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Clean Enzymatic Oxidation of 12α-Hydroxysteroids to 12-Oxo-Derivatives Catalyzed by Hydroxysteroid Dehydrogenase

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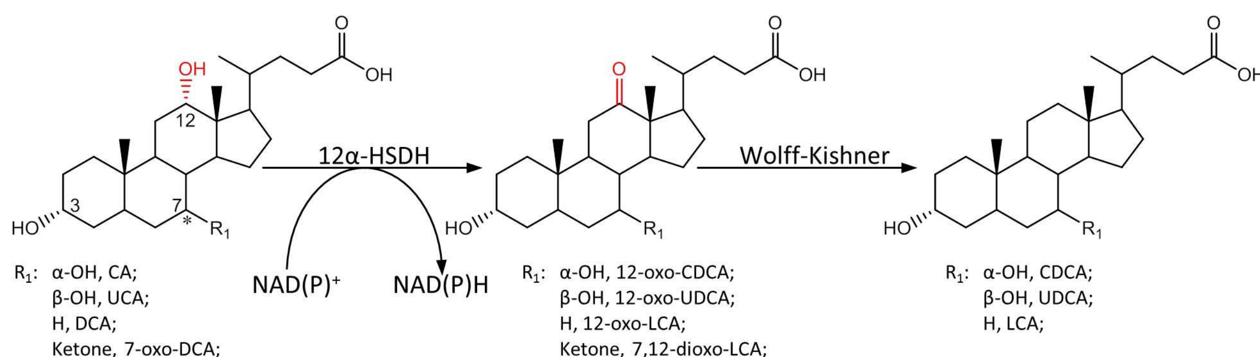
Abstract: The C12 specific oxidation of hydroxysteroids is an essential reaction required for the preparation of pharmaceutical ingredients like ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA), which can be synthesized by Wolff-Kishner reduction of the obtained 12-oxo-hydroxysteroids. 12α-hydroxysteroid dehydrogenases (12α-HSDHs) have been shown to perform this reaction with high yields, under mild conditions and without the need of protection and deprotection steps, required in chemical synthesis. Here, the recombinant expression and biochemical characterization of the nicotinamide adenine dinucleotide (NAD^+)-dependent HSDH from *Eggerthella lenta* (*El*12α-HSDH) are reported. This enzyme shows comparable properties with the well-known nicotinamide adenine dinucleotide phosphate ($NADP^+$)-dependent enzyme from *Clostridium sp.* 48–50. In order to perform a viable and atom efficient enzymatic hydroxysteroid oxidation, NAD(P)H oxidase (NOX) was employed as cofactor regeneration system: NOX uses oxygen (O_2) as sacrificial substrate and produces only water as side product. 10 mM of cholic acid was fully and selectively converted to 12-oxo-CDCA in 24 h. The possibility to employ this system on UCA and 7-oxo-deoxycholic acid (7-oxo-DCA) as substrates was additionally investigated. The performance of the *El*12α-HSDH was evaluated also in combination with a "classical" regeneration system (oxaloacetate/malate dehydrogenase) showing full conversion in 4 h. Finally, the feasibility of a catalytic aerobic-NAD^+-dependent enzymatic oxidation was shown on a preparative scale (oxidation of CA to 12-oxo-CDCA) employing the *El*12α-HSDH-NOX system in a segmented-flow-reactor.

Keywords: Bile acids; NAD^+-dependent; 12α-hydroxysteroid dehydrogenases; NAD(P)H oxidase; Flow-reactor

Ursodeoxycholic acid (UDCA) and chenodeoxycholic acid (CDCA) are widely used as pharmaceutical ingredients.[1] UDCA can be employed to treat gallstones,[2] to improve the digestion of fatty acids, to reduce cholesterol absorption and, in cases of cholestatic diseases, to stimulate the liver functions.[3] CDCA has been used for the same treatments, but its side effects made the use in clinical treatment less desiderable than UDCA.[4] However, in the last years several pharmaceutical properties of this compound have been discovered and explored (e.g. CDCA is used for the treatment of Cerebrotendinous Xanthomatosis (CTX), a rare genetic metabolic disorder).[5]

Nowadays, these two compounds are produced by chemical C12 dehydroxylation of cholic acid (CA) in a 5-step synthesis: after the protection of the carboxylic group, a selective protection of the 3- and 7-OH groups with acetic anhydride and pyridine is performed (92% yield). The removal of the 12-OH group is achieved by a redox route. Oxidation with CrO_3 (98% yield) and subsequent reduction of the formed keto group by Wolff-Kishner reduction (82% yield) finally give CDCA with an overall yield of 65%.[6]

The selective oxidation of the 12-OH group can be achieved by using a 12α-hydroxysteroid dehydrogen-



Scheme 1. Oxidation of 12α-OH group of hydroxysteroids catalysed by 12α-HSDH.

ase (12α-HSDH).[7] Using this enzyme, the 12-hydroxy group of CA can be specifically oxidized to the corresponding ketone (forming 12-oxo-CDCA) without the need of protection steps or the toxic Cr(VI). The product can afterwards undergo Wolff-Kishner reduction to form CDCA (Scheme 1). The advantages of enzymatic oxidation include the reduction of waste and mild reaction conditions with high conversion and yield utilizing catalysts easily produced by microorganisms.[8] The 12α-HSDHs belong to the family of oxidoreductases with NAD^+ or $NADP^+$ as electron acceptor. The $NADP^+$-dependent activity is distributed among the strains of the genus *Clostridium*, while the NAD^+-dependent activity has been observed and reported in *Eubacterium sp.*

Several reports describe the $NADP^+$-dependent 12α-HSDH from *Clostridium sp.* 48–50 (*C*12α-HSDH) as biocatalyst for the production of 12-oxo hydroxysteroids.[9] However, the use of a NAD^+ -dependent enzyme for this reaction would be more desirable: in comparison to $NADP^+$, NAD^+ is more stable, naturally more abundant, cheaper and easier to regenerate by employing additional enzymes and sacrificial substrates.[10] In addition, several studies have recently reported the development of a fully NAD^+-dependent process for the production of 12-oxo-UDCA,[11] opening up the possibility that the same cofactor could be used for both epimerization (employing NAD^+-dependent 7α- and 7βHSDH) and the 12-OH oxidation step.

A NAD^+-dependent 12α-HSDH from unknown source has been commercialized by Genzyme Biochemicals and employed in several biocatalytic studies,[8c] however no DNA or protein sequences were annotated for this enzyme.

In this work, the gene corresponding to the NAD^+-dependent 12α-HSDH from *Eggerthella lenta* (*El*12α-HSDH) was characterized. Although this enzymatic activity was identified earlier,[12] its biocatalytic potential was not investigated to date. The identified gene coding for the *El*12α-HSDH (GenBank: WP_114518444.1) showed 57% of sequence identity with the $NADP^+$-dependent *C*12α-HSDH (GenBank: WP_044992937), 53% with the $NADP^+$-dependent enzyme from *Eggerthella* CAG:298 (GenBank: CDD59475.1) and differs from the reported enzyme Elen-2515 (GenBank: ACV56470.1) by a single amino acid (P41S). Multiple sequence analyses (BLASTp), 3D structure modelling (using SWISS-MODEL) and docking analyses (AutoDock VINA)[13] were carried out in order to investigate the protein sequence/structure relationship of this enzyme. Particularly, the $NADP^+$ binding motif (G39 and R40) in *C*12α-HSDH, is exchanged to a NAD^+ binding motif (D39 and L40) (Supplementary Figure 1). This change in the amino acid sequence is frequently involved in the determination of the cosubstrate specificity of these enzymes.[11] The 3D model of the *El*12α-HSDH structure showed a homotetrameric oligomeric state, conserved among the group of SDR oxidoreductases, confirming the predicted NAD^+-binding (Figure 1A). Docking analysis of CA in the active site of the *El*12α-HSDH showed correct positioning of the 12α-OH group with respect to the catalytic residues (S145 and Y158) and the nicotinamide cofactor (Figure 1B). Therefore, the bioinformatic data suggested that the sequence was coding for a NAD^+-dependent 12α-HSDH.

In order to evaluate the catalytic performances of the *El*12α-HSDH and directly compare them with the one of the $NADP^+$-dependent system reported in literature, *C*12α-HSDH and El12α-HSDH were recombinantly expressed in *E. coli* BL21 (DE3) cells as N-His-tagged proteins and purified by a single HiTrap chelating chromatography step (≥95% purity, as determined by SDS-PAGE analysis, Supplementary Figure 2).

*El*12α-HSDH was expressed in higher volumetric yield than the *C*12α-HSDH (215 and 26 $mg/L_{culture}$, respectively), making it more suitable for its industrial employment (Purification tables are provided in Supplementary Table 1). *El*12α-HSDH and *C*12α-HSDH showed enzymatic activity (under standard conditions) of 59.2 and 30.1 $U mg^{-1}$, respectively.

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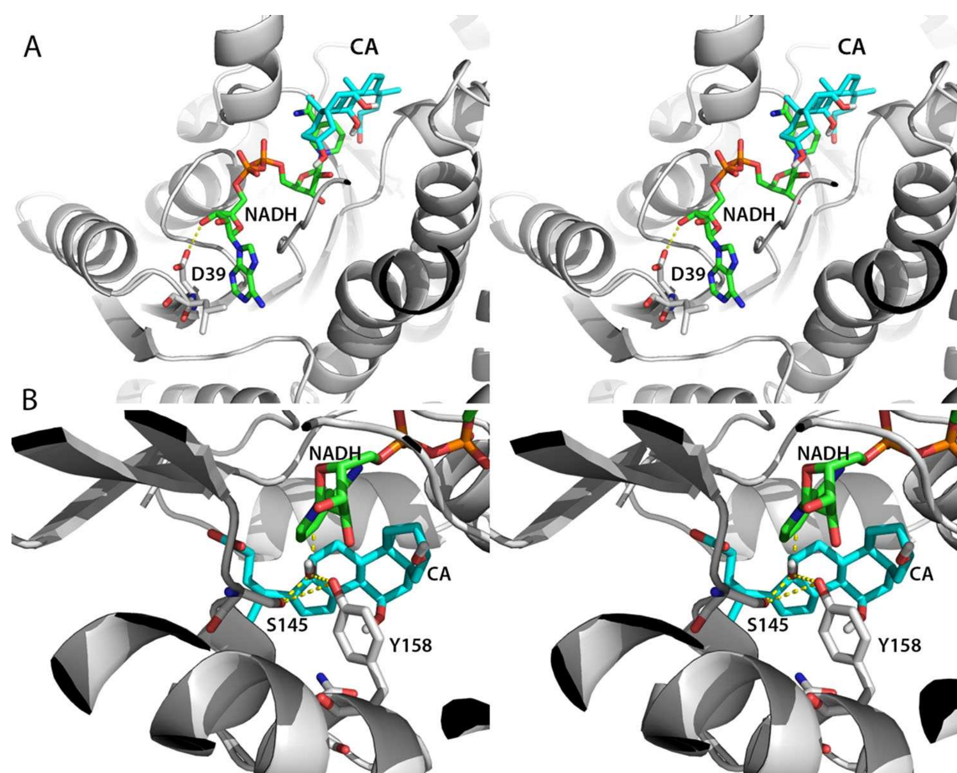

Figure 1. (A) Hypothetical binding mode of the NADH inside the 3D model of *El*12α-HSDH active site: the putative formation of a hydrogen bond between the aspartate side-chain (D39) and the 2'-OH group of ribose (distance 1.9–2.3 Å). (B) Docking analysis of CA in the catalytic pocket of *El*12α-HSDH. The 3D structure model of the *El*12α-HSDH was obtained using SWISS-MODEL (https://swissmodel.expasy.org/interactive), employing the crystal structure of the putative SDR from *Burkholderia xenovorans* (PDB ID: 5JY1.1) as template. Docking analyses were performed employing AutoDock Vina [13] (see Supplementary Material).

Table 1. Kinetic parameters of the recombinant *C*12α-HSDH and *El*12α-HSDH.

		k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m
*C*12α-HSDH	CA[a]	97.7 ± 2.4	0.121 ± 0.014	807.3 ± 113.2
	7-oxo-DCA[a]	62.7 ± 0.2	0.252 ± 0.022	248.9 ± 22.4
	UCA[a]	113.8 ± 2.8	0.239 ± 0.025	476.0 ± 61.4
	DCA[a]	198.9 ± 11.5	0.548 ± 0.117	362.9 ± 98.4
	$NADP^{+}$[b]	78.6 ± 2.4	0.073 ± 0.009	1077 ± 165
*El*12α-HSDH	CA[a]	146.1 ± 4.0	0.636 ± 0.062	229.8 ± 28.6
	7-oxo-DCA[a]	90.0 ± 2.3	0.461 ± 0.047	195.3 ± 24.8
	UCA[a]	87.0 ± 3.4	0.399 ± 0.060	218.1 ± 41.3
	DCA[a]	27.5 ± 1.5	0.673 ± 0.130	40.9 ± 10.1
	NAD^{+}[b]	122.4 ± 10.5	0.919 ± 0.164	133.2 ± 35.2

[a] Kinetic parameters determined in presence of 1 mM of $NAD(P)^{+}$;
[b] Kinetic parameters determined in presence of 1 mM of CA. Activities were spectrophotometrically measured by following the production of NAD(P)H at 340 nm. All the activities were performed in presence of 50 mM KPi buffer, pH 8.0 and 10% MeOH at 25 °C. k_{cat} values were calculated considering a MW of 111 and 113 kDa for *C*12α-HSDH and *El*12α-HSDH, respectively.

Notably, the position of the His-tag plays a crucial role in the activity of these enzymes: when a C-His-*C*12α-HSDH was expressed and purified, it showed a low enzymatic activity (0.1 U mg^{-1}). This loss of activity was probably due to the C-His-Tag "masking" of the C-terminal proline residue, which has an essential role in the binding of bivalent cations necessary for the enzyme oligomerization. Similar behaviour was observed in other C-terminal proline proteins.[14] Additionally, the C-His-Tag was located

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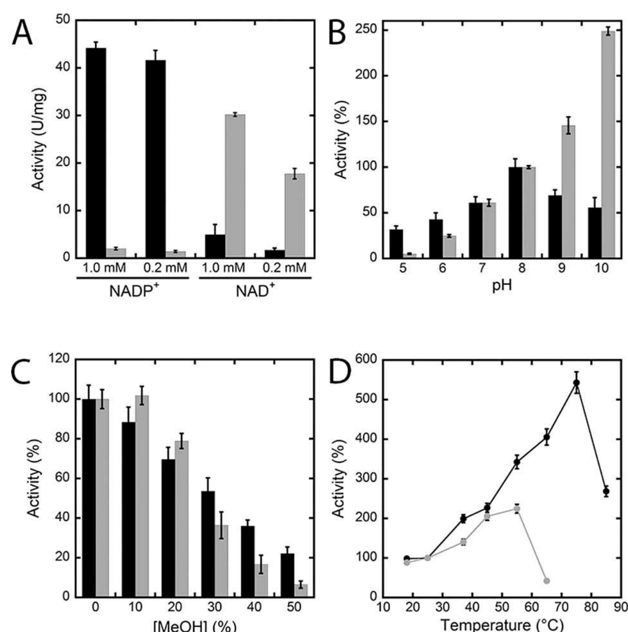

Figure 2. Activity of *C*12α-HSDH (black) and *El*12α-HSDH (grey) under different conditions. (A) activity in presence of different concentrations of nicotinamide cofactors, 1 mM CA, 10% MeOH and 50 mM KPi pH 8.0. (B) Effect of pH on the enzymatic activity at 25 °C. The value at pH 8.0 is taken as 100%. (C) Effect of MeOH on the enzymatic activity at pH 8.0, 25 °C. The value in absence of MeOH is taken as 100%. (D) Effect of temperature on the enzymatic activity at pH 8.0. The value at 25 °C is taken as 100%. In all the cases, activities were determined in presence of 1 mM CA and 1 mM NAD(P)$^+$ by measuring the NAD(P)$^+$ reduction at 340 nm.

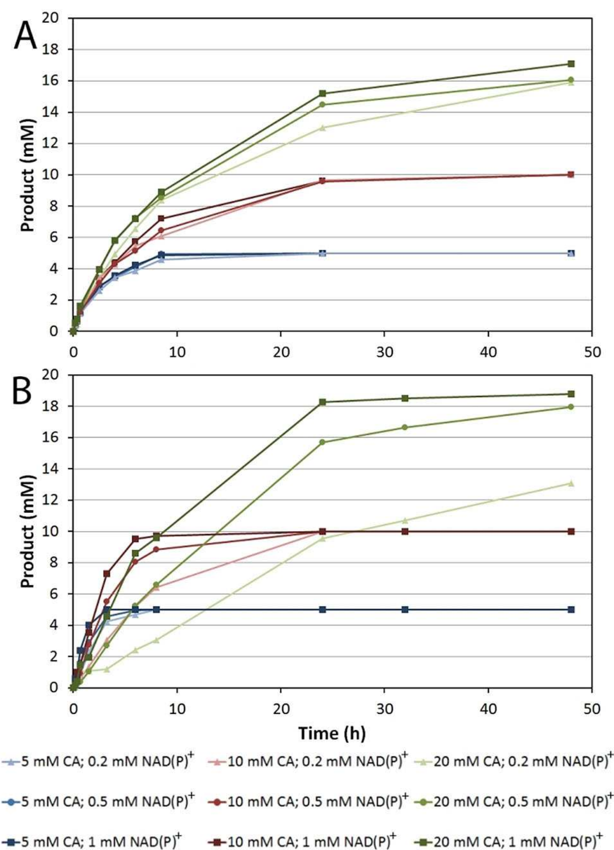

Figure 3. Bioconversion time-courses employing (A) *C*12α-HSDH and (B) *El*12α-HSDH of: (green lines) 20 mM, (red lines) 10 mM and (blue lines) 5 mM of CA in presence of (from the lightest to the darkest tone) 0.2, 0.5 and 1 mM NAD(P)$^+$. All reactions were carried out employing 0.1 U/mL of enzyme (2.6 μg mL^{-1} and 3.5 μg mL^{-1} of *C*12α-HSDS and *El*12α-HSDS, respectively) and 0.5 mg mL^{-1} of NOX in 50 mM KPi, pH 8.0 and 10% MeOH at 25 °C.

close to the active site of the enzyme, restricting the accessibility of the substrate in the catalytic pocket (further details are provided in Supplementary Figure 3 and Supplementary Table 2).

Kinetic parameters of the two enzymes were evaluated with different substrates (CA, 7-oxo-deoxycholic acid (7-oxo-DCA), UCA and deoxycholic acid (DCA)) in the presence of β-nicotinamide cofactors (NAD$^+$ and NADP$^+$, for *El*12α-HSDH and *C*12α-HSDH, respectively) (Table 1).

The *C*12α-HSDH showed higher affinity to both substrate and cosubstrate. On the other hand, *El*12α-HSDH displayed higher V_{max} for CA and 7-oxo-DCA: in biocatalytic reactions, the influence of the K_m value on the performances of the enzymes is of lower relevance as concentrations are typically higher (< 5 mM). Indeed, to achieve similar conversions, larger amounts of *El*12α-HSDS where therefore necessary (Table 2).

The enzymatic activities in the presence of the not-preferred cofactor were also assayed and, as predicted by the *in silico* analysis of the sequence/structural relationship, *El*12α-HSDH shows a strict NAD$^+$-dependent activity (Figure 2A). Notably, since the K_m value for NAD$^+$ is 0.91 mM, a decrease of the activity was observed when lower amounts of cofactor were employed.

The activity and the stability of the obtained enzymes were evaluated at different pH values, different MeOH concentrations and temperatures. Unlike the *C*12α-HSDH, which showed a pH optimum at pH 8.0, the optimal pH for *El*12α-HSDH activity was 10 (Figure 2B). However, stability studies showed that this enzyme was less stable at this pH value, retaining 43% of its initial activity when incubated for 24 hour at pH 10 (Supplementary Figure 4A). Thus, we conclude that the optimal working pH for both enzymes is 8.0.

Interestingly, *El*12α-HSDH was less hampered by the cosolvent, retaining 100% of its activity in presence of 10% of MeOH (Figure 2C). Additionally, no significant decrease of activity was observed after 24 h of

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Table 2. Bioconversion table.

Entry	Enzyme	Reaction	[Substrate] (mM)	Regeneration system	Scale (mg)	Conversion$_{(time)}$ (%)$_{(h)}$	
1	*C*12α-HSDH	CA→12-oxo-CDCA	10	NOX	10	$54_{(6)}$	$96_{(24)}$
2	*C*12α-HSDH	CA→12-oxo-CDCA	20	NOX	20	$36_{(6)}$	$72_{(24)}$
3	*C*12α-HSDH	CA→12-oxo-CDCA	20[a]	NOX	20	$32_{(6)}$	$65_{(24)}$
4	*El*12α-HSDH	CA→12-oxo-CDCA	10	NOX	10	$80_{(6)}$	$>99_{(24)}$
5	*El*12α-HSDH	CA→12-oxo-CDCA	20	NOX	20	$26_{(6)}$	$78_{(24)}$
6	*El*12α-HSDH	CA→12-oxo-CDCA	20[a]	NOX	20	$12_{(6)}$	$48_{(24)}$
7	*C*12α-HSDH	7-oxo-DCA→7,12-dioxo-LCA	10	NOX	10	$10_{(6)}$	$34_{(24)}$
8	*C*12α-HSDH	UCA→12-oxo-UDCA	10	NOX	10	$28_{(6)}$	$76_{(24)}$
9	*C*12α-HSDH	DCA→12-oxo-LCA	10	NOX	10	$43_{(6)}$	$56_{(24)}$
10	*El*12α-HSDH	7-oxo-DCA→7,12-dioxo-LCA	10	NOX	10	$12_{(6)}$	$94_{(24)}$
11	*El*12α-HSDH	UCA→12-oxo-UDCA	10	NOX	10	$3_{(6)}$	$20_{(24)}$
12	*El*12α-HSDH	DCA→12-oxo-LCA	10	NOX	10	$2_{(6)}$	$3_{(24)}$
13	*El*12α-HSDH	CA→12-oxo-CDCA	10	OxAc+MDH	10	$>99_{(2)}$	
14	*El*12α-HSDH	7-oxo-DCA→7,12-dioxo-LCA	10	OxAc+MDH	10	$85_{(2)}$	$>99_{(4)}$
15	*El*12α-HSDH	UCA→12-oxo-UDCA	10	OxAc+MDH	10	$56_{(2)}$	$>99_{(4)}$
16	*El*12α-HSDH	DCA→12-oxo-LCA	10	OxAc+MDH	10	$53_{(2)}$	$>99_{(4)}$
17	*El*12α-HSDH	CA→12-oxo-CDCA	10	OxAc+MDH	200	$>99_{(4)}$	
18	*El*12α-HSDH	7-oxo-DCA→7,12-dioxo-LCA	10	OxAc+MDH	1000	$>99_{(4)}$	
19	*El*12α-HSDH	UCA→12-oxo-UDCA	10	OxAc+MDH	200	$>99_{(4)}$	
20	*El*12α-HSDH	DCA→12-oxo-LCA	10	OxAc+MDH	200	$>99_{(4)}$	
21	*C*12α-HSDH	CA→12-oxo-CDCA	10	NOX	25	$34_{(6)}$[b]	
22	*C*12α-HSDH	CA→12-oxo-CDCA	10	NOX	25	$23_{(6)}$[c]	
23	*El*12α-HSDH	CA→12-oxo-CDCA	10	NOX	25	$18_{(6)}$[b]	
24	*El*12α-HSDH	CA→12-oxo-CDCA	10	NOX	25	$9_{(6)}$[c]	
25	*El*12α-HSDH	CA→12-oxo-CDCA	10	NOX	300	$78_{(6)}$[d]	$>99_{(24)}$[d]
26	*El*12α-HSDH	CA→12-oxo-CDCA	20	NOX	600	$28_{(6)}$[d]	$68_{(24)}$[d]

[a] $[NAD(P)+] = 0.2$ mM;
[b] Reaction in autoclave under oxygen pressure (3 bar);
[c] Reaction in autoclave under ambient atmosphere;
[d] Reaction in flow-reactor. All the reactions were carried out employing 0.1 U mL^{-1} of enzyme (2.6 μg mL^{-1} and 3.5 μg mL^{-1} of *C*12α-HSDS and *El*12α-HSDS, respectively), 0.5 mg mL^{-1} of NOX and 0.5 mM NAD(P)$^+$ in 50 mM KPi, pH 8.0 and 10% MeOH at 25 °C. In all the cases we observe a reaction selectivity of 100%. Reaction times are shown in the brackets.

incubation of the enzyme under the same conditions (Supplementary Figure 4B).

Finally, the influence of the temperature on the enzymatic activity of these two enzymes was evaluated. As previously reported, *C*12α-HSDH is a thermostable protein, showing a temperature optimum of 75 °C and retaining 97% of its initial activity after 24 h of incubation at 37 °C. On the other hand, *El*12α-HSDH showed a temperature optimum of 55 °C (Figure 2D), but its activity was halved after incubation at 37 °C for 24 h. However, both enzymes were stable when incubated at 25 °C for 24 h (Supplementary Figure 4C).

The biocatalytic properties of the two 12α-HSDHs were evaluated in batch mode reactions. In order to regenerate the oxidised nicotinamide cofactors, NAD(P)H oxidase (NOX), a commercial enzyme that uses O_2 for the oxidation of NAD(P)H was applied. In comparison with other classical regeneration systems, the NOX system has the advantage that O_2 as sacrificial substrate is gaseous and generates only water as side product, leading to cleaner reactions and simplifying the downstream processes.[15] As observed from the biochemical characterization, NOX shows comparable activity with both NADH and NADPH, (198 and 223 mU/mg$_{\text{freeze-dried powder}}$, respectively). In order to demonstrate the applicability of this enzymatic cascade (Supplementary Scheme 1A), bioconversion reactions were set up with 0.1 U mL^{-1} of 12α-HSDHs, NOX (0.5 mg mL^{-1}), different amounts of CA (5, 10 and 20 mM) and NAD(P)$^+$ (0.2, 0.5 and 1 mM).

The *C*12α-HSDH-NOX system was able to fully convert 5 and 10 mM of CA into 12-oxo-CDCA in 8 and 48 h, respectively (Figure 3A). However, when higher concentration (20 mM) of CA where employed only 85% of conversion to the desired product was achieved in 48 h. Notably, the reaction rates observed were not influenced by the different concentrations of NADP$^+$ employed in the reactions, which can be explained by the low K_m of the enzyme for the cofactor

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(73 μM), making the enzymatic reactions proceed at rates close to V_{max} even at low cosubstrate concentrations (Table 2; entries 1–3).

On the other hand, in the presence of 1 mM NAD^+, *El*12α-HSDH converted 5 and 10 mM of CA in 12-oxo-CDCA in 6 and 24 h, respectively (Figure 3B). Under the same conditions, 20 mM of substrate were converted to the desired product (94% conversion). Different to its $NADP^+$-dependent homologue, the reaction rate decreased when lower amounts of cosubstrate were applied. However, except when 20 mM of CA were incubated in the presence of 0.2 mM $NAD(P)^+$, the catalytic performances of the *El*12α-HSDH were better than those of the *C*12α-HSDH, leading to higher conversions and lower reaction times (Table 2, entries 4–6). Again, the disadvantage in the use of the *El*12α-HSDH is partially compensated by the high volumetric production of these recombinant enzyme.

Having established the time course of the reaction, experiments focused on the scope of the enzymatic system (Table 2).

The systems *C*12α-HSDH-NOX and *El*12α-HSDH-NOX were applied for the preparation of 7,12-dioxo-LCA, 12-oxo-UDCA and 12-oxo-LCA (using 7-oxo-DCA, UCA and DCA as substrate, respectively; entries 7–12). In comparison to the reaction with CA as substrate, lower conversions and rates were observed with both enzymatic systems. However, when a "classical" NAD^+-regeneration system (oxaloacetate and malate dehydrogenase (MDH – Supplementary Scheme 1B) was used, all three substrates were fully converted in 4 h by *El*12α-HSDH (entries 13–16). The same performance was observed on preparative scale (entries 17–20). The comparison between the regeneration systems suggests that the rate limiting enzyme of 12α-HSDH-NOX cascade is the cofactor regeneration by NOX.

Since NOX uses O_2 as electron acceptor, the poor solubility of this gas in aqueous environment can limit the $NAD(P)^+$-regeneration rate. In order to investigate the O_2 limitation, reactions were carried out in an autoclave (with 3 bar of pure O_2 gas; entries 21 and 23). Increased conversions were obtained when comparing the reaction under O_2 pressure with control reactions (in autoclave under atmospheric pressure; entries 22 and 24). However, with both enzymes, conversion values lower than those obtained in the rotatory shaker were observed, suggesting that the stirring method employed in the autoclave reactor is not optimal (possibly because of the mechanical stress that leads to the inactivation of the biocatalysts).

A promising technology that enables high O_2 transfer rates without strain on the enzymatic structure is the flow-reactor.[16] Preparative reactions employing *El*12α-HSDH-NOX system was recirculated for 24 h in a flow-system (Supplementary Figure 5). This system (entry 25–26) performed equally well as the batch reactions on the rotatory shaker (analytical scale; entry 4–5, respectively). 10 mM of CA were fully converted into 12-oxo-CDCA in 24 h. However, when a high substrate loading was applied (20 mM CA) only 13.6 mM of product were obtained.

These data show the feasibility of a NAD^+-dependent process for the specific oxidation of 12-OH hydroxysteroids to 12-oxo-hydroxysteroids catalysed by recombinantly expressed *El*12α-HSDH. The biosynthetic potential of this enzyme is similar to the one of the widely used *C*12α-HSDH, with the advantage of using a cheaper and more stable cosubstrate, NAD^+.

The combination of 12α-HSDH and NOX represents a promising system for this biocatalytic conversion: *C*12α-HSDH and *El*12α-HSDH, coupled with NOX for $NAD(P)^+$ regeneration, showed similar TTN and ToF (330000 and 2.6 s^{-1} vs. 250000 and 2.9 s^{-1} for the *C*12α-HSDH and *El*12α-HSDH, respectively). However, when *El*12α-HSDH was coupled with a classical regeneration system for NAD^+, a ToF of 17.4 s^{-1} was calculated, confirming that the low activity of NOX is limiting the reaction rate of these systems. Future research will be conducted in order to find a more robust O_2 dependent $NAD(P)^+$ regeneration system.

Green metrics values were calculated in order to compare the different synthetic routes.

For the pure chemical route[6] we calculated an atom efficiency[17] of 49% and an E-factor[18] of 55.4.

In addition to these undesired values, the route requires the use of the highly toxic and carcinogenic CrO_3. The combination of 12α-HSDH and NOX showed high atom efficiency (96%) and a low E-factor (2.5). In comparison, the "classical" MDH regeneration system showed a lower (but still acceptable) atom efficiency (75%) and an E-factor value of 4.4. These calculations do not take in account the waste produced for the production and purification of the enzymes and chemicals or for the downstream process. The alternative chemo/enzymatic route for the production of 12-oxo-UDCA (chemical oxidation of CA to dehydrocholic acid (DHCA) followed by reduction with 3α- and 7β-HSDHs in presence of formate dehydrogenase[19]) is less atom economic (57%). Although the E-factor value of this system (5.2) is comparable with the 12α-HSDH-MDH system proposed here, the chemical oxidation is still performed employing toxic and environmentally hazardous reagents, making it less desirable for an industrial scale.

In conclusion, the clean oxidation of several 12α-hydroxysteroids to 12-oxo derivatives by employing 12α-HSDH-NOX system has been shown. This one-step enzymatic transformation avoids the use of protection groups and toxic oxidants (CrO_3) required by the chemical synthesis and, in comparison with other enzymatic route, does not need sacrificial substrates that

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complicate downstream processing. In addition, the substitution of a $NADP^+$- for a NAD^+-dependent enzymatic system was achieved. Combined, these factors improve the sustainability of CDCA and UDCA production.

Experimental Section

Cloning, Recombinant Expression and Purification of 12α Hydroxysteroid Dehydrogenases (12α-HSDHs)

The synthetic cDNAs encoding for the *El*12α-HSDH and for the *C*12α-HSDH were designed by *in silico* back translation of the amino acid sequence reported in the GenBank database (Accession no. WP_114518444.1 and AET80684.1, respectively). The genes were subcloned into the pET28a(+) plasmid, using *Nco*I (CCATGG) and *Xho*I (CTCGAG) restriction sites resulting in 6.1-kb constructs (pET28-*El*12α-HSDH and pET28-*C*12α-HSDH). Six codons (encoding for six additional histidines) were added to the 5′-end of the 12α-HSDH gene during the subcloning process. The codon usage of the synthetic gene was optimized for expression in *Escherichia coli* and produced by BaseClear.

HSDH enzymes were expressed in BL21(DE3) *E. coli* cells and purified by HiTrap chelating affinity chromatograpy employing the same procedure described in a preceding paper.[11a]

Activity and Kinetic Measurements

12α-HSDHs enzymatic activities in the crude extract and of the purified enzyme were determined at 25 °C using 1.0 mM CA, 1.0 mM $NAD(P)^+$, in 50 mM KPi buffer, pH 8.0 and 10% methanol (v/v). The production of NAD(P)H was followed at 340 nm (extinction coefficient of NAD(P)H is 6,220 $M^{-1}\cdot cm^{-1}$). One unit (U) was defined as the amount of enzyme producing 1 μmol of product per minute at 25 °C and at pH 8.0. Blank measurements were performed in absence of CA, $NAD(P)^+$ and enzyme.

Biocatalytic Reactions

All bioconversions were carried out employing 0.1 $U mL^{-1}$ of purified *C*12α-HSDS (2.6 $\mu g mL^{-1}$) or *El*12α-HSDS (3.5 $\mu g mL^{-1}$), 0.5 $mg mL^{-1}$ of NAD(P)H oxidase PRO-NOX (001) (Prozomix Limited, Haltwhistle, UK – NOX), 10% MeOH and 50 mM KPi buffer at pH 8.0. Analytical scale conversions were carried out in a 15 mL tube filled with 2 mL of reaction mixture. Reactions were incubated at 25 °C on rotator shaker (45 rpm) (IntelliMixer, Neolab Migge GmbH, Heidelberg, DE). Reaction in autoclave were performed employing 6 mL of reaction mixture in 10 mL metal vessels (HP Chemscan 8, HEL Ltd, Borehamwood, UK), magnetically stirred (200 rpm), at 25 °C under 3 bar of pure oxygen pressure. Control reactions were performed in autoclave vessels under atmospheric pressure. Analytical and preparative reaction employing the "classical" NAD^+-regeneration system were performed in presence of 30 mM oxaloacetate, L-malate dehydrogenase from pig heart (6 $U mL^{-1}$, 5 $\mu g mL^{-1}$, Sigma Aldrich, Saint Louis, USA) and 2.6 $\mu g mL^{-1}$ of *C*12α-HSDS or 3.5 $\mu g mL^{-1}$ of *El*12α-HSDS. Samples (50 μL) were withdrawn at different times, diluted with 200 μL of water/CH_3CN/TFA (70/30/0.1) solution and centrifuged at 14000 xg for 2 min. 10 μL of the obtained samples were analysed by HPLC (Supplementary Material).

Flow Reactor Experiments

Silicon reactor coils (2 mm ID) with a volume of 60 mL were constructed. The reaction mixture (75 mL) containing 10 mM CA, 0.5 mM of NAD^+, 0.1 $U mL^{-1}$ of *El*12α-HSDH and 0.5 $mg mL^{-1}$ of NOX was introduced (3.5 mL min^{-1}) *via* a peristaltic pump (120 U, Watson Marlow, Falmouth, UK), while the pure oxygen flow (5 mL min^{-1}) was controlled by a mass flow controller (EL-FLOW, Bronkhorst, Ruurlo, NL), resulting in a segmented flow. The reaction mixture was recirculated for 24 h (at 21 °C) and, at fixed times analysed by HPLC.

Abbreviations

CA, Cholic acid; CDCA, Chenodeoxycholic acid; DCA, Deoxycholic acid; DHCA, dehydrocholic acid; LCA, Lithocholic acid; UCA, Ursocholic acid; UDCA, Ursodeoxycholic acid; HSDH, Hydroxysteroid dehydrogenase; MDH, Malate Dehydrogenase; NOX, NAD(P)H oxidase; OxAc, Oxaloacetate; SDR, Short-chain Dehydrogenase/Reductase; ToF, Turnover frequency; TTN, Total turnover number.

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