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A Simple Efficient Synthesis of [23,24]-$^{13}C_2$-Labeled Bile Salts as NMR Probes of Protein–Ligand Interactions

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Abstract—The synthesis of [23,24]-$^{13}C_2$-labeled bile salts is achieved through a steroidal side chain degradation and isotopic regeneration strategy. Three common bile acids were degraded to the corresponding C_{22} aldehyde by an oxidative decarboxylation followed by ozonolysis. The side chain was subsequently regenerated via a Horner–Emmons reaction using an ylide generated from $^{13}C_2$-labeled bromoacetic acid. These compounds were used as probes of protein–bile salt interactions using two- and three-dimensional NMR techniques.

Bile salts are amphipathic surfactants that facilitate the absorption of dietary fats, fat-soluble vitamins, and cholesterol in the lumen of the small intestine. However, this is not the limit of their biological significance. Bile salts recently have been shown to facilitate divalent cation absorption across biological membranes. This process is associated with bile salt induced apoptosis.[1] Moreover, bile salts have been shown to have effects at the transcriptional level via interactions with the farnesol X receptor, a bile salt-activated nuclear receptor.[2,3] The enterohepatic circulation effectively recycles bile salts with efficiencies of over 90%.[4] Human ileal bile acid binding protein (I-BABP) is thought to function in this recycling through binding interactions occurring in the absorptive enterocytes of the distal small intestine. In the course of studying this protein we found that ^{15}N- or ^{13}C-labeled bile salts are useful as site-specific probes of bile salt–protein interactions. In these studies, bile salts conjugated to either ^{15}N or [1′,2′]-$^{13}C_2$ enriched glycine at the C-24 carboxylic acid were complexed with I-BABP to probe binding interactions using 2-D and 3-D NMR techniques. This study showed that I-BABP binds bile salts with low intrinsic affinity, but a remarkably high degree of positive cooperativity.[5] The method of isotope incorporation used in this binding study is restrictive, limiting all studies to only conjugated bile salts. Ideally, we would like to compare and contrast site-specific binding constants, and local interactions using both conjugated and unconjugated bile salts. Additionally, our lab is working towards obtaining an NMR structure of a ternary complex of bile salt bound to I-BABP. The molecular details of this structure would be better refined by bile salt–protein NOE restraints involving the C-23 and C-24 positions of the conformationally flexible steroidal side chain than if only NOE data from the glycine moiety were obtained.

Routes for the synthesis of [24]-^{13}C-labeled bile salts have previously been reported, and these compounds are commercially available. However, this labeling strategy is not amenable to multi-dimensional NMR techniques since the carboxyl carbon at C-24 lacks any directly bonded protons. The vast majority of 2-D and 3-D NMR experiments use ^1H as the detected nucleus due to the large sensitivity enhancement with respect to direct detection using either ^{13}C or ^{15}N. Therefore, C-24 enriched bile salts are only amenable to low sensitivity ^{13}C detection experiments. This becomes more problematic when collecting isotope-directed $^1H\{^1H\}$ NOESY data aimed at solving a 3-D structure of a ternary complex of bile salt bound to I-BABP. Here we report the synthesis of three [23,24]-$^{13}C_2$-labeled bile salts in greater than 40% overall yield and illustrate their utility as probes of I-BABP–bile salt interactions.

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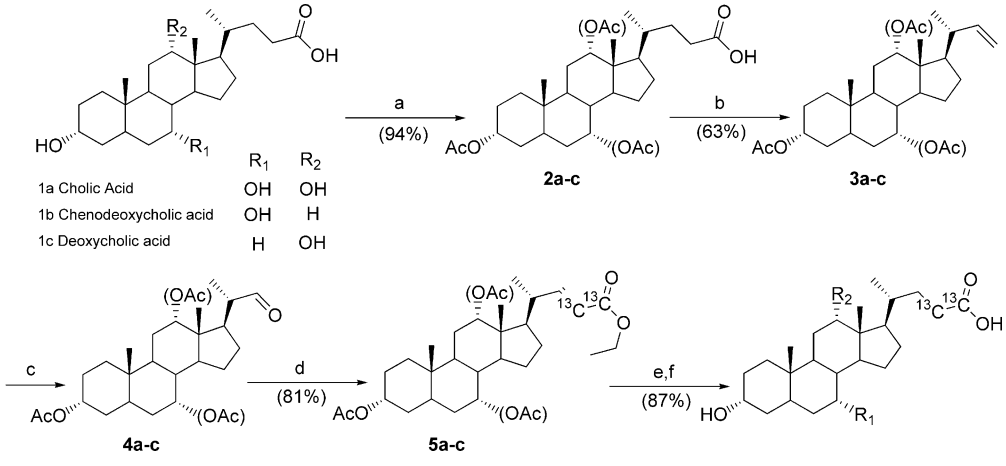
The methodology presented here is applicable to most cholane steroids. However, only the three most physiologically abundant bile salts are presented: cholic acid, chenodeoxycholic acid, and deoxycholic acid. The general approach taken was to begin with the desired steroid, degrade the steroidal side chain by two carbons, and then rebuild the side chain with ^{13}C enriched carbons (Scheme 1). The scheme has six steps, but due to the high yield of each individual step, the overall yield is high. In addition, all of the steps are sufficiently rapid that the compounds can be synthesized in a reasonably short period of time. Previous synthetic schemes decarboxylated, acetylated or formylated cholanic acids to the 23-chloronorcholanes followed by a subsequent conversion to the C-24 nitrile using ^{13}C-labeled sodium cyanide.[6] Hydrolysis of the nitrile yielded C-24 labeled steroids. The 23,24 scheme presented here has comparably high yields, but affords the distinct advantage of incorporation of a two-carbon spin system versus an isolated carbon spin system. The two-carbon spin system affords more flexibility for NMR experiments since one can take advantage of ^{1}H–^{13}C and/or ^{13}C–^{13}C couplings.

Protection of the hydroxyl groups as acetates **2** generally proceeds to completion using acetic anhydride in pyridine with catalytic amounts of dimethylaminopyridine.[7] The degradation of the 24-carboxylic acid is achieved via an oxidative decarboxylation to yield the 24-nor-Δ^{22} norcholene **3** as previously reported.[8] The olefin is subsequently degraded to the C-22 aldehyde **4** via ozonolysis. Conversion of the ozonide to an aldehyde was achieved using zinc in acetic acid. The aldehyde can be subsequently used without further purification in a Horner–Emmons reaction to regenerate the side chain. Triethyl phosphonoacetate, formed from heating equimolar amounts of $^{13}C_2$-enriched ethyl bromoacetate and triethyl phosphite at 150 °C for 2 h, is converted to the sodium salt using 1.0 equiv of sodium hydride in dry benzene.[9] The addition of the aldehyde to the highly reactive ylide forms the α,β-unsaturated ester **5** in less than 5 min at room temperature and in very high yield. The Wittig-type reaction was also explored to rebuild the side chain, but the Horner–Emmons variation held two distinct advantages. The Horner–Emmons triethyl phosphonoacetate can be formed in quantitative yield versus the triphenylphosphonium salt, which in our hands required purification and could not be formed in greater than 70% yield. Further, the Wittig reaction would never proceed in greater than 65% yield, even under high temperature conditions. The resultant α,β-unsaturated ester from the Horner–Emmons reaction could then be hydrogenated with palladium on carbon and converted to the [23,24]-$^{13}C_2$-labeled bile salt via saponification. The bile salts were then crystallized to purity as previously summarized.[10]

To illustrate the utility of these compounds as probes of protein–bile salt interactions, NMR studies were performed to attain structural information on the bile salt–I-BABP complex. In preliminary studies directed at solving the NMR structure of a ternary complex of bile salt bound to I-BABP it was determined that the most kinetically stable and well resolved complex was a 1:1 ratio of glycocholic acid (GCA)/glycochenodeoxycholic acid (GCDA) in a final bile salt/protein ratio of 3:1. The [23,24]-$^{13}C_2$-enriched bile salts were first conjugated to glycine using previously reported methodology,[11] then complexed to I-BABP in a 1.5:1.5:1 GCA/GCDA/I-BABP ratio. This system proved to be amenable to NMR data collection, providing high quality spectra as illustrated in Figure 1. Proton–carbon HSQC Spectra for a 1:1 ratio of GCA/GCDA in buffer reveal a single degenerate peak corresponding to the geminal protons of C-23 (not shown). Upon addition of protein, the geminal proton resonances resolve into separate peaks representing different bound states of the bile salt in addition to the unbound state, as shown in Figure 1A. Peak U represents both unbound GCA and GCDA as a single degenerate peak as assigned by spectra of otherwise identical control samples lacking protein. Upon binding, the two geminal protons at C-23 of GCA become diastereotopically resolved. These resonances were assigned using a sample containing labeled GCA and unlabeled GCDA. Therefore, each geminal proton is in a unique chemical environment that is in slow exchange on the NMR timescale (~10 ms). This observation implies that the bile salt side chain is rigidly



Scheme 1. (a) Ac_2O, pyridine, DMAP; (b) $Pb(OAc)_4$, $Cu(OAc)_2$; (c) O_3, CH_2Cl_2, −78 °C, then Zn dust, AcOH; (d) triethyl phosphonoacetate-[1,2]-$^{13}C_2$, NaH; (e) Pd/C, EtOH, H_2 (45 psi); (f) NaOH.

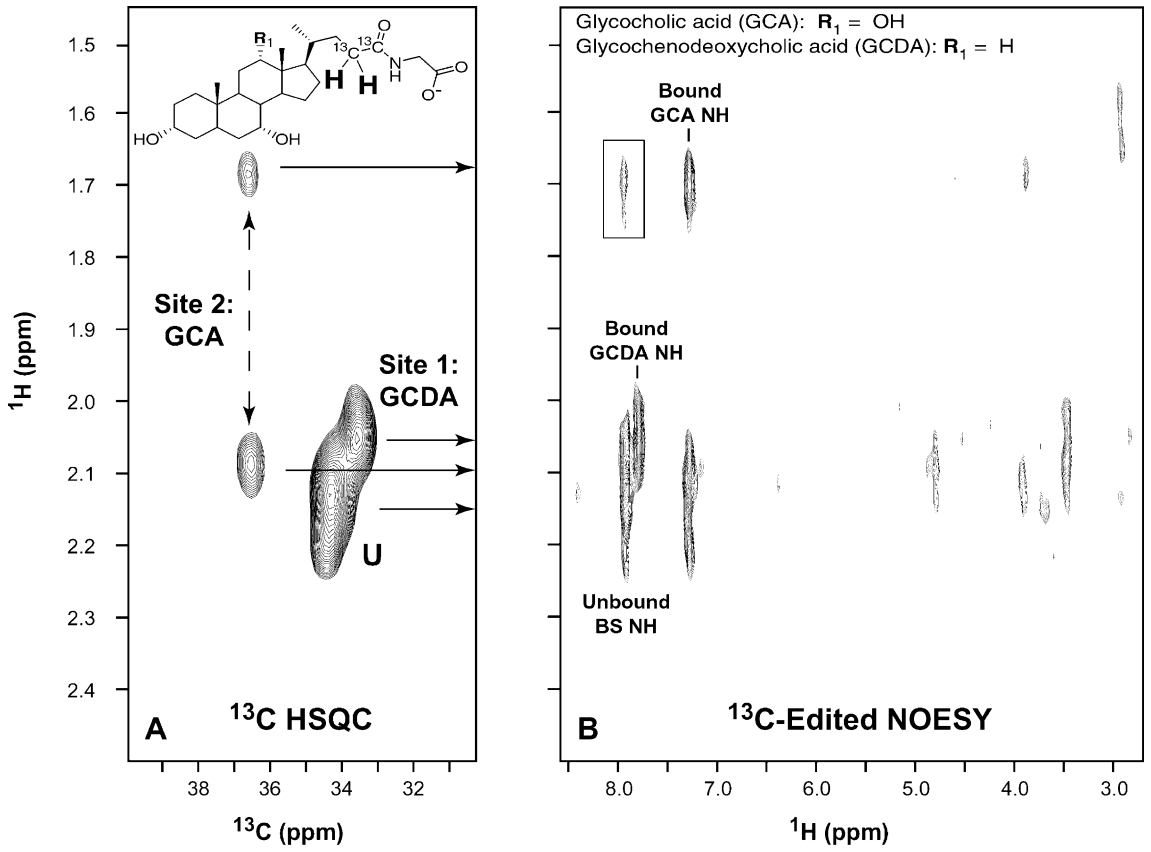


Figure 1. NMR spectra of a ternary complex of [23,24]-$^{13}C_2$-enriched glycochenodeoxycholic and glycocholic acids bound to human ileal bile acid-binding protein (I-BABP). **A**) 2-D ^{1}H–^{13}C HSQC spectrum showing bound glycochenodeoxycholic acid (site 1: GCDA) and bound glycocholic acid (site 2: GCA). Unbound bile salts (GCA and GCDA) appear as a single degenerate peak (U). Bound GCA shows two distinct diastereotopic protons for the geminal C-23 protons. Bound GCDA exhibits a single peak for the C-23 geminal proteins. Arrows point to NOE interaction partners shown in panel B. **B**) 2-D ^{13}C- edited NOESY of labeled bile salts bound to I-BABP. GCA and GCDA show strong NOEs to their own respective glycine-conjugated amide proton. In addition, NOEs are also present between bound GCA and GCDA (boxed). Potential protein–bile salt NOEs are also shown ranging in chemical shift from 6.5 to 2.5 ppm. Spectra collected under following conditions: mixing time = 200 ms, 1.6 mM I-BABP, 20 mM K_2PO_4, 50 mM KCl, 0.05% NaN_3, pH 6.3, 20 °C. 1.5:1.5:1 ratio of GCDA/GCA/I-BABP.

anchored to the protein, perhaps through a pairwise interaction. In contrast, the geminal protons of GCDA remain degenerate, suggesting that the side chain of GCDA is in free rotation on the NMR timescale. After peak assignment, a ^{13}C-edited NOESY experiment was performed to identify bile salt–protein interactions that would help place the bile salt in the binding cavity of the protein. The most prominent NOE peaks arose from intra-bile salt connections arising from the geminal protons at C-23 to the bile salt amide hydrogens. In addition, inter-bile salt NOE peaks were detected, which helps to restrain the bile salts in the I-BABP binding cavity. Many weaker cross peaks were also visible, indicating potential bile salt–protein cross peaks. The cross peaks occur in regions ranging from aromatic to aliphatic chemical shifts. These NOE data represent the first structural data for a doubly ligated bile salt–I-BABP complex. Further studies are necessary to assign the protein–bile salt cross peaks. These studies are in progress. Detailed experimental is available online.[12]

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