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Stereoselective enone reductions by *Saccharomyces carlsbergensis* old yellow enzyme

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Abstract

A series of 2- and 3-alkyl-substituted 2-cyclohexenones were shown to be substrates for the old yellow enzyme of *Saccharomyces carlsbergensis* expressed in *Escherichia coli* cells. Chemo- and stereoselective alkene reductions were observed, and the absolute configurations of the products could be predicted from the X-ray crystal structure of the protein. No competing carbonyl reductions were detected. These results support the notion that enzymes of this family may be useful in stereoselective organic synthesis.

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1. Introduction

Asymmetric hydrogenation by chiral rhodium or ruthenium phosphines has resulted in an impressive number of enantioselective alkene reductions during the last 20 years [1,2]. Despite the tremendous progress in this area, however, high stereoselectivities nearly always depend on olefin proximity to highly polar functional groups such as amides, acids and alcohols. Attempts to generalize these procedures to alkenes conjugated with less polar groups such as aldehydes, ketones, esters or nitro groups have been much less successful, although a handful of notable exceptions have been reported [3–6]. The lack of generally applicable hydrogenation catalysts for these “difficult” alkenes, the need to prepare complex ligands and the requirement for high pressures have motivated us to explore biocatalytic alternatives. Enzymatic alkene reductions might be one useful solution to this problem, particularly those mediated by whole cells (for examples, see [7–12]).

While several isolated enzymes have been reported to reduce α,β-unsaturated cyclic ketones producing optically active saturated ketones [13,14], those of the old yellow enzyme (OYE) family have been characterized most thoroughly. Christian and Warburg isolated the first family member from brewers’ bottom yeast (*Saccharomyces carlsbergensis*) in 1932. The name “old yellow enzyme” came from its color (due to bound FMN) and the need to distinguish it from a second yellow protein isolated a few years later [15]. While its ligand binding properties were investigated extensively [16], it was not until 1995 that its catalytic ability (reducing “activated” alkenes in the presence of NADPH) was discovered [17].

Work by the Massey and Karplus groups [18–21] are consistent with a proposed mechanism in which net *anti*-addition of H_2 occurs by hydride transfer from the flavin with concomitant protonation by the side-chain of Tyr 196 (Fig. 1). Hydrogen bonds contributed by His 191 and Asn 194 position and activate the carbonyl oxygen. Despite their potential utility in stereoselective organic transformations [22], there is only a single reported example [23], which employed a homolog of the *S. carlsbergensis* old yellow enzyme. This report details our first examples that start to define the substrate- and stereoselectivity of the *S. carlsbergensis* old yellow enzyme and point to ways in which it can be employed in chiral building block production.

2. Experimental

2.1. General methods

All reagents and solvents were purchased from commercial suppliers and used as received. Thin-layer chromatography (TLC) was performed using precoated silica gel plates

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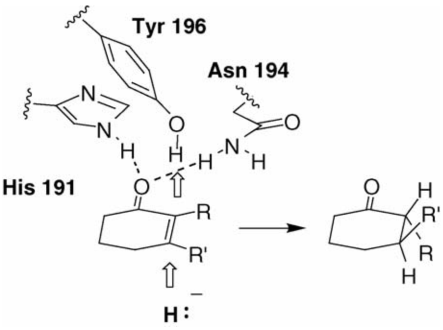


Fig. 1. Schematic diagram of the *S. carlsbergensis* old yellow enzyme active site. Hydride is donated by reduced FMN (not shown for clarity) from the β-face with simultaneous α-face protonation by the phenol of Tyr 196.

(EMD Chemicals; Darmstadt). Products were purified by flash chromatography on Purasil silica gel 230–400 mesh (Whatman; Clifton, NJ). Biotransformation reactions were monitored by GC/MS using DB-17 (0.25 mm × 30 m; Fisher Scientific; Pittsburgh, PA) and Chirasil-Dex CB (0.25 mm × 25 m; Varian; Lake Forest, CA) columns for achiral and chiral separations, respectively. The absolute configurations of products and the order of elution during chiral-phase GC analysis were determined by comparisons with authentic standards [24]. The plasmid encoding *S. carlsbergensis* old yellow enzyme (pOYE-pET3b) was obtained from Professor Vincent Massey's group and used to transform *Escherichia coli* strain BL21(DE3) (Novagen; La Jolla, CA) by electroporation. LB medium used for routine cultivation and biotransformations contained 1% Bacto–Tryptone, 0.5% Bacto–Yeast Extract and 1% NaCl.

*2.2. General procedure for preparation of 3-alkyl-2-cyclohexenones **1a–e***

The appropriate Grignard reagent was added to 3-isobutoxy-2-cyclohexenone (prepared from cyclohexane-1,2-dione and *i*-BuOH), then the crude product was hydrolyzed in aqueous acid [25]. The enones were purified by silica gel column chromatography.

*2.3. General procedure for preparation of 2-alkyl-2-cyclohexenones **3a,b***

Friedel–Crafts acylation of the appropriate carboxylic acid with glutaryl chloride in the presence of aluminum chloride yielded the 2-alkylcyclohexane-1,3-dione [26], which was converted to the corresponding 2-alkyl-3-isobutoxy-2-cyclohexenone by reaction with *i*-BuOH and *p*-TsOH [27]. The resulting vinylogous esters were reduced by LAH to afford the desired 2-alkyl-2-cyclohexenones [28].

2.4. General procedure for enone reductions by whole cells of E. coli BL21(DE3)(pOYE-pET3b)

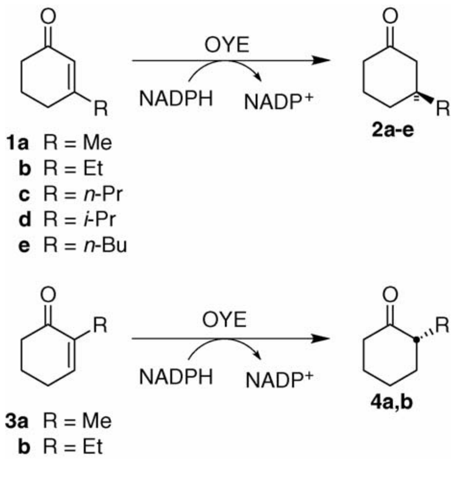
A 500 μL aliquot from an overnight culture of BL21(DE3)(pOYE-pET3b) with an OD_{600} value between 4 and 5 was added to 50 mL of LB medium supplemented with 200 μg/mL ampicillin in a 500 mL Erlenmeyer flask. The culture was shaken at 150–200 rpm at 37 °C until it reached an OD_{600} value between 0.4 and 0.5, then isopropylthio-β-D-galactoside (IPTG) was added to a final concentration of 0.10 mM. The culture was shaken at 150 rpm at room temperature for an additional 30 min, then the ketone and a stoichiometric quantity of β-cyclodextrin were added and shaking was continued at room temperature at 150 rpm. Samples for GC analysis were prepared by vortex mixing 50 μL of the reaction mixture with 50 μL of EtOAc for ca. 30 s. A 1 μL portion of the organic phase was analyzed by GC. At the conclusion of the reactions, the mixture was extracted with EtOAc (3× 30 mL), then the combined organic extracts were dried with $MgSO_4$ and concentrated by rotary evaporator.

2.5. Derivatization of cyclohexanones with (2R,3R)-(-)-2,3-butanediol

The stereochemical purities of all reduction products were determined by chiral-phase GC after ketalization with optically pure 2,3-butanediol. Derivatizations were carried out by heating a mixture of 1.0 equiv of crude biotransformation product, 2.0 equiv of (2*R*,3*R*)-(-)-2,3-butanediol and a catalytic amount of *p*-TsOH in 1.5 mL of CH_2Cl_2 at reflux for 2 h. A 1 μL aliquot was directly analyzed by GC. Samples of racemic ketones **2a–e** and **4a,b** were derivatized and analyzed by GC to demonstrate baseline resolution of enantiomers.

3. Results and discussion

Of the series of 2- and 3-alkyl-substituted cyclohexenones used in this study (Scheme 1), only **1a** was commercially available. The remaining substrates were synthesized by standard methods [25–28]. All reductions were carried out on 20 mg scales with growing cells of an *E. coli* strain that overproduced the old yellow enzyme from *S. carlsbergensis*. Flavoprotein expression was induced by adding isopropylthio-β-D-galactoside (IPTG) when the cultures reached the early logarithmic phase of growth. After 30 min, the ketone and a



Scheme 1.

Table 1
Reductions of alkyl-substituted 2-cyclohexenones by engineered *E. coli* cells overexpressing *S. carlsbergensis* old yellow enzyme

Ketone	*R*	Conversion (%)	ee (%)	Configuration
1a	Me	100	94	S
1b	Et	76	94	S
1c	*n*-Pr	25	89	S
1d	*i*-Pr	18	90	S
1e	*n*-Bu	NR[a]	–	–
3a	Me	100	96	R
3b	Et	16	90	R

[a] No reaction.

stoichiometric quantity of β-cyclodextrin (to promote substrate solubility in the aqueous reaction medium) were added. The reactions were allowed to proceed at room temperature until the bioconversions ceased. Contols suggested that the enones were ultimately toxic to one or more of the reaction components after extended periods.

Only the smallest substrates – **1a** and **3a** – were reduced completely after 24 h under these conditions (Table 1). Larger ketones were not completely reduced, even after 48 h and the conversion did not proceed further after this time. Larger substituents gave lower extents of conversion. A simple affinity purification strategy has been described for old yellow enzyme [29], and substituting the isolated enzyme (along with an NADPH regeneration system) for whole *E. coli* cells offers a straightforward way to overcome enone toxicity. Attempted reductions of 2-*exo*-methylene cyclohexenones gave very low extents of conversions under these reaction conditions (data not shown). In all cases, only alkene reductions were observed; no alcohol products were detectable.

All enone reductions proceeded with ≥89% ee values (Table 1). Product enantiomeric purities were assessed by GC under conditions that allowed baseline resolution of the racemic materials following ketalization with (2*R*,3*R*)-2,3-butanediol. Product absolute configurations were revealed by comparison with authentic standards available from earlier studies [24].

4. Conclusion

Sketching the rough outlines of old yellow enzyme's substrate- and stereoselectivities was the goal of this initial study, which employed a homologous series of simple alkyl-substituted enones. The enzyme displayed gratifying enantioselectivity. Moreover, the absolute configurations of the products could be predicted reliably from a simple model derived from X-ray crystallography data (Fig. 1). Because of hydrogen bonding with the carbonyl oxygen and the requirement that the β-carbon lie above N_5 of the flavin, reduced FMN always delivered hydride to the *re* face of the bound cyclohexenones while the solvent-derived hydrogen was always introduced to the *si* face.

Increasing the extents of conversion, particularly for larger substrates, is a key challenge that must be overcome before the enzyme can be considered synthetically useful. Nonetheless, the present results underscore the high potential of *S. carlsbergensis* old yellow enzyme and related proteins [30] in stereoselective organic synthesis.

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