# Identification of *Candida tenuis* xylose reductase as highly selective biocatalyst for the synthesis of aromatic α-hydroxy esters and improvement of its efficiency by protein engineering†

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**Wild-type *Candida tenuis* xylose reductase and two Trp-23 mutants thereof catalyze NADH-dependent reduction of a homologous series of aromatic α-keto esters with absolute pseudo *re*-face stereoselectivity and broad tolerance for the substituent on the aromatic ring, producing the corresponding *R*-alcohols in high yield.**

Chiral α-hydroxy esters are valuable target structures for organic synthesis. Mandelic acid ester and derivatives thereof (**1a–1g**; Fig. 1) have attracted special attention because they are bioactive compounds or key intermediates in the preparation of pharmaceuticals.[1] Asymmetric reduction of α-keto precursor compounds (**2a–2g**; Fig. 1) is a promising route to synthesize them.[2] Enzyme-catalyzed hydrogen transfer often outperforms counterpart chemo-catalytic reductions of prochiral ketones in terms of enantioselectivity. It usually proceeds at a reasonable rate and with low environmental burden. However, the range of substrates converted and unpredictable dependences of enzyme activity and selectivity on the substrate structure sometimes limit the scope of reductases for biocatalytic applications.[3]

Although candidate catalysts for a particular transformation may be identified from enzyme[4] or genomic[5] libraries by extensive screening, it is often preferable to use a small set of practical reductase systems that exhibit complementary patterns of activity and stereoselectivity and have had their structure–function relationship well characterized. We introduce here xylose reductase from the yeast *Candida tenuis* (CtXR)[6] as a powerful hydrogen transfer biocatalyst for the synthesis of aromatic α-hydroxy esters. Considering its physiological function,[7] CtXR was a very unlikely α-keto ester reductase. However, we show that the enzyme displays excellent stereochemical selectivity and broad substrate acceptance during NADH-dependent reduction of **2a**, **2b**, **2d**, **2g**, thus providing a useful combination of properties for organic synthesis. CtXR belongs to a large superfamily of NAD(P)-dependent oxidoreductases, the aldo-keto reductases,[8] and salient features of how it recognizes and converts carbonyl substrates have been elucidated at the molecular level (see Scheme 1).[6a,9] The enzyme is therefore tractable by structure guided protein engineering, and we report on two site-directed mutants of the reductase that showed up to eightfold enhanced specificity for the target keto ester substrates. Energy-minimised docking of the open-chain xylose into the active site of CtXR bound to $NADP^+$ revealed a probable mode of substrate binding. The modeling predicts steric conflicts between the side chain of Trp-23 and carbonyl group substituents replacing the aldehydic hydrogen (Scheme 1).[6a,9] The replacement of Trp-23 by phenylalanine and tyrosine caused a relative better utilization of ketones than xylose, reflected by both a strong

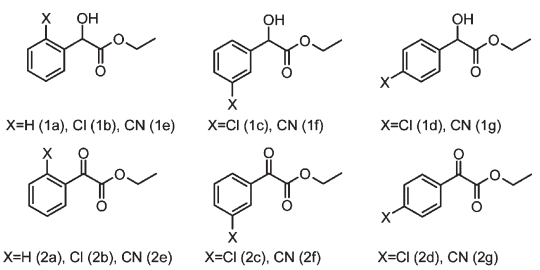


**Fig. 1** Aromatic α-hydroxy esters and their α-keto precursor compounds which are substrates of NADH-dependent reduction by *Candida tenuis* xylose reductase.

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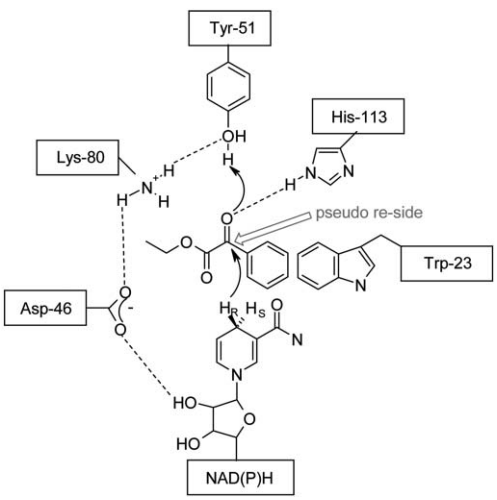


**Scheme 1** Proposed orientation of **2a** in the active site of CtXR and stereochemical course of carbonyl group reduction by enzyme-bound NADH. Dashed lines show hydrogen bonds or electrostatic interactions. The indole ring of Trp-23 is close to the reactive carbonyl group of xylose,[6a] and its replacement by smaller groups in Tyr or Phe promotes reaction of **2a** compared with the natural substrate xylose.[9]

**Table 1** Catalytic efficiencies[a] ($k_{cat}/K_m$ $M^{-1}s^{-1}$) and stereoselectivities[b] (e.e. %) of wild-type CtXR and Trp-23 mutants thereof for NADH-dependent reduction of a series of aromatic α-ketoesters. The corresponding $k_{cat}/K_m$ values for xylose are shown for comparison[9]

| Substrate | Wild-type $k_{cat}/K_m$ (e.e.) | W23F $k_{cat}/K_m$ (e.e.) | W23Y $k_{cat}/K_m$ (e.e.) |
|---|---|---|---|
| D-xylose | 136 | 2.4 | 0.8 |
| **2a** | 269 (>99.9 *R*) | 911 (>99.9 *R*) | 636 (>99.9 *R*) |
| **2b** | 576 (99.9 *R*) | 4029 (99.9 *R*) | 3158 (99.8 *R*) |
| **2c** | 53 | 369 | 186 |
| **2d** | 2211 (>99.9 *R*) | 13835 (>99.9 *R*) | 11056 (>99.9 *R*) |
| **2e** | 27 | 208 | 128 |
| **2f** | 21 | 131 | 74 |
| **2g** | 197 (99.7 n.d.[c]) | 955 (99.7 n.d.[c]) | 717 (99.7 n.d.[c]) |

[a] From initial rate data recorded at varied concentration of α-keto ester substrate. [b] The α-keto esters (1.5 or 5 mM) were incubated in the presence of excess NADH (2.5 or 6 mM) and enzyme (1–50 μM) in 50 mM potassium phosphate buffer, pH 7.0. HPLC analysis of the products was performed using authentic standards of the respective alcohol antipodes. [c] A single enantiomeric product was obtained. However, only racemic ethyl 4-chloromandelate was available as standard. Details of the experimental procedures are described in the Supporting Information.

decrease in efficiency for xylose reduction and an increase in efficiency for ketone reduction.[9]

Table 1 summarizes results of a steady-state kinetic analysis of NADH-dependent reduction of **2a–2g** by wild-type CtXR and W23F and W23Y mutants.[10] Specificity constants ($k_{cat}/K_m$) for reduction of xylose are shown for comparison. (Values of $k_{cat}/K_m$ for xylose and **2a** were determined previously and are taken from ref. 9). The series of α-keto esters were converted by the three enzymes with efficiencies that were up to 14000-fold higher than the corresponding observable $k_{cat}/K_m$ values for reaction with xylose.[9,11] Replacement of Trp-23 by Phe or Tyr significantly enhanced α-keto ester reductase activity of the wild-type. As shown in Table 1, W23F was the best improved of the two mutants, exhibiting three- to eightfold increased catalytic efficiencies for reduction of the chosen substrates in comparison with wild-type. In a homologous series of chloro- or cyano-substituted derivatives of **2a**, reduction of **2d** and **2g** harbouring the substituent in the *para*-position on the aromatic ring was strongly preferred. Structure–activity correlations of kinetic substituent effects on $k_{cat}/K_m$ for carbonyl group reduction by CtXR suggested that the intrinsic chemical reactivity of the substrate, reflected by the ability to stabilise a partial positive charge on the reactive carbon, determined the speed of the enzymatic reaction.[9,12] Considering the evidence from our previous studies, the results in Table 1 indicate that direct resonance effects of the substituent on the reaction center probably provide most of this electronic stabilization in the conversion of **2b–2g** (Scheme 2).

Table 1 also reports enantiomeric excess (e.e.) values for alcohol products obtained by enzymatic reduction and measured in reaction samples at a substrate conversion of 70% or greater (up to 95%). Note that isolation of products was beyond the scope of this study and can be done according to literature.[2,4a] W23F and W23Y retain the very high stereoselectivity of the wild-type enzyme. In contrast to conversions of aromatic α-keto esters which yielded *R*-configured alcohols, the opposite enantiomer, *S*-ethyl lactate (e.e. > 99.9%), was obtained upon reduction of ethyl pyruvate (90% conversion). These results suggest a stereochemical course of the reaction catalyzed by CtXR (Scheme 1) where the 4-pro-*R* hydride of NADH[13] attacks the carbonyl carbon from its pseudo *re*-face, which according to Prelog's generalization on the enantioselectivity of reductases is determined by the relative steric requirements of the substituents of the prochiral ketone.[14] A binding mode of aromatic α-keto esters is thus supported in which the carbonyl group is positioned above the nicotinamide ring of NADH and the phenyl ring points towards the substrate binding pocket of the enzyme (Scheme 1). With the reactive carbonyl group locked in place by active-site residues, the relatively open and flexible structure of the substrate binding site of CtXR[6a] underpins acceptance of various substituents at different positions on the aromatic ring without, however, compromising the stereoselectivity of reduction. Among the many isolated reductases tested for the synthesis of aromatic α-hydroxy esters,[15] this property of CtXR seems to be shared only by ketoamide reductase from *Saccharomyces cerevisiae*,[16] which like CtXR belongs to the aldo-keto reductase superfamily, and 7α-hydroxysteroid dehydrogenases from *Bacteroides fragilis*,[17] a member of the short-chain dehydrogenase/reductase superfamily. However, because a structural basis for these two enzymes is lacking, rational engineering of their substrate specificities, as done here for CtXR, would seem to be currently out of reach. Studies of lactate dehydrogenase are relevant in showing the improvement of a biocatalyst for the reduction of α-keto acids by protein engineering.[18] However, the relatively narrow substrate specificity of the lactate dehydrogenase makes mandelate dehydrogenase[2e,19] a more suitable choice for the conversion of α-keto acids with bulky side chains such as the aromatic ring.

In conclusion, we report development of an unanticipated activity of CtXR towards aromatic α-keto esters into a synthetic application for which well established reductase systems are lacking. Because substrate binding site plasticity is a characteristic property of aldo-keto reductases, results for CtXR may promote the more widespread structure-based systematic examination of these reductases for synthetic purposes.

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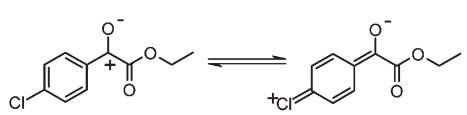

**Scheme 2** Resonance structures of **2d**. The cationic center is adjacent to the aromatic ring, so substituents can undergo direct stabilizing resonance interaction with it, as shown in the resonance hybrid on the right hand side.

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