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Synthesis of [3,4-$^{13}C_2$]-Enriched Bile Salts as NMR Probes of Protein−Ligand Interactions

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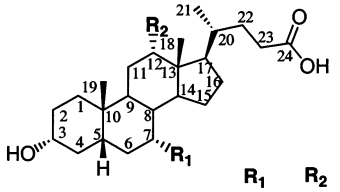
Synthetic methodology that allows for incorporation of isotopic carbon at the C-3 and C-4 positions of bile salts is reported. Three [3,4-$^{13}C_2$]-enriched bile salts were synthesized from either deoxycholic or lithocholic acid. The steroid 3α-OH group was oxidized and the A-ring was converted into the Δ^4-3-ketone. The C-24 carboxylic acid was next converted into the carbonate group and selectively reduced to the alcohol in the presence of the A-ring enone. Following protection of the 24-OH group, the Δ^4-3-ketone was converted into the A-ring enol lactone. Condensation of the enol lactone with [1,2-$^{13}C_2$]-enriched acetyl chloride and subsequent Robinson annulation afforded a [3,4-$^{13}C_2$]-enriched Δ^4-3-ketone that was subsequently converted back into a 3α-hydroxy-5β-reduced bile steroid. C-7 hydroxylation, when necessary, was achieved via conversion of the Δ^4-3-ketone into the corresponding $\Delta^{4,6}$-dien-3-one, epoxidation of the Δ^6-double bond, and hydrogenolysis/hydrogenation of the 5,6-epoxy enone system. The [3,4-$^{13}C_2$]-enriched bile salts were subsequently complexed to human ileal bile acid binding protein (I-BABP), and ^{1}H-^{13}C HSQC spectra were recorded to show the utility of the compounds for investigating the interactions of bile acids with I-BABP.

Introduction

Bile salts are amphipathic steroidal detergents that facilitate the absorption of dietary fats, fat-soluble vitamins, and cholesterol in the lumen of the small intestine. Bile salts, the fundamental constituent of bile, also represent the major end product of cholesterol metabolism in the body.[1] Their synthesis and subsequent excretion in the feces represent a significant mechanism for the elimination of excess cholesterol.[2] The body produces two classes of primary bile salts which differ only by the presence or absence of hydroxyl groups on the C-12 steroid position: cholic acid (3α,7α,12α OH) and chenodeoxycholic acid (3α,7α OH) (Figure 1). Further diversity in the bile salt pool results from microbial transformations occurring in the intestine. These transformations include dehydroxylation, epimerization, oxidation, hydroxylation, and reduction.[3] In humans, deoxycholic acid (3α,12α OH) and lithocholic acid (3α OH) exemplify the secondary bile salts that result from microbial dehydroxylations at the C-7 position.

The vast majority of bile salts are efficiently recycled via a process termed the enterohepatic circulation.[4] Ileal



	R_1	R_2
Cholic Acid	OH	OH
Chenodeoxycholic acid	OH	H
Deoxycholic acid	H	OH

FIGURE 1. Chemical structures (showing steroid numbering) of the three most physiologically abundant bile salts.

bile acid binding protein (I-BABP; also known as ileal lipid binding protein,[5] gastrotropin,[6] or I-15P[7]) was originally isolated from porcine ileal mucosa[6,8,9] and has been implicated in the enterohepatic circulation through bile salt binding interactions occurring in the ileal enterocytes.

Previously, our group has published results showing two distinct binding sites for bile salts bound to I-BABP using [^{15}N]-enriched, [1′,2′-$^{13}C_2$]-enriched, and [23,24-

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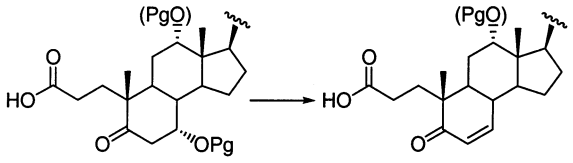

FIGURE 2. The keto acid elimination reaction showing substitution at C-7 results in elimination to form the B-ring enone (Pg = protecting group).

$^{13}C_2$]-enriched bile salts.[10,11] However, we were unable to assign the structural topology of the bound ligands because the structural data were obtained with bile salts isotopically enriched in the side chain, providing no direct information on the location of the steroid rings. Consequently, the synthesis of [3,4-$^{13}C_2$]-enriched bile salts was undertaken because of the potential for these compounds to provide structural information on the location of the steroid A-ring when bound to I-BABP. Overall, the NMR data collected from the side chain and A-ring ^{13}C-labeled bile salts has the potential to provide a high-resolution NMR structure of bile salts bound to I-BABP.

Turner reported the methodology that has been generally used for the synthesis of C-3 and C-4 isotopically labeled steroids.[12] In short, this is achieved via conversion of an A-ring enol lactone to the corresponding labeled Δ^4-3-ketone either through a Reformatski reaction or a series of reactions that include an acylation of the A-ring enol lactone, saponification, decarboxylation, and Robinson annulation to reform the A-ring. Both routes utilize isotopically labeled acetyl chloride as the carbon source. Recently, the Vierhapper group reported a modified methodology that allowed for the incorporation of isotopic carbon into the C-2 as well as C-3 and/or C-4 positions.[13,14] Although the work presented by Vierhapper et al. is a new route, the general strategy employed is similar to that presented by Turner almost 50 years ago. Here we show application of the Turner strategy to the synthesis of isotopically labeled bile salts and NMR spectra for their binding interactions with I-BABP.

The Turner strategy is routinely used with steroids having limited functionality in the steroid tetracycle and C-17 side chain.[12,15] The bile salt system presents a greater challenge due to the amount of functionality in the steroid tetracycle and the C-24 carboxylic acid. Additionally, a strategy that incorporates isotopic carbon into the bile salt A-ring must take into account both the stereo- and regiochemistry of the three possible hydroxyl groups at C-3, C-7, and C-12, as well as the cis A,B-ring fusion.

Initial studies in our laboratory found that a hydroxyl group at the C-7 position was incompatible with necessary intermediates formed along any of our explored pathways. The keto acid shown in Figure 2 readily undergoes elimination at the C-7 position to form the B-ring enone. This elimination forced us to develop strategies that utilized starting materials lacking a C-7 hydroxyl group. Where necessary, this hydroxyl group is introduced after the isotopic carbon is incorporated into the A-ring. An additional problem is that several bile salts lack the hydroxyl group at the C-12 position. We explored two possible approaches to developing a scheme that allowed for flexibility of hydroxylation at the C-12 position. First Fieser,[16] and later Hoffman,[17] reported a five-step chemical procedure for the conversion of cholic acid to chenodeoxycholic acid. Although this chemistry for the 12-OH removal is relatively efficient, it adds five steps to an already lengthy synthesis. The second approach, the one reported herein, was to develop two synthetic routes in parallel where one route used a starting material containing a 12-OH, while the other route lacked this group.

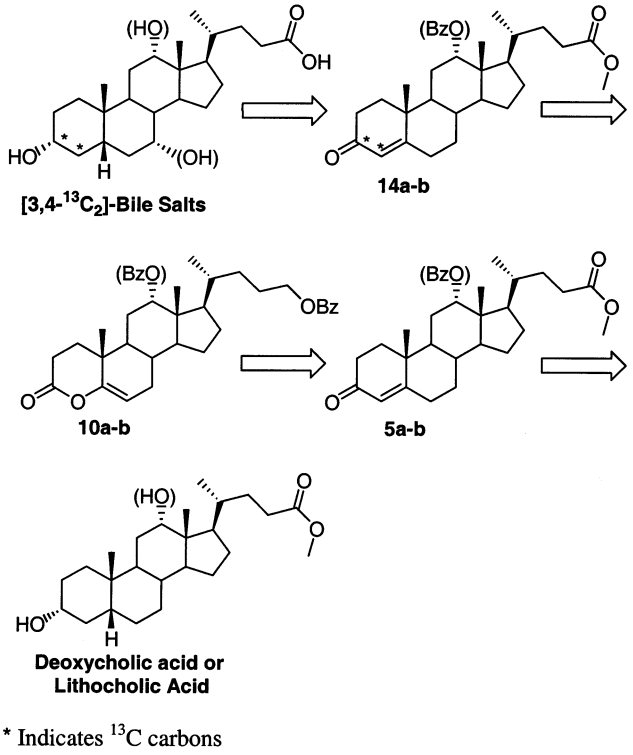

FIGURE 3. Retrosynthetic analysis of [3,4-$^{13}C_2$]-enriched bile salts. The asterisk indicates ^{13}C carbons.

Synthesis of [3,4-$^{13}C_2$]-Enriched Bile Salts

Deoxycholic (3α, 12α OH) and lithocholic acid (3α OH) are used as starting materials for preparing the labeled bile salts with or without the 12-OH. A brief retrosynthesis is diagramed in Figure 3. The route begins with conversion of the 3α-OH group into A-ring enones **5a**,**b** and protection of the 12-OH and C-24 carboxylic acid. The enol lactones **10a**,**b** are prepared via an ozonolysis and subsequent lactonization. Acylation with isotopically enriched acetyl chloride followed by subsequent decarboxylation, ring closure, and modification of the C-24 functional group yields enones **14a**,**b**. The enones are

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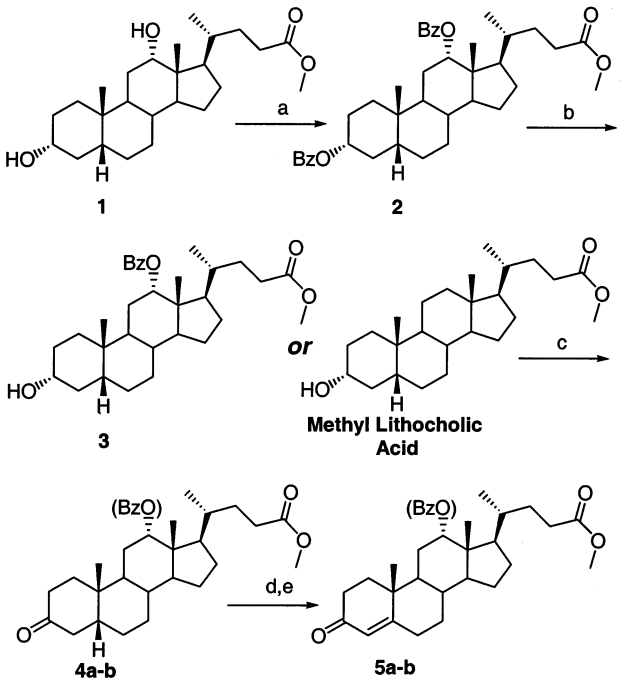
SCHEME 1[a]



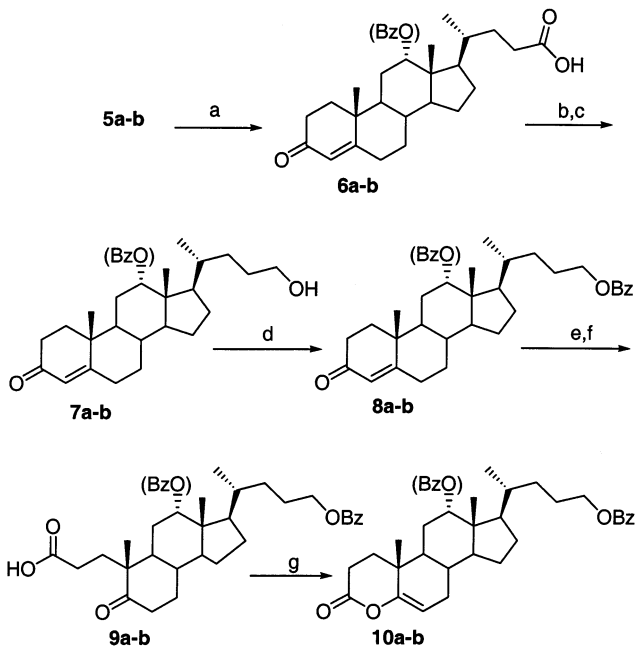
[a] Reagents and conditions: (a) benzoyl chloride, pyridine, 90 °C (96%); (b) NaOMe, MeOH, 50 °C; (c) Jones reagent (**4a**, 81%; **4b**, 91%); (d) Br_2, AcOH; (e) Li_2CO_3, LiBr, DMF, 150 °C (**5a**, 70%; **5b**, 68%).

then converted to isotopically enriched bile salts through incorporation of the C-7 hydroxyl (if necessary), hydrogenation of the Δ^4 double bond, reduction of the C-3 ketone, and removal of the protecting groups.

The most difficult aspect of the overall scheme involved the choice of the protecting groups for the functionalities at the C-12 and C-24 positions. Due to the lack of adequate carboxylic acid protecting groups, we chose to reduce the C-24 carboxylic acid to the primary alcohol and to protect this functionality. The major obstacle to choosing the hydroxyl protecting groups lay in the A-ring lactonization and subsequent acylation reactions. The lactonization must be achieved through harsh acidic conditions, while the enolate generation and subsequent acylation must be carried out under strongly basic conditions. This is problematic since the majority of well-characterized alcohol protecting groups are either acid or base labile. This would not be as significant a problem if the A-ring enol lactone were stable enough to undergo an exchange of protecting groups at these positions. However, when this was attempted with a variety of protecting groups the enol lactone was consistently lost. This forced us to find a protecting group that was stable enough to withstand both the harsh acidic and basic conditions, but also easily removed at the end of the synthetic route. After an exhaustive examination of protecting groups (ethers, esters, and silyl ethers), the benzoate ester was chosen. The benzoate, although base labile, is able to withstand the enolate generation conditions for the time needed to complete acylation of the enol lactone.

As shown in Scheme 1, the C-24 carboxylic acid of deoxycholic acid was initially esterified (MeOH, HCl) to give methyl ester **1** and the hydroxyl groups were then

SCHEME 2[a]



[a] Reagents and conditions: (a) NaOH, EtOH (**6a**, 92%; **6b**, 94%); (b) ethyl chloroformate, Et_3N, THF; (c) $NaBH_4$, H_2O (**7a**, 79%; **7b**, 72%); (d) benzoyl chloride, Et_3N (**8a**, 92%; **8b**, 92%); (e) O_3, −15 °C; (f) HOOH, AcOH; (g) Ac_2O, $HClO_4$ (**10a**, 62%; **10b** 58%).

protected as benzoates **2**. The C-3 benzoate was cleaved in the presence of the C-12 benzoate, using sodium methoxide in methanol with complete selectivity to yield product **3** that was then oxidized to ketone **4a** with Jones reagent. Similarly, Jones oxidation of methyl lithocholic acid yields ketone **4b**. It has previously been shown that 5β-reduced C-3 ketones undergo selective α-bromination at the C-4 position over the C-2 position.[18] Hence, bromination at C-4 followed by HBr elimination gave enones **5a**,**b**. An attempt was made to take enone **5a** or the corresponding free acid through the Turner methodology, but the attempt failed due to acylation of the side chain or precipitation of the dianion. Consequently, a method to selectively reduce the methyl ester in the presence of the enone was developed to avoid this difficulty.

Previously several groups have had success reducing acids in the presence of esters by first converting the acid to a carbonate and then reducing it with sodium borohydride.[19] Using this methodology, we found that the carboxylic acid group can also be selectively reduced in the presence of the enone functionality. As shown in Scheme 2, the carboxylic acid groups of enones **6a**,**b** were efficiently converted into the primary alcohol groups of enones **7a**,**b** via reduction of the intermediate carbonates. The 24-OH was then protected as the benzoate ester and benzoates **8a**,**b** were subjected to ozonolysis, followed by overnight workup with hydrogen peroxide, to afford the keto acids **9a**,**b**. These keto acids were subsequently lactonized with acetic anhydride in ethyl acetate containing a catalytic amount of perchloric acid.[20]

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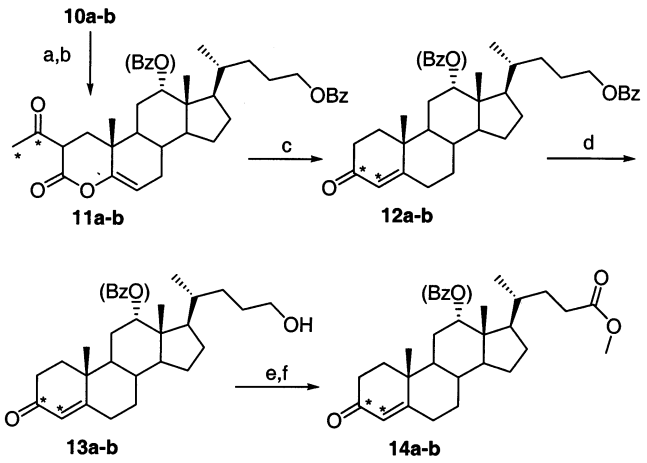
SCHEME 3[a]



[a] Reagents and conditions: (a) LiHMDS, THF, −78 °C; (b) [1,2-$^{13}C_2$]-acetyl chloride; (c) AcOH, HCl (**12a**, 58%; **12b**, 56%); (d) KOH, EtOH; (e) Jones reagent; (f) MeOH, cat. HCl (**14a**, 87%; **14b**, 92%). The asterisk indicates ^{13}C carbons.

The enol lactones **10a**,**b** were then acylated through enolate generation at C-2 with lithium hexamethyldisilazane and subsequent condensation with [1,2-$^{13}C_2$]-acetyl chloride. The acylated intermediates **11a**,**b** were then immediately refluxed in acetic acid containing HCl. In one pot, the enol lactone was hydrolyzed, the corresponding β-keto acid was decarboxylated, and the compounds underwent Robinson annulation to afford the [3,4-$^{13}C_2$]-enriched A-ring enones **13a**,**b**. The 24-benzoates were then saponified (selectively when 12-benzoate is present) with aqueous KOH. The 24-OH groups were oxidized with Jones reagent to the carboxylic acids and then esterified with methanolic HCl (Scheme 3).

As illustrated in Scheme 4, [3,4-$^{13}C_2$]-deoxycholic acid was formed from a series of reactions beginning with compound **14a**. It has been shown previously that a Δ^4-3-ketone can be selectively hydrogenated to form the cis A,B-ring fusion through hydrogenation under basic conditions.[18] We found that palladium on calcium carbonate in a basic ethanol solution gave us the best results (80:20 cis:trans A,B-ring fusion). The mixture of diastereomers was easily separated with silica chromatography to afford the cis A,B-ring fused ketone **16**. Stereospecific reduction of the 3-ketone with lithium tri-*tert*-butoxyaluminohydride gave the 3α-hydroxy steroid product and saponification of the benzoate and methyl ester protecting groups afforded [3,4-$^{13}C_2$]-deoxycholic acid. This chemistry could also be applied to compound **14b** to afford enriched lithocholic acid; however, this was not attempted.

As shown in Scheme 5, cholic acid and chenodeoxycholic acid are formed from compounds **14a** and **14b**, respectively, through incorporation of a 7α-hydroxyl group followed by a stereoselective hydrogenation and reduction similar to that shown in Scheme 4. The initial approach we took to incorporate the C-7 hydroxyl group involved isomerization of the Δ^4-double bond to the Δ^5 position thereby generating an allylic position at C-7 that could be subsequently oxygenated. Although the double bond migration was successful and several of the attempts to incorporate either a hydroxyl or ketone at C-7 were successful, the stereoselective hydrogenation of the Δ^5-double bond consistently afforded the 5α-reduced steroid instead of the 5β-reduced product.

The synthetic route was then altered to incorporate the C-7 hydroxyl group via a stereoselective epoxidation followed by a regiospecific opening. The regiospecific incorporation of the Δ^6-double bond to form the dienone system and corresponding epoxidation has been accomplished with other steroids.[21−24] Moreover, Lai et al. reported methodology on a similar system that regiospecifically opened the C−O epoxide bond on the allylic carbon and hydrogenated the Δ^4-double bond in one pot using palladium on carbon in pyridine.[21] We found that these reactions proceeded with complete stereo- and regiospecificity to convert steroids **14a**,**b** into ketones **20a**,**b**. Asymmetric reduction of the ketones **20a**,**b** gave the alcohols **21a**,**b** and saponification converted these steroids into [3,4-$^{13}C_2$]-cholic acid and [3,4-$^{13}C_2$]-chenodeoxycholic acid, respectively.

NMR Spectra of Bile Salt/Protein Complexes

As our major reason for synthesizing these compounds is to facilitate the study of their interactions with I-BABP, the NMR spectroscopic properties of [3,4-$^{13}C_2$]-GCA and [3,4-$^{13}C_2$]-GCDA bound to I-BABP are critical for assessing the utility of these compounds in future structural studies. Two-dimensional ^{1}H/^{13}C heteronuclear single quantum correlation (HSQC) spectra were used to selectively observe the protons that are directly attached to the isotopic carbon. The [3,4-$^{13}C_2$]-enriched bile salts were first conjugated to glycine (their predominant form in vivo) by using previously reported methodology,[25] then incubated with I-BABP in a 3:1 (GCA:I-BABP or GCDA:I-BABP) ratio. The ^{1}H/^{13}C HSQC spectra for GCA (Panel A) and GCDA (Panel B) are shown in Figure 4. In the absence of protein the [3,4]-$^{13}C_2$-bile salts give rise to three resonances, which correspond to the 4α- and 4β-geminal protons and the 3β-proton.

Upon complexation to the protein, the three resonances segregate into three sets of three peaks representing the unbound and two bound states of the bile salt, as shown in Figure 4. Peaks labeled U correspond to the unbound bile salt, as assigned by spectra of otherwise identical control samples lacking protein. Peaks labeled 1 and 2 correspond to bile salt in each of two binding sites. The assignments were confirmed by ^{13}C-edited TOCSY and NOESY experiments. It is of interest that both GCA and GCDA reside in magnetically similar environments when bound to the protein. This is particularly evident with the 4β-hydrogen in both GCA and GCDA. This hydrogen has a chemical shift of 1.30 ppm in the unbound form, which shifts to −0.37 ppm upon binding to Site 2 for both bile salts. This large decrease in chemical shift is likely caused by the close proximity to an aromatic side chain. The fact that there is very little overlap between any of the unbound or bound resonances is important since this

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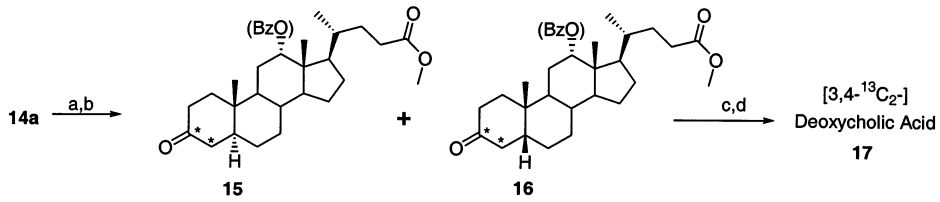
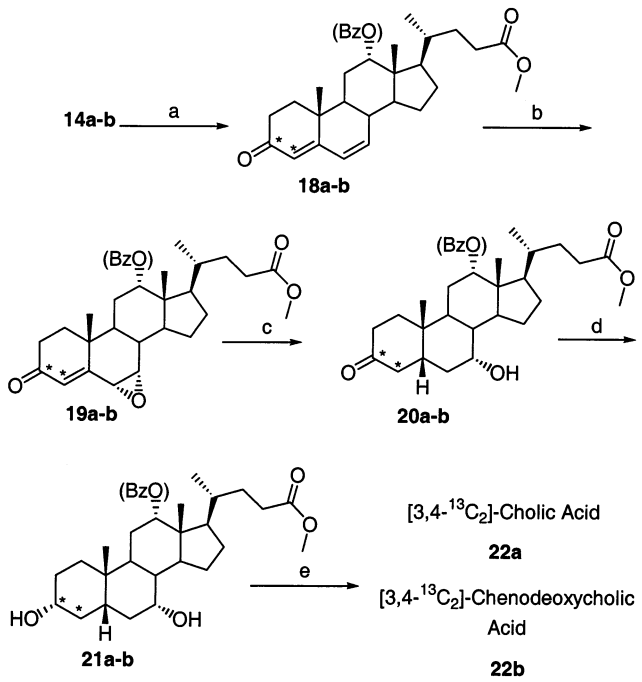
SCHEME 4[a]



[a] Reagents and conditions: (a) $Pd/CaCO_3$, KOH, H_2 (1 atm); (b) MeOH, cat. HCl (63%); (c) lithium tri-*tert*-butoxyaluminohydride, THF, −78 °C; (d) KOH, EtOH (65%). The asterisk iIndicates ^{13}C carbons.

SCHEME 5[a]



[a] Reagents and conditions: (a) *p*-chloranil (**18a**, 79%; **18b**, 74%); (b) *m*-chloroperbenzoic acid (**19a**, 63%; **19b**, 59%); (c) Pd/C, pyridine, H_2 (60 psi) (**20a**, 89%; **20b**, 72%); (d) lithium tri-*tert*-butoxyaluminohydride, THF, −78 °C; (e) KOH, EtOH (**22a**, 72%; **22b**, 76%). An asterisk indicates ^{13}C carbons.

will lead to unambiguous assignment of protein−bile salt contacts for the determination of the NMR structure of the protein complex.

Conclusions

Here we have reported the synthesis of [3,4-$^{13}C_2$]-enriched bile salts and illustrated their utility as structural probes of bile salt interactions with I-BABP. Of particular note with the synthetic methodology presented is the selective reduction shown in Scheme 2. This reaction has previously been used several times to reduce carboxylic acids in the presence of esters, but to our knowledge this is the first report of this methodology being used to reduce an acid in the presence of an enone. Further, the ability to reduce an acid in the presence of an enone has only been reported in the literature a few times regardless of the methodology employed.[26] This relatively unexplored reaction could prove to be widely useful if the extent of the selectivity was further defined.

Our goal in synthesizing these compounds was to be able to collect structural data to establish the topology for bile salts binding to I-BABP. This report represents a major step in that direction. Upon binding, the three reporter protons are well-resolved and reside in unique areas of the spectrum. Of particular promise is the 4β-proton from Site 1. Because this resonance resides in a very unpopulated area of the I-BABP NMR spectrum, unambiguous assignment of ligand−protein interproton distance restraints should be relatively straightforward. Therefore the [3,4-$^{13}C_2$]-bile salts provide an important and useful set of tools for determining the three-dimensional NMR structure of human I-BABP complexed with two bile salts.

Materials and Methods

Melting points were determined on a Kofler micro hot stage and are uncorrected. NMR spectra were recorded at ambient temperature in $CDCl_3$ or CD_3OD with a 5 mm probe operating at 300 (^1H) or 75 MHz (^{13}C). For ^1H NMR and ^{13}C NMR spectra, the internal references were TMS (δ 0.00) and $CDCl_3$ (δ 77.00), respectively. IR spectra were recorded as films on a AgCl plate. Solvents were either used as purchased or dried and purified by standard methodology. M−H−W laboratories, Phoenix, AZ, carried out elemental analyses. Flash chromatography was performed with silica gel (32−63 μm) purchased from Scientific Adsorbents, Atlanta, GA.

(3α,5*β*,12α)-12-(Benzoyloxy)-3-hydroxycholan-24-oic acid methyl ester (3): Sodium methoxide was prepared by reacting Na metal (5.6 g, 243.9 mmol) with MeOH (700 mL). The dibenzoate **2** (50 g, 81.3 mmol) was added and the solution was heated to 50 °C. After 30 min, the dibenzoate was completely dissolved. The reaction was then allowed to stir at 50 °C for an additional 1.5 h, cooled for 5 min in an ice bath, and quenched through the careful addition of 25% H_2SO_4 (25 mL). The methanol was removed in vacuo and monobenzoate **3** was oxidized without purification or characterization.

(5*β*,12α)-12-(Benzoyloxy)-3-oxo-cholan-24-oic acid methyl ester (4a): Monobenzoate **3** (81.3 mmol) was dissolved in acetone (500 mL) and cooled to 0 °C in an ice bath. Jones reagent was added dropwise until an orange color persisted. The reaction was allowed to stir for an additional 10 min at 0 °C before 2-propanol (20 mL) was added to quench the reaction. The acetone was then removed in vacuo and the residue dissolved in EtOAc and washed with water. The organic layer was dried over Na_2SO_4 and the solvent removed in vacuo to afford a gummy residue that was chromatographed over silica gel (15:85, EtOAc:hexanes) to yield the product (33.6 g, 81% from dibenzoate **2**) as a white gummy solid that crystallized upon sitting for several days. Compound **4a**: mp 100−105 °C; TLC R_f 0.67 (1:1, EtOAc:hexanes); IR 1714, 1728, 1738 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.78 (d, 3H, J = 6.3 Hz), 0.81 (s, 3H), 0.99 (s, 3H), 2.61 (t, 1H, J = 13.8 Hz), 3.57 (s, 3H), 5.35 (br, 1H), 7.41−7.58 (m, 3H), 7.97−8.01 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.42, 17.29, 22.24, 23.35, 25.32, 25.88, 26.28, 27.20, 30.62, 30.76, 34.14, 34.57, 34.78, 35.32, 36.45, 36.63, 42.03, 43.88,

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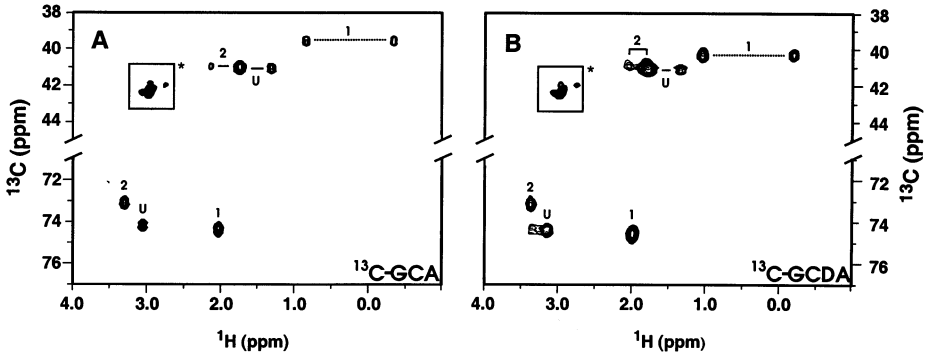

FIGURE 4. 2D $^1H-^{13}C$ HSQC spectrum of a ternary complex of [3,4-$^{13}C_2$]-enriched glycocholic (A) and glycochenodeoxycholic (B) acids bound to human ileal bile acid-binding protein (I-BABP). Unbound bile salts appear as three peaks denoted by "U", which correspond to the 3β-proton and the 4α- and 4β-protons. Upon binding, the bile salt resonances segregate into two binding sites indicated by "1" or "2". Spectra were collected under the following conditions: 1.85 mM I-BABP, 20 mM K_2PO_4, 10 mM NaCl 135 mM KCl, 0.05% NaN_3, pH 7.2, 10 °C, 3:1 mole ratio of bile salt:I-BABP. The box with an asterisk indicates protein resonances arising from natural abundance carbon-13.

45.42, 47.81, 49.82, 51.27, 128.41, 128.47, 128.48, 129.14, 129.15, 129.36, 132.97, 165.62, 174.32, 212.61. Anal. Calcd for $C_{32}H_{44}O_5$: C, 75.56; H, 8.72. Found: C, 75.66; H, 8.47.

(12α)-12-(Benzoyloxy)-3-oxo-chol-4-en-24-oic acid methyl ester (5a): Ketone **4a** (30 g, 59.0 mmol) was dissolved in acetic acid (300 mL) and stirred at room temperature. Bromine (10.4 g, 64.9 mmol) was then dissolved in acetic acid (20 mL) and added dropwise over 1 h. The reaction was allowed to stir for an additional 10 min, then poured into a separatory funnel containing EtOAc (1 L) and washed with water and saturated $NaHCO_3$. The organic layer was dried (Na_2SO_4) and the solvent removed in vacuo. The yellowish solid was then dissolved in DMF (500 mL) and Li_2CO_3 (30 g) and LiBr (15 g) were added. The mixture was heated to 150 °C for 2 h, then cooled to room temperature, and the DMF was removed with high vacuum. The yellowish white residue was suspended in EtOAc (750 mL) and carefully quenched with 1 M HCl (100 mL) causing copious amount of CO_2 gas to be formed. The suspension was then transferred to a separatory funnel and additional 1 M HCl (500 mL) was carefully added until gas evolution ceased, the layers were separated, and the organic layer was washed with water. The organic layer was dried (Na_2SO_4) and the solvent removed in vacuo to afford a gummy yellow solid that was chromatographed on silica gel (25:75, EtOAc:hexanes) to yield a white solid (21.0 g, 70%): mp 87−91 °C; TLC R_f 0.56 (1:1 EtOAc:hexanes); IR 1670, 1717, 1734 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.80 (d, 3H, J = 6.3 Hz), 0.87 (s, 3H), 1.18 (s, 3H), 3.61 (s, 3H), 5.38 (br, 1H), 5.71 (s, 1H), 7.43−7.58 (m, 3H), 7.99−8.02 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.48, 17.20, 17.41, 23.52, 25.80, 27.25, 30.74, 30.94, 31.70, 32.71, 33.70, 34.70, 35.58, 37.98, 45.28, 46.28, 47.83, 48.19, 49.24, 51.46, 75.74, 124.07, 128.55, 128.56, 129.43, 129.44, 130.53, 133.07, 160.47, 160.77, 174.54, 199.28. Anal. Calcd for $C_{32}H_{42}O_5$: C, 75.86; H, 8.36. Found: C, 75.79; H, 8.42.

(12α)-12-(Benzoyloxy)-3-oxo-chol-4-en-24-oic acid (6a): Enone **5a** (20.0 g, 39.5 mmol) was dissolved in ethanol (300 mL) and 10% NaOH (150 mL) was then added slowly so as not to precipitate the ester. The resultant yellowish solution was allowed to stand at room temperature for 1.5 h. HCl (2.5 M, 200 mL) was then added causing the solution to become cloudy. The ethanol was removed in vacuo and the resultant aqueous mixture was extracted with EtOAc. The organic layers were combinedand dried and the EtOAc removed in vacuo to afford a slightly yellow solid (17.8 g, 92%). The acid was reduced without further purification or characterization.

3-Oxo-chol-4-en-24-oic acid (6b): Enone **5b** (10.0 g, 25.9 mmol) was dissolved in ethanol (200 mL) and 10% NaOH (100 mL) was then added slowly so as to maintain a clear solution. The yellowish solution was allowed to stand at room temperature for 1.5 h. HCl (2.5 M, 100 mL) was then added very carefully causing the acid to precipitate. The acid was collected to yield a slightly yellow solid (9.1 g, 94%). The acid was reduced without further purification or characterization.

(12α)-24-Hydroxy-12-(benzoyloxy)-chol-4-en-3-one (7a): Acid **6a** (15.0 g, 30.4 mmol) was dissolved in anhydrous THF (300 mL) and cooled to 0 °C. Triethylamine (15 mL) was then added followed by ethyl chloroformate (9 mL) causing the solution to become milky. This milky solution was stirred for an additional 1.5 h at 0 °C under nitrogen. Sodium borohydride (7.5 g) dissolved in water (100 mL) was then carefully added during 1 min causing the evolution of copious amounts of gas. The reaction was allowed to stir for 2 min after the initial gas evolution stopped (total of 4−6 min after addition of sodium borohydride) and was then carefully quenched by the addition of 1 M HCl (100 mL) causing another large evolution of gas. The mixture was then transferred to a separatory funnel with the aid of ethyl acetate (200 mL), the organic layer was washed with 1 M HCl and dried (Na_2SO_4), and the solvent was removed in vacuo to afford a colorless gum that was chromatographed the same day on silica gel (50:50, EtOAc:hexanes) to yield product **7a** as a white solid (11.5 g, 79%): mp 101−105 °C; TLC R_f 0.31 (1:1, EtOAc:hexanes); IR 1672, 1714, 3469 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.76 (d, 3H, J = 6.6 Hz), 0.80 (s, 3H), 1.12 (s, 3H), 3.48 (d of t, 2H, J = 6.6, 1.8 Hz), 5.32 (br, 1H), 5.65 (s, 1H), 7.37−7.54 (m, 3H), 7.92−7.99 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 12.47, 17.19, 23.50, 25.79, 27.20, 27.32, 29.27, 30.51, 30.72, 31.60, 31.70, 32.71, 33.67, 34.63, 34.93, 35.57, 37.96, 47.81, 47.98, 63.33, 75.82, 124.02, 128.51, 128.52, 129.42, 129.43, 130.52, 133.06, 162.61, 165.69, 199.36. Anal. Calcd for $C_{31}H_{42}O_4$: C, 77.79; H, 8.84. Found: C, 77.95; H, 8.62.

(12α)-12,24-Bis(benzoyloxy)-chol-4-en-3-one (8a): Alcohol **7a** (10.0 g, 20.9 mmol) was dissolved in anhydrous THF (200 mL) followed by the addition of triethylamine (8.74 mL; 62.7 mmol) and benzoyl chloride (7.28 mL; 62.7 mmol) causing the solution to become milky. The solution was stirred under nitrogen for 5 h. The THF was then removed in vacuo and the residue was suspended in EtOAc (200 mL), washed with saturated bicarbonate, and dried (Na_2SO_4), and the solvent was removed in vacuo to afford a yellowish oil that was chromatographed on silica gel (25:75, EtOAc:hexanes) to yield product **8a** as a white solid (11.2 g, 92%): mp 97−100 °C; TLC R_f 0.63 (1:1 EtOAc:hexanes); IR 1673, 1717 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.86 (d, 3H, J = 6.6 Hz), 0.88 (s, 3H), 1.19 (s, 3H), 4.23 (t, 2H, J = 6.9), 5.40 (br, 1H), 5.71 (s, 1H), 7.38−7.60 (m, 6H), 7.96−8.10 (m, 4H); ^{13}C NMR ($CDCl_3$) δ 12.47, 14.07, 17.19, 17.72, 22.60, 23.50, 25.21, 25.80, 27.32, 31.54, 31.70, 31.83, 32.71, 33.70, 34.78, 35.58, 37.96, 45.26, 47.86, 48.21, 49.26, 65.33, 75.82, 124.05, 124.12, 128.28, 128.29, 128.51, 128.55, 129.45, 129.49, 130.53, 132.76, 132.99, 165.68, 166.59, 170.37,

199.20. Anal. Calcd for $C_{38}H_{46}O_5$: C, 78.32; H, 7.96. Found: C, 78.35; H, 7.67.

(12α)-12,24-Bis(benzoyloxy)-4-oxachol-5-en-3-one (10a): Enone **8a** (10.0 g, 17.2 mmol) was dissolved in EtOAc (250 mL) and AcOH (50 mL) and cooled to −15 °C in a dry ice/acetone bath. The solution was then ozonized for 45 min while maintaining the bath temperature between −10 and −20 °C until a slight blue color persisted. O_2 was then bubbled through the solution until the blue color was lost and the solution was allowed to warm to room temperature. A solution of AcOH (10 mL), H_2O, (20 mL), and 30% hydrogen peroxide (5 mL) was added and the solution was stirred overnight at room temperature. The clear solution was transferred to a separatory funnel in EtOAc (250 mL) and the organic layer was washed with H_2O and dried (Na_2SO_4). Solvent removal in vacuo gave a colorless oil that was directly converted to the enol lactone by the addition of 500 mL of a solution of 1 mM $HClO_4$ with 10% acetic anhydride in EtOAc. The solution was allowed to stand for 10 min becoming light brown in color. It was transferred to a separatory funnel, washed with saturated bicarbonate, and dried (Na_2SO_4), and the solvent was removed in vacuo. The residual acetic anhydride was removed with high vacuum and gentle heating. The crude enol lactone was then chromatographed on silica gel (25:75, EtOAc:hexanes) to afford a clear viscous oil that solidified upon standing (6.2 g, 62%). The slightly unstable enol lactone **10a** was characterized by ^{1}H NMR then acylated without further characterization. Product **10a**: ^{1}H NMR ($CDCl_3$) δ 0.87 (s, 3H), 0.88 (d, 3H, J = 6.6 Hz), 1.10 (s, 3H), 2.55(m, 2H), 4.22 (t, 2H, J = 6.3), 5.26 (m, 1H), 5.42 (br, 1H), 7.38−7.57 (m, 6H), 7.98−8.11 (m, 4H).

[3,4-$^{13}C_2$]-(12α)-12,24-Bis(benzoyloxy)-chol-4-en-3-one (12a): Under strictly anhydrous conditions the enol lactone **10a** was acylated in three batches. Typically, 1,1,1,3,3,3-hexamethyldisilazane (0.87 mL, 4.10 mmol) was dissolved in THF (50 mL) and cooled to −78 °C in a dry ice/acetone bath followed by the addition of *n*-butyllithium (1.57 mL of a 2.5 M solution, 3.93 mmol). This was allowed to stir for 20 min at −78 °C. The enol lactone (2.0 g, 3.42 mmol) dissolved in THF (15 mL) was cannulated into the LiHMDS solution over 1 min. The enolate was allowed to form for no longer than 7 min followed by the addition of [1,2-$^{13}C_2$]-acetyl chloride (0.27 mL, 3.76 mmol). The solution was then allowed to warm to room temperature and after the THF was removed in vacuo, AcOH (50 mL) and concentrated HCl (4 mL) were added. This solution was then heated to reflux for 18 h causing the solution to become brown. After cooling, EtOAc (300 mL) was added and the solution was transferred to a separatory funnel and washed with H_2O, saturated bicarbonate, and brine. The organic layer was dried (Na_2SO_4) and the solvent was removed in vacuo to afford a brown oil that was chromatographed on silica gel (25:75, EtOAc:hexanes) to afford a white solid (1.16 g, 58% from the enol lactone). This compound was compared to compound **8a** and found to have the identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR ($CDCl_3$) δ 124.1 (d, 1C, C-4, $^1J_{CC}$ = 54.9 Hz), 199.2 (d, 1C, C-3, $^1J_{CC}$ = 54.9 Hz).

[3,4-$^{13}C_2$]-24-(Benzoyloxy)-chol-4-en-3-one (12b): Under anhydrous conditions the enol lactone **10b** was acylated in two batches by using the procedure described for the acylation of steroid **10a**. The brown oil obtained was chromatographed on silica gel (20:80, EtOAc:hexanes) to afford product **12b** as a white solid (1.12 g, 56% from the enol lactone **10b**). This compound was compared to compound **8b** and found to have the identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR ($CDCl_3$) δ 123.8 (d, 1C, C-4, $^1J_{CC}$ = 51.2 Hz), 199.2 (d, 1C, C-3, $^1J_{CC}$ = 51.2 Hz).

[3,4-$^{13}C_2$]-(12α)-12-(Benzoyloxy)-3-oxo-chol-4-en-24-oic acid methyl ester (14a): In two batches enone **12a** (1.0 g, 1.71 mmol) was dissolved in absolute ethanol (100 mL) followed by the careful addition of 10% w/v KOH (10 mL) causing the solution to turn pale yellow. The reaction was allowed to stir at room temperature for 1 h while being carefully monitored by TLC. Upon reaction completion, 1 M HCl (20 mL) was added to acidify the reaction. The ethanol was removed in vacuo leaving a water emulsion that was transferred to a separatory funnel in EtOAc (100 mL). The organic layer was separated and dried, and the solvent was removed in vacuo. The crude residue was dissolved in acetone, Jones reagent was added until an orange color persisted, and 2-propanol (5 mL) was added to quench the reaction. The acetone was then removed in vacuo and the residue was dissolved in EtOAc (100 mL) and washed with water. The organic layer was dried (Na_2SO_4) and the solvent removed in vacuo to afford a gummy residue that was dissolved in a solution of acetyl chloride (0.1 mL) in MeOH (100 mL) and stirred overnight. The MeOH was removed in vacuo and the gummy residue was chromatographed on silica gel (25:75, EtOAc:hexanes) to afford product **14a** as a white solid (0.76 g, 87%). This compound was compared to compound **5a** and found to have the identical melting point and NMR spectrum except for C-3 and C-4. ^{13}C NMR ($CDCl_3$) δ 124.0 (d, 1C, C-4, $^1J_{CC}$ = 49.8 Hz), 199.3 (d, 1C, C-3, $^1J_{CC}$ = 49.8 Hz).

[3,4-$^{13}C_2$]-3-Oxo-chol-4-en-24-oic acid methyl ester (14b): In two batches enone **12b** (1.0 g, 2.15 mmol) was dissolved in 100% ethanol (100 mL) followed by the careful addition of 10% w/v KOH (10 mL) causing the solution to turn pale yellow. The reaction was allowed to stir at room temperature for 2 h. The resultant alcohol was then worked up, oxidized, and protected as above to afford a yellow residue that was chromatographed on silica gel (20:80, EtOAc:hexanes) to afford produce **14b** as a white solid (0.77 g, 92%). This compound was compared to compound **5b** and found to have the identical melting point and NMR spectrum except for C-3 and C-4. ^{13}C NMR ($CDCl_3$) δ 123.8 (d, 1C, C-4, $^1J_{CC}$ = 52.5 Hz), 199.8 (d, 1C, C-3, $^1J_{CC}$ = 52.5 Hz).

[3,4-$^{13}C_2$]-(5α,12α)- and [3,4-$^{13}C_2$]-(5*β*,12α)-12-(Benzoyloxy)-3-oxo-cholan-24-oic acid methyl ester (15, 16): Enone **14a** (200 mg, 0.39 mmol) was dissolved in ethanol (10 mL) followed by the addition of KOH (0.1 g) in H_2O (0.2 mL) and Pd/$CaCO_3$ (20 mg, 10% Pd content) causing the solution to turn a pale yellow. The solution was then stirred under H_2 gas (1 atm) overnight. The mixture was then acidified with 0.5 M HCl (2 mL) filtered over a pad of Celite and the solvent removed under high vacuum. The resultant residue, which was a mixture of the C-24 acid and methyl ester, was dissolved in methanolic HCl to esterify the C-24 carboxylic acid. The resultant yellowish residue was found to be a mixture of diastereomers at the C-5 position. NMR was used to determine an approximate 80:20 ratio of the desired 5*β*- to the undesired 5α-reduced steroid through monitoring of the 4α-proton resonance that is shifted downfield in the 5*β*-reduced steroid.[27] The diastereomers were separated on silica gel (15:85, EtOAc:hexanes) to afford **16** as a white solid (126 mg, 63%). This compound was compared to compound **4a** and found to have the identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR ($CDCl_3$) δ 41.6 (d, 1C, C-4, $^1J_{CC}$ = 37.8 Hz), 212.5 (d, 1C, C-3, $^1J_{CC}$ = 37.8 Hz).

[3,4-$^{13}C_2$]-Deoxycholic acid (17): Ketone **16** (100 mg, 0.20 mmol) was dissolved in THF (10 mL) and cooled to −78 °C. Lithium tri-*tert*-butoxyaluminohydride (0.22 mL of a 1.0 M solution, 0.22 mmol) was added and the reaction was monitored by TLC. After 2 h, the reaction was complete and the solution was allowed to warm to room temperature. The THF was removed in vacuo and the residue dissolved in EtOAc (50 mL) and washed with 0.5 M HCl, 0.5 M NaOH, and water. The organic layer was dried (Na_2SO_4) and the solvent was removed in vacuo to afford a colorless oil that was dissolved in EtOH (10 mL) and heated to reflux. A 20% solution of KOH (10 mL) was added and the reaction was refluxed for 18 h. The reaction was allowed to cool, and carefully acidified with 3.5 M HCl (~10 mL) causing a solid to precipitate. The solid

(27) Han, M.; Zorumski, C. F.; Covey, D. F. *J. Med. Chem.* **1996**, *39*, 4218−32.

was collected and chromatographed on silica gel (0.5:99.5, AcOH:EtOAc) and crystallized as previously summarized[28] to afford steroid **17** (50 mg, 65%). This compound was compared to commercial samples of deoxycholic acid and was found to have an identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR (CD_3OD) δ 41.3 (d, 1C, C-4, $^1J_{CC}$ = 40.1 Hz), 74.2 (d, 1C, C-3, $^1J_{CC}$ = 40.1 Hz).

[3,4-$^{13}C_2$]-(12α)-12-(Benzoyloxy)-3-oxo-chola-4,6-dien-24-oic acid methyl ester (18a): Enone **14a** (500 mg, 0.99 mmol) was dissolved in AcOH (4 mL) and toluene (1 mL) followed by the addition of tetrachloro-1,4-benzoquinone (*p*-chloranil, 755 mg, 1.49 mmol). The solution was heated to reflux for 1 h, cooled, diluted with EtOAc (100 mL), transferred to a separatory funnel, and washed with saturated bicarbonate then H_2O. The organic layer was dried (Na_2SO_4) and the solvent removed in vacuo to afford a brown residue that was chromatographed on silica gel (27:73, EtOAc:hexanes) to afford product **18a** as a white solid (395 mg, 79%): mp 149−153 °C; TLC R_f 0.66 (1:1, EtOAc:hexanes); IR 1648, 1717, 1735 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.83 (d, 3H, J = 6.6 Hz), 0.91 (s, 3H), 1.13 (s, 3H), 3.62 (s, 3H), 5.27 (br, 1H), 5.73 (d, 1H, $^1J_{HC}$ = 159 Hz), 6.09 (d, 1H, J = 9.9 Hz), 6.12 (d, 1H, J = 9.9 Hz), 7.41−7.52 (m, 3H), 7.89−8.00 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 121.5 (d, 1C, C-4, $^1J_{CC}$ = 55.2 Hz), 198.7 (d, 1C, C-3, $^1J_{CC}$ = 55.2 Hz). Anal. Calcd for $C_{32}H_{40}O_5$: C, 76.16; H, 7.99. Found: C, 76.42; H, 7.97.

[3,4-$^{13}C_2$]-3-Oxo-chola-4,6-dien-24-oic acid methyl ester (18b): Dienone **18b** was prepared from enone **14b** (500 mg, 0.99 mmol) by using the procedure given for the preparation of dienone **18a**. Chromatography (silica gel; 25:75, EtOAc:hexanes) gave product **18b** as a white solid (368 mg, 74%): mp 146−151 °C (lit.[29] mp for unlabeled isotopomer 150−154 °C); ^{13}C NMR (CDCl3) δ 120.3 (d, 1C, C-4, $^1J_{CC}$ = 48.3 Hz), 199.3 (d, 1C, C-3, $^1J_{CC}$ = 48.3 Hz).

[3,4-$^{13}C_2$]- (6α,7α,12α)-12-(Benzoyloxy)-6,7-epoxy-3-oxo-chol-4-en-24-oic acid methyl ester (19a): Dienone **18a** (350 mg, 0.69 mmol) was dissolved in a cold solution of CH_2Cl_2 (10 mL) followed by the addition of 70% *m*-chloroperbenzoic acid (220 mg, 1.28 mmol). This suspension was allowed to stir at 4 °C for 48 h while being closely monitored by TLC. Upon completion, calcium hydroxide (1 g) was added, and the reaction was stirred for 10 min then filtered over a pad of Celite that was then washed with CH_2Cl_2. The solvent was removed in vacuo to afford a clear residue that was chromatographed on silica gel (30:70, EtOAc:hexanes) to afford a white solid (230 mg, 63%). Product **19a**: mp 92−94 °C; TLC R_f 0.59 (1:1, EtOAc:hexanes); IR 1657, 1717, 1735 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.81 (d, 3H, J = 6.6 Hz), 0.90 (s, 3H), 1.11 (s, 3H), 3.42 (d, 1H, J = 3.6 Hz), 3.49 (d, 1H, J = 3.6 Hz), 3.62 (s, 3H), 5.27 (br, 1H), 6.13 (d, 1H, $^1J_{HC}$ = 159 Hz), 7.41−7.52 (m, 3H), 7.89−8.00 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 131.5 (d, 1C, C-4, $^1J_{CC}$ = 55.2 Hz), 198.3 (d, 1C, C-3, $^1J_{CC}$ = 55.2 Hz). Anal. Calcd for $C_{32}H_{40}O_6$: C, 73.82; H, 7.74. Found: C, 73.91; H, 7.91.

[3,4-$^{13}C_2$]-(6α,7α)-6,7-Epoxy-3-oxo-chol-4-en-24-oic acid methyl ester (19b): Steroid **19b** was prepared from dienone **18b** (350 mg, 0.91 mmol) by the same procedure described for the preparation of compound **19a**. Chromatography (silica gel; 25:75, EtOAc:hexanes) gave compound **19b** as a white solid (213 mg, 59%): mp 148−151 °C; TLC R_f 0.60 (1:1, EtOAc:hexanes); IR 1653 1734 cm^{-1}; ^1H NMR ($CDCl_3$) δ 0.83 (s, 3H), 0.84 (d, 3H, J = 6.3 Hz), 1.09 (s, 3H), 3.38 (d, 1H, J = 3.6 Hz), 3.49 (d, 1H, J = 3.6 Hz), 3.67 (s, 3H), 5.27 (br, 1H), 6.13 (d, 1H, $^1J_{HC}$ = 156 Hz); ^{13}C NMR ($CDCl_3$) δ 132.5 (d, 1C, C-4, $^1J_{CC}$ = 55.5 Hz), 199.1 (d, 1C, C-3, $^1J_{CC}$ = 55.5 Hz). Anal. Calcd for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.21; H, 9.11.

[3,4-$^{13}C_2$]-(5β,7α,12α)-12-(Benzoyloxy)-7-hydroxy-3-oxo-cholan-24-oic acid methyl ester (20a): Epoxide **19a** (200 mg, 0.38 mmol) was dissolved in freshly distilled pyridine followed by the addition of 10% Pd/C (20 mg). The mixture was then hydrogenated on a Parr apparatus (60 psi) for 18 h. The mixture was then filtered over a pad of Celite and the solvent removed in vacuo with the aid of high vacuum. The colorless residue was chromatographed on silica gel (30:70, EtOAc:hexanes) to yield a white solid (179 mg, 89%) that was reduced and saponified without characterization.

[3,4-$^{13}C_2$]-(5β,7α)-7-Hydroxy-3-oxo-cholan-24-oic acid methyl ester (20b): Steroid **20b** was prepared from epoxide **19b** (200 mg, 0.50 mmol) in the manner described for the preparation of steroid **20a**. Chromatography (silica gel, 25:75, EtOAc:hexanes) gave product **20b** as a white solid (145 mg, 72%) that was reduced and saponified without characterization.

[3,4-$^{13}C_2$]-Cholic acid (22a): Ketone **20a** (150 mg, 0.29 mmol) was converted to cholic acid by using the methodology described for the conversion of compound **16** to deoxycholic acid. Chromatography (silica gel; 0.5:10:89.5, AcOH:MeOH:CH_2Cl_2) and crystallization as previously summarized[28] gave **22a** (84 mg, 72%). This compound was compared to commercial samples of cholic acid and was found to have an identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR (CD_3OD) δ 42.3 (d, 1C, C-4, $^1J_{CC}$ = 42.3 Hz), 73.2 (d, 1C, C-3, $^1J_{CC}$ = 42.3 Hz).

[3,4-$^{13}C_2$]-Chenodeoxycholic acid (22b): With use of the procedure described for the preparation of steroid **17**, ketone **20b** (130 mg, 0.32 mmol) was converted into steroid **22b**. Chromatography (silica gel, 0.5:99.5, AcOH:EtOAc) and crystallization as previously summarized[28] gave **22b** (96 mg, 76%). This compound was compared to commercial samples of chenodeoxycholic acid and was found to have an identical melting point and ^{13}C NMR spectrum except for C-3 and C-4. ^{13}C NMR (CD_3OD) δ 41.6 (d, 1C, C-4, $^1J_{CC}$ = 40.4 Hz), 74.3 (d, 1C, C-3, $^1J_{CC}$ = 40.4 Hz).

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Supporting Information Available: Experimental procedures for compounds **2**, **4b**, **5b**, **7b**, **8b**, and **10b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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