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Stereoselective Enzymatic Synthesis of Chiral Alcohols with the Use of a Carbonyl Reductase from *Candida magnoliae* with Anti-Prelog Enantioselectivity

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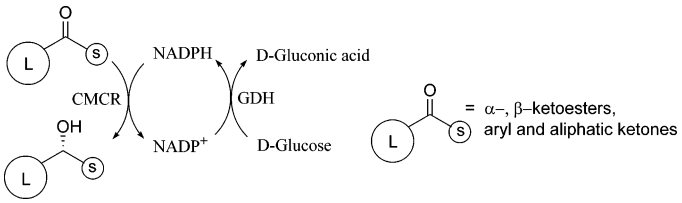
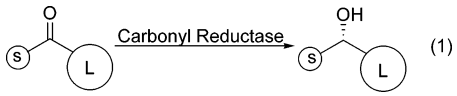
Received February 16, 2006



In our effort to search for carbonyl reductases with anti-Prelog enantioselectivity, the activity and enantioselectivity of a carbonyl reductase from *Candida magnoliae* have been examined with various ketones of diverse structures. This carbonyl reductase catalyzed the reduction of a series of ketones, α- and *β*-ketoesters, to anti-Prelog configurated alcohols in excellent optical purity. The usefulness of this carbonyl reductase has been demonstrated by synthesis of several chiral alcohol intermediates of pharmaceutical importance.

Introduction

Enantiometrically pure alcohols including α- and *β*-hydroxy-esters are important and valuable intermediates in the synthesis of pharmaceuticals and other fine chemicals. A variety of synthetic methods have been developed to obtain optically pure alcohols, α- and *β*-hydroxy acids, and their derivatives. Among these methods, a straightforward approach is the reduction of prochiral ketones to chiral alcohols. In this context, a variety of chiral metal complexes have been developed as catalysts in the asymmetric ketone reductions.[1−3] However, in many cases difficulties remain in the process operation, and obtaining sufficient enantiomeric purity and productivity.[2,3] In addition, residual metal in the products originated from the metal catalyst presents another challenge because of ever more stringent regulatory restrictions on the level of metals allowed in pharmaceutical products.[4] An alternative to the chemical asymmetric reduction processes is the biocatalytic transformation with isolated enzymes or whole-cell microorganisms that offers advantages such as mild and environmentally benign reaction conditions, high chemo-, regio-, and stereoselectivity, and being void of residual metal in the products.[5−7] Therefore, great efforts have been made to develop biocatalytic ketone reduction processes, and many examples with isolated carbonyl reductases have been described.[8−12] The stereoselectivity of carbonyl reductase-catalyzed ketone reduction is usually dependent on the steric situation of substrates, and may be predicted with "Prelog's rule".[13,14] The majority of known carbonyl reductases used for the enantioselective ketone reduction follow Prelog's rule as shown in eq 1.[10,13]



(1)

Because of the equal importance of both enantiomers, there is a great demand for the carbonyl reductases with anti-Prelog

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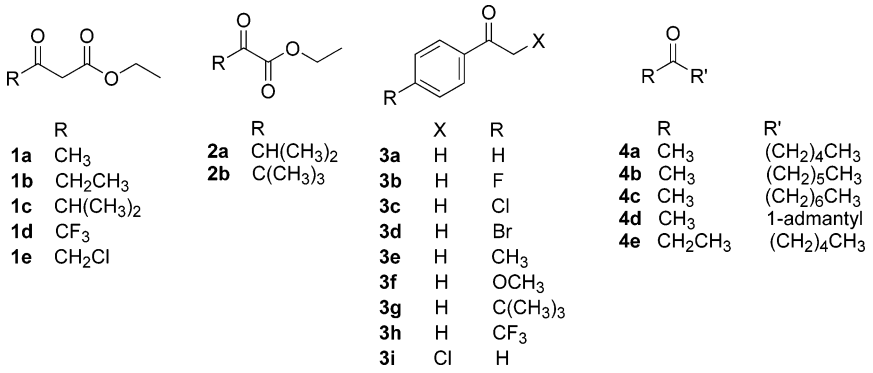
10.1021/jo0603328 CCC: $33.50
Published on Web 05/04/2006

CHART 1



stereopreference, which lead to the formation of anti-Prelog configurated alcohols. Recently a carbonyl reductase gene from *Candida magnoliae* was cloned and the encoded protein was found to catalyze the reduction of ethyl 4-chloro-3-oxobutanoate to give ethyl (*S*)-4-chloro-3-hydroxybutanoate in optically pure form.[15−18] Since the chloromethyl group is smaller in size than $CH_2COOC_2H_5$, but has higher priority in the CIP rules for R/S configuration assignment, we reasoned that the carbonyl reductase from *Candida magnoliae* (CMCR) might not obey Prelog's rule in the ketone reduction to afford anti-Prelog configurated alcohols. To initiate our effort to search for carbonyl reductases with anti-Prelog enantioselectivity, the activity and enantioselectivity of CMCR have been evaluated toward the reduction of various ketones including α- and *β*-ketoesters (Chart 1). This carbonyl reductase indeed reduced a diversity of ketones to anti-Prelog configurated alcohols in excellent enantiomeric purity. To explore the synthetic potential of this carbonyl reductase, it was then applied to the synthesis of several pharmaceutically important chiral alcohol intermediates.

Results and Discussion

The carbonyl reductase gene from *Candida magnoliae* (Genbank Accession No. AB036927) was overexpressed in *E. coli* and the encoded protein was purified and lyophilized by following the literature procedure.[15] This lyophilized enzyme was stable for months at 4 °C without significant loss of activity. The specific activity of the carbonyl reductase from *Candida magnoliae* (CMCR) toward the reduction of ketones in Chart 1 was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm at room temperature. Activity assay with the control cell-free extract, which was obtained by expression of pET15b vector without CMCR gene in *E. coli* Rosetta2(DE3)pLysS strain, did not show any activity toward all the substrates tested. The specific activities for different ketone substrates are presented in Tables 1−3.

From Table 1 it can be seen that the carbonyl reductase from *Candida magnoliae* efficiently catalyzed reduction of the tested

TABLE 1. Specific Activity and Enantioselectivity of Carbonyl Reductase from *Candida magnoliae* toward the Reductions of α- or *β*-Ketoesters

α-/*β*-ketoester	specific activity[a]	ee (%)[b]
1a (CH_3)	100	>99 (R)[c]
1b (C_2H_5)	682	>99 (R)
1c ($CH(CH_3)_2$)	1545	>99 (S)
1d (CF_3)	209	>99 (S)
1e (CH_2Cl)	6614	97 (S)
2a ($CH(CH_3)_2$)	1000	>99 (R)
2b ($C(CH_3)_3$)	118	99 (R)

[a] The unit of specific activity was nmol·min^{-1}·mg^{-1}. [b] The ee value was measured by chiral GC analysis. [c] The absolute configuration of product alcohol was determined by comparing the retention time with that of standard samples.

α- or *β*-ketoesters and the catalytic activity was dependent on the substrate structure. It was interesting that the specific activity of CMCR for reduction of *β*-ketoesters increased as the alkyl group became larger (**1a** → **1b** → **1c**). The chloro substituent at the 4-position of ethyl 3-oxo-butyrate also greatly enhanced the enzyme activity. In contrast to *β*-ketoesters, CMCR showed less activity for the reduction of α-ketoester (**2b)** with a bulky *tert*-butyl group than ethyl 3-methyl-2-oxo-butyrate (**2a)**.

Table 2 showed that the carbonyl reductase (CMCR) was active for a series of acetophenone derivatives. The substituents at the para position of acetophenone derivatives greatly affected the enzyme catalytic activity. The electron-withdrawing substituents enhanced the enzyme activity (**3b**, **3c**, **3d**, and **3h**), while the substrates with an electron-donating substituent were less active than the unsubstituted acetophenone (**3e**, **3f**, and **3g**). The acetophenone derivatives with a substituent at the ortho or meta position were much less active (data not shown). α-Chloroacetophenone (**3i**) showed much higher activity than acetophenone (**3a**) and was an excellent substrate for the carbonyl reductase from *Candida magnoliae*. The aliphatic ketones were also good substrates for this carbonyl reductase, and chain length exerted some effect on the enzyme activity as shown in Table 3.

The enantioselectivity of the carbonyl reductase from *Candida magnoliae* toward the reduction of ketones in Chart 1 was studied by using a NADPH regeneration system consisting of D-glucose dehydrogenase (GDH) and D-glucose (Scheme 1).[19]

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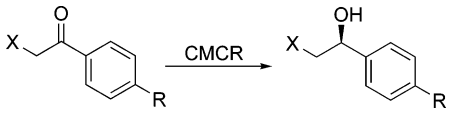
TABLE 2. Specific Activity and Enantioselectivity of Carbonyl Reductase from *Candida magnoliae* toward the Reduction of Aryl Ketones



ketone (X, R)	specific activity[a]	ee (%)[b]
3a (H, H)	65	99 (R)[c]
3b (H, F)	864	>99 (R)
3c (H, Cl)	455	>99 (R)
3d (H, Br)	682	>99 (R)
3e (H, CH_3)	59	99 (R)
3f (H, OCH_3)	18	>99 (R)
3g (H, $C(CH_3)_3$)	10	99 (R)
3h (H, CF_3)	1136	>99 (R)
3i (Cl, H)	818	>99 (S)

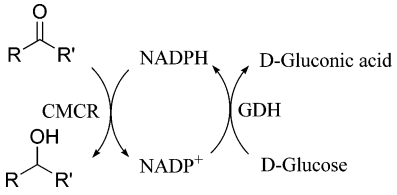
[a] The unit of specific activity was nmol·min^{-1}·mg^{-1}. [b] The ee value was measured by chiral GC analysis. [c] The absolute configuration of product alcohol was determined by comparing the retention time with that of standard samples, or by the sign of optical rotation.

TABLE 3. Specific Activity and Enantioselectivity of Carbonyl Reductase from *Candida magnoliae* toward the Reduction of Aliphatic Ketones

ketone (R, R′)	specific activity[a]	ee (%)[b]
4a (CH_3, *n*-C_5H_{11})	200	>99 (R)[c]
4b (CH_3, *n*-C_6H_{13})	95	>99 (R)
4c (CH_3, *n*-C_7H_{15})	73	>99 (R)
4d (CH_3, 1-adamantyl)	18	>99 (R)
4e (C_2H_5, *n*-C_5H_{11})	15	>99 (R)

[a] The unit of specific activity was nmol·min^{-1}·mg^{-1}. [b] The ee value was measured by chiral GC analysis. [c] The absolute configuration of product alcohol was determined by comparing the retention time with that of standard samples, or by the sign of optical rotation.

SCHEME 1



The enantiomeric excess (ee) values of the product alcohols were determined by chiral GC analysis. The absolute configuration of product alcohols was assigned by comparing either the retention time with standard sample or the specific rotation with reported data. The standard samples were from either commercial sources or the preparation in our previous studies.[9,10,20,21] The results are summarized in Tables 1−3. It can be seen that the carbonyl reductase from *Candida magnoliae* efficiently catalyzed the reduction of various ketones including α- and *β*-ketoesters to give anti-Prelog configurated alcohols in excellent optical purity (≥97% ee). More importantly, aliphatic ketones were also reduced to the corresponding alcohols in optically pure form (Table 3). It is usually difficult to achieve such high enantioselectivty in the aliphatic ketone reduction by metal-catalyzed hydrogen transfer or hydrogenation.[22,23] This indicated that the carbonyl reductase from *Candida magnoliae* was indeed an oxidoreductase with excellent anti-Prelog enantioselectivity for a diversity of ketones.

TABLE 4. Isolated Yields, Enantiomeric Excess, and Optical Rotation of the Product Alcohols in the Preparative Scale

ketones	yield (%)	ee (%)	$[\alpha]^{22}_D$[a]
2a	87	>99 (R)	−9.5
2b	91	99 (R)	−30.3
3b	89	>99 (R)	+45.2
3h	94	>99 (R)	+29.3
3i	92	>99 (S)	+49.5
4b	92	>99 (R)	−9.4
4d	95	>99 (R)	+15.7[b]
4e	90	>99 (R)	−8.8

[a] $[\alpha]^{22}_D$ was measured in $CHCl_3$ (*c* 1). [b] $[\alpha]^{22}_D$ was measured as 1-(1-adamantyl)ethyl acetate.

Many chiral alcohols are important intermediates in the synthesis of pharmaceuticals and agrichemicals. For example, optically active 2-hydroxy-3-methylbutyrate is an important chiral synthon in the preparation of a potent, selective, and cell-penetrable inhibitor of caspase 3.[24] (*R*)-2-Hydroxy-3,3-dimethylbutyrate is a key component P3 of thrombin inhibitor identified by Merck.[25] Chiral 2-chloro-1-phenylethanol is a key synthon for the preparation of a large group of anti-depressants and potential cocaine-abuse therapeutic agents.[26] Optically pure 1-(4′-flurophenyl)ethanol and 1-(4′-trifluromethylphenyl)ethanol have been used to synthesize m2 muscarinic antagonists and the modulators of the CCR-5 chemokine receptor for treating patients with HIV.[27−29] To further explore the potential application of CMCR in the synthesis of these pharmaceutucally important chiral alcohols, the reduction of ethyl 3-methyl-2-oxo-butanoate (**2a**), ethyl 3,3-dimethyl-2-oxo-butanoate (**2b**), α-chloroacetophenone (**3i**), 4′-fluoroacetophenone (**3b**), 4′-trifluoromethylacetophenone (**3h**) ,and aliphatic ketones (**4b**, **4d**, and **4e**) was carried out in a preparative scale. The isolated yields, enantiomeric excess, and optical rotation of the product alcohols are summarized in Table 4. From Table 4 it can be seen that these chiral alcohols were obtained in essentially optically pure form in excellent yields. This demonstrated the

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usefulness of the carbonyl reductase from *Candida magnoliae* as an enzyme catalyst in the synthesis of chiral alcohol intermediates.

In conclusion, the carbonyl reductase from *Candida magnoliae* (CMCR) was an efficient enzyme catalyst for the enantioselective reduction of a diversity of ketones including aliphatic, aromatic ketones, α- and β-ketoesters. More importantly, this carbonyl reductase showed anti-Prelog enantioselectivity, which was complementary to most of the known ketoreductases, and would find more applications in the synthesis of chiral alcohol intermediates of pharmaceutical and agricultural interest.

Experimental Section

Gene Expression and Purification of the Carbonyl Reductase from *Candida magnoliae* (CMCR). The carbonyl reductase gene from *Candida magnoliae* (Genbank Accession No. AB036927) was cloned by gene assembly techniques.[30] Ten oligonucleotides ranging from 100 to 120 nucleotides were designed on the basis of the nucleotide sequence of the *C. magnoliae* carbonyl reductase gene,[15] the open reading frame of which is composed of 852 nuclotides (284 amino acid residues). The *Nco* I and *Bam* HI sites were franked to the open reading frame for easy cloning into expression vector pET15b (Novagen). The plasmid DNA containing CMCR gene was transformed into the *E. coli* Rosetta2(DE3)pLysS strain. Overnight culture was diluted into fresh LB medium containing ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL) and incubated at 37 °C until the optical density reached 0.6 at 595 nm. The expression was induced by addition of IPTG to 0.5 mM and the culture was incubated at 30 °C for another 6 h. Cells were harvested by centrifugation at 4100 rpm at 4 °C for 30 min. The cell pellet was resuspended in 100 mM potassium phosphate buffer (pH 6.5) and the cells were disrupted by EmulsiFlex-C5 Homogenizer. The cell-free extract was mixed with an equal volume of PEI solution (0.25% polyethyleneimine MW 40−60 K, 6% NaCl, 100 mM Borax, pH 7.4) to remove lipids.[31] The supernatant was precipitated with 25% ammonium sulfate and the precipitate was discarded. The remaining supernatant was precipitated with 55% ammonium sulfate. The resulting precipitate was collected after centrifugation and dissolved in potassium phosphate buffer (10 mM, pH 7.0, 2 mM 2-mercaptoethanol). The lysate was desalted by gel filtration into potassium phosphate buffer (10 mM, pH 7.0, 2 mM 2-mercaptoethanol) and lyophilized to afford the CMCR enzyme as white powder with a protein content of 86% measured with Bradford assay. The expression vector pET15b without the CMCR gene was also expressed in Rosetta2(DE3)pLysS. The cell-free extract was purified by the same procedure and used as a control in the activity assay.

Activity Assay of the Carbonyl Reductase from *Candida magnoliae* (CMCR). The activity of the carbonyl reductase from *Candida magnoliae* toward the reduction of ketones in Chart 1 was determined by spectrophotometrically measuring the oxidation of NADPH at 340 nm ($\epsilon = 6.22\ mM^{-1}\ cm^{-1}$) in the presence of an excess amount of ketones. The activity was measured at room temperature in a 96-well plate, in which each well contained ketone (6.25 mM) and NADPH (0.25 mM) in potassium phosphate buffer (100 mM, pH 6.5, 180 μL). The reaction was initiated by the addition of the carbonyl reductase (20 μL solution containing 2−40 μg of enzyme). The specific activity was defined as the number of nanomoles of NADPH converted in 1 min by 1 mg of enzyme ($nmol \cdot min^{-1} \cdot mg^{-1}$).

Enantioselectivity of the Reduction of Ketones Catalyzed by the Carbonyl Reductase from *Candida magnoliae* (CMCR). The enantioselectivity of the enzymatic reduction of ketones was studied with an NADPH recycle system.[19] The general procedure was as follows: D-glucose (4 mg), D-glucose dehydrogenase (0.5 mg), NADPH (0.5 mg), the carbonyl reductase (CMCR, 0.5 mg), and the ketone solution in DMSO (50 μL, 0.25 M) were mixed in a potassium phosphate buffer (1 mL, 100 mM, pH 6.5) and the mixture was shaken overnight at room temperature. The mixture was extracted with methyl *tert*-butyl ether (1 mL). The organic extract was dried over anhydrous sodium sulfate and subjected to chiral GC analysis to determine the enantiomeric excess.[21] The absolute configuration of product alcohols was identified by comparing the chiral GC data with the standard samples, or by comparing the specific rotation of the product alcohols with the literature data.

Preparative Procedures. The preparative synthesis was carried out as follows: D-Glucose (1.0 g), d-glucose dehydrogenase (10 mg), NADPH (10 mg), and ketoreductase (10 mg) were mixed in a potassium phosphate buffer (50 mL, 100 mM, pH 6.5). To the mixture was added a ketone solution (500 mg in 2.0 mL of DMSO). The mixture was stirred at room temperature and the pH was controlled at 6.5−6.6 with addition of 0.5 M NaOH solution until conversion was complete (usually overnight). The mixture was extracted with ether or methyl *tert*-butyl ether. The organic extract was dried over anhydrous sodium sulfate and removal of the solvent gave product alcohol, which was identified by comparison of ^{1}H and ^{13}C NMR with literature data.[12,25,32,33] The absolute configurations of the product alcohols were determined by comparison of the specific rotation data with those in the literature.[12,32,34,35]

Supporting Information Available: General methods and chrial GC chromatograms of all product alcohols and their racemates. This material is available free of charge via the Internet at http://pubs.acs.org.

JO0603328

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