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Stereocomplementary Bioreduction of β-Ketonitrile without Ethylated Byproduct

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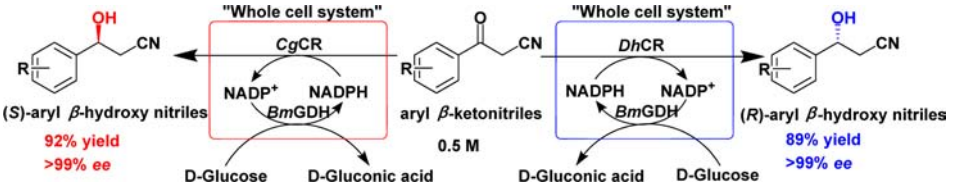
ABSTRACT



α-Ethylation is competing with the biocatalytic reduction of aromatic β-ketonitriles in a whole-cell system. Use of two newly mined robust and stereocomplementary carbonyl reductases in a biphasic system has completely eliminated the competing byproduct. For the first time, both enantiomers of fluoroxetine precursors were obtained at 0.5 M with >99% *ee* and excellent chemoselectivity, without addition of any external cofactors.

Optically pure β-hydroxynitriles are important building blocks for the synthesis of a variety of natural compounds and pharmaceutically relavant products because of their facile conversion into chiral 1,3-amino alcohols, β-hydroxy acids, etc.[1] The most important application has been found to be the precursors of the popular serotonin/norepinephrine reuptake inhibitors (SNRIs), commercially known as Prozac (fluoxetine), Straterra (atomoxetine), and nisoxetine. These medicines are employed for relieving people from inception and other disorders, including anxiety, chronic pain, alcoholism, migraine headaches, urinary incontinence, sleep and memory disorders, obesity, and bulimia.[2]

Several chemical routes have been developed for yielding chiral β-hydroxy nitriles, including asymmetric aldol-type reactions with acetonitrile and benzaldehyde, β-boration of α,β-unsaturated nitriles followed by oxidation, borane reductions, and hydrogen transfer of β-ketonitriles.[3] Silica-immobilized Ru-TsDPEN catalysts were developed by Tu et al. for asymmetric transfer hydrogenation of aryl ketones with relatively high activity and 97% *ee*.[3f] Iridium diamine catalysts were designed by Carreira et al. for the reduction of aryl ketonitrile with 47−95% *ee*.[3h] However, the main disadvantage of these strategies is the time- and cost-intensive synthesis of catalysts employed and the toxicity issues especially when considering the use of heavy metals for the synthesis of pharmaceuticals. It is often difficult to achieve a very high enantioselectivity for chemical routes.

Asymmetric synthesis of optically active compounds employing biocatalysts represents an effeicent alternative to chemical strategies. A classic example is the kinetic resolution of racemic β-hydroxy nitrile catalyzed by a lipase or nitrilase.[1c,4] However, these enzymes are inherently limited to a theoretical yield of 50% when resolving the racemate. The oxidoreductase-catalyzed asymmetric reduction of β-ketonitriles provides another route to preparing β-hydroxy nitrile with almost 100% yield. However, a common side

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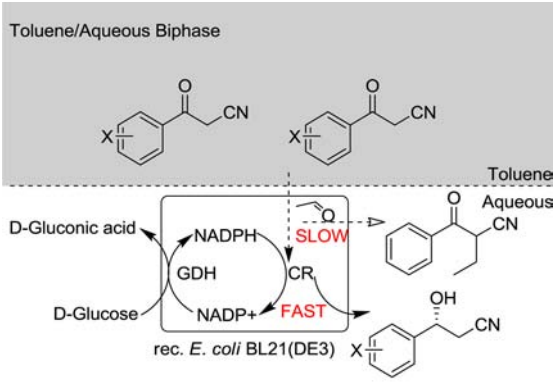
Scheme 1. Asymmetric Reduction of Benzoylacetonitrile with Recombinant *E. coli* BL21(DE3) Cells Harboring Carbonyl Reductase and Glucose Dehydrogenase



reaction of α-ethylation of β-ketonitriles employing "whole-cell system" biocatalysts was diadvantageous for the application in the scale-up synthesis of chiral β-hydroxy nitrile. This competing α-ethylation was even found in the recombinant *E. coli* BL21(DE3) system overexpressing carbonyl reductases from baker's yeast.[2a,5] This ethylated product was supposed to come from aldol type condensation between acetaldehyde produced by the metabolism of cells and the α-cynaoketon of substrates, followed by reduction of the resultant alkene.[5a] Although the ethylated byproduct can be avoided by lowering the reaction temperature to 4 °C[5f] or employing purified carbonyl reductase,[5g] low catalytic efficiency, the requirement of expensive nicotiamide cofactors, the tedious purification procedure, and the low stability of isolated enzyme posed new issues.

To eliminate the concomitant α-ethylated byproduct and improve the productivity of β-hydroxy nitriles, we then made an effort to search for new carbonyl reductases with higher catalytic efficiency and employed a biphasic system of aqueous/organic solvent for the bioreduction of β-ketonitrile with recombinant "designer cells" coexpressing carbonyl reductase (CR) and glucose dehydrogenase (GDH).[6] Biphasic systems are usually employed to improve the substrate solubility and to avoid the spontaneous hydrolysis and substrate inhibition.[7] Additionally, employment of recombinant whole cells, especially those coexpressing reductase with glucose dehydrogenase, could achieve high reduction efficiency and internal cofactor regeneration.[8] We thus hope that the use of these newly designed whole cells in the biphasic system can make the bioreduction process independent of metabolic activity and avoid the formation of a byproduct.

Exploring nature's diversity is a promising approach to discovering novel and efficient biocatalysts with desired properties.[9] A carbonyl reductase toolbox, consisting of 30 oxidoreductases, has been designed and developed using a genome mining strategy as previously described.[10] These reductases show moderate identities (40−70%) with known oxidoreductases. Of these oxidoreductases, 17 belong to the short-chain dehydrogenase/reductase (SDR) family, 7 belong to the aldo/keto reductase family (AKR), and 6 belong to the zinc-containing alcohol dehydrogenase family (ADH_SF_Zn-type). Six oxidoreductases from this toolbox were selected because of their higher specific activity shown in the asymmetric reduction of aryl ketones (Table S2). Among them, two reductases, *Dh*CR from *Debaryomyceshansenii* (GenBank accession No. CAG87931) and *Cg*CR from *Candida glabrata* (No. CAG60239), displayed complementary enantioselectivity toward benzoylacetonitrile. In addition, both reductases exhibited excellent chemical selectivity in reducing the carbonyl group, without forming any ethylated byproduct as reported in the literature.[5g] Therefore, *Dh*CR and *Cg*CR were selected for further characterization in asymmetric reduction of benzoylacetonitrile, whose products (either (*R*)- or (*S*)-β-hydroxynitrile) are very important chiral synthons for the production of many pharmaceuticals including fluoroxetine.

To establish an efficient route for asymmetric bioreduction of benzoylacetonitrile, a glucose dehydrogenase from *Bacillus megaterium* (*Bm*GDH)[11] was introduced for the internal regeneration of the cofactor ($NADP^+$/NADPH) and coexpressed in *E. coli* with *Dh*CR and *Cg*CR, respectively. The asymmetric reductions of five different benzoylacetonitriles with high *ee* values by recombinant *Dh*CR−*Bm*GDH and *Cg*CR−*Bm*GDH whole cells are shown in Table 1. A higher conversion rate was found with *Cg*CR (data not shown) than *Dh*CR. However, in the case of substrates **3**, **4**, and **5** catalyzed by *Dh*CR,

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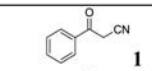
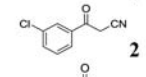
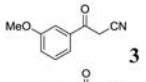
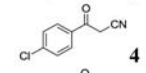
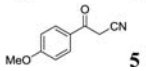
Table 1. Relative Activity of *Dh*CR and *Cg*CR towards Various Aryl β-Ketonitriles and the Percentage of α-Ethylated Byproduct Detected in Aqueous System

substrate	*Dh*CR		*Cg*CR	
	relative activity [%][a)]	by-product [%][b)]	relative activity [%][a)]	by-product [%][b)]
 1	100	0.0	310	0.0
 2	85.4	2.8	350	0.0
 3	68.9	4.0	273	0.0
 4	192	0.0	408	0.0
 5	75.0	6.8	258	0.0

[a] The reductase activity was spectrophotometrically estimated with 1 mM aryl β-ketonitriles, 0.1 mM NADPH, and a proper amount of purified enzymes at 30 °C. The activity of *Dh*CR toward benzoylacetonitrile, 60 nmol/min/mg, was considered as 100%. [b] Reactions were carried out with 10 mM benzoylacetonitrile derivatives (dissolved in DMSO), 10 g/L recombinant whole cells of *Bm*GDH−*Dh*CR or *Bm*GDH−*Cg*CR with 1.5 equiv glucose in 0.5 mL PBS (pH 6.5, 100 mM) at 30 °C, 900 rpm for 12 h (Thermomixer compact, Eppendorf). Samples were analyzed by GC equipped with Chiralsil-CP column.

Table 2. Asymmetric Reduction of Aryl β-Ketonitriles in Toluene/Aqueous System

	*Bm*GDH−*Dh*CR[a)]				*Bm*GDH−*Cg*CR[a)]			
substrate	time [h]	conversion [%]	*ee*[b)] [%]	yield [%]	time [h]	conversion [%]	*ee*[b)] [%]	yield [%]
1	6	>99	>99/*R*	85	4	>99	>99/*S*	89
2	15	>99	>99/*R*	82	12	>99	>99/*S*	87
3	24	>99	>99/*R*	84	24	>99	>99/*S*	85
4	6	>99	>99/*R*	84	6	>99	>99/*S*	85
5	12	>99	>99/*R*	82	12	>99	>99/*S*	87

[a)] Reaction was carried out at 10 g/L substrate concentration dissolved in 5 mL of toluene, 1.5 equiv of glucose, and a proper amount of dried *Bm*GDH−*Dh*CR (50 g/L) or *Bm*GDH−*Cg*CR (20 g/L) whole cells in 5 mL of PBS buffer (pH 6.5, 100 mM) at 25 °C until completion. [b)] *ee* was measured using chiral GC, the absolute configuration was determined according to optical rotation data, and the standard product samples were prepared by reduction with $NaBH_4$.

some ethylated byproducts (4−9%) were detected. No ethylated product was detected in the reactions mediated by *Cg*CR. The relatively lower specific activity of *Dh*CR (20−30% of *Cg*CR) to β-ketonitrile derivatives may account for the appearance of the ethylated byproduct. The unreduced substrates in aqueous phase were liable to be ethylated by the cell metabolism of *E. coli*. Inspired by the purpose of avoiding the competing α-ethylation of the unreduced substrate in the aqueous phase, an organic phase was introduced. As shown in Scheme 1, in the biphasic system, the majority of the substrate was located in the organic phase. The substrate in the organic phase

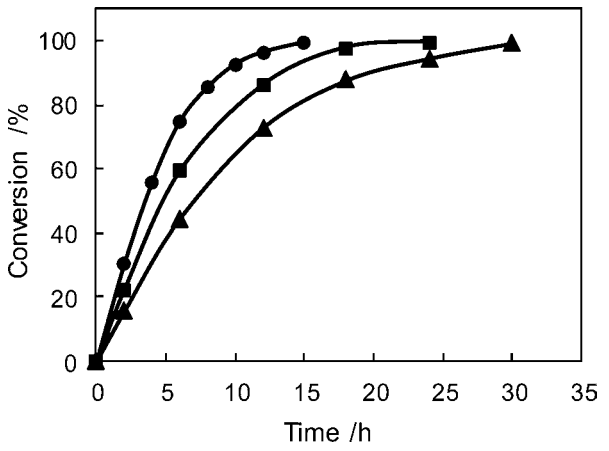


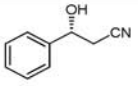
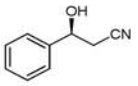
Figure 1. Asymmetric reduction of benzoylacetonitrile with recombinant *Bm*GDH−*Dh*CR whole cells in aqueous/toluene biphasic system. (●) 30.0 g/L, (■) 50.0 g/L, (▲) 72.5 g/L. Reactions were carried out with 0.30, 0.50, and 0.725 g of benzoylacetonitrile dissolved in 5.0 mL of toluene, 0.50 g of *Dh*CR-*Bm*GDH whole cells, and 1.50 equiv of glucose in 5.0 mL of PBS buffer (pH6.5) at 25 °C, 180 rpm with titration of 2.0 M NaOH to control pH at 6.5 until completion.

diffused into the aqueous phase increasingly and then was asymmetrically reduced by the carbonyl reductase.

Ten different organic solvents with different Log *P* values were examined for their performance as the organic phase. As shown in Table S3, with ethyl butyrate, ethyl hexanoate, butyl acetate, or dibutyl phathlate, the β-ketonitrile was efficiently extracted into the organic layer. And *Dh*CR, *Cg*CR, and *Bm*GDH also showed high residual activities in all of the above-mentioned organic solvents except dibutyl phathlate. Taking the optimized partition coefficient, low toxicity, and moderate boiling point into account, toluene was chosen as the organic phase. To our delight, as shown in Table 2, none of the ethylated byproduct was detected with this biphasic system employing recombinant whole cells harboring pET28-*Bm*GDH-*Dh*CR. Within 24 h, all the tested substrates were asymmetrically and chemoselectively reduced into optically pure form with >82% yield.

To work as an efficient tool in organic synthesis, it is essential to establish a bioreduction process with high substrate loadings and most importantly, without the requirement of external cofactor addition. We then sought to investigate the application of *Dh*CR in the synthesis of (*R*)-3-hydroxy-3-phenylpropanenitrile, a key synthon for fluoroxetine, at high substrate loading. As shown in Figure 1, within 30 h, as much as 0.5 M benzoylacetonitrile (145 g/L in toluene phase) was asymmetrically reduced into optically pure (*R*)-3-hydroxy-3-phenylpropanenitrile without external cofactor addition. Compared with other reported bioreduction systems in Table 3, these two enantiocomplemetary reductases in the toluene/aqueous biphasic system provide several additional advantages, including high substrate loading, no ethylated byproduct, no addition of external cofactor, and easy operation, which make them more competitive and

Table 3. Comparative Preparation of Optically Active 3-Hydroxy-3-phenylpropanenitrile Using Biocatalytic Asymmetric Reduction

product	biocatalyst	form	concn [g/L]	cofactor [mM]	time [h]	yield [%]	*ee* [%]	by-prod [%][d]	sty[e] [g·L^{-1}·d^{-1}]	ref
	*Cm*CR	I. E.[a]	1.62	0.218	60	85	98/*R*	0	0.55	5g
	YOL039w	W. C.[b]	0.96	0	24	96[c]	97/*R*	6	0.96	5e
	***Bm*GDH-*Dh*CR**	**W. C.**	**145**	**0**	**30**	**89**	**>99/*R***	**0**	**51.6**	**This work**
	Ymr226c	I. E	3.09	0.218	48	90	>99/*S*	0	1.39	5h
	Baker's Yeast	W. C.	0.11	0	24	10	98/*S*	90	0.11	5e
	Curvularia lunata	W. C.	1.00	0	12	22	78/*S*	50	0.44	5d
	YGL151w	W. C.	1.01	0	24	96[c]	99/*R*	4	1.01	5e
	***Bm*GDH-*Cg*CR**	**W. C.**	**145**	**0**	**24**	**92**	**>99/*S***	**0**	**66.7**	**This work**

[a] I. E.: isolated enzyme. [b] W. C.: Whole cells. [c] Conversion rate. [d] Percentage of α-ethylated byproduct. [e] Space-time yield.

promising tools in the preparation of both enantiomers of chiral alcohols. This study also provides an effective proof for the discovery of naturally evolved biocatalysts.

In summary, to screen for enzymes with high specific activity, enantioselectivity, and substrate/product tolerance for the asymmetric reduction of aromatic β-ketonitriles, two stereocomplementary carbonyl reductases *Dh*CR and *Cg*CR were identifid with desired properties from a newly established oxidoreductase toolbox. Effective asymmetric reduction of aromatic β-ketonitrile was achieved with *Dh*CR and *Cg*CR. Ethylated byproducts were successfully eliminated in a toluene/aqueous biphasic system employing the recombinant *E. coli* whole cells coexpressing reductase and *Bm*GDH. As much as 145 g/L benzoylacetonitrile in the toluene phase was reduced into optically pure (*R*)-3-hydroxy-3-phenylpropanenitrile without external cofactor addition and with no ethylated byproduct. Either the high catalytic activity of reductases or the use of a biphasic system was considered as the key to eliminate the competing α-ethylated byproduct when using a whole cell system. Protein engineering of these two carbonyl reductases to further improve their catalytic activity are now underway in our laboratory, in order to make these two newly discovered reductases very competitive and promising tools for the preparation of key building blocks of fluoroxetine.

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Supporting Information Available. General experimental procedures and spectra data. This material is avaiable free of charge via the Internet at http://pubs.acs.org.

The authors declare no competing financial interest.