Large-scale Enzymatic Synthesis of 12-Ketoursodeoxycholic Acid from Dehydrocholic Acid by Simultaneous Combination of 3α-Hydroxysteroid Dehydrogenase from *Pseudomonas testosteroni* and 7β-Hydroxysteroid Dehydrogenase from *Collinsella aerofaciens*

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Dedicated to Professor Heribert Offermanns on the occasion of his 75th birthday

12-Keto-UDCA is an important optically active component for the drug ursodeoxycholic acid (UDCA). Starting from the three-keto compound dehydrocholic acid, the carbonyl groups at position 3 and 7 have to be reduced stereo- and regioselectively. In this case we applied two hydroxysteroid dehydrogenases for this purpose, the NAD-dependent 3α-HSDH from *Pseudomonas testosteroni* and the NADP-dependent 7β-hydroxysteroid dehydrogenase from *Collinsella aerofaciens*. Both enzymes can be produced in high yields by an *Escherichia coli* strain as recombinant proteins. In order to avoid impurities by the 7α-hydroxysteroid dehydrogenase of *Escherichia coli*, a mutant strain with an inactivated 7α-enzyme was applied for producing the three enzymes. For bioconversion, the dehydrogenases can be used as crude enzyme samples and are applied simultaneously. A 1.8 L batch of 100 mM DHCA incubated at pH = 8.0 and 25 °C resulted in 80 g crude product with a quite high purity of $\geq$ 99.5 % as judged by HPLC analysis.

Key words: Bile Acid, Hydroxysteroid Dehydrogenase, Short-chain Dehydrogenase, Ursodeoxycholic Acid

Introduction

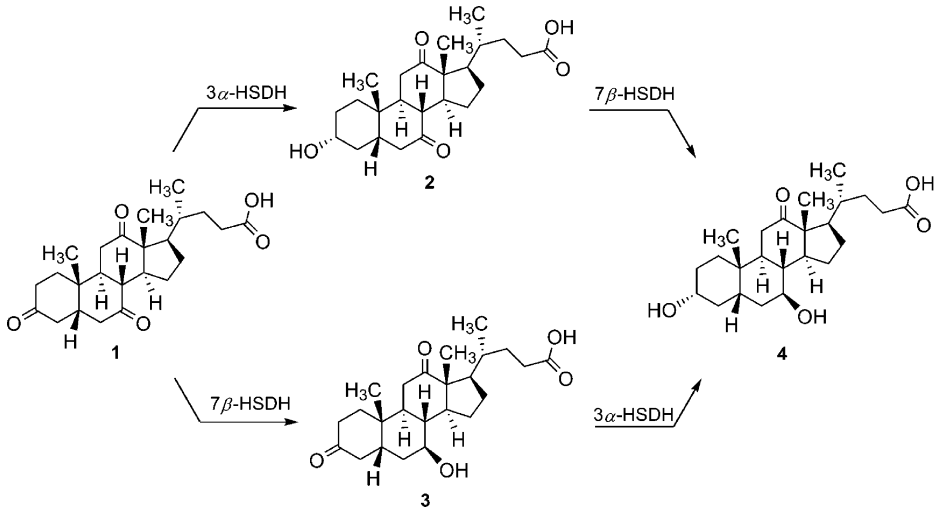
Bile acids are important biological compounds for the digestion and adsorption of fats, fatty acids and lipid-soluble vitamins. As an example of the existing drugs, ursodeoxycholic acid (UDCA), which is widely used in cholesterol gall-stones dissolution therapy [1 – 4], is industrially prepared on a ton scale, either by several chemical steps or by a combination of enzymatic and chemical steps. An important target compound in the synthesis of ursodeoxycholic acid is 12-ketoursodeoxycholic acid, which can be converted by a Wolff-Kishner reduction into ursodeoxycholic acid. One route for the synthesis of 12-ketoursodeoxycholic acid starts from cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid), which can be converted by two oxidative steps catalyzed by 7α- and 12α-hydroxysteroid dehydrogenases (HSDHs), followed by a 7β-HSDH-catalyzed reductive step [5, 6]. A further route starts from 7-ketolithocholic acid which is converted into ursodeoxycholic acid by reducing the 7-keto group by 7β-HSDH [7, 8].

Here we describe the complete conversion of dehydrocholic acid (DHCA) into 12-ketoursodeoxycholic acid at high substrate concentrations (up to 100 mM) by using two hydroxysteroid dehydrogenases (3α- and 7β-HSDH) for reduction of the keto groups in 3- and 7-position.

Results and Discussion

Cloning and heterologous overexpression of the hydroxysteroid dehydrogenases and glucose dehydrogenase

The complete route leading to ursodeoxycholic acid starting from cholic acid consists of a consecutive



Scheme 1. The enzyme-catalyzed conversion of DHCA (dehydrocholic acid) **1** to 12-keto-ursodeoxycholic acid applying 3α- and 7β-hydroxysteroid dehydrogenases (HSDHs) simultaneously. The mono-reduced intermediates **2** (7,12-diketo-UDCA) as the product of 3α-HSDH, and **3** (3,12-diketo-UDCA) can be detected by HPLC after reduction by 7β-HSDH.

combination of an oxidative chemical step to oxidize the three hydroxy groups (3α, 7α, 12α), followed by a biocatalytic treatment of DHCA (dehydrocholic acid or 3α,7α,12α-triketocholanic acid) to reduce the keto groups at C-3 and C-7, and followed by a further chemical step, the Wolff-Kishner reduction of 12-ketoursodeoxycholic acid to delete the keto group at C-12. In order to reduce the two keto groups at C-3 and C-7, NAD-dependent 3α-HSDH from *Pseudomonas testosteroni* [9 – 11] and NADP-dependent 7β-hydroxysteroid dehydrogenase from *Collinsella aerofaciens* [8] were used simultaneously (Scheme 1). The reduced coenzymes were regenerated by glucose dehydrogenase and glucose. The enzymes were produced in recombinant form using *Escherichia coli* BL21(DE3) as the host. The key advance is the use of an *E. coli* host strain where the gene coding for 7α-hydroxysteroid dehydrogenase has been deleted (BL21(DE3) Δ7α-HSDH). This avoids the formation of the undesired by-product 12-ketochenodeoxycholic acid (3α,7α-dihydroxy-12-ketocholanoic acid).

In order to express the dehydrogenases, standard cloning techniques were used to create three different recombinant plasmids. All used hydroxysteroid dehydrogenases were cloned without any modification. For the recombinant overexpression of the dehydrogenases, *E. coli* BL21(DE3) Δ7α-HSDH was used as host, leading to a high level of recombinant protein for all three strains. The activities of the different crude extracts were studied comparatively using DHCA respectively glucose as substrate. The functional expression of the enzymes was determined by measuring photometrically the activities in cell-free extracts. The GDH activity was found to be 197 U mL^{-1} (19.9 U mg^{-1}), the 3α-HSDH activity 35 U mL^{-1} (3.1 U mg^{-1}), and

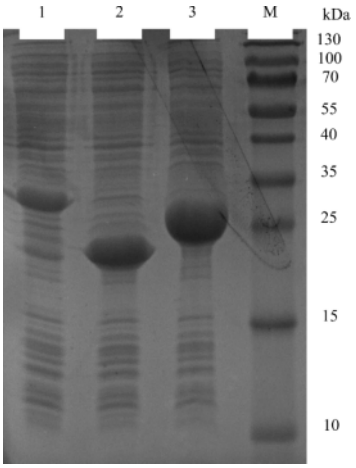


Fig. 1. SDS-PAGE analysis of the enzymes applied for the enzyme-catalyzed conversion of DHCA. Lane M = marker proteins, lane 1 = crude extract from recombinant GDH, lane 2 = crude extract from recombinant 3α-HSDH, lane 3 = crude extract from recombinant 7β-HSDH.

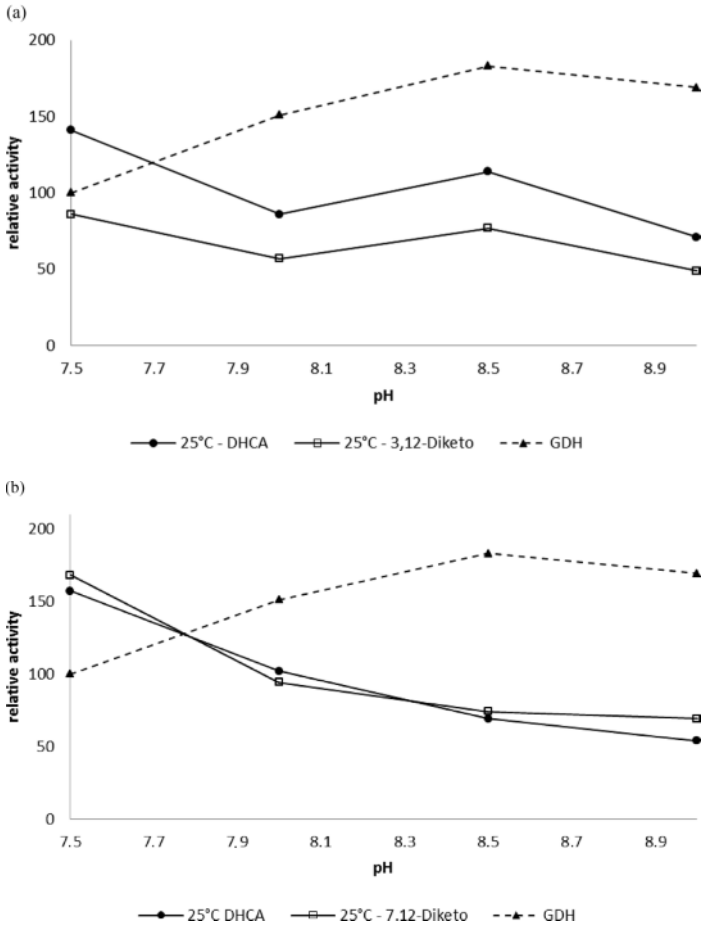


Fig. 2. Influence of pH on the activity of 3α-HSDH (a) and 7β-HSDH (b) for dehydrocholic acid (DHCA) and the mono-reduced intermediates (3,12-diketo-UDCA for 3α-HSDH and 7,12-diketo-UDCA for 7β-HSDH). Activity was measured at 25 °C.

the 7β-HSDH activity 134 U mL^{-1} (5.6 U mg^{-1}) for expression in LB. The activities of the expression in autoinduction medium (AI) was up to 3-fold higher.

The overexpression was judged by SDS gel electrophoresis (Fig. 1). The main bands in lanes 1 to 3 (Fig. 1) are in good accordance with the molecular masses of dehydrogenases (calculated molecular masses: GDH = 28.1 kDa, 3α-HSDH = 26.4 kDa, 7β-HSDH = 28.8 kDa).

Biochemical characterization of 3α- and 7β-hydroxysteroid dehydrogenases

– Dependence of the hydroxysteroid dehydrogenase activities on pH

The influence of pH on the enzyme activity and stability is a crucial parameter for preparative applications of these enzymes. Especially when using enzymes such as hydroxysteroid dehydrogenases in the reductive and a further enzyme for regeneration in the oxidative direction it is necessary to find an optimal pH range for the enzymes. Furthermore, the influence of the pH on the solubility of the substrates, the intermediates (3,12- and 7,12-diketo compounds) and the product have to be considered. Due to the pK_a values, these bile acids are soluble at alkaline pH values of > 7.8. Thus, we measured the pH dependence of the hydroxysteroid dehydrogenases in the range of 7.5 – 9.0 with the substrate DHCA and the corresponding mono-reduced intermediates (the 3,12-diketo compound with 3α-HSDH and the 7,12-diketo compound with 7β-HSDH) (Figs. 2a and 2b). For GDH it is known from the literature that the pH optimum for the oxidation of glucose lies at 8.5 with a residual activity of about 40 % at 7.5 [12]. Fig. 2 shows that the activities of both

hydroxysteroid dehydrogenases decrease with increasing pH value in the range of 7.5 up to 9.0, whereas glucose dehydrogenase shows an opposing effect. For preparative application of this coupled dehydrogenase system a pH of 8.0 was used to convert DHCA into 12-ketoursodeoxycholic acid which takes the solubility of the bile acids and the residual activities of the three dehydrogenases into consideration.

– Determination of kinetic parameters of the hydroxysteroid dehydrogenases

The kinetic parameters of the substrates DHCA and 3,12-diketo-UDCA were determined photometrically for 3α-HSDH, and that of DHCA and 7,12-diketo-UDCA for 7β-HSDH. Michaelis-Menten kinetic curves are given for these four parameters in Figs. 3a–3d. For 7β-HSDH both K_M values are in the same order of magnitude, and a slight substrate excess inhibition was observed for both substrates. Compared to the three-keto compound DHCA (dehydrocholic acid) which can be converted with a v_{max} of 8.4 U mg^{-1}, the mono-reduced intermediate compound, 7,12-diketo-UDCA, is reduced with a significantly lower reaction rate of 3.2 U mg^{-1}. For 3α-HSDH it is very difficult to determine exact K_M values, since both substrates show a quite strong substrate excess inhibition. The apparent K_M values are

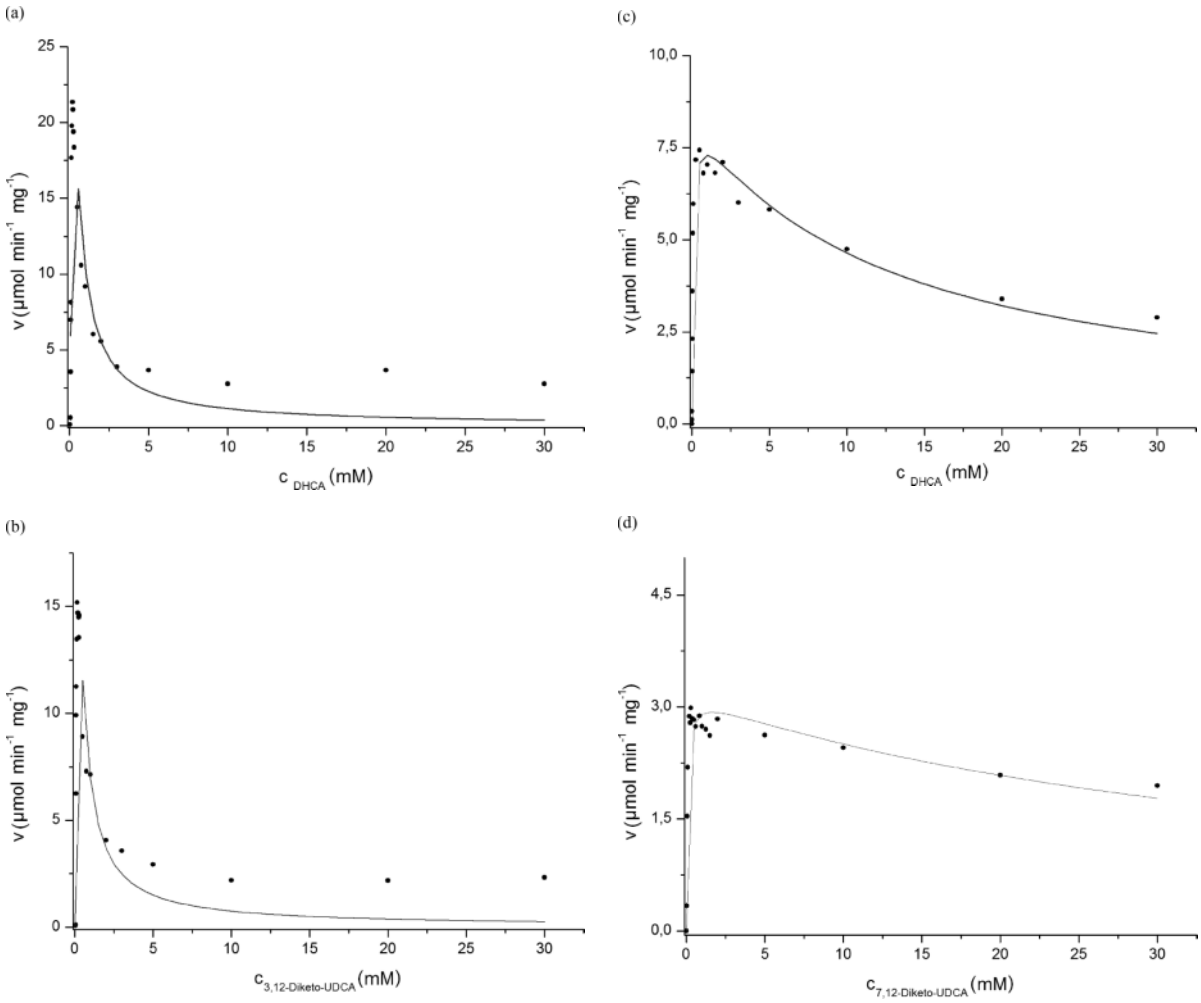


Fig. 3. Michaelis-Menten graphs for 3α- (a and b) and 7β-hydroxysteroid dehydrogenases (c and d) with the substrates DHCA (a and c) and 3,12-diketo-UDCA for 3α-HSDH (b) and 7,12-diketo-UDCA for 7β-HSDH (d).

Enzyme	Substrate	v_{max} (U mg^{-1})	K_M (mM)	K_I (mM)
7β-HSDH	DHCA (dehydrocholic acid)	8.4 ± 0.5	0.07 ± 0.015	12.4 ± 3.2
7β-HSDH	7,12-diketo-UDCA (3α-hydroxy-7,12-dioxo-5β-cholanoic-24 acid)	3.2 ± 0.2	0.07 ± 0.02	38.3 ± 14.5
3α-HSDH	DHCA (dehydrocholic acid)	25[a]	0.2[a]	9[a]
3α-HSDH	3,12-diketo-UDCA (7β-hydroxy-3,12-dioxo-5β-cholanoic-24 acid)	18[a]	0.15[a]	7[a]

[a] Data taken from Figs. 3a and 3b.

Table 1. Kinetic characterization of 7β- and 3α-HSDH for dehydrocholic acid and the mono-reduced intermediates 7,12-diketo-UDCA (for 7β-HSDH) and 3,12-diketo-UDCA (for 3α-HSDH).

in the same order of magnitude for both substrates (0.2 mM for DHCA and 0.15 mM for 3,12-diketo-UDCA) which holds true also for the the v_{max} values (25 U mg^{-1} for DHCA and 18 U mg^{-1} for 3,12-diketo-UDCA). Table 1 summarizes the calculated kinetic data for 7β-HSDH and apparent kinetic data for 3α-HSDH.

Biotransformation of DHCA

In order to produce 12-keto-UDCA on a gram-scale, the enzyme-catalyzed reduction of DHCA was carried out in 1.8 L aqueous buffered solution. 100 mM of DHCA was converted by 1.5 U mL^{-1} of recombinant 3α- and 1.5 U mL^{-1} of 7β-HSDH in the presence of a slight excess of glucose dehydrogenase (5 U mL^{-1}) for regeneration of the coenzymes. Samples were taken periodically up to 19 h and analyzed by HPLC for monitoring the process.

Fig. 4 shows that during the first 90 min the concentration of DHCA decreases with a nearly linear reaction rate of 1 mM min^{-1}. Only small amounts of the mono-reduced intermediate 7,12-diketo-UDCA were observed whereas the intermediate 3,12-diketo-UDCA, formed by the primary reduction step catalyzed by 7β-HSDH, accumulates to a significantly higher degree. This means that 3α-HSDH is less effective against the mono-reduced compound than 7β-HSDH against the 3α-HSDH product, which corresponds to the results measuring enzyme activities at different pH values (Fig. 2). Nevertheless, nearly 80 % of the product 12-keto-UDCA is formed at a high rate of about 0.67 mM min^{-1} during 3 h. After completion of the bioconversion a crude product was obtained by precipitation. 80 g of the product with a high purity of $\geq$ 99.5 % (HPLC analysis; Fig. 5) was obtained by this bioconversion step applying two hydroxysteroid dehydrogenases for reduction of DHCA, glucose dehydrogenase for regeneration of NADH or NADPH, and glucose as the hydrid donor.

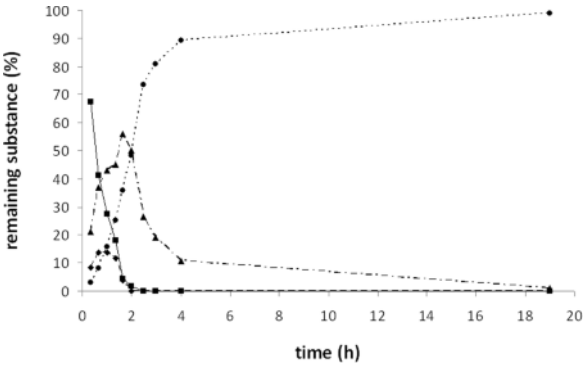


Fig. 4. Cell-free biotransformation of 100 mM dehydrocholic acid (DHCA) by 3α- and 7β-hydroxysteroid-dehydrogenases in the presence of 0.1 mM $NADP^+$ and 0.25 mM NAD^+, respectively on a 1.8 L scale monitored over 19 h. Concentrations of DHCA (dehydrocholic acid) (—■—), 3,12-diketo-UDCA (–·△·–), 7,12-diketo-UDCA (––♦––), and 12-keto-UDCA (···●···) were determined by HPLC.

Conclusion

Two hydroxysteroid dehydrogenases, namely NAD-dependent 3α-hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* and NADP-dependent 7β-specific dehydrogenase from *Collinsella aerofaciens* can be applied for the regio- and stereoselective synthesis of 12-keto-UDCA from the precursor DHCA. A 1.8 L batch reaction to convert 100 mM DHCA was carried out at pH = 8.0 and 25 °C. HPLC analysis confirm a complete biotransformation of DHCA into the product 12-keto-UDCA, which means that 80 g crude product with a quite high purity of $\geq$ 99.5 % was obtained. The conversion of higher concentrations are limited so far by the solubility of the components and a substrate excess inhibition. Furthermore, a strong

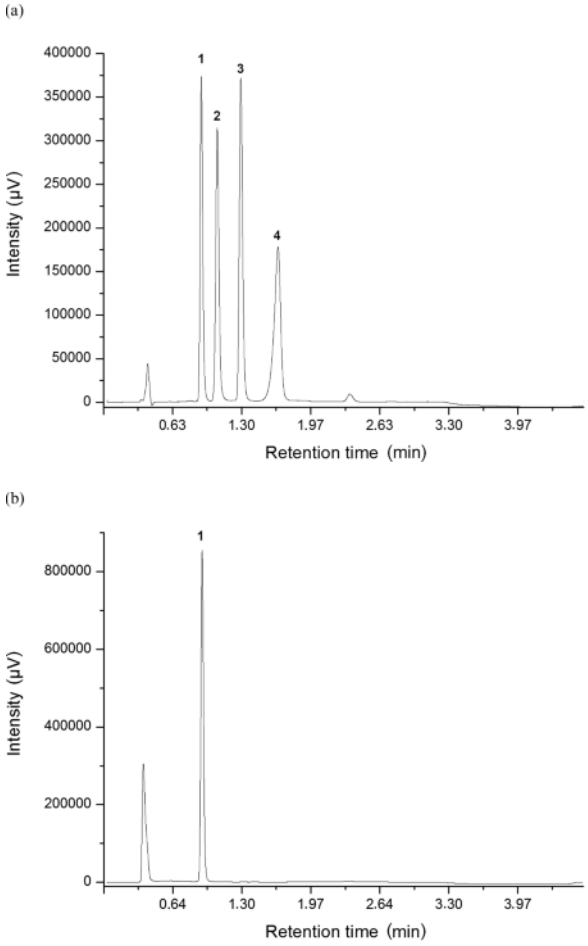


Fig. 5. HPLC chromatogram of the enzyme-catalytically produced 12-keto-UDCA. a: the chromatogram of standard substances with retention times in min given in parentheses: 1 = UDCA (0.90), 2 = 3,12-diketo-UDCA (1.10), 3 = 7,12-diketo-UDCA (1.3), 4 = DHCA (1.6); b: the chromatogram of the product after enzyme-catalyzed conversion of DHCA followed by acid precipitation and drying over-night at 60 °C. The chromatogram confirms a purity of $\geq 99\,\%$.

buffer capacity is required at higher concentration due to the production of two acid equivalents per mol DHCA.

Experimental Section

Materials

If not specified otherwise, all chemicals were purchased from Sigma Aldrich (Buchs/Switzerland). Bile acids were purchased from PharmaZell GmbH (Raubling/Germany). Centrifugation was carried out using the centrifuges RC5BPlus (Sorvall, Waltham, MA/USA), Mikro22 and Rotina 35 R (Hettich, Tuttlingen/Germany). For analytical methods, HPLC-colums purchased from Phenomenex (Aschaffenburg/Germany) and Merck (Darmstadt/Germany) were used. Restriction enzymes were purchased from Fermentas (St. Leon-Rot/Germany).

Bacterial strains and culture conditions

Escherichia coli strain DH5α (Novagen, Madison WI USA) was grown at 37 °C in LB-medium containing appropriate antibiotics. *E. coli* strain BL21(DE3)Δ7α-HSDH (PharmaZell GmbH, Raubling/Germany) was grown at 37 °C in LB-Medium containing appropriate antibiotics. After the cells reached the exponential growth phase ($OD_{600} = 0.5 - 0.7$) they were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and kept at 25 °C.

Cloning and creation of expression constructs

For cloning of the 7β-hydroxysteroid-dehydrogenase gene, the plasmid pET22b_7β-HSDH (PharmaZell GmbH, Raubling/Germany) was used as template for PCR introducing the restriction sites *Nde*I and *xho*I (up: 5′-AACCAACCA**CATATG**AACCTGAGGGAGAAGTAC-3′ down: 5′-ATAATAA**GAGCTC**TTAGTCGCGGTAGAA-CGACCC-3′). The restriction endonuclease sites for *Nde*I and *xho*I were in bolt and the methionine/stop-codons were underlined. The amplification was performed using standard PCR techniques, and the created *Nde*I-*xho*I fragment was subcloned into the vector pET21a_7β-HSDH. This vector is based on the commercially available pET21a(+). The *gdh* gene was amplified using genomic DNA from *Bacillus subtilis* as template and primers with recognition sites for the restriction enzymes *Eco31*I/*Nco*I and *not*I (up: 5′-ACCAACCA**GGTCTCAC**ATGTATCCGGATTTAAA-3′, down: 5′-ATAATAA**GCGGCCGC**TTAACCGCGGCC-TGC-3′). The restriction endonuclease sites for *Eco31*I/*Nco*I and *not*I were in bolt and the methionine/stop-codons were underlined. The amplification was performed using standard PCR techniques, the created *Eco31*I/*Nco*I-*not*I fragment was subcloned into the vector pACYCDuet. For cloning of the 3α-hydroxysteroid-dehydrogenase gene, the plasmid pET22b_3α-HSDH (PharmaZell GmbH, Raubling/Germany) was used as template for PCR introducing the restriction sites *Nde*I and *xho*I (up: 5′-AACCAACCA**CATATG**TCCATCATCGTGATAAG-3′ down: 5′-ATAATA**CTCGAG**TTAGAACTGTGTCGGGCG-CAT-3′). The restriction endonuclease sites for *Nde*I and *xho*I were in bolt and the methionine/stop-codons were underlined. The amplification was performed using standard PCR techniques, the created *Nde*I-*xho*I fragment was cloned into the vector pET21a(+). The inserted DNA was

sequenced to confirm the correct in-frame DNA sequence and the absence of any mutations.

Heterologous expression of the hydroxysteroid dehydrogenase

Starting cultures of *E. coli* BL21(DE3) Δ7α-HSDH cells carrying the recombinant plasmid were cultivated overnight at 37 °C in 5 mL LB medium [13], containing 100 μg mL^{-1} ampicillin (pET-Vector) or 36 μg mL^{-1} chloramphenicol (pACYCDuet-Vector), respectively. The cultures were used to inoculate the main cultures in LB medium or autoinduction (AI) medium (24 g L^{-1} yeast extract, 12 g L^{-1} casein hydrolysate, 5 g L^{-1} glycerol in 100 mM KP$_i$ buffer pH = 7, 2 g L^{-1} lactose, 0.5 g L^{-1} glucose) at a final concentration of 0.05 optical density at 600 nm (OD_{600}). The cultures were grown at 37 °C. When the OD_{600} reached a value between 0.5 and 0.7 the production of the recombinant steroid dehydrogenase and GDH was induced by the addition of isopropyl thio-β-D-galactoside (IPTG) to a final concentration of 0.5 mM. The cultures were shaken for 20 h at 25 °C, respectively for 72 h at 15 °C (AI), and harvested by centrifugation.

Preparation of cell-free extracts

The bacterial cultures were harvested by centrifugation at 18,000 × g for 20 min at 4 °C. A cell suspension (20 %) was prepared in 50 mM KPi buffer pH = 8. Cells were disrupted by three sonification cycles of 1 min (30 % – 40 % power output) with cooling periods in-between. The lysed cells were centrifuged at 18,000 × g for 30 min at 4 °C, and the supernatant was used for determination of HSDH and GDH activities. Protein concentrations were determined according to Bradford using BSA as a standard [14].

Spectrophotometrical assays for the determination of the glucose dehydrogenase and hydroxysteroid dehydrogenases activities

Continuous assays using UV absorbance at 340 nm were employed to monitor the NADPH concentration during reduction or oxidation catalyzed by steroid dehydrogenase or GDH. One unit of activity was defined as the amount of enzyme which catalyzes the oxidation of 1 μmol NAD(P)H per minute (HSDH) or reduction of 1 μmol $NADP^+$ (GDH) under standard conditions (25 °C, pH = 8). For the determination of HSDH activity, dehydrocholic acid was used as standard substrate. The assay mixture contains 970 μL substrate solution (10 mM substrate in 50 mM KPi buffer pH = 8), 20 μL NAD(P)H (12.5 mM) in distilled water and 10 μL enzyme solution. For the determination of GDH activity, D-glucose was used as substrate, standard conditions were 25 °C and pH = 8. The assay mixture contains 970 μL substrate solution (100 mM substrate in 50 mM KPi buffer, pH = 8), 20 μL $NAD(P)^+$ (25 mM) and 10 μL enzyme solution. The reaction was started by addition of the enzyme solution, and the amount of NAD(P)H was measured over 1 min.

Determination of kinetic parameters

To determine the kinetic parameters (K_M und v_{max}), the standard activity assay for reduction was used. All parameters were kept constant, only the investigated parameter (substrate concentration) was modified. Kinetic parameters were calculated using a non-linear fitting algorithm (software ORIGIN 7G).

Protein analysis

Protein overexpression was monitored by SDS polyacrylamide gel electrophoresis. The gels were stained with coomassie brilliant blue, and molecular mass was determined by comparison with standard markers (Invitrogen, Darmstadt/Germany).

Biotransformation of DHCA

For the biotransformation 100 mM substrate in 500 mM KPi-buffer, pH = 8.0, 0.1 mM $NADP^+$, 0.25 mM NAD^+, 1 mM $MgCl_2$, 1.5 U mL^{-1} of HSDHs and 5 U mL^{-1} of glucose dehydrogenase were incubated at 25 °C. Cultivation was done in glass vials at 25 °C with a stirring bar in it and a stirrer speed of 800 rpm. Samples (100 μL) were taken periodically and diluted with methanol-water (9 : 1, v/v) (200 μL) for HPLC analysis. Concentration was determined using a Kinetex column (Phenomenex, Aschaffenburg/Germany). For analysis an isocratic program was used. After finishing the biotransformation, the solution was acidified with 3 M hydrochloric acid to pH = 1. After filtration the precipitated product was dried over-night at 60 °C followed by another HPLC analysis.

Chromatography

HPLC analysis was performed on a Kinetex column, 75 × 4.6 mm, protected by a "security guard" column (both Phenomenex, Aschaffenburg/Gemany) on a HPLC LC-2010AHT System (Shimadzu/Japan) at a flow rate of 2 mL min^{-1}. The mobile phase consisted of two eluents. Eluent A was HPLC-acetonitrile, and eluent B was distilled water (pH = 2.6 adjusted with 85 % orthophosphoric acid). The isocratic program was eluent A 65 % and eluent B 35 %. The system was monitored by UV detection at 200 nm.

Acknowledgement

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