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Preparation and Properties of Antisera against Lithocholic Acid Linked to Bovine Serum Albumin at the C-1β and C-6 Positions

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The preparation and antigenic properties of lithocholic acid–bovine serum albumin conjugates in which the haptens are coupled to the carrier protein through a 6α-hemisuccinoyl, 6-*O*-carboxymethyloximino or 1β-hemisuccinoyl bridge are described. The antisera elicited by both the former conjugates showed relatively poor ability to discriminate lithocholic acid from ursodeoxycholic and chenodeoxycholic acids, whereas that from the latter revealed a high affinity constant ($K_a = 6.4 \times 10^7$ M^{-1}) and excellent specificity to lithocholic acid, exhibiting no significant cross-reactivity with any of the major human bile acids.

Keywords——lithocholic acid; radioimmunoassay; hapten; antiserum; cross-reaction; specificity; methyl 1β-hemisuccinoyloxylithocholate; 6α-hemisuccinoyloxylithocholate; 6-*O*-carboxymethyloxyiminolithocholate

The circulating plasma level of lithocholic acid (LCA), which is formed from chenodeoxycholic acid by bacterial 7α-dehydroxylation in the intestine, is regarded as an important index of liver damage, since this bile acid has been shown to be hepatotoxic.[1] Radioimmunological determinations of LCA have been developed to clarify this possibility because of the increasing use of the agent for the dissolution of cholesterol gallstones.[2,3] The antisera hitherto used in radioimmunoassay (RIA) have been made by immunizing animals with hapten-bovine serum albumin (BSA) conjugates coupled only through the carboxylic side chain[2] or the 3α-hydroxyl group.[3,4] Therefore, the resulting antibodies cross-reacted substantially with structurally similar bile acids. No information is available to define the importance of the site of conjugation although some general comments can be made by extrapolation from data provided by steroid RIA. Conjugation at the C-1 and C-6 positions which leaves the functional groups in LCA should elicit antisera with enhanced specificity, since the structures of bile acids are commonly characterized by the 3α-, 7α- and 12α-hydroxyl groups and also the side chain. The present paper describes the synthesis of new types of haptens for LCA-immunoassay, the preparation of the BSA conjugates, and the properties of antisera raised by administration of these immunogens.

Materials and Methods

Chemicals and Reagents——[^{3}H]Sodium borohydride (5 Ci/mmol) was supplied by Amersham International (Amersham, Bucks., U.K.). BSA and complete Freund's adjuvant were purchased from Sigma Chemical Co. (St. Louis, MO) and Iatron Laboratories (Tokyo), respectively. The reference bile acids were either synthesized in our laboratory or were obtained commercially. All solvents and chemicals were of analytical reagent grade.

Synthesis of Haptenic Derivatives——Apparatus: All melting points were taken on a Mitamura micro hot-stage apparatus and are uncorrected. The ultraviolet (UV) spectra were recorded on a Shimadzu model UV-200 spectrometer and the optical rotations were obtained with a Union Giken-201 polarimeter in MeOH. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were measured on a Hitachi model R-40 spectrometer (90 MHz) in $CDCl_3$.

Chemical shifts are given as δ value with tetramethylsilane as an internal standard (s, singlet; d, doublet; t, triplet; m, multiplet). Column chromatography was carried out on Merck silica gel (70—230 mesh). All organic solvents were dried over anhydrous Na_2SO_4.

tert-Butyldimethylsilylation of Methyl 3α, 6α-Dihydroxy-5β-cholan-24-oate (**1**): Imidazole (250 mg) and *tert*-butyldimethylsilyl chloride (83 mg) were added to a solution of methyl 3α, 6α-dihydroxy-5β-cholan-24-oate (**1**, 1.0 g) in *N*,*N*-dimethylformamide (DMF, 2 ml) and the mixture was stirred at room temperature for 2 h. After addition of H_2O, the resulting mixture was extracted with ether and the organic layer was washed with H_2O, dried and evaporated *in vacuo*. The crude product was submitted to column chromatography on silica gel using hexane–AcOEt (5 : 1) as an eluent. The less polar eluate was recrystallized from aqueous MeOH to give methyl 3α, 6α-dihydroxy-5β-cholan-24-oate bis(*tert*-butyldimethylsilyl) ether (**2**, 325 mg) as colorless needles. mp 93—95 °C. $[\alpha]_D^{15}$ +1.07° (c=0.93). ^{1}H-NMR: 0.04 (12H, s, OSi $(CH_3)_2\times 2$), 0.61 (3H, s, 18-CH_3), 0.88 (24H, s, 19- and 21-CH_3, and *tert*-$C_4H_9\times 2$), 3.45 (1H, m, 3β-H), 3.64 (3H, s, $COOCH_3$), 3.92 (1H, m, 6β-H). *Anal.* Calcd for $C_{37}H_{70}O_4Si_2$: C, 69.97; H, 11.11. Found: C, 69.80; H, 11.11. The more polar eluate was recrystallized from aqueous MeOH to give methyl 3α,6α-dihydroxy-5β-cholan-24-oate 3-*tert*-butyldimethylsilyl ether (**3**, 640 mg) as colorless needles. mp 96—100 °C. $[\alpha]_D^{15}$ +13.1° (c=0.94). ^{1}H-NMR: 0.05 (6H, s, OSi $(CH_3)_2$), 0.62 (3H, s, 18-CH_3), 0.87 (15H, s, 19- and 21-CH_3, and *tert*-C_4H_9), 3.50 (1H, m, 3β-H), 3.63 (3H, s, $COOCH_3$), 4.00 (1H, m, 6β-H). *Anal.* Calcd for $C_{31}H_{56}O_4Si$: C, 71.24; H, 10.83. Found: C, 71.48; H, 10.83.

Methyl 3α-*tert*-Butyldimethylsilyloxy-6α-hemisuccinoyloxy-5β-cholan-24-oate (**4**): A solution of **3** (500 mg) and succinic anhydride (150 mg) in pyridine (3 ml) was heated at 90 °C for 3 h. After evaporation of the pyridine *in vacuo*, the residue was diluted with ether and the insoluble material was filtered off. The filtrate was washed with H_2O, dried, and evaporated. The crude product was chromatographed using hexane–AcOEt (2 : 1) as an eluent. The eluate gave **4** (490 mg) as a colorless noncrystalline material. ^{1}H-NMR: 0.05 (6H, s, OSi $(CH_3)_2$), 0.62 (3H, s, 18-CH_3), 0.85 (15H, s, 19- and 21-CH_3, and *tert*-C_4H_9), 2.62 (4H, m, $CO(CH_2)_2CO$), 3.52 (1H, m, 3β-H), 3.63 (3H, s, $COOCH_3$), 5.08 (1H, m, 6β-H).

Methyl 6α-Hemisuccinoyloxy-3α-hydroxy-5β-cholan-24-oate (**5**): A mixture of 5 N HCl (0.5 ml) and **4** (400 mg) in acetone (10 ml) was stirred at room temperature for 30 min. After evaporation of the acetone *in vacuo*, the residue was extracted with AcOEt. The organic layer was washed with brine, dried, and evaporated. The crude product was chromatographed using $CHCl_3$–MeOH (100 : 1) as an eluent. The eluate was recrystallized from acetone–MeOH to give **5** (315 mg) as colorless needles. mp 166—167.5 °C. $[\alpha]_D^{15}$ −59.7° (c=0.10). ^{1}H-NMR: 0.65 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 2.59 (4H, m, $CO(CH_2)_2CO$), 3.60 (1H, m, 3β-H), 3.63 (3H, s, $COOCH_3$), 5.15 (1H, m, 6β-H). *Anal.* Calcd for $C_{29}H_{46}O_7$: c, 68.85; H, 9.13. Found: C, 68.74; H, 9.15.

Methyl 6-(*O*-Carboxymethyloxyimino)-3α-hydroxy-5β-cholan-24-oate (**7**): A solution of carboxymethoxylamine hemihydrochloride (215 mg) in 2 N KOH (1.2 ml) was added to a solution of methyl 3α-hydroxy-6-oxo-5β-cholan-24-oate[5] (**6**, 200 mg) in EtOH (10 ml) and the mixture was refluxed for 6 h. After evaporation of EtOH, the residue was adjusted to pH 12 with 1 N NaOH and extracted with AcOEt. The aqueous layer was acidified with 5% HCl and extracted with AcOEt. The organic layer was washed with H_2O, dried, and evaporated. The crude product was chromatographed using $CHCl_3$–MeOH (20 : 1) as an eluent. The eluate gave **7** (177 mg) as a colorless noncrystalline material. $[\alpha]_D^{15}$ −29.1° (c=0.17). ^{1}H-NMR: 0.65 (3H, s, 18-CH_3), 0.83 (3H, s, 19-CH_3), 0.92 (3H, d, J=5 Hz, 21-CH_3), 3.65 (1H, m, 3β-H), 3.68 (3H, s, $COOCH_3$), 4.54 (2H, s, $NOCH_2COOH$). *Anal.* Calcd for $C_{27}H_{43}NO_6\cdot 1/2H_2O$: C, 66.09; H, 9.30. Found: C, 66.36; H, 9.08.

Methyl 3α-*tert*-Butyldimethylsilyloxy-1β-hydroxy-5β-cholan-24-oate (**9**): Imidazole (62 mg) and *tert*-butyldimethylsilyl chloride were added to a solution of methyl 1β, 3α-dihydroxy-5β-cholan-24-oate[6] (**8**, 150 mg) in DMF (3 ml). After being held at room temperature for 2h, the resulting mixture was diluted with H_2O and extracted with ether. The organic layer was washed with H_2O, dried, and evaporated. The residue was chromatographed using hexane–AcOEt (15 : 1) as an eluent and recrystallized from aqueous MeOH to give **9** (170 mg) as colorless needles, mp 95—96.5 °C. $[\alpha]_D^{15}$ +28.1° (c=0.89). ^{1}H-NMR: 0.06 (6H, s, OSi $(CH_3)_2$), 0.73 (3H, s, 18-CH_3), 0.87 (15H, s, 19- and 21-CH_3, and *tert*-C_4H_9), 3.63 (3H, s, $COOCH_3$), 3.86 (1H, t, J=3 Hz, 1α-H), 4.25 (1H, m, 3β-H). *Anal.* Calcd for $C_{31}H_{56}O_4Si$: C, 71.48; H, 10.83. Found: C, 71.46; H, 10.91.

Methyl 3α-*tert*-Butyldimethylsilyloxy-1β-hemisuccinoyloxy-5β-cholan-24-oate (**10**): Succinic anhydride (150 mg) and 4-dimethylaminopyridine (20 mg) were added to a solution of **9** (50 mg) in pyridine (3 ml) and the mixture was refluxed for 35 h. After evaporation of the pyridine *in vacuo*, the residue was diluted with ether and the insoluble material was removed by filtration. The filtrate was washed with H_2O, dried, and evaporated to dryness *in vacuo*. The residue was chromatographed using hexane-AcOEt (5 : 1) as an eluent to give **10** (40 mg) as colorless semicrystals. ^{1}H-NMR: 0.04 (6H, s, $OSi(CH_3)_2$), 0.64 (3H, s, 18-CH_3), 0.89 (15H, s, 19- and 21-CH_3, and *tert*-C_4H_9), 2.72 (4H, s, $CO(CH_2)_2CO$), 3.63 (3H, s, $COOCH_3$), 3.88 (1H, m, 3β-H), 5.12 (1H, m, 1α-H).

Methyl 1β-Hemisuccinoyloxy-3α-hydroxy-5β-cholan-24-oate (**11**): A mixture of 5N HCl (0.3 ml) and **10** (40 mg) in acetone (1.5 ml) was stirred at room temperature for 30 min. After evaporation of the acetone *in vacuo*, the resulting solution was extracted with AcOEt. The organic layer was washed with H_2O, dried, and evaporated to dryness *in vacuo*. The residue was chromatographed with benzene-acetone (1 : 1) as an eluent and the eluate was recrystallized from acetone to give **11** (25 mg) as colorless needles. mp 105.5—107.5 °C. $[\alpha]_D^{15}$ −5.11° (c=1.76). ^{1}H-

NMR: 0.63 (3H, s, 18-CH_3), 0.90 (6H, s, 19- and 21-CH_3), 2.62 (4H, s, $CO(CH_2)_2CO$), 3.62 (3H, s, $COOCH_3$), 3.98 (1H, m, 3β-H), 5.12 (1H, m, 1α-H). *Anal.* Calcd for $C_{29}H_{46}O_7$: C, 68.74; H, 9.15. Found: C, 68.55; H, 9.26.

Conjugation of Haptens with BSA——A solution of a hapten (**5**, **7** or **11**, 40 mg) in dry dioxane (0.7 ml) was treated with tri-*n*-butylamine (0.02 ml) and isobutyl chlorocarbonate (0.01 ml) at 11 °C and the whole 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 10 min. This procedure was repeated until the free bile acid was removed completely. The precipitate was dissolved in 5% $NaHCO_3$ and dialyzed in the same manner as mentioned above. Lyophilization of the solution afforded the bile acid–BSA conjugate (*ca.* 60 mg) as a fluffy powder. The values of molar ratio of the hapten to the protein in the conjugates were spectrophotometrically (at 375 nm) determined by the use of the color reaction with 83% H_2SO_4 to be 28 for **5**, 20 for **7**, and 8 for **11**.

Preparation of [3-^{3}H]Lithocholic Acid——Radioactivity Counting: Radioactivity was counted with a Aloka model 903 liquid scintillation spectrometer employing EX-H(Dozin Chemical Institute, Kumamoto, Japan) as a scintillator. The radio thin-layer scannograph used was a Berthold 2D-TLC scanner LB 276 (Berlin, West Germany).

Preparation Method: [^{3}H]Sodium borohydride (100 mCi) was added to an ice-cold solution of 3-oxo-5β-cholan-24-oic acid (0.08 mmol) in 1 N NaOH (0.1 ml), and the whole was allowed to stand at 0°C for 3 h. After addition of 10% HCl (3 ml), the resulting solution was extracted with CH_2Cl_2. After evaporation of the solvent to dryness *in vacuo*, the residue was chromatographed with hexane–ether–acetone (2 : 2 : 1) as an eluent. The eluate was further subjected to preparative thin layer chromatography (TLC) using benzene–acetone (5 : 2) as a developing solvent. The band corresponding to the spot of authentic LCA was scraped off and eluted with MeOH. The tracer was kept in a refrigerator. An aliquot containing 126500 cpm was diluted with inert LCA (24.7 mg) and recrystallized from MeOH three times. The successive specific activities were 5061, 5178, 5170 cpm/mg, indicating a radiohomogeneity of 100%. Another aliquot of the tracer was scanned following the above TLC and showed a single radioactive peak that coincided with unlabeled LCA. The specific activity of the synthesized material was approximately 0.55 Ci/mmol.

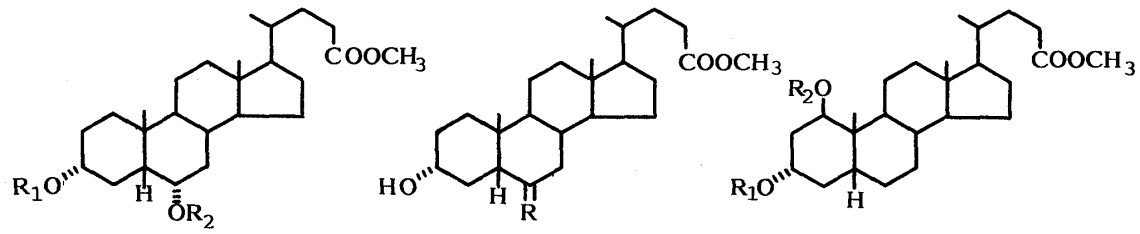
Immunization of Rabbits——Two female domestic rabbits were used for immunization with each conjugate. The antigen (2 mg) was dissolved in sterile isotonic saline (1 ml) and emulsified with complete Freund's adjuvant (1 ml). The emulsion was injected into rabbits subcutaneously at multiple sites on the 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 the booster injection. The sera were separated by centrifugation at 3000 rpm for 15 min at 4°C and stored at −20 °C. The antisera were thawed with 0.01 M phosphate buffer (pH 7.4) containing 0.9% NaCl, 0.1% gelatin, and 0.01% NaN_3.

Assay Procedure——A standard curve was constructed by setting up duplicate centrifuge tubes (7 ml) containing 0, 1, 2.5, 5.6, 10, 25, 50 and 100 ng of unlabeled LCA in the above buffer (0.1 ml) and [^{3}H]LCA (10000 dpm) in the buffer (0.1 ml). The diluted antiserum (0.1 ml) was added to this solution, and the mixture was incubated at 4 °C overnight. After addition of dextran (0.05% w/v)–charcoal (1.5% w/v) solution (0.5 ml), 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 supernatant was transferred by decantation into a vial containing a scintillation cocktail (10 ml), and the radioactivity was counted for 5 min in each sample.

Cross-Reaction Study——The specificity of antisera raised against the LCA–BSA conjugates was tested by determining the reactivities with fourteen kinds of purified compounds related to LCA (Table I). The relative amounts required to reduce the initial binding of [^{3}H]LCA by half, where the mass of unlabeled LCA was arbitrarily taken as 100% were calculated from the standard curve.

Results and Discussion

Initially, preparation of the hapten possessing the half ester at the C-6 position was undertaken by utilizing methyl hyodeoxycholate (**1**) as a starting material. Difficulties were encountered in a selective acylation of the 6α-hydroxyl group because the relative reactivity for acylation is in the order of 3α > 6α,[7] and a route was thus required to generate the 3α-hydroxyl group without removal of the ester. For this purpose, a *tert*-butyldimethylsilyl group was employed. Treatment of **1** with *tert*-butyldimethylsilyl chloride in pyridine and DMF in the presence of imidazole afforded the 3-monosilyl ether (**3**) accompanied with the corresponding the 3,6-bissilyl ether (**2**) in a ratio of 2 : 1. Refluxing of **3** with succinic anhydride–pyridine and subsequent acid hydrolysis gave the desired hapten, methyl 6α-hemisuccinoyloxylithocholate (**5**). The structure of **5** was confirmed by the downfield shift of the 6β-proton signal to 5.15 ppm in the ^{1}H-NMR spectrum. Next, the hapten having a different bridge mode at the same position, the 6-*O*-carboxymethyloxyimino derivative (**7**), was prepared by condensation



1: $R_1=R_2=H$
2: $R_1=R_2=SiMe_2\cdot tert\text{-}Bu$
3: $R_1=SiMe_2\cdot tert\text{-}Bu$, $R_2=H$
4: $R_1=SiMe_2\cdot tert\text{-}Bu$, $R_2=CO(CH_2)_2COOH$
5: $R_1=H$, $R_2=CO(CH_2)_2COOH$

6: $R=O$
7: $R=NOCH_2COOH$

8: $R_1=R_2=H$
9: $R_1=SiMe_2\cdot tert\text{-}Bu$, $R_2=H$
10: $R_1=SiMe_2\cdot tert\text{-}Bu$, $R_2=CO(CH_2)_2COOH$
11: $R_1=H$, $R_2=CO(CH_2)_2COOH$

Chart 1

of 6-oxolithocholate (**6**) with carboxymethoxylamine hemihydrochloride in refluxing methanol. Finally, the 1β-hemisuccinoyloxy hapten (**11**) was synthesized from methyl 1β, 3α-dihydroxy-5β-cholan-24-oate (**8**), which was prepared from LCA in our laboratory.[6] *tert*-Butyldimethylsilylation of **8** did take place selectively at the C-3 position to give the 3-monosilyl ether (**9**) in a quantitative yield. Subsequent acylation with succinic anhydride and 4-dimethylaminopyridine in pyridine, following acid hydrolysis gave the methyl 1β-hemisuccinoyloxylithocholate (**11**). The haptenic derivatives were covalently coupled to BSA by using mixed anhydride method of Erlanger *et al.*[8] A satisfactory number of hapten moieties was bound to BSA in each conjugate.

As a ligand required for the RIA, tritium labeled LCA was prepared by reduction of 3-oxo-5β-cholan-24-oic acid with [^{3}H]-sodium borohydride. The product was purified from a mixture with the epimeric 3β-hydroxy compound by silica gel column chromatography followed by preparative TLC. Reverse isotope dilution with the carrier LCA showed the product to be 100% radio-homogeneous, with a specific activity of approximately 0.55 Ci/mmol.

Rabbits were immunized with these conjugates emulsified with complete Freund's adjuvant. The titer was determined from the ability of the antibody at several dilutions after each bleeding to bind a certain amount of labeled [^{3}H]LCA. Approximately 40—50% of the tracer was bounded by a 1 : 100 dilution in all antisera. The binding decreased linearly with dilution on a logarithmic scale from the 1 : 100 to the 1 : 1000 dilution in all antisera. No substantial difference in affinity or specificity was observed between antisera elicited in two rabbits for each conjugate.

The dose response curves were constructed with 1 : 100 dilution of the antisera. When logit transformation was used to construct the curve, plots of logit percent bound radioactivity *vs.* logarithm of the amount of unlabeled LCA showed a linear relationship in the range of 1.0 to 100 ng.

The binding affinity was determined by incubation of a constant amount of antisera with increasing amounts of the labeled antigen. The ratio of the bound to the free antigen (B/F) observed with each antiserum plotted against the concentration of antigen according to the method of Scatchard.[9] These antisera exhibited high affinity for LCA with association constants of 4.3×10^7, 6.7×10^7, and 6.4×10^7 M^{-1}, respectively.

The specificity of the resulting antisera was assessed by testing the ability of related compounds to compete for binding sites on the antibody. The percent cross-reactions of antisera were determined by the method of Abraham.[10] The cross-reactions of anti LCA antisera with fourteen kinds of closely related compounds are listed in Table I.

The antiserum elicited by the 1β-hemisuccinate–BSA conjugate was the most specific

TABLE I. Percent Cross-Reactivities of Anti-lithocholic Acid Antisera with Selected Bile Acids

Bile acid	% cross-reactivity (50%)		
	6α-HS[a]	6-CMO[a]	1β-HS[a]
Lithocholic acid	100	100	100
Ursodeoxycholic acid	15.5	93.8	0.25
Chenodeoxycholic acid	14.1	4.69	1.52
Deoxycholic acid	0.19	0.02	0.09
Cholic acid	<0.01	<0.01	<0.01
Glycolithocholic acid	1.32	0.21	0.05
Glycoursodeoxycholic acid	3.11	10.00	0.03
Glycochenodeoxycholic acid	5.09	0.17	0.04
Glycodeoxycholic acid	0.02	<0.01	<0.01
Glycocholic acid	<0.01	<0.01	<0.01
Taurolithocholic acid	0.36	0.02	0.03
Tauroursodeoxycholic acid	4.67	6.00	0.02
Taurochenodeoxycholic acid	<0.01	<0.01	<0.01
Taurodeoxycholic acid	<0.01	<0.01	<0.01
Taurocholic acid	<0.01	<0.01	<0.01

a) 6α-HS = methyl 6α-hemisuccinoyloxylithocholate–BSA; 6-CMO = methyl 6-*O*-carboxymethyloximinolithocholate–BSA; 1β-HS = methyl 1β-hemisuccinoyloxylithocholate–BSA.

among these three antisera and was found to be sufficiently specific for LCA. There was no significant cross-reactivity with any of the major human bile acids though the cross reactions of ursodeoxycholic and chenodeoxycholic acids exhibited slightly higher value (0.25% and 1.25%) as compared with the other bile acids (ranging from less than 0.01% to 0.09%). On the other hand, both antisera elicited from the 6α-hemisuccinate and the 6-*O*-carboxymethyloxime showed remarkably high cross-reactivities to these bile acids among the bile acids tested (15.5% and 14.1% for the former; 93.8% and 4.69% for the latter, respectively). The lower discriminating ability of these antisera may be ascribable to the site of conjugation, since LCA is coupled with BSA through the C-6 position adjacent to the C-7 hydroxyl group.

In this work, we have shown that specific antiserum capable of recognizing the ring structure and also the side chain can be obtained by immunizing animals with an antigen in which LCA is coupled to the carrier protein through the C-1β position rather than the C-6. To the best of our knowledge, hydroxylation at the 1β-position in the biotransformation of LCA has not yet been reported, contrary to the case of other primary bile acids.[6,11,12] Thus, the 1β position appears to be a suitable site for attachment of a carrier protein in the preparation of immunogens for RIA. Application of this haptenic design to other biologically important bile acids will be investigated in the future.

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