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Stereoselective, Biocatalytic Reductions of α-Chloro-β-keto Esters

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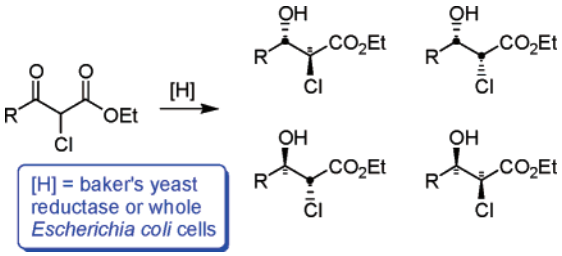
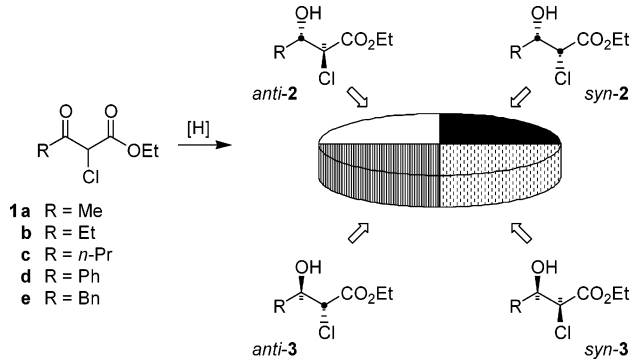


Eighteen known and putative reductases from baker's yeast (*Saccharomyces cerevisiae*) were tested for the ability to reduce a series of α-chloro-β-keto esters. In nearly all cases, it was possible to produce at least two of the four possible α-chloro-β-hydroxy ester diastereomers with high optical purities. The utility of this approach was demonstrated by reducing ethyl 2-chloroacetoacetate to the corresponding *syn*-(2*R*,3*S*)-alcohol on a multigram scale using whole cells of an *Escherichia coli* strain overexpressing a single yeast reductase identified from the screening studies.

Homochiral glycidic esters are versatile intermediates that can be converted into a variety of high-value products. Optically active glycidates can be prepared by a number of routes including asymmetric Darzens reactions, chiral alkene oxidation methodologies, and ring closure of homochiral α-halo-β-hydroxy esters (see ref 1 and references cited therein). We were particularly interested in the last strategy because asymmetric reductions of α-chloro-β-keto esters might afford each of the four possible glycidate precursors via dynamic kinetic resolution processes from single, inexpensive starting materials (Scheme 1). Here, we explore the potential of individual reductase enzymes from baker's yeast (*Saccharomyces cerevisiae*) as solutions to the problem of obtaining homochiral glycidate precursors.

Reductions of α-chloro-β-keto esters by whole cells of commercial baker's yeast generally produce disappointing mixtures of alcohol diastereomers.[2−5] Recent work has

SCHEME 1



revealed that the yeast genome encodes a large number of reductases,[6] and it seemed likely that their simultaneous participation was mainly responsible for the modest stereoselectivities commonly observed in yeast-mediated ketone reductions.[7−9] In response, we have adapted a fusion protein strategy[10] that allows the properties of yeast reductases to be assessed individually, so that enzymes yielding homochiral products can be uncovered.[11,12] Moreover, after a reductase with the desired properties has been identified, whole *Escherichia coli* cells expressing the same protein can be employed for bioconversions on preparative scales using glucose fed-batch conditions.[13] Cellular metabolic pathways supply NADPH and the whole cells display very high stereoselectivities because they express only a single yeast reductase.

Results and Discussion

A series of five α-chloro-β-keto esters **1a**−**e** was used in this study (Scheme 1). Those not commercially available were synthesized from the corresponding β-keto esters by treatment with sulfuryl chloride.[14] Eighteen yeast reductases were isolated as fusion proteins with glutathione *S*-transferase using previously described methods.[12] The collection of enzymes included members of the aldose reductase, D-hydroxy acid dehydrogenase, medium-chain dehydrogenase, and short-chain dehydrogenase superfamilies. Each α-chloro-β-keto ester was tested as a substrate for each reductase in the presence

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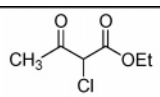
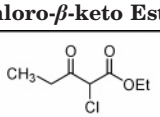
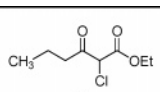
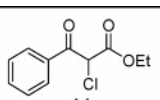
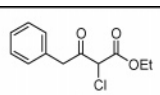
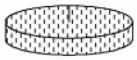
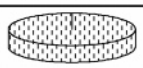
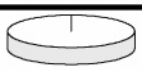
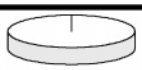
TABLE 1. Biocatalytic Reductions of α-Chloro-*β*-keto Esters[c]

Yeast Gene	 1a	 1b	 1c	 1d	 1e
YJR096w			---[a]		---
YDL124w			---		---
YBR149w				---	---
YOR120w			---	---	---
YHR104w		---	---	---	---
YDR368w				---	
YGL185c			---		---
YNL274c			---		---
YPL275w		---	---	---	---
YPL113c			---	---	---
YLR070c		---	---	---	---
YAL060w				---	---
YGL157w				---	
YDR541c			---	---	---
YGL039w					
YNL331c					---
YCR107w				---	---
YOL151w		---	---	---	---
Yeast Cells	[b]		[b]	[b]	

[a] <20% conversion after 24 h; [b] Reference 3. [c] Yeast enzymes are referred to by their systematic names and grouped by superfamilies. Product compositions from reactions that proceeded to at least 20% conversion within 24 h are shown in pie charts (white, *anti*-**2**; black, *syn*-**2**; light gray, *syn*-**3**; dark gray, *anti*-**3**).

of NADPH, which was supplied by a cofactor regeneration system. For comparison, parallel reductions were also carried out with commercial baker's yeast cells for the two cases where literature data were not available.[15] Samples were analyzed by chiral-phase GC under conditions that allowed resolution of all four diastereomeric alcohols in racemic standards prepared by $NaBH_4$ reduction. Individual stereoisomers were linked to the appropriate GC peak by isolating alcohols from enzymatic conversions that afforded only single products. The relative configurations of these materials were determined by NMR through the combined use of ^3J(H−H) homonuclear and ^nJ(C−H) long-range heteronuclear coupling constants,[16,17] and their absolute configurations were assigned from the differences in chemicals shifts between the (*R*)- and (*S*)-α-methoxy-α-phenylacetic esters.[18,19] Where literature data were available, optical rotation values were also used to determine absolute

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stereochemistry; these assignments were consistent with those made by NMR in all cases.

Comparing the outcomes of reactions using whole baker's yeast cells with those employing isolated yeast reductases clearly demonstrates the utility of examining individual biocatalysts (Table 1). Not only did the purified yeast reductases deliver higher stereoselectivities in most cases, they also produced diastereomers not observed in reductions employing commercial yeast cells. This may result from low expression of some reductases under the physiological conditions prevailing in commercial baker's yeast, and this highlights an important advantage of using isolated reductases, rather than relying on whole yeast cells. Alternative methods to increase expression levels of desirable reductases, such as adding specific enzyme inhibitors, are more difficult to optimize and control (for examples, see refs 9, 20, and 21). It should also be noted that the screening reactions could be carried out rapidly, and a complete data set was typically obtained for each substrate within 48 h.

The smallest substrate, **1a**, was accepted by all of the yeast reductases examined, although the stereoselectivities of these reactions were relatively poor except for YOR120w and YGL157w, which afforded *syn*- and *anti*-**2a** as the major products, respectively. In all cases, however, only L-alcohols were formed. This behavior parallels our earlier observations from reactions in which ethyl acetoacetate was used as a substrate for the same collection of yeast fusion proteins.[12] The behavior of higher homologue **1b** provides an interesting contrast. In four cases, D-alcohols were the major products. This is significant because D-alcohols are observed much less commonly in biocatalytic reductions and enzymes that deliver this enantioselectivity are correspondingly important. Six enzymes examined accepted **1d** as a substrate: four afforded only *syn*-**3d** while the remaining two produced mainly *syn*-**2d**. Benzyl-substituted β-keto ester **1e** was reduced by three enzymes, with very high stereoselectivities in two cases.

The studies summarized in Table 1 delineated the subset of enzymes that *might* be useful in producing specific α-chloro-β-hydroxy ester diastereomers. To show that these enzymes could in fact be used for reactions on preparatively useful scales, **1a** was reduced by whole cells of an *E. coli* strain overexpressing the YOR120w protein in a 1 L laboratory-scale fermenter. Cells were grown in rich medium under inducing conditions and then resuspended in phosphate buffer supplemented with glucose. The reduction of **1a** was carried out under aerobic conditions (dissolved oxygen maintained at 75%), and the pH was kept constant at 5.6. The ketone substrate was added portionwise to a final concentration of 27 mM. After reduction, the product was recovered by extraction and chromatographically purified to yield 3.7 g of *syn*-**3c** (98% ee) in 89% isolated yield. We have used a similar approach for reductions of **1d** and **1e** with equal success.[22]

Taken together, our results have demonstrated that reductase enzymes uncovered by an analysis of the yeast genome can deliver important chiral building blocks for organic synthesis. At least two of the four possible α-chloro-β-hydroxy ester diastereomers could be produced in high optical purities in most cases. The major deficiency in the present collection is a lack of stereoselective reductases with D-specificities. Biocatalysts with these properties might be identified by including enzymes from additional organisms in our collection of fusion proteins, and the increasing pace of the genome-sequencing project bodes well for expanding the utility of our chemoenzymatic approach.

Experimental Section

General Procedures. Standard media and techniques for growth and maintenance of *E. coli* were used, and LB medium contained 1% Bacto-Tryptone, 0.5% Bacto-Yeast Extract, and 1% NaCl. GST-fusion proteins were isolated as described previously.[12] Glucose-6-phosphate dehydrogenase (Sigma type XV from baker's yeast) was used for NADPH regeneration. Ketones **1a**−**e** were prepared by treating the corresponding β-keto esters with sulfuryl chloride.[14]

NMR spectra were recorded with a 5 mm indirect detection probe at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are reported at 25 °C in ppm relative to TMS. Optical rotations were measured from $CHCl_3$ solutions at room temperature. GC analyses were carried out with a 0.32 mm × 30 m DB-17 column for nonchiral separations and a 0.25 mm × 25 m Chirasil-Dex CB or a 0.25 × 25 m Chirasil-L-Val column for enantiomer separations. GC samples were prepared by vortex mixing of 200 μL of the reaction mixture with an equal volume of Et_2O and then removing the organic layer for analysis. Racemic alcohols were prepared from ketones **1a**−**e** by reduction with $NaBH_4$ and GC conditions providing resolution of all products were used for analyzing products from enzymatic reductions. In cases where insufficient resolution was obtained, alcohol products were acetylated prior to GC analysis.

General Procedure for Ketone Reductions Using Purified Yeast GST-Fusion Proteins. Reaction mixtures contained $NADP^+$ (0.20 μmoles, 0.15 mg), glucose 6-phosphate (14 μmoles, 4.3 mg), glucose 6-phosphate dehydrogenase (5 μg), α-chloro-β-keto ester substrate (5 mM), and purified GST-fusion protein (10−100 μL, containing 5−50 μg) in 1.0 mL of 100 mM KP_i, pH 7.0. Reactions were incubated at 30 °C and sampled for GC analysis periodically.

To determine relative and absolute configurations of alcohols, bioconversions affording single products were scaled up 10- or 20-fold from the procedure described above. After nearly all of the substrate had been consumed, the reaction mixture was extracted with Et_2O (3 × (5 × reaction volume)). The combined organic extracts were washed with brine (1 volume) and water (1 volume), dried with $MgSO_4$, and concentrated in vacuo. If required, the alcohol product was purified by flash column chromatography prior to spectral analysis.

Optical rotation data for isolated α-chloro-β-hydroxy esters: *syn*-**2a** (YOR120w), $[\alpha]_D = +11$, *c* 2.0, lit.[23] $[\alpha]_D = +12.4$, *c* 1 ($CHCl_3$); *syn*-**2b** (YDR368w), $[\alpha]_D = +8.0$, *c* 0.98; *syn*-**3d** (YDL124w), $[\alpha]_D = -3.0$, *c* 3.5, lit.[3] $[\alpha]_D = -3.0$, *c* 1.7 ($CHCl_3$); *syn*-**2e** (YDR368w), $[\alpha]_D = +24$, *c* 0.7; *anti*-**2a** (YGL157w), $[\alpha]_D = +4.0$, *c* 1.1; *anti*-**2b** (YGL157w), $[\alpha]_D = -0.17$, *c* 0.25; *anti*-**2c** (YGL157w), $[\alpha]_D = -9.8$, *c* 1.7, lit.[3] for enantiomer $[\alpha]_D = +8.5$, *c* 1 ($CHCl_3$); *anti*-**2e** (YGL157w), $[\alpha]_D = -2.2$, *c* 3.0.

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Reduction of 1a by Engineered *E. coli* Cells. A single colony of *E. coli* BL21(DE3)(pIK30) was used to inoculate 40 mL of LB medium containing 40 μg/mL of kanamycin. After shaking overnight at 37 °C, the preculture was added to 4 L of the same medium in a New Brunswick M19 fermenter. The culture was grown for 2 h at 37 °C with a stir rate of 800 rpm and an air flow of 0.3 vessel volumes per minute (vvm) until it reached $O.D._{600} = 0.59$, and then it was cooled over 15 min to 30 °C. A sterile solution of isopropylthio-β-D-galactoside was added to achieve a final concentration of 0.10 mM, and the culture was kept under the same conditions for an additional 6 h. Cells were collected by centrifugation (6000*g* for 10 min at 4 °C) and stored overnight at 4 °C. Half of the cells were resuspended in 1 L of 10 mM KP_i (pH 5.6) containing 4 g/L glucose. The bioconversion was carried out in a B. Braun Biostat B at 32 °C with pH maintained at 5.6 using 3 M KOH. The dissolved oxygen was maintained at 75% saturation using a fixed air flow of 0.25 vvm and variable stirring rate. Portions of neat **1a** (0.20 or 0.25 mL) were added approximately every 30 min over a total of 6.3 h to provide a final value of 27 mmol (4.4 g after 16 additions). Portions of glucose (4 g) were added from a 20% stock solution after 2.6 and 4.0 h to maintain an approximately steady-state concentration of the carbon source. Consumption of **1a** slowed significantly after 6 h. After 8 h, the reaction mixture was gently extracted with CH_2Cl_2 (3 × 250 mL; 1 day for each portion) so that emulsion formation was avoided. The combined organic extracts were dried with $MgSO_4$, concentrated and subjected to flash column chromatography (85:15 cyclohexanes−ether) to yield residual **1a** (0.25 g) and syn-**2a** (3.7 g, 89% yield based on recovered starting material). Chiral-phase GC analysis showed that syn-**2a** was isolated in 98% ee and both GC and ^{1}H NMR confirmed its chemical purity.

NMR Analysis of Reduction Products. Complete ^{1}H and ^{13}C chemical shift assignments for alcohols derived from α-chloro-β-keto esters **1a**−**e** were based on the ^{1}H−^{1}H, ^{1}H−^{13}C one-bond, and ^{1}H−^{13}C long-range couplings seen in the ^{1}H and the G-BIRD-HSQMBC spectra.[24] Absolute configurations were determined based on the differences in chemical shifts between the (*R*)- and (*S*)-α-methoxy-α-phenylacetic (MPA) esters as described by Seco et al.[18,19]

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Supporting Information Available: Details of the NMR analyses that allowed the assignments of relative and absolute stereochemistries of alcohol products, an alternative version of Table 1, in which the data are presented in numerical form, and a description of the apparatus used to extract *syn*-**2a** on a preparative scale. This material is available free of charge via the Internet at http://pubs.acs.org.

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