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Preparation of New Haptens for Use in Immunoassay of Glycine-Conjugated Bile Acids[1)]

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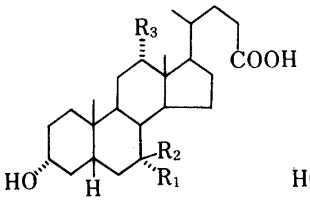
The *N*-succinimidyl esters of various glycine-conjugated bile acid 3-hemisuccinates and 3-hemiglutarates have been prepared for use in immunoassay. Unconjugated bile acids were condensed with *p*-nitrophenol to form the activated esters. Subsequent reaction with 2,2,2-trichloroethyl glycinate provided the glycine-conjugated trichloroethyl esters. On treatment with succinic anhydride and glutaric anhydride in pyridine, the glycine-conjugated bile acids were led to the 3-hemisuccinates and 3-hemiglutarates, respectively. Condensation of these half esters with *N*-hydroxysuccinimide was effected by the use of water-soluble carbodiimide to provide the *N*-succinimidyl esters. Elimination of the protecting group in the side chain was achieved by reduction with zinc dust in tetrahydrofuran–potassium dihydrogen phosphate solution, yielding the desired haptens.

Keywords——bile acid; glycine-conjugate; immunoassay; immunogen; hapten; 3-hemisuccinate; 3-hemiglutarate; *N*-succinimidyl ester

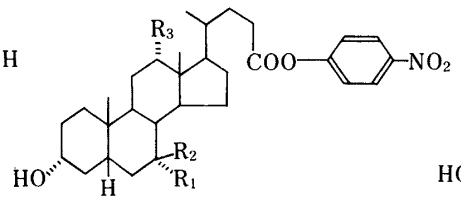
In recent years, considerable attention has been paid to the metabolism of bile acids in man in connection with hepatobiliary diseases, and the formation and dissolution of cholesterol gallstones. Numerous methods are at present available for the determination of bile acids in biological fluids. The immunoassay method is favorable for routine work with respect to sensitivity and simplicity. However, the procedures hitherto developed are still unsatisfactory as regards reliability owing to the lack of specific antisera. The antisera used have been elicited with immunogens in which the hapten was linked to the carrier through the carboxyl group in the side chain.[2)] It is well substantiated that the site through which a hapten is linked to an immunogenic carrier significantly influences the specificity of antibody raised against the hapten–carrier conjugate. The use of a steroid hapten coupled through a position remote from the side chain seems to be promising for producing a specific antibody which is capable of discriminating closely related unconjugated and conjugated bile acids. Therefore, the preparation of haptenized glycine-conjugated bile acids possessing an activated ester bridge at C-3 has been undertaken. The present paper deals with the synthesis of the *N*-succinimidyl esters of glycolithocholate, glycochenodeoxycholate, glycodeoxycholate, glycocholate and glycoursodeoxycholate 3-hemisuccinates and 3-hemiglutarates.

Our effort was initially directed to the synthesis of the *N*-succinimidyl esters of glycolithocholate 3-hemisuccinate and 3-hemiglutarate. The trichloroethyl group, which is readily removable under acidic conditions, was utilized for protecting the carboxyl group of the side chain.[3)] First, lithocholate (**1**) was converted to the *p*-nitrophenyl ester (**2**) by the known method.[4)] When **2** was condensed with 2,2,2-trichloroethyl glycinate[5)] in pyridine, trichloroethyl lithocholylglycinate (**3**) was formed in satisfactory yield. Subsequent treatment with succinic anhydride and glutaric anhydride in pyridine afforded the 3-hemisuccinate (**4a**) and 3-hemiglutarate (**4b**) in reasonable yields, respectively. The downfield shifts of the 3β-

proton signal to 4.74 and 4.71 ppm in the nuclear magnetic resonance (NMR) spectra upon acylation assisted the assignment of the structures **4a** and **4b**. Condensation of the half esters (**4a**, **4b**) with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide[6] provided the *N*-succinimidyl esters (**5a**, **5b**). Contrary to expectation, however, reductive elimination of the protecting group with zinc dust in 90% acetic acid[3] failed owing to concomitant cleavage of the *N*-succinimidyl ester bond. Therefore, the method of Just *et al.*[7] was employed. Upon being treated with zinc dust in tetrahydrofuran–potassium dihydrogen phosphate solution, **5a** and **5b** provided the desired *N*-succinimidyl esters of glycolithocholate 3-hemisuccinate (**6a**) and 3-hemiglutarate (**6b**), respectively.



1, 7, 13, 19, 25



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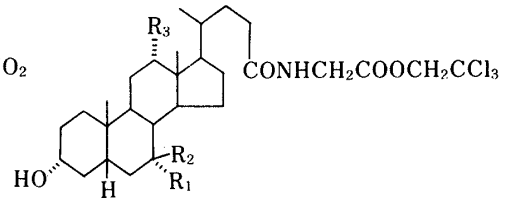
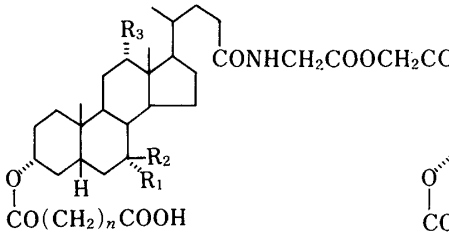
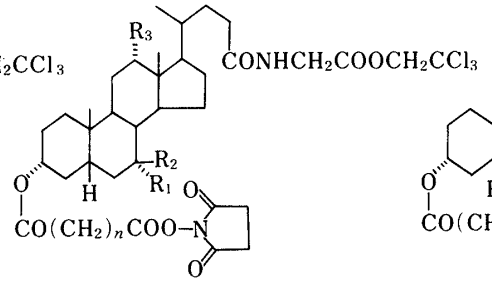


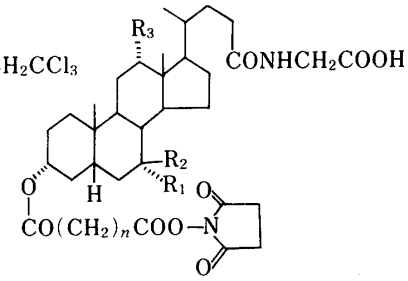
3, 9, 15, 21, 27



4, 10, 16, 22, 28



5, 11, 17, 23, 29



6, 12, 18, 24, 30

1–6: $R_1=R_2=R_3=H$
7–12: $R_1=OH$, $R_2=R_3=H$
13–18: $R_1=R_2=H$, $R_3=OH$
19–24: $R_1=R_3=OH$, $R_2=H$
25–30: $R_1=R_3=H$, $R_2=OH$

a: $n=2$, **b**: $n=3$

Chart 1

Next, we focused on the preparation of similar types of haptens from chenodeoxycholate (**7**), deoxycholate (**13**), cholate (**19**) and ursodeoxycholate (**25**). The relative ease of acylation of the hydroxyl groups in dihydroxy and trihydroxy bile acids has been demonstrated to be in the order $3\alpha > 7\alpha > 12\alpha$.[8] In addition, the 7$\beta$-hydroxyl group is known to be more reactive than the C-7 epimer, but much less reactive than the 3α-hydroxyl function (due to steric hindrance). Accordingly, the possibility of selective acylation appears to be promising, when bile acids are treated with dicarboxylic acid anhydride under mild conditions.

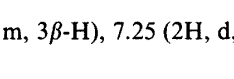
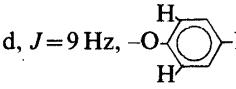
Unconjugated bile acids (**7**, **13**, **19**, **25**) were first led to the *p*-nitrophenyl esters (**8**, **14**, **20**, **26**). The activated esters were readily transformed into the trichloroethyl esters of glycine-conjugates (**9**, **15**, **21**, **27**). Subsequent acylation with succinic anhydride– and glutaric anhydride–pyridine under mild conditions did take place selectively at the 3α-hydroxyl group, yielding the 3-hemisuccinates (**10a**, **16a**, **22a**, **28a**) and 3-hemiglutarates (**10b**, **16b**, **22b**, **28b**), respectively. Condensation with *N*-hydroxysuccinimide in the presence of water-soluble carbodiimide provided the *N*-succinimidyl esters (**11a**, **b**, **17a**, **b**, **23a**, **b**, **29a**, **b**) which, on

reduction with zinc dust in tetrahydrofuran–potassium dihydrogen phosphate solution, provided the desired *N*-succinimidyl esters of glycine-conjugated 3-hemisuccinates (**12a**, **18a**, **24a**, **30a**) and 3-hemiglutarates (**12b**, **18b**, **24b**, **30b**).

The availability of these haptens should permit the preparation of carrier conjugates as well as enzyme-labeled antigens. The antigenic properties of these haptenized immunogens will be investigated in the future.

Experimental

All melting points were taken on a micro hot-stage apparatus and are uncorrected. Optical rotations were determined with a JASCO model DIP-180 polarimeter. Proton nuclear magnetic resonance (^{1}H-NMR) spectra were recorded on a JEOL FX-100 spectrometer at 100 MHz (with tetramethylsilane as an internal standard).

4-Nitrophenyl Lithocholate (2)——A solution of lithocholic acid (**1**) (1 g), *p*-nitrophenol (400 mg) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide·HCl (870 mg) in 95% dioxane (4 ml) was stirred at room temperature overnight. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (18 g). Elution with hexane–AcOEt (2 : 1) and recrystallization of the eluate from acetone–hexane gave **2** (845 mg) as colorless needles. mp 156—158 °C. $[\alpha]_D^{17}$ +19.8° (c=0.18, $CHCl_3$). NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 0.99 (3H, d, J=5 Hz, 21-CH_3), 3.64 (1H, m, 3β-H), 7.25 (2H, d, J=9 Hz, ), 8.25 (2H, d, J=9 Hz, ). *Anal.* Calcd for $C_{30}H_{43}NO_5$: C, 72.40; H, 8.71; N, 2.81. Found: C, 72.17; H, 8.87; N, 2.98.

***N*-Lithocholylglycine 2,2,2-Trichloroethyl Ester (3)**——A solution of **2** (600 mg) and 2,2,2-trichloroethyl glycinate·HBr (520 mg) in pyridine (4 ml) was stirred at room temperature overnight. The resulting solution was diluted with AcOEt, washed successively with 5% HCl, 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (18 g). Elution with hexane–AcOEt (1 : 1) and recrystallization of the eluate from acetone–hexane gave **3** (500 mg) as colorless needles. mp 149.5—150 °C. $[\alpha]_D^{24}$ +18.6° (c=0.35, $CHCl_3$). NMR ($CDCl_3$) δ: 0.63 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.93 (3H, d, J=5 Hz, 21-CH_3), 3.60 (1H, m, 3β-H), 4.17 (2H, d, J=5 Hz, NCH_2CO), 4.76 (2H, s, OCH_2CCl_3), 6.01 (1H, t, J=5 Hz, CONH). *Anal.* Calcd for $C_{28}H_{44}Cl_3NO_4$: C, 59.52; H, 7.85; Cl, 18.82; N, 2.48. Found: C, 59.38; H, 7.93; Cl, 18.57; N, 2.52.

***N*-Lithocholylglycine 2,2,2-Trichloroethyl Ester 3-Hemisuccinate (4a)**——A solution of **3** (400 mg) and succinic anhydride (400 mg) in pyridine (1.5 ml) was heated at 80—100 °C for 1 h. After addition of H_2O (1 ml), the resulting solution was allowed to stand at room temperature for 30 min, then extracted with AcOEt. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (18 g). Elution with $CHCl_3$–MeOH (20 : 1) and recrystallization of the eluate from aq. acetone gave **4a** (395 mg) as colorless leaflets. mp 145—146 °C. $[\alpha]_D^{24}$ +34.0° (c=0.39, $CHCl_3$). NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.94 (3H, d, J=5 Hz, 21-CH_3), 2.60 (4H, m, $COCH_2CH_2CO$), 4.17 (2H, d, J=5 Hz, NCH_2CO), 4.74 (1H, m, 3β-H), 4.76 (2H, s, OCH_2CCl_3), 6.04 (1H, t, J=5 Hz, CONH). *Anal.* Calcd for $C_{32}H_{48}Cl_3NO_7$: C, 57.78; H, 7.27; Cl, 15.99; N, 2.11. Found: C, 57.50; H, 7.23; Cl, 15.78; N, 2.02.

***N*-Lithocholylglycine 2,2,2-Trichloroethyl Ester 3-(3-Succinimidoxycarbonyl)propionate (5a)**——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (160 mg) and *N*-hydroxysuccinimide (78 mg) were added to a solution of **4a** (300 mg) in 95% dioxane (1 ml), and the whole was stirred at room temperature for 3 h. The resulting solution was diluted with AcOEt, washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The crude product was recrystallized from acetone–hexane to give **5a** (315 mg) as colorless leaflets. mp 158—160 °C. $[\alpha]_D^{24}$ +26.5° (c=0.28, $CHCl_3$). NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 0.93 (3H, d, J=5 Hz, 21-CH_3), 2.52—3.04 (4H, m, $COCH_2CH_2CO$), 2.80 (4H, s, succinimidyl), 4.18 (2H, d, J=5 Hz, NCH_2CO), 4.74 (1H, m, 3β-H), 4.76 (1H, s, OCH_2CCl_3), 5.96 (1H, t, J=5 Hz, CONH). *Anal.* Calcd for $C_{36}H_{51}Cl_3N_2O_9$: C, 56.73; H, 6.75; Cl, 13.96; N, 3.68. Found: C, 56.77; H, 6.84; Cl 13.72; N, 3.75.

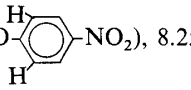
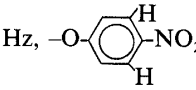
***N*-Lithocholylglycine 3-(3-Succinimidoxycarbonyl)propionate (6a)**——A solution of **5a** (250 mg) in tetrahydrofuran (12 ml)–0.5 M KH_2PO_4 (4 ml) was stirred with Zn dust (2 g) at room temperature for 30 min. The precipitate was removed by filtration through celite (10 g) on a sintered glass funnel and washed with AcOEt and $CHCl_3$–MeOH (10 : 1). The filtrate and washings were combined, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (18 g). Elution with $CHCl_3$–MeOH (6 : 1) and recrystallization of the eluate from MeOH gave **6a** (115 mg) as colorless leaflets. mp 188—190 °C. $[\alpha]_D^{24}$ +34.6° (c=0.17, $CHCl_3$). NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.67 (3H, s, 18-CH_3), 0.76—1.04 (6H, 19-CH_3 and 21-CH_3), 2.50—3.08 (4H, m, $COCH_2CH_2CO$), 2.84 (4H, s, succinimidyl), 3.82 (2H, s, NCH_2CO), 4.72 (1H, m, 3β-H). *Anal.* Calcd for $C_{34}H_{50}N_2O_9$: C, 64.74; H, 7.99; N, 4.44. Found: C, 64.50; H, 8.12; N, 4.40.

***N*-Lithocholylglycine 2,2,2-Trichloroethyl Ester 3-Hemiglutarate (4b)**——Treatment of **3** (400 mg) with glutaric

anhydride (600 mg) in the manner described for **4a** gave **4b** (360 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.64 (3H, s, 18-CH_3), 0.88—1.00 (6H, 19-CH_3 and 21-CH_3), 2.35 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 4.19 (2H, d, J=5 Hz, NCH_2CO), 4.71 (1H, m, 3β-H), 4.77 (2H, s, OCH_2CCl_3), 6.11 (1H, t, J=5 Hz, CONH).

***N*-Lithocholylglycine 2,2,2-Trichloroethyl Ester 3-(4-Succinimidoxycarbonyl)butyrate (5b)**——**4b** (200 mg) was treated in the manner described for **5a**. The crude product was chromatographed on silica gel (18 g). Elution with hexane–acetone (1 : 3) gave **5b** (170 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-CH_3), 0.76—1.02 (6H, 19-CH_3 and 21-CH_3), 2.70 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.82 (4H, s, succinimidyl), 4.19 (2H, d, J=5 Hz, NCH_2CO), 4.72 (1H, m, 3β-H), 4.78 (2H, s, OCH_2CCl_3), 5.93 (1H, t, J=5 Hz, CONH).

***N*-Lithocholylglycine 3-(4-Succinimidoxycarbonyl)butyrate (6b)**——**5b** (170 mg) was treated in the manner described for **6a**. The crude product was rinsed with hexane–ether (5 : 1), then recrystallized from MeOH to give **6b** (115 mg) as colorless leaflets. mp 164—165 °C. $[\alpha]_D^{18}$ +27.6° (c=0.15, $CHCl_3$). NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.66 (3H, s, 18-CH_3), 0.90—1.02 (6H, 19-CH_3 and 21-CH_3), 2.71 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.84 (4H, s, succinimidyl), 3.94 (2H, s, NCH_2CO), 4.70 (1H, m, 3β-H). *Anal.* Calcd for $C_{35}H_{52}N_2O_9$: C, 65.19; H, 8.13; N, 4.35. Found: C, 65.28; H, 7.96; N, 4.17.

4-Nitrophenyl Chenodeoxycholate (8)——Chenodeoxycholic acid (**7**) (2 g) was treated in the manner described for **2**. The crude product was chromatographed on silica gel (18 g). Elution with hexane–AcOEt (1 : 1) and recrystallization of the product from acetone–hexane gave **8** (1.7 g) as colorless leaflets. mp 164—166 °C. $[\alpha]_D^{18}$ +5.4° (c=0.28, $CHCl_3$). NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.00 (3H, d, J=5 Hz, 21-CH_3), 3.47 (1H, m, 3β-H), 3.94 (1H, m, 7β-H), 7.25 (2H, d, J=9 Hz, ), 8.25 (2H, d, J=9 Hz, ).

Anal. Calcd for $C_{30}H_{43}NO_6 \cdot 1/4H_2O$: C, 69.53; H, 8.46; N, 2.70. Found: C, 69.55; H, 8.69; N, 2.78.

***N*-Chenodeoxycholylglycine 2,2,2-Trichloroethyl Ester (9)**——A solution of **8** (1.5 g) and 2,2,2-trichloroethyl glycinate·HBr (800 mg) in pyridine (6 ml) was stirred at room temperature overnight. The resulting solution was diluted with AcOEt, washed successively with 5% HCl, 5% $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (18 g). Elution with hexane–AcOEt (1 : 3) gave **9** (1.05 g) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), 0.96 (3H, d, J=5 Hz, 21-CH_3), 3.46 (1H, m, 3β-H), 3.84 (1H, m, 7β-H), 4.20 (2H, d, J=5 Hz, NCH_2CO), 4.78 (2H, s, OCH_2CCl_3), 6.07 (1H, t, J=5 Hz, CONH).

***N*-Chenodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemisuccinate (10a)**——A solution of **9** (500 mg) and succinic anhydride (500 mg) in pyridine (2 ml) was stirred at 37 °C for 24 h. After addition of H_2O (1 ml), the resulting solution was allowed to stand at room temperature for 30 min and extracted with AcOEt. The organic phase was washed with H_2O, dried over anhydrous Na_2SO_4, and evaporated down. The residue was chromatographed on silica gel (60 g). Elution with $CHCl_3$–MeOH (15 : 1) gave **10a** (450 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.67 (3H, s, 18-CH_3), 0.84—1.04 (6H, 19-CH_3 and 21-CH_3), 2.55—2.68 (4H, m, $COCH_2CH_2CO$), 3.83 (1H, m, 7β-H), 4.19 (2H, d, J=5 Hz, NCH_2CO), 4.59 (1H, m, 3β-H), 4.76 (2H, s, OCH_2CCl_3), 6.01 (1H, t, J=5 Hz, CONH).

***N*-Chenodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(3-Succinimidoxycarbonyl)propionate (11a)**——1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide·HCl (270 mg) and *N*-hydroxysuccinimide (140 mg) were added to a solution of **10a** (300 mg) in 95% dioxane (3 ml), and the whole was stirred at room temperature for 4 h. The resulting solution was diluted with AcOEt, washed with H_2O, and dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure gave **11a** (270 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-CH_3), 0.82—1.04 (6H, 19-CH_3 and 21-CH_3), 2.52—3.04 (4H, m, $COCH_2CH_2CO$), 2.80 (4H, s, succinimidyl), 3.82 (1H, m, 7β-H), 4.16 (2H, d, J=5 Hz, NCH_2CO), 4.56 (1H, m, 3β-H), 4.75 (2H, s, OCH_2CCl_3), 5.95 (1H, t, J=5 Hz, CONH).

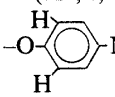
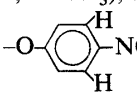
***N*-Chenodeoxycholylglycine 3-(3-Succinimidoxycarbonyl)propionate (12a)**——A solution of **11a** (270 mg) in tetrahydrofuran (15 ml)–0.5 M KH_2PO_4 (8 ml) was stirred with Zn dust (2.5 g) at room temperature for 40 min. The precipitate was removed by filtration through celite (10 g) on a sintered glass funnel and washed with AcOEt. The filtrate and washings were combined, dried over anhydrous Na_2SO_4 and evaporated down. The residue was chromatographed on silica gel (15 g). Elution with $CHCl_3$–MeOH (6 : 1) and recrystallization of the product from MeOH gave **12a** (190 mg) as colorless leaflets. mp 182—184 °C. $[\alpha]_D^{18}$ +20.4° (c=0.17, dioxane). NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.67 (3H, s, 18-CH_3), 0.90—1.04 (6H, 19-CH_3 and 21-CH_3), 2.60—3.00 (4H, m, $COCH_2CH_2CO$), 2.84 (4H, s, succinimidyl), 3.81 (3H, 7β-H and NCH_2CO), 4.59 (1H, m, 3β-H). *Anal.* Calcd for $C_{34}H_{50}N_2O_{10} \cdot 1/4H_2O$: C, 62.70; H, 7.82; N, 4.30. Found: C, 62.76; H, 7.84; N, 4.48.

***N*-Chenodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemiglutarate (10b)**——**9** (550 mg) was treated with glutaric anhydride (750 mg) in the manner described for **4a**. Elution with $CHCl_3$–MeOH–AcOH (30 : 1 : 0.05) gave **10b** (380 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.65 (3H, s, 18-CH_3), 0.84—1.02 (6H, 19-CH_3 and 21-CH_3), 2.34 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 3.84 (1H, m, 7β-H), 4.19 (2H, d, J=5 Hz, NCH_2CO), 4.58 (1H, m, 3β-H), 4.75 (2H, s, OCH_2CCl_3), 5.95 (1H, t, J=5 Hz, CONH).

***N*-Chenodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(4-Succinimidoxycarbonyl)butyrate (11b)**——**10b** (270 mg) was treated in the manner described for **5a** to give **11b** (300 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.66 (3H, s, 18-CH_3), 0.84—1.02 (6H, 19-CH_3 and 21-CH_3), 2.69 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.81 (4H, s,

succinimidyl), 3.83 (1H, m, 7β-H), 4.19 (2H, d, $J=5$ Hz, NCH_2CO), 4.58 (1H, m, 3β-H), 4.77 (2H, s, OCH_2CCl_3), 5.90 (1H, t, $J=5$ Hz, CONH).

***N*-Chenodeoxycholylglycine 3-(4-Succinimidoxycarbonyl)butyrate (12b)**——**11b** (300 mg) was treated in the manner described for **6a** to give **12b** (260 mg) as colorless semicrystals. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.68 (3H, s, 18-CH_3), 0.80—1.04 (6H, 19-CH_3 and 21-CH_3), 2.71 (4H, t, $J=7$ Hz, $COCH_2CH_2CH_2CO$), 2.86 (4H, s, succinimidyl), 3.82 (3H, 7β-H and NCH_2CO), 4.55 (1H, m, 3β-H).

4-Nitrophenyl Deoxycholate (14)——Deoxycholic acid (**13**) (1 g) was treated in the manner described for **2**. The residue was chromatographed on silica gel (18 g). Elution with hexane–AcOEt (1 : 1) gave **14** (900 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.04 (3H, d, $J=5$ Hz, 21-CH_3), 3.61 (1H, m, 3β-H), 3.97 (1H, m, 12β-H), 7.24 (2H, d, $J=9$ Hz, ), 8.24 (2H, d, $J=9$ Hz, ).

***N*-Deoxycholylglycine 2,2,2-Trichloroethyl Ester (15)**——**14** (1.8 g) was treated in the manner described for **3**. The residue was chromatographed on silica gel (60 g). Elution with hexane–AcOEt (1 : 5) gave **15** (1.3 g) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.99 (3H, d, $J=5$ Hz, 21-CH_3), 3.59 (1H, m, 3β-H), 3.96 (1H, m, 12β-H), 4.18 (2H, d, $J=5$ Hz, NCH_2CO), 4.78 (2H, s, OCH_2CCl_3), 6.17 (1H, t, $J=5$ Hz, CONH).

***N*-Deoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemisuccinate (16a)**——**15** (850 mg) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (60 g). Elution with $CHCl_3$–MeOH–AcOH (30 : 2 : 0.1) gave **16a** (950 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.92 (3H, s, 19-CH_3), 0.99 (3H, d, $J=5$ Hz, 21-CH_3), 2.62 (4H, s, $COCH_2CH_2CO$), 4.01 (1H, m, 12β-H), 4.19 (2H, d, $J=5$ Hz, NCH_2CO), 4.77 (2H, s, OCH_2CCl_3), 4.50—5.00 (1H, m, 3β-H), 6.22 (1H, t, $J=5$ Hz, CONH).

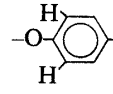
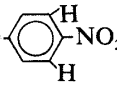
***N*-Deoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(3-Succinimidoxycarbonyl)propionate (17a)**——**16a** (300 mg) was treated in the manner described for **5a**. The crude product was chromatographed on silica gel (18 g). Elution with benzene–AcOEt (1 : 7) and recrystallization of the product from acetone–hexane gave **17a** (270 mg) as colorless leaflets, mp 96—97 °C. $[\alpha]_D^{15}$ +32.7° ($c=0.28$, $CHCl_3$). NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 0.99 (3H, d, $J=5$ Hz, 21-CH_3), 2.84 (4H, s, succinimidyl), 2.60—3.00 (4H, m, $COCH_2CH_2CO$), 3.93 (1H, m, 12β-H), 4.18 (2H, d, $J=5$ Hz, NCH_2CO), 4.77 (2H, s, OCH_2CCl_3), 4.52—5.00 (1H, m, 3β-H), 6.00 (1H, t, $J=5$ Hz, CONH). *Anal.* Calcd for $C_{36}H_{51}Cl_3N_2O_{10}$: C, 55.56; H, 6.61; Cl, 13.67; N, 3.60. Found: C, 55.30; H, 6.67; Cl, 13.54; N, 3.59.

***N*-Deoxycholylglycine 3-(3-Succinimidoxycarbonyl)propionate (18a)**——**17a** (200 mg) was treated in the manner described for **6a**. The crude product was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (10 : 1) and recrystallization of the product from MeOH gave **18a** (100 mg) as colorless needles. mp 112—115 °C. $[\alpha]_D^{18}$ +30.0° ($c=0.25$, $CHCl_3$). NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.96 (3H, s, 19-CH_3), 1.01 (3H, d, $J=5$ Hz, 21-CH_3), 2.86 (4H, s, succinimidyl), 2.48—3.04 (4H, m, $COCH_2CH_2CO$), 3.86 (2H, s, NCH_2CO), 3.94 (1H, m, 12β-H), 4.72 (1H, m, 3β-H). *Anal.* Calcd for $C_{34}H_{50}N_2O_{10}\cdot H_2O$: C, 61.43; H, 7.88; N, 4.21. Found: C, 61.50; H, 7.83; N, 4.06.

***N*-Deoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemiglutarate (16b)**——**15** (630 mg) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (60 g). Elution with $CHCl_3$–MeOH–AcOH (30 : 2 : 0.1) gave **16b** (440 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.00 (3H, d, $J=5$ Hz, 21-CH_3), 2.36 (4H, t, $J=7$ Hz, $COCH_2CH_2CH_2CO$), 3.99 (1H, m, 12β-H), 4.20 (2H, d, $J=5$ Hz, NCH_2CO), 4.70 (1H, m, 3β-H), 4.77 (2H, s, OCH_2CCl_3), 6.06 (1H, t, $J=5$ Hz, CONH).

***N*-Deoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(4-Succinimidoxycarbonyl)butyrate (17b)**——**16b** (300 mg) was treated in the manner described for **5a**. The residue was chromatographed on silica gel (15 g). Elution with benzene–AcOEt (1 : 5) gave **17b** (290 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.98 (3H, s, 19-CH_3), 1.01 (3H, d, $J=5$ Hz, 21-CH_3), 2.70 (4H, t, $J=7$ Hz, $COCH_2CH_2CH_2CO$), 2.83 (4H, s, succinimidyl), 3.97 (1H, m, 12β-H), 4.21 (2H, d, $J=5$ Hz, NCH_2CO), 4.79 (2H, s, OCH_2CCl_3), 4.52—4.96 (1H, m, 3β-H), 5.98 (1H, t, $J=5$ Hz, CONH).

***N*-Deoxycholylglycine 3-(4-Succinimidoxycarbonyl)butyrate (18b)**——**17b** (240 mg) was treated in the manner described for **6a**. The residue was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (10 : 1) gave **18b** (110 mg) as colorless semicrystals. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.94 (3H, s, 19-CH_3), 1.01 (3H, d, $J=5$ Hz, 21-CH_3), 2.72 (4H, t, $J=7$ Hz, $COCH_2CH_2CH_2CO$), 2.86 (4H, s, succinimidyl), 3.88 (2H, s, NCH_2CO), 3.97 (1H, m, 12β-H), 4.70 (1H, m, 3β-H).

4-Nitrophenyl Cholate (20)——Cholic acid (**19**) (1 g) was treated in the manner described for **2**. The residue was chromatographed on silica gel (35 g). Elution with hexane–AcOEt (1 : 100) and recrystallization of the product from acetone gave **20** (820 mg) as colorless leaflets. mp 190—192 °C. $[\alpha]_D^{16}$ +21.3° ($c=0.26$, $CHCl_3$). NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.88 (3H, s, 19-CH_3), 1.05 (3H, d, $J=5$ Hz, 21-CH_3), 3.42 (1H, m, 3β-H), 3.83 (1H, m, 7β-H), 3.96 (1H, m, 12β-H), 7.24 (2H, d, $J=9$ Hz, ), 8.23 (2H, d, $J=9$ Hz, ). *Anal.* Calcd for $C_{30}H_{43}NO_7$: C, 68.03; H, 8.18; N, 2.64. Found: C, 67.94; H, 8.35; N, 2.59.

***N*-Cholylglycine 2,2,2-Trichloroethyl Ester (21)**——**20** (800 mg) was treated in the manner described for **3**. The residue was chromatographed on silica gel (35 g). Elution with acetone–hexane (1 : 1) gave **21** (615 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.88 (3H, s, 19-CH_3), 0.99 (3H, d, J=5 Hz, 21-CH_3), 3.41 (1H, m, 3β-H), 3.82 (1H, m, 7β-H), 3.95 (1H, m, 12β-H), 4.15 (2H, d, J=6 Hz, NCH_2CO), 4.76 (2H, s, OCH_2CCl_3), 6.89 (1H, t, J=6 Hz, CONH).

***N*-Cholylglycine 2,2,2-Trichloroethyl Ester 3-Hemisuccinate (22a)**——**21** (1 g) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (60 g). Elution with $CHCl_3$–MeOH–AcOH (30 : 2 : 0.1) gave **22a** (750 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.00 (3H, d, J= 5 Hz, 21-CH_3), 2.61 (4H, s, $COCH_2CH_2CO$), 3.85 (1H, m, 7β-H), 4.01 (1H, m, 12β-H), 4.19 (2H, d, J=5 Hz, NCH_2CO), 4.55 (1H, m, 3β-H), 4.78 (2H, s, OCH_2CCl_3), 6.69 (1H, t, J=5 Hz, CONH).

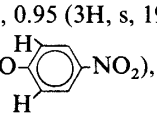
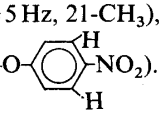
***N*-Cholylglycine 2,2,2-Trichloroethyl Ester 3-(3-Succinimidoxycarbonyl)propionate (23a)**——**22a** (450 mg) was treated in the manner described for **5a**. The residue was chromatographed on silica gel (16 g). Elution with acetone–AcOEt (10 : 1) gave **23a** (450 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), 1.01 (3H, d, J=5 Hz, 21-CH_3), 2.56—3.00 (4H, m, $COCH_2CH_2CO$), 2.83 (4H, s, succinimidyl), 3.81 (1H, m, 7β-H), 3.92 (1H, m, 12β-H), 4.17 (2H, d, J=5 Hz, NCH_2CO), 4.56 (1H, m, 3β-H), 4.76 (2H, s, OCH_2CCl_3), 6.12 (1H, t, J=5 Hz, CONH).

***N*-Cholylglycine 3-(3-Succinimidoxycarbonyl)propionate (24a)**——**23a** (250 mg) was treated in the manner described for **6a**. The residue was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (4 : 1) gave **24a** (115 mg) as a colorless amorphous substance. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.01 (3H, d, J=5 Hz, 21-CH_3), 2.87 (4H, s, succinimidyl), 2.48—3.04 (4H, m, $COCH_2CH_2CO$), 3.81 (3H, 7β-H and NCH_2CO), 3.95 (1H, m, 12β-H), 4.60 (1H, m, 3β-H).

***N*-Cholylglycine 2,2,2-Trichloroethyl Ester 3-Hemiglutarate (22b)**——**21** (1.2 g) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (90 g). Elution with $CHCl_3$–MeOH–AcOH (36 : 2 : 0.1) gave **22b** (850 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 0.91 (3H, s, 19-CH_3), 1.00 (3H, d, J=5 Hz, 21-CH_3), 2.35 (4H, m, $COCH_2CH_2CH_2CO$), 3.85 (1H, m, 7β-H), 4.00 (1H, m, 12β-H), 4.20 (2H, d, J=5 Hz, NCH_2CO), 4.56 (1H, m, 3β-H), 4.77 (2H, s, OCH_2CCl_3), 6.36 (1H, t, J=5 Hz, CONH).

***N*-Cholylglycine 2,2,2-Trichloroethyl Ester 3-(4-Succinimidoxycarbonyl)butyrate (23b)**——**22b** (400 mg) was treated in the manner described for **5a**. The residue was chromatographed on silica gel (16 g). Elution with hexane–AcOEt (15 : 1) gave **23b** (310 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.90 (3H, s, 19-CH_3), 1.01 (3H, d, J=5 Hz, 21-CH_3), 2.69 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.82 (4H, s, succinimidyl), 3.84 (1H, m, 7β-H), 3.98 (1H, m, 12β-H), 4.20 (2H, d, J=5 Hz, NCH_2CO), 4.58 (1H, m, 3β-H), 4.78 (2H, s, OCH_2CCl_3), 6.10 (1H, t, J=5 Hz, CONH).

***N*-Cholylglycine 3-(4-Succinimidoxycarbonyl)butyrate (24b)**——**23b** (300 mg) was treated in the manner described for **6a**. The residue was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (4 : 1) gave **24b** (200 mg) as colorless semicrystals. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.93 (3H, s, 19-CH_3), 1.01 (3H, d, J=5 Hz, 21-CH_3), 2.71 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.85 (4H, s, succinimidyl), 3.81 (3H, m, 7β-H and NCH_2CO), 3.95 (1H, m, 12β-H), 4.56 (1H, m, 3β-H).

4-Nitrophenyl Ursodeoxycholate (26)——Ursodeoxycholic acid (**25**) (3 g) was treated in the manner described for **2**. The crude product was chromatographed on silica gel (60 g). Elution with hexane–AcOEt (1 : 3) gave **26** (3.03 g) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.70 (3H, s, 18-CH_3), 0.95 (3H, s, 19-CH_3), 1.01 (3H, d, J=5 Hz, 21-CH_3), 3.32—3.76 (2H, m, 3β-H and 7α-H), 7.25 (2H, d, J=9 Hz, ), 8.25 (2H, d, J=9 Hz, ).

***N*-Ursodeoxycholylglycine 2,2,2-Trichloroethyl Ester (27)**——**26** (3 g) was treated in the manner described for **3**. The residue was chromatographed on silica gel (60 g). Elution with hexane–AcOEt (1 : 5) gave **27** (2.4 g) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.84—1.02 (6H, 19-CH_3 and 21-CH_3), 3.36—3.76 (2H, m, 3β-H and 7α-H), 4.24 (2H, d, J=5 Hz, NCH_2CO), 4.78 (2H, s, OCH_2CCl_3), 6.07 (1H, t, J=5 Hz, CONH).

***N*-Ursodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemisuccinate (28a)**——**27** (770 mg) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (90 g). Elution with $CHCl_3$–MeOH (10 : 1) gave **28a** (480 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.84—1.04 (6H, 19-CH_3 and 21-CH_3), 2.44—2.76 (4H, m, $COCH_2CH_2CO$), 3.58 (1H, m, 7α-H), 4.20 (2H, d, J=5 Hz,NCH_2CO), 4.40—4.84 (1H, m, 3β-H), 4.79 (2H, s, OCH_2CCl_3), 6.08 (1H, t, J=5 Hz, CONH).

***N*-Ursodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(3-Succinimidoxycarbonyl)propionate (29a)**——**28a** (480 mg) was treated in the manner described for **5a**. The residue was chromatographed on silica gel (16 g). Elution with hexane–AcOEt (1 : 5) gave **29a** (280 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.84—1.04 (6H, 19-CH_3 and 21-CH_3), 2.60—3.04 (4H, m, $COCH_2CH_2CO$), 2.83 (4H, s, succinimidyl), 3.60 (1H, m, 7α-H), 4.20 (2H, d, J=5 Hz, NCH_2CO), 4.44—4.96 (1H, m, 3β-H), 4.78 (2H, s, OCH_2CCl_3), 5.97 (1H, t, J=5 Hz, CONH).

***N*-Ursodeoxycholylglycine 3-(3-Succinimidoxycarbonyl)propionate (30a)**——**29a** (280 mg) was treated in the manner described for **6a**. The crude product was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (6 : 1) gave **30a** (110 mg) as colorless semicrystals. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.94—1.08

(6H, 19-CH_3 and 21-CH_3), 2.86 (4H, s, succinimidyl), 2.52—3.06 (4H, m, $COCH_2CH_2CO$), 3.52 (1H, m, 7α-H), 3.80 (2H, s, NCH_2CO), 4.72 (1H, m, 3β-H).

***N*-Ursodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-Hemiglutarate (28b)**——**27** (1500 mg) was treated in the manner described for **4a**. The residue was chromatographed on silica gel (90 g). Elution with $CHCl_3$–MeOH (10 : 1) gave **28b** (300 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.69 (3H, s, 18-CH_3), 0.94—1.04 (6H, 19-CH_3 and 21-CH_3), 2.36 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 3.53 (1H, m, 7α-H), 4.20 (2H, d, J=5 Hz, NCH_2CO), 4.40—4.88 (1H, m, 3β-H), 4.73 (2H, s, OCH_2CCl_3), 6.09 (1H, t, CONH).

***N*-Ursodeoxycholylglycine 2,2,2-Trichloroethyl Ester 3-(4-Succinimidoxycarbonyl)butyrate (29b)**——**28b** (300 mg) was treated in the manner described for **5a**. The residue was chromatographed on silica gel (15 g). Elution with hexane–AcOEt (1 : 5) gave **29b** (250 mg) as colorless semicrystals. NMR ($CDCl_3$) δ: 0.68 (3H, s, 18-CH_3), 0.84—1.02 (6H, 19-CH_3 and 21-CH_3), 2.68 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.83 (4H, s, succinimidyl), 3.54 (1H, m, 7α-H), 4.17 (2H, d, J=5 Hz, NCH_2CO), 4.44—4.93 (1H, m, 3β-H), 4.77 (2H, s, OCH_2CCl_3), 5.98 (1H, t, J=5 Hz, CONH).

***N*-Ursodeoxycholylglycine 3-(4-Succinimidoxycarbonyl)butyrate (30b)**——**29b** (250 mg) was treated in the manner described for **6a**. The residue was chromatographed on silica gel (10 g). Elution with $CHCl_3$–MeOH (10 : 1) gave **30b** (100 mg) as colorless semicrystals. NMR ($CDCl_3$–CD_3OD (2 : 1)) δ: 0.70 (3H, s, 18-CH_3), 0.84—1.04 (6H, 19-CH_3 and 21-CH_3), 2.71 (4H, t, J=7 Hz, $COCH_2CH_2CH_2CO$), 2.87 (4H, s, succinimidyl), 3.50 (1H, m, 7α-H), 3.83 (2H, s, NCH_2CO), 4.66 (1H, m, 3β-H).

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References and Notes

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