ACS OMEGA

http://pubs.acs.org/journal/acsodf

Article

Optimization and Impurity Control Strategy for Lithocholic Acid Production Using Commercially Plant-Sourced Bisnoralcohol

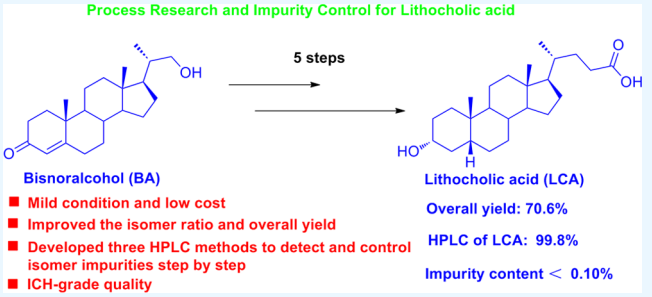
Chunling Zeng, Shitang Xu, Zhenlong Yin, Yue Cui, Xinhua Xu,* and Ningbo Li*

Cite This: *ACS Omega* 2023, 8, 23130−23141

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: In this study, lithocholic acid (LCA) was prepared using commercially available plant-sourced bisnoralcohol (BA), and the overall yield of the product was 70.6% for five steps. To prevent process-related impurities, the isomerizations of catalytic hydrogenation in the C4−C5 double bond and reduction of the 3-keto group were optimized. The double bond reduction isomerization was improved (5β-H:5α-H = 97:3) using palladium−copper nanowires (Pd−Cu NWs) instead of Pd/C. The reduction of the 3-keto group was 100% converted to a 3α-OH product by 3α-hydroxysteroid dehydrogenase/carbonyl reductase catalysis. Moreover, the impurities during the optimization process were comprehensively studied. Compared with the reported synthesis methods, our developed method significantly improved the isomer ratio and overall yield, affording ICH-grade quality of LCA, and it is more cost-effective and suitable for large-scale production of LCA.



INTRODUCTION

In recent years, lithocholic acid (LCA, Figure 1) and its derivatives have attracted considerable scholarly attention owing to their biological and pharmaceutical activities. They are good inhibition effects for epithelial apoptosis in the intestine, protein tyrosine phosphatase 1B or α-2,3-sialyltransferase,[1,2] and TGR5 receptor or vitamin D receptor activation.[3−5] Additionally, lithocholic acid has excellent antibacterial,[6] antiaging,[7] and antitumor activities.[8−11] Several studies have shown that LCA can be used as a starting material for the synthesis of ursodeoxycholic acid in the presence of the cytochrome P450 monooxygenase or a few microbial organisms,[12−16] which is a clinical first-line drug for treating primary biliary cirrhosis and cholecystitis.[17−19] Thus, given these unique and favorable properties of LCA, the development of an efficient approach for the synthesis of LCA has drawn considerable attention in recent years.

Researchers have discovered some synthetic methods for LCA, which are mainly divided into the following two categories: (1) the use of commercially available domestic animal bile-based materials, such as cholic acid, deoxycholic acid, chenodeoxycholic acid, and hyodeoxycholic acid (Figure 1), as starting materials for the synthesis of LCA[20,21] and (2) the use of commercially plant-sourced materials as starting materials (bisnoralcohol BA, Figure 1) for the synthesis of LCA.[22] However, animal-sourced materials have some potential risks, such as avian influenza, bovine spongiform encephalopathy, and swine influenza.[23,24] Moreover, the target LCA is often obtained after a multistep operation with existing low yields and difficult separation of isomers, which limits their further applications. Recently, Qiu et al.[22] used plant-sourced bisnoralcohol as the starting material, owing to its low cost, availability, and accessibility, to develop a five-step synthetic route for LCA (68% overall yield) (Scheme 1A). Although great success has been achieved using this synthetic route, the control and analysis of the chiral isomers about the hydrogenation at the C4−C5 position of compound 3 and reduction of the 3-keto group in compound **4** remain vague and need to be improved. For example, Qiu et al.[22] used Pd/C and borohydride to reduce the C4−C5 double bond in compound **3** and the 3-keto group in compound **4**. However, the results of hydrogenation at the C4−C5 position (5β-H:5α-H = 92:8) and reduction at the 3-keto group (3α-OH:3β-OH = 95:5) need to be improved to meet the requirement for LCA drug synthesis. Although high efficiency of the reduction of compound **3** was achieved using Raney Ni and H_2 for LCA synthesis (Scheme 1A, f), the hydrogenation is a dangerous reaction (Raney Ni under H_2 (4.0 MPA) for 48 h at 90 °C) because of its harsh conditions and the simultaneous generation of two chiral sites, which makes isomerization and impurity analyses remain ambiguous.

Received: April 14, 2023
Accepted: May 30, 2023
Published: June 14, 2023

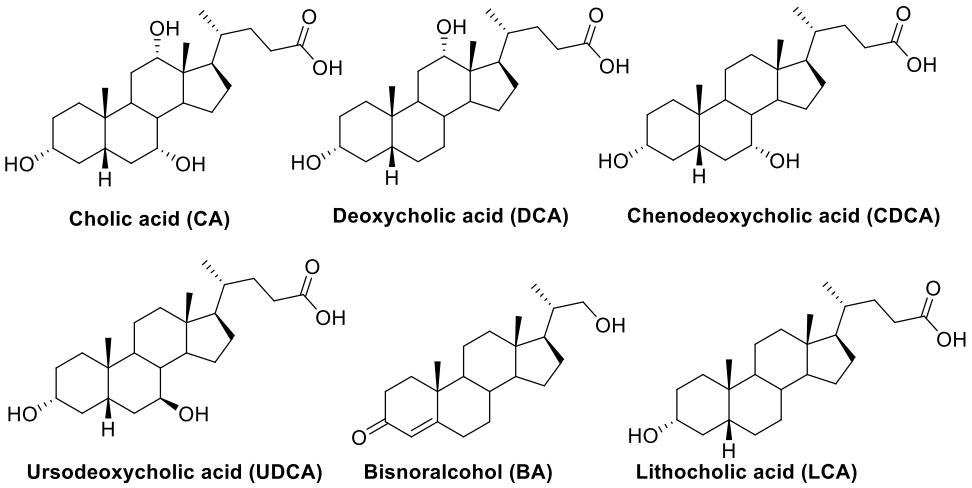
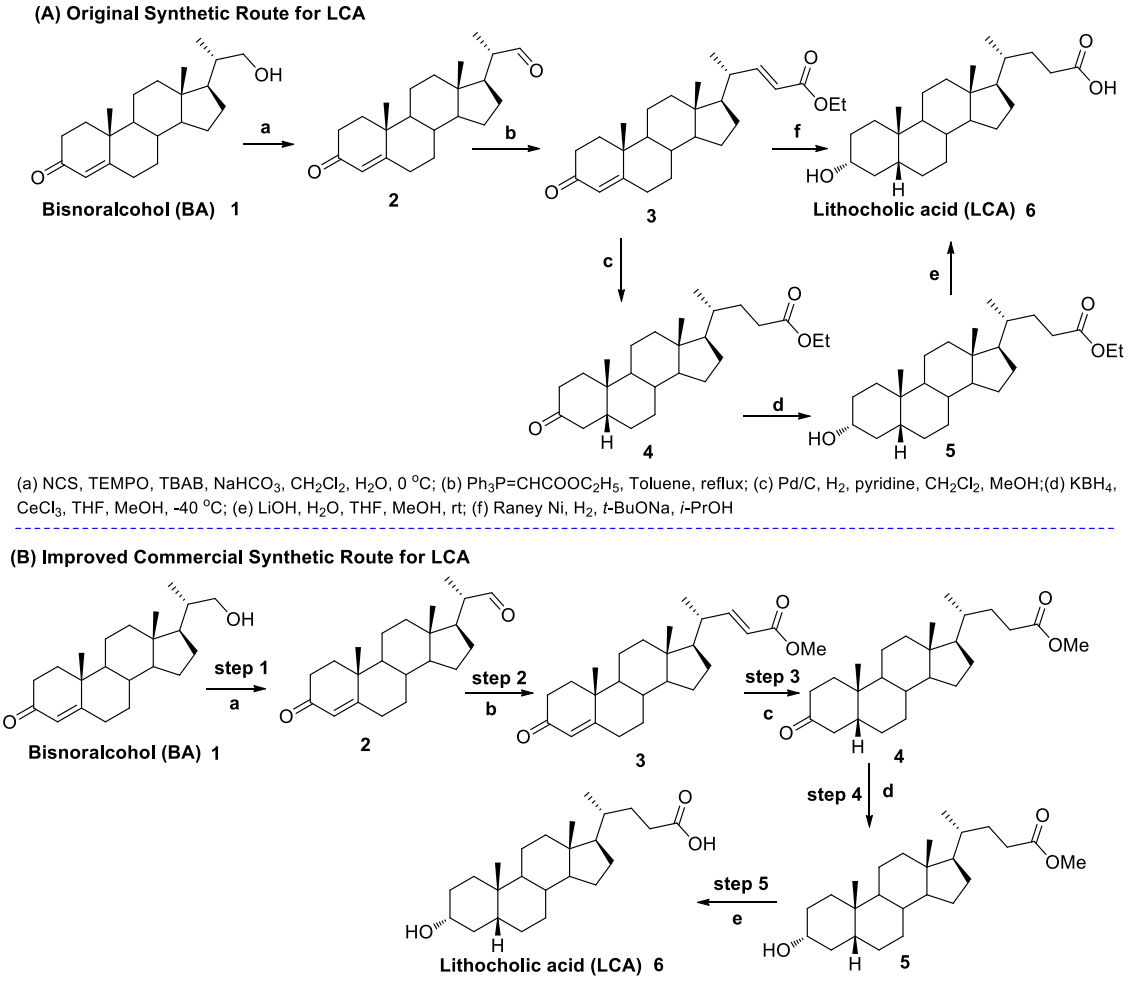


Figure 1. Structures of bile acids, bisnoralcohol, and lithocholic acid.

Scheme 1. Synthetic Route for LCA



In this study, we provided an optimized route for preparing LCA using commercially available plant-sourced BA (Scheme 1B). In this process, palladium−copper nanowires were used instead of Pd/C for the hydrogenation of the C4−C5 of compound **2** with H_2, and the isomer ratio was improved to 97:3. Particularly, the reduction of the 3-keto group could be 100% converted to a 3α-OH product through 3α-hydroxysteroid dehydrogenase/carbonyl reductase catalysis. The process-related impurities were also studied in detail during the optimization process. Moreover, the above synthesis methods can be used for large-scale LCA synthesis.

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

Scheme 2. Synthetic Route for LCA by Raney Ni and H_2

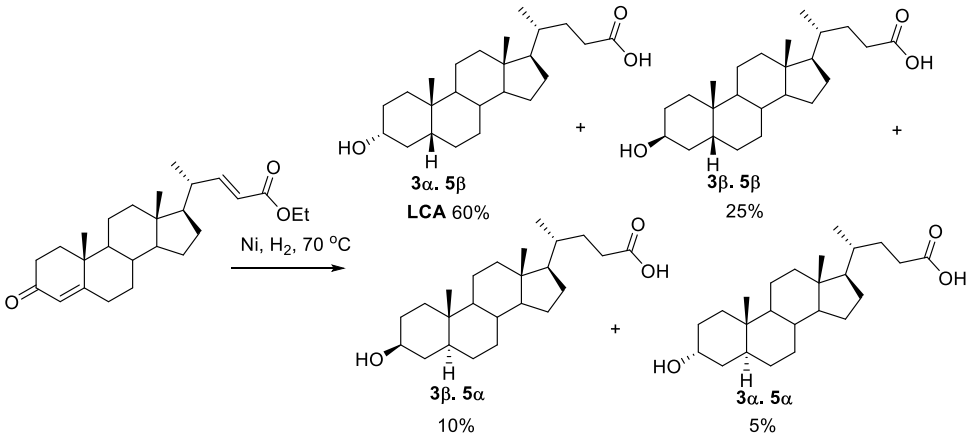
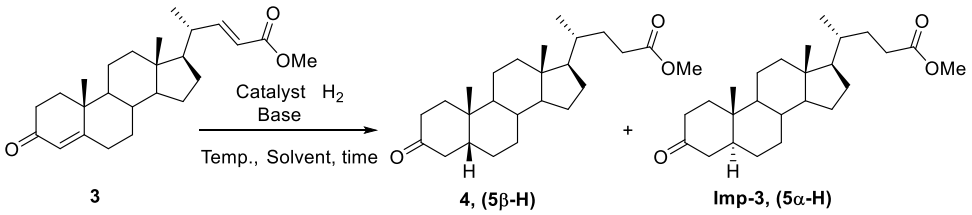


Table 1. Optimization of Reaction Conditions for Hydrogenation and Reduction of Compound 3[a]



entry	cat. (mg)	base (equiv)	solvent (v:v)	temp. (°C)	time (h)	yield (%)[b]	4:Imp-3[c]
1	10%wt Pd/C (5.0)	pyridine (1.0)	MeOH/DCM (1:1)	25	12	64	88:12
2	10%Pd/$CaCO_3$ (5.0)	pyridine (1.0)	MeOH/DCM (1:1)	25	12	47	80:20
3	$PdCl_2(PPh_3)_2$ (5.0)	pyridine (1.0)	MeOH/DCM (1:1)	25	12	0	-
4	$Pd(PPh_3)_4$ (5.0)	pyridine (1.0)	MeOH/DCM (1:1)	25	12	0	-
5	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH/DCM (1:1)	25	12	72	92:8
6	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH/DCM (2:1)	25	12	76	92:8
7	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH/DCM (4:1)	25	12	84	94:6
8	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH/DCM (5:1)	25	12	69	93:7
9	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH/DCM (1:2)	25	12	68	82:13
10	Pd−Cu NWs (5.0)	pyridine (1.0)	MeOH	25	12	75	85:15
11	Pd−Cu NWs (5.0)	pyridine (1.0)	DCM	25	12	40	88:12
12	Pd−Cu NWs (5.0)	CH_3COONH_4 (1.0)	MeOH/DCM (4:1)	25	12	72	90:10
13	Pd−Cu NWs (5.0)	Et_3N (1.0)	MeOH/DCM (4:1)	25	12	89	93:7
14	Pd−Cu NWs (5.0)	cholamine (1.0)	MeOH/DCM (4:1)	25	12	78	89:11
15	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	25	12	92	93:7
16	Pd−Cu NWs (7.0)	Et_3N (1.0)	MeOH/DCM (4:1)	25	12	90	93:7
17	Pd−Cu NWs (6.0)	Et_3N (0.75)	MeOH/DCM (4:1)	25	12	84	93:7
18	Pd−Cu NWs (6.0)	Et_3N (1.2)	MeOH/DCM (4:1)	25	12	91	93:7
19	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	40	12	83	90:10
20	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	10	12	92	97:3
21	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	10	9	92	97:3
22	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	10	7	83	97:3
23[d]	Pd−Cu NWs (3 g)	Et_3N (13 g)	MeOH/DCM (4:1)	10	18	91	96.8:3.2
24[e]	Pd−Cu NWs (6.0)	Et_3N (1.0)	MeOH/DCM (4:1)	10	9	91/89/85	97:3

[a]Reaction conditions: compound 3 (0.26 mmol, 0.1 g), catalyst, 1 atm of H_2, base, solvent: 5 mL. [b]The yield is crude (**4** and Imp-3). [c]The ratio of the isomer was determined by HPLC equipped with an RI detector. [d]Compound 3 (50 g), solvent: 500 mL. [e]Recycled three times: 91% (run 1), 89% (run 2), and 85% (run 3).

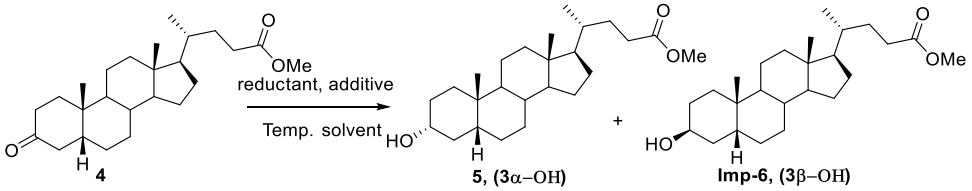
RESULTS AND DISCUSSION

First, we verified the synthetic route using Raney Ni and H_2 to reduce compound **3** for the preparation of LCA, and the results are summarized as follows (Scheme 2): the product was a mixture of different isomers. Although LCA isomers dominated, they were difficult to isolate. As a result, this synthetic route is not advisable.

Hence, we focused on improving the isomer ratio of the hydrogenation of the C4−C5 and reducing the 3-keto group. Our optimized route for synthesizing LCA is shown in Scheme 1B. Bisnoralcohol was chosen as the starting material, and its side chain hydroxyl group was oxidized to an aldehyde in the presence of TEMPO, NCS, TBAB, and $NaHCO_3$ to generate compound **2** with 97% yield (high-performance liquid

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

Table 2. Optimization of Reaction Conditions for Reduction of Compound 4[a]



entry	reductant (g, equiv)	addictive (g, equiv)	solvent	temp. (°C)	yield (%)[b]	5/Imp-6[c]
1	$NaBH_4$ (0.1, 1.03)	/	THF/MeOH (1:1, v-v)	25	89	79/21
2	$NaBH_4$ (0.1, 1.03)	AcOH (0.01, 0.06)	THF/MeOH (1:1, v-v)	25	89	85/15
3	$NaBH_4$ (0.1, 1.03)	H_2O (0.01, 0.22)	THF/MeOH (1:1, v-v)	25	93	88/12
4	$NaBH_4$ (0.1, 1.03)	$CaCl_2{\cdot}6H_2O$ (0.1, 0.18)	THF/MeOH (9:1, v-v)	25	92	91/9
5	$NaBH_4$ (0.1, 1.03)	$MnCl_2{\cdot}H_2O$ (0.1, 0.27)	THF/MeOH (9:1, v-v)	25	89	92/8
6	$NaBH_4$ (0.1, 1.03)	$MnCl_2{\cdot}H_2O$ (0.1, 0.27)	THF/H_2O (9:1, v-v)	25	89	93/7
7	$NaBH_4$ (0.1, 1.03)	$CeCl_3{\cdot}7H_2O$ (0.1, 0.10)	THF/H_2O (9:1, v-v)	25	90	94/6
8	KBH_4 (0.15, 1.08)	$CeCl_3{\cdot}7H_2O$ (0.1, 0.10)	THF/H_2O (9:1, v-v)	25	93	94/6
9	KBH_4 (0.15, 1.08)	$MnCl_2{\cdot}H_2O$ (0.1, 0.27)	THF/H_2O (9:1, v-v)	10	95	96/4
10[d]	KBH_4 (22.5, 2.16)	$MnCl_2{\cdot}H_2O$ (7.5, 0.27)	THF/H_2O (9:1, v-v)	10	96	97/3

[a]Reaction conditions: compound **4** (1.0 g), catalyst, Lewis acid, solvent: 10 mL, 3 h. [b]The yield is crude (5 and Imp-6). [c]The ratio of the isomer was determined by HPLC equipped with an RI detector. [d]Compound **4** (75.0 g), solvent, 500 mL.

chromatography (HPLC) Purity 98.7%). Compound **3** was prepared through a Wittig reaction with 98% yield (HPLC Purity 99.6%). We used methyl chloroacetate to prepare the Wittig reagent because it was much cheaper than ethyl bromoacetate. The choice of methyl chloroacetate had a better cost advantage than Qiu's reported method,[22] and the first two optimizations were mainly based on economic considerations. The isomerization and impurity analysis of steps 3 and 4 are discussed in detail.

We used 3-oxo-4,22-diene-5β-cholanoic acid-24 methyl ester (3) as the starting material to optimize the reaction parameters, and the results are summarized in Table 1. Based on previous reports,[22] we used the mixed solvent of methylene chloride and methanol to improve the isomerization. Thus, different catalysts were synthetically investigated using pyridine as a base under a mixed solvent condition. The result showed that the Pd/C and $Pd/CaCO_3$ exhibited low yields and a moderate isomer ratio, and $PdCl_2(PPh_3)_2$ and $Pd(PPh_3)_4$ did not facilitate the reaction (Table 1, entries 1−4). We recently synthesized Pd−Cu nanowires (Pd−Cu NWs) with high catalytic activity.[25,26] Our finding showed that Pd−Cu NWs were the best catalyst for producing 4 with a good yield and isomer ratio (Table 1, entry 5). We used Pd−Cu NWs as a catalyst for exploring parameters owing to good catalytic performance. For solvents, different volume ratios of methanol and methylene chloride were examined (Table 1, entries 6−11), and the results showed that the optimal volume ratio of methanol to dichloromethane was 4:1. Then, the effects of other bases, such as CH_3COONH_4, Et_3N, and cholamine on the reaction were investigated (Table 1, entries 12−14). A high yield and isomer ratio were obtained in the presence of 1.0 equiv Et_3N. In addition, we investigated the amount of the Pd−Cu NW catalyst and Et_3N base suitable for the reaction. The result showed that the optimal amount of the catalyst and base was 6 mg and 1.0 equiv, respectively (Table 1, entries 15−18). In addition, the reaction temperature and time were optimized (Table 1, entries 19−22). The yield and isomer ratio (yield: 92%, 4: Imp-3 = 97:3) were further improved by reducing the temperature to 10 °C for 9 h (Table 1, entry 21). Finally, the optimum reaction conditions were as follows: 3-oxo-4,22-diene-5β-cholanoic acid-24 methyl ester (3) (0.25 mmol), 1 atm of H_2, Pd−Cu NWs (6 mg), and Et_3N (1.0 equiv) at 10 °C under a mixed solvent condition (methanol: DCM = 4:1, v/v) for 9 h. Compared with the reported methods (Scheme 1A), the reaction conditions, product yield, and isomer ratio of our method were greatly improved (the best result in the literature showing 92:8).[22] Moreover, the reaction was directly enlarged to a scale of 50 g, generating 4 and Imp-3 with a yield of 91% and an isomer ratio of 96.8:3.2 (Table 2, entry 23). Fortunately, the crystal structure of 5β-H isomer (4) was confirmed using X-ray analysis (Figure 2). In addition, the Pd−Cu NW catalyst was

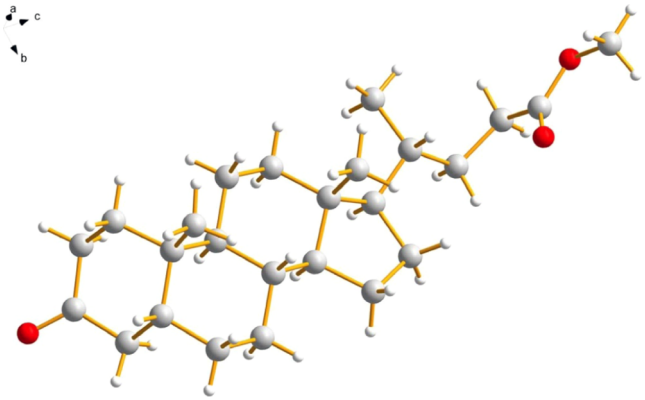


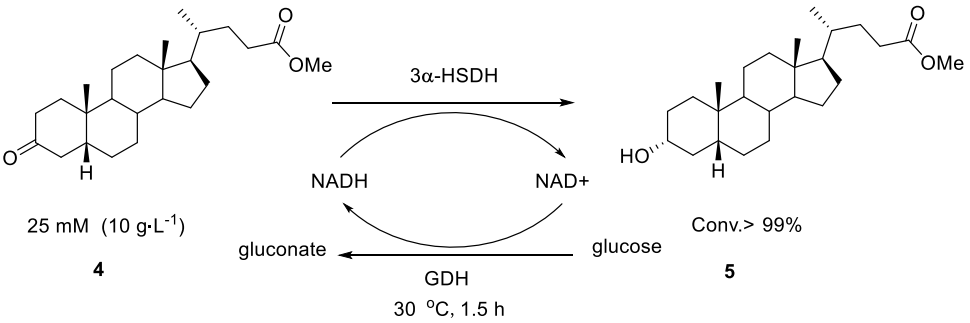
Figure 2. Single-crystal X-ray structure of compound **4**.

separated by sample filtration and could be recycled three times. The catalytic activity did not decrease significantly, and the isomerization ratio remained unchanged.

We also investigated the fourth step of 3-keto group reduction condition optimization (Table 2). First, we reduced the 3-keto group using sodium borohydride as a conventional reducing reagent in a mixed solvent of THF and methanol (1:1, v/v) at room temperature for 2 h. Although this reaction was highly efficient, the selectivity of 3α-OH was moderate at a ratio of approximately 4:1 (Table 2, entry 1). We added a small amount of acetic acid or water to the reaction solution, and the

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

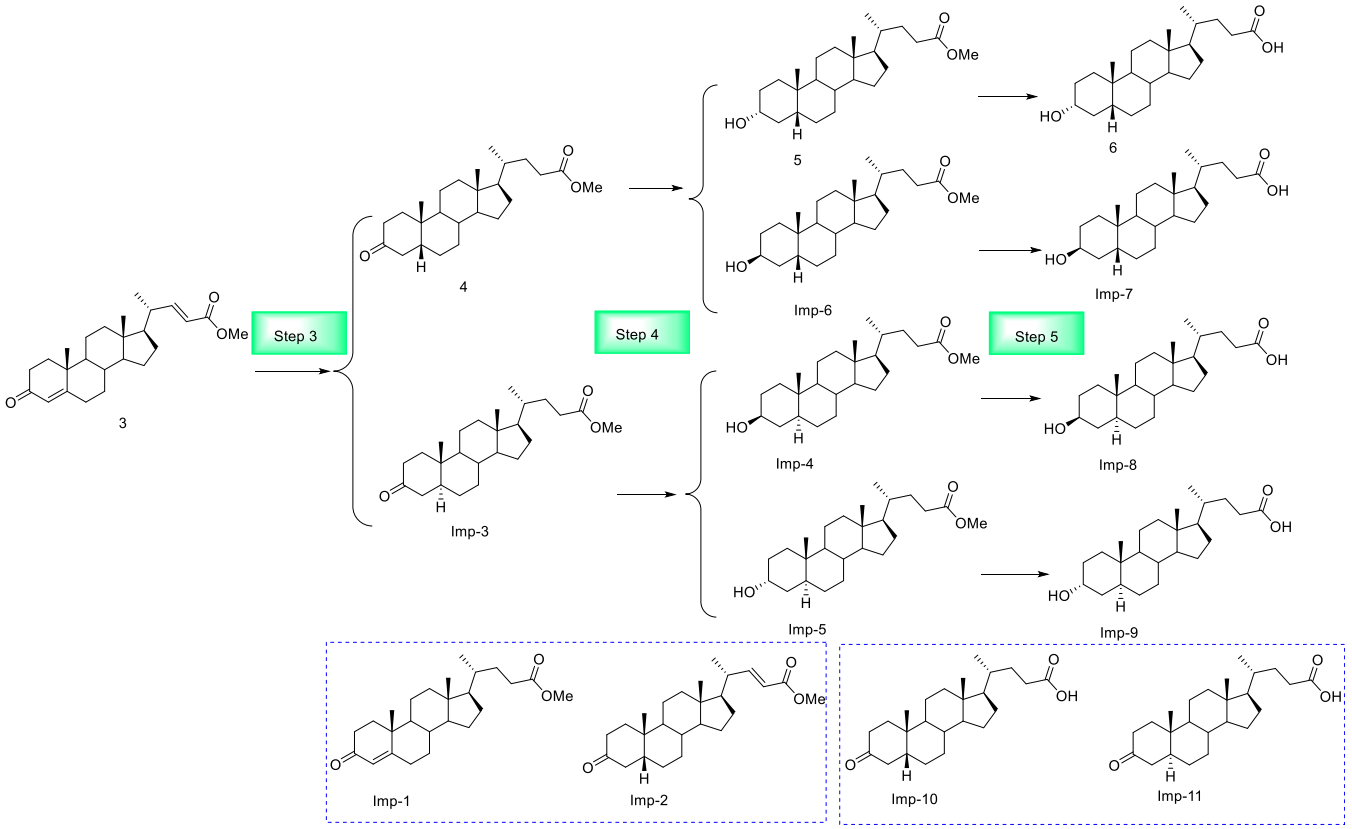
Table 3. Optimization of Reaction Conditions for Reduction of Compound 4 by 3α-HSDH[a]



entry	solvent	3α-HSD[b](400 g/L)	GDH[b] (400 g/L)	gluconate/H_2O	NAD+ (g)	yield (%)[b]	conversion[c]
1	*t*-butanol (25 mL)	5 mL	5 mL	5 g/15 mL	0.025	89	100% (1 h)
2	Isopropanol (25 mL)	5 mL	5 mL	5 g/15 mL	0.025	65	80% (1 h)
3	Ethyl acetate (25 mL)	5 mL	5 mL	5 g/15 mL	0.025	/	20% (1 h)
4	*t*-butanol (25 mL)	4 mL	4 mL	5 g/15 mL	0.025	92	100% (1.5 h)
5	*t*-butanol (25 mL)	3 mL	3 mL	5 g/15 mL	0.025	93	100% (1 h)
6	*t*-butanol (30 mL)	3 mL	3 mL	5 g/15 mL	0.025	80	90% (1 h)
7	*t*-butanol (35 mL)	3 mL	3 mL	5 g/15 mL	0.025	92	100% (1 h)
8	*t*-butanol (35 mL)	3 mL	3 mL	4.5 g/15 mL	0.015	93	100% (1 h)
9	*t*-butanol (35 mL)	3 mL	3 mL	4.0 g/15 mL	0.015	95	100% (1 h)
10[d]	*t*-butanol (350 mL)	30 mL	30 mL	45g/150 mL	0.15	95	100% (1.5 h)

[a]Charged with compound **4** (5 g); the pH (6.8−7.2) was adjusted with sodium hydroxide solution (1 mol/L), 30 °C. [b]The concentration of cells was about 400 g/L. [c]Determined by HPLC. [d]Charged with compound **4** (50 g).

Scheme 3. Impurity Analysis for Steps 3−5



reaction became more intense, and the selectivity of 3α-OH was slightly improved (Table 2, entries 2 and 3). Lewis acids, including $CaCl_2·6H_2O$, $CeCl_3·7H_2O$, and $MnCl_2·H_2O$, were added to the reaction solution, which significantly improved the 3α-OH selectivity. In addition, we found that the mixed system of THF/H_2O could improve the selectivity of 3α-OH more than that of THF/MeOH (Table 2, entries 4−7). Furthermore, other reducing reagents, Lewis acids, and reaction temperature were examined (Table 2, entries 8 and 9). The results showed that potassium borohydride was used

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

Table 4. Control Strategy and Impurity Limit of Process-Related Impurity

intermediate	crude product	control strategy[a]	retention time (min)	impurity limit
4		recrystallized twice, first in MeOH and then in ethyl acetate:	12.563	
	Imp-1 (ND)[b]		12.125	Imp-1 <0.10%
	Imp-2 (ND)[b]	Imp-3 (1.35%)	11.993	Imp-2 <0.10%
	Imp-3 (2.6%)[a]		13.402	Imp-3 <1.5%
5		recrystallized with ethyl acetate: 4 (0.07%)	10.825	
	4 (0.2%)[a]		12.563	4 <0.10%
	Imp-3 (0.6%)		13.402	Imp-3 <0.10%
	Imp-4 (ND)[b]		10.476	Imp-4 <0.10%
	Imp-5 (0.5%)[a]		11.277	Imp-5 <0.10%
	Imp-6 (ND)[b]		10.244	Imp-6 <0.10%
6 (LCA)		recrystallized with MeOH/DCM (10:1):	8.658	
	Imp-7 (ND)[b]		6.787	Imp-7<0.10%
	Imp-8 (ND)[b]		6.524	Imp-8 <0.10%
	Imp-9 (ND)[b]	Imp-10 (0.07%)	9.216	Imp-9 <0.10%
	Imp-10 (0.08%)[a]		9.352	Imp-10 <0.10%
	Imp-11 (ND)[b]		8.936	Imp-11 <0.10%

[a]Content assay of impurities in product using the peak area normalization method. [b]Not detected on HPLC.

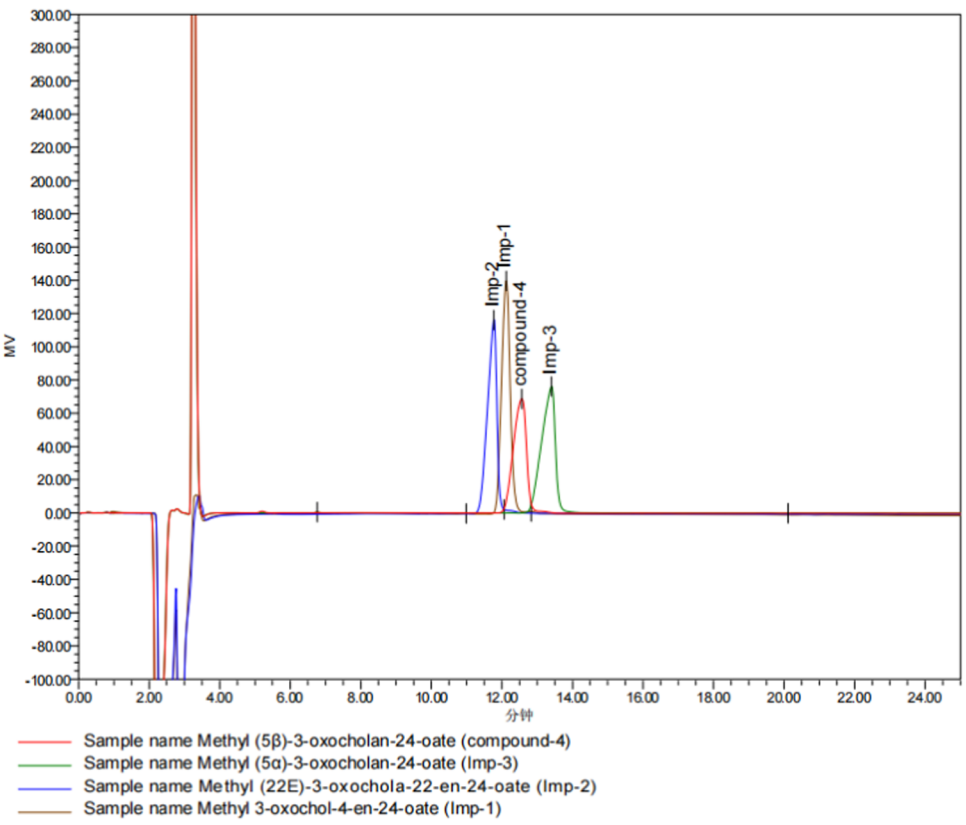


Figure 3. HPLC spectrum of compound **4** and Imp-1−3 (method 2; Imp-2: 11.773, Imp-1: 12.125, compound **4**: 12.563, Imp-3: 13.402).

as the reduction reagent and manganese chloride was used as the additive. The reaction yield and selectivity were the highest when the temperature was 10 °C. Finally, the selectivity of 3α-OH was increased to 96.7%, and the yield was 95% (Table 2, entry 9). In addition, the reaction was directly enlarged to a scale of 75 g, generating **5** and Imp-6 with 96% yield and an isomer ratio of 97:3. Compared to the reported method,[22] the optimized method not only had mild conditions but also improved the yield and isomer ratio.

In general, a 100% isomerization product is undoubtedly the most desirable outcome for drug synthesis, 3α-hydroxysteroid dehydrogenase (3α-HSDH) from *Comamonas* testosteroni stereoselectively reduced prochiral ketones to chiral secondary alcohols using reduced nicotinamide adenine dinucleotide (NADH) as a cofactor, which was regenerated with a recycling system comprising glucose dehydrogenase (GDH) and glucose. The structure and reaction mechanism of the 3α-HSDH is clear.[27−32] 3α-HSDH and GDH were heterologously expressed in *Escherichia coli* BL21 (DE3). Based on the stability of the enzyme and the cost of subsequent industrialization, the 3α-HSDH and the GDH used were both high-density cultured *E. coli* (under the condition of 50−

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

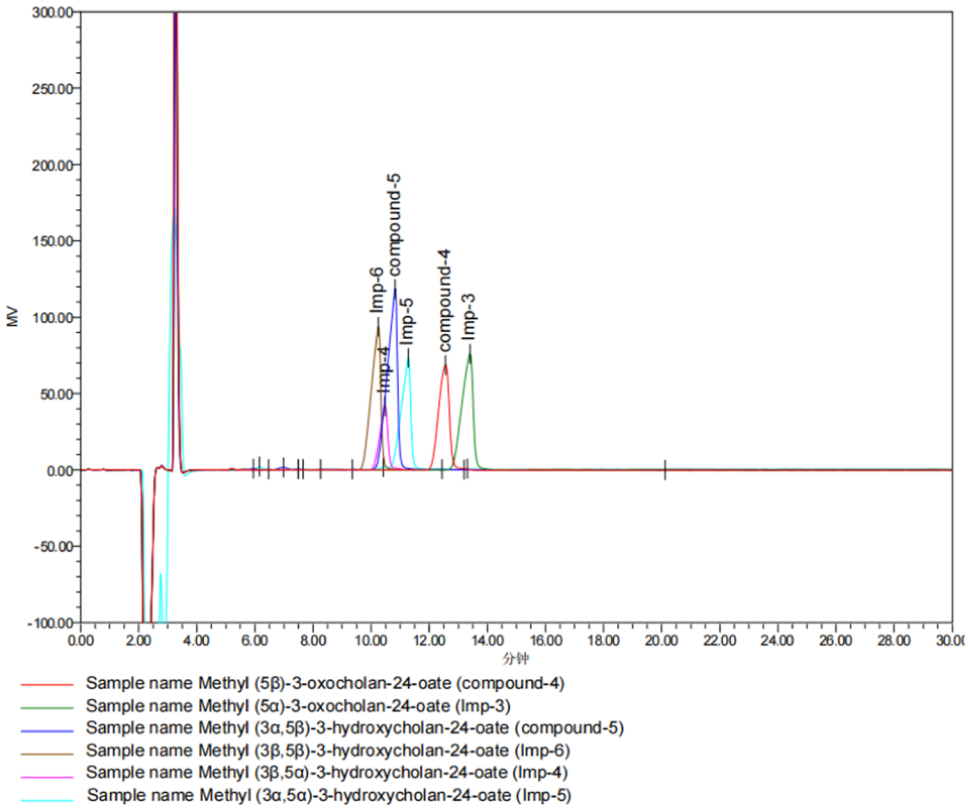


Figure 4. HPLC spectrum of compounds **4** and **5** and impurities 3−6 (method 2; Imp-6: 10.244, Imp-4: 10.476, Compound **5**: 10.825, Imp-5: 11.277, Compound **4**: 12.563, Imp-3: 13.402).

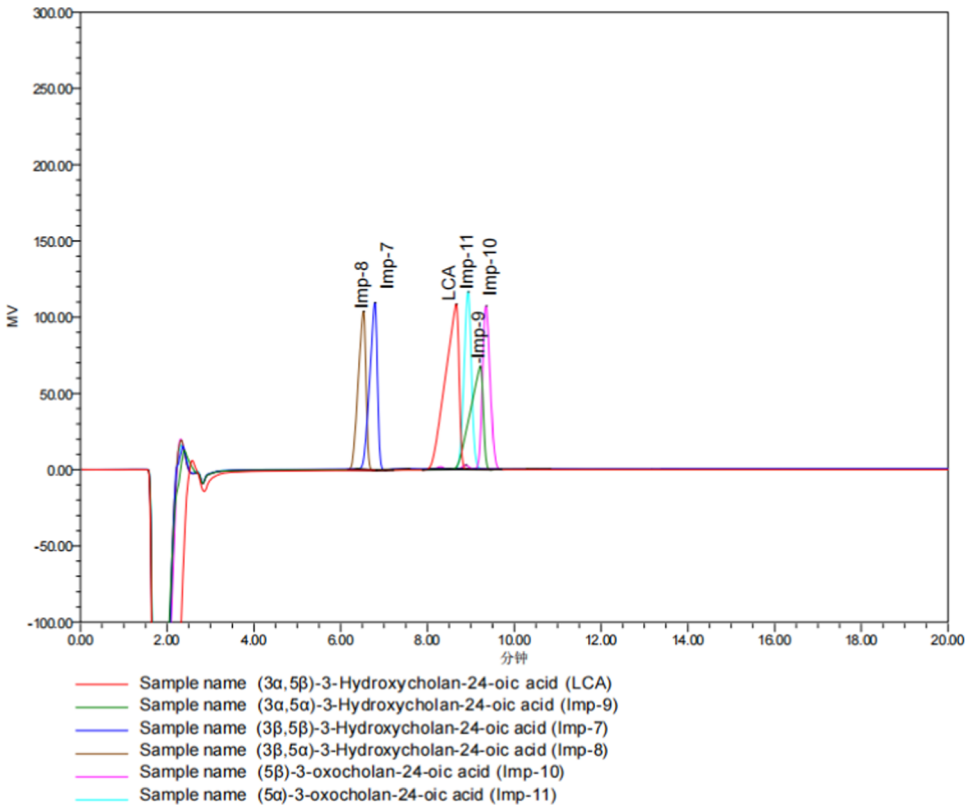


Figure 5. HPLC spectrum of LCA and Imp-7−11 (method 3; Imp-8: 6.524, Imp-7: 6.787, LCA: 8.658, Imp-11: 8.936, Imp-9: 9.216, Imp-10: 9.352).

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

60% concentration of *tert*-butanol/water, the cells would automatically break, thereby releasing the enzyme). Under the optimum conditions (Table 3, entries 1−9), 25 mM (10 g/L) compound **4** was smoothly reduced by the crude enzymes of 3α-HSDH and GDH in a 600 mL scale biotransformation reactor, achieving 100% conversion and generating compound **5** with >99% isomer ratio (Table 3, entry 10).

The analysis and control of impurities play a crucial role in drug synthesis. Our primary objective was to obtain a high-quality LCA and develop a robust process that allows for quality control. Process-related impurities or intermediates share similar physical properties and structures. In Scheme 3, we demonstrated impurity analysis, while Table 4 presents the impurity limit and control strategy for process-related impurities. By effectively managing the 5-hydrogen and 3-hydroxy isomers during the reaction, we were able to successfully control impurities. The key to achieving quality control was managing the contents of Imp-3, which resulted from hydrogenation and reduction of compound **3**. In step 3, we limited Imp-3 to less than 1.5% by double recrystallization (first in MeOH and then in ethyl acetate). By recrystallizing ethyl acetate once more in step 4, we were able to limit Imp-3 and Imp-5 (3-keto reduction products of Imp-3) to 0.10% (Table 4).

The control of the configuration of 5-H and 3-OH impurities is difficult. The development of analytical methods for isomer impurities is also difficult because of their similar polarity. Three HPLC methods were developed to control the quality of LCA through directional preparation and analysis of impurities. Analytical method 1 revealed that compounds **1**−**3**, with ultraviolet (UV) absorption, had a good separation (compound **1**, RT: 14.113; compound **2**, RT: 20.536; and compound **3**, RT: 24.632). During the hydrogenation of step 3, Imp-1 and Imp-2 might be formed due to incomplete hydrogenation and 5α-H chiral isomer Imp-3 might also be produced. In the actual reaction, Imp-1 and Imp-2 were not detected in compound **4** (Table 4), which might be due to the hydrogenation reaction of two enone-ketone conjugated structures (3-ketone-4,5-ene, 22,23-ene-24 carboxyl-methyl ester) in the palladium−copper catalyst system that is easy to take place at atmospheric pressure. Compound **4** has good separation with Imp-3 (diastereomer, Figure 3; method 2, RT: 12.465; Imp-3, RT: 13.402).

In the enzyme-catalyzed process of step 4 (Scheme 3), we successfully reduced compound **4** to target compound **5**. While compound **5** showed good separation from compound **4** and Imp-3 (Figure 4, method 2), the separation between compound **5** and diastereomer impurities 4−6 was not ideal. These impurities could potentially produce new impurities (Imp-7, Imp-8, Imp-9) in step 5. To address this, we prepared the hydrolysis products of the diastereomers (Imp-7, Imp-8, Imp-9) and coupled them with impurities 10 and 11 (hydrolysis products of compound **4** and Imp-3) to further confirm the impurity residue of the final product. The data analysis (Figure 5, method 3) revealed no 3β-isomer impurities in the final product LCA, which was obtained through recrystallization at a purity of 99.8%. The results provide further evidence of the stereoselectivity of 3α-HSDH for the reduction of the 3-ketone compared to hydride reducing agents like KBH_4.

CONCLUSIONS

In this study, lithocholic acid (LCA) was prepared via Tempo oxidation, Witting reaction, hydrogenation at atmospheric pressure, enzymatic reduction, and hydrolysis using bisnoralcohol (BA) as a starting material. High-quality lithocholic acid was obtained by optimizing the catalytic hydrogenation and reduction conditions using Pd−Cu NWs and 3α-HSDH and controlling the ratio of 5-hydrogen and 3-hydroxy chiral isomers. HPLC was used to determine the purity of lithocholic acid. The purity and the total yield of LCA were improved to 99.8% and 70.8%, respectively. Due to the similar solubility and polarity of chiral isomers, impurities in drugs were difficult to detect and remove using conventional purification. We developed three analytical methods to detect and stepwise control impurities involved in the synthetic route of LCA and its intermediates to meet the initial raw material requirements of the active pharmaceutical ingredient (API) preparation standard. Compared to the reported method, the optimized route significantly improved the isomer ratio and overall yield and was more suitable for industrial production.

EXPERIMENTAL SECTION

General information. Commercially available reagents and solvents were used as received. Bisnoralcohol, NAD^+, 3α-HSDH, and GDH were supplied by Hunan Norchem Pharmaceutical Co., Ltd. The 400 and 100 MHz ^1H and ^{13}C NMR spectra were recorded with a Bruker Ascend-400 spectrometer using $CDCl_3$, DMSO-d_6, or CD_3OD and tetramethylsilane as the solvent and internal reference, respectively. Reactions were monitored using TLC with F_{254} silica-gel precoated sheets (Merck Darmstadt, Germany) and treated with oxidizing solutions (phosphomolybdic reagent). Electrospray ionization mass spectrometry (ESI-MS) was carried out on a Waters Alliance e2695-ACQUITY QDa. The Pd−Cu NW catalyst was prepared according to the literature method.[25]

HPLC Analysis Method 1. Chromatographic separation of compounds **1**−**3** was achieved using a reversed-phase column (C18, 4.6 × 250 mm, 5 μm, Waters e2695−2489 equipped with a 2489 ultraviolet/visible (UV/Vis) detector at 241 nm) at a flow of 1 mL/min and a column temperature of 30 °C. The mobile phase comprised acetonitrile (solvent A) and 0.1% of phosphoric acid (solvent B). The gradient pattern includes 0 min, 50% B; 15 min, 20% B; 25 min, 0% B; and 50.1 min, 50% B. The injection volume was set as 20 μL.

HPLC Analysis Method 2. Chromatographic separation of compounds **4** and **5** and Imp-1−6 was achieved using a reversed-phase column (C18, 4.6 × 250 mm, 3.5 μm, Waters e2695−2489 equipped with a 2414 RI detector) at a flow of 0.8 mL/min and a column temperature of 40 °C. The mobile phase comprised monopotassium phosphate solution (3.4 g/L), acetonitrile, and phosphoric acid (30:70:0.1, v/v/v). The injection volume was set as 30 μL.

HPLC Analysis Method 3. Chromatographic separation of compound **6** and Imp-7−11 was achieved using a reversed-phase column (C18, 4.6 × 250 mm, 5 μm, Waters e2695−2489 equipped with a 2414 RI detector) at a flow of 1 mL/min and a column temperature of 40 °C. The mobile phase comprised acetonitrile, water, and trifluoroacetic acid (70:30:0.1, v/v/v). The injection volume was set as 50 μL.

Typical Experimental Procedure for the Synthesis of (20S)-3-Oxopregn-4-ene-20-carboxaldehyde (2). Sodium

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

bromide (7.2 g, 70.0 mmol) and sodium bicarbonate (5.0 g, 59.5 mmol) were dissolved in H_2O (200.0 mL) in a 500 mL beaker and stirred to obtain a uniform solution. Dichloromethane (600.0 mL), compound **1** (bisnoralcohol, 200.0 g, 605.1 mmol), and TEMPO (1.0 g, 6.4 mmol) were transferred into a 2000 mL four-neck round-bottom flask equipped with a magnetic stirrer, addition funnel, and thermometer. The mixture was stirred to obtain a uniform solution, which was added to the above previously prepared aqueous solution. The resultant mixed solution was stirred at 0 °C; afterward, sodium hypochlorite aqueous solution (10%, 500.0 g, 671.7 mmol) was dropwise added for 2 h. Then, the obtained solution was stored at 0 °C for 0.5 h. The reaction was detected by TLC (toluene:ethyl acetate = 1:1), then quenched by adding sodium thiosulfate aqueous solution (containing sodium 20.0 g of thiosulfate and 180.0 g of water), and stirred at 20 °C for 30 min. The nonoxidative property of the solution was examined using starch potassium iodide test paper. Water (100 mL) was added to the mixed solution, stirred for 10 min, and allowed to stand for separation. Then, the aqueous phase was extracted once with dichloromethane (100 mL), and the organic phases were combined and washed once with water (200 mL). The organic phase was concentrated to a small volume and replaced with n-heptane (100 mL × 3), cooled to 0−5 °C, stood for 1 h, and filtered. The filter cake was dried at 55 °C to obtain a white solid (192.8 g, yield: 97.0%, HPLC 98.7%, method 1). The spectroscopic data were in agreement with the reported data.[33]

Bisnoralcohol. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.70 (d, J = 1.8 Hz, 1H, H-4), 3.61 (dd, J = 10.5, 3.2 Hz, 1H, H-22), 3.34 (dd, J = 10.5, 6.9 Hz, 1H, H-22), 2.46−2.20 (m, 4H), 2.05−1.96 (m, 2H), 1.87−1.76 (m, 2H), 1.72−1.26 (m, 9H), 1.23−1.18 (m, 1H), 1.16 (s, 3H), 1.11 (dt, J = 11.7, 5.9 Hz, 1H), 1.02 (d, J = 6.6 Hz, 3H), 1.00−0.85 (m, 3H), 0.71 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.8 (C-3), 171.8 (C-5), 123.8 (C-4), 67.9, 55.7, 53.8, 52.5, 42.6, 39.5, 38.8, 38.7, 35.7, 35.7, 34.0, 33.0, 32.1, 27.7, 24.4, 21.1, 17.5, 16.8, 12.1.

Compound **2**. Light-yellow powder, Mp: 159−160 °C, (ref 22 Mp: 155−157 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.54 (d, J = 3.2 Hz, 1H, H-22), 5.70 (d, J = 1.8 Hz, 1H, H-4), 2.49−2.17 (m, 5H), 2.06−1.29 (m, 12H), 1.26−1.18 (m, 2H), 1.16 (s, 3H), 1.10 (d, J = 6.8 Hz, 3H), 1.08−0.88 (m, 2H), 0.74 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 204.9 (C-22), 199.5 (C-3), 171.2 (C-5), 123.9 (C-4), 55.2, 53.8, 51.0, 49.5, 43.1, 39.4, 38.6, 35.8, 35.7, 34.0, 32.9, 32.0, 27.1, 24.6, 21.0, 17.5, 13.5, 12.4; Ms (ESI) m/z: $C_{22}H_{32}O_2$ $[M + H]^+$, calcd for 329.24; found, 329.24.

Typical Experimental Procedure for the Synthesis of Methyl (22E)-3-Oxochola-4,22-dien-24-oate (3). Preparation of Witting reagent: toluene (600.0 mL), water (600.0 mL), triphenylphosphine (200.0 g, 762.5 mmol), potassium iodide (12.8 g, 77.1 mmol), and methyl chloroacetate (87.0 g, 801.7 mmol) were transferred into a 3000 mL three-neck round-bottom flask equipped with a mechanical stirrer. The temperature of the mixed solution was raised to 70−75 °C and stirred for 3 h. TLC analysis (PE/EA 5:1) revealed the complete consumption of triphenylphosphine. The system was cooled to 25−35 °C; then, water (600.0 mL) was added, stirred for 30 min, and allowed to stand for 30 min. Afterward, the organic layer was separated, and the aqueous phase was extracted with toluene (200 mL × 2). The temperature of the system was regulated to 5−10 °C. And the aqueous phase was adjusted to pH 8−9 by slowly adding sodium hydroxide solution (30.0 g of NaOH and 400.0 mL of H_2O), and a large number of solids were precipitated. The solid was filtered and dried at 40 °C (245.3 g, yield: 86.8%).

THF (600.0 mL) and compound **2** (100.0 g, 304.4 mmol) were transferred to a 1000 mL round-bottom flask and stirred to obtain a uniform solution, and Witting reagent (150.0 g, 404.5 mmol) was added to the mixed solution. The temperature of the system was raised to a reflux state (65 °C), and the sample was reacted for 18 h. The reaction was detected using TLC (PE:EA = 5:1). Water (50.0 mL) was added to the sample after it was cooled to 40 °C, and the mixture was concentrated under reduced pressure at 55 °C to form a viscous solid. The viscous solid system was transferred to a 3000 mL four-neck reaction flask containing acetone (900.0 mL) and mechanically stirred to form at a temperature range of 50−55 °C to obtain a uniform and clear solution. Then, water (900.0 mL) was slowly added to the obtained solution and stirred for 30 min to precipitate a large number of off-white solids. Afterward, the sample was slowly allowed to cool to 20−25 °C for 1 h for crystallization. Then, the sample was filtered and washed twice with a small amount of acetone/water (1:1, 30 mL each time). The solid was dried at 50 °C (109.5 g, yield: 93.5%, HPLC 99.6%, method 1). The spectroscopic data were in agreement with the reported data.[34]

Compound **3**. White powder, Mp: 148.5−150.5 °C, (ref 34 Mp: 146.5−148.5 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.81 (dd, J = 15.6, 9.0 Hz, 1H, H-22), 5.73 (d, J = 16.2 Hz, 1H, H-23), 5.71 (s, 1H, H-4), 3.70 (s, 3H, $-OCH_3$), 2.47−2.20 (m, 5H), 2.00 (dq, J = 12.1, 3.9, 3.4 Hz, 2H), 1.88−1.35 (m, 8H), 1.23 (dt, J = 12.4, 4.5 Hz, 3H), 1.17 (s, 3H), 1.07 (d, J = 6.7 Hz, 3H), 1.04−0.87 (m, 3H), 0.73 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 199.5 (C-3), 171.3 (C-5), 167.4 (C-24), 154.7 (C-22), 123.8 (C-4), 118.7 (C-23), 55.7, 54.9, 53.7, 51.4, 42.7, 39.7, 39.4, 38.6, 35.7, 35.6, 34.0, 32.9, 31.9, 28.0, 24.2, 21.0, 19.2, 17.4, 12.2; Ms (ESI) m/z: $C_{25}H_{36}O_3$ $[M + H]^+$, calcd for 385.27; found, 385.20.

Typical Experimental Procedure for the Synthesis of Methyl (5β)-3-Oxocholan-24-oate (4). Compound **3** (100.0 g, 260.0 mmol), triethylamine (13.0 g), dichloromethane (400.0 mL), and methanol (100.0 mL) were transferred to a 1000 mL round-bottom flask and stirred at a room temperature, and then Pd−Cu NWs (6.0 g) were added. The system was first replaced with nitrogen three times and then with hydrogen three times, and the reaction was performed under pressure in a hydrogen balloon (the pressure was about 20−30 cm water column) (TLC developing solvent was PE:EA = 5:1). After the reaction was completed, the system was replaced with nitrogen, and the catalyst was recovered by filtration, followed by washing thoroughly with ethyl acetate and water. The filtrate was concentrated to a viscous state. Subsequently, methanol (500 mL) was added to dissolve the sample at 50 °C and was concentrated, and the remaining volume was 100−120 mL. When the temperature was lowered to 10−15 °C, the solid was suction-filtered. The solid was repurified and recrystallized with ethyl acetate (89.6 g, yield: 88.7%, HPLC 98.0%, 5α-isomer 1.3%, method 2). The spectroscopic data were in agreement with the reported data.[6]

Compound **4**. White powder, Mp: 121.0−122.0 °C, (ref 35 Mp: 119 °C); ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.64 (s, 3H, $-OCH_3$), 2.67 (dd, J = 15.1, 13.3 Hz, 1H, 4α-H), 2.32 (tq, J = 15.2, 5.3 Hz, 2H), 2.25−2.09 (m, 2H), 2.00 (dtd, J = 14.8, 5.4, 2.8 Hz, 3H), 1.92−1.71 (m, 4H), 1.63−1.01 (m, 17H), 0.99 (s, 3H), 0.90 (d, J = 6.5 Hz, 3H), 0.66 (s, 3H); ^{13}C NMR (100

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

MHz, $CDCl_3$) δ 213.4 (C-3), 174.7 (C-24), 56.4, 55.9, 51.5, 44.3, 42.8, 42.4, 40.7, 40.0, 37.2, 37.0, 35.5, 35.3, 34.9, 31.0, 28.2, 26.6, 25.8, 25.7, 24.2, 22.7, 21.2, 18.3, 12.1; Ms (ESI) m/z: $C_{25}H_{40}O_3$ $[M + H]^+$, calcd for 389.30; found, 389.21.

Crystal data for 4: $C_{25}H_{40}O_3$; Mr = 388.57, monoclinic, space group P 21/c, a = 9.753 (5) Å, b = 7.6611 (18) Å, c = 14.684 (5) Å; V = 1093.7 (6) Å ^{3}; T = 273.15 K; Z = 2; reflections collected/unique, 5442/2921 R_{int} = 0.0523, R_1 = 0.0426, wR_2 = 0.1093; GOF = 1.048; CCDC-2251977 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.

Typical Experimental Procedure for the Synthesis of Methyl (3α,5β)-3-Hydroxycholan-24-oate (5). Method A (Potassium Borohydride Reduction). Tetrahydrofuran (500.0 mL), water (50.0 mL), and compound **4** (75.0 g, 193.0 mmol) were transferred into a 1000 mL round-bottom flask and stirred at 5 °C, and then anhydrous manganese chloride (7.5 g, 59.6 mmol) was added to the mixture. When T ≤ 5 °C, potassium borohydride (22.5 g, 417.1 mmol) was added to the system in batches (about 1.0 g every 5 to 7 min) for about 2 h, and the reaction was detected with TLC (PE:EA = 5:1). When the temperature was set to 5 °C, the mixture was adjusted to pH 6−7 with 10% hydrochloric acid. Then, the mixture was concentrated at 40−50 °C until no fraction was observed, and water was added to the concentrated mixture (150.0 mL). Then, combined organic phases were extracted with dichloromethane (150.0 mL × 2) and washed once with water (150.0 mL). The organic phase was concentrated. Then, isopropyl ether (750.0 mL) was added to the concentrated organic phase, and the temperature of the system was raised to a reflux state (68−70 °C) to dissolve the solid. Afterward, the system was gradually cooled to 0−10 °C, stirred for 1 h, and filtered to obtain compound **5** (71.5 g, yield: 94.5%, HPLC 3α-isomer: 96.7%, 3β-isomer: 2.7%, method 2).

Method B (Enzymatic Reduction). At room temperature, *tert*-butanol (350.0 mL), compound **4** (50.0 g, 128.7 mmol), and glucose solution containing glucose (45.0 g) and water (150.0 mL) were added into a 1000 mL round-bottom flask and stirred at 30 °C. Oxidized NAD^+ (0.15 g) was added to the mixed solution. Then, the pH of the solution was adjusted to 6.8−7.2 with sodium hydroxide solution (1 mol/L) using an autocompensator. After the temperature and pH were stabilized, GDH (30.0 mL, 400 g/L) and 3α-HSDH (30.0 mL, 400 g/L) were added to the solution, and then the pH of the system was adjusted to 6.8−7.2 with the autocompensator using sodium hydroxide solution (1 mol/L). Finally, when the pH of the solution remained constant, the reaction was completely detected with TLC (PE:EA = 5:1). After the reaction was completed, the resultant solution was heated to 50−60 °C and stirred for 30 min. Then, *tert*-butanol was concentrated and recovered under low pressure and replaced with water (100 mL × 3). Afterward, the temperature of the system was reduced to 5 °C, and the sample was stirred for 30 min, filtered, washed with water, and dried at 50 °C (51.6 g, HPLC 3α-isomer: 96.8%, no 3β-isomer, method 2).

Recrystallization: ethyl acetate (1000 mL, containing about 16 mL/g of compound **5** at room temperature), activated carbon (5.0 g, which has decolorization and filtration aid), and the above solids were added into a 1000 mL flask. The solution was stirred at 40 °C for 30 min and filtered, and the filtrate was concentrated to a small volume. The filtrate formed a white crystal and dried at 50 °C (47.8 g, yield: 95.1%, HPLC 3α-isomer: 98.5%, no 3β-isomer, method 2). The spectroscopic data were in agreement with the reported data.[6]

*Compound **5**.* White powder, Mp: 126.5−128.0 °C, (ref 36 Mp: 125.4 °C) ^{1}H NMR (400 MHz, $CDCl_3$) δ 3.65 (s, 3H, $-OCH_3$), 3.61 (dq, J = 10.6, 5.2, 4.7 Hz, 1H, 3β-H), 2.38−2.30 (m,1H), 2.24−2.17 (m, 1H), 2.00−1.90 (m, 1H), 1.90−0.94 (m, 26H), 0.91 (s, 3H), 0.90 (d, J = 8.0 Hz, 3H), 0.63 (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 174.8 (C-24), 71.8 (C-3), 56.5, 56.0, 51.5, 42.7, 42.1, 40.4, 40.2, 36.5, 35.9, 35.4, 34.6, 31.1, 31.0, 30.6, 28.2, 27.2, 26.4, 24.2, 23.4, 20.8, 18.3, 12.0; Ms (ESI) m/z: $C_{25}H_{42}O_3$ $[M + H]^+$, calcd for 391.31; found, 391.14.

*Typical Experimental Procedure for the Synthesis of (3α,5β)-3-Hydroxycholan-24-oic Acid (**6**).* Water (60.0 mL) and sodium hydroxide (4.8 g, 120 mmol) were added to a 500 mL round-bottom flask and stirred to obtain a uniform mixture. Then, methanol (150 mL) and compound **5** (30.0 g 76.8 mmol) were added to the mixture. The suspension was heated to 45 °C for 12 h, and the completion of the reaction was confirmed using TLC (PE:EA = 2:1). Then, water (150 mL) was added after the completion of the reaction. The mixture was adjusted to pH 3 with 10% hydrochloric acid (45 mL) and then stirred for 0.5 h. Afterward, the sample was filtered, washed with water until the sample became neutral, and dried at 55 °C to form a white solid (28.4 g, HPLC 98.2%). Recrystallization: The solid was recrystallized with methanol/dichloromethane (10:1) to obtain LCA (26.7 g, yield: 92.3%, HPLC 99.8%, method 3). The spectroscopic data were in agreement with the reported data.[21]

*Compound **6**.* White powder, Mp: 186.0−187.5 °C, (ref 22 Mp: 185−186 °C) ^{1}H NMR (400 MHz, 90% CD_3OD + 10% $CDCl_3$) δ 3.56 (tt, J = 10.8, 4.6 Hz, 1H, 3β-H), 2.38−2.30 (m,1H), 2.24−2.16 (m, 1H), 2.02−1.98 (m, 1H), 1.95−0.97 (m, 26H), 0.95 (d, J = 5.7 Hz, 3H), 0.94 (s, 3H), 0.68 (s, 3H); ^{13}C NMR (100 MHz, 90% CD_3OD+10% $CDCl_3$) δ 177.0 (C-24), 71.1 (C-3), 56.5, 56.0, 42.6, 42.1, 40.5, 40.1, 35.8, 35.8, 35.3, 35.2, 34.4, 31.0, 30.8, 29.8, 28.0, 27.1, 26.3, 24.0, 22.9, 20.7, 17.7, 11.5; Ms (ESI) m/z: $C_{24}H_{40}O_3$ $[M - H]^-$, calcd for 375.30; found, 375.46; HRMS (ESI) m/z: $C_{24}H_{40}O_3$ $[M + NH_4]^+$, calcd for 394.3316; found, 394.3311.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c02548.

Details of preparation experiments of impurities and copies of NMR spectra; HPLC of compounds **1**−**6** and Imp-1−11; and MS of compounds **2**−**6** and impurities 1−11 (PDF)

FAIR data, including the primary NMR FID files, for compounds **1**−**10** and impurities 1−11 (ZIP)

■ AUTHOR INFORMATION

Corresponding Authors

Xinhua Xu − *College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China;* orcid.org/0000-0001-8975-2943; Phone: (+86) 731-88821546; Email: xhx1581@hnu.edu.cn

Ningbo Li − *School of Basic Medical Sciences, Shanxi Medical University, Taiyuan 030001, China*; Email: ningboli@sxmu.edu.cn

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141

Authors

Chunling Zeng − *College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China*
Shitang Xu − *College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China*
Zhenlong Yin − *College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China*
Yue Cui − *College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.3c02548

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors thank the National Natural Science Foundation of China (21802093), the Natural Science Foundation of Shanxi Province (202203021211240), and the Ph.D. Start-up Foundation of Shanxi Medical University (03201501) for financial support. They also thank Emmanuel A., Ph.D. from Editorbar Language Editing, Beijing, China (www.editorbar.com), for editing the English text of a draft of this manuscript.

■ REFERENCES

(1) Chang, K.-H.; Lee, L.; Chen, J.; Li, W. S. Lithocholic acid analogues, new and potent alpha-2,3-sialyltransferase inhibitors. *Chem. Commun.* **2006**, *6*, 629−631.
(2) He, H.-B.; Gao, L.-X.; Deng, Q.-F.; Ma, W.-P.; Tang, C.-L.; Qiu, W.-W.; Tang, J.; Li, J.-Y.; Li, J.; Yang, F. Synthesis and biological evaluation of 4,4-dimethyl lithocholic acid derivatives as novel inhibitors of protein tyrosine phosphatase 1B. *Bioorg. Med. Chem. Lett.* **2012**, *22*, 7237−7242.
(3) Nehring, J. A.; Zierold, C.; DeLuca, H. F. Lithocholic acid can carry out in vivo functions of vitamin D. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 10006−10009.
(4) Adachi, R.; Honma, Y.; Masuno, H.; Kawana, K.; Shimomura, L.; Yamada, S.; Makishima, M. Selective activation of vitamin D receptor by lithocholic acid acetate, a bile acid derivative. *J. Lipid Res.* **2005**, *46*, 46−57.
(5) Ishizawa, M.; Matsunawa, M.; Adachi, R.; Uno, S.; Keda, K.; Masuno, H.; Shimizu, M.; Iwasaki, K.-I.; Yamada, S.; Makishima, M. Lithocholic acid derivatives act as selective vitamin D receptor modulators without inducing hypercalcemia. *J. Lipid Res.* **2008**, *49*, 763−772.
(6) do Nascimento, P. G. G.; Lemos, T. L. G.; Almeida, M. C. S.; de Souza, J. M. O.; Bizerra, A. M. C.; Santiago, G. M. P.; da Costa, J. G. M.; Coutinho, H. D. M. Lithocholic acid and derivatives: Antibacterial activity. *Steroids* **2015**, *104*, 8−15.
(7) Ward, J. B. J.; Lajczak, N. K.; Kelly, O. B.; O'Dwyer, A. M.; Giddam, A. K.; Gabhann, J. N.; Franco, P.; Tambuwala, M. M.; Jefferies, C. A.; Keely, S.; et al. Ursodeoxycholic acid and lithocholic acid exert anti-inflammatory actions in the colon. *Am. J. Physiol.: Gastrointest. Liver Physiol.* **2017**, *312*, G550−G558.
(8) Luu, T. H.; Bard, J.-M.; Carbonnelle, D.; Chaillou, C.; Huvelin, J.-M.; Bobin-Dubigeon, C.; Nazih, H. Lithocholic bile acid inhibits lipogenesis and induces apoptosis in breast cancer cells. *Cell. Oncol.* **2018**, *41*, 13−24.
(9) Mikó, E.; Vida, A.; Kovacs, T.; Ujlaki, G.; Trencsenyi, G.; Marton, J.; Sari, Z.; Kovacs, P.; Boratko, A.; Hujber, Z.; et al. Lithocholic acid, a bacterial metabolite reduces breast cancer cell proliferation and aggressiveness. *Biochim. Biophys. Acta, Bioenerg.* **2018**, *1859*, 958−974.
(10) Kovács, P.; Csonka, T.; Kovacs, T.; Sari, Z.; Ujlaki, G.; Sipos, A.; Karanyi, Z.; Szeocs, D.; Hegedus, C.; Uray, K.; et al. Lithocholic Acid, a Metabolite of the Microbiome, Increases Oxidative Stress in Breast Cancer. *Cancers* **2019**, *11*, 1255.
(11) Trah, J.; Arand, J.; Oh, J.; Pagerols-Raluy, L.; Trochimiuk, M.; Appl, B.; Heidelbach, H.; Vincent, D.; Saleem, M. A.; Reinshagen, K.; et al. Lithocholic bile acid induces apoptosis in human nephroblastoma cells: a non-selective treatment option. *Sci. Rep.* **2020**, *10*, No. 20349.
(12) Kollerov, V. V.; Monti, D.; Deshcherevskaya, N. O.; Lobastova, T. G.; Ferrandi, E. E.; Larovere, A.; Gulevskaya, S. A.; Riva, S.; Donova, M. V. Hydroxylation of lithocholic acid by selected actinobacteria and filamentous fungi. *Steroids* **2013**, *78*, 370−378.
(13) Acevedo-Rocha, C. G.; Gamble, C. G.; Lonsdale, R.; Li, A. T.; Nett, N.; Hoebenreich, S.; Lingnau, J. B.; Wirtz, C.; Fares, C.; Hinrichs, H.; et al. P450-Catalyzed Regio- and Diastereoselective Steroid Hydroxylation: Efficient Directed Evolution Enabled by Mutability Landscaping. *ACS Catal.* **2018**, *8*, 3395−3410.
(14) Li, A. T.; Acevedo-Rocha, C. G.; D'Amore, L.; Chen, J. F.; Peng, Y. Q.; Garcia-Borras, M.; Gao, C. H.; Zhu, J. M.; Rickerby, H.; Osuna, S.; et al. Regio- and Stereoselective Steroid Hydroxylation at C7 by Cytochrome P450 Monooxygenase Mutants. *Angew. Chem., Int. Ed.* **2020**, *59*, 12499−12505.
(15) Grobe, S.; Badenhorst, C. P. S.; Bayer, T.; Hamnevik, E.; Wu, S. K.; Grathwol, C. W.; Link, A.; Koban, S.; Brundiek, H.; Grossjohann, B.; et al. Engineering Regioselectivity of a P450 Monooxygenase Enables the Synthesis of Ursodeoxycholic Acid via 7 beta-Hydroxylation of Lithocholic Acid. *Angew. Chem., Int. Ed.* **2021**, *60*, 753−757.
(16) Peng, Y. Q.; Gao, C. H.; Zhang, Z. L.; Wu, S. J.; Zhao, J.; Li, A. T. A Chemoenzymatic Strategy for the Synthesis of Steroid Drugs Enabled by P450 Monooxygenase-Mediated Steroidal Core Modification. *ACS Catal.* **2022**, *12*, 2907−2914.
(17) Paumgartner, G.; Beuers, U. Ursodeoxycholic acid in cholestatic liver disease: Mechanisms of action and therapeutic use revisited. *Hepatology* **2002**, *36*, 525−531.
(18) Parés, A.; Caballeria, L.; Rodes, J. Excellent long-term survival in patients with primary biliary cirrhosis and biochemical response to ursodeoxycholic acid. *Gastroenterology* **2006**, *130*, 715−720.
(19) Chascsa, D.; Carey, E. J.; Lindor, K. D. Old and new treatments for primary biliary cholangitis. *Liver Int.* **2017**, *37*, 490−499.
(20) Hoehn, W. M.; Mason, H. L. The Preparation and Degradation of Lithocholic Acid. *J. Am. Chem. Soc.* **1940**, *62*, 569−570.
(21) He, X.-L.; Xing, Y.; Gu, X.-Z.; Xiao, J.-X.; Wang, Y.-Y.; Yi, Z.; Qiu, W.-W. The synthesis and antitumor activity of lithocholic acid and its derivatives. *Steroids* **2017**, *125*, 54−60.
(22) He, L.-M.; Li, C.-C.; Jiang, C.-Y.; Zhang, J.-Z.; Gu, X.-Z.; Qiu, W.-W. Synthesis of Lithocholic Acid from Plant-sourced Bisnoralcohol. *Org. Prep. Proced. Int.* **2022**, *54*, 355−362.
(23) Corfield, L.; Granne, I. Ethical and practical considerations in prescribing animal-derived medication. *Postgrad. Med. J.* **2012**, *88*, 497−498.
(24) Daher, M.; Chaar, B.; Saini, B. Impact of patients' religious and spiritual beliefs in pharmacy: From the perspective of the pharmacist. *Res. Soc. Adm. Pharm.* **2015**, *11*, E31−E41.
(25) Lv, J.-J.; Wang, Z.-J.; Feng, J.-J.; Qiu, R.; Wang, A.-J.; Xu, X.-H. Facile synthesis of highly active Pd-Cu nanowires catalyst through a simple wet-chemical strategy for ligand-free Suzuki cross coupling reaction. *App. Catal., B* **2016**, *522*, 188−193.
(26) Yi, R.; Wang, Z.-J.; Liang, Z.; Xiao, M.; Xu, X.-H.; Li, N.-B. Expeditious and highly efficient synthesis of propargylamines using a Pd-Cu nanowires catalyst under solvent-free conditions. *Appl. Organomet. Chem.* **2019**, *33*, No. e4917.
(27) Mobus, E.; Maser, E. Molecular cloning, overexpression, and characterization of steroid-inducible 3alpha-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas* testosteroni. A novel member of the short-chain dehydrogenase/reductase superfamily. *J. Biol. Chem.* **1998**, *273*, 30888−30896.
(28) Bianchini, E.; Chinaglia, N.; Dean, M.; Giovannini, P. P.; Medici, A.; Pedrini, P.; Poli, S. Regiospecific oxidoreductions

catalyzed by a new Pseudomonas paucimobilis hydroxysteroid dehydrogenase. *Tetrahedron* **1999**, *55*, 1391−1398.
(29) Grimm, C.; Maser, E.; Mobus, E.; Klebe, G.; Reuter, K.; Ficner, R. The crystal structure of 3 alpha-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas* testosteroni shows a novel oligomerization pattern within the short chain dehydrogenase/reductase family. *J. Biol. Chem.* **2000**, *275*, 41333−41339.
(30) Xiong, G. M.; Maser, E. Regulation of the steroid-inducible 3 alpha-hydroxysteroid dehydrogenase/carbonyl reductase gene in *Comamonas* testosteroni. *J. Biol. Chem.* **2001**, *276*, 9961−9970.
(31) Hwang, C.-C.; Chang, Y. H.; Hsu, C. N.; Hsu, H. H.; Li, C. W.; Pon, H. I. Mechanistic roles of Ser-114, Tyr-155, and Lys-159 in 3 alpha-hydroxysteroid dehydrogenase/carbonyl reductase from *Comamonas* testosteroni. *J. Biol. Chem.* **2005**, *280*, 3522−3528.
(32) Bertuletti, S.; Ferrandi, E. E.; Marzorati, S.; Vanoni, M.; Riva, S.; Monti, D. Insights into the Substrate Promiscuity of Novel Hydroxysteroid Dehydrogenases. *Adv. Synth. Catal.* **2020**, *362*, 2474−2485.
(33) Peng, H. D.; Wang, Y. Y.; Jiang, K.; Chen, X. R.; Zhang, W. L.; Zhang, Y. A.; Deng, Z. X.; Qu, X. D. A Dual Role Reductase from Phytosterols Catabolism Enables the Efficient Production of Valuable Steroid Precursors. *Angew. Chem., Int. Ed.* **2021**, *60*, 5414−5420.
(34) Linker, M.; Kreiser, W. Synthesis of methyl (20R,22E)- and (20S,22E)-3-oxochola-1,4,22-trien-24-oate. *Helv. Chim. Acta* **2002**, *85*, 1096−1101.
(35) Yosief, T.; Rudi, A.; Kashman, Y. Asmarines A−F, Novel Cytotoxic Compounds from the Marine Sponge Raspailia Species. *J. Nat. Prod.* **2000**, *63*, 299−304.
(36) Yu, D. D.; Sousa, K. M.; Mattern, D. L.; Wagner, J.; Fu, X.; Vaidehi, N.; Forman, B. M.; Huang, W. Stereoselective synthesis, biological evaluation, and modeling of novel bile acid-derived G-protein coupled Bile acid receptor 1 (GP-BAR1, TGR5) agonists. *Bioorg. Med. Chem.* **2015**, *23*, 1613−1628.

https://doi.org/10.1021/acsomega.3c02548
ACS Omega 2023, 8, 23130−23141