-cholen-24-oyl-glycine–BSA conjugate which had a linkage through the C-19 position distant from the functional groups at both C-3 and at C-24.

Methyl 3β-acetoxy-19-oxo-5-cholen-24-oate (I), described previously,[10] was taken as a starting material. Alkaline hydrolysis of the 19-aldehyde derivative gave the corresponding

3β-alcohol II which was converted to the glycine ester III with *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ).[17] The glycine ester III was treated with carboxymethoxylamine hemihydrochloride in pyridine[18] to give the 19-oxime derivative, methyl 3β-hydroxy-19-oxo-5-cholen-24-oyl-glycinate 19-(*O*-carboxymethyl)oxime (IV). Chart 1 shows the synthetic route to the hapten. Compound IV was covalently linked to BSA by the mixed anhydride method under usual conditions.[19] The hapten–BSA conjugate thus prepared was used for immunization of animals.

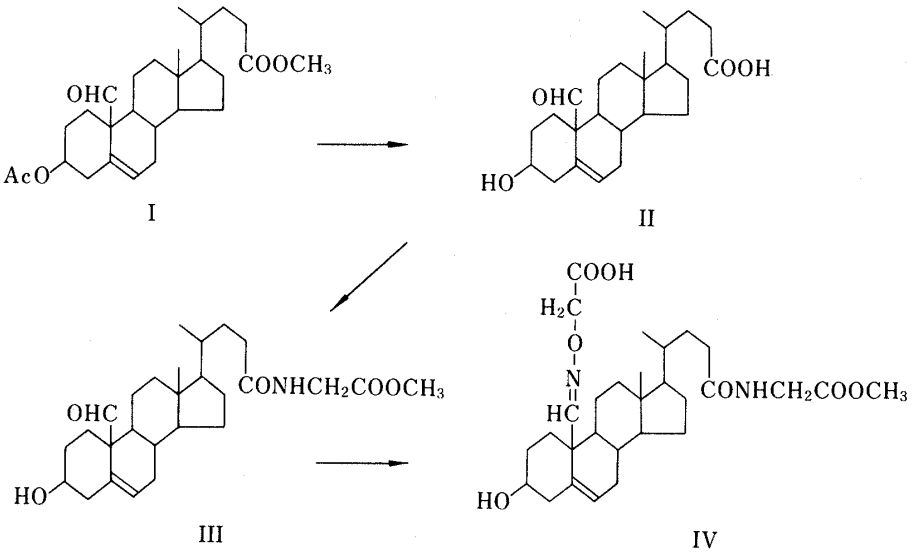

Chart 1. Synthesis of Hapten

The tyrosine conjugate of compound IV was synthesized for use as a radioligand. Compound IV was converted to the tyrosine ethyl ester conjugate V with dicyclohexylcarbodiimide and hydroxybenzotriazole.[20] Alkaline hydrolysis of the tyrosine ethyl ester V with sodium hydroxide gave the tyrosine conjugate, 3β-hydroxy-19-oxo-5-cholen-24-oyl-glycine 19-(*O*-carboxymethyl)oxime-tyrosine conjugate (VI). The tyrosine conjugate VI was radioiodinated by the method of Hunter and Greenwood.[21]

All rabbits immunized with the hapten–BSA conjugate produced antisera with high titer in 6 months' time. Titer ranged from 1 : 24000 to 34000. No substantial difference in affinity or specificity was observed among antisera elicited in three rabbits with the antigen.

A standard curve was constructed with 1 : 24000 dilution of antiserum. The response was linear from 1 ng/ml to 1 μg/ml.

Table I. Cross-Reactivity Data of 19-Linked Anti-(3β-hydroxy-5-cholen-24-oyl-glycine)serum

Compound	% cross-reactivity
3β-Hydroxy-5-cholen-24-oyl-glycine	100.0
Sodium 3β-hydroxy-5-cholen-24-oyl-taurinate	0.1
3β-Hydroxy-5-cholen-24-oic acid	0.4
Lithocholylglycine	<0.1
Deoxycholylglycine	<0.1
Chenodeoxycholylglycine	<0.1
Ursodeoxycholylglycine	<0.1
Cholylglycine	<0.1
Cholesterol	<0.3

The specificity of the antiserum was assessed by testing the ability of related compounds to compete for the binding site on the antibody.[22] The results on cross-reactivity of the antiserum are listed in Table I. It is evident from the data that this antiserum is highly specific to 3β-hydroxy-5-cholen-24-oyl-glycine. There were no significant cross-reactions with various bile acids. This low cross-reactivity with bile acids (5β-cholanic acid) suggests that the antibody can recognize the difference in configuration of the A and B rings of the steroid. The cross-reactivity with cholesterol was below 0.3%. Minder *et al.* reported that the cross-reactivity with cholesterol was 5.6% using the hapten coupled to a carrier protein through the carboxylic acid on the side chain.[16] It is clear that the site of conjugation plays an important role in producing specific antisera.[23] The antiserum obtained in the present study is much more satisfactory with respect to specificity than that reported previously.[16] Development of a simplified and reliable radioimmunoassay system for 3β-hydroxy-5-cholen-24-oyl-glycine in biological fluids will be the subject of a future communication.

Experimental

Reagents——Na[^{125}I] was kindly donated by Daiichi Radioisotope Laboratories (Tokyo, Japan). BSA was purchased from Sigma Chemical Co. (St. Louis, MO), and reference bile acids from Steraloids Inc. (Wilton NH). Freund's complete adjuvant was obtained from Difco Laboratories (Detroit, MI) and other reagents from Wako Pure Chemical Industries (Osaka, Japan).

Synthesis of Hapten

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Nuclear magnetic resonance (NMR) spectra were run on a JEOL PS-100 spectrometer at 100 MHz, using tetramethylsilane as an internal standard. Infrared (IR) spectra were recorded on a Jasco IRA-1 spectrometer. Elemental analyses were performed by the staff of the Central Analytical Laboratory of Kyushu University.

Methyl 3β-Hydroxy-19-oxo-5-cholen-24-oyl-glycinate (III)——A solution of I (1.0 g) in tetrahydrofuran (THF) (30 ml) was added dropwise to a solution of sodium hydroxide (500 mg) in aqueous methanol (50 ml). The mixture was stirred at room temperature overnight, then poured into ice-water, and neutralized with 1 N HCl to give 3β-hydroxy-19-oxo-5-cholen-24-oic acid (II). A suspension of methyl glycinate hydrochloride (1.2 g) in ethyl acetate containing triethylamine (1.34 ml) was stirred at 25 °C for 30 min, II (2.65 g) and EEDQ (2.4 g) were then added to the solution. After stirring at 25 °C for 15 min, the suspension was refluxed on an oil bath for 3 h. The resulting suspension was cooled to room temperature and filtered. The filtrate was washed with 0.5 N HCl and water, and evaporated *in vacuo*. The residue was chromatographed on silica gel with chloroform to give III (2.2 g) which was recrystallized from ethyl acetate–petroleum ether as colorless needles; mp 165—167 °C. IR ν_{max} (Nujol) 3520, 3240, 2700, 1730, 1640, 1550, 1220 cm^{-1}; NMR (pyridine-d_5, δ) 0.52 (3H, s, 18-C$\underline{H}_3$), 3.60 (3H, s, COOC$\underline{H}_3$), 3.80 (1H, br, 3-$\underline{H}$), 4.30 (2H, d, N–C$\underline{H}_2$–COO), 5.84 (1H, t, 6-$\underline{H}$), 9.12 (1H, t, N$\underline{H}$), 9.84 (1H, s, 19-C$\underline{H}$O) ppm. *Anal.* Calcd for $C_{27}H_{41}NO_5$: C, 70.55; H, 8.99; N, 3.05. Found: C, 70.38; H, 9.00; N, 3.00.

Methyl 3β-Hydroxy-19-oxo-5-cholen-24-oyl-glycinate 19-(*O*-Carboxymethyl)oxime (IV)——To a solution of III (200 mg) in pyridine was added carboxymethoxylamine hemihydrochloride (200 mg). The mixture was stirred at 70 °C for 3 h. The solvent was evaporated *in vacuo*, and the residue was chromatographed on silica gel with chloroform to give IV (164 mg) which was recrystallized from ethyl acetate as colorless needles; mp 157—159 °C. IR ν_{max} (Nujol) 3440, 3240, 2700, 1640, 1560, 1230 cm^{-1}; NMR (pyridine-d_5, δ) 0.62 (3H, s, 18-C$\underline{H}_3$), 3.60 (3H, s, COOC$\underline{H}_3$), 3.82 (1H, br, 3-$\underline{H}$), 4.32 (2H, d, N–C$\underline{H}_2$–COO), 5.60 (1H, t, 6-$\underline{H}$), 7.38 (1H, s, 19-C$\underline{H}$O), 9.10 (1H, t, N$\underline{H}$) ppm. *Anal.* Calcd for $C_{29}H_{44}N_2O_7$: C, 65.39; H, 8.33; N, 5.26. Found: C, 65.01; H, 8.38; N, 5.28.

Preparation of Antigen——The 19-oxime derivative IV (100 mg) and tri-*n*-butylamine (0.0435 ml) were dissolved in THF (2.5 ml). After the solution was cooled to 0 °C, isobutyl chloroformate (0.0235 ml) was added, the mixture was allowed to stand at 0 °C for 30 min, and added in one portion to a well-stirred, cooled solution of BSA (260 mg) in a mixture of water (6.8 ml), THF (6.8 ml) and 1 N sodium hydroxide (0.21 ml). Stirring was continued for another 30 min, and the solution was adjusted to pH 9.0 with 1 N sodium hydroxide. Stirring and cooling were continued for a total of 4 h. The solution was dialyzed against water and adjusted to pH 4.5 with 1 N HCl. The resulting precipitate, after storage in the cold for 24 h, was collected by centrifugation, and suspended in water (30 ml). The suspension was redissolved by adding a minimal quantity of saturated sodium bicarbonate, and the clear solution was lyophilized to give the conjugate (210 mg). The molar steroid/protein ratio of the conjugate was 24 by spectrometry (at 280 nm in 0.1 M borate buffer, pH 9.0).

Immunization Procedures——Three female New Zealand white rabbits were used for immunization. The antigen (2 mg) was dissolved in 0.5 M phosphate buffer (1 ml, pH 7.4) and emulsified with Freund's complete adjuvant (1 ml). The emulsion (0.5 ml) was intradermally injected into a rabbit at multiple sites over the back, and given intradermal

injections monthly. The rabbits were bled 6 months after the initial injection.

Synthesis of Tracer

Methyl 3β-Hydroxy-19-oxo-5-cholen-24-oyl-glycinate 19-(*O*-Carboxymethyl)oxime–Tyrosine Ethyl Ester Conjugate (V)——A mixture of IV (210 mg), tyrosine ethyl ester (98 mg), hydroxybenzotriazole (124 mg) and dicyclohexylcarbodiimide (105 mg) was stirred under ice-cooling for 1 h, and allowed to stand at room temperature overnight. The resulting solution was filtered and the filtrate was evaporated *in vacuo*. The residue was chromatographed on silica gel with chloroform to give V (115 mg) as an amorphous yellow material; NMR (pyridine-d_5, δ) 0.56 (3H, s, 18-CH$_3$), 1.08 (3H, t, CH$_2$–CH$_3$), 3.32 (2H, d, CH–CH$_2$–), 3.60 (3H, s, COOCH$_3$), 3.82 (1H, br, 3-H), 4.12 (2H, m, CH$_2$–CH$_3$), 4.30 (2H, d, NH–CH$_2$–COO), 4.84 (2H, s, O–CH$_2$–COO), 5.60 (1H, t, 6-H), 7.20 (4H, m, aromatic), 8.36 (1H, d, NH–CH), 9.10 (1H, t, NH) ppm.

3β-Hydroxy-19-oxo-5-cholen-24-oyl-glycine 19-(*O*-Carboxymethyl)oxime–Tyrosine Conjugate (VI)——A solution of V (100 mg) in THF was added to a solution of sodium hydroxide (50 mg) in aqueous methanol (5.0 ml). The mixture was stirred at room temperature overnight, poured into ice-water, and neutralized with 1 N HCl. The precipitate was collected by filtration; NMR (pyridine-d_5, δ) 0.57 (3H, s, 18-CH$_3$), 3.32 (2H, d, CH–CH$_2$–), 3.80 (1H, br, 3-H), 4.48 (2H, d, NH–CH$_2$–COO), 4.86 (2H, s, O–CH$_2$–COO), 5.60 (1H, t, 6-H), 7.20 (4H, m, aromatic), 8.12 (1H, d, NH–CH), 8.92 (1H, t, NH) ppm.

[^{125}I]-Iodination of VI——To a solution of VI (10 μg) in ethanol (10 μl) and Na[^{125}I] (1 mCi) was added Chloramine-T (60 μg) in 0.5 M phosphate buffer (pH 7.4) (10 μl) and the mixture was stirred on a vortex for 50 s. Sodium metabisulfite (240 μg) in 0.5 M phosphate buffer (pH 7.4) (20 μl) was added to stop the reaction. The solvent was evaporated *in vacuo* and the residue was purified by preparative thin-layer chromatography on a silica gel plate (60F 254, 0.25 mm layer, Merck) in chloroform–methanol–acetic acid (3 : 1 : 0.1). Scanning the thin-layer plate in a radiochromatogram scanner (Aloka TRM-1B) revealed a major peak of radioactive material (*Rf*: 0.20) which was eluted with methanol. After removal of silica gel particles by centrifugation, the eluate was stored at 4 °C. The specific radioactivity of the ligand was 48.5 Ci/mmol.

Synthesis of Standard Samples

Methyl 3β-Hydroxy-5-cholen-24-oyl-glycinate (VII)——A suspension of methyl glycinate hydrochloride (14 mmol) in ethyl acetate containing triethylamine (2 ml) was stirred at 25 °C for 30 min, 3β-hydroxy-5-cholen-24-oic acid (10 mmol) and EEDQ (14 mmol) were then added to the solution. After stirring at 25 °C for 15 min, the suspension was refluxed on an oil bath for 3 h. The resulting suspension was cooled to room temperature and filtered. The filtrate was washed with 0.5 N HCl and water, and then evaporated *in vacuo*. The residue was chromatographed on silica gel with chloroform to give VII which was recrystallized from ethanol as colorless needles; mp 202—204 °C. IR ν_{max} (Nujol) 3520, 3280, 1760, 1650, 1550, 1210 cm^{-1}; NMR (pyridine-d_5, δ) 0.62 (3H, s, 18-CH$_3$), 1.04 (3H, s, 19-CH$_3$), 3.60 (3H, s, COOCH$_3$), 3.84 (1H, br, 3-H), 4.32 (2H, d, N–CH$_2$–COO), 5.42 (1H, t, 6-H), 9.10 (1H, t, NH) ppm. *Anal.* Calcd for $C_{27}H_{43}NO_4$: C, 72.77; H, 9.73; N, 3.14. Found: C, 72.68; H, 9.79; N, 3.18.

3β-Hydroxy-5-cholen-24-oyl-glycine (VIII)——A solution of VII (1.0 g) in THF was added to a solution of sodium hydroxide (500 mg) in aqueous methanol (50 ml). The mixture was stirred at room temperature overnight, then poured into ice-water, and neutralized with 1 N HCl. The precipitate was recrystallized from aqueous ethanol to give colorless needles of VIII (0.8 g); mp 213—215 °C. IR ν_{max} (Nujol) 3480, 3280, 1740, 1620, 1580, 1220 cm^{-1}; NMR (pyridine-d_5, δ) 0.62 (3H, s, 18-CH), 1.04 (3H, s, 19-CH$_3$), 3.82 (1H, br, 3-H), 4.48 (2H, d, N–CH$_2$–COO), 5.40 (1H, t, 6-H), 8.96 (1H, t, NH) ppm. *Anal.* Calcd for $C_{26}H_{41}NO_4 \cdot H_2O$: C, 69.45; H, 9.64; N, 3.12. Found: C, 69.25; H, 9.53; N, 3.12.

Sodium 3β-Hydroxy-5-cholen-24-oyl-taurinate (IX)——Taurine (138 mg) and triethylamine (0.18 ml) were added to a stirred solution of 3β-hydroxy-5-cholen-24-oic acid (374 mg) and EEDQ (346 mg) in 2 ml of *N,N*-dimethylformamide, and the resulting suspension was heated at 90 °C until a clear solution was formed. The solution was stirred at 90 °C for 15 min longer and then cooled to room temperature with stirring. The solution was poured into 20 ml of stirred, chilled, anhydrous ether in an ice bath. The suspension was filtered. The collected solid was dissolved in methylene chloride and filtered. The clear filtrate was poured into chilled anhydrous ether in an ice bath. After 0.5 h at 0 °C, the precipitate was collected. The precipitate was dissolved in 0.2 N methanolic sodium hydroxide. The solution was diluted with anhydrous ether. After a period at 0 °C, the precipitate was collected; mp 231—233 °C. NMR (CD_3OD, δ) 0.66 (3H, s, 18-CH$_3$), 1.01 (3H, s, 19-CH$_3$), 2.95 (2H, t, –CH$_2$–S–), 3.56 (1H, br, 3-H), 3.59 (2H, t, N–CH$_2$), 5.36 (1H, t, 6-H) ppm. *Anal.* Calcd for $C_{26}H_{42}NNaO_5S \cdot 2H_2O$: C, 58.08; H, 8.62; N, 2.60. Found: C, 57.99; H, 8.10; N, 2.71.

Quantitation of Antibody Titers——Approximately 100 pg (20000 dpm) of the radioligand in 100 μl of borate-gelatin buffer (BGB, consisting of 0.1 M borate buffer at pH 8.0 containing 0.1% gelatin and 0.01% sodium azide) was added to each radioimmunoassay (RIA) tube containing 1000 μl of BGB. After addition of duplicate 100 μl aliquots of an appropriate antiserum dilution, mixing and incubation at 37 °C for 90 min, bound and unbound radioligands were separated by adding 500 μl of dextran-coated charcoal suspension (500 mg of charcoal and 50 mg of Dextran T-70 in 100 ml of BGB) at 4 °C. The counts in the charcoal pellets were determined, after decanting off the supernatant.

Assay Procedures——A standard curve was constructed by setting up duplicate RIA tubes containing 100 μl of 3β-hydroxy-5-cholen-24-oyl-glycine ranging in concentration from 100 pg/ml to 10 μg/ml. Aliquots of 100 μl of

antiserum (1 : 2000 working dilution), 100 μl of radioligand (20000 dpm) and 1000 μl of BGB 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 methyl 3β-hydroxy-19-oxo-5-cholen-24-oyl-glycinate 19-(*O*-carboxymethyl)oxime–BSA conjugate were tested by cross-reaction studies with various bile acids. The relative amount required to reduce the initial binding of radioligand by half, where the mass of non-labelled 3β-hydroxy-5-cholen-24-oyl-glycine was arbitrarily chosen as 100%, was calculated from the standard curve.

Nomenclature——The following trivial names are used in the present paper: Cholesterol: 5-cholesten-3β-ol, lithocholylglycine: 3α-hydroxy-5β-cholan-24-oyl-glycine, deoxycholylglycine: 3α,12α-dihydroxy-5β-cholan-24-oyl-glycine, chenodeoxycholylglycine: 3α,7α-dihydroxy-5β-cholan-24-oyl-glycine, ursodeoxycholylglycine: 3α,7β-dihydroxy-5β-cholan-24-oyl-glycine, cholylglycine: 3α,7α,12α-trihydroxy-5β-cholan-24-oyl-glycine.

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