DOI: 10.1002/adsc.200800727

One-Pot Multienzymatic Synthesis of 12-Ketoursodeoxycholic Acid: Subtle Cofactor Specificities Rule the Reaction Equilibria of Five Biocatalysts Working in a Row

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Received: November 21, 2008; Published online: March 17, 2009

Dedicated to Prof. Karl "Kalle" Hult on the occasion of his 65th birthday.

Abstract: The hydroxysteroid dehydrogenases (HSDHs)-catalyzed one-pot enzymatic synthesis of 12-ketoursodeoxycholic acid (3α,7β-dihydroxy-12-oxo-5β-cholanoic acid), a key intermediate for the synthesis of ursodeoxycholic acid, from cholic acid has been investigated. This goal has been achieved by alternating oxidative and reductive steps in a one-pot system employing HSDHs with different cofactor specificity, namely NADH-dependent HSDHs in the oxidative step and an NADPH-dependent 7β-HSDH in the reductive one. Coupled *in situ* regeneration systems have been exploited not only to allow the use of catalytic amounts of the cofactors, but also to provide the necessary driving force to opposite reactions (i.e., oxidation and reduction) acting on different sites of the substrate molecule. Biocatalysts suitable for the set-up of this process have been selected and their kinetic behaviour in respect of the reactions of interest has been evaluated. Finally, the process has been studied employing the enzymes both in free and compartmentalized form.

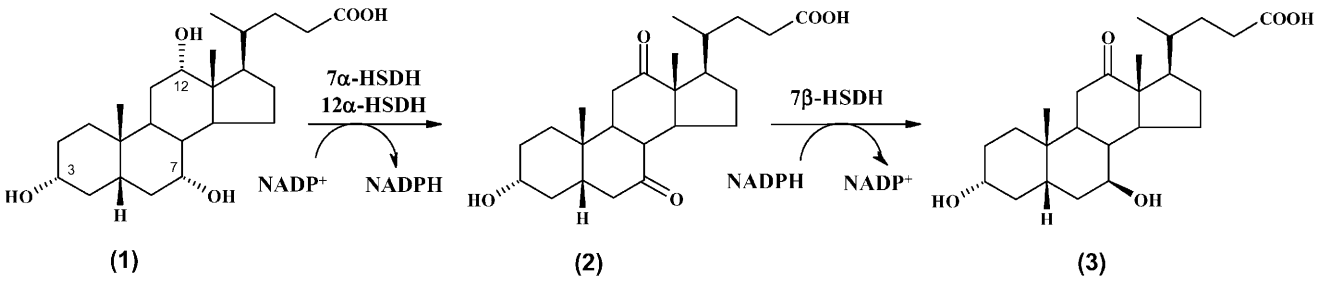
Keywords: bile acids; hydroxysteroid dehydrogenases; one-pot reaction; oxido-reductions; regioselectivity

Introduction

One-pot multistep enzyme-catalyzed processes involve the use of different biocatalysts for two or more sequential transformations without the isolation of intermediates. Recently, these systems have gained significant attention since they can be effective in reducing operation time, costs and environmental impact. These goals can be achieved by optimizing the overall process instead of the individual steps and by improving the synthetic efficiency with a decreased number of required laboratory operations and reduced quantities of chemicals and solvents used.[1] Moreover, mimicking biosynthetic pathways in living cells, reactions suffering from equilibrium constraints can be driven towards product formation if the latter is continually removed in a subsequent reaction step. Product/substrate inhibition effects can be avoided as well.

Among the most studied multistep enzymatic processes, oxidation/reduction reactions catalyzed by different dehydrogenases coupled with *in situ* cofactor regeneration systems are of particular interest as they allow the use of catalytic quantities of the expensive nicotinamide cofactors $NAD(P)^+$/NAD(P)H.[2] Specifically, hydroxysteroid dehydrogenases (HSDHs), mainly obtained from bacterial sources, catalyze the oxidoreduction of the hydroxy/keto groups of steroids with remarkably high regio- and stereoselectivity, and have been employed for the selective modification of neutral steroids, bile acids and other steroid derivatives.[3] The selective oxidations and reductions catalyzed by HSDHs are reversible reactions, but this limitation can be satisfactorily overcome by coupling them with suitable regeneration systems which can provide the driving force to shift the overall equilibrium towards the desired product.[4–7]

Several years ago, we reported the HSDH-catalyzed two-steps enzymatic synthesis of 12-ketoursodeoxycholic acid (3α,7β-dihydroxy-12-oxo-5β-cholanoic acid, Scheme 1, **3**), a key intermediate for the synthesis of ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid) by a Wolff–Kishner reduction.[8]

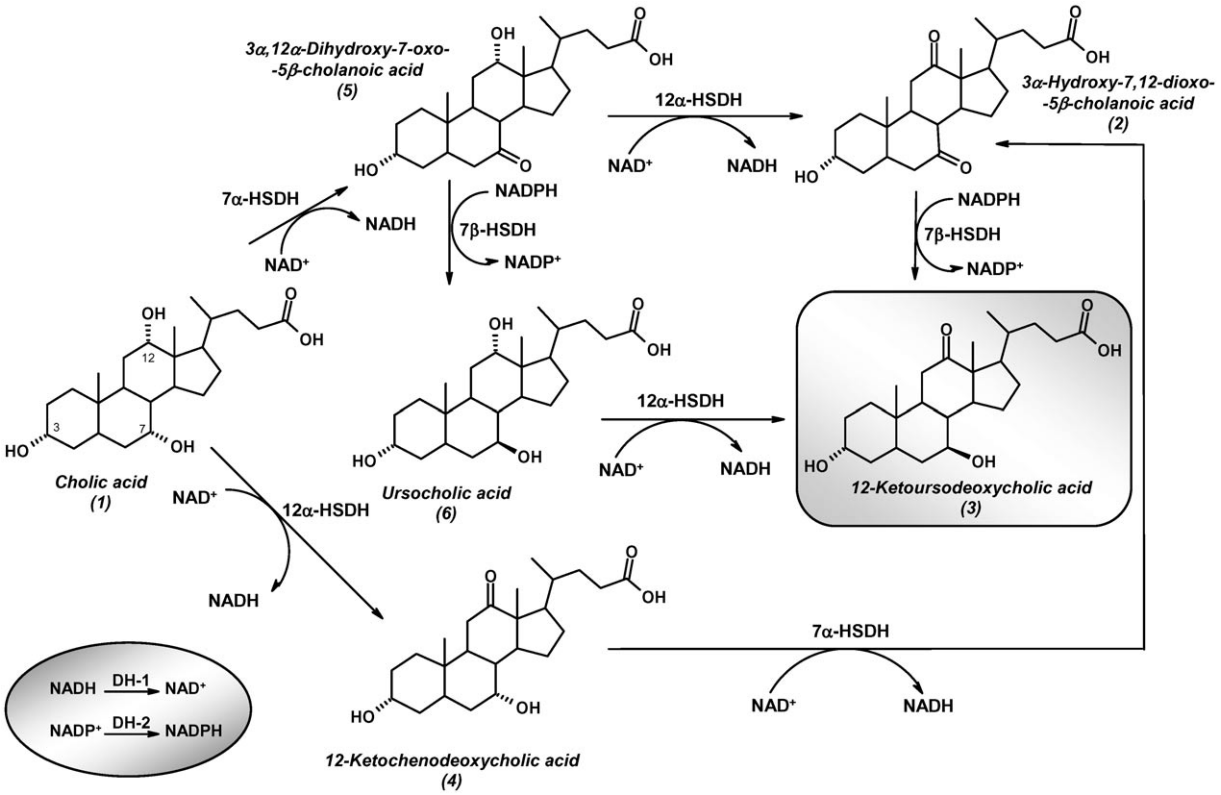


Scheme 1. Enzymatic synthesis of 12-ketoursodeoxycholic acid (**3**) from cholic acid (**1**) through the isolated intermediate 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**).

This latter biliary acid is used as a drug in cholesterol gallstone dissolution therapy and for the treatment of patients with primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC), the most common chronic cholestatic liver diseases in adults.[9]

In this earlier work cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid, **1**) was first oxidized to 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**) by NADPH-dependent 7α-HSDH and 12α-HSDH. After purification, this intermediate product was submitted to a regio- and stereoselective reduction catalyzed by an NADPH-dependent 7β-HSDH. NADP(H) *in situ* regeneration in the two steps was carried out with α-ketoglutarate/glutamate dehydrogenase and glucose/glucose dehydrogenase, respectively.[8] All these enzymatic activities were NADPH-dependent, this hampering their concurrent use in a single-step biotransformation of cholic acid (**1**) to **3**. In fact, a mixture of **1**, **3** and of the intermediate products 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**), 12-ketochenodeoxycholic acid (3α,7α-dihydroxy-12-oxo-5β-cholanoic acid, **4**), 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (**5**), and ursocholic acid (3α,7β,12α-trihydroxy-5β-cholanoic acid, **6**, see Scheme 2) is expected to occur in the absence of a suitable driving force leading the synthesis towards the desired product.

In the present work, we have investigated the possibility of performing these oxidative and reductive steps in a one-pot system by employing HSDHs with different cofactor specificity, namely NADH-depen-



Scheme 2. Alternative pathways and possible intermediates in the one-pot multienzymatic synthesis of 12-ketoursodeoxycholic acid (**3**) from cholic acid (**1**).

dent HSDHs in the oxidative step and an NADPH-dependent 7β-HSDH in the reductive one. In this new concept (suitable HSDHs with different cofactor specificity were not available in the 1990s), coupled *in situ* regeneration systems have been exploited not only to allow the use of catalytic amounts of the cofactors, but also to provide the necessary driving force to opposite reactions (i.e., oxidation and reduction) acting on different sites of the substrate molecule. In fact, in principle, these two steps should occur simultaneously if properly driven by distinct regeneration systems for the NAD^+ and NADPH cofactors and if the enzymes used are sufficiently selective for the respective cofactors (recently, a similar approach has been successfully used by Kroutil and co-workers in concurrent enzymatic oxidations and reductions for the stereoinversion and deracemization of secondary alcohols[10]). Biocatalysts suitable for the set-up of the one-pot process have been selected and their kinetic behaviour in respect of the reactions of interest has been evaluated. Moreover, the process has been studied employing the enzymes both in free and compartmentalized form.

Results and Discussion

A One-Pot/Five-Enzymes System: Identification and Characterization of the Biocatalysts

The first task was the identification of the five enzymes required for testing the one-pot process, namely NADH-dependent 7α-HSDH and 12α-HSDH, NADPH-dependent 7β-HSDH and two additional dehydrogenases for NAD^+ and NADPH regeneration, respectively. We both checked enzyme availability (from commercial sources or by in-lab production), and evaluated their substrate and coenzyme specificity by kinetic measurements. In fact, a detailed analysis of the different possible pathways in the multienzymatic synthesis of **3** (Scheme 2) made relevant the specification of possible bottle-necks in the cascade conversion.

A commercially available NADH-dependent microbial 12α-HSDH (unknown source, commercialized by Genzyme Biochemicals Ltd.), showing a main band of about 29 kDa by SDS-PAGE, was submitted to kinetic analysis for determination of Michaelis (K_m) and specificity constants (k_{cat}/K_m), not only for the starting substrate (**1**), but also for the possible bile acids intermediates and for the NAD^+ cofactor.

As shown in Table 1 (entries 1–7), all the K_m values were below the millimolar range, making this enzyme suitable for preparative biotransformations. Moreover, specificity constants for the 7α-hydroxy (cholic acid, **1**), 7-oxo- (3α,12α-dihydroxy-7-oxo-5β-cholanoic acid, **5**) and 7β-hydroxy derivatives (ursocholic acid, 3α,7β,12α-trihydroxy-5β-cholanoic acid, **6**) in the oxidation reactions were quite similar, so that 12α-HSDH could equally accept the different intermediates shown in Scheme 2. Similar K_m and k_{cat}/K_m values were determined for NAD^+, whereas this enzyme showed no activity in the presence of NADP(H), both in oxidative and reductive reactions.

Concerning 7α-HSDH, since the NADH-dependent enzyme from *Escherichia coli* used in the past[6] was

Table 1. Kinetic parameters of 12α-HSDH "*Genzyme*" and 7α-HSDH from *B. fragilis* for substrates and coenzymes.[a]

Enzyme	Substrate or coenzyme	K_m (mM)	k_{cat} [sec^{-1}]	k_{cat}/K_m [sec^{-1} mM^{-1}]
12α-HSDH	Cholic acid (**1**)[b]	0.107	1.91×10^5	1.79×10^6
	3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid (**5**)[b]	0.130	1.01×10^5	7.82×10^5
	Ursocholic acid (**6**)[b]	0.315	9.52×10^4	3.02×10^5
	12-Ketochenodeoxycholic acid (**4**)[c]	0.169	8.76×10^4	5.18×10^5
	12-Ketoursodeoxycholic acid (**3**)[c]	0.547	8.12×10^3	1.48×10^4
	3α-Hydroxy-7,12-dioxo-5β-cholanoic acid (**2**)[c]	0.124	2.04×10^5	1.65×10^6
	NAD^+[d]	0.220	5.18×10^5	2.35×10^6
7α-HSDH	Cholic acid (**1**)[b]	0.115	9.95×10^4	8.65×10^5
	12-Ketochenodeoxycholic acid (**4**)[b]	0.160	1.16×10^5	7.28×10^5
	3α,12α-Dihydroxy-7-oxo-5β-cholanoic acid (**5**)[c]	0.516	3.22×10^2	6.24×10^2
	3α-Hydroxy-7,12-dioxo-5β-cholanoic acid (**2**)[c]	1.190	2.14×10^4	1.80×10^4
	NAD^+[d]	0.100	1.83×10^5	1.83×10^6

[a] Kinetic parameters (K_m and k_{cat}) values were determined with the Solver tool of Microsoft Excel from Michaelis–Menten plots obtained using electrophoretically pure samples of 12α- and 7α-HSDH.
[b] Oxidations were carried out at 20 °C in 0.1 M potassium phosphate buffer, pH 9.0, using 0–15 mM bile acid and 0.2 mM NAD^+ as a cofactor.
[c] Reductions were carried out at 20 °C in 0.1 M potassium phosphate buffer, pH 9.0, using 0–15 mM bile acid and 0.2 mM NADH as a cofactor.
[d] Kinetic parameters for NAD^+ were determined in the oxidation reactions of a 2.5 mM cholic acid solution in 0.1 M potassium phosphate buffer, pH 9.0, using 0–3 mM coenzyme at 20 °C.

no more available from commercial sources, we focused our attention on the recently cloned thermostable 7α-HSDH from *Bacteroides fragilis*.[11] This enzyme was efficiently expressed in recombinant form in *E. coli* Rosetta2(DE3)pLysS and partially purified for biocatalytic applications by heat treatment and ammonium sulfate precipitation. An electrophoretically homogeneous sample with a main protein band of about 27 kDa in SDS-PAGE analysis was prepared by subsequent hydrophobic interaction chromatography and was subjected to kinetic determinations (Table 1, entries 8–12). This HSDH showed a higher specificity for the substrates of the oxidative reactions, cholic acid (**1**) and 12-ketochenodeoxycholic acid (**4**), being practically equivalent both in terms of K_m and k_{cat}/K_m values. It was catalytically inactive in the presence of NADP(H).

NADPH-dependent 7β-HSDH from *Clostridium absonum* DSM 599 was expressed under induction of chenodeoxycholic acid in anaerobic conditions as previously described.[12] In order to obtain suitable amounts of the enzyme, production was scaled-up to 1 L culture broth and carried out under a nitrogen atmosphere. 7β-HSDH was partially purified by DEAE and dye-binding chromatography with an effective separation from the co-expressed NADPH-dependent 7α-HSDH.

In order to evaluate the substrate specificity of *C. absonum* 7β-HSDH, K_m and maximum velocity (V_{max}) values of the partially purified enzyme towards two 7-oxo bile acids (**2** and **5**) in the reduction reactions were determined. The enzyme showed similar V_{max} in these reactions (3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**), 1.04 μmol min^{-1}; 3α,12α-dihydroxy-7-oxo-5β-cholanoic acid (**5**), 1.54 μmol min^{-1}), and a K_m value for **5** that was only slightly higher than the one for **2** (35 and 9 μM, respectively), thus suggesting that both reduction pathways shown in Scheme 2 could be equally effective in preparative reaction conditions. Concerning the cofactor specificity, 7β-HSDH was active only in the presence of NADPH, showing a K_m value of 21 μM in reduction reactions of **5** carried out at substrate saturation concentrations.

Regeneration of NAD^+ was achieved, as previously described,[7] using a coupled enzymatic process with a commercially available NADH-dependent lactate dehydrogenase (LDH) from rabbit muscle and pyruvate as a cosubstrate. A test reaction catalyzed by 12α-HSDH *Genzyme*, *B. fragilis* 7α-HSDH and rabbit muscle LDH in the presence of catalytic amounts of NAD^+ (0.4 mM) showed the quantitative conversion of a 0.1 M solution of **1** into **2** (see Experimental Section for details).

As far as *in situ* regeneration of NADPH is concerned, different commercially available enzymatic systems were evaluated, namely the glucose/glucose dehydrogenase (GDH) from *Thermoplasma acidophilum*, the formate/formate dehydrogenase (FDH) from *Pseudomonas* sp. 101 and the malate/malate dehydrogenase (MDH) from chicken liver.

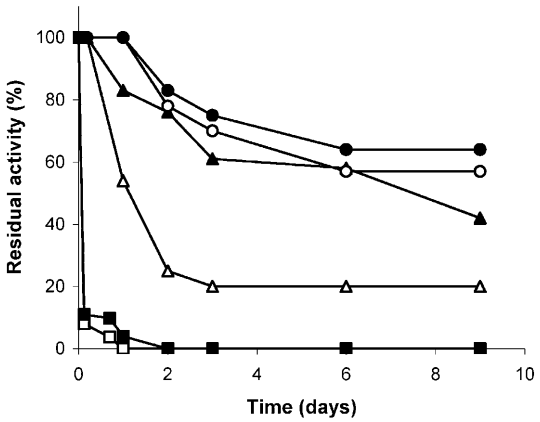


Figure 1. Stability of NADPH-dependent GDH, FDH and MDH in 50 mM potassium phosphate buffer, pH 8.0, in the presence (full symbols) or in the absence (empty symbols) of 12.5 mM cholic acid at 28 °C. Symbols: ●/○, GDH; ▲/△, FDH; ■/□, MDH.

As bile acids show detergent properties that might significantly denature proteins, initially the stabilities of NADPH-dependent GDH, FDH and MDH were evaluated in the presence or in the absence of 12.5 mM cholic acid (Figure 1). MDH was quickly inactivated even in the absence of cholic acid, whereas – quite surprisingly – higher stabilities of both GDH and FDH in the presence of cholic acid (64% and 42% residual activity after 9 days, respectively) rather than in its absence (57% and 20% residual activity) were observed.

NADPH-dependent GDH and FDH were then tested in the coupled enzymatic reduction of **2** to **3** catalyzed by 7β-HSDH using catalytic amounts of cofactor, both giving quantitative conversions.

Finally, GDH and FDH cofactor specificities were evaluated. Whereas the FDH from *Pseudomonas* sp. 101 showed only a limited preference for $NADP^+$ over NAD^+ (K_m values of 0.15 and 1 mM, respectively),[13] the *T. acidophilum* GDH confirmed to be highly (but not exclusively) NADPH-dependent, giving K_m values of 0.113 mM for $NADP^+$ and >30 mM for NAD^+.[14] These data and the higher stability shown under the reaction conditions, prompted us to use the *T. acidophilum* GDH in the proposed one-pot multienzymatic system.

One-Pot Multienzymatic Synthesis of 12-Ketoursodeoxycholic Acid: Concurrent Oxidations and Reduction in a Catalytic Cascade Process

Multienzymatic one-pot synthesis of **3** was carried out on a 5-mL scale in the presence of catalytic amounts

of NAD^+ and $NADP^+$ under different reaction conditions, by changing the relative ratio of 7α-, 12α- and 7β-HSDH activities and the cholic acid (**1**) concentration (4–100 mM).

Reaction outcomes were monitored by TLC hourly by comparison with standards of the desired product and of the possible reaction intermediates. It was found that the one-pot synthesis of **3** indeed took place, complete conversion of a 12.5 mM solution of **1** into **3** being achieved after about 5 h when using 0.5, 2 and 1 $U\,mL^{-1}$ of 7α-, 12α- and 7β-HSDH, respectively, and 4 $U\,mL^{-1}$ of both LDH and GDH (Figure 2, lane A). Higher substrate concentrations gave incomplete conversions even after longer incubation times, thus suggesting the possible occurrence of inhibition effects on one or more of the used enzymes. Using 12.5 mM initial cholic acid concentration, three consecutive conversion cycles could be carried out in a membrane reactor without adding fresh enzymes.

However, an unexpected change in the bioconversion outcome was disappointingly found when the reaction was conducted for a longer time, i.e., 24 h. In fact, as shown in Figure 2 (lane B), the former desired product **3** was slowly reduced by 12α-HSDH back to ursocholic acid (**6**), thus suggesting an unexpected lack of cofactor specificity for one or more of the used enzymes.

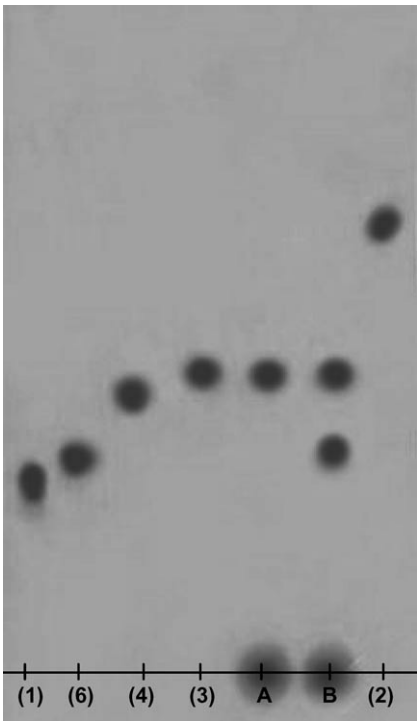


Figure 2. TLC analysis of the one-pot cascade conversion of cholic acid (**1**) to 12-ketoursodeoxycholic acid (**3**). **A**: reaction after 5 h; **B**: reaction after 24 h. Reference compounds: **1**, cholic acid; **6**, ursocholic acid; **4**, 12-ketochenodeoxycholic acid; **3**, 12-ketoursodeoxycholic acid; **2**, 3α-hydroxy-7,12-dioxo-5β-cholanoic acid. The R_f of the intermediate compound **5** (3α,12α-dihydroxy-7-oxo-5β-cholanoic acid) was identical to the one of compound **4**.

As in the previous kinetic determinations 12α-HSDH was active only in the presence of NAD(H), the only possible explanation relied on the unexpected GDH-catalyzed generation of NADH in addition to the desired NADPH regeneration.

Different attempts of decreasing the unspecific driving force of the GDH-catalyzed reduced cofactors regeneration in this cascade system by lowering glucose concentration or GDH activity were not satisfactory, the whole process becoming very slow and not completely selective anymore. Therefore, it was decided to test the use of compartmentalized enzymes. In fact, if the observed reduction of **3** to **6** during extended reaction time was indeed determined by the copresence in the same reactor of 12α-HSDH and GDH, we reasoned that it could have been possible to overcome this problem by physically separating them.

Sequential Synthesis of 12-Ketoursodeoxycholic Acid with Compartmentalized Enzymes

The first attempt of performing the one-pot bioconversion of **1** to **3** using separately compartmentalized oxidative and reductive systems was carried out by using the so-called "tea-bags" system[15] in a sequential one-pot approach.

Practically speaking, the enzymes involved in the double oxidation reaction, namely the NADH-dependent 7α-HSDH, 12α-HSDH and LDH, were dissolved together into a 0.5 mL disposable dialyzer (OX-tea-bag), while a second dialyzer contained 7β-HSDH and GDH (RED-tea-bag). The reaction mixture (8 mL) contained all the low-molecular-weight compounds needed for the synthesis of **3**, namely the starting substrate (**1**), the two co-substrates needed for cofactors regeneration (pyruvate and glucose) and catalytic amounts of both cofactors (NAD^+ and $NADP^+$) dissolved in 50 mM potassium phosphate buffer, pH 8.0.

The reaction was started by putting the OX-tea-bag into the reaction mixture and stirring vigorously for 24–48 h. Regardless of the presence of different amounts of the three enzymes (2.5–16 U, 10–32 U and 20–64 U for 7α-HSDH, 12α-HSDH and LDH, respectively), bioconversions were completed after a relatively long time (24–30 h), thus suggesting possible diffusional limitations. At the end of the oxidation step, the OX-tea-bag was removed from the reaction solution and substituted by the RED-tea-bag containing 5 U 7β-HSDH and 30 U GDH. Again, complete conversion of **2** to **3** was obtained after about 24 h

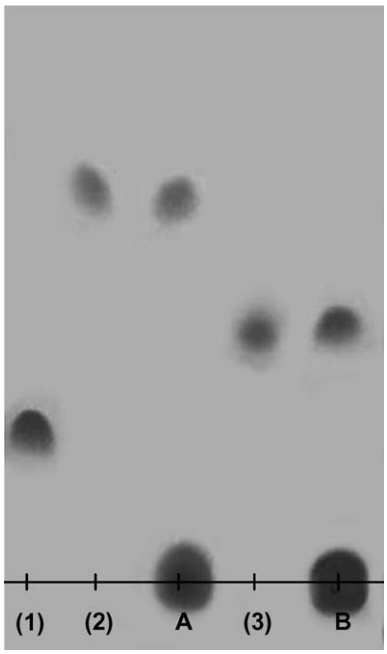


Figure 3. TLC analysis of the one-pot sequential synthesis of **3** using compartmentalized enzymes. **1**, cholic acid; **2**, 3α-hydroxy-7,12-dioxo-5β-cholanoic acid; **3**, 12-ketoursodeoxycholic acid; **A**, oxidative step after 24 h; **B**, reductive step after 24 h.

(Figure 3), but, unlike what was previously observed with free enzymes, no formation of back-side products, ursocholic acid (**6**), was observed, even after 100 hours incubation.

These results confirmed our previous hypothesis that the undesired observed reduction of **3** to **6** could be overcome by physically separating the "oxidative" and the "reductive" enzymes and, although the "tea-bags" system proved to be not sufficiently effective because of the very long conversion times (probably related to diffusional limitations), it provided a proof-of-principle of the feasibility of the sequential one-pot synthesis of **3** using compartmentalized enzymes.

The same concept was finally applied by compartmentalizing the "oxidative" enzymes (7α-HSDH, 12α-HSDH and LDH) and the "reductive" ones (7β-HSDH and GDH) into two distinct membrane reactors (Figure 4).

As in the tea-bags system, the reaction mixture containing all the needed non-enzymatic reagents was added to the first reactor and left for 15 h at 28 °C. After this time, a TLC control showed the complete conversion of **1** to **2**. The mixture was therefore submitted to ultrafiltration and the filtered solution was directly transferred to the second reactor. After 15 h, the reduction reaction was complete and the pure product **3** could be recovered by ultrafiltration and acid precipitation.

The five biocatalysts used for the multienzymatic synthesis of **3** were kept in their respective reactors at the end of the conversion cycle and tested for residual activity. As shown in Figure 5, all the enzymes retained most of their activity after this first cycle. Therefore, the sequential bioconversion could be repeated, the biocatalysts stabilities being monitored at the end of each cycle.

Six consecutive cycles (the fifth one over the weekend) were carried out using the initially loaded enzymes, all of them giving quantitative conversion of **1** into **3** and no formation of by-products.

As far as enzymes stability concerns, quite surprisingly, it was observed that the "weakest" enzyme was the thermostable 7α-HSDH, whereas the other dehydrogenases retained about 25–50% of their initial activity after 8 days of use and six ultrafiltration steps (Figure 5).

Conclusions

It was shown that the one-pot synthesis of **3** can be achieved using HSDHs with different cofactor specificity in oxidation and reduction reactions occurring in the same reaction mixture and properly driven by different cofactor regeneration systems. Nonetheless, it

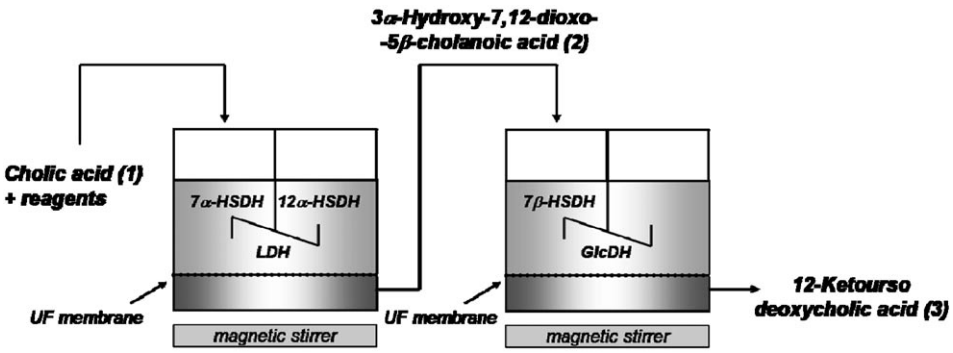


Figure 4. Schematic representation of the sequential synthesis of **3** in membrane reactors.

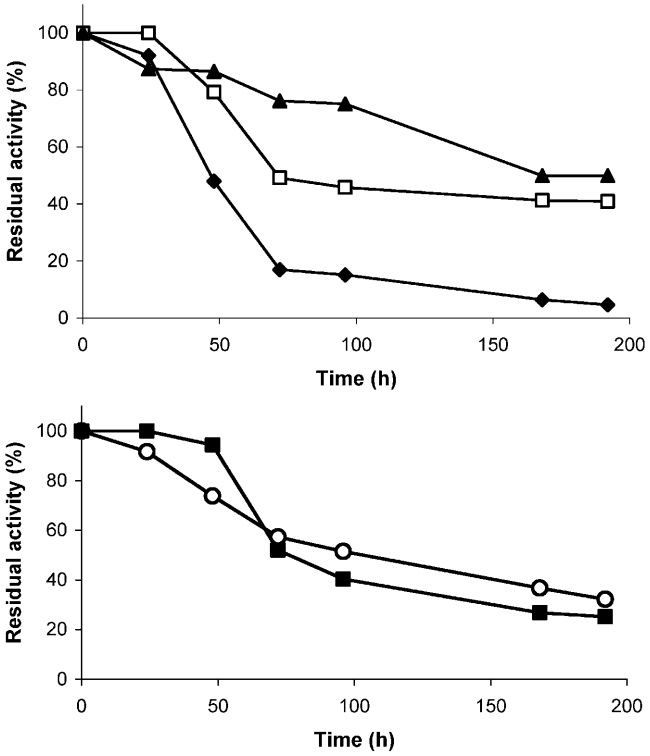


Figure 5. Stability of the five enzymes used in the sequential process in membrane reactors; (*top*) "oxidative" reactor; (*bottom*) "reductive" reactor. Symbols: ◆, 7α-HSDH; □, 12α-HSDH; ▲, LDH; ○, 7β-HSDH; ■, GDH.

was demonstrated that all the involved biocatalysts should be exclusively selective for the respective cofactors in order to perform this multienzymatic synthesis in a cascade process. In this specific case, the establishment of undesired reaction equilibria (likely due to a limited cofactor specificity of the GDH for NADPH) leading to a non-specific bioconversion process and, consequently, to reduced product yields and purity, was overcome by uncoupling the oxidative and reductive biocatalysts. However, we may foresee that, thanks to the ongoing advances in biocatalysts engineering, a new GDH with exclusive NADPH-specificity will become available in the near future, and with this GDH at hand the physical separation of the oxidative and reductive biocatalysts could be avoided.

Experimental Section

Materials

12α-Hydroxysteroid dehydrogenase (12α-HSDH, EC 1.1.1.176) was obtained from Genzyme Biochemicals Ltd. (Kent, England). Glucose dehydrogenase (GDH, EC 1.1.1.47) from *Thermoplasma acidophilum*, lactate dehydrogenase (LDH, EC 1.1.1.27) from rabbit muscle, malate dehydrogenase (MDH, EC 1.1.1.40) from chicken liver, NAD(H), NADP(H), sodium pyruvate, glucose and Reactive Red 120™ were purchased from Sigma; cholic acid and pure standards of cholic acid derivatives were obtained from Prodotti Chimici e Alimentari S.p.A (Basaluzzo, Italy). NADPH-specific formate dehydrogenase (FDH, EC 1.2.1.2) from *Pseudomonas* sp. 101 overexpressed in recombinant *E. coli* was from Julich Chiral Solutions GmbH (Germany). All other reagents were of analytical grade.

Expression and Purification of NADH-Dependent 7α-HSDH from *Bacteroides fragilis*

Plasmid JS2.2 containing the gene coding for the NADH-dependent 7α-hydroxysteroid dehydrogenase (7α-HSDH, EC 1.1.1.159) from *Bacteroides fragilis* was provided by one of us (Prof. Ling Hua). Freshly transformed *E. coli* Rosetta2-(DE3)pLysS harboring JS2.2 was cultured overnight in 20 mL of LB medium containing 100 μg mL^{-1} ampicillin and 34 μg mL^{-1} chloramphenicol at 37 °C and 220 rpm. The culture was used to inoculate 1 L of fresh medium and growth of recombinant *E. coli* was carried out in the same conditions until $OD_{600} = 1$. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM. After induction of expression of the heterologous gene, the culture was kept at 30 °C for 5 h, then the cells were harvested by centrifugation (30 min, 5000 rpm) and lysed by ultrasonication in 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM DTT and 100 μL mL^{-1} Protease Inhibitor Cocktail (Sigma).

After centrifugation (40 min, 15000 rpm), the crude protein extract was heat-treated in a water-bath at 60 °C for 30 min and centrifuged at 15000 rpm for 30 min. The residual proteins were precipitated from the supernatant with 60% sat. ammonium sulfate.

Further purification of 7α-HSDH from *B. fragilis* to electrophoretic homogeneity was performed by hydrophobic interaction chromatography as described by Bennett et al.[10a]

Production and Partial Purification of NADPH-Dependent 7β-HSDH from *Clostridium absonum*

Clostridium absonum DSM 599 (ATCC 27555) was from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH) and routinely maintained at −80 °C after overnight growth in cooked meat broth at 37 °C and anaerobic conditions.

C. absonum was grown on brain heart infusion (BHI) medium which was submitted to repeated vacuum-nitrogen cycles after autoclaving for oxygen removal. Starter cultures (100 mL) were inoculated with 1 mL cooked meat culture and grown overnight at 37 °C under nitrogen, then added to 900 mL of fresh medium in the same conditions. Chenodeoxycholic acid was added to a final concentration of 0.4 mM when the cells reached $OD_{600} = 0.4$ and cultures were kept for additional 2.5 h ($OD_{600} < 0.9$). After recovery by centrifugation (4500 rpm for 30 min), cells were resuspended in 20 mM potassium phosphate buffer, pH 8.0, 1 mM DTT, containing 100 μL mL^{-1} of Protease Inhibitor Cocktail (Sigma), and lysed by ultrasonication. The cell-free extract was recovered by centrifugation (15000 rpm for 20 min) and dialysed against 20 mM potassium phosphate buffer, pH 8.0, 1 mM EDTA, 1 mM DTT, 5% (v/v) glycerol (buffer A). Enzyme solution (25 mL) was then applied to a Fractogel

EMD DEAE-650 (S) (Merck) column (16 mm × 110 mm) previously equilibrated with buffer A, at a 1 mL min^{-1} flow rate.

After loading, the column was washed with 200 mL of the equilibration buffer and bound proteins were eluted by a linear gradient from 0 to 0.25 M NaCl in the same buffer within 4 h at 1 mL min^{-1} flow rate. Protein concentration was continuously monitored at 280 nm.

Fractions containing the 7β-hydroxysteroid dehydrogenase (7β-HSDH) activity were collected and loaded onto a Reactive Red 120™ (3000CL, Sigma) column (2 × 16 cm) equilibrated with buffer A containing 0.1 M NaCl at a flow rate of 1 mL min^{-1}. After loading the column was washed with 20 mL of the equilibration buffer, then 7β-HSDH was eluted with 40 mL of buffer A containing 0.4 M NaCl. The column was extensively washed with 1 M NaCl before reusing.

Enzyme and Protein Assays

Dehydrogenase activities were assayed by spectrophotometrically measuring the oxidation of NAD(P)H or reduction of NAD(P)$^+$ at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) and 20 °C on a Jasco V-530 UV/VIS spectrophotometer.

Specifically, assay mixtures in a total volume of 1 mL were: a) NADH-dependent 12α-HSDH: 0.2 mM NAD$^+$, 2.5 mM cholic acid (or deoxycholic acid) in 0.1 M potassium phosphate buffer, pH 9.0; b) NADH-dependent 7α-HSDH: 0.2 mM NAD$^+$, 2.5 mM cholic acid (or chenodeoxycholic acid) in 0.1 M potassium phosphate buffer, pH 9.0; c) NADPH-dependent 7β-HSDH: 0.2 mM NADP$^+$, 2.5 mM ursodeoxycholic acid in 0.1 M potassium phosphate buffer, pH 9.0; d) NADPH-dependent GDH: 0.2 mM NADP$^+$, 50 mM glucose in 0.1 M potassium phosphate buffer, pH 7.0; e) NADH-dependent LDH: 0.2 mM NADH, 10 mM pyruvate in 0.1 M potassium phosphate buffer, pH 7.0; f) NADPH-dependent formate dehydrogenase: 0.2 mM NADP$^+$, 0.2 M ammonium formate, pH 7.0; g) NADPH-dependent malate dehydrogenase: 0.2 mM NADP$^+$, 50 mM sodium malate, pH 7.0. One unit of activity is defined as the enzyme activity that reduces (or oxidizes) 1 μmol of NAD(P)(H) per min under the assay conditions described above.

Protein concentration was determined according to the method of Bradford (Bio-Rad Protein Assay),[16] using bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis

Enzyme purity was monitored by SDS-PAGE (15% T, 4% C) according to the method of Laemmli.[17] The gels were stained with Coomassie brilliant blue and molecular mass under denaturing conditions was determined by comparison with standard markers (Bio-Rad).

Enzyme Stability

NADPH-dependent GDH, FDH and MDH were dissolved in 1 mL of 50 mM potassium phosphate buffer, pH 8.0, in the presence or in the absence of 12.5 mM cholic acid at 28 °C. At scheduled times enzyme activity was assayed as described above. Enzyme stability was monitored for 9 days.

Oxidation of Cholic Acid (1) to 3α-Hydroxy-7,12-dioxo-5β-cholanoic Acid (2) and NAD$^+$ Regeneration

The reaction was carried out in a 3 mL solution containing 50 mM potassium phosphate buffer, pH 8.0, 0.1 M cholic acid, 0.35 M sodium pyruvate, 0.4 mM NAD$^+$, 1 mg mL^{-1} NaN_3, 5 U mL^{-1} NADH-dependent 7α-HSDH from *B. fragilis*, 5 U mL^{-1} NADH-dependent 12α-HSDH (Genzyme) and 15 U mL^{-1} NADH-dependent LDH from rabbit muscle (Sigma) at 28 °C for 24 h.

Reaction progress was monitored by TLC on precoated silica gel 60 F_{254} plates (Merck) using chloroform-methanol-acetic acid, 10:1:0.08, as eluting system. Plates were sprayed with the Komarowsky's reagent.[18]

Reduction of 3α-Hydroxy-7,12-dioxo-5β-cholanoic Acid (2) to 12-Ketoursodeoxycholic Acid (3) and NADPH Regeneration

NADPH regeneration catalyzed by NADPH-dependent GDH from *T. acidophilum* (Sigma) was tested in a 5 mL reaction containing 50 mM potassium phosphate buffer, pH 8.0, 12.5 mM 3α-hydroxy-7,12-dioxo-5β-cholanoic acid, 0.1 M glucose, 0.1 mM NADP$^+$, 1 mg mL^{-1} NaN_3, 2 U mL^{-1} GDH from *T. acidophilum* and 0.5 U mL^{-1} NADPH-dependent 7β-HSDH from *C. absonum*.

NADPH-specific formate dehydrogenase from *Pseudomonas* sp. 101 (Julich Chiral Solutions GmbH) was tested in parallel in a 5 mL reaction containing 50 mM potassium phosphate buffer, pH 8.0, 12.5 mM 3α-hydroxy-7,12-dioxo-5β-cholanoic acid, 0.1 M ammonium formate, 0.1 mM NADP$^+$, 1 mg mL^{-1} NaN_3, 2 U mL^{-1} FDH and 0.5 U mL^{-1} NADPH-dependent 7β-HSDH from *C. absonum*.

Both reactions were kept at 28 °C for 24 h and monitored by TLC as described above.

Multienzymatic Synthesis of 12-Ketoursodeoxycholic Acid (3)

Cascade process: 12-ketoursodeoxycholic acid (**3**) synthesis was carried out at 28 °C in a 25 mm diameter Amicon stirred ultrafiltration cell (model 8010, cell capacity: 10 mL) equipped with a polyethersulphone membrane (NMWL 5,000, Millipore) and monitored by TLC as described above.

Reaction mixture in a total volume of 5 mL was: 50 mM potassium phosphate buffer, pH 8.0, 12.5 mM cholic acid (**1**), 0.1 M glucose, 50 mM sodium pyruvate, 0.1 mM NAD$^+$, 0.1 mM NADP$^+$, 1 mg mL^{-1} NaN_3, 0.5 U mL^{-1} *B. fragilis* 7α-HSDH (NADH-dependent), 2 U mL^{-1} 12α-HSDH (NADH-dependent) from *Genzyme*, 4 U mL^{-1} LDH from rabbit muscle, 1 U mL^{-1} *C. absonum* 7β-HSDH (NADPH-dependent) and 4 U mL^{-1} *T. acidophilum* GDH.

At the end of each conversion cycle (after about 2–5 h), the solution was ultrafiltered. The retained solution (0.5 mL) was stored at 4 °C overnight and then used for a new biocatalytic cycle by addition of 4.5 mL of fresh reaction mixture containing all the reagents described above with the exception of the enzymes.

Sequential process in a "tea-bags" reactor: Synthesis of 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**) was achieved by putting a 0.5 mL disposable dialyzer (Spectra/Por® RC, regenerated cellulose membrane, MWCO 8,000 Da, Sigma) containing different amounts of *B. fragilis* 7α-HSDH, 12α-

HSDH "*Genzyme*" and LDH from rabbit muscle (OX-tea-bag), into a 10 mL glass tube filled with 8 mL of 50 mM potassium phosphate buffer, pH 8.0, 12.5 mM cholic acid (**1**), 0.1 M glucose, 50 mM sodium pyruvate, 0.1 mM NAD^+ and 0.1 mM $NADP^+$. The double oxidation reaction was kept at 28 °C under vigorous stirring until complete conversion of cholic acid into the desired product, then the OX-tea-bag was transferred to a fresh reaction mixture for a new conversion cycle.

The produced 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**) was then submitted to reduction to 12-ketoursodeoxycholic acid (**3**) by action of *C. absonum* 7β-HSDH (5 U, concentrated by ultrafiltration or ammonium sulfate precipitation) and *T. acidophilum* GDH (30 U), both compartmentalized in the same 0.5 mL disposable dialyzer (RED-tea-bag).

Both reactions were monitored by TLC as described above.

Sequential process in membrane reactors: Oxidation of cholic acid (**1**) to 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**) was carried out at 28 °C in a 44.5 mm diameter Amicon stirred ultrafiltration cell (model 8050, cell capacity: 50 mL) equipped with a polyethersulphone membrane (NMWL 5,000, Millipore) and containing 10 mL of 50 mM potassium phosphate buffer, pH 8.0, 12.5 mM cholic acid, 0.1 M glucose, 50 mM sodium pyruvate, 0.1 mM NAD^+, 0.1 mM $NADP^+$, 1 mg mL^{-1} NaN_3, 0.5 U mL^{-1} *B. fragilis* 7α-HSDH, 2 U mL^{-1} 12α-HSDH "*Genzyme*" and 4 U mL^{-1} LDH from rabbit muscle.

After 15 h the solution was ultrafiltered. The retained solution (0.5 mL) was used for a new biocatalytic cycle, which was started by addition of 9.5 mL of fresh reaction mixture containing all the above described components (with the exception of the enzymes).

5 mL of the ultrafiltered solution containing 3α-hydroxy-7,12-dioxo-5β-cholanoic acid (**2**) was transferred to a second 50 mL membrane reactor containing 7.5 U *C. absonum* 7β-HSDH and 20 U *T. acidophilum* GDH and kept at 28 °C for 15 h. At the end of the conversion cycle, the solution was ultrafiltered. The retained solution (0.5 mL) was used for a new reduction cycle by addition of 4.5 mL of the 3α-hydroxy-7,12-dioxo-5β-cholanoic acid solution recovered from the oxidation reactor.

Following this protocol, biocatalysts compartmentalized in both reactors were reused for six consecutive cycles. Operative stability of each enzyme was evaluated by testing the residual activity at the end of each cycle spectrophometrically, as previously described.

The six final reaction mixtures were mixed and the final product, 12-ketoursodeoxycholic acid (**3**), was isolated by acid precipitation (109 mg, 73% i.y.). Its identity was confirmed by TLC and ^{1}H NMR by comparison with an authentic sample. ^{1}H NMR (DMSO-d_6, selected data): $\delta = 3.298$ (2 H, m, H-3β and H-7α); 0.945 and 0.946 (3H each, s each, CH_3-18 and CH_3-19); 0.729 (3 H, d, $J = 6$ Hz, CH_3-21).

Acknowledgements

This work was performed in the frame of the COST Action CM0701 "Cascade Chemoenzymatic Processes - New Synergies Between Chemistry and Biochemistry". The contribution by Prodotti Chimici Alimentari S.p.A. (fellowships to E.E.F. and I.Z.) is gratefully acknowledged.

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