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Flavin Oxidoreductase-Mediated Regeneration of Nicotinamide Adenine Dinucleotide with Dioxygen and Catalytic Amount of Flavin Mononucleotide for One-Pot Multi-Enzymatic Preparation of Ursodeoxycholic Acid

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Abstract. Ursodeoxycholic acid (UDCA), a pharmaceutical ingredient widely used in clinics, can be prepared from chenodeoxycholic acid (CDCA) by the epimerization of the 7α-OH group. In this study, a nicotinamide adenine dinucleotide (NAD^+) regeneration system was developed by using flavin oxidoreductase (FR) and flavin mononucleotide (FMN). Only catalytic amount of FMN is required for the effective NAD^+ recycling. FR/FMN system was then applied in the oxidation of CDCA to 7-ketolithocholic acid (7-keto-LCA) by NAD^+-dependent 7α-hydroxysteroid dehydrogenase (*Bs*-7α-HSDH) from *Brevundimonas* sp., which showed extremely high enzyme activity toward CDCA (k_{cat}/K_m = 8050 $s^{-1}\cdot mM^{-1}$). When *Escherichia coli* whole cells coexpressing *Bs*-7α-HSDH and FR genes were used as biocatalyst, CDCA (50 mM) was completely converted to 7-keto-LCA with the turnover number of FMN being 227 and 58.8 $g\cdot L^{-1}\cdot d^{-1}$ space-time yield of 7-keto-LCA. For the reduction of 7-keto-LCA, nicotinamide adenine dinucleotide phosphate (NADPH)-dependent 7−β−hydroxysteroid dehydrogenase (*Cm*-7β-HSDH) from *Clostridium* sp. Marseille was employed with alcohol dehydrogenase from *Thermoanaerobacter brockii* (*Tb*ADH) and *iso*-propanol as co-factor regeneration system. When *E. coli* whole cells coexpressing *Cm*-7β-HSDH and *Tb*ADH genes were used as biocatalyst, 40 mM 7-keto-LCA was reduced to UDCA with 26.8 $g\cdot L^{-1}\cdot d^{-1}$ space-time yield. The oxidation and reduction were then carried in a one-pot concurrent mode, 12.5 mM CDCA was completely converted to UDCA. The epimerization of CDCA to UDCA proceeded to completion at the substrate concentration of 30 mM in the one-pot sequential process. Therefore, the complete conversion of CDCA to UDCA in one-pot has been realized by employing 7α-HSDH and 7β-HSDH of different co-factor specificities with independent co-factor recycling systems. The cholic acids, especially UDCA, exert inhibitive effect on the activities of these enzymes, preventing the complete epimerization of 7α-OH at higher substrate loading. This inhibition issue should be solvable by engineering the involved enzymes, that is currently pursued in our laboratory.

Keywords: NAD^+ regeneration; chenodeoxycholic acid (CDCA); one-pot reaction; ursodeoxycholic acid (UDCA); epimerization

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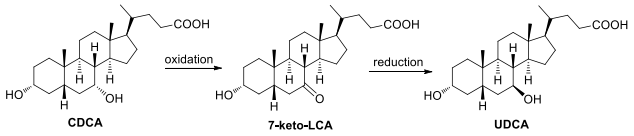
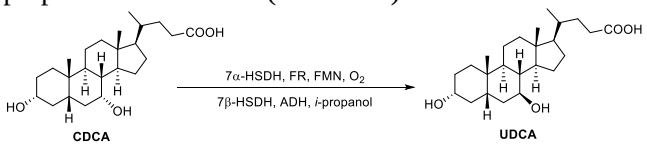
Introduction

Ursodeoxycholic acid (UDCA) is a pharmaceutical ingredient widely used in clinics for the treatment of gallstone disease and liver disease associated with cystic fibrosis [1]. Latest development in the synthesis of UDCA has been summarized [2]. Cholic acid (CA) from bovine bile is the most available starting material, but the most direct synthesis of UDCA is using chenodeoxycholic acid (CDCA) as the starting material. CDCA is widely distributed in the bile acids of poultry or can be prepared from cholic acids. Conversion of CDCA to UDCA involves two transformations: the oxidation of 7α-OH group to carbonyl group, and the reduction of 7-carbonyl group to 7β-OH group (Scheme 1) [3]. Both chemical and biocatalytic approaches have been explored for the 7-OH epimerization [2]. For the biocatalytic process, 7α-hydroxysteroid dehydrogenases (7α-HSDH) and 7β-hydroxysteroid dehydrogenases (7β-HSDH) have been used for the oxidative and reductive steps, respectively. The epimerization of CDCA to UDCA have been achieved by using a redox-neutral cascade reaction with two nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent dehydrogenases. However, the conversion was limited by the intrinsic reaction equilibrium and a mixture of CDCA, UDCA and 7-ketolithocholic acid (7-keto-LCA) was obtained [3d]. Therefore, sequential two-step process has been adopted to enforce the complete epimerization of CDCA to UDCA, in which the enzymes for the oxidation step were inactivated by heat-treatment [4] or removed as "tea-bag" [5] before the reduction was initiated. In this context, we adopted an alternative approach [5,6] to addressing this equilibrium issue, in which cofactor-specific 7α-HSDH and 7β-HSDH of different cofactors were employed with suitable co-factor recycling systems.



Scheme 1 Preparation of UDCA from CDCA through 7-keto-LCA

For the regioselective oxidation of 7α-hydroxyl group of CDCA, some chemical oxidizing reagents, such as N-bromosuccinimide (NBS) [7], *o*-iodoxybenzoic acid (IBX) [8], pyridinium chlorochromate (PCC) [9], NaClO [10], ceric ammonium nitrate (CAN) in conjunction with sodium bromate ($NaBrO_3$) [11] have been reported. Although chemical oxidation has been widely studied, but there are at least one of the problems such as relatively higher temperature, use of organic solvent, over oxidation of product or toxic heavy metal-based oxidizing agents. On the other hand, 7α-HSDH catalyzes the regio- and stereoselective oxidative reaction of 7α-hydroxyl group of CA derivatives. 7α-HSDHs have been found in *Escherichia coli* [12], *Clostridium absonum* [13], *Pseudomonas* sp. B-083 [14], Acinetobacter *calcoaceticus lwoffi* [15], *Eubacterium* sp. strain VPI 12708 [16] and *Bacteroides fragilis* [17]. Some of them have been used in the preparation of 7-oxo bile acid and proved to be a powerful biocatalyst [3d, 18]. Glutamate dehydrogenase/α-ketoglutarate [17a], lactate dehydrogenase/pyruvate [3d, 18b], alcohol dehydrogenase/acetone or acetaldehyde [19] have been developed for the *in situ* regeneration of cofactor $NAD(P)^+$ in the transformation of CDCA to 7-keto-LCA employing 7α-HSDHs. No over-oxidation of the product was reported because of the excellent selectivity of 7α-HSDHs. In these systems, usually 2-3 equivalents of α-ketoglutarate or pyruvate, or large excess of acetone or acetaldehyde was required as the co-substrate to drive the reaction to completion, raising the operation cost. Laccase/Meldola's blue system showed its potential for the oxidation of CA, with 0.005 eq of Meldola's blue being added into the reaction, but the acidic pH should be selected at the expense of enzyme activity of 7α-HSDH because laccase showed low enzyme activity at neutral to alkaline pH [20]. Nicotinamide adenine dinucleotide (NADH) oxidases (NOX) have been employed to regenerate NAD^+ with dioxygen (O_2) being reduced to hydrogen peroxide (H_2O_2) [21] or water (H_2O) [22], but NOX have the same problem with laccase/Meldola's blue system, the relative low enzyme activity of NOX made it becoming the rate limiting step. Light-emitting diode (LED) light sources promote the hydride transfer from NAD(P)H to FMN [23], but the transmittance of light would become a problem when the reaction is scaled up to tons production. Flavin oxidoreductase (FR) has been reported with high activity at a wide range of pH toward oxidation of NADH [24] and/or NADPH [25] by flavin mononucleotide (FMN). However, until now FR/FMN system has not been used to regenerate the cofactor NAD^+ or $NADP^+$. Flavin oxidoreductase (MgFR) from *Mycobacterium goodii* X7B [26] showed 678 U/mg (pH 7.0) specific activity toward the hydride transfer from NADH to FMN [24], yielding NAD^+ and the reduced FMN ($FMNH_2$) [27]. The fast autoxidation of $FMNH_2$ [28] suggests that it may be not necessary to use the stoichiometric amount of FMN. Herein, we report an effective FR-mediated regeneration of NAD^+ by using O_2 and catalytic amount of FMN as the oxidizing agent, which is coupled with a highly active NAD^+-dependent 7α-HSDH from *Brevundimonas* sp. to achieve the efficient conversion of CDCA to 7-keto-LCA, followed by the reduction using 7β-HSDH and alcohol dehydrogenase from *Thermoanaerobacter brockii* (*Tb*ADH) [29] in one-pot fashion for the preparation of UDCA (Scheme 2).



Scheme 2 One-pot multi-enzymatic preparation of UDCA

Results and Discussion

Screening of 7α-HSDHs and 7β-HSDHs

Many 7α-HSDHs have been identified as the biocatalyst for the oxidation of CDCA to 7-keto-LCA. For example, 7α-HSDH (*Ec*-7α-HSDH) from *Escherichia coli* HB101 showed good catalytic capacity toward CA or CDCA with NAD^+ as cofactor [12]. Recently, an increasing number of reports on the bioconversion with 7β-HSDH has also been appeared [3]. In order to search for more effective 7α/β-HSDHs, *Ec*-7α-HSDH or *Cae*-7β-HSDH from *Collinsella aerofaciens* ATCC 25986 was used as the template, respectively, for searching new candidates and neighbor-joining trees were constructed with MEGA software (Fig. S1). Seven 7α-HSDH genes and four new together with four reported 7β-HSDH genes were selected and overexpressed in *E. coli* (Fig. S2).

The activity of crude 7α-HSDHs and 7β-HSDHs were measured, and the results are shown in Table S1. *Bs*-7α-HSDH from *Brevundimonas* sp. and *Cm*-7β-HSDH from *Clostridium* sp. Marseille showed the highest enzyme activity, and thus selected for the following studies. The recombinant *Bs*-7α-HSDH and *Cm*-7β-HSDH together with *Mg*FR and *Tb*ADH were purified (Tables S2-S5, Fig. S3-S6). The pH, temperature dependence and kinetic parameters of the purified *Bs*-7α-HSDH and *Cm*-7β-HSDH enzymes were determined (Table S6 and Fig. S7-S8).

Flavin oxidoreductase-catalyzed regeneration of NAD^+ for the oxidation of CDCA

FRs catalyze the reduction of free flavins (riboflavin, FMN, or FAD) by using reduced pyridine nucleotides, NADPH, or NADH. *Mg*FR from *Mycobacterium goodii* X7B [26] showed strict specificity toward NADH with high enzyme activity (678 U/mg), and thus was tested for the regeneration of NAD^+ in the *Bs*-7α-HSDH-catalyzed oxidation of CDCA. *E. coli* (BsMg) cells co-expressing *Bs*-7α-HSDH and *Mg*FR were used as biocatalyst in the following studies (Fig. S9).

The amount of FMN required for the effective cycling of NAD^+ was examined, and the results are summarized in Table 1. The oxidation reaction was finished in 30 min when 10 mM CDCA, 10 mM FMN and 0.15 mM NAD^+ were used (Table 1, entry 1). The results showed that even 0.022 equivalent of FMN was used, the oxidation reaction proceeded successfully in 1 h (Table 1, entry 4), suggesting that it is not necessary to add stoichiometric amount of FMN, due to the fast auto-oxidation of reduced FMN [28]. When the oxidation of CDCA was carried out at 75 mM substrate concentration with shaking or stirring in an open reaction system, the dissolved O_2 was enough for the oxidation step. Without addition of extra NAD^+ into the system the reaction time was prolonged from 1 h to 6 h (Table 1, entry 5), implying that the endogenous NAD^+ of *E. coli* could be used as the cofactor. Without extra addition of FMN, the reaction proceeded with low conversion (Table 1, entry 6).

The influence of substrate addition mode was investigated. Feeding the substrate in batches shortened the reaction time from 6 to 4 h at substrate concentration of 25 mM (Table 1, entries 7 and 8). Without addition of external NAD^+, the reaction conversion was only 94%, and the complete conversion of CDCA required longer reaction time of 30 h (Table 1, entry 9). When substrate loading was increased to 50 mM and no extra NAD^+ was added, only 62% conversion of CDCA was achieved (Table 1, entry 11). At the substrate loading of 50 mM, addition of CDCA in batches exerted remarkable effect on the reaction rate (Table 1, entries 10 and 12). Under this reaction condition, doubling the concentration of co-substrate FMN shortened the reaction time from 6 to 5 h (Table 1, entries 12 and 13). When the substrate concentration was further increased to 75 mM (30 g/L), the reaction was finished in 16 h. When the cell-free extracts were used as the biocatalyst, the oxidation reaction proceeded faster, shortening the reaction time dramatically (Table 2, entry 14). The whole-cell transformation was conducted in 100 mL at the concentration of 50 mM CDCA. The reaction was completed in 8 h, and 7-keto-LCA was obtained with 94% yield by adjusting the pH of the reaction mixture. In this case, only 0.0044 equivalent of co-substrate FMN was added with its turnover number being 227, and 58.8 $g \cdot L^{-1} \cdot d^{-1}$ space-time yield was attained (Table 1, entry 15).

Table 1. Optimization of the reaction conditions for the preparation of 7-keto-LCA [a].

Entry	Substrate loading (mM)	NAD^+/FMN (mM)	Conv. (%)	Reaction time (h)
1	10	0.15/10	> 99	0.5
2	10	0.15/0.88	> 99	0.5
3	10	0.15/0.44	> 99	1
4	10	0.15/0.22	> 99	1
5	10	0/0.22	> 99	6
6	10	0.15/0	16	24
7	25	0.15/0.22	> 99	6
8	25 [b]	0.15/0.22	> 99	4
9	25 [b]	0/0.22	94 (> 99)	24 (30)
10	50	0.15/0.22	> 99	12
11	50	0/0.22	62	30
12	50	0.15/0.22	> 99	6 (4 [c])
13	50	0.15/0.44	> 99	5
14	75	0.15/0.44	> 99	16 (10 [c])
15	50 [d]	0.15/0.22	> 99 (94 [e])	8

[a] 10 mg/mL wet cells, CDCA was added once, pH 8.0; [b] CDCA was added in batches; [c] cell-free extracts of 10 mg/mL wet cells; [d] the reaction was conducted at 100 mL volume; [e] isolated yield.

Compared with the previous reported cofactor regeneration systems used for the preparation of 7-keto-LCA (Table 2), we used recombinant *E. coli*

cells co-expressing 7α-HSDH and FR genes instead of partial or purified enzyme as the biocatalyst, and only catalytic amount of FMN was required. Our results demonstrate that the FR/FMN is an effective cofactor regeneration system for the alcohol dehydrogenase-catalyzed reactions.

Table 2 Comparison of biotransformation conditions of 7α-HSDH-catalyzed oxidations

Entry	Substrate loading (mM)	Co-substrate (mM)	Catalyst form
1	CA 12.5	α-ketoglutarate 50	partial purified enzyme [30]
2	CDCA 10	sodium pyruvate 12	crude enzyme [4b]
3	CDCA 50	Meldola's blue 0.3	purified enzyme [20]
4	CDCA 50	FMN 0.22	recombinant *E. coli* (This work)

Optimization of the reduction reaction conditions

*Tb*ADH/*i*-propanol (IPA) was selected for the regeneration of NADPH in the *Cm*-7β-HSDH-catalyzed reduction of 7-keto-LCA. *E. coli* (CmTb) cells co-expressing *Cm*-7β-HSDH and *Tb*ADH genes were used as the biocatalyst for the reduction of 7-keto-LCA (Fig. S10).

Different concentrations of cosubstrate IPA (0.1-1 M) were examined for the reduction (Table 3, entry 1-4), and 1 M IPA was chosen for the reaction. The endogenous $NADP^+$ of *E. coli* was sufficient for the complete reduction of 10 mM 7-keto-LCA, although longer reaction time was needed. When the concentration of 7-keto-LCA was increased to 40 mM, the reduction was completed in 20 h. The reaction time was shortened to 14 h when 7-keto-LCA was added in batches. The reduction of 7-keto-LCA was carried out at laboratory preparative scale, and UDCA was isolated with 93% yield by adjusting the pH of the reaction mixture (Table 3, entry 8).

Table 3. Optimization of the reaction conditions for the preparation of UDCA from 7-keto-LCA [a].

Entry	Substrate loading (mM)	$NADP^+$ (mM)/ IPA (M)	Conv. (%)	Reaction time (h)
1	10	0.15/0.1	> 99	2
2	10	0.15/0.25	> 99	1.5
3	10	0.15/0.5	> 99	1
4	10	0.15/1	> 99	1
5	10	0/1	> 99	16
6	20	0.15/1	> 99	4
7	40	0.15/1	> 99	20
8	40 [b]	0.15/1	> 99 (93 [c])	14

[a] 50 mg/mL wet cells, 7-keto-LCA was added once, 37°C, pH 7.0; [b] 7-keto-LCA was added in batches; [c] 50 mL reaction was conducted, isolated yield.

Sequential and concurrent one-pot preparation of UDCA from CDCA

From the above results, it could be seen that FR effectively mediated the regeneration of NAD^+ by using catalytic amount of FMN and O_2 as the oxidizing agent, leading to efficient conversion of CDCA to 7-keto-LCA by coupling with a highly active NAD^+-dependent 7α-HSDH. The reduction of 7-keto-LCA to UDCA was achieved by using 7β-HSDH and *Tb*ADH/IPA as cofactor recycling system. Therefore, the oxidation and reduction reactions were performed in sequential and concurrent one-pot fashion with an aim to realize the complete epimerization of CDCA to UDCA in a single step. The *E. coli* (BsMg) and *E. coli* (CmTb) whole cells were used as the biocatalysts, and results are summarized in Table 4.

Table 4. Sequential and concurrent one-pot preparation of UDCA from CDCA [a].

Entry	Substrate loading (mM)	Conv. (%) oxidation/reduction	Reaction time (h)
1	10	> 99/> 99	0.5/1.5
2	10 [b]	> 99/65 (70)[d]	4/24 (30)
3	10 [c]	> 99/85 (95)[d]	3/24 (30)
4	20	> 99/> 99	1/7
5	30	> 99/> 99	4/20
6	40	> 99/60	8/24
7	10	> 99	2
8	20	50	24
9	12.5	> 99	12

[a] 20 mg/mL wet cells, 0.15 mM NAD^+, 0.15 mM $NADP^+$, 0.44 mM FMN, 1.0 M IPA were added, 37°C, pH 7.0. Entries 1-6, the reactions were carried out sequentially, entries 7-9, the reactions were performed concurrently. [b] Without addition of external NAD^+ and $NADP^+$. [c] 0.075 mM NAD^+ and $NADP^+$. [d] The conversion of reduction step after 30 h.

For the one-pot sequential reaction, 30 mM CDCA was completely transformed to 7-keto-LCA, which was then completely reduced to UDCA (Table 4, entry 5). When the concentration of CDCA was increased to 40 mM, CDCA was completely oxidized to 7-keto-LCA in 8 h. However, the reduction reaction could not proceed to completion, resulting in a 60% conversion of 7-keto-LCA to UDCA (Table 4, entry 6). When the amount of cofactors was reduced to 0.075 mM or no external cofactor was added, the oxidation proceeded to completion in a longer time, but the reduction was not completed in 30 h even at 10 mM substrate concentration (Table 4, entries 2 and 3). It seems that the reduction requires higher cofactor concentration than the oxidation. For the one-pot concurrent reaction with whole cells of BsMg and CmTb being added at the same time, 12.5 mM (5 g/L) of CDCA was completely epimerized into UDCA (Table 4, entry 9). Addition of 20 mM CDCA into the reaction mixture resulted in an incomplete

epimerization with 50% conversion, and a mixture of substrate CDCA, intermediate 7-keto-LCA and product UDCA was obtained (Table 4, entry 8). The sequential and concurrent one-pot preparation of UDCA from CDCA were performed at 50 mL scale. The results showed that in sequential reaction mode 30 mM CDCA was transformed to UDCA with 92% isolated yield, and in concurrent reaction mode 12.5 mM CDCA was epimerized to UDCA with 78% isolated yield.

It has been reported that cholic acid and its derivatives exert dramatic inhibition effect on the enzyme activity of 7α-HSDH at mM concentration [12, 31]. We thus proposed that the low conversion of CDCA to UDCA at higher concentration in the one-pot process might be due to the inhibition effects of the bile acids on these enzymes. As such, the influence of all the reaction components (FMN, H_2O_2, CDCA, 7-keto-LCA, UDCA, IPA, and acetone) in the one-pot reaction mixture on the enzyme activity of *Mg*FR, *Bs*-7α-HSDH, *Tb*ADH, and *Cm*-7β-HSDH were investigated. FMN (2 mM), H_2O_2 (100 mM), IPA (1.0 M) showed no dramatic influence on the activity of these enzymes. CDCA, 7-keto-LCA, UDCA exerted inhibition effects on the tested enzymes. Especially, when 20 mM UDCA was added into the reaction mixture, only 17%, 16%, 20% remaining enzyme activities of *Mg*FR, *Cm*-7β-HSDH, *Tb*ADH were observed, respectively (Fig. S11). Therefore, the inhibition of UDCA on the enzyme activity is responsible for the incomplete epimerization at higher substrate concentration. Further studies are thus required to address this inhibition issue.

Conclusion

Because HSDHs catalyze the reversible oxidation/reduction reactions, it is inevitable that 7α-HSDH and 7β-HSDH would interfere each other in the one-pot enzymatic epimerization of CDCA to UDCA if they do not have different co-factor specificities. As such, *Bs*-7α-HSDH and *Cm*-7β-HSDH, which are exclusively selective for NAD^+ or $NADP^+$, respectively, were selected in this study. *Mg*FR with strict specificity toward NADH was used for the regeneration of NAD^+, and NADPH-specific *Tb*ADH was chosen for the recycling of NADPH. It should be noted that only a catalytic amount of FMN is needed for an effective regeneration of NAD^+, avoiding the high cost of FMN mediator. Therefore, the complete conversion of CDCA to UDCA was achieved in one-pot epimerization either in sequential or concurrent mode. Although the inhibition of UDCA on the enzyme activity prevents the efficient epimerization of CDCA to UDCA at high substrate concentration, the present study offers an approach to enabling the epimerization proceeding to completion in the one-pot setting. The inhibition issue of the CA derivatives on the enzyme activity should be addressable by engineering the enzymes or searching for new enzymes from the Nature, and/or by removing the product UDCA from the reaction mixture, that are currently pursued in our laboratory.

Experimental Section

General

All chemicals including CA, CDCA and 7-keto-LCA were obtained from commercial sources and the cofactors were obtained from Roche. The enzyme activities toward the reduction of ketones were measured using a SpectraMax M2 microplate reader (Molecular Devices). The ^1H NMR spectra were measured on Brucker Avance using CD_3OD as the solvent. Materials used for culture media including peptone, yeast extract, and agar were purchased from Becton, Dickinson, and Company (BDX).

Gene cloning and expression

In order to identify more potential 7α-HSDHs and 7β-HSDHs for the synthesis of UDCA from CDCA, *Ec*-7α-HSDH (GenBank: P0AET8) and *Cae*-7β-HSDH (GenBank: WP_006236005.1) were used as the templates for BLASTP search in NCBI at default settings (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Phylogenetic tree and alignment of amino acid sequences of 7α-HSDHs and 7β-HSDHs were presented in Fig. S1. The optimized gene sequences of 7α-HSDHs and 7β-HSDHs from the selected strains were synthesized and cloned into *Nde*I and *Xho*I sites of pET21a vector. The plasmids were introduced into *E. coli* BL21(DE3), resulting in recombinant strains.

The flavin oxidoreductase (*Mg*FR) gene (GenBank: EU154996.1) was amplified using genomic DNA from *Mycobacterium goodie* X7B as template and primers with recognition sites for the restriction enzymes *Nde*I and *Xho*I were used for the construction of the enzyme with C-terminal 6×His-tag in pRSFDuet-1. The constructed vector was named pRSFDuet-*Mg*FR. The DNA sequence were confirmed by sequencing. The plasmid was introduced into *E. coli* BL21 (DE3), resulting in recombinant strain pRSFDuet-*Mg*FR-BL21 (DE3). The recombinant *E. coli* BL21(DE3) carrying alcohol dehydrogenase *Tb*ADH was preserved in our laboratory. Recombinant *E. coli* BL21(DE3) was propagated in Luria-Bertani medium containing 100 μg/mL ampicillin or 50 μg/mL kanamycin. When the OD600 reached a value between 0.6 and 0.8, the production of the recombinant proteins was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.1 mM. The cultures were shaken for 20 h at 25°C and the cells were harvested by centrifugation.

Activity assay of crude enzymes

Recombinant *E. coli* cells were resuspended in potassium phosphate buffer (50 mM, pH 8.0) and disrupted by ultrasonic. Cell debris was precipitated by centrifuge at 10,000 × g for 20 min. The supernatants (cell-free extracts) were used for activity assays with the corresponding substrates and the

expressed proteins were analyzed by SDS-PAGE. The enzyme activity of reduction/oxidation was assayed by spectrophotometrically measuring the absorbance of NAD(P)H at 340 nm (ε=6.22 mM^{-1} cm^{-1}). The specific activity U was defined as the number of micromoles of $NAD(P)^+$ or NAD(P)H converted in 1 min by 1 mg of enzyme ($\mu mol \cdot min^{-1} \cdot mg^{-1}$).

For 7α-HSDH, the assay mixture (0.2 mL) contained 2.5 mM CDCA, 0.5 mM NAD^+ and appropriate amount of crude enzyme in 50 mM potassium phosphate buffer (pH 8.0).

For *Mg*FR, the assay mixture (0.2 mL) contained 0.3 mM FMN, 0.5 mM NADH and appropriate amount of enzyme in potassium phosphate buffer (50 mM, pH 8.0).

For 7β-HSDH, the assay mixture (0.2 mL) contained 2.5 mM 7-keto-LCA, 0.5 mM NADPH and appropriate amount of crude enzyme in 50 mM potassium phosphate buffer (pH 8.0).

For *Tb*ADH, the assay mixture (0.2 mL) contained 100 mM IPA, 0.5 mM $NADP^+$ and appropriate amount of crude enzyme in 50 mM potassium phosphate buffer (pH 8.0).

The influence of the reaction components on the enzyme activity of *Bs*-7α-HSDH, *Mg*FR, *Cm*-7β-HSDH and *Tb*ADH

For the influence of the reaction components, different final concentrations (0-20 mM) of CDCA, 7-keto-LCA, UDCA, (0-2 mM) FMN, (0-100 mM) H_2O_2, (0-1.2 M) IPA, and (0-100 mM) acetone were respectively added into the assay mixture of *Bs*-7α-HSDH, *Mg*FR, *Cm*-7β-HSDH and *Tb*ADH, the activity of was determined by the assay procedure mentioned above.

Construction of co-expression vector and cultivation of strains

pACYCDuet™-1 vector carrying chloramphenicol resistance gene (CmR) was designed for the co-expression of two target ORFs . The *Mg*FR and Bs-7α-HSDH gene was placed in the first and second MCS, respectively. The *Tb*ADH and *Cm*-7β-HSDH genes were placed in the first and second MCS of pRSFDuet™-1 vector (kanaR), respectively. Thus two binary expression vectors pACYCDuet-*Mg*FR-Bs-7α-HSDH (BsMg) and pRSFduet-*Tb*ADH-Cm-7β-HSDH (CmTb) were obtained. The plasmids were introduced into *E. coli* BL21 (DE3). The cultivation of *E. coli* BL21 was conducted as mentioned above.

Optimization for the oxidation of CDCA

Into 5 mL broth (pH 8.0) containing *E. coli* (BsMg) cells (about 50 mg), 0.1 g/L (0.15 mM) NAD^+, different concentration of cosubstrate FMN, and different concentrations of substrate CDCA were added in one-time or in several batches. The reactions were monitored by thin-layer chromatography (TLC) or HPLC at 206 nm using methanol/water (75/25) as mobile phase with Agela® ODS-4 column (250 mm × 4.6 mm ID, 5 μm).

Preparation of 7-keto-LCA

Into 100 mL broth (pH 8.0) containing *E. coli* (BsMg) cells, 0.1 g/L (0.22 mM) FMN, 20 g/L (50 mM) CDCA was added in 6 h, the reaction conversion was monitored from time to time with TLC and HPLC. After the reaction was finished, pH of the reaction mixture was adjusted to 9.0. The mixture was centrifuged to remove the *E. coli* (BsMg) cells, and the pH of the resulting solution was adjusted to 6.0. The precipitate was collected by centrifugation, and washed with 0.01 N HCl. The product was dried and obtained with 94% yield (1.9 g), characterized by NMR analysis. NMR data were consistent with reference [8] ^{1}H NMR (CD_3OD, 600 MHz) δ (ppm) 0.70 (s, 3H), 0.95 (d, *J* = 3.0 Hz, 3H), 1.22 (s, 3H), 1.01-1.26 (m, 4H), 1.27-1.37 (m, 2H), 1.38-1.68 (m, 6H), 1.77-1.88 (m, 4H), 1.88-1.97 (m, 2H), 2.04 (m, 1H), 2.18 (m, 2H), 2.32 (m, 1H), 2.53 (t, *J* = 9.6 Hz, 1H), 2.98 (q, *J* = 6.0, 12.0 Hz, 1H), 3.51 (m, 1H). ^{13}C NMR (CD_3OD, 125 MHz) δ (ppm) 215.1, 178.2, 71.9, 56.4, 50.7, 50.5, 47.6, 46.5, 44.5, 43.9, 40.4, 38.3, 36.6, 36.4, 35.3, 32.4, 32.1, 30.7, 29.4, 25.9, 23.6, 22.9, 18.9, 12.6.

Optimization for the reduction of 7-keto-LCA

Into 5 mL potassium phosphate buffer containing *E. coli* (CmTb) cells (about 250 mg), 0.1 g/L (0.15 mM) $NADP^+$, different concentration of cosubstrate IPA, and different concentrations of substrate CDCA were added in one-time or in several batches (Table 3). The reaction were monitored by thin-layer chromatography (TLC) or HPLC.

Preparation of UDCA from 7-keto-LCA

Into 50 mL potassium phosphate buffer (pH 7.0) containing cell-free extract of wet strain (BsMg, 0.5 g), 0.1 g/L $NADP^+$, 6% v/v IPA, 12 g/L (30 mM) 7-keto-LCA was added in 4 h, the reaction was performed at 37°C, and monitored from time to time with TLC and HPLC. After the reaction was finished, UDCA was purified as mentioned above with 93% yield (0.55 g), and characterized by NMR analysis. NMR data were consistent with reference [3d]. ^{1}H NMR (CD_3OD, 600 MHz) δ (ppm) 0.62 (s, 3H), 0.84-0.89 (m, 6H), 0.90-1.25 (m, 8H), 1.27-1.52 (m, 10H), 1.61-1.93 (m, 6H), 2.01-2.26 (m, 2H), 3.85 (m, 1H), 4.42 (m, 1H). ^{13}C NMR (CD_3OD, 100 MHz) δ (ppm) 178.2, 72.7, 72.0, 57.6, 56.7, 44.9, 44.6, 44.1, 40.8, 38.7, 37.1, 36.8, 36.2, 35.3, 32.5, 32.1, 31.1, 29.7, 28.0, 24.1, 22.5, 19.1, 12.8.

One-pot sequential preparation of UDCA from CDCA

Into 5 mL potassium phosphate buffer (pH 7.0) containing *E. coli* (BsMg) cells (about 100 mg), 0.15 mM NAD^+, 0.44 mM FMN, and different concentrations of substrate CDCA were added, the reaction were performed at 37°C, and monitored by TLC. After CDCA were completely transformed to 7-keto-LCA, *E. coli* (CmTb) cells (about 100 mg), 1.0 M IPA, 0.15 mM $NADP^+$ were added to the reaction, and monitored by TLC and HPLC.

Into 50 mL potassium phosphate buffer (pH 7.0) containing *E. coli* (BsMg) cells (about 1.0 g), 0.15 mM NAD^+, 0.44 mM FMN were added, 30 mM CDCA was added in three batches, after CDCA were completely transformed to 7-keto-LCA (4 h), *E. coli* (CmTb) cells (about 1.0 g), 1.0 M IPA, 0.15 mM

$NADP^+$ were added to the reaction, and monitored by TLC. After 20 h, the reaction was finished. UDCA was purified as mentioned above, with 92% yield (0.54 g).

One-pot concurrent reaction of preparation of UDCA from CDCA

Into 5 mL phosphate buffer (pH 7.0) containing *E. coli* (BsMg) cells (about 100 mg), *E. coli* (CmTb) cells (about 100 mg), 0.15 mM NAD^+, 0.15 mM $NADP^+$, 0.44 mM FMN, 1.0 M IPA and different concentrations of substrate CDCA were added, the reaction were performed at 37°C, and monitored by TLC and HPLC.

Into 50 mL phosphate buffer (pH 7.0) containing *E. coli* (BsMg) cells (about 1.0 g), *E. coli* (CmTb) cells (about 1.0 g), 0.15 mM NAD^+, 0.15 mM $NADP^+$, 0.44 mM FMN, 1.0 M IPA and 12.5 mM CDCA were added, the reaction were performed at 37°C, and monitored by TLC and HPLC. After 20 h, CDCA was completely transformed to UDCA. UDCA was purified as mentioned above in 78% yield (0.19 g).

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FULL PAPER

Flavin Oxidoreductase-Mediated Regeneration of Nicotinamide Adenine Dinucleotide with Dioxygen and Catalytic Amount of Flavin Mononucleotide for One-Pot Multi-Enzymatic Preparation of Ursodeoxycholic Acid

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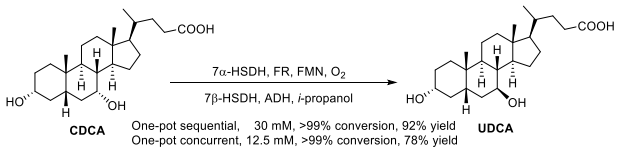

chenodeoxycholic acid (CDCA); ursodeoxycholic acid (UDCA); hydroxysteroid dehydrogenase (HSDH); flavin reductase (FR); flavin mononucleotide (FMN); alcohol dehyfrogenase (ADH)