CHEMICAL INVESTIGATIONS INTO THE

PHYSIOLOGY OF BILE ACID SKELETONS

by

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Submitted in partial fulfillment of the requirements

For the degree of Doctor of Philosophy

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January 2013
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(date) 11/29/2012

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<tbody>
<tr>
<td>ASBT</td>
<td>Apical Sodium-dependent bile acid transporter</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
</tr>
<tr>
<td>CYP7A1</td>
<td>Cholesterol 7 alpha-hydroxylase</td>
</tr>
<tr>
<td>CMC</td>
<td>Critical micelle concentration</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FXR</td>
<td>Farnsoid X receptor</td>
</tr>
<tr>
<td>HRMS</td>
<td>High resolution mass spectrometry</td>
</tr>
<tr>
<td>IBX</td>
<td>2-Iodoxybenzoic acid</td>
</tr>
<tr>
<td>IR</td>
<td>Infra red spectrometry</td>
</tr>
<tr>
<td>ITC</td>
<td>Isothermal titration calorimetry</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>NaAC</td>
<td>Sodium allo cholate</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
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<tr>
<td>NaACDC</td>
<td>Sodium allo chenodeoxycholate</td>
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<td>NaADC</td>
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<td>NaC</td>
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<td>NaCDC</td>
<td>Sodium chenodeoxycholate</td>
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<tr>
<td>NaDC</td>
<td>Sodium deoxycholate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance spectrometry</td>
</tr>
<tr>
<td>Ost</td>
<td>Organic solute transporter</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SAR</td>
<td>Structure-activity relationship</td>
</tr>
<tr>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UDCA</td>
<td>Ursodeoxycholic acid</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet spectrometry</td>
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ABSTRACT

Chemical Investigations into the Physiology of
Bile Acid Skeletons

by

QINGJIANG LI

Bile acids are a class of surfactants that facilitate the digestion of dietary lipids and fat soluble vitamins in the intestines of vertebrates. Since they are the end products of cholesterol metabolism, bile acids biosynthesis is the major pathway to remove excess of cholesterol, which is another major biologic role of bile acids. A recycling process called enterohepatic circulation increases the efficiency of bile acid usage during digestion and meanwhile, by reabsorption of bile acids modified by colon bacteria, increases the structural diversity of bile acids in vertebrates. While most of bile acid structural variations occur on the appendages, one variation, the 5α versus 5β A/B ring junction, is on the core skeleton. The cause of this variation on the skeleton is especially intriguing considering bile acid is the only major human steroid family bearing 5β A/B ring junction. Further, a clear trend was found correlating bile acid skeletal variation to the evolution of vertebrates. In this thesis, we discuss our preliminary research on the evolutionary significance of the skeleton in several aspects of bile acid functions.

First, three 5α bile acids were synthesized using the classic strategy of epimerizing α-carbon of hydroxyl group. This methodology features a shorter synthetic route and better overall yield compared to previous reports with the same synthetic
targets. Consequently, after a comprehensive analysis of the detergent properties of $5\alpha$ and $5\beta$ bile acids using isothermal titration calorimetry, a conclusion that $5\beta$ bile acids are superior to $5\alpha$ bile acids as detergents was drawn in consistent with the hypothesis of bile acids skeleton evolution in vertebrates. The skeletal significance in bile acid cytotoxicity was evaluated by induction of apoptosis to colon cancer cells using flow cytometry and confocal microscopy. The results were complicated by a recovery mechanism of bile acid induced apoptosis and did not correlate to bile acid evolution. The investigation of bile acids’ ability to traverse cellular membranes was undertaken using radioactively labeled of bile acids. A new methodology for $\alpha,\beta$-unsaturation of the side chain of bile acids was developed and the mechanism of the key step, dehydrogenation by selenium dioxide, was thoroughly studied. Isotopic labeling experiments will be performed in the future and the radioactive bile acids with different skeletons will be sent to Wake Forest University for membrane penetration assay.
ACKNOWLEDGEMENTS

First of all, I would like to express my deep and respectful gratitude to my supervisor, Professor Gregory Tochtrop, for his continuous support and guidance throughout the years of my Ph. D. education. His mentorship was paramount in providing a well rounded experience consistent with my long-term career goals. He has continuously provided me with enthusiasm, vision, and wisdom, and inspired me from beginning to end. He also created an environment that gave me the flexibility to explore new ideas, while helping me to make critical decisions whenever the project hit an obstacle. He is an outstanding researcher to have as a role model for a graduate student.

I also want to thank Professor Brian Cobb and his wife Lori Cobb, for their generous help on cell culture and apoptosis study. My knowledge in biology was very limited when I started the apoptosis study. They were always there, gave me advice and guided me through the assay from every aspect. Without their help, it would be impossible for me to complete this project.

My thanks also are due to all members of the Tochtrop research group, especially Dr. Yong Han, who was always there to give advice and discuss every aspect of life. It has been a privilege to work together with all these intelligent and friendly people in my lab. Here I send my gratitude to Yong Han, Brian Werry, Tonibelle Gatbonton-Schwager, Sushabhan Sadhukhan, Jianye Zhang, Vasily Ignatenko, Emily Barker, Chuan Shi, Katie Doud, Roozbeh Eskandari, and Mohsen Badiee. I would like to take this opportunity to express my gratitude to the staff of the Department of Chemistry, Dr. Dale Ray, June Ilhan, and Jim Sill.

My most heartfelt thanks must go to my family, my parents and my wife in particular. They have always been there, providing me the support and the strength to get
through all the frustrations and difficult times. It is their unconditional love and support
that are behind every bit of my achievement and success.

This research was supported by a National Science Foundation grant MCB–
0844801 to G.P.T.
Chapter 1

Introduction:

Background and Significance
Bile acid and cholesterol

Bile acids are synthesized in hepatocytes from cholesterol, and the biosynthesis of bile acids represents the major pathway to remove excess cholesterol. Their production is tightly controlled by nuclear receptor transcription factors, including the liver X receptors (LXR-α and LXR-β) and the Farnesoid X receptor (FXR).1-2 LXRαs, activated by specific oxysterol derivatives, control the activation of 7α-hydroxylase (CYP7A1), which is the rate limiting enzyme in bile acid synthesis (Scheme 1). This enzyme converts cholesterol to 7-hydroxycholesterol, which is subsequently oxidized and converted to cholesteryl-CoA or chenodeoxycholyl-CoA. Through multiple reactions, these CoAs are converted to cholic acid and chenodeoxycholic acid respectively.3-5

Scheme 1. Bile acid synthesis in the liver.
In addition to the removal of excess cholesterol, bile acids have been reported to be the primary ligands of nuclear receptors regulating the expression of important genes in cholesterol homeostasis.\(^6\) This suggests that the synthesis of bile acids is crucial in multiple aspects of cholesterol regulation. As a result, bile acid research has experienced a renaissance recently, with a primary focus on elucidating the extremely complex signaling network of bile acid homeostasis. The complexity of bile acid regulation is derived not only from the number of organs involved in bile acid flow, but also from the great variety of their chemical structures.

Figure 1. Characteristic bile acids structure (using cholic acid as an example).

Bile acids are a heterogeneous mixture of over 100 isoforms that share certain common features. As shown in Figure 1 (cholic acid, one of the most common bile acids in humans), they all have a rigid steroidal skeleton, which defines the hydrophobic beta-face with axial methyl groups. Typical bile acids contain multiple hydroxyl groups on the steroidal face opposite to the methyl groups, defining the hydrophilic alpha-face. A side chain containing a terminal carboxylic acid group or alcohol is attached to the five-membered D-ring, and is subject to conjugation with glycine or taurine to improve its solubility in physiological environments. One hydrophobic face and one hydrophilic face make bile acids amphipathic, which is critical when considering their major biologic role is to aid the digestion of dietary lipids, including fat soluble vitamins, via detergent action.
Figure 2. Bile acids structure complexity. Variations are on the number and position of hydroxyl groups, stereochemistry of A, B ring fusion, length of side chain and functional groups at the end.

For different bile acids, the position and stereochemistry of hydroxyl groups may vary as well as the length of the side chain (Figure 2). Some naturally occurring bile acids have carbonyl groups instead of hydroxyl groups on the skeleton. The great number of bile acids and bile alcohols can be explained by the divergent evolution of multiple biochemical pathways of bile acid synthesis from a poorly soluble membrane lipid to a highly water soluble amphipathic detergent.

The enterohepatic circulation

As previously mentioned, other than structural diversity, the difficulty of studying bile acid regulation is also derived from the number of organs involved in bile acid flow in humans. After being synthesized and conjugated with glycine or taurine in the liver, they are stored in the gall bladder. After a meal, they are excreted into the intestine, where they aid in the absorption of dietary lipids. Instead of being excreted through the feces, over 95% of bile acids are reabsorbed in the distal ileum and transported to the portal venous circulation and eventually to the liver where they are recycled back into the bile acid pool.
Some bile acids escape reabsorption, and subsequently travel to the large intestine where modifications can occur. These modifications are carried out by the diverse bacteria residing in the large intestine and these modified bile acids can subsequently be passively reabsorbed, returned to the liver, and combined into the bile acid pool. The entire process whereby bile acids excreted from the gall bladder are recycled is called enterohepatic circulation (Figure 3). There are two net effects of
enterohepatic circulation. First, the efficiency of bile acids usage is greatly improved, since they are reused up to 20 times per day. Second, complexity of the bile acid pool composition is increased, as a small portion of primary bile acids are modified and integrated back into the bile acid pool as secondary bile acids.

**Allo bile acids**

Bile acids that are directly synthesized in the liver are termed as primary bile acids, whereas all other bile acids from modifications by colon bacteria are called secondary bile acids. As a result of diversified biochemical pathways, different species may have different primary bile acids. Cholic acid and chenodeoxycholic acid are the two primary bile acids in humans. Bacterial modifications of primary bile acids in the colon produce a great variety of secondary bile acids including the allo bile acids. As shown in Figure 4, allo bile acids, demarcated by a \(5\alpha\) configuration (trans A/B ring fusion), result from the only skeletal modification by colon bacteria, whereas most modifications involve hydroxyl groups. They are dominant primary bile acids in some lower vertebrates such as reptiles but only trace amounts can be found sporadically in higher vertebrates. No allo bile acid are directly synthesized from cholesterol in higher vertebrates and the only natural source is bacterial modification of \(5\beta\) configuration bile acids.\(^8\) However, \(5\beta\) analogs (cis A/B ring fusion) are more widely distributed in mammals and other high vertebrates. From an evolutionary point of view, allo bile acids (\(5\alpha\)) can be thought of as precursors to the \(5\beta\) bile acids. Considering bile acids are the only major \(5\beta\) steroids found in high vertebrates,\(^9\) the reason why they are chosen by nature is especially intriguing. On the other hand, the production of \(5\alpha\) bile acids suggests that they are still important in certain physiological roles and would not fade away in evolution.
Figure 4. 3-D skeletal conformation of cholic acid vs. allo cholic acid.

Although only one stereo center is inverted from 5β to 5α bile acid, the 3-D structure of the skeleton changes dramatically. As shown in Figure 4, allo cholic acid has a planar scaffold while the 5β-hydrogen of cholic acid makes the A-ring protrude from the planar skeleton. Therefore, significant differences in their properties can be expected, including physical properties as detergents and binding affinity with proteins or nuclear receptors. An environment for their comparison is also provided since they can all be found in humans. A thorough comparison of these properties can potentially provide insight to the driving force behind this evolution.

Scope of project

The driving force of this evolution can possibly lie in any area of bile acid functions, such as biosynthesis, transportation, surfactant properties or toxicity. 5β Bile acids are expected to be superior to their 5α analogs in one or multiple aspects to justify the natural selection in higher vertebrates. A comprehensive investigation into every aspect mentioned above is necessary in order to accurately evaluate these two classes of molecules and find the possible factors that drive their evolution. As a result, this thesis covers multiple chemical and physiological topics relevant to bile acids.

Synthesis of allo bile acids
Allo bile acids are dominant primary bile acids only in some birds and reptiles. In higher vertebrates, where 5β-reduced bile acids are obtained in relatively large amounts for research or medicinal purpose, allo bile acids are modified in very limited amounts by colon bacteria. Because of this, the only practical way to obtain allo bile acids in large amounts is organic synthesis. Since the identification of allo cholic acid by Anderson and Haselwood in 1962, several syntheses have been reported. The most recent reports, which are almost 20 years old, suffer from iterative protection-deprotection, and non-selective substitution-elimination reactions. Several new reactions that can be utilized in allo bile acid synthesis have been developed since then. We decided to develop our own methodology for allo bile acid synthesis using such reactions. This work comprises Chapter 2.

**Measurement of Critical Micelle Concentration (CMC)**

The major physiological role of bile acids is to act as detergent, aiding the solubilization of dietary fat and other lipids, including fat soluble vitamins. The key factor in the evaluation of a detergent is the determination of its CMC and other thermodynamic data during formation of micelles. There are several ways to measure CMC of a given detergent, including the maximum bubble pressure method and hydrophobic dye solubilization method. These methods work well with simple surfactants, such as SDS, with well-defined hydrophilic and hydrophobic domains. The micelle formation of simple surfactants is abrupt because monomers can be well organized and tightly packed when they assemble to construct the micelles. Large entropy changes ensure that the spontaneous formation of micelles happens rapidly. However, this is not the case for bile acids. Termed facial amphiphiles, bile acids have a hydrophilic face with multiple hydroxyl groups and a hydrophobic face demarcated by the methyl groups on the other side of scaffold (Figure 3). Because of this, the self-
assembly pattern of these molecules is much more complicated compared to SDS and the micelle formation is more gradual since no unified micelle shape can be expected. Consequently, a more informative method, Isothermal Titration Calorimetry (ITC), was used to measure the CMC of both classes of bile acids. ITC has the advantage of monitoring the complete process of micelle formation over a period of time rather than only observing the inflection point of a particular equilibrium, as the methods mentioned above do. Further, ITC can also provide enthalpy and entropy of micelle formation. A comprehensive data set is crucial for evaluation of detergent properties for such a complicated surfactant. This work comprises Chapter 3.

Cytotoxicity and apoptosis induction of bile acids

During cholestasis, a medical condition characterized by impaired bile flow, the accumulation of bile acids in the liver can cause hepatocellular injury, development of cirrhosis, and even death from liver failure. This cytotoxicity of bile acid in the liver has been attributed to their detergent properties, mainly based on the observation that hydrophobic bile acids are more toxic than hydrophilic ones. But recent research has shown that bile acid induced apoptosis through specific signaling pathways, other than detergent properties, is more likely to be the key event during hepatobiliary disease.

In contrast to necrosis, apoptosis is a programmed cell death process that confers advantages in an organism’s life cycle. Not all the bile acids can cause apoptosis, some may even be cytoprotective. The rule that hydrophobic bile acids are generally more toxic is not always true either. Actually, bile acid cytotoxicity is a much more complex issue than a simple rule. For example, with only the difference of the stereochemistry of one hydroxyl group, chenodeoxycholic acid is strongly toxic while ursodeoxycholic acid is cytoprotective and promotes cell proliferation. It is intriguing that a minor change on
the structure can cause complete overturn of the cytotoxicity. Curiously, there is no systematic structure-activity relationship study of bile acids in this field available at the present time.

Inspired by the dramatic cytotoxicity changes caused by inverting the stereochemistry of one hydroxyl group of ursodeoxycholic acid, we would predict that inversion of \(5\beta\) configuration can also cause significant cytototoxic changes of bile acids. A comparison between \(5\alpha\) and \(5\beta\) bile acids in apoptosis induction can provide the first example of SAR study on the bile acids scaffold. A detailed comparison of bile acid induced apoptosis assay on a colon cancer cell line, HT-29, will be discussed in Chapter 4.

*Radioactive labeling of bile acids and new methodology for \(\alpha, \beta\)-unsaturation of carboxylic acid*

Each molecule of bile acid has to be reused multiple times during the digestion of each meal. This exerts a significant challenge for bile acid reabsorption and transportation. In order to finish multiple recycling processes in a few hours, bile acid reabsorption and transportation must be very efficient. On the first step of recycling, bile acid molecules have to travel through multiple cell membranes from the intestinal lumen to the portal vein. The ability to traverse the cell membrane obviously affects bile acid reabsorption and thereby affects the overall efficiency of enterohepatic circulation. With different core skeletons, \(5\alpha\) and \(5\beta\) bile acids are expected to exhibit different ability to transport. The different ability to transport across membrane will affect not only the efficiency of recycling, and subsequently, the composition of the overall bile acid pool, but also their availability inside cells to take action. Our collaborator’s laboratory at Wake Forest University has a unique assay system to grow cells with monolayers of both
apical and basolateral membranes with bile acid transporters. Active uptake of bile acids by intestinal epithelial cells requires two transporters, Apical Sodium-dependent Bile acid Transporter (ASBT) and Organic solute transporter α/β (Ost α/β), to cross apical and basolateral membranes respectively. It is critical to have those transporters transfected in the monolayer cells.

Evaluation of bile acids’ ability to cross the monolayer membrane with transporters requires direct detection on both sides of the membrane. The detection of bile acids can be realized by labeling with radioactive isotopes. Here we discuss our strategy of tritium labeling of bile acids in Scheme 2.

Scheme 2. Synthetic design of isotopic labeling on bile acids.

Other than overall yield and step efficiency, several more principles guided us to the strategy of unsaturation of a carboxylic acid on the side chain followed by catalytic tritiation. First, isotope labeling has to be the last step because it is radioactive. Second, in order to avoid proton exchange with water and ensure accurate quantification of molecules on each side of membrane, the labeling isotopes cannot be on oxygen atoms.

The key reaction in our methodology is selenium dioxide dehydrogenation of a trifluoromethyl ketone, which is easily converted to carboxylic acid, to introduce a carbon-carbon double bond into the structure. Compared to the traditional method to make α, β-unsaturated carboxylic acids (Scheme 3), this methodology has several advantages. First, it circumvents the harsh basic conditions in the deprotonation step.
Second, it has no extra steps of protection or deprotection and thereby improves the step efficiency. Third, it revives an old chemistry of selenium dioxide, known for its versatility in performing multiple conversions on similar substrate. The detail of each step of reactions, the scope of this methodology and mechanistic study of selenium dioxide chemistry, along with the progress of making radioactive bile acids will be discussed in Chapter 5.

**Scheme 3. Traditional method for α, β-unsaturation of carboxylic acid.**

![Scheme 3](image)

**Summary**

As the only major steroids with a cis A/B ring junction\(^9\), 5β bile acids are also the final products of bile acids evolution throughout vertebrate species. When compared with 5α reduced ring precursors, the allo bile acids, 5β bile acids should have significant advantages. However, they are far from being perfect, given the cytotoxicity and carcinogenic effect of some bile acids, especially hydrophobic ones. A comprehensive comparison of two subfamilies provides the driving force of this evolution and potentially helps us move forward to develop an even better bile acid in attempting to solve medicinal problems of bile acid systems.

New synthetic methodology of allo bile acid (Chapter 2) is the first step and provides enough allo bile acids to start the investigation. A comprehensive comparison of two subfamilies of bile acids with distinct skeletal structures in aspects of detergent
properties (Chapter 3), apoptosis induction of a colon cancer cell line (Chapter 4), $\alpha$, $\beta$-unsaturation and isotopic labeling methodology for purpose of membrane permeability study (Chapter 5) will be discussed afterwards. An overall conclusion and insight into bile acids evolution based on the results of our investigation will be summarized at the end (Chapter 6).
References


22. Heuman, D. M.; Mills, A. S.; McCall, J.; Hylemon, P. B.; Pandak, W. M.; Vlahcevic, Z. R., Conjugates of ursodeoxycholate protect against cholestasis and
hepatocellular necrosis caused by more hydrophobic bile salts: in vivo studies in the rat. 


Chapter 2

An Efficient Synthesis of Allo Bile Acids
Introduction

Bile acids are ubiquitous small molecules across vertebrates that facilitate digestion by acting as surfactants in the lumen of the small intestine. The allo bile acids represent a subfamily of this class of physiologic molecules that is primarily demarcated by an AB trans ring fusion (also referred to as 5α-reduced see Figure 1). They occur widely in lower vertebrates, including various fish, birds and reptiles, but are also found sporadically in higher vertebrates and mammals (including humans). Since the identification of the farnesoid X receptor (FXR) as an endogenous nuclear receptor for the bile acids, there has been renewed interest in this class of physiologic molecules. Given the central role of 5α-reduced C24 cholanic acids in gaining access to a major subset of these bile acids, efficient methods for their synthesis will always represent important research tools.

Figure 1. Numbered and 3-D representation of allo cholic acid

No significant biological feedstocks are currently available that are rich in 5α-reduced bile acids. Consequently, the primary route for access to these molecules has been via chemical synthesis. To this point, since the identification of allo-cholic acid by Anderson and Haslewood in 1962, several syntheses have been reported. Our laboratory has recently become interested in studying the allo-bile acids in the context of their ability to modulate cellular signaling through FXR, and after analyzing the literature we came to the conclusion that the available methods and are not general across the
spectrum of allo bile acids, and would further preclude access to select members of the allo-family. Further, recent reports suffer from iterative protection-deprotection, and non-selective substitution-elimination reactions.7

**Scheme 1. Synthetic route to allo bile acids.**

Results and discussion

Conversely, the approach reported here can be applied across the bile acid spectrum, and is highly efficient. As shown in Scheme 1, our synthesis begins with a regioselective C-3 oxidation of methylated 5β-reduced bile acids using Ag₂CO₃ adsorbed on Celite.9 The selective nature of this reaction is most likely due to relative steric accessibility of the C-3 position interacting with the Celite solid surface. Subsequently, any additional hydroxyl groups are protected as methoxymethyl ethers.10 2-Iodoxybenzoic acid (IBX) smoothly and regioselectively performs the dehydrogenation to give the Δ⁴ enone.11

Stereochemistry of a Birch reduction of the Δ⁴ moiety proceeds as expected based on the model put forth by Stork whereby the radical anion intermediate protonates
axially.\textsuperscript{12} From a technical point of view, achieving correct stoichiometry in the Birch reduction is absolutely crucial for success. Excess lithium will readily reduce the C-24 ester in addition to the C-3 carbonyl. In this molecule, the sequence of reactivity towards the Birch conditions proved to be $\Delta^4$ enone, C-24 ester, C-24 aldehyde (from the C-24 ester), and finally the C-3 ketone. If the amount of lithium is strictly controlled it is possible to obtain any of the sequential intermediates, though for this study we were only interested in the 5\(\alpha\)-reduced product. The consequence of understanding this sequential reactivity is that we do not have to saponify the C-24 ester prior to Birch conditions as is traditionally performed.\textsuperscript{8} It is also noteworthy that all other reduction methods tried, including sodium borohydride and catalytic hydrogenation, selectively gave 5-\(\beta\) reduced isomer, presumably directed by steric effects of the 7\(\alpha\)-protected hydroxyl. Literature precedent predicted a smooth and selective reduction of the C-3 ketone to the 3\(\alpha\)-hydroxy compound using potassium tri-sec-butylborohydride.\textsuperscript{13} Removal of the protecting groups afforded the allo bile acids in very good yields over seven steps: 28\% to 35\% depending on the bile acid (see Table 1).
Table 1. Yields of the intermediate steps of the allo bile acid synthesis

<table>
<thead>
<tr>
<th>Product</th>
<th>yield (%)</th>
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<tr>
<td>2a, R1=R2=OH</td>
<td>98</td>
</tr>
<tr>
<td>2b, R1=H, R2=OH</td>
<td>98</td>
</tr>
<tr>
<td>2c, R1=OH, R2=H</td>
<td>98</td>
</tr>
<tr>
<td>3a, R3=R4=MOMO</td>
<td>77</td>
</tr>
<tr>
<td>3b, R3=H, R4=MOMO</td>
<td>82</td>
</tr>
<tr>
<td>3c, R3=MOMO, R4=H</td>
<td>82</td>
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<tr>
<td>4c, R3=MOMO, R4=H</td>
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<tr>
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<tr>
<td>6c, R3=MOMO, R4=H</td>
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<td>7c, R1=OH, R2=H</td>
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In summary, an efficient synthesis of 5-α reduced bile acids has been developed. This methodology not only gives the highest overall yields in shortest route so far, but also provided insights into selective epimerization at ring conjunctions. Hypotheses presented on the stereoselectivity of varies hydrogenation methods can serve as the guidance for reduction of enone ring conjunctions.

Experimental section
General. All reactions were carried out under inert atmospheric conditions, except as noted. TLC was done on Hard Layer, Organic Binder TLC-plates with a fluorescent indicator and visualized by UV light (254 nm) or phosphomolybdic acid stain, and purchased from Dynamics Adsorbents (Atlanta, GA). Flash chromatography was performed on silica gel (230-400 mesh, 60 Å) purchased from Dynamics Adsorbents (Atlanta, GA). Solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the $^1$H and $^{13}$C-NMR spectra, respectively. The internal references were TMS (δ 0.00) and CDCl$_3$ (δ 77.2) for $^1$H and $^{13}$C spectra, respectively. Only $^1$H NMR spectra are reported for known compounds, while IR, $^1$H NMR, $^{13}$C NMR, MS, HRMS and melting temperatures are reported for all new compounds. Coupling constants are reported in Hz and multiplicities are indicated as follows: s (singlet); d (doublet); t (triplet); m (multiplet).

**General procedure of methyl esterification of bile acids**

To a solution of appropriate bile acid (free acid or sodium salt) (0.1mol) in 200 mL of methanol, 1 mL of sulfuric acid was added. The resulting mixture was then stirred overnight and monitored by TLC. Removal of the solvent under vacuum was followed by dissolving the residue in ethyl acetate. The solution was then washed with water, and brine before being dried over sodium sulfate. The solvent was removed in vacuo to provide a slightly yellowish solid (I) in 99% yield with over 95% purity confirmed by $^1$H NMR.
Methyl 3α, 5β, 7α, 12α-cholan-24-oate (Ia, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 3.92 (t, $J = 2.8$ Hz, 1H), 3.79 (d, $J = 2.4$ Hz, 1H), 3.60 (s, 3H), 3.40 (m, 1H), 2.32-2.13 (m, 4H), 1.91-1.29 (m, 23H), 0.92 (d, $J = 6.0$ Hz, 3H), 0.83 (s, 3H), 0.63 (s, 3H).

Methyl 3α, 5β, 7α-cholan-24-oate (Ib, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 3.85 (s, 1H), 3.66 (s, 3H), 3.46 (m, 1H), 2.40-2.20 (m, 3H), 1.98-1.10 (m, 24H), 1.02-0.92 (m, 7H), 0.66 (s, 3H).
Methyl 3α, 5β,12α-cholan-24-oate (Ic, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 3.98 (s, 1H), 3.66 (s, 3H), 3.61 (m, 1H), 2.41-2.19 (m, 2H), 1.87-1.12 (m, 24H), 0.97 (d, $J = 6.0$ Hz, 3H), 0.91 (s, 3H), 0.68 (s, 3H).

General procedure for oxidation of 3-hydroxyl group

To a solution of Ia (4.22g, 10.0 mmol) in 200 mL toluene, freshly made Ag$_2$CO$_3$ on Celite (50% wt, 12.1g, 22.0 mmol) was added in portions. Sometimes the suspension of Ag$_2$CO$_3$ on Celite has to be prerefluxed for 0.5h to be activated and completely remove the water. The suspension was refluxed and the water was removed by a Dean-Stark apparatus. The reaction was monitored by TLC until the starting material was consumed. The mixture was filtered to remove the solid, and the filtrate was concentrated in vacuo to provide a yellowish solid (II, 4.1g, 98% yield) with over 95% purity. Further purification was done by flash chromatography with eluent EA/Hex =1/2.
Methyl 5β, 7α, 12α-3-oxo-cholan-24-oate (IIa, known compound) \(^1\)H NMR (CDCl\(_3\), 400MHz) \(\delta\) 4.03 (s, 1H), 3.93 (s, 1H), 3.67 (s, 3H), 3.41 (t, \(J = 14.4\) Hz, 1H), 2.45-1.15 (m, 25H), 1.00 (s, 3H), 0.99 (d, \(J = 7.2\) Hz, 3H), 0.73 (s, 3H).

Methyl 5β, 7α-3-oxo-cholan-24-oate (IIb, known compound) \(^1\)H NMR (CDCl\(_3\), 400MHz) \(\delta\) 3.93 (t, \(J = 2.6\) Hz, 1H), 3.67 (s, 3H), 3.40 (t, \(J = 14.2\) Hz, 1H), 2.44-1.17 (m, 26H), 1.01 (s, 3H), 0.94 (s, \(J = 6.4\) Hz, 3H), 0.70 (s, 3H).
Methyl 5β, 12α-3-oxo-cholan-24-oate (IIc, known compound) $^1$H NMR (CDCl$_3$, 400MHz) δ 4.04 (t, $J = 1.4$ Hz, 1H), 3.67 (s, 3H), 2.73 (t, $J = 14.4$ Hz, 1H), 2.42-1.09 (m, 26H), 1.01 (s, 3H), 0.98 (s, $J = 6.4$ Hz, 3H), 0.72 (s, 3H).

**General procedure of MOM protection**

Il (0.82g, 2.0 mmol) was added to a solution of dimethoxymethane (4.2mL, 24.0 mmol) in 100 mL of anhydrous CHCl$_3$. Phosphorous pentoxide (about 3g) was added in portions over a period of 0.5h with vigorous stirring. The resulting mixture was stirred at room temperature for additional 4h until the starting material was consumed as shown by TLC. The reaction mixture was poured into 400 mL of ice water, and separation of the organic layer was followed by washing with 1M HCl (1×20mL), water (2×20mL), and brine (1×20mL), and then dried over anhydrous sodium sulfate. The solvent was removed in vacuo to provide a yellowish oil, which was then purified by flash chromatography with eluent EA/Hex =1/4. The product was obtained as colorless oil (III 0.78g, yield 77%).
Methyl 7α, 12α-7, 12-Dimethoxymethoxy-3-oxo-5β-cholan-24-oate (IIIa) ¹H NMR (CDCl₃, 400MHz) δ 4.75-4.54 (m, 4H), 3.82 (s, 1H), 3.69-3.68 (m, 1H), 3.67 (s, 3H), 3.40 (s, 3H), 3.38 (s, 3H), 2.48-1.06 (m, 24H), 1.02 (s, 3H), 0.95 (d, J = 6.4 Hz, 3H), 0.73 (s, 3H).

Methyl 7α-7-methoxymethoxyl-3-oxo-5β-cholan-24-oate (IIIb, known compound) ¹H NMR (CDCl₃, 400MHz) δ 4.67 (d, J = 6.8 Hz, 1H), 4.54 (d, J = 6.8 Hz, 1H), 3.68-3.67 (m, 4H), 3.37 (s, 3H), 2.40-1.16 (m, 26H), 1.02 (s, 3H), 0.94 (d, J = 6.4 Hz, 3H), 0.69 (s, 3H).
Methyl 12α-12-methoxymethoxyl-3-oxo-5β-cholan-24-oate (IIIc) 

^1H NMR (CDCl₃, 400MHz) \( \delta \) 4.73 (d, \( J = 6.8 \) Hz, 1H), 4.65 (d, \( J = 6.4 \) Hz, 1H), 3.82 (t, \( J = 2.8 \) Hz, 1H), 3.67 (s, 3H), 3.38 (s, 3H), 2.73 (t, \( J = 13.2 \) Hz, 1H), 2.43-1.07 (m, 5H), 1.02 (s, 3H), 0.94 (d, \( J = 6.4 \) Hz, 3H), 0.73 (s, 3H).

^13C NMR (CDCl₃, 100MHz) \( \delta \) 213.4, 174.6, 72.7, 51.4, 47.9, 47.1, 46.4, 44.1, 42.2, 37.0, 36.7, 35.5, 35.0, 34.3, 33.6, 30.9, 30.7, 28.8, 27.3, 26.4, 25.3, 23.5, 22.3, 17.2, 12.6 ppm;

IR (KBr plate) 2938, 2862, 1737, 1713, 1437, 1376, 1162 cm⁻¹; MS (TOF, ES) m/z
(relative intensity %) 245 (3), 422 (8), 427 (M-MOM+Na\(^+\), 100); HRMS (TOF, ES), Calcd. \(\text{C}_{25}\text{H}_{40}\text{O}_{4}\) (M-MOM+Na\(^+\)) 427.2824, found 427.2821.

**General procedure of IBX oxidation**

Freshly made IBX\(^3\) (0.62g, 4.4mmol) was added to a solution of IIIa (1.02g, 2.0mmol) in 100mL DMSO. The resulting mixture was heated to 40 \(^\circ\)C for 3 days after a catalytic amount of trifluoacetic acid (30% mol) was added. The starting materials was still not completely consumed but prolonged reaction time and excess of IBX proved to be ineffective for better conversion. The resulting suspension was filtered through a pad of celite and the filtrate was dissolved in 200mL ethyl acetate. The solution was thoroughly washed with water (5×100mL) and brine (1×100mL). The organic layer was separated and dried over anhydrous Na\(_2\)SO\(_4\). Flash chromatography was performed using eluent EA/Hex =1/4 to isolate the product IVa as a yellowish oil (0.7g, yield 70% and additional 15% for starting materials), which would solidify slowly.
Methyl 7α, 12α-7, 12-Dimethoxymethoxy-3-oxo-5β-chol-4-en-24-oate (IVa)  $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 5.76 (s, 1H), 4.71-4.62 (m, 4H), 3.80 (s, 1H), 3.76 (s, 1H), 3.67 (s, 3H), 3.40 (s, 1H), 3.35 (s, 3H), 2.67-2.62 (m, 1H), 2.45-2.23 (m, 5H), 2.05-1.26 (m, 15H), 1.19 (s, 3H), 0.94 (d, $J = 5.6$ Hz, 3H), 0.74 (s, 3H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 198.5, 174.2, 168.4, 126.0, 96.3, 95.7, 80.2, 74.8, 55.8, 55.4, 51.1, 45.80, 45.78, 42.0, 39.7, 39.5, 38.2, 37.6, 35.1, 34.9, 33.6, 30.6, 27.2, 25.5, 23.0, 17.2, 16.8, 12.1 ppm; IR (KBr plate) 3051, 2948, 2824, 2363, 2335, 1737, 1665, 1618, 1438, 1147, 1100, 1039 cm$^{-1}$; MS (TOF, ES) m/z (relative intensity %) 413 (3), 445 (3), 507 (M+H$^+$, 8), 529 (M+Na$^+$, 100); HRMS (TOF, ES), Calcd. C$_{29}$H$_{46}$O$_7$ (M+Na$^+$) 529.3141, found 529.3148.
Methyl 7α-7-methoxymethoxyl-3-oxo-5β-chol-4-en-24-oate (IVb, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 5.75 (s, 1H), 4.68-4.60 (m, 2H), 3.76 (s, 1H), 3.67 (s, 3H), 3.35 (s, 3H), 2.66-2.61 (dd, $J = 3.2$, 15.2 Hz, 1H), 2.46-2.32 (m, 5H), 2.05-1.13 (m, 20H), 0.93 (d, $J = 6.4$ Hz, 3H), 0.71 (s, 3H).
Methyl 12α-12-methoxymethoxyl-3-oxo-5β-chol-4-en-24-oate (IVc)

$^1$H NMR (CDCl$_3$, 400MHz) δ 5.66 (s, 1H), 4.63-4.59 (m, 2H), 3.74 (t, $J = 2.6$ Hz, 1H), 3.60 (s, 3H), 3.31 (s, 3H), 2.36-2.16 (m, 6H), 1.96-1.21 (m, 15H), 1.10 (s, 3H), 1.06-1.02 (m, 2H), 0.86 (d, $J = 6.0$ Hz, 3H), 0.68 (s, 3H). $^{13}$C NMR (CDCl$_3$, 100MHz) δ 199.4, 174.6, 171.2, 123.8, 96.8, 81.0, 56.1, 51.4, 47.9, 47.5, 46.2, 46.1, 38.1, 35.6, 35.3, 33.9, 32.9, 31.6, 31.0, 30.9, 27.5, 25.9, 23.7, 17.5, 17.2, 12.5 ppm; IR (KBr plate) 2947, 2886, 1737, 1675, 1613, 1437, 1143, 1039 cm$^{-1}$; MS (TOF, ES) m/z (relative intensity %) 245 (3), 335 (4), 385 (22), 447 (M$^+$, 13), 469 (M$^++$Na$^+$, 100); HRMS (TOF, ES), Calcd. C$_{27}$H$_{42}$O$_5$ (M$^++$Na$^+$) 469.2930, found 469.2943.

**General procedure for Birch reduction**

A solution of IVa (102mg, 0.2mmol) in 10mL dry THF was added to 30mL liquid NH$_3$ at -78 °C. The resulting mixture was vigorously stirred while small pieces of lithium (3mg, 4mmol) were added. t-BuOH was added via a syringe subsequently as the hydrogen source and the resulting reaction system was stirred at -78 °C for 5 min before quenched by 1mL of saturated NH$_4$Cl solution in water. The solution was allowed to warm to room temperature to remove the ammonia. The mixture was extracted by ethyl
acetate (3×20mL) and the organic phase was washed with brine (1×10mL). The organic layer was dried over anhydrous Na₂SO₄ before flash chromatography was performed using eluent EA/Hex =1/4. Colorless oil Va (66mg, 65%) was obtained and solidified in hours to provide a white solid.
Methyl 7α, 12α-7, 12-Dimethoxymethoxyl-3-oxo-5α-cholan-24-oate (Va) ¹H NMR (CDCl₃, 400MHz) δ 4.72-4.59 (m, 4H), 3.67 (s, 1H), 3.76 (3, 3H), 3.62 (s, 1H), 3.40 (s, 3H), 3.35 (s, 3H), 2.37-1.11 (m, 24H), 1.00 (s, 3H), 0.93 (d, J = 6.8 Hz, 3H), 0.71 (s, 3H). ¹³C NMR (CDCl₃, 100MHz) δ 211.8, 174.7, 96.6, 96.2, 80.7, 74.6, 56.0, 55.7, 51.5, 46.3, 46.2, 44.3, 42.3, 39.81, 39.78, 39.6, 38.1, 38.0, 35.5, 34.0, 31.0, 30.9, 29.7, 27.6, 26.2, 23.4, 17.6, 12.5, 10.5 ppm; IR (KBr plate) 2928, 1737, 1713, 1442, 1143, 1100, 1039 cm⁻¹; MS (TOF, ES) m/z (relative intensity %) 245 (3), 335 (8), 413 (6), 531 (M+Na⁺, 100); HRMS (TOF, ES), Calcd. C₂₉H₄₈O₇ (M+Na⁺) 531.3298, found 531.3292.

Methyl 7α-7-methoxymethoxyl-3-oxo-5α-cholan-24-oate (Vb) ¹H NMR (CDCl₃, 400MHz) δ 4.67 (d, J = 6.8 Hz, 1H), 4.60 (d, J = 6.8 Hz, 1H), 3.66 (3, 3H), 3.63 (s, 1H), 3.35 (s, 3H), 2.36-2.26 (m, 5H), 2.05-1.76 (m, 6H), 1.65-1.05 (m, 15H), 1.01 (s, 3H), 0.92 (d, J = 6.4 Hz, 3H), 0.67 (s, 3H).
Methyl 12α-methoxymethoxy-3-oxo-5α-cholan-24-oate (Vc)

$^1$H NMR (CDCl$_3$, 400MHz) \( \delta \) 4.70 (d, \( J = 6.8 \) Hz, 1H), 4.67 (d, \( J = 6.8 \) Hz, 1H), 3.78 (s, 1H), 3.66 (3, 3H), 3.39 (s, 3H), 2.39-1.07 (m, 26H), 1.00 (s, 3H), 0.92 (d, \( J = 5.6 \) Hz, 3H), 0.72 (s, 3H).

$^{13}$C NMR (CDCl$_3$, 100MHz) \( \delta \) 211.8, 174.6, 96.8, 81.3, 56.0, 51.4, 48.4, 47.3, 46.6, 46.3, 46.2, 44.6, 38.2, 38.0, 35.4, 35.3, 35.2, 31.3, 31.0, 30.9, 28.8, 27.6, 26.3, 23.7, 17.5, 12.5, 11.3 ppm; IR (KBr plate) 2947, 2876, 2359, 1737, 1713, 1447, 1171, 1147, 1100, 1043 cm$^{-1}$; MS (TOF, ES) m/z (relative intensity %) 335 (2), 333 (25), 332 (100), 331 (10), 330 (10), 329 (25), 328 (20), 307 (10), 285 (10), 284 (25), 283 (100), 282 (5), 261 (25), 260 (2), 239 (10), 238 (20), 237 (100), 236 (5).
General procedure for 3-keto reduction

A solution of Va (508mg, 1.0mmol) in 30mL dry THF was stirred at -78°C under argon protection while K-selectride (1.2mL, 1M in THF, 1.2mmol) was slowly added via a syringe. The reaction mixture was stirred at -78 °C for 2 hours. The reaction was monitored TLC and quenched by 1mL of saturated NH₄Cl solution in water after starting materials disappeared. The solution was allowed to warm to room temperature and THF was removed under vacuum. The mixture was extracted by ethyl acetate (2×20mL) and the organic phase was washed with water (1×10mL) and brine (1×10mL). The organic layer was dried over anhydrous Na₂SO₄ before flash chromatography was performed using eluent EA/Hex =1/2. The product VIa was obtained as a white solid (450mg, 88%).
Methyl 3α, 5α, 7α, 12α-7, 12-Dimethoxymethoxyl-cholan-24-oate (VIa) ¹H NMR (CDCl₃, 400MHz) δ 4.73-4.60 (m, 4H), 4.04 (s, 1H), 3.75 (s, 1H), 3.66 (3, 3H), 3.60 (s, 1H), 3.41 (s, 3H), 3.37 (s, 3H), 2.41-1.02 (m, 25H), 0.93 (d, J = 6.0 Hz, 3H), 0.80 (s, 3H), 0.69 (s, 3H). ¹³C NMR (CDCl₃, 100MHz) δ 174.7, 96.3, 95.9, 80.4, 74.8, 66.4, 56.0, 55.6, 51.5, 46.2, 46.1, 42.5, 40.2, 39.8, 35.7, 35.5, 33.4, 31.84, 31.80, 31.04, 30.96, 28.8, 27.6, 25.3, 23.3, 17.6, 12.5, 10.2 ppm; IR (KBr plate) 3470, 2928, 1732, 1447, 1143, 1095, 1039 cm⁻¹; MS (TOF, ES) m/z (relative intensity %) 245 (2), 288 (4), 413 (2), 533 (M+Na⁺, 100); HRMS (TOF, ES), Calcd. C₂₉H₅₀O₇ (M+Na⁺) 533.3454, found 533.3464.
Methyl 3α, 5α, 7α-7-methoxymethoxyl-cholan-24-oate (VIb) \(^1\)H NMR (CDCl\(_3\), 400MHz) \(\delta\) 4.69 (d, \(J = 6.8\) Hz, 1H), 4.61 (d, \(J = 6.8\) Hz, 1H), 4.04 (s, 1H), 3.66 (s, 3H), 3.61 (s, 1H), 3.38 (s, 3H), 2.37-1.03 (m, 27H), 0.92 (d, \(J = 6.4\) Hz, 3H), 0.78 (s, 3H), 0.64 (s, 3H). \(^{13}\)C NMR (CDCl\(_3\), 100MHz) \(\delta\) 174.7, 95.9, 75.1, 66.4, 55.62, 55.56, 51.4, 50.0, 46.0, 42.5, 39.6, 39.2, 36.0, 35.6, 35.3, 33.3, 31.87, 31.86, 31.0, 30.9, 28.8, 28.0, 23.6, 20.5, 18.2, 11.8, 10.2 ppm; IR (KBr plate) 3399, 2933, 2862, 2364, 1732, 1447, 1376, 1167, 1039 cm\(^{-1}\); MS (TOF, ES) m/z (relative intensity %)
Methyl 3α, 5α, 12α-12-methoxymethoxyl-cholan-24-oate (VIc)  

$^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 4.64 (d, $J = 6.8$ Hz, 1H), 4.59 (d, $J = 6.8$ Hz, 1H), 3.97 (t, $J = 2.6$ Hz, 1H), 3.70 (t, $J = 2.9$ Hz, 1H), 3.59 (s, 3H), 3.34 (s, 3H), 2.34-2.12 (m, 2H), 1.82-0.89 (m, 24H), 0.85 (d, $J = 6.0$ Hz, 3H), 0.70 (s, 3H), 0.63 (s, 3H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 174.7, 96.6, 81.0, 66.4, 56.0, 51.4, 48.6, 47.6, 46.2, 39.1, 35.9, 35.7, 35.52, 35.50, 32.0, 31.7, 31.1, 31.0, 28.9, 27.6, 25.5, 23.7, 12.6, 11.0 ppm; IR (KBr plate) 3465, 2933, 2367, 2253, 1740, 1447, 1371, 1257, 1146, 1105, 1044, 918, 733 cm$^{-1}$; MS (TOF, ES) m/z (relative intensity %) 245 (3), 371 (4), 473 (M+Na$^+$, 100); HRMS (TOF, ES). Calcd. C$_{25}$H$_{42}$O$_4$ (M+Na$^+$) 473.3243, found 473.3260.

General procedure for deprotection of MOM and methyl ester

A solution of VIa (510mg, 1.0mmol) in 30mL MeOH was stirred at room temperature while 10mL of 1N HCl solution was slowly added. The reaction mixture was refluxed for 2 hours until the starting material was consumed according to TLC. To the
cooled (room temperature) reaction mixture was added 10mL of 2N KOH solution in water. The reaction was then refluxed overnight (8h) and cooled to room temperature. The organic solvent was removed under vacuum and the aqueous solution was acidified with 1N HCl. White solid precipitated out and was filtered. Alternatively, for extremely fine precipitates, the suspension was centrifuged at 10,000 × g. The solid was washed with cold water and then collected and dried under vacuum. The product VIIa was obtained as a white solid (375mg, overall yield 92% over two steps).

3α, 5α, 7α, 12α-cholan-24-oic acid (VIIa, known compound) ¹H NMR (CD3OD, 400MHz) δ 3.97 (t, J = 3.0 Hz, 1H), 3.93 (s, 1H), 3.78 (d, J = 2.8 Hz, 1H), 2.37-2.11 (m, 3H), 1.96-1.29 (m, 24H), 1.14-1.09 (m, 1H), 1.01 (d, J = 6.4 Hz, 3H), 0.81 (s, 3H), 0.71 (s, 3H).
3α, 5α, 7α-cholan-24-oic acid (VIIb, known compound) $^1$H NMR (CD$_3$OD, 400MHz) δ 3.97 (t, $J = 2.6$ Hz, 1H), 3.77 (d, $J = 2.0$ Hz, 1H), 2.36-1.04 (m, 29H), 0.95 (d, $J = 6.4$ Hz, 3H), 0.81 (s, 3H), 0.69 (s, 3H).

3α, 5α, 12α-cholan-24-oic acid (VIIc, known compound) $^1$H NMR (CD$_3$OD, 400MHz) δ 3.96 (apparently triplet, $J = 2.8$ Hz, 2H), 2.38-2.17 (m, 2H), 1.82-1.19 (m, 27H), 1.01 (d, $J = 6.4$ Hz, 3H), 0.81 (s, 3H), 0.72 (s, 3H).
References


Chapter 3

Critical Micelle Concentration Measurement of Bile Salts

Using Isothermal Titration Calorimetry
Introduction

Small molecules are not typically thought of as evolutionary markers in vertebrates. Further, the chemical community has generally chosen not to address issues germane to the evolution of small molecules in biological systems, but has rather focused on either dogmatic DNA-based evolution or the design of self-replicating chemical systems. We would argue this to be unfortunate when considering how alkaloids, flavonoids, terpenoids, and steroids have successfully been used in plant biology to study taxonomy.\textsuperscript{1,2} Further, a comprehensive understanding of the role of small molecules and their unarguable importance in biological systems demands an understanding of their co-evolution with macromolecules. A possible reason for the bias in these studies could be linked to the fact that many of the most thoroughly studied biological small molecules (such as the co-enzymes) are ubiquitous across species, and there is little basis for devising evolutionary hypotheses. Another reason may be related to the dearth of examples of heterogeneous classes of small molecules with unified function.

Bile acids are ubiquitous small molecules across Animalia that facilitate digestion by acting as detergents in the lumen of the small intestine. Interestingly, the chemical composition of the bile acid pool varies significantly across species, and even within a species. This variance includes both perturbations to the steroidal core skeleton in addition to major variations in the hydroxylation patterns (see Figure 1 and the review\textsuperscript{3} for a comprehensive treatment of bile acid structures). Hofmann\textsuperscript{3} has put forward the hypothesis that the variable steroid hydroxylation patterns seen in bile acids represent an adaptation to the dehydroxylation processes carried out by intestinal flora. Inspired by these ideas we recently became interested in better understanding the evolution of the bile acid core skeleton.
The vast majority of bile acids in higher vertebrates have a $5\beta$-reduced steroidal skeleton (cis AB ring fusion).\(^3\) Interestingly, bile acids represent the only major $5\beta$-reduced steroids found in higher vertebrates. The allo bile acids are a subfamily of bile acids that are primarily demarcated by a $5\alpha$-reduced ring fusion (trans AB ring fusion). They occur widely in lower vertebrates, including various fish, birds and reptiles, but are also found sporadically in higher vertebrates (including humans).\(^4\) In one sense, allo bile acids can be thought of as evolutionary precursors to the $5\beta$-reduced bile acids (cis AB ring fusion) which comprise the vast majority of the bile acid pool in higher vertebrates and mammals. The significance of this in the context of evolution is especially intriguing and the reason why cis AB rings are chosen by nature can lie in expression of protein related to bile acids synthesis, transportation, or, more likely, their detergent properties. As part of our work on understanding the origins of this class of molecules, we became interested in seeking the evolutionary driving forces that yielded $5\beta$-reduced bile acids and started from evaluation of their detergent properties.

Although only one stereo center is perturbed when comparing $5\alpha$-reduced and $5\beta$-reduced steroids, the impact on 3-D structure is dramatic. As shown in Figure 1, the $5\alpha$-reduced ring fusion confers a planar steroidal scaffold (consequently why the allo bile acids are sometimes termed ‘flat’ bile acids) with clearly delineated hydrophobic and hydrophilic faces. In contrast, the $5\beta$-reduced ring fusion affords a bent scaffold with
slightly less defined hydrophilic and hydrophobic faces. A careful examination of the literature failed to answer the question of what, if any, evolutionary advantage the 5β-reduced scaffold holds over the 5α-reduced bile acids and why the 5β-reduced bile acids might be preferred for higher vertebrates. As a first step towards answering this question, we have conducted a careful thermodynamic analysis of the detergent properties of both sets of bile acids and compared the detergent properties of the allo bile acids to their 5β-reduced counterparts. In consideration of the structural complexity of bile acids and the micelles formed, Isothermal Titration Calorimetry (ITC) was chosen to evaluate the detergent properties. ITC can provide a set of data including Critical Micellar Concentration (CMC), along with a spectrum-like graph recording the enthalpy change, from which all thermodynamic data can be derived, during the process of micellization. The spectrometric visualization of this process, whose importance is often underestimated, and the thermodynamic data are crucial in evaluating a complicated system like this. As a result of this work, we propose that the evolution from a 5α-reduced to 5β-reduced scaffold affords molecules that possess properties that better resemble traditional amphipathic detergents.

Materials and methods

Reagents and materials

The allo-bile acids were prepared using a seven-step synthetic procedure. Briefly, 5β-reduced bile acids were unsaturated to the Δ4 enone using IBX and converted to the 5α-reduced bile acids via Birch reduction. 5β-reduced bile acids were purchased from Sigma-Aldrich and used without further purification.
Isothermal Titration Calorimetry (ITC)

Experiments to measure the enthalpy changes of demicellization and critical micellization concentration were conducted using a VP-ITC titration microcalorimeter (Microcal, Northampton, MA). 5µL x 50 aliquots of concentrated bile salts solution were injected into the sample cell at intervals of 3 min. The heat of each injection was plotted vs. bile salt concentration in the sample cell. The concentration of bile salt in the syringe was chosen such that the CMC could be reached in the sample cell at the approximate midpoint of each experiment. Each measurement was repeated three times at each temperature, 25 °C, 30 °C and 37 °C. Data analysis was carried out using ORIGIN version 8.1. An integration of the obtained ITC data gave the reaction enthalpy as a function of total bile acid concentration in the reaction cell. These data points were then fit to a Boltzmann equation using Origin’s sigmoidal non-linear least squares regression method. From this the value for \( \Delta H_{\text{demic}} \) (enthalpy of demicellization) was determined from the difference between the largest enthalpy at the beginning of injection and the prolonged asymptote of the curve at the end, and the value for the CMC was determined from the derivative of the curve to identify the inflection point.

Results

Critical Micelle Concentration (CMC) and enthalpy of demicellization

Our first step in addressing this complex question was to better understand the comparative detergent properties of 5α- vs. 5β-reduced bile acids. A standard for comparing detergents is the critical micellar concentration (CMC). In simplistic terms, CMC is a reflection of the quality of a detergent. At concentrations below the CMC,
detergents exist as monomers in solution. Above the CMC, detergents aggregate and facilitate the solvation of insoluble particles and hydrophobic molecules. Given the primary role of bile acids in the solvation and transportation of dietary fats and fat-soluble vitamins, a comparison of detergent properties, such as CMC and energetics of micellization, is a critical first step in understanding this evolution.

Bile acids, termed facial amphiphiles, have a hydrophilic face with multiple hydroxyl groups, and a hydrophobic face demarcated by the C-18, and C-19 methyl groups. Compared to classical amphiphilic detergents, such as sodium dodecyl sulfate (SDS), the formation of bile acid micelles is believed to be much more complex due to their unique self-assembly pattern(s). It is generally agreed that bile acid micelle formation involves multiple steps of varied assembly geometry, and varied aggregation numbers, all of which are concentration dependent. Further, the aggregation number is smaller and more ambiguous, resulting in a more gradual transition from monomer to micelle (as compared to SDS). The result of these properties is that on its own, CMC determination of bile acids is less informative than it might be for typical detergents. Consequently, we concluded that traditional methods for measuring CMC, such as maximum bubble pressure measurements or the hydrophobic dye solubilization method would be less informative and would not readily provide the information we would need to begin to understand the evolutionary advantages of 5α- vs. 5β-reduced bile acids.

CMC determination via isothermal titration calorimetry (ITC) is a relatively new method that has the advantage of not only providing CMC, but simultaneously measures the enthalpy associated with the micellization/demicellization (ΔH_{mic}/ΔH_{demic}) process. The method also provides an appreciation of whether the transition from monomer to micelle is abrupt or gradual, which can provide additional insights into detergent properties. During the experiment, a concentrated solution of bile salt (significantly above the predicted CMC) is prepared in an injection syringe and titrated over multiple
injections into the calorimeter cell filled with water or buffer. The micellized solution of bile acid will demicellize until the CMC is reached in the reaction cell. Other than CMC, the enthalpy of demicellization ($\Delta H_{\text{demic}}$) is directly observed as illustrated in Figures 2-4. The Gibbs free energy, entropy and heat capacity of demicellizaion can also be calculated as discussed below.

**Figure 2.** ITC titration of 50 injections of 5µl sodium cholate (left column) and sodium allo cholate (right column) at 37°C: (A, B) calorimetric plot of heat flow vs. time; (C, D) reaction enthalpy vs. total concentration in sample cell; (E, F) Plot of the first derivative of the non linear fit to curve C, D respectively.
In Figure 2, a comparison of sodium cholate to sodium allo cholate is illustrated (see Figures 3 and 4 for analogous comparisons of sodium allo chenodeoxycholate and sodium allo deoxycholate). Instead of using direct interpolated values as Blume et al. previously reported,\textsuperscript{14-16} the $\Delta H_{\text{demic}}$ is calculated after the data is fit to a Boltzmann sigmoid equation using Origin. The CMC is defined as the inflection point of the sigmoid fit to the integrated molar enthalpy, which can be best visualized by the first derivative plot (Panel E & F, Figure 2). The $\Delta H_{\text{demic}}$ is defined as the difference of the two horizontal asymptotes given by the fitted Boltzmann equation. The sigmoidal fitting has two advantages over the numerical interpolating method. First, the titration curve can be extrapolated mathematically to more accurately define the asymptotes, which consequently affords a more accurate $\Delta H_{\text{demic}}$ determination. Bile acid micellization is concentration dependent\textsuperscript{10} and titration with higher concentration of bile salts results in different titration curves, especially for allo-bile acids. As a result, the old way to determine $\Delta H_{\text{demic}}$ is not applicable. The data was partially fitted with the Boltzmann equation to make the high concentration end of the curve more accurate since extrapolation is needed only at this end. Furthermore, the fitting provides a more accurate determination of CMC by taking the first derivative of the best fit line, instead of interpolating from a first derivative plot, which avoids discontinuity of titration data. This is also necessary when the titration curve is gradual and numerical interpolating method become inaccurate because heat change is relatively small.
Figure 3. ITC titration of 50 injections of 5µl sodium chenodeoxycholate (left column) and sodium allo chenodeoxycholate (right column) at 37°C: (A, B) calorimetric plot of heat flow vs. time; (C, D) reaction enthalpy vs. total concentration in sample cell; (E, F) Plot of the first derivative of the non linear fit to curve C, D respectively.
Figure 4. ITC titration of 50 injections of 5µl sodium deoxycholate (left column) and sodium allo deoxycholate (right column) at 37°C: (A, B) calorimetric plot of heat flow vs. time; (C, D) reaction enthalpy vs. total concentration in sample cell; (E, F) Plot of the first derivative of the non linear fit to curve C, D respectively.
The entropy and Gibbs free energy of demicellization

Using the ITC data, two methods can be used to calculate the Gibbs free energy and the concomitant entropy of demicellization ($\Delta S_{\text{demic}}$): the mass action model or pseudophase separation model. The mass action model requires an accurate simulation or experimental determination of aggregation number, which is not well defined for bile acids given the complex stepwise models for aggregation that are best described by Small. This phenomenon can be partially visualized in both Figure 2 and Figure 3 where hints of a biphasic demicellization process can be seen when examining the initial injections of the titration. Further, depending on the bile acid composition and temperature at which titration data is collected, very complex multiphasic demicellization curves are possible. Because of the requisite determination of aggregation number in the mass action model, we chose to use the pseudophase separation model. In this model, the relationship of free energy and CMC is defined with Equation 1, where the effective micellar charge fraction, “$f$” can be simply defined as the fraction of counterions bound to the micelles. It can be approximated to be one in an ionic detergent comprised of weak acid salt with a bulky structure, which eliminates the repulsion of cations associated to acid anions, as is the case with bile acids.

$$\Delta G^\circ_{\text{demic}} = -(1 + f)RT \ln \text{CMC} \tag{1}$$

Consequently, the free energy can be calculated with the simplification in Equation 2.

$$\Delta G^\circ_{\text{demic}} = -2RT \ln \text{CMC} \tag{2}$$

Given that our analysis is isothermal, the entropy of demicellization can be calculated intuitively.

$$\Delta S^\circ_{\text{demic}} = (\Delta H^\circ_{\text{demic}} - \Delta G^\circ_{\text{demic}})/T \tag{3}$$
Our bile acid analysis focused on three most predominant hydroxylation patterns in bile acids: C-3, C-7, & C-12 (cholic acid); C3 & C7 (chenodeoxycholic acid); and C3 & C12 (deoxycholic acid) in both the 5β- and 5α-reduced forms. We chose three separate temperatures that corresponded to physiologically relevant temperatures across a wide variety of species (25, 30 and 37 ºC) and performed full thermodynamic analyses of the demicellization process (Table 1). In analyzing the differences between cholic acid and allo cholic acid, in addition to the analogous comparisons for chenodeoxycholic acid and deoxycholic acid, two trends emerge. First, the self assembly of the 5β-reduced bile acids is enthalpically more favorable by 0.9-2.7 kJ/mol depending on the temperature and bile acid structure. Interestingly, this does not necessarily translate to a CMC trend. Second, the transition from monomer to micelle is significantly broader for the allo bile acids. This can most effectively be seen by examining the first derivative plot of the demicellization process (Panel E & F, Figures 2, 3, & 4), and is most pronounced for the ubiquitous bile acid, chenodeoxycholic acid. This second point was counterintuitive when we considered the structural differences between the molecules. With the 5α-reduced scaffold, allo bile acids possess more highly demarcated hydrophobic and hydrophilic faces. Because of this, we predicted that the transition from monomer to micelle would be more abrupt as compared to their 5β-reduced counterparts. The opposite proved to be true, probably because allo bile acids can form more stable dimmers and further aggregation to micelles is therefore more energetically demanding. Bile acids with 5β-reduced scaffold afforded molecules behaved as more traditional detergents in completing the transition from monomers to micelles more swiftly. This is an important point, and may give clues to the evolutionary driving forces that afforded the 5β-reduced scaffold.
Table 1. CMC and thermodynamic values of bile acids demicellization ± standard error

<table>
<thead>
<tr>
<th>Compound</th>
<th>( T(\degree C) )</th>
<th>CMC ((\text{mM}))</th>
<th>( \Delta G_{\text{demic}}^{\circ} ) ((\text{kJ/mol}))</th>
<th>( \Delta H_{\text{demic}} ) ((\text{kJ/mol}))</th>
<th>( \Delta S_{\text{demic}} ) ((\text{J/mol*K}))</th>
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<tr>
<td>NaC</td>
<td>25.00</td>
<td>11.3±0.7</td>
<td>22.2±0.3</td>
<td>-0.96±0.1</td>
<td>-77.8±1.3</td>
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<td></td>
<td>30.00</td>
<td>19.3±1.2</td>
<td>19.6±0.3</td>
<td>0.58±0.03</td>
<td>-62.7±0.9</td>
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<td></td>
<td>37.00</td>
<td>19.7±0.3</td>
<td>19.5±0.1</td>
<td>1.50±0.05</td>
<td>-58.1±0.3</td>
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<tr>
<td>NaAC</td>
<td>25.00</td>
<td>13.7±1.1</td>
<td>21.3±0.4</td>
<td>-1.81±0.05</td>
<td>-77.4±1.5</td>
</tr>
<tr>
<td></td>
<td>30.00</td>
<td>13.8±0.5</td>
<td>21.2±0.2</td>
<td>-1.66±0.04</td>
<td>-75.6±0.7</td>
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<tr>
<td></td>
<td>37.00</td>
<td>14.1±0.9</td>
<td>21.1±0.3</td>
<td>0.44±0.07</td>
<td>-66.8±1.0</td>
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<tr>
<td>NaCDC</td>
<td>25.00</td>
<td>4.35±0.2</td>
<td>27.0±0.2</td>
<td>-1.34±0.04</td>
<td>-94.9±0.6</td>
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<td>30.00</td>
<td>7.72±0.3</td>
<td>24.1±0.2</td>
<td>0.98±0.04</td>
<td>-76.3±0.6</td>
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<tr>
<td></td>
<td>37.00</td>
<td>8.03±0.08</td>
<td>23.9±0.05</td>
<td>2.90±0.08</td>
<td>-67.8±0.2</td>
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<td>NaACDC</td>
<td>25.00</td>
<td>6.87±0.1</td>
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<td>-91.0±0.6</td>
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<td>30.00</td>
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<td>-85.0±0.5</td>
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<td>37.00</td>
<td>8.72±1.0</td>
<td>23.5±0.06</td>
<td>1.44±0.09</td>
<td>-74.2±1.6</td>
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<td>NaDC</td>
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<td>30.00*</td>
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<tr>
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<td>37.00</td>
<td>8.74±0.2</td>
<td>23.5±0.1</td>
<td>3.40±0.16</td>
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<td>NaADC</td>
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<td>7.62±0.1</td>
<td>24.2±0.08</td>
<td>-1.04±0.06</td>
<td>-84.6±0.4</td>
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<tr>
<td></td>
<td>30.00*</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>37.00</td>
<td>6.96±0.4</td>
<td>24.6±0.3</td>
<td>1.66±0.2</td>
<td>-74.3±0.7</td>
</tr>
</tbody>
</table>

* These temperature/bile acid conditions afforded multiphasic titrations that were not interpretable using the described model.

**Discussion**

As a test case, if we carefully compare the demicellization process of allo cholic acid to cholic acid and then subsequently compare these to SDS (see Paula et al.\(^5\) for a thermodynamic analysis of SDS) we find that the energetic aspects and phase transition profile of cholic acid more closely resembles that of SDS. For example the trend for enthalpy of demicellization at physiologic temperature is 0.44 kJ/mol; 1.50 kJ/mol; and 8.6 kJ/mol for allo cholic acid, cholic acid and SDS respectively. Further, when we carefully analyze the phase transition of cholic acid compared to allo cholic acid (Panel E & F, Figure 2) we see that the primary transition from monomer to micelle occurs from approximately 10 mM and is basically completed by 30 mM (affording a CMC of 19.7)
for cholic acid. This is contrasted to allo cholic acid, which begins the demicellization process at approximately 5 mM and is still in micelle monomer transition throughout the titration. These trends hold true for all the bile acids analyzed, and are most striking for the ubiquitous bile acid chenodeoxycholic acid. Taken together, the difference in enthalpy of micelle formation and the phase transition properties clearly points to the 5β-reduced bile acids possessing properties that more closely mirror traditional detergents like SDS. Interestingly, the properties discussed here do not correlate to a clear CMC trend, but based on our results, we would conclude that CMC is not as important as the phase transition properties and enthalpic components of self assembly in terms of evolutionary driving forces or even what makes a good detergent. In a nontraditional detergent system like bile acids, self assembly is much more complex and gradual than SDS and CMC is no longer the gold standard for detergent evaluation.

It is difficult to rationalize these observed trends, in terms of a model of micellization, in the absence of structural data of the micelles. However, given the clear trends we illustrate here, we predict there will be a shared structural underpinning of 5α-vs. 5β-reduced bile acids that is related to the evolutionary driving force that led to 5β-reduced bile acids. Obtaining this type of structural data is currently underway in our lab, and will be the subject of future publications. Regardless, this report sets a solid experimental foundation for answering a fundamentally interesting question about the evolution of a class of small molecules with new observations on the micellization properties of these ubiquitous biological detergents. Further, we have clearly illustrated the insight that is made possible by using an analytical technique that allows for a complete energetic analysis and a spectrum-like visualization of the the micellization process.
References


Chapter 4

Bile acid induced apoptosis in human colon cancer cell line HT-29
Introduction

As previously mentioned, impaired bile acid pool regulation can cause many problems including colon cancer, and cholestasis that leads to liver failure.\(^1\)-\(^2\) Many of these observations can be directly attributed to cytotoxicity, a phenomenon that is highly structure-dependent. For example, chenodeoxycholic acid (CDCA, Figure 1) is highly toxic towards hepatocyte cell lines while ursodeoxycholic acid (UDCA) is reported to be cytoprotective to liver cells and has been used to treat cholestatic liver diseases.\(^3\)-\(^5\) The most basic correlation between cellular toxicity of bile acids has been shown to be roughly proportional to their hydrophobicity.\(^6\)-\(^8\) This observation leads to the assumption that bile acid cytotoxicity is derived from direct detergent-like action that damages the cellular membranes, which subsequently causes necrosis. However, cytotoxicity of bile acids does not always correlate with hydrophobicities\(^9\) and their biologic concentrations are too low, even during cholestasis, to cause major necrosis from cellular membrane disruption. Actually, recent studies indicated that the cytotoxicity of hydrophobic bile acids at biologic concentrations is mainly due to induction of apoptosis instead of necrosis.\(^2\)

![Figure 1. Structural comparison of CDCA and UDCA](image)

Apoptosis is a programmed cell death and controlled self destruction. In contrast to necrosis, apoptotic cells can undergo death cleanly without damaging neighboring cells
and therefore normally being conferred as beneficial. However, it can also be detrimental if apoptosis does not occur in a carefully controlled fashion, as what happens in the liver during cholestasis.\textsuperscript{10-12} There are several hallmarks of apoptosis, including plasma membrane alteration, mitochondrial permeability transition, activation of caspases and DNA fragmentation.\textsuperscript{13} These hallmarks also define different phases of apoptosis and can all be monitored to discriminate apoptotic cells from healthy cell populations.

The induction of apoptosis by bile acids in hepatocytes has been well documented,\textsuperscript{2,14-18} while studies on colon cancer cell lines are rare. However, there is a large body of evidence for bile acids as carcinogens in colorectal cancer.\textsuperscript{19-22} Considering the high concentration of bile acids that colorectal tissues are exposed to, the investigation of bile acid induced apoptosis to colon cancer cells is as important as to hepatocytes. In addition, the colon is the environment where bacteria reside and secondary bile acids are produced. Comparing cytotoxicity of 5α and 5β bile acids in the colon system is especially promising since it can potentially answer why 5α bile acids are still needed rather than vanish in evolution. The colon cancer cell line, HT-29, was consequently chosen for the apoptosis assay.

**Materials and Methods**

**Cell Culture**

HT-29 human colon adenocarcinoma cells were purchased from American Type Culture Collection (Manassas, VA). Cells were seeded in flasks containing Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco, Invitrogen) supplemented with 10% heat inactivated fetal calf serum (FCS) and 1% penicillin at 37 °C in a humidified 5% CO\textsubscript{2} atmosphere. They were split and kept in similar conditions once 70% confluency is reached.
Pretreatment of apoptosis assay

Cells were seeded in 12-well plates with cover and grown till reaching 50-70% confluency. Then they were treated with culture solution containing specified concentrations of bile salts for the indicated time. DMEM solution was removed, carefully so as not to remove suspended cells, and each well was washed with PBS followed by Trypsin/EDTA treatment for 5 min to make cells detach. Suspended cells were transferred to 15 mL centrifuge tubes and trypsin/EDTA solution was removed after centrifuging at 200g for 5 min. The cells were resuspended in 0.5 mL of PBS buffer before being fixed by adding 4.5 mL of 70% (v/v with water) cold ethanol to the suspension. Cells can be stored in ethanol solution at -20 ºC for several weeks. The fixed cell suspension was centrifuged at 400g for 5 min and the ethanol supernatant was removed. The cells were washed with 5 mL of PBS and centrifuged at 400 g for another 5 min. The supernatant was removed and the cells were resuspended in 1 mL of propidium iodide (PI) solution. The resulting solution was incubated in the dark for at least 30 min at room temperature to ensure effective staining.23

Flow cytometry

The stained cells in suspension were filtered through meshes into sample tube to remove clogs formed during treatment with bile salts. Count of 10,000 cells for each sample were analyzed on Accuri (BD Biosciences, San Jose, CA) C6 Flow Cytometer. Quantitation of normal and apoptotic cells were performed using BD Accuri™ C6 software that was preinstalled on the flow cytometer.

Confocal Microscopy

HT-29 cells were incubated within a chambered dish (Lab-Tek II) containing 1.5 mL of DMEM media. After two hours, the cells were found attached to the chamber. Bile
salts dissolved in 0.5 mL of DMEM media were added to the dishes right before Leica (Leica Microsystems Inc, IL) TCS SP5 confocal microscope started recording the video with proper settings of resolution and  

**Results and Discussion**  

Two subfamilies of bile acids with different skeletal structures were tested for induction of apoptosis in colon cancer cell line HT-29 using flow cytometry. Propidium iodide stained DNA strands in healthy cells show two strong peaks in the diagraphs in Figure 2, representing the G1 and G2 peaks respectively. In late phase of apoptosis, DNA degradation causes a decrease of fluorescence intensity and the corresponding sub-G1 peaks to appear. The relative ratio of healthy cells versus the apoptotic cells can be calculated by comparing G1+G2 peaks vs. sub-G1 peaks.

*Figure 2.* Apoptosis assay by Flow Cytometry. HT-29 cells were treated with DMSO (Panel A, Control) and sodium allo deoxycholate in DMSO (Panel B) for 60 min and analyzed with flow cytometer after staining with propidium iodide. PI was exited at 488 nm and fluorescence intensity was measured to discriminate healthy cells against apoptotic cells with DNA degradation. Peaks on the right indicate healthy cells in G1 and
G2 phases. Peak on the left indicates apoptotic cells with lower fluorescence intensity caused by DNA degradation.

Figure 3 indicates that sodium cholate (NaC) has no significant induction of apoptosis even at 500 μM, which is much higher than any relevant biological concentration, even during cholestasis. However, sodium allo cholate (NaAC) started to show significant apoptosis induction at 250 μM at 60 min. At 500 μM, the DNA degradation occurred as early as 30 min.

**Figure 3.** Comparison of apoptosis induction by sodium cholate and sodium allo cholate using Flow Cytometry. Three concentrations for each bile salt with DMSO were tested at four time points. Each value is the average of two samples for every data point and error bars are as shown.
In the case of sodium chenodeoxycholate (NaCDC) and sodium allochenodeoxycholate (NaACDC), the results are opposite. In cells treated with 100 μM of NaCDC, DNA degradation started within 30 min, while no significant DNA degradation was observed for NaACDC in 2h at the same concentration. Treated with 500 μM of NaACDC, cellular DNA degradation started as early as 30 min, but the percentages of apoptotic cells were much lower than NaCDC treated cells at all time points checked. Among all the bile acids analyzed, CDCA is the most toxic in terms of apoptosis induction.

**Figure 4.** Comparison of apoptosis induction by sodium chenodeoxycholate and sodium allo chenodeoxycholate using Flow Cytometry. Three concentrations for each bile salt in DMSO were tested at four time points. Values are average of two samples for each data points and error bars are as shown.
Sodium deoxycholate (NaDC) and sodium allo deoxycholate (NaADC) are generally less toxic than chenodeoxycholate or allo chenodeoxycholate. NaADC is generally more effective in apoptosis induction than NaDC as moderate DNA degradation happened at concentration of 250 μM. Hardly any effect of NaDC was observed at the same concentration.

The overall conclusion is that NaCDC is most toxic, but NaAC and NaADC are more toxic than their 5β counterparts. To colon cancer cells, NaC and NaDC are superior to their 5α analogs while NaCDC is worse than its 5α analog. It should be pointed out that at high concentrations of bile salts tested, the cells tended to aggregate when trypsinized, presumably because of the morphological change of cells during apoptosis. Cells became irregular in shape as a result of morphological alterations and aggregated. These aggregates had to be removed by filtration to protect the flow tunnel of the cytometer from being blocked. But the removal of these clogs could potentially bias the results of our analysis since most of the cells removed were suffering from apoptosis. As a result, the percentages of apoptosis by bile salts at high concentrations obtained in this experiment were expected to be lower than the actual values. As shown in Figure 3-5, in some cases apoptosis percentages at 500 μM of bile salts treatment for 120 min are less than cells treated for 90 min or less. This is not accurate but resulted from cell aggregation.
**Figure 5.** Comparison of apoptosis induction by sodium deoxy cholate and sodium allo deoxy cholate using Flow Cytometry. Three concentrations for each bile salt in DMSO were tested at four time points. Values are average of two samples for each data points and error bars are as shown.

To study the morphological changes in early phase apoptosis induced by bile salts, more importantly, to verify the results of flow cytometry with PI staining, cells were observed visually by confocal laser scanning microscopy. They were seeded in chambered dishes (Lab-Tek II) and allowed to adhere by being incubated for two hours in standard conditions. A video was taken for two hours after bile salts solution in DMSO/DMEM media was slowly added. Pictures were captured at specific time points in the 2 hour period.
Early phase morphological alterations characterizing apoptosis were observed in cells treated with almost all bile salts in 30 min at a concentration as low as 50 μM. Figure 6 gives an example of this observation. When NaACDC or NaCDC in DMEM media were added, the majority of cells presented normally at time 0. After 30 min, approximately 25% of cells showed blebbing or shrinkage of cell membranes.

Figure 6. Morphological alterations during apoptosis induced by bile salts: (Panel A, B) microscopic images of cells treated with 50 uM NaACDC at time 0 and 30 min; (Panel C, D) images of cells treated with 50 uM NaCDC at time 0 and 30 min.
Based on the confocal microscopy observations, bile salts showed toxicity at a much lower concentration than flow cytometry data. The main reason is that DNA degradation, which is used as a standard to differentiate apoptotic cells from normal cells in flow cytometry, occurs at the late stage of apoptosis. But morphologic changes observed by microscopy occur at the early phase of apoptosis. Depends on cell types, the process of apoptosis can last as long as 12 hours. Therefore, measuring different events on the timeline of cell death from apoptosis at the same time will result in different outcomes, and the results from flow cytometry and confocal microscopy were expected to be different. The differences also proved that an observation of early phase apoptosis is critical for accurate evaluation bile acid cytotoxicity. Consequently, we started to explore methods to evaluate early phase apoptosis quantitatively.

Microscopy is capable of visualizing the process, but is not able to quantify the extent of toxicity. For quantification of apoptotic cells at early phases, we need to go back to flow cytometry. By switching the staining method, apoptotic cells at early phases can also be discriminated and subsequently counted by flow cytometry. The most common dye for early stage apoptosis assay is Annexin V. It binds to phosphatidylserine, which is kept at the cytosolic side of membrane in healthy cells. When cells undergo apoptosis, phosphatidylserine is translocated to the cell surface and can be bound by Annexin V from outside of live cells. Utilizing this feature of early apoptotic cells, Annexin V, labeled with fluororescent tag, such as fluorescein isothiocyanate (FITC), can be used to label apoptotic cells for flow cytometry analysis of apoptosis at early stage.

However, the importance of Annexin V assay is greatly impaired by another fact. That is, early phase morphological changes like blebbing or translocation of phosphatidylserine does not always result in cell death. Apoptosis can be reversed at an early stage and only the late phase of apoptosis indicates cell death. Some potential
toxic bile acids were reported to activate survival pathways and promote cell proliferation. To die or survive depends on the overall balance of the apoptosis and survival pathway. This has been well studied in the case of ursodeoxycholic acid,\textsuperscript{3-5, 27} and we had similar observations of cells recovery from early apoptosis.

When treated with sodium cholate, HT-29 cells showed obvious recovery after blebbing and shrinkage in two hour period (Figure 5). Blebbing of cells numbered as 1, 2, and 3 started in 20 min after sodium cholate was added (Panel B, Figure 5). Cell 3 was fully recovered after another 20 min while cells 1 and 2 were still struggling (Panel C), and it took them 20 min more to stop blebbing (Panel D). Cell number 4 started blebbing at 60 min time point (Panel D) and recovered after 30 min (Panel E).

![Figure 7. HT-29 cells recover from early phase of apoptosis induced by 200 μM of sodium cholate.](image)

As a result of this recovery mechanism, quantification of apoptotic cells at early phase using Annexin V assay is of only moderate significance. Either monitoring DNA degradation or translocation of phosphatidylserine, one method can only quantify apoptotic cells at one phase. However, those two phases are not necessarily correlated with each other because the early phase of apoptosis can be reversed.

Based on the results of flow cytometry experiments, 5-β bile acids are not always superior to 5-α bile acids. Only NaAC and NaADC are more toxic than their 5-β counterparts, while NaACDC is less toxic. Sodium chenodeoxy cholate, a primary bile salt in humans proved to be the most toxic of all bile salts tested. This suggests that cytotoxicity is not the reason that nature chose 5-β bile acids as predominant in high vertebrates.

In summary, 5α and 5β bile acid induced apoptosis of colon cancer cells was evaluated using two methods. Confocal microscopy is a method for observation of the early phase of apoptosis visually, while flow cytometry with DNA staining by PI quantitatively calculates the percentage of apoptotic cells at the late stage. Unfortunately, no general rule of bile acid cytotoxicity corresponding to the evolutionary trend can be found based on our apoptosis assays. As the final product of bile acid evolution, a primary bile acid, CDCA, was found to be most toxic. The early phase of apoptosis induced by NaC was found to be reversible, possibly through a similar cytoprotective mechanism with UDCA. This observation is important as it indicates that cholic acid can potentially be another lead in pharmaceutical research targeting the treatment of cholestasis. On the other hand, it also made our apoptosis study of bile acids more complicated. Unless a detailed mechanistic study on bile acid interplay during apoptosis is done, it is unlikely that any evolutionary insights can be found in this area.
References


Chapter 5

Isotopic labeling of bile acids and

new synthetic methodology for $\alpha, \beta$-unsaturation of carboxylic acids
Introduction

The ability of small molecules to penetrate cellular membranes is important in pharmaceutical research as it affects their availability in cells where they function. For bile acids, this ability can also affect the efficiency of its recycling via the enterohepatic circulation. Since bile acids that cannot be reabsorbed efficiently will be excreted through the colon, the composition of the bile acid pool will also be affected by different bile acids’ rates of crossing cellular membranes. Bile acids with different skeletons are expected to have different rates in crossing membranes and in this chapter, we present our progress on evaluating the abilities of $5\alpha$ and $5\beta$ bile acids to traverse cellular membranes.

As mentioned in the introductory chapter, our collaborator’s laboratory has developed a unique assay system to grow mono layers of cell membrane with bile acid transporters transfected in the appropriate cellular orientations. The objective in our lab is to label the bile acids with radioactive isotopes for the evaluation of the ability of various bile acids to traverse cellular membranes in our collaborator’s lab. We desired an approach that would be general across both $5\alpha$ and $5\beta$-reduced bile acids, could be performed in the final step before assay, and would be orthogonal to any functionality across the bile acid spectrum. Our strategy was to therefore develop methodology for the dehydrogenation of the bile acid side chain, which would be followed by catalytic tritiation as the last step.

In the process of synthesizing $\alpha, \beta$-unsaturated bile acids, we found that traditional methods, either through substitution-elimination (see Scheme 1) or Wittig reaction, are troublesome and unreliable on substrates with highly functionalized structures. Consequently, we set out to design a new method for $\alpha, \beta$-unsaturation of carboxylic acids optimized for complex molecules like bile acids.
Scheme 1. Traditional method of $\alpha$, $\beta$-unsaturation of carboxylic acids.

Oxidation with selenium dioxide was a prosperous area in the 1960’s because of its capability of achieving a variety of organic conversions, as shown in Table 1. While the versatility of selenium dioxide is highly advantageous synthetically, it is also a double-edged sword as it makes reactions hard to manipulate and results in low yields with multiple byproducts. Historically, this greatly limited its utility in organic syntheses. In this chapter, however, we argue that with a better understanding of the mechanism(s) involved, the chemoselectivity of this reaction could be improved.

Table 1. Oxidative conversions by selenium dioxide.

<table>
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<th>Substrate</th>
<th>Products that has been identified</th>
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<td>R$_1$</td>
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<tr>
<td>R$_1$</td>
<td>R$_1$ Y, OH Y</td>
</tr>
<tr>
<td>R$_1$</td>
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<tr>
<td>Ar</td>
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Using selenium dioxide to generate a carbon-carbon double bond adjacent to carbonyl group, which has been reported on steroid rings,\textsuperscript{6,7} can greatly improve the efficiency of $\alpha,\beta$-unsaturation of carbonyl compounds. Unfortunately, this reaction has not been reported for carboxylic acids, and only a trace amount of enone was observed on reactions with acyclic mono carbonyl compounds. Moderate yields could be obtained on cyclic ketones,\textsuperscript{8} presumably because the carbonyl group is more approachable than linear ketones. Since the conversion from ketones to carboxylic acids is well documented, the challenge to utilize this reaction in our methodology fell to how we could manipulate the reaction to work on acyclic ketones.

After a careful literature research, we found that generally accepted mechanisms inferred that the enol form of the ketone-enol equilibrium is likely the reactive species in reactions with selenium dioxide.\textsuperscript{9} By activating the carbonyl group in a way that favors the enol form, introduction of an electron withdrawing group on ketone would be hypothesized to facilitate the reaction. The trifluoromethyl ketone functionality was chosen for several reasons. First, the strong electron withdrawing effect of trifluoromethyl group affords ketones that favor the enol form. Second, the group can be readily converted to a carboxylic acid under very mild basic conditions. Finally, trifluoromethyl ketone can be furnished by reacting acid with trifluoroacetic anhydride using Reeves’ method in a straightforward manner.\textsuperscript{10}

**Results and discussion**

*Synthesis of $\alpha,\beta$-unsaturated bile acids*

Bile acids are a class of amphiphiles with multiple hydroxyl groups. Presumably, when treated with trifluoroacetic anhydride in presence of base, all the hydroxyl groups should also be protected with concomitant conversion of acid to trifluoromethyl ketone.
After selenium dioxide reaction, the enone product will be hydrolyzed under basic conditions and all trifluoromethyl esters should be removed as well as the trifluoromethyl ketone, leaving all hydroxyl groups unprotected. Our strategy is outlined in scheme 2.

As shown in Table 2, using Reeves’ method, all three bile acids reacted with excess of trifluoroacetic anhydride in the presence of pyridine produced trifluoromethyl ketone with all hydroxyl groups protected as anticipated. As we expected, dehydrogenation of these ketones with 1.5 equivalent of selenium dioxide went smoothly to provide enones in good yields. Several solvent combinations, including dioxane/water, ethanol, and isopropanol, were tried and only tert-butanol gave us the desired products.

Scheme 2. Strategy of α, β-unsaturated bile acids synthesis

The reaction mixture had to be refluxed for 3 days before the substrates were consumed completely, and less selenium dioxide resulted in incomplete conversion of trifluoromethyl ketone. Inconsistent with previous reports\textsuperscript{11-12} of similar reactions, the addition of acid had no significant accelerating effect on the reaction rate. α, β-unsaturated bile acids were obtained in good yields after being hydrolyzed in 0.2 M solution of KOH in MeOH/H\textsubscript{2}O.

Scope of selenium dioxide dehydrogenation
With all three bile acids successfully synthesized, we studied the scope of this methodology. As shown in Table 2, trifluoromethyl ketones with an aryl ring on the β position (Table 2, entry 4) work well to afford the enone in good yields and subsequent hydrolysis went smoothly. Electron withdrawing groups such as carbonyl group on β position accelerated the dehydrogenation reaction significantly (Table 2, entry 5). Reaction between selenium dioxide and 5,5,5-trifluoro-1-phenylpentane-1,4-dione (entry 5) was complete in 8 hours, which is 3 times faster than 1,1,1-trifluoro-4-phenylbutan-2-one (entry 4). Reaction of α-substituted ketones with selenium dioxide produced α-hydroxylated product rather than enone (entry 6). For compounds with γ-hydrogen the dehydrogenation by selenium dioxide did occur, but did not stop at the enone stage. Further oxidation at the γ-position introduced a hydroxyl group, which subsequently attacked the carbonyl group to generate a stable hemiketal (entry 7, 8). Two functional groups were furnished in cascade reactions and the product is also important as many metabolites of lipids in humans bearing a 4-hydroxy-2-ene functional group, such as 4-hydroxynonenal.\(^{13-15}\) It is noteworthy that only E isomers were observed in acyclic products. In order to cyclize into the hemiketal, the stereochemistry of the double bond had to be inverted. Isomerization of carbon-carbon double bond was driven by two forces. First, the strong electron withdrawing effect of the trifluoromethyl group favors enol form over the enone form. Second, the cyclic hemiketal is so stable that it cannot be converted back to ketone (Scheme 3).
Table 2. Yields for three steps of $\alpha, \beta$-unsaturation of carboxylic acids

$$\text{R} \cdots \text{COOH} \xrightarrow{\text{TFA/Py, TFA}} \text{R} \cdots \text{COF_3} \xrightarrow{\text{HNO/ACOH, H_2O}} \text{R} \cdots \text{COF_3} \xrightarrow{\text{1MNOH, MeOH-H_2O}} \text{R} \cdots \text{COOH}$$

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</table>

a. Another method$^{16}$ was used, see experimental section for detail.
b. Reaction mixture was stirred at 60 °C for 1h then heated to 100 °C for 48 h.
c. Reaction completed in 8 h.
Scheme 3. Tautomerization of trifluoromethyl enone

Actually, this hemiketal product is so stable with a five-member ring that strong acidic conditions were required to hydrolyze and no ring opening product was observed due to cyclization during the work-up. However, in the presence of selenium dioxide under acidic conditions, the product can be converted to 1,4 dione, which can be isolated (Scheme 4). The best yield on attempts to stop the cascade reaction at the enone stage by adding selenium dioxide in portions was approximately 35%.

Mechanistic study of selenium dioxide dehydrogenation

Scheme 4. Dehydrogenation of substrate with γ-hydrogen

The versatility of selenium dioxide also makes mechanistic studies more difficult. Since multiple reactions can occur on one substrate, it is hard to decipher whether they react via the same intermediate. To date, three mechanisms that have been
proposed for dehydrogenation: the direct $\beta$-deprotonation mechanism proposed by Speyer, Sharpless mechanism and Corey-Schaefer mechanism (Scheme 4). The first two mechanisms agreed that the enol form was the active species that reacts with selenium dioxide, while Corey et al. proposed the enolization was assisted by selenium dioxide.

Scheme 5. Mechanistic study of dehydrogenation by selenium dioxide

The fact that the 1, 4 diketone reacted faster than monoketone (entry 5 over entry 4 in Table 2) caused us to prefer the direct $\beta$-hydrogen transfer mechanism and we consequently designed an isotope labeling experiment to test the hypothesis. A $\beta$ deuterated fatty acid was prepared in a short synthesis described below, and converted to trifluoromethyl ketone to react with selenium dioxide. The reaction rate was monitored by NMR and compared with that of similar fatty acid without deuterium. We reasoned that if the reaction proceeded through direct $\beta$-hydrogen transfer, we would observe a first order isotope effect on the reaction rate.
As shown in scheme 5, 1-dodecyne was cross coupled with ethyl diazoacetate catalyzed by copper (I) iodide\(^\text{18}\) in excellent yield. Subsequently, the ester was deuterated with deuterium gas in the presence of 5% platinum on carbon. The deuterated ester was then hydrolyzed with potassium hydroxide solution in water before it was treated with trifluoroacetic anhydride to be converted to trifluoromethyl ketone.

**Scheme 6. Synthesis of deuterated trifluoromethyl myristyl ketone**

\[ \text{C}_{12}\text{H}_{25} + \text{N}_{2}\text{CHCO}_{2}\text{Et} \xrightarrow{\text{CuI/MeCN}} \text{C}_{12}\text{H}_{25} \text{CO}_{2}\text{Et} \]

The deuterated ketone was refluxed in tert-butanol with selenium dioxide and the reaction rate was monitored with NMR at 24h intervals to compare with that of the undeuterated compound. The reaction rate was calculated by the integral of the peaks of starting materials vs. that of the product (Figure 1). The triplet (asymmetric triplet for deuterated compound as shown in panel B) at approximately 2.8 ppm is the peak of \(\alpha\)-hydrogen of the substrates and 2 peaks at 5.9 ppm to 6.4 ppm stand for alkene hydrogens of the products. Unfortunately, no significant difference in reaction rates was observed. This suggested that the removal of \(\beta\)-hydrogen to form carbon-carbon double bond is not likely to be the rate-limiting step therefore excluded the \(\beta\)-hydrogen transfer mechanism.

The fact that 1, 4 diketone reacts faster than mono ketone is probably thermodynamically driven, as the longer conjugation system of the dienol intermediate, which is the proposed key intermediate in Corey mechanism, lowers the activation energy of the reaction.
The main disagreement between the other two mechanisms is on the reactive species that initiates the reaction with selenium dioxide. The Sharpless mechanism starts with a direct electrophilic attack of selenium dioxide to enol based on an indirect observation of carbon-selenium species, while Corey proposed that the enolization of the ketone was assisted by selenium dioxide as a Lewis acid (Scheme 5). The Corey mechanism seems more reasonable to us as no significant increase of the reaction rate was observed when acetic acid was added. In the Sharpless mechanism, electrophilic attack of selenium dioxide to the enol would be the rate limiting step and addition of acetic acid should accelerate the reaction because these conditions accelerate the enol-ketone tautomerization. To further verify this observation, 1.2 eq. of Ti(OiPr)$_4$, a strong
Lewis acid to coordinate with carbonyl group, was stirred with the trifluoromethyl ketone for 1 h before selenium dioxide was added, but no reaction occurred. This can serve as a substantial evidence that the reaction initiates with ketone instead of enol. In addition, they proposed a syn elimination of intermediate 3 (Scheme 5) to justify why the E isomer was the major product. However, in E2 reactions, syn eliminations only happen on substrates with rigid structures where the leaving group and the potential leaving hydrogen are held in cis position, therefore the possibility of an anti E2 elimination is excluded. Otherwise, E2 elimination always prefers anti elimination. Only E alkenes were obtained in our experiments while syn elimination is unlikely to happen on linear substrates, which is another arguement against the Sharpless mechanism. Further, this mechanism cannot explain the formation of α-hydroxylation product (entry 6, Table 2) under the same reaction conditions.

Corey et al. proposed enolization of ketone was assisted by selenium dioxide, which serves as a Lewis acid. The main argument against this mechanism was presented by Sharpless et al., who stated that selenium II ester (intermediate 5 in Scheme 4) would hydrolyze very fast to alcohol before proceeding to elimination.

After considering the three mechanisms from the literature above, we propose a new mechanism by combining selenium dioxide assisted enolization and β-deprotonation. As shown in Scheme 7, high valence selenium reacts with trifluoromethyl ketone to form the enol ester. The enol ester undergoes 1, 4-elimination directly instead of the nucleophilic attack of the carbon-carbon double bond to form selenium ester. Absence of an isotope effect would be rationalized by proposing that in this multi-step process, 1,4-elimination is not the rate limiting step. In the case of an α-substituted substrate, such as entry 6 in Table 2, 1,4-elimination is prohibited in the most energetically stable conformation since the selenium ester is trans to the β-hydrogens. The nucleophilic attack to α position occurs instead when 1,4-elimination is restricted.
Subsequent hydrolyzation of selenium II ester afford alcohol as product instead of enone. This explains the formation of $\alpha$-hydroxylation product.

**Scheme 7. Proposed mechanism of dehydrogenation by selenium dioxide**

![Scheme 7](image)

**Conclusion**

In summary, a three step method for the $\alpha$, $\beta$-unsaturation of carboxylic acids that features the shortest synthetic route and good substrate scope was developed. An undeveloped versatile reaction has been thoroughly reinvestigated and used as the key step of our methodology for $\alpha$, $\beta$-unsaturation of carboxylic acid. Careful thought was given to the introduction of a trifluoromethyl ketone, which made the utilization of this reaction possible. The intermediates, trifluoromethyl enone and $\gamma$-hydroxyl enone, are as important as the final products, especially in medicinal chemistry. With the electron withdrawing trifluoromethyl group, the enones are more reactive towards electrophiles to undergo 1,2-addition, 1,4-addition or Diels-Alder reaction\textsuperscript{20} to enrich the library of trifluoromethyl ketones, which are reported to be potent enzyme inhibitors.\textsuperscript{21-24} The mechanism of selenium dioxide dehydrogenation reaction was studied and a plausible mechanism was presented based on our investigation and previous research. The better understanding of the mechanism could lead to a renaissance of this reaction considering
its versatility in accomplishing multiple conversions. Research targeting at synthesis of biologically important compounds utilizing selenium dioxide chemistry is currently underway in our lab.
Experimental section

General. Unless otherwise stated, solvents and reagents were of commercially available analytical grade quality and used as received without any further purification. $^1$H and $^{13}$C-NMR spectra were recorded on a Varian Inova spectrometer (at the Department of Chemistry, Case Western Reserve University) operating at 400 MHz and 100 MHz for the $^1$H and $^{13}$C-NMR spectra, respectively. The internal references were TMS ($\delta$ 0.00) and CDCl$_3$ ($\delta$ 77.2) for $^1$H and $^{13}$C spectra, respectively. Only $^1$H NMR spectra are reported for known compounds, while IR, $^1$H NMR, $^{13}$C NMR, MS, HRMS and melting temperatures are reported for all new compounds. Coupling constants are reported in Hz and multiplicities are indicated as follows: s (singlet); d (doublet); t (triplet); m (multiplet).

Conversion of carboxylic acid to trifluoromethyl ketone

To a solution of carboxylic acid (10 mmol) in 50 mL of toluene at room temperature, 3.1 mL (22 mmol, 2.2 equiv) of trifluoroacetic anhydride (TFAA) was added. The resulting mixture was cooled to 0-5 °C before pyridine (2.0 mL, 25mmol, 2.5 equiv) was added slowly. The mixture was then heated to required temperature for indicated time in table 2. The reaction mixture was cooled to 0 °C and 20 mL of water was added slowly. The temperature was then brought up to 45 °C and maintained for 2 h. After cooling down, the aqueous phase of the mixture was separated and washed with ethyl acetate (20mL x 2). The combined organic phase was subsequently washed with water (20mL x 2) and brine (20mL x 1) and dried over anhydrous sodium sulfate. Removal of solvent in vacuum gave dark brown oil, which was further purification by flash chromatography to provide yellowish oil. For bile acid substrates, 1.0 more equiv of
TFAA and pyridine was added for each hydroxyl group present on the skeleton and the product solidifies to white solid over days.

Trifluoromethyl 3α, 7α, 12α-tri(trifluoroacetate)-5β-cholan-24-one (II-1) $^1$H NMR (CDCl$_3$, 400MHz) 5.34 (m, 1H), 5.17 (m,
1H), 4.77 (m, 1H), 2.78-2.59 (m, 2H), 2.16-1.13 (m, 22H), 1.00 (s, 3H), 0.84 (d, J = 6.4 Hz, 3H), 0.81 (s, 3H); 13C NMR (CDCl3, 100MHz) δ 191.6 (q, 2JF-C = 36 Hz), 156.6 (q, 2JF-C = 43 Hz), 156.5 (q, 2JF-C = 43 Hz, 2C), 115.5 (q, 1JF-C = 332 Hz), 114.5 (q, 1JF-C = 296 Hz), 114.44 (q, 1JF-C = 296 Hz), 114.40 (q, 1JF-C = 296 Hz), 79.8, 75.7, 47.3, 45.2, 42.5, 40.1, 37.7, 34.1, 34.0, 33.5, 33.0, 30.8, 28.2, 27.8, 26.9, 25.7, 24.9, 22.6, 22.0, 17.4, 11.8 ppm; IR (neat) 2951, 1778, 1650, 1221, 1166 cm\(^{-1}\); HRMS (TOF, ES), Calcd. C\(_{31}\)H\(_{36}\)F\(_{12}\)O\(_7\) (M + Na\(^+\)) 773.2167, found 773.2175.

Trifluoromethyl 3\(\alpha\), 12\(\alpha\)-di(trifluoroacetate)-5\(\beta\)-cholan-24-one (II-2) \(^1\)H NMR (CDCl3, 400MHz) δ 5.17 (m, 1H), 4.77 (m, 1H), 2.78-2.59 (m, 2H), 2.10-1.08 (m, 24H), 0.95 (s, 3H), 0.81 (d, J = 6.4 Hz, 3H), 0.79 (s, 3H). 13C NMR (CDCl3, 100MHz) δ 191.8 (q, 2JF-C = 36 Hz), 156.9 (q, 2JF-C = 41 Hz), 156.6 (q, 2JF-C = 41 Hz), 115.6 (q, 1JF-C = 294 Hz), 114.5 (q, 1JF-C = 297 Hz, 2C), 78.3, 76.7, 55.5, 49.9, 42.8, 40.4, 39.0, 37.8, 34.9, 34.5, 34.3, 34.0, 33.5, 33.3, 30.9, 28.2, 27.8, 26.0, 23.3, 22.3, 20.5, 18.0, 11.5 ppm; IR (KBr plate) 2944, 2875, 1779, 1220, 1167 cm\(^{-1}\); HRMS (TOF, ES), Calcd. C\(_{29}\)H\(_{37}\)F\(_9\)O\(_5\) (M +Na\(^{+}\)) 659.2395, found 659.2394.
Trifluoromethyl 3α, 7α-di(trifluoroacetate)-5β-cholan-24-one (II-3)  $^1$H NMR (CDCl$_3$, 400MHz) δ 5.32 (m, 1H), 4.90 (m, 1H), 2.76-2.58 (m, 2H), 1.98-1.08 (m, 24H), 0.95 (s, 3H), 0.81 (d, $J$ = 6.4 Hz, 3H), 0.79 (s, 3H).  $^{13}$C NMR (CDCl$_3$, 100MHz) δ 191.6 (q, $^2J_{F-C}$ = 34 Hz, 1C), 156.9 (q, $^2J_{F-C}$ = 43 Hz, 1C), 156.6 (q, $^2J_{F-C}$ = 42 Hz, 1C), 115.4 (q, $^1J_{F-C}$ = 312 Hz, 1C), 114.6 (q, $^1J_{F-C}$ = 284 Hz, 1C), 114.44 (q, $^1J_{F-C}$ = 286 Hz, 1C), 80.8, 78.6, 49.3, 47.4, 45.1, 41.6, 35.4, 34.2, 34.15, 33.8, 32.9, 31.4, 27.9, 27.1, 26.5, 25.6, 25.5, 25.4, 23.1, 22.6, 17.3, 12.1 ppm; IR (neat) 2951, 1778, 1161 cm$^{-1}$; C$_{29}$H$_{37}$F$_9$O$_5$ (M +Na$^+$) 659.2395, found 659.2394.

Conversion of 4-oxo-4-phenylbutanoic acid to trifluoromethyl ketone

To a solution of 4-oxo-4-phenylbutanoic acid (1.78 g, 10 mmol) in anhydrous dichloromethane (20 mL) was added oxalyl chloride (3.6 mL, 12 mmol) and 30μL of DMF. The reaction mixture was stirred at room temperature for 2 h. Solvent and excess oxalyl chloride were removed under reduced pressure. The residue was dissolved in dry
dichloromethane (30 mL). Trifluoroacetic anhydride (6 mL, 40 mmol) and pyridine (4 mL, 50 mmol) were added consecutively to this solution at -20ºC. The reaction mixture was kept at -20 ºC for another 4 h and the temperature was brought up to 0 ºC for another 0.5 h before water (10 mL) was added. The organic phase was separated and washed with water and brine. Removal of solvent under reduced pressure afforded the crude product in brown oil and further purification was done by chromatography to provide a brown solid.
5,5,5-trifluoro-1-phenylpentan-1,4-dione (II-5) 

$^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 7.61-7.59 (m, 2H), 7.42-7.38 (m, 3H), 5.34 (t, $J = 2.7$ Hz, 1H), 3.42 (t, $J = 2.7$ Hz, 2H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 175.9, 153.9, 129.6, 128.6, 124.7, 107.5, 98.32, 98.30, 97.6, 34.6 ppm;
1,1,1-trifluoro-3-methyl-4-phenylbutan-2-one (II-6, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 7.33-7.15 (m, 5H), 3.32-3.23 (m, 1H), 3.12 (dd, $J = 6.4$, 13.6 Hz, 1H), 2.66 (dd, $J = 8.0$, 13.6 Hz, 1H), 1.21 (d, $J = 6.4$ Hz, 3H).

1,1,1-trifluoropentadecan-2-one (II-7) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 2.71 (t, $J = 7.2$ Hz, 2H), 1.67 (m, 2H), 1.32-1.26 (m, 20H), 0.88 (t, $J = 6.8$ Hz, 3H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 191.5 (q, $^2J_{F-C} = 32.0$ Hz), 115.6 (q, $^1J_{F-C} = 291$ Hz, 1C), 36.3, 32.0, 31.9, 29.70, 29.69, 29.66, 29.59, 29.42, 29.40, 29.38, 29.23, 29.21, 28.8,
22.7, 22.4, 14.1 ppm; IR (neat) 2928, 2856, 1763, 1208, 1150 cm⁻¹ HRMS (FT-ICR), Calcd. C₁₅H₂₆F₃O (M-H⁻) 279.1941, found 279.1941.

1,1,1-trifluoro-5-phenylpentan-2-one (II-8, known compound) ¹H NMR (CDCl₃, 400MHz) δ 7.16-7.32 (m, 5H), 2.72-2.63 (m, 4H), 2.17-1.95 (m, 2H). NMR (CDCl₃, 100MHz) δ 191.3 (q, J_F-C = 38 Hz, 1C), 140.6, 128.5, 128.4, 126.3, 119.9, 116.9, 114.0, 36.4, 34.4, 23.8 ppm; HRMS (TOF, ES), Calcd. C₁₁H₁₁F₃O (M-H⁻) 215.0689, found 215.0689.

Dehydrogenation of trifluoromethyl ketone by selenium dioxide
The mixture of trifluoromethyl ketone (1.0 mmol) and selenium dioxide (1.5 mmol) in 20 mL of tert-butanol was refluxed (about 85 °C) for 3 days. Black precipitates appeared after several hours. After cooling down to room temperature, the solvent was removed under vacuum. The residue was resolved in dichloromethane and the resulting solution was filtered through a pad of celite to remove selenium species. Crude product was obtained by removal of the solvent and further purification was done by flash chromatography.

Trifluoromethyl 3α, 7α, 12α-tri(trifluoroacetate)-5β-cholan-22-en-24-one (III-1) \(^1\)H NMR (CDCl\(_3\), 400MHz) \(\delta\) 7.11 (dd, \(J = 15.9\)Hz, 8.6 Hz, 1H), 6.34 (d, \(J = 15.7\) Hz, 1H), 5.33 (t, \(J = 2.7\) Hz, 1H), 5.16 (m, 1H), 4.77 (m, 1H), 2.40 (m, 1H), 2.21-1.14 (m, 19H), 1.05 (d, \(J = 6.9\) Hz, 3H), 1.00 (s, 3H), 0.85 (s, 3H). \(^1\)C NMR (CDCl\(_3\), 100MHz) \(\delta\) 180.6 (q, \(^2J_{F-C} = 36\) Hz, 1C), 159.3, 157.0-156.2 (m, 3C), 119.7, 116.0 (q, \(^1J_{F-C} = 193\) Hz, 1C), 114.3 (q, \(^1J_{F-C} = 189\) Hz, 3C), 79.3, 77.5, 75.6, 46.4, 45.6, 42.4, 40.1, 39.6, 37.7, 34.1, 34.09, 33.5, 30.8, 28.4, 26.6, 25.8, 25.0, 22.7, 22.1, 18.3, 12.3 ppm; IR (neat) 2956, 2878, 1777, 1726, 1625, 1167 cm\(^{-1}\); HRMS (TOF, ES), Calcd. C\(_{31}\)H\(_{34}\)F\(_{12}\)O\(_7\) (M+Na\(^+\)) 769.2005, found 769.2006.
Trifluoromethyl 3α, 7α-di(trifluoroacetate)-5β-cholan-22-en-24-one (III-2)  

1H NMR (CDCl₃, 400MHz) 7.17 (dd, J = 16.3Hz, 9.8 Hz, 1H), 6.34 (d, J = 16.4 Hz, 1H), 5.11 (m, 1H), 4.81 (m, 1H), 2.40 (m, 1H), 2.14-1.56 (m, 15H), 1.41-1.26 (m, 6H), 1.15 (d, J = 6.9 Hz, 3H), 0.97 (s, 3H), 0.72 (s, 3H).  

13C NMR (CDCl₃, 100MHz) δ 180.1 (q, ²J₇-F-C = 34 Hz, 1C), 161.0, 157.0 (q, ²J₅-F-C = 42 Hz, 1C), 156.6 (q, ²J₅-F-C = 42 Hz, 1C), 119.4, 116.2 (q, ¹J₇-F-C = 289 Hz, 1C), 114.5 (q, ¹J₉-F-C = 285 Hz, 2C), 78.2, 76.6, 54.4, 49.7, 43.3, 40.6, 40.3, 38.9, 37.9, 34.62, 34.61, 34.4, 34.0, 33.5, 30.9, 27.7, 26.0, 23.4, 22.4, 20.5, 18.7, 11.9 ppm; IR (neat) 2946, 2876, 1778, 1625, 1220, 1165 cm⁻¹; HRMS (TOF, ES), Calcd. C₂₉H₃₅F₉O₄ (M+Na⁺) 657.2239, found 657.2304.
Trifluoromethyl 3α, 12α-di(trifluoroacetate)-5β-cholan-22-en-24-one (III-3) 1H NMR (CDCl₃, 400MHz) 7.12 (dd, \( J = 16.0 \)Hz, 9.2 Hz, 1H), 6.34 (d, \( J = 16.0 \) Hz, 1H), 5.32 (m, 1H), 4.91 (m, 1H), 2.40 (m, 1H), 1.99-1.09 (m, 21H), 1.02 (d, \( J = 6.4 \) Hz, 3H), 0.96 (s, 3H), 0.83 (s, 3H). 13C NMR (CDCl₃, 100MHz) \( \delta 179.9 \) (q, \( ^2J_{F-C} = 35 \) Hz, 1C), 160.0, 157.2-156.1 (m, 2C), 118.0-113.1 (m, 3C), 119.6, 80.4, 78.5, 49.0, 46.6, 45.5, 45.1, 41.5, 39.7, 35.4, 34.35, 34.32, 34.29, 34.25, 33.82, 33.81, 33.78, 31.4, 26.9, 26.4, 25.6, 25.49, 25.46,
23.1, 22.7, 18.2, 12.6 ppm; IR (neat) 2951, 1778, 1627, 1161 cm⁻¹; HRMS (TOF, ES), Calcd. C₂₉H₃₅F₉O₅ (M+Na⁺) 657.2239, found 657.2245.

1,1,1-trifluoro-4-phenylbut-3-en-2-one (III-4, known compound) ¹H NMR (CDCl₃, 400MHz) δ 7.98 (d, J = 16.0 Hz, 1H), 7.65 (m, 2H), 7.50-7.44 (m, 3H), 7.02 (d, J = 16.0 Hz, 1H).

1,1,1-trifluoro-3-hydroxy-3-methyl-4-phenylbutan-2-one (III-5) ¹H NMR (CDCl₃, 400MHz) δ 7.32-7.26 (m, 3H), 7.16-7.14 (m, 2H), 3.30 (d, J = 13.6 Hz, 1H), 2.96 (d, J = 13.6 Hz, 1H), 2.74 (broad, 1H), 1.56 (s, 3H).
(E)-5,5,5-trifluoro-1-phenylpent-2-ene-1,4-dione (III-5, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 8.04-8.00 (m, 3H), 7.67-7.63 (m, 1H), 7.58-7.51 (m, 2H), 6.91 (d, $J = 16$Hz, 1H). $^{13}$C NMR (CDCl$_3$, 100MHz) $\delta$ 189.2, 138.3, 136.3, 134.1, 131.4, 128.94, 128.90 ppm.
1,1,1-trifluoropentadec-3-en-2-one (III-9) 

$^1$H NMR (CDCl$_3$, 400MHz) δ 7.34 (dt, $J = 7.2$ Hz; 15.6Hz, 1H), 6.41 (d, $J = 15.6$Hz, 1H), 2.33 (m, 2H), 1.52 (m, 2H), 1.35-1.27 (m, 16H), 0.88 (t, $J = 6.8$ Hz, 3H). 

$^{13}$C NMR (CDCl$_3$, 100MHz) δ 179.8 (q, $^2J_{F-C} = 33.4$ Hz), 157.0, 121.3, 116.2 (q, $^1J_{F-C} = 289$ Hz, 1C), 33.3, 31.9, 29.6, 29.48, 29.46, 29.33, 29.31, 29.2, 27.6, 22.7, 14.1 ppm; IR (neat) 2927, 2855, 1727, 1628, 1203, 1148 cm$^{-1}$; HRMS (TOF, ES), Calcd. C$_{15}$H$_{25}$F$_3$O (M+Na$^+$) 301.1755, found 301.1747.
5-decyl-2-(trifluoromethyl)-2,5-dihydrofuran-2-ol (III-7) obtained as 2 pairs of diastereomers, and unable to separate. $^1$H NMR (CDCl$_3$, 400MHz) δ 6.34 (d, $J = 6.0$ Hz, 1H), 5.81 (m, 1H), 5.05 (m, 0.5 H), 4.93 (m, 0.5 H), 1.68-1.30 (m, 5H), 0.88 (t, $J = 6.4$ Hz, 3H). $^{13}$C NMR (CDCl$_3$, 100MHz) δ 179.8 (q, $^2J_{F-C} = 35$ Hz, 1C), 157.1, 121.3, 116.2 (q, $^1J_{F-C} = 290$ Hz, 1C), 33.3, 31.9, 29.6, 29.4, 29.32, 29.30, 29.1, 27.6, 22.6, 14.1 ppm; IR (neat) 3053, 2985, 2305, 1265 cm$^{-1}$; HRMS (TOF, ES), Calcd. C$_{11}$H$_6$F$_3$O$_2$ (M+Na$^+$) 317.1704, found 317.1730.
5-phenyl-2-(trifluoromethyl)-2,5-dihydrofuran-2-ol (III-8) obtained as 2 pairs of diastereomers, and unable to separate. $^1$H NMR (CDCl$_3$, 400MHz) δ 7.39-7.29 (m, 5H), 6.40-6.38 (m, 1 H), 6.04 (broad singlet, 0.5 H, first pair of diastereomer), 6.00-5.96 (m, 0.5 H, first pair of diastereomer), 5.96-5.94 (m, 0.5 H, second pair of diastereomer), 5.89-5.88 (m, 0.5 H, second pair of diastereomer), $^{13}$C NMR is very complex since it’s a mixture of two stereoisomers. IR (neat) 3426, 3059, 1266, 1183 cm$^{-1}$; HRMS (TOF, ES), Calcd. C$_{11}$H$_9$F$_3$O$_2$ (M+Na$^+$) 253.0447, found 253.0389.
1,1,1-trifluoropentadec-3-ene-2,5-dione (III-10) ¹H NMR (CDCl₃, 400MHz) δ 6.66 (d, J = 3.2 Hz, 1H), 6.04 (d, J = 3.0Hz, 1H), 2.64 (t, J = 7.6Hz, 2H), 1.65 (m, 2H), 1.32-1.26 (m, 14H), 0.88 (t, J = 7.2 Hz, 3H). ¹³C NMR (CDCl₃, 100MHz) δ 159.7, 140.2 (q, ²J_F-C = 42.0 Hz), 119.3 (q, ¹J_F-C = 264 Hz, 1C), 112.2 (q, ³J_F-C = 2.2 Hz, 1C), 105.7, 31.9, 29.6, 29.5, 29.32, 29.27, 29.1, 27.9, 27.6, 22.7, 14.1 ppm; IR (neat) 2923, 2856, 1708, 1615, 1559, 1463, 1381, 1175, 1159, 1130 cm⁻¹; HRMS (TOF, ES), Calcd. C₁₅H₂₃F₃O (M+Na⁺) 317.1704, found 317.1757.
$3\alpha, 7\alpha, 12\alpha$-cholan-22-en-24-oic acid (IV-1, known compound) $^1$H NMR (CD$_3$OD, 400MHz) $\delta$ 6.07-6.00 (m, 1H), 5.36 (dd, $J = 4.3, 15.4$ Hz, 1H), 3.94 (s, 1H), 3.80 (s, 1H), 3.41-3.35 (m, 1H), 2.37-2.15 (m, 2H), 2.02-1.07 (m, 22H), 1.00 (d, $J = 5.5$ Hz, 3H), 0.90 (s, 3H), 0.70 (s, 3H).

$3\alpha, 7\alpha$-cholan-22-en-24-oic acid (VI-2, known compound) $^1$H NMR (CD$_3$OD, 400MHz) $\delta$ 6.07-6.00 (m, 1H), 5.36 (dd, $J = 4.3, 15.4$ Hz, 1H), 3.80 (s, 1H), 3.41-3.35 (m, 1H), 2.37-2.15 (m, 2H), 2.02-1.07 (m, 23H), 1.00 (d, $J = 5.5$ Hz, 3H), 0.90 (s, 3H), 0.70 (s, 3H).

$3\alpha, 12\alpha$-cholan-22-en-24-oic acid (VI-3, known compound) $^1$H NMR (CD$_3$OD, 400MHz) $\delta$ 6.07-6.00 (m, 1H), 5.36 (dd, $J = 4.3, 15.4$ Hz, 1H), 3.94 (s, 1H), 3.41-3.35 (m, 1H), 2.37-2.15 (m, 2H), 2.02-1.07 (m, 23H), 1.00 (d, $J = 5.5$ Hz, 3H), 0.90 (s, 3H), 0.70 (s, 3H).

ethyl tetradec-3-ynoate (V, known compound) $^1$H NMR (CDCl$_3$, 400MHz) $\delta$ 4.19 (q, $J = 7.2$ Hz, 2H), 3.25 (t, $J = 2.4$ Hz, 2H), 2.19 (m, 2H), 1.49 (m, 2H), 1.39-1.26 (m, 17H), 0.88 (t, $J = 7.2$ Hz, 3H).
4,4,5,5-tetraduterium-1,1,1-trifluoropentadecan-2-one (VI)

\[ \text{\textsuperscript{1}H NMR (CDCl\textsubscript{3}, 400MHz) \ensuremath{\delta} 2.71 \text{ (asymmetric triplet, } J = 6.4 \text{ Hz, } 2\text{H}), 1.26 \text{ (apparent singlet, } 18\text{H}), 0.88 \text{ (t, } J = 6.8 \text{ Hz, } 3\text{H).}} \]

\[ \text{\textsuperscript{13}C NMR (CDCl\textsubscript{3}, 150MHz) \ensuremath{\delta} 191.6 \text{ (q, } J_{\text{F-C}} = 35 \text{ Hz), 115.0 \text{ (q, } J_{\text{F-C}} = 292 \text{ Hz, } 1\text{C), 36.3-36.2 \text{ (m, } 1\text{C),}} \]

\[ 31.9, 29.66, 29.63, 29.60, 29.36, 29.35, 29.32, 29.1-28.9 \text{ (m, } 1\text{C), 28.7-28.0 \text{ (m, } 1\text{C), 22.7, 22.4-21.7 \text{ (m, } 1\text{C), 14.1 ppm; IR (neat) 2928, 2856, 1763, 1208, 1150 cm\textsuperscript{-1}; \text{\textsuperscript{19}F NMR (CDCl\textsubscript{3}, 600MHz, trifluorotoluene as standard) \ensuremath{\delta} -79.9. HRMS (TOF, ES), Calcd. C\textsubscript{15}H\textsubscript{22}D\textsubscript{4}F\textsubscript{3}O \text{(M-H\textsuperscript{+})} 283.2192, found 283.2192.}} \]
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Chapter 6

Conclusions and future directions
Project goals

Bile acids are a class of ubiquitous small molecules aiding the digestion of lipids and fat soluble vitamins in vertebrates. From C27 bile alcohols, C27 bile acids to C24 bile acids, a clear trend of evolution in bile acid structures is present corresponding to the evolution of vertebrates itself.1 However, very little was known about the driving force behind bile acid evolution before this thesis project was begun. Among all the structural variations in bile acids, only one variation, cis versus trans A/B ring junction, is with respect to the skeleton. The overall goal of this project was to, from an evolutionary point of view, investigate into the significance of the bile acid skeleton with respect to bile acids function. We set out to compare two subfamilies of bile acids with different skeletons in several aspects of bile acids functions, including detergent properties, cytotoxicity and cross membrane ability, in hoping to find any evolutionary insights of bile acid in some of these areas.

Synthesis of allo bile acids

Our strategy for allo bile acid synthesis was via oxidation of the 3-hydroxy group to C-3 ketone, which was followed by dehydrogenation to produce an enone as the key intermediate. Birch reduction of the carbon-carbon double bond of the enone produces 5α-hydrogen-reduced product exclusively due to a favored axial protonation of the radical intermediate. While utilizing the classical strategy of inverting the stereochemistry of the α-carbon, our efforts were focused on eliminating extra steps and finding the most efficient route. The last report of an allo bile acid synthesis was approximately ten years ago and suffered from iterative protection/ deprotection, substitution/ elimination to furnish an enone intermediate for subsequent reduction. We optimized a new route whereby we were able to selectively oxidize the C-3 hydroxy
group without protecting other variant hydroxyl groups. Further, we were able to refine direct α-dehydrogenation of the C-3 ketone, providing a great opportunity to circumvent the traditional substitution/elimination method. Using these new methods, we developed the shortest route to invert the 5-carbon stereochemistry and produce allo bile acids from commercially available 5-β bile acids. As a result of the shortened route, the overall yields were significantly improved as well.  

**Evaluation of the detergent properties of bile acids**

As amphiphiles, aiding digestion by detergent action is the primary physiologic role of bile acid. Evaluation of a detergent consists of several types of measurements, including the measurement of the critical micelle concentration and all thermodynamic parameters involved in the process of micelle formation. Acquiring a complete set of data is especially important for evaluating a detergent like bile acid, because the formation of bile acid micelles is much more complex than that of a simple amphiphile with well-defined hydrophilic and hydrophobic domains. By measuring the enthalpic change during the whole process of demicellization using isothermal titration calorimetry, all parameters can be obtained in a single experiment.

Intuitively, allo bile acids should be a better detergent considering the flat scaffold favors the tight assemble of monomers. However, our experiments showed that the micellization of 5-β bile acids completed more rapidly and was enthalpically more favored. Both the transition pattern and enthalpy change of micellization suggested that 5-β bile acids are better detergents. It should be pointed out that those two observations are beyond the reach of traditional methods of CMC measurement and detergent property evaluation. Although the conclusion of our experiments is consistent with the evolutionary trend of bile acids, we are unable to explain this observation at this point.
without further structural information of the micelle. Very little is known about the micelle structure of bile acid or how they interact with dietary lipids. Both structural and thermodynamic studies of bile acid micelle formation are more accurate in the presence of dietary lipids because in physiologic environments, they always coexist and interact. As a preliminary test, we didn’t involve any dietary lipid in the titration, but in the next step of our investigation, typical dietary lipids will be tested. Comparison of the thermodynamic data with or without lipids will not only give insights on how they interact with each other, but also help solve the puzzle of micelle structure when combined with computational studies.

**Bile acid induced apoptosis in colon cancer cells**

Bile acid induced apoptosis has been found to be related to a number of cholestatic diseases and colorectal cancer. The large intestine, where primary bile acids are converted to secondary bile acids, is exposed to high concentration of bile acids, especially during digestion. Investigations into the significance of the bile acid skeleton with respect to colon-specific toxicity are promising for providing insight into the effect these molecules could have on the resident tissues where they are produced.

Apoptosis is highly structure-dependent and minor structural variations, such as the inversion of one stereo center, can possibly turn a cytotoxic bile acid into a cytoprotective one. With the purpose of elucidating the significance of bile acid skeletal conformation in apoptosis induction, our assay of 5α and 5β bile acids induced apoptosis consisted of two parts. First, we used confocal microscopy to monitor the early phase morphological alterations visually, while flow cytometry assisted by DNA staining measured the percentage of late phase apoptotic cells. Colon cancer cells HT-29 showed morphological alterations in 50uM of bile acids but DNA degradation was only observed
in concentration as high as 250uM. NaCDC, a primary bile acid in humans, was found to be the most toxic in terms of induction of apoptosis. This was beyond our expectations and cannot be explained by evolution trends of bile acids. Detailed investigation into the mechanism of bile acid induced apoptosis is needed before any structure-activity correlations can be proposed.

**Isotopic labeling of bile acid and methodology for α, β-unsaturation carboxylic acid**

Bile acids’ ability to cross cellular membrane is important because it affects the efficiency of bile acid recycling via the enterohepatic circulation and the availability of bile acid inside cells to functionsignal. If bile acids have different rates of penetrating cell membrane, their composition in the colon is not necessarily the same as compared with the composition in the liver. For the same reason, concentrations of bile acids inside cells that trigger the apoptosis process are not necessarily the same as concentrations that cells are exposed to. By radioactive labeling, bile acids can be traced and the rate of cell membrane penetration can therefore be monitored. Our collaborator’s laboratory has an assay system to grow mono layers of cell membrane mimicking the apical and basolateral membranes with bile acid transporters properly transfected. This taken together with our qualification in synthesizing radioactively labeled bile acids, abilities of bile acids with different skeletons to cross membrane can be evaluated. We started the study with the radioactive label of both subfamilies of bile acids.

Our synthetic route of radioactive labeling features α, β-unsaturation of the carboxylic acid side chain followed by catalytic tratiation. α, β-unsaturation of the bile acids was done in three steps, protection, dehydrogenation and deprotection. Dehydrogenation with selenium dioxide, known but underdeveloped chemistry with great versatility, was utilized by introducing the trifluoromethyl ketone as an electron
withdrawing group. Utilization of selenium dioxide has been greatly limited because its versatility caused difficulties in controlling the outcomes. We carefully studied the substrate scope and mechanism of this reaction after the unsaturation of bile acids and provided guidelines to improve the reactivity of substrate. After our systematic study, we expect this area of research to become revived again.

The more we understand the selenium dioxide chemistry, the more research areas we realize to be explored. In the future, research on utilizing this reaction will be focused on taking advantage of the versatility to make biologically important compounds. Introduction of multiple functional groups in single reaction, which is the hallmark of selenium dioxide chemistry, has always been a prosperous area. Another direction of selenium dioxide research is to develop more reactive selenium species to further broaden the substrate scope of this reaction to circumvent the necessity of the trifluoromethyl ketone as an electron withdrawing group. Based on our mechanistic study, the key factor for this reaction to work is the formation of enol assisted by selenium dioxide. Enolization of ketone can be favored by introducing electron withdrawing group on α position of ketone, as with the trifluoromethyl group. It can also be favored by increasing the electron deficiency of the lewis acid, selenium dioxide in this reaction. Stronger electron withdrawing group or element will increase the electron deficiency of selenium atom and thereby facilitate the enolization.

After α, β-unsaturated bile acids are synthesized, the subsequent catalytic tritiation to introduce radioactive tritium atoms will be performed and the products will be sent to Wake Forest University for membrane penetration analysis.
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Appendix

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