Microbial Cell Factories

REVIEW

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Synthesis of TUDCA from chicken bile: immobilized dual-enzymatic system for producing artificial bear bile substitute

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Abstract

Bear bile, a valuable animal-derived medicinal substance primarily composed of tauroursodeoxycholic acid (TUDCA), is widely distributed in the medicinal market across various countries due to its significant therapeutic potential. Given the extreme cruelty involved in bear bile extraction, researchers are focusing on developing synthetic bear bile powder as a more humane alternative. This review presents an industrially practical and environmentally friendly process for producing an artificial substitute for bear bile powder using inexpensive and readily available chicken bile powder through an immobilized 7α-,7β-HSDH dual-enzymatic syste. Current technology has facilitated the industrial production of TUDCA from Tauodeoxycholic acid (TCDCA) using chicken bile powder. The review begins by examining the chemical composition, structure, and properties of bear bile, followed by an outline of the pharmacological mechanisms and manufacturing methods of TUDCA, covering chemical synthesis and biotransformation methods, and a discussion on their respective advantages and disadvantages. Finally, the process of converting chicken bile powder into bear bile powder using an immobilized 7α-Hydroxysteroid Dehydrogenases(7α-HSDH) with 7β- Hydroxysteroid Dehydrogenases (7β-HSDH) dual-enzyme system is thoroughly explained. The main objective of this review is to propose a comprehensive strategy for the complete synthesis of artificial bear bile from chicken bile within a controlled laboratory setting.

Keywords Tauroursodeoxycholic acid, Artificial Bear Bile, 7α-Hydroxysteroid dehydrogenases, 7β- Hydroxysteroid dehydrogenases, Chicken bile powder, Bear bile powder

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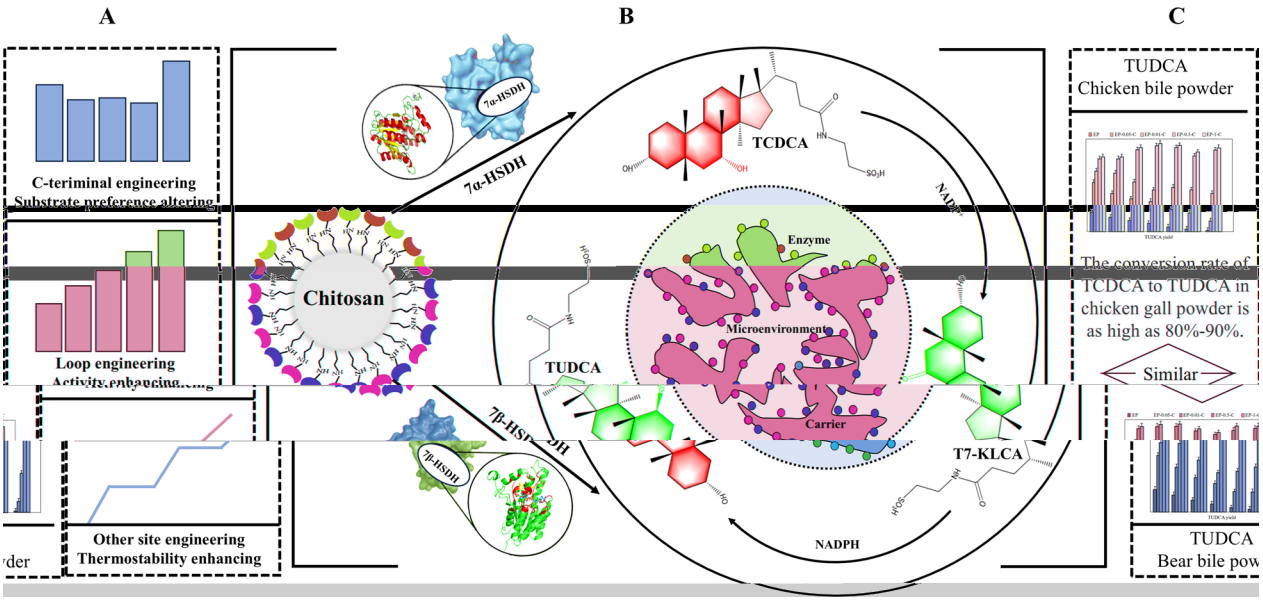
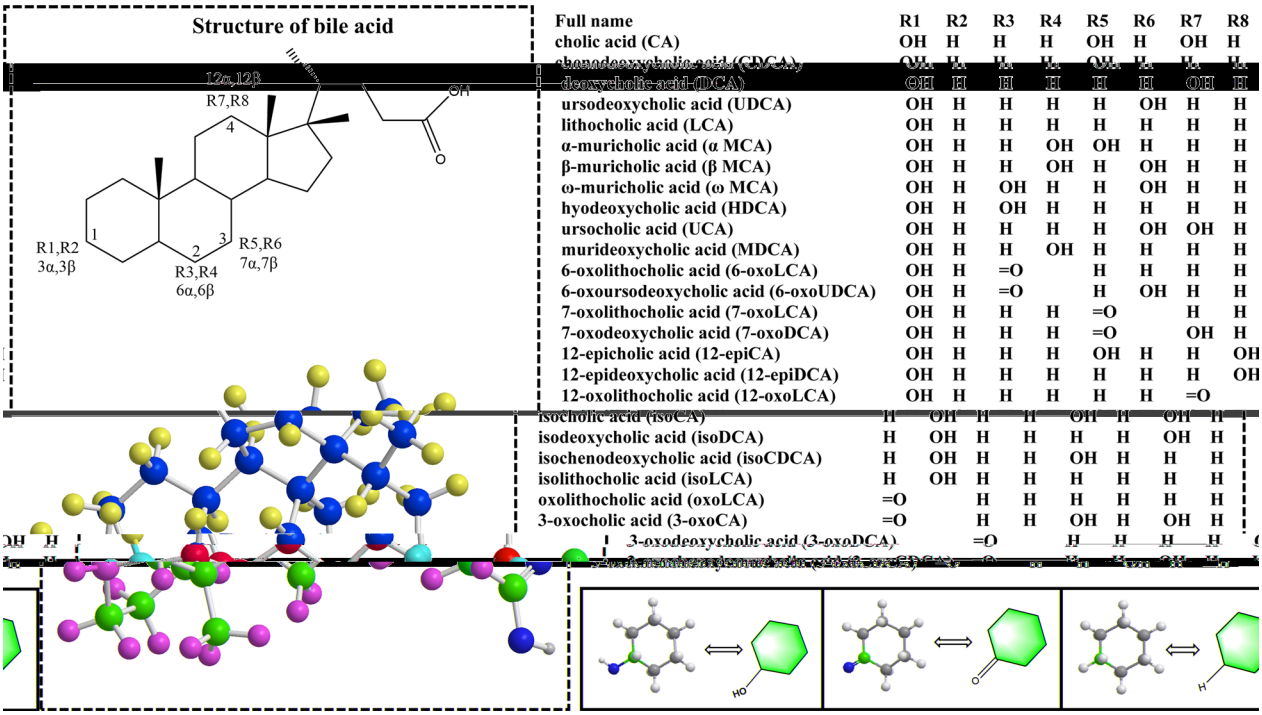
Graphical Abstract



Introduction

Animal bile has long been utilized to treat ailments in traditional Chinese medicine(TCM). Bear bile is the most well-known animal bile-based medication. It has a more than 1,000-year therapeutic history and is well-known in many nations [1–9]. Bear bile is the dried gallbladder bile of the Asiatic black bear (Selenarctos thibetanus), the brown bear (Ursus arctos) [10, 11] and other bear species, obtained from the Asiatic black bear by "live bile extraction" [6, 12].Although this cruel practice has stimulated a wave of condemnation across the world [6], bear bile, as an ingredient in over 100 traditional Chinese medicinal preparations, has not been effectively replaced by synthetic ursodeoxycholic acid (UDCA) [6], bile from other animals [7, 8] (such as cattle, chickens, and pigs), or herbs from TCM [8, 13, 14] (such as *Gardenia jasminoides*, *Scutellaria baicalensis*, *Coptis chinensis*, *Phellodendron amurense*, *Andrographis paniculata*, and *Rheum palmatum*).

Bile acids, which are synthesized from cholesterol in the liver [15, 16], initially yield chenodeoxycholic acid (CDCA) and cholic acid (CA) (Fig. 1). Following this, bile acids undergo conjugation with glycine or taurine before being stored and concentrated in the gallbladder. These bile acids, synthesized in the liver, are referred to as primary bile acids. Following a meal, bile acids are released into the duodenum to aid in the digestion of dietary fats and oils as well as facilitating the absorption of lipid-soluble vitamins [17–20]. In the ileum, conjugated bile acids are reabsorbed and transported via the portal blood back to the liver, a process known as enterohepatic circulation (Fig. 2). This process conserves over 95% of the bile acid pool [15]. After being synthesized in the liver, bile acids are secreted into the capillary bile ducts via the bile-salt export pump (BSEP) located in hepatocytes [21–23]. Simultaneously, bile acids combine with organic anions, organic cations, and reduced glutathione to form divalent anions. These substances, along with cholesterol and phospholipids, are then transported into the bile ducts by multidrug resistance related protein 2 (MRP2) and multidrug resistance transporter1 (MDRT1), also known as MDR1 [24, 25]. Transporters located on the basolateral side of the hepatocyte basement membrane act as a pathway for the release of bilirubin and bile acids in cases of cholestasis. The primary basolateral transport systems include MRP3 and MRP4 from the multidrug resistance related protein (MRP) family, as well as organic anion transporting peptide 2 (OATP2) and organic solute transporting peptide (OSTP) also known as organic solute transporters alpha/beta (OSTα/β) [24, 26]. These transporters facilitate the movement of bile acids and other organic anions into the systemic circulation. In a healthy state, bile is stored in the gallbladder and released into the intestinal lumen post-meals [27]. It is then reabsorbed at the end of the ileum through the apical sodium-dependent bile acid transporter (ASBT) and transported to the portal vein via the basolateral



Full name	R1	R2	R3	R4	R5	R6	R7	R8
cholic acid (CA)	OH	H	H	H	OH	H	OH	H
chenodeoxycholic acid (CDCA)	OH	H	H	H	OH	H	H	H
deoxycholic acid (DCA)	OH	H	H	H	H	H	OH	H
ursodeoxycholic acid (UDCA)	OH	H	H	H	H	OH	H	H
lithocholic acid (LCA)	OH	H	H	H	H	H	H	H
α-muricholic acid (α MCA)	OH	H	H	OH	OH	H	H	H
β-muricholic acid (β MCA)	OH	H	H	OH	H	OH	H	H
ω-muricholic acid (ω MCA)	OH	H	OH	H	H	OH	H	H
hyodeoxycholic acid (HDCA)	OH	H	OH	H	H	H	H	H
ursocholic acid (UCA)	OH	H	H	H	H	OH	OH	H
murideoxycholic acid (MDCA)	OH	H	H	OH	H	H	H	H
6-oxolithocholic acid (6-oxoLCA)	OH	H	=O		H	H	H	H
6-oxoursodeoxycholic acid (6-oxoUDCA)	OH	H	=O		H	OH	H	H
7-oxolithocholic acid (7-oxoLCA)	OH	H	H	H	=O		H	H
7-oxodeoxycholic acid (7-oxoDCA)	OH	H	H	H	=O		OH	H
12-epicholic acid (12-epiCA)	OH	H	H	H	OH	H	H	OH
12-epideoxycholic acid (12-epiDCA)	OH	H	H	H	H	H	H	OH
12-oxolithocholic acid (12-oxoLCA)	OH	H	H	H	H	H	=O	
isocholic acid (isoCA)	H	OH	H	H	OH	H	OH	H
isodeoxycholic acid (isoDCA)	H	OH	H	H	H	H	OH	H
isochenodeoxycholic acid (isoCDCA)	H	OH	H	H	OH	H	H	H
isolithocholic acid (isoLCA)	H	OH	H	H	H	H	H	H
oxolithocholic acid (oxoLCA)	=O		H	H	H	H	H	H
3-oxocholic acid (3-oxoCA)	=O		H	H	OH	H	OH	H
3-oxodeoxycholic acid (3-oxoDCA)			=O		H	H	H	H

Fig. 1 Structure of bile acid.The basic structure of bile acids mainly consists of a steroid core and a five-carbon side chain. The steroid core is composed of three six-carbon rings and one five-carbon ring. When R1 to R8 are combined with different chemical groups, different types of bile acids are formed. For example, UDCA is formed by the isomerization of the seven-position hydroxyl group of CDCA, and different bile acids have different functions and biological activities

OSTα/β and MRP3 [28–30]. Intracellularly, ileal bile acid binding protein (IBABP) helps transport bile acids to the basement membrane side and reduces their toxicity to ileal cells. MRP2 is located in the luminal membrane of small intestinal cells and is responsible for re-secreting bile acids into the intestine lumen. Once in the portal vein, hepatocytes reabsorb the bile acids through the Na^+-dependent taurocholic cotransporting polypeptide (NTCP) and organic anion transport polypeptide 1 (OATP1) [31, 32], before being transported to the luminal membrane of the small intestine. OATP1 is also involved in the re-uptake of bile acids into hepatocytes.

Daily, approximately 70–82% of bile acids in the bile acid pool are reabsorbed via ileal active transport [32–34]. The presence of more hydroxyl groups in bile acids enhances transport speed, with trihydroxy bile acids showing a 6 to 8 times higher uptake rate than monohydroxy bile acids [35, 36]. Conjugated bile acids are transported 4–6 times faster than non-conjugated bile acids, and T-bound bile acids are transported more swiftly than G-bound bile acids [37]. Additionally, there is competitive inhibition among different types of bile acids during the transport process.

Passive transport of bile acids is influenced by intestinal pH and the dissociation coefficient of bile acids. Free bile acids are reabsorbed passively at a faster rate than G-bound bile acids, while ionic T-bound bile acids are primarily reabsorbed through active transport. Moreover, the presence of a higher number of hydroxyl groups leads to lower membrane permeation efficiency [38]; the arrangement of hydroxyl groups also impacts passive reabsorption, with 7α hydroxycholic acid being more readily reabsorbed passively than 7β hydroxycholic acid [39, 40]. The length of the side chain does not affect passive reabsorption.

UDCA has a long history of being used in the treatment of liver disease, with its origins dating back to the Tang Dynasty in China. It was not until 1927 that a Japanese scientist successfully isolated pure UDCA crystals from bear bile and officially named it UDCA(urso, derived from the Latin word for 'bear') [41, 42]. CDCA is the precursor of UDCA. Due to its low hepatotoxicity, Western countries have recognized its medicinal value and started producing it in large quantities. In the late 1980s, with the emergence of laparoscopic cholecystectomy, the use of UDCA gradually declined [43, 44]. However, its medicinal value was rediscovered when it was found to improve biochemical parameters in primary biliary cholangitis and slow down disease progression. Subsequently, UDCA was certified by the U.S. Food and

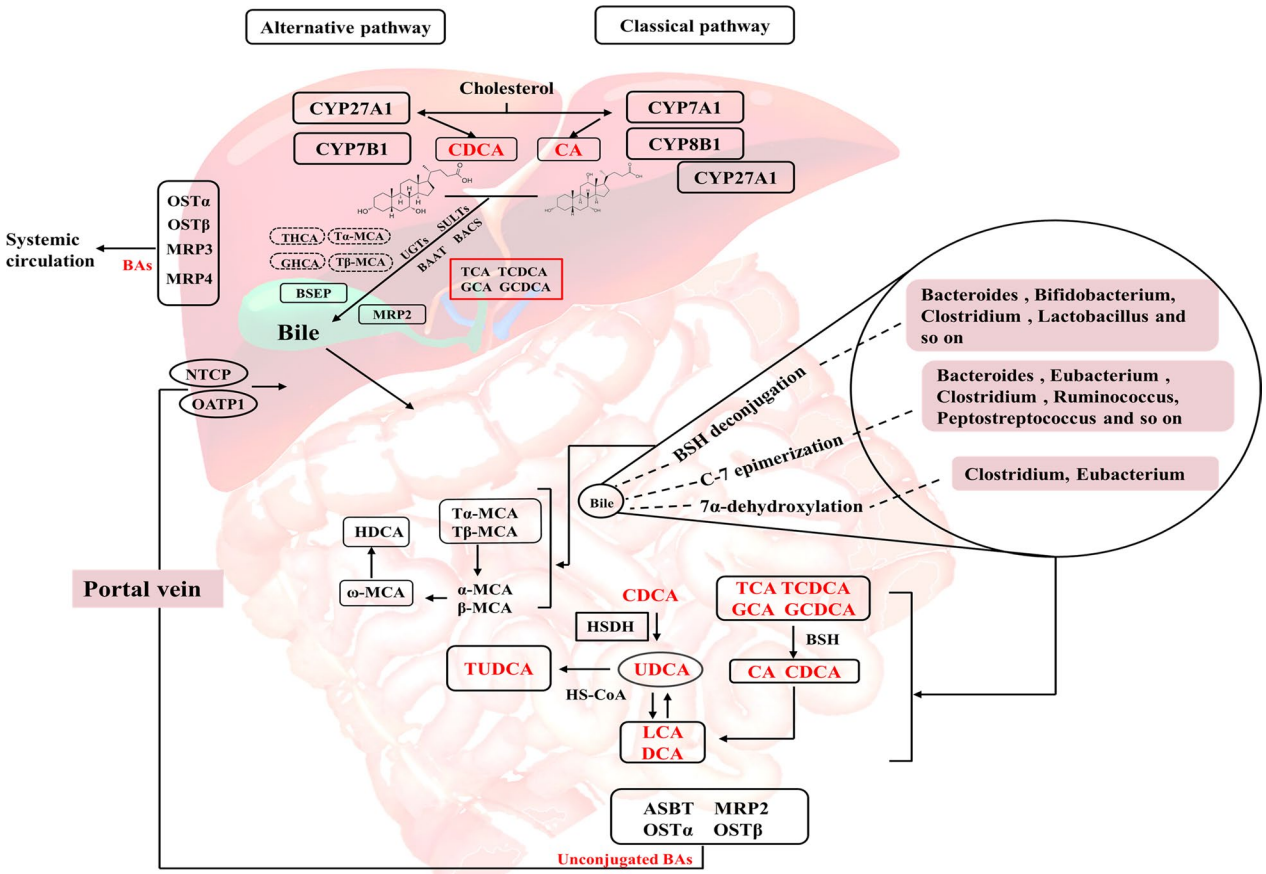


Fig. 2 Bile acid biosynthesis, transport and metabolism.The synthesis pathways of bile acids involve both the classical pathway and the alternative pathway, with key enzymes such as CYP7A1 and CYP8B1. Once bile acid is produced, it is transported to the capillary bile ducts via bile salt export pumps and further moved with the assistance of various drugs. Bile acids are stored in the gallbladder, reabsorbed through ASBT, and then transported back to the portal vein via OSTα/β and MRP3 in the terminal ileum. In the enterohepatic circulation, IBABP and MRP2 play crucial roles. The synthesis and recycling of bile acids is a complex process involving multiple enzymes and transporters, which is essential for maintaining bile acid balance and bile formation in the body

Drug Administration (FDA) as a first-line drug for treating PBC [45].

TUDCA is a unique natural bile acid amide that is produced in the body by combining UDCA and taurine. It stands out from UDCA due to the additional taurine group, which increases its polarity. TUDCA exhibits a faster dissolution rate and higher solubility compared to UDCA. Research indicates that TUDCA is not only safer but also more effective in treating various diseases [46, 47].This compound has shown promise in various pharmaceutical applications, including its positive impact on hepatobiliary diseases, its potential for enhancing treatment outcomes in Alzheimer's and Parkinson's diseases [2, 4, 48, 49], as well as its ability to prevent diseases associated with apoptosis. TCDCA is the precursor of TUDCA, and the two form an isomer pair. Importantly, bear bile contains a relatively high content of TUDCA (23–40%), while the content of TUDCA in other animals' bile is very low (< 1%) [50–52]. Interestingly, the content of TCDCA in the bile of poultry such as chicken, ducks, and geese is higher than 45% [53–55], while the content of their other components is very similar to that of bear bile. It is well-known that in vivo TCDCA is converted into TUDCA through a five-step process for the enterohepatic circulation of bile acid [56–63] that involves the deconjugation of TCDCA by bile salt hydrolase (BSH), the 7α- or 7β-dehydroxylation of CDCA, the ligation of UDCA and coenzyme A (CoA) thioesters by bile acid CoA ligase (BAL), and the formation of TUDCA by the catalysis of bile acid CoA:amino acid N-acyltransferases (BAT). In the past, TUDCA was prepared by chemical synthesis, and compared to chemical epimerization, biotransformation of TUDCA from TCDCA is a mild and environmentally friendly process [64–67].

This review article provides a comprehensive overview of the latest research progress on the discovery, pharmacological mechanisms and biosynthesis of UDCA and TUDCA. The focus was on the biosynthetic pathway of TUDCA prepared from chicken bile powder by a two-step enzymatic method of co-immobilizing 7α-HSDH and 7β-HSDH with chitosan. Initially, a unique dual enzyme coupling system was established to produce artificial bear bile from chicken bile, with the goal of preserving and utilizing scarce medicinal resources within the context of the enterohepatic cycle of bile acids. Subsequently, the emergence of artificial bear bile powder as a potential substitute for natural bear bile powder using readily available materials has become a prominent area of interest in current research and development. Furthermore, this study involved the identification and characterization of several 7α- and 7β-hydroxysteroid dehydrogenases (7α- and 7β-HSDH) responsible for TUDCA biosynthesis, achieved through the construction of the genome of intestinal microbial elements from black bears. The structural elucidation of 7α- and 7β-HSDH enzymes using X-ray crystallography and molecular modification techniques facilitated the discovery of high enzyme activity mutants. Moreover, the development of immobilized 7α- and 7β-HSDH enzymes using chitosan-modified epoxy resin as a carrier enhanced their thermal and cyclic stability. TCDCA was successfully converted into TUDCA from chicken bile, and industrial production has been achieved (*Shanghai Kaibao Pharmaceutical Co and Chongqing Jize Biotechnology Co*), laying the foundation for the potential development of bile powder alternative drugs [68–70].

Advances in bile acid research: mechanisms and pharmacological effects of UDCA

Bile acids are crucial for the digestion and absorption of lipids and also play a role in modulating various physiological functions in the body. UDCA, a particular bile acid known for its distinctive characteristics, has garnered considerable interest in medical research. This review highlights the latest developments in bile acid studies, particularly emphasizing the mechanisms and therapeutic effects of ursodeoxycholic acid (UDCA). UDCA has been recognized as an important therapeutic compound exhibiting various actions across several disorders. Grasping its mechanisms of action and pharmacological properties is vital for enhancing its clinical use.

Advances in bile acid research and pharmaceutical applications

Bile acids were first isolated in 1838, but their precise chemical structures were not elucidated until almost a century later [68]. The synthesis of cortisone by *H. Sarett's* [71–73] using deoxycholic acid (DCA) and the observation by *rheumatologist Philip Hench* [74–76] that cortisone alleviated symptoms of rheumatoid arthritis played pivotal roles in advancing bile acid research. Subsequent discovery that fungi could synthesize significant quantities of cortisone from plant saponins led to a decline in DCA-related studies [77–79]. Despite fluctuations in interest over time, bile acid research remains crucial in the pharmaceutical field. Ongoing scientific investigations continue to focus on bile acids, with the potential to pave the way for novel avenues in drug development.

Research on bile acids has seen a resurgence with advancements in chromatography and mass spectrometry technology [80–84]. A study revealed that CDCA effectively reduces cholesterol saturation and dissolves gallstones, sparking renewed interest in bile acids. Initially, UDCA was favored over CDCA for its ability to dissolve cholesterol stones, but the rise of laparoscopic cholecystectomy led to UDCA's decline [85]. However, UDCA's therapeutic potential was later recognized in improving biochemical indicators of primary biliary cholangitis [86–88] and slowing disease progression [89], establishing its enduring medicinal value.

The role of bile acids in metabolic regulation: FXR and TGR5 signaling

In recent years, research on bile acids has expanded, uncovering their role as signaling molecules that interact with the nuclear receptor farnesoid X receptor (FXR) and the membrane receptor TGR5 [90, 91]. These molecules not only regulate homeostasis but also impact various physiological activities, including sugar, fat, and energy metabolism. The mechanisms involved include blocking NTCP to prevent bile acid uptake into hepatocytes, protecting hepatocytes from bile acid-induced ER stress and mitochondrial damage, and creating cytokine receptor inhibitors to hinder neutrophil chemotaxis (Fig. 3). Obeticholic acid [91, 92], a derivative of CDCA and an FXR agonist, has been shown to significantly improve blood sugar, serum triglyceride, and total cholesterol levels [93]. Studies indicate that it reduces bile acid pools by inhibiting CYP7A1, leading to a decrease in intracellular bile acid levels and a reduced risk of triggering an inflammatory response. Additionally, modified versions of FGF19 and all-trans retinoic acid seem to play a role in inhibiting CYP7A1 function [92, 94].

Traditional Chinese medicine claims that bear bile can clear heat, promote choleretics, calm the liver, and detoxify, among other effects, and may have a beneficial impact on liver protection and vision enhancement. Research indicates that UDCA, when combined with

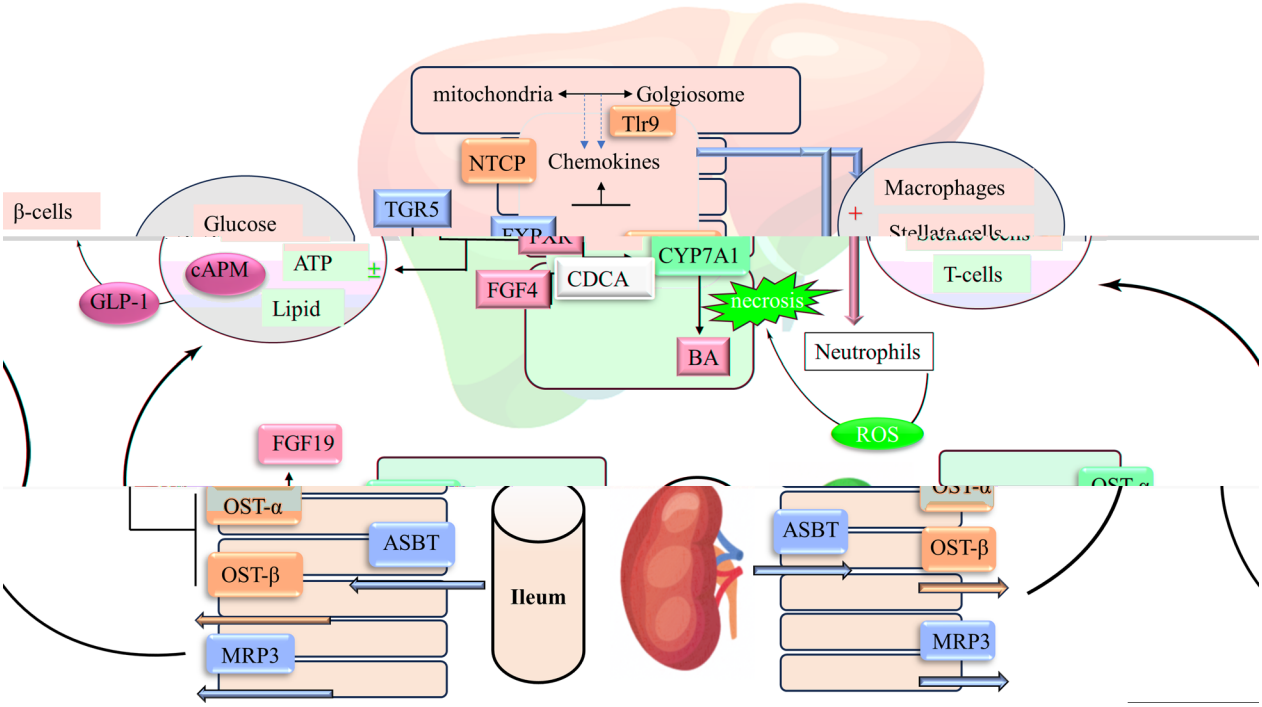


Fig. 3 Bile acid signaling and control of metabolism.Bile acids are actively reabsorbed in the intestine through ASBT, with the majority being reabsorbed in the distal ileum. ASBT shows a preference for transporting T- or G-conjugated bile acids, particularly dihydroxy bile acids. Furthermore, ASBT is negatively regulated by FXR and SHP in both humans and mice, with bile acids inhibiting ASBT expression via the FXR-SHP pathway. Additionally, FGF15 plays a role in regulating the inhibitory effects on ASBT

glycine or taurine, can inhibit the intestinal FXR signaling pathway [95, 96], leading to increased bile acid reabsorption and faster enterohepatic circulation. Furthermore, studies show that reduced levels of FGF15/19 [97–99] can enhance the expression of hepatic bile acid transporters, decrease bile acid pools, and facilitate bile acid excretion through feces, thus maintaining bile acid balance. These findings broaden our understanding of UDCA's pharmacological mechanisms and offer new avenues for future research on bile acid metabolism.

The role of UDCA in enhancing bile efflux and transport in liver cells

UDCA can enhance the function and expression of Cl-/HCO3- cotransporter AE2, leading to improved bile efflux [100]. TUDCA can elevate calcium ion levels in liver cells, triggering PKC-α activation and facilitating bile outflow [101]. Moreover, TUDCA can enhance the expression of bile transporters on the bile capillary membrane by activating MAPK. Additionally, UDCA can upregulate the expression of bile acid transporters in hepatocytes, thereby enhancing bile acid efflux [2, 102]. Overall, UDCA plays a crucial role in regulating bile efflux and bile acid transport, facilitating bile extracellular secretion and vesicle exocytosis.

The protective effect of continuous UDCA administration on liver toxicity

Accumulation of bile in the liver to a toxic concentration can result in severe damage and potentially progress to cirrhosis [103, 104]. However, Continuous administration of UDCA can improve bile acid composition, thereby reducing the toxicity of endogenous bile acids [105, 106]. This treatment can effectively improve the bile acid composition and protect the liver.

The immunomodulatory effects of UDCA in patients with primary biliary cholangitis and primary sclerosing cholangitis

Research indicates [107, 108] that the major histocompatibility complex (MHC) is expressed in liver cells of patients with primary biliary cholangitis (PBC) or primary sclerosing cholangitis (PSC), and UDCA can decrease this expression. UDCA functions by activating the glucocorticoid receptor (GR) and inhibiting nitric oxide synthase activit [109, 110]. Clinical trials [111–113] have demonstrated that following UDCA treatment,

there is a reduction in various antibodies levels in patients, such as serum immunoglobulin M13 and anti-mitochondrial antibodies. Consequently, UDCA exhibits anti-inflammatory and immunomodulatory properties in the management of patients with PBC or PSC.

The cytoprotective effects of UDCA on cell membrane stability and apoptosis

UDCA is a compound known for its cytoprotective effects, primarily focused on maintaining cell membrane stability and anti-apoptotic properties [114–116]. Research indicates that UDCA can effectively counteract cell membrane damage caused by hydrophobic bile acids through potential binding to various regions of the cell membrane [117]. Furthermore, UDCA has been observed to reduce mitochondrial membrane permeability [118], leading to an anti-apoptotic impact. Both in vitro and in vivo studies have demonstrated the significant protective and anti-apoptotic capabilities of UDCA, positioning it as a promising treatment option for preventing cell membrane damage and apoptosis. Moreover, UDCA has been shown to inhibit the formation of MPTP, decrease ROS production, and elevate GSH levels, thereby enhancing the antioxidant response in liver cells [119, 120]. Additionally, UDCA can impede P53 activity and promote proteasomal degradation to reduce P53's half-life. Furthermore, TUDCA can alleviate ER stress in cells and hinder apoptosis progression. Interestingly, some studies suggest that UDCA may have a pro-apoptotic effect on liver cancer cells by inducing alkaline sphingomyelinase expression, ultimately restraining cell proliferation [121, 122]. Overall, UDCA exhibits multifaceted effects on regulating cell apoptosis and proliferation, positioning it as a potential therapeutic candidate for liver diseases and liver cancer.

Comparative study on artificial and natural bear bile powder composition and pharmacological effects

Bile acids, which are essential components of bile, are synthesized by the liver from cholesterol through two main pathways: the classical pathway and the alternative pathway [123, 124]. The key enzyme that controls the rate of bile acid synthesis in the classical pathway is cholesterol 7α-hydroxylase [125–127], located in the endoplasmic reticulum of hepatocytes. This pathway primarily produces CA) and CDCA.On the other hand, the alternative pathway, known as the acidic pathway, converts cholesterol to CDCA with the help of CYP27A1 and CYP7B1 enzymes [128, 129].

Currently, artificial bear bile powder is primarily prepared using chemical compounding and biological transformation methods. The chemical compounding method prepares artificial bear bile powder by mixing different chemical ingredients [130]. This method can more accurately control the proportion of ingredients, making the final product closer to the ingredients of natural bear bile. Biotransformation methods include enzymatic methods and microbial transformation methods. The enzyme catalysis method achieves the synthesis of chemical substances through enzymatic reactions [131, 132]. For instance, *Wang Bochu* and others [133] pointed out that chenodeoxycholic acid can be converted into UDCA under the action of 7α-hydroxysteroid dehydrogenase and oxidative coenzyme. Cholic acid and the microbial transformation method uses microorganisms to perform such transformations, such as using immobilized cells of anaerobic bacteria [134] to synthesize ursodeoxycholic acid.

Enzymatic synthesis and microbial transformation are considered safer alternatives to chemical synthesis due to their avoidance of toxic chemical reagents [135]. Moreover, these methods can establish a simulated enterohepatic circulation expression system to produce bile acid's active ingredients, resulting in higher medicinal efficacy. As a result, enzymatic synthesis and microbial transformation are generally preferred for synthesizing pharmaceutical ingredients in bile [136]. Despite enzyme synthesis being more environmentally friendly and healthier than chemical synthesis, improving enzyme catalytic performance through enzyme engineering presents a difficult and intricate challenge.

The chemical composition of artificial bear bile powder closely resembles that of natural bear bile powder, allowing its pharmacological effects to match those of traditional bear bile [137]. Research indicates that artificial bear bile powder contains similar levels of TUDCA,TCDCA other key components, leading to comparable sedative and choleretic effects as natural bear bile powder [138]. Based on pharmacodynamic study findings, it can be inferred that artificial bear bile powder can serve as a viable substitute for natural bear bile powder.

Understanding the synthesis of UDCA and TUDCA: enzyme cascade and chemical approaches in enterohepatic circulation

This review article discusses the synthetic methods of UDCA and TUDCA. UDCA is a widely utilized drug for the treatment of hepatobiliary diseases, while TUDCA exhibits distinct pharmacological activities[139]. The article elaborates on the synthesis pathways for both substances, detailing the synthesis of UDCA from animal cholic acid and steroid compounds as starting materials, as well as the chemical and biological synthesis methods for TUDCA.

The complex enzymatic cascade involved in UDCA and TUDCA biosynthesis in enterohepatic circulation

Intestinal microorganisms can provide key enzymes for the bile acid synthesis pathway-enterohepatic circulation [16]. Enterohepatic circulation represents a sophisticated physiological process involving the synthesis of primary bile acids, which are produced by hepatocytes using cholesterol as a foundational substrate [140]. Once synthesized, these primary bile acids undergo metabolism within the liver, where they are conjugated with either glycine or taurine. The resultant conjugated bile acids then move through the cellular structures known as microtubules to be stored in the gallbladder. In this organ, bile acids are concentrated and eventually released into the small intestine. These bile acids play a crucial role in aiding the digestion and absorption of dietary fats and fat-soluble vitamins by facilitating their solubilization. In the small intestine, bile acids are actively engaged in the processes of fat digestion and the absorption of essential fat-soluble vitamins. In particular, within the terminal ileum, these bile acids are taken up through active transport mechanisms. It is estimated that around 95% of the bile acids that are absorbed are subsequently transported mixed with plasma proteins back to the liver, illustrating the efficiency of enterohepatic circulation. This complex process involves numerous enzymes, including bile salt hydrolase (BSH) and several hydroxysteroid dehydrogenases (3α-HSDH, 3β-HSDH, 7α-HSDH, 7β-HSDH, 12α-HSDH, and 12β-HSDH), which perform critical roles in modifying bile acids. Specifically, BSH is responsible for the hydrolysis of conjugated bile acids into their free acid forms, while the hydroxysteroid dehydrogenases mentioned facilitate the isomerization of hydroxyl groups present in the bile acids, a series of reactions that are characterized by their reversibility. The in vivo synthesis of TUDCA nvolves a sequence of five distinct enzymatic steps, each facilitated by specific enzymes, as depicted in Fig. 4. During this process, bile salt hydrolase (BSH) acts on TCDCA to convert it into CDCA, which undergoes further enzymatic modification by 7α-HSDH and 7β-HSDH to yield UDCA. This UDCA is then transformed into TUDCA through the actions of bile acid-CoA ligase (BAL) and bile acid-N-acetyltransferase [141]. Additionally, literature suggests that 7α-HSDH exhibits activity against TCDCA in vitro, highlighting its importance. Notably, the in vitro reactions do not necessitate the involvement of cellular membrane crossing, allowing for a direct catalytic transformation of TCDCA into TUDCA through

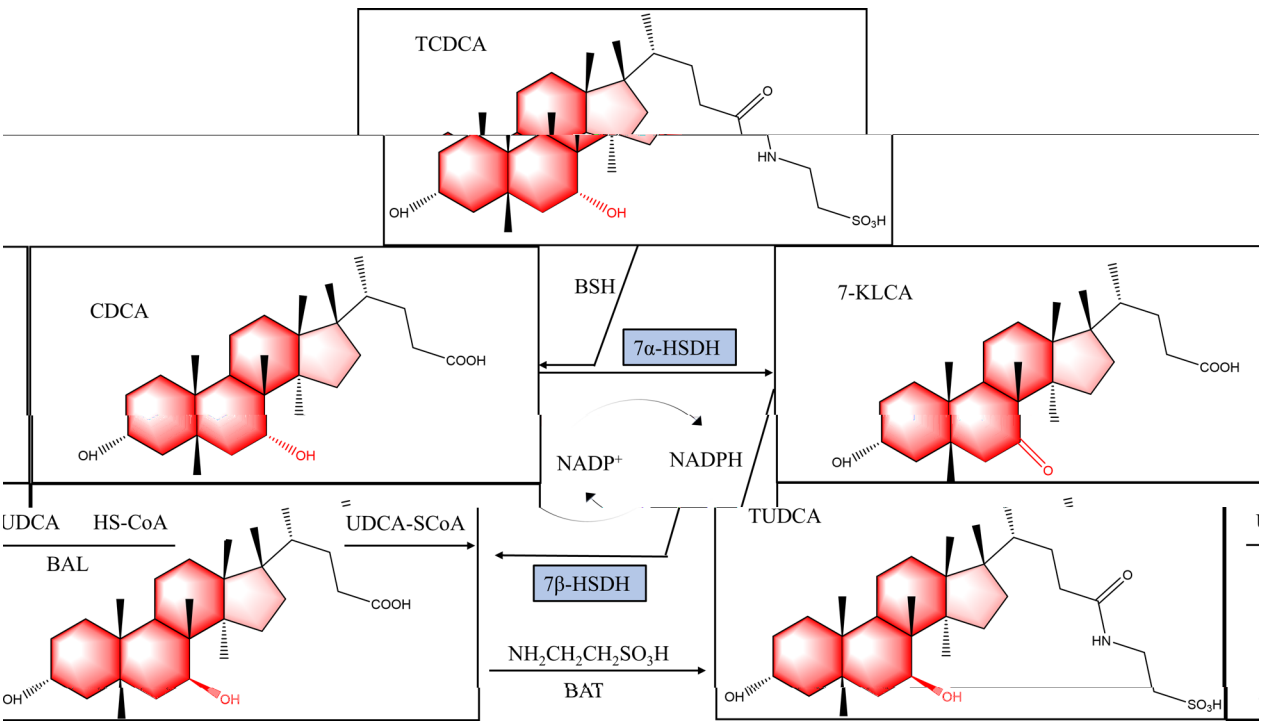


Fig. 4 The process of transformation from TCDCA to TUDCA in vivo. BSH catalyzes the hydrolysis of TCDCA to produce CDCA. Subsequently, CDCA is acted upon by 7α- and 7β-HSDH to generate UDCA. UDCA is then converted into BAL, leading to the formation of TUDCA under the catalysis of N-acetyltransferase

hydroxyl isomerization, irrespective of hydrolysis and independent of taurine's binding to the 7α- and 7β-HSDH enzymes [9].

Chemical synthesis method of TUDCA: mixed anhydride-phenol ester method and condensation agent method

The mixed anhydride-phenolic ester method has historically been one of the prevalent techniques for synthesizing TUDCA in prior research endeavors [142–144]. This method operates on a core principle that entails the initial transformation of UDCA into a mixed anhydride or an activated phenolic ester. Following this, the converted compound is then reacted with taurine, resulting in the formation of TUDCA. Despite its common use, this synthesis approach requires the employment of highly toxic reagents, including sulfoxide dichloride and trifluoroacetic anhydride, which pose significant safety hazards. Moreover, the mixed anhydride-phenolic ester method is characterized by an intricate sequence of numerous reaction steps, contributing to its complexity and inefficiency. This convoluted process often yields a relatively low overall output of the desired product, which is a considerable drawback. Additionally, the substantial generation of byproducts during the reaction exacerbates the challenges faced during the subsequent separation and purification stages, further complicating the synthesis process. These factors collectively highlight the limitations of this method in the efficient and safe production of TUDCA.

The condensation agent method involves the direct combination of UDCAwith Taurine, resulting in the formation of an amide bond that ultimately synthesizes TUDCA [46, 145]. While this approach is considered straightforward in terms of procedure, it comes with significant drawbacks. One major concern is the high cost associated with the condensation agents required for the reaction. Additionally, there is a risk of side reactions occurring, particularly racemization, which can compromise both the purity and yield of the final product. These factors highlight the need for careful consideration when employing this method in the synthesis of TUDCA.

Synthesis of UDCA from androstenedione

The synthesis of UDCA, is achieved by employing steroidal compounds like androstenedione as initial raw materials [146]. This approach involves a series of multi-step chemical reactions that transform these starting materials into the desired compound. One notable advantage of this method is the use of easily accessible raw materials, which can facilitate the overall synthesis process. However, the complexity of this method cannot be understated, as it involves multiple reaction steps that require careful and precise control of the reaction conditions. Additionally, the process necessitates elaborate separation and purification techniques to ensure the final product is of high purity and quality.

Conversion of TCDCA to TUDCA in chicken bile: insights from enzyme modification and optimization in gut microbiota

TUDCA is a secondary bile acid formed from UDCA and taurine, known for its low toxicity, strong hydrophilicity, and high bioavailability [147, 148]. It plays a significant pharmacological role in addressing various diseases associated with metabolic abnormalities. Research indicates that TUDCA can enhance Alzheimer's disease [149–151], avert atherosclerosis [152], and manage hepatobiliary diseases [153, 154], while also alleviating intestinal inflammation [155] and retinal degeneration [156, 157], ultimately promoting overall health benefits for individuals.

The process of TUDCA synthesis in the body primarily involves several steps. Initially, liver cells utilize cholesterol to synthesize the primary bile acid CDCA, which is then combined with glycine and taurine to form GCDCA and TCDCA [158, 159]. These compounds are subsequently excreted from the digestive tract. Upon reaching the intestine, GCDCA and TCDCA are enzymatically converted back into CDCA, which is further transformed into UDCA by microorganisms [160]. UDCA then enters the bloodstream through reabsorption. Subsequently, UDCA is taken back into liver cells, where it is converted into TUDCA and GUDCA before being secreted into the digestive tract to initiate a new enterohepatic circulation process (Fig. 1).

Differences in bile acid functions among different animals are primarily due to variations in bile acid composition [161]. Bear bile powder is highly valued for its medicinal properties due to its high content of TUDCA and TCDCA, whereas poultry and chicken gall predominantly contain TCDCA. Hyodeoxycholic acid (HDCA) is a unique component found in pig gallbladders. Interestingly, our analysis revealed that TCDCA and TUDCA share a very similar structure, differing only in 7 key components [138, 162, 163]. Furthermore, the non-bile acid content of poultry chicken bile powder closely resembles that of bear bile powder. Although chicken bile powder has been historically used for its medicinal benefits, it is now less commonly utilized [164]. Therefore, repurposing discarded chicken bile powder as the primary material for producing analogues of bear bile powder offers a sustainable approach to resource utilization, effectively transforming waste into valuable substances.

TUDCA has demonstrated effectiveness in treating various diseases. The in vivo production of TUDCA

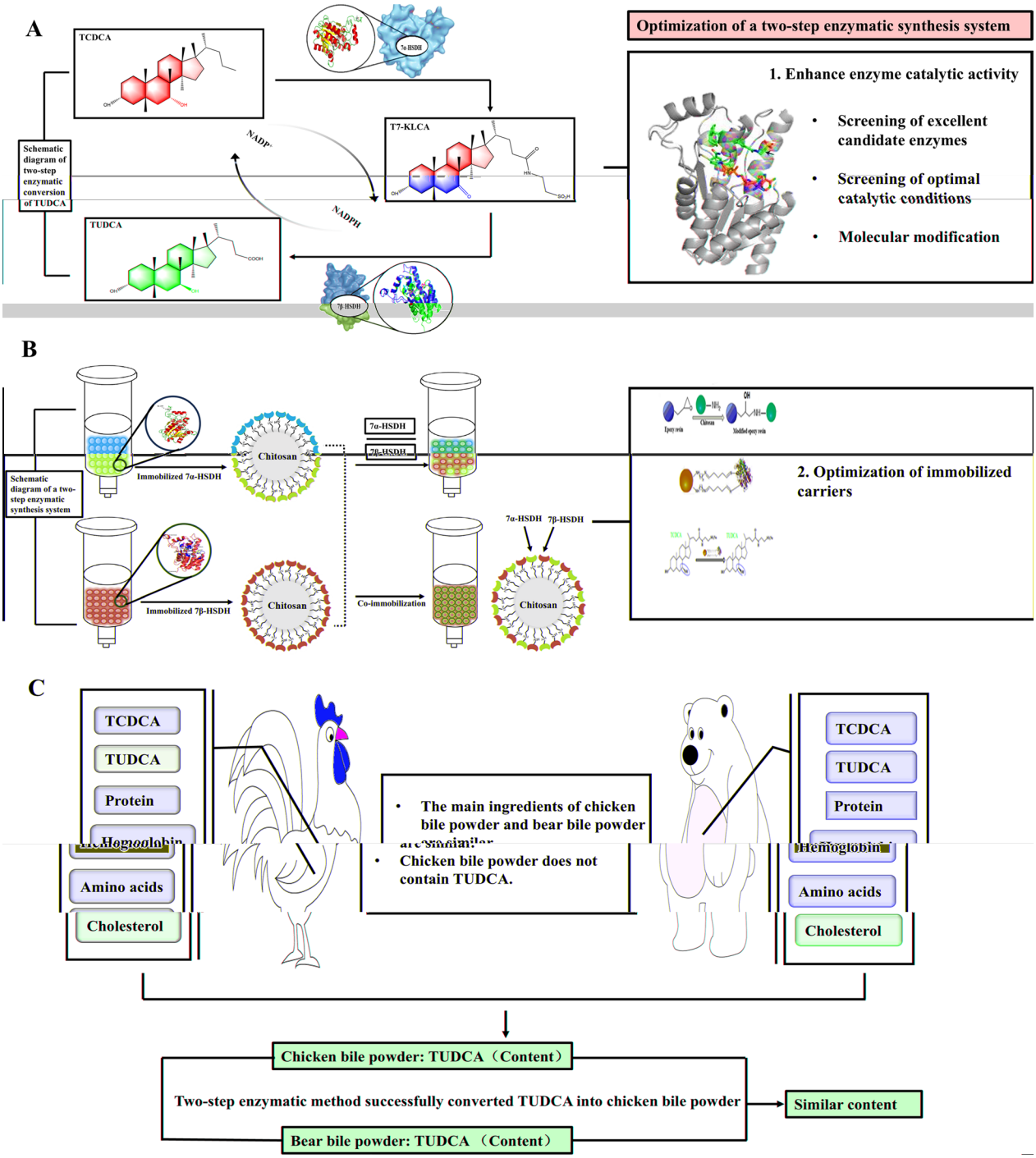


Fig. 5 Converting TCDCA to TUDCA in chicken bile. **A** Schematic diagram of two-step enzymatic conversion of TUDCA [61, 70, 165, 168]. TCDCA is catalyzedby 7α- and 7β-hydroxysteroid dehydrogenases, which facilitate an epimerization reaction of the C7 hydroxyl group, resulting in the conversion to TUDCA. By screening or molecular modification to identify 7α- and 7β-hydroxysteroid dehydrogenases with enhanced catalytic activity, and by determining their optimal reaction conditions, the conversion efficiency of TCDCA can be improved.54. **B** Immobilized 7α-, 7β-HSDH coupled system [68, 175]: Schematic diagram of a two-step enzymatic synthesis system: Utilizing knowledge from enzyme engineering, pharmaceutical engineering, and biochemical reaction engineering, the key enzymes involved in bile acid metabolism, namely 7α- and 7β-hydroxysteroid dehydrogenase, were successfully immobilized. A dual-enzyme coupling system comprising both 7α- and 7β-HSDH was constructed. Furthermore, optimizing the immobilization conditions can enhance the catalytic efficiency of this dual-enzyme coupling system. **C** Two-step enzymatic method successfully converted TUDCA into chicken bile powder: based on A and B, the optimal coupled immobilized enzymes of 7α- and 7β-HSDH were obtained. This two-step enzyme coupling system was employed to catalyze the complex substrate, chicken bile powder, resulting in a TUDCA content that closely approximates that found in bear bile powder

involves a complex five-step process catalyzed by five enzymes [165] within the enterohepatic circulation of bile salts. In 2015, *Ji Qingzhi* and colleagues [68] introduced a novel in vitro method for preparing TUDCA. This method entails the direct epimerization of TCDCA to TUDCA catalyzed by immobilized 7α- HSDH with 7β-HSDH in a dual-enzyme coupling system. 7α- HSDH with 7β-HSDH were immobilized on modified chitosan microspheres using single and co-immobilization techniques. A comparison of the TUDCA yields from the two methods revealed that the batch reaction catalyzed by the dual-enzyme coupling system achieved a high TUDCA yield of 62.3%. This enzymatic technology underscores the in vitro synthesis of TUDCA via a dual-enzyme coupling system, offering potential applications for the synthesis of TUDCA and other valuable bile acid derivatives. Since the inception of in vitro TUDCA synthesis using a dual-enzyme coupling system, numerous researchers [166–168] have dedicated their efforts to refining and optimizing this method. In recent years, there has been significant progress in enhancing the catalytic efficiency of key enzymes, particularly focusing on the molecular structures of 7α- HSDH with 7β-HSDH to optimize their activity (Fig. 5). Through the discovery of new enzymes and molecular modifications, a more efficient dual-enzyme coupling system was developed, successfully extracted from chicken bile. TCDCA was successfully converted into TUDCA from chicken bile, and industrial production has been achieved. These accomplishments have established a strong foundation for the development of alternative medicines to replace bear bile powder.

Metagenomic analysis of black bear fecal samples reveals novel 7α-HSDH with 7β- HSDH

The research explored the gut microbiome of black bears in China by analyzing fecal samples from regions like Heilongjiang, Sichuan, and Yunnan using metagenomic sequencing. The sequencing data is accessible in the NCBI Short Reads Archive under accession number SRP079591 [169]. This study sheds light on the microbial communities and metabolic functions in black bear feces. Scientists identified new 7α- and 7β-HSDHs in Asian black bear feces through metagenomics, expanding knowledge of their gut microbiome. Enzymes responsible for these discoveries were cloned and expressed in E. coli, revealing eight 7α-HSDHs and one 7β-HSDH. Among these, J-1–1 showed significant activity towards TCDCA, indicating its role in bile acid metabolism. Y1-b-1 (7β-HSDH) exhibited optimal pH and temperature at 9.0 and 30 °C, respectively, highlighting its efficiency with TCDCA. These HSDHs were categorized as basophilic and mesophilic enzymes, with St-2–1 identified as an acidophilic mesophilic enzyme with an optimum pH of 5.5, showcasing the diverse functionalities of these enzymes in the Asian black bear gut microbiome [9] (Fig. 5A).

Enhancing thermostability and activity of steroid dehydrogenases

The CA 7α-HSDH [10] crystal structure contains the enzyme, coenzyme $NADP^+$, and substrate TCDCA, while the CA 7β-HSDH structure includes only the enzyme and coenzyme [163]. In a different study [170], researchers used AutoDock software to dock TUDCA to CA 7β-HSDH [171], revealing an interesting spatial orientation difference compared to TCDCA in CA 7α-HSDH [172, 173]. Molecular dynamics simulations identified flexible sites for improving thermal stability, leading to the selection of Asp201 in the LOOP structure of CA 7β-HSDH for saturation mutagenesis. Among the tested mutants, only the Asp201Cys mutant showed a significant increase in activity and Tm value. Researchers have also engineered mutants of 7α-HSDH to enhance catalytic activity, with notable success in the presence of Mg^{2+}. Additionally, mutants like A26V, I222V, and P212A were developed to improve thermostability for industrial applications, showing promising results in activity retention and Tm value enhancement.

The study delved into the effects of St-2–2 mutations at the carboxyl terminus (C-terminal), including various mutations such as K262R, K261Q, A259L, I255Q, K262R/K261Q/A259L, and K262R/K261Q/A259L/I255Q, on enzyme function. Results indicated that the I255Q mutant displayed increased activity towards certain substrates but decreased activity towards others. Conversely, the three-site directed mutant K262R/K261Q/A259L showed enhanced activity compared to the wild type, with no significant differences observed for specific substrates. These findings suggest that modifications in the C-terminal region of SDRs members can significantly impact substrate specificity, influencing preferences for certain substrates.Moreover, certain mutants (K262R, A259L, I255Q, and K262R/K261Q/A259L) demonstrated improved thermostability, especially the I255Q mutant which exhibited higher activity levels after heat treatment compared to the wild type. Overall, the study stresses the crucial role of the C-terminal in determining substrate specificity and enzyme activity in St-2–2 mutations. It emphasizes how targeted modifications in this region could potentially improve enzymatic function and stability, offering insights into enhancing enzyme performance for various applications. Continued research in this field could lead to the development of more efficient and stable enzymes through targeted alterations in the C-terminus of SDRs members, further advancing our understanding of the impact of mutations on enzyme efficiency (Fig. 5A).

Optimization of immobilized 7α-HSDH with 7β-HSDH double enzyme carriers based on multi-parameter collaboration in microenvironment and its application

A double-enzyme-coupled system was established by researchers [11], utilizing immobilized 7α-HSDH with 7β-HSDH on carriers like chitosan and epoxy resin. Both enzymes were co-immobilized in the carrier, with 7α-HSDH catalyzing the reaction of TCDCA with $NADP^+$ to produce T-7-KLC and NADPH, which were then converted to TUDCA and $NADP^+$ by 7β-HSDH. This coupling of reactions allowed for the regeneration of cofactors ($NADP^+$ and NADPH) in the process [68].

The double-enzyme-coupled system includes two forms: one consisting of a mix of immobilized 7α-HSDH microspheres and 7β-HSDH microspheres, and the other containing co-immobilized 7α- and 7β-HSDH microspheres. The latter form shows higher productivity and yield of TUDCA, partially explained by the close proximity of 7α- and 7β-HSDH, reducing diffusional limitations during the reaction. Immobilized enzyme carriers were designed with varying concentrations of chitosan-modified epoxy resins to create different microenvironments with medium hydrophobicity, crowding, and charge conditions. Manipulating these conditions can enhance the activities and thermal stabilities of immobilized 7α-HSDH and 7β-HSDH. Increasing crowding by reducing pore size of the carrier improves enzyme activity, as well as enhances thermal stability and cycling stability.In particular, among the chitosan-modified epoxy resin carriers, the catalytic efficiency of double enzyme immobilized in EP-0.5-C was found to be least affected after 7 successive reaction cycles [12]. The conversion rate of TCDCA only decreased from 85.45 ± 0.36% to 84.32 ± 0.55%, while the yield of TUDCA decreased from 55.02 ± 2.07% to 51.54 ± 0.67% [68]. This indicates that carefully controlling the microenvironment of immobilized enzymes through different carrier compositions can have a significant impact on enzyme activity and stability over multiple reaction cycles [174].

Dual-enzyme co-immobilization for efficient TCDCA conversion

In the study conducted by *Ji Qingzhi* and colleagues [68], three methods for immobilization were put forward: firstly, step-by-step catalysis with immobilized enzymes; secondly, mixed catalysis using immobilized enzymes; and thirdly, co-immobilization of dual enzymes for catalysis. The findings indicated that co-immobilization of dual enzymes yielded the most promising results, as assessed by the yield of TUDCA and the conversion rate of TCDCA. A comparative analysis of the three techniques—step-by-step catalysis with immobilized enzymes, mixed catalysis with immobilized enzymes, and dual-enzyme co-immobilization—showcased the following results: step-by-step catalysis achieved a conversion rate of 72.76% for TCDCA and a yield of just 22.08% for TUDCA. In comparison, the immobilized enzyme mixture and dual-enzyme co-immobilization techniques attained TCDCA conversion rates of 80.12% and 90.40%, with corresponding TUDCA yields of 41.23% and 62.49%. Therefore, the dual-enzyme co-immobilization technique is recognized as the most efficient approach for immobilizing 7α-, 7β-HSDH in facilitating the conversion of TCDCA. As shown in Fig. 5B.

Large-scale production of artificial bear bile powder using co-immobilized enzymes

The production of Artificial Bear Bile Powder (ABBP) involved using a chicken bile powder solution with 7α- and 7β-HSDHs co-immobilized on EP-0.5-C to create a bear bile powder analogue [175–177]. TCDCA was successfully converted into TUDCA from chicken bile, and industrial production has been achieved (Shanghai Kaibao Pharmaceutical Co and Chongqing Jize Biotechnology Co), laying the foundation for the potential development of bile powder alternative drugs [68, 178, 179]. By carefully controlling the ratio of 7α-HSDH to 7β-HSDH, a quantitative transformation of TCDCA to TUDCA was achieved in the complex substrate of chicken bile powder solution [178]. Notably, the intermediate T-7-KLCA was not detected in the reaction process, showcasing the efficiency of the transformation. Furthermore, the results of the production process showed that the levels of TCDCA, TUDCA, total protein, and bilirubin in the reaction product closely resembled those found in natural bear bile powder (NBBP) [137]. However, it was observed that the levels of total amino acids and total cholesterol in the reaction product were higher compared to NBBP. This indicates that while the production of ABBP successfully replicated the key components of natural bear bile powder, there were slight differences in certain parameters. Overall, the study demonstrated the feasibility of efficiently producing ABBP using enzymatic transformation processes. Relevant studies have demonstrated that there is no significant difference in the pharmacodynamics of ABBP and NBBP [180, 181]. Overall, the study introduced a innovative dual-enzyme system for the mass production of synthetic bear bile using chicken bile. This was achieved through a combination of technologies including metagenomics, enzyme crystallography, enzyme design, and enzyme manipulation. The synthetic bear bile derived from chicken bile is comparable to natural bear bile in terms of both chemical composition and pharmacodynamics. The advancement in biotechnology is immensely advantageous in efforts to decrease the practice of "live bear bile extraction" and to

safeguard the Asian black bear population. As shown in Fig. 5C.

Conclusions

This article provides a comprehensive review of the latest research advancements concerning the discovery, pharmacological mechanisms, and biosynthesis of ursodeoxycholic acid (UDCA) and TUDCA. The study specifically investigates the biosynthetic pathway of TUDCA utilizing chicken bile powder as the raw material, and employs chitosan for the co-immobilization of 7α-hydroxysteroid dehydrogenase (7α-HSDH) and 7β-hydroxysteroid dehydrogenase (7β-HSDH) through a two-step enzymatic method. This review outlines the identification and characterization of several 7α- and 7β-HSDH enzymes that are pivotal for TUDCA biosynthesis. It highlights that, based on the X-ray crystal structures of these enzymes, molecular modification techniques have yielded mutant 7α- and 7β-HSDH enzymes exhibiting enhanced catalytic activity. Furthermore, the immobilized 7α- and 7β-HSDH enzymes, developed using chitosan-modified epoxy resin as a carrier, were employed to construct a stable and efficient dual-enzyme coupling system capable of biotransforming complex substrates in situ. A product resembling natural bear bile powder was synthesized from a chicken bile powder solution. The dual-enzyme coupling system, as described in this review, demonstrates significant efficacy in converting taurochenodeoxycholic acid (TCDCA) into TUDCA, achieving a high conversion rate while maintaining an appropriate immobilization ratio and conditions, thereby promoting the production of the target product. Finally, the process of transforming chicken bile powder into bear bile powder using the immobilized 7α-HSDH and 7β-HSDH dual-enzyme system is elaborated in detail. In conclusion, this review presents a biopreparative method based on a dual-enzyme coupling system (immobilized 7α- and 7β-HSDH) as a sustainable approach for the complete synthesis of artificial bear bile from chicken bile within a controlled laboratory environment.

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Author contributions
CRediT authorship contribution statement ShijinTang: Investigation, Methodology, Data curation, Formal analysis, Writing-original draft;Yinping Pan: Data curation, Methodology; Qiong Yang: Writing-review & editing; Deshuai Lou: Writing-review & editing;Jun Tan: Writing-review & editing; Liancai Zhu: Supervision, Conceptualization, Methodology; Shaoyong Liu: Supervision, Conceptualization, Methodology; Bochu Wang: Supervision, Methodology, Funding acquisition.

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No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare no competing interests.

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