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A New Dehydrogenase from *Clostridium acetobutylicum* for Asymmetric Synthesis: Dynamic Reductive Kinetic Resolution Entry into the Taxotère Side Chain

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Abstract

An NADP-dependent alcohol dehydrogenase from *Clostridium acetobutylicum* (CaADH) has been expressed and characterized. CaADH enantioselectively reduces aromatic α -, β - and γ -keto esters to the corresponding D-hydroxy esters and provides a building block for the Taxotère side chain (95% yield, 95% de, 99% ee) by dynamic reductive kinetic resolution (DYRKR).

Biocatalytic synthesis with purified enzymes, including aldolases,¹ epoxide hydrolases,² Baeyer-Villigerases,³ amine oxidases,⁴ lipases,⁵ and alcohol dehydrogenases,⁶ is emerging as a viable alternative to traditional asymmetric synthesis. The recent transaminase-based asymmetric synthesis of the JanuviaTM core by the Merck-Codexis team highlights the viability of such approaches, and was recognized with the 2010 Presidential Green Challenge Award.⁷

This strategem tracks with a long-standing interest in our group in using enzymes in asymmetric synthesis.^{8,9} Of late, that focus has been especially on alcohol dehydrogenases (ADHs), both for asymmetric carbonyl reduction¹⁰ and for installation of a center α - to a carbonyl, via dynamic reductive kinetic resolution (DYRKR), using a hyperthermophilic archaeal dehydrogenase.¹¹

In seeking other sources of synthetically useful DH's, we were struck by properties of the microorganism, *Clostridium acetobutylicum*. The organism has both acidogenic and solventogenic growth phases,¹² and is known to produce high titres of organic solvent in the latter. Indeed, n-butanol is one of the most abundant solvents produced, leading to great interest in *Clostridial* strain fermentation,¹³ and importation of *Clostridial* genes into other hosts toward bio-butanol production.¹⁴ Particularly notable in this latter endeavor (yeast host) are the efforts of Keasling and his team at DOE's Joint BioEnergy Institute.¹⁵ This suggested to us that *Clostridia* likely both possess enzymes capable of reducing organic carbonyl functionalities, and of operating in a mixed aqueous, alcoholic solvent milieu.

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[†]Electronic Supplementary Information (ESI) available: Exptl details, NMR spectra and chiral HPLC traces.

Indeed, early studies at UMIST revealed the presence of such alicyclic/aromatic carbonyl reduction activity in *C. acetobutylicum*.¹⁶ Now the entire *C. acetobutylicum* genome has been sequenced,¹⁷ and consists of a 3.94 MB chromosome and a 192 KB megaplasmid that encodes six different genes annotated as DHs, one of which (GI: 15004706) has 40% homology to a β -ketoacyl reductase from *Ralstonia eutropha*, an enzyme capable of enantioselective carbonyl reduction.¹⁸ Henceforth, this enzyme will be labeled simply *Clostridium acetobutylicum* ADH (*CaADH* - 251 AAs, 28.5 KD – calcd MW and 9.5 – calcd pI). This short chain NADPH-dependent DH is clearly distinct from the established NADH-dependent butanol DH that has been well characterized for some time.¹⁹ *CaADH* was expressed in recombinant form in *E. coli* and purified via a hexahistidine-tag.

As can be seen in the SDS-PAGE overview (Figure 2), abundant expression of the target protein was seen in the *E. coli* host, and the (his)₆-tag, provided a useful handle for purification via Co²⁺-NTA agarose affinity chromatography. Even though the growth temperature was reduced to 25°C, after induction with IPTG, and held there for 16 h, considerable protein (likely aggregated *CaADH*) was seen in the pellet. That said, we were delighted to obtain over 5 mg of soluble, homogeneous protein, of high specific activity (58 U/mg-benzaldehyde reduction) per liter of culture.

Next, substrate specificity was evaluated, by sequential screening against batteries of aldehyde (Figure 3) and ketone (Figure 4) substrate candidates. Several clear trends emerged. On the one hand, aromatic aldehydes and ketones possessing a carbonyl group directly conjugated with a benzenoid ring are, in general, quite good substrates. Simple aliphatic aldehydes are much poorer substrates. Insertion of one or two methylene units between the aromatic ring and the aldehyde carbonyl group, i.e. as in phenylacetaldehyde and hydrocinnamaldehyde, respectively, greatly depletes substrate activity.

Within the apparently preferred Ar-C=O substructure, there appears to be great sensitivity to aryl ring electronics. At the extremes, the electronic rich aromatic aldehyde, furfural, shows a full order of magnitude slower reduction than benzaldehyde, whereas 4-pyridine carboxaldehyde is that fastest substrate yet identified for this ADH. Interestingly, however, movement of the ring nitrogen to the 3-position leads to a better substrate, than its placement at the 2-position, clearly indicating that the inherent electronics of the substrate can be overridden in the active site. One possible explanation that an active site general acid is present capable of protonating a ring N when it is presented at the 3- or 4-position, relative to the carbonyl, but incapable of doing so at the 2-position.

Retaining the well-tolerated aroyl group, we next appended a (CH₂)_nCO₂R moiety, to scan for the ability of this *CaADH* to handle ω -keto esters (Figure 4, violet bars). Good activity was seen with the set of α -, β - and γ -keto esters ($n = 0-2$). Moreover, it was established that all three of these biocatalytic reductions proceed with excellent facial selectivity, providing an efficient (84–93% isolated yields) and enantioselective (90–99% ee; D-configuration by optical rotation-see SI) entry into the corresponding α - (**2**), β - (**4**) and γ -hydroxy esters (**6**) (Figure 5). While other biocatalytic entries into **2**,^{21, 22} **4**,^{22, 23} and **6**²⁴ are known,²⁵ the observed *CaADH* enantioselectivity is comparable, and the *CaADH* substrate scope compares favorably to these other systems.

Inversion of such D- α - and β -hydroxy esters to the corresponding esters of L- α -phenylglycine²⁶ and L- β^3 -phenylalanine²⁷ is known. As is depicted in Figure 5, the former amino acid serves as a building block for peptides with anti-thrombotic²⁸ and anti-arthritis²⁹ potential. The L- β^3 -phenylalanine building block, in turn, is useful for the assembly of β -peptides,³⁰ whereas the D- β -hydroxy ester obtained directly from the CaADH reduction serves as a precursor for the important anti-depressant, fluoxetine.³¹ Finally, the D- γ -hydroxy ester itself is a valuable chiron for the assembly of cryptophycin,³² and L- γ^4 -homophenylalanine is as useful monomer for γ -peptides.³³

We next sought to perform a similar reduction on α -halo- β -keto ester **13**, in an effort to install two stereocenters. This strategy, pioneered by Stewart³⁴ (yeast) provides an efficient chemoenzymatic entry into the phenylisoserine side chain of the taxoid chemotherapeutics. In the event, CaADH performed exceptionally, delivering the desired α -chloro- β -hydroxy ester, **14**, with proper relative (99% de) and absolute stereochemistry (95% ee) under DYRK conditions (Fig. 6). Pleasingly, this level of stereochemical control was maintained on a gram scale. D-Glucose serves as biorenewable reductant regenerating NADPH under the aegis of GDH from *T. acidophilum*.

Conclusions

In light of these results, it will be of interest to further mine the genome of this solventotolerant bacterium and further explore the potential of this *Clostridium acetobutylicum* ADH. This work was supported by the National Science Foundation (CHE-0911732) and the NCSER. R.W.C. thanks the Am. Chem. Soc. for a SURF Fellowship. (We thank the NSF (CHE-0091975, MRI-0079750) and the NIH (SIG-1-510-RR-06307) for NMR, and the NIH (RR016544) for lab renovation.

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Figure 1. Map of the *C. acetobutylicum* 192 KB megaplasmid, generated with Microbial Gene Viewer²⁰(arrow indicates CaADH gene locus)

Figure 2. SDS-PAGE gel illustrating the purification of CaADH: Outside lanes = molecular markers (MM); then l to r: (i) crude supernatant; (ii) crude pellet; (iii) eluent from Co^{2+} -column; (iv) 6 x loading of (iii)

Figure 3.

SAR survey of aldehyde substrates for CaADH (all at 10 mM in 100 mM K_2PO_4/KH_2PO_4 pH 7). Rates are normalized to that for benzaldehyde (100%). Note that the rate for 4-pyridine carboxaldehyde is off scale.

Figure 4.

SAR survey of ketone substrates for CaADH, revealing a predilection for reducing w-keto ester substrates

Figure 5. High facial selectivity observed for the CaADH-mediated reduction of α -, β - and γ -keto esters. The resulting D- ω -hydroxy esters are valuable chiral synthetic building blocks.

Figure 6. Efficient DYRKR entry into the side chain for taxoid anti-cancer drugs, including taxol and taxotère.