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Synthetic Applications Of The Carbonyl Reductases Isolated From *Candida parapsilosis* And *Rhodococcus erythropolis*

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Abstract: Synthetic applications of the novel carbonyl reductases isolated from *Candida parapsilosis* and *Rhodococcus erythropolis* are reported. A number of different carbonyl compounds such as 3-, 4- and 5-ketoesters, ethyl 4-chloro 3-oxobutanoate, pyruvic aldehyde dimethyl acetal, ethyl α-methyl 3-oxobutanoate and acetophenone are reduced on the preparative scale to the corresponding (S)-hydroxy compounds with high enantiomeric excess (» 94%) and yield (» 83%). Product concentrations in the range of 30 to 200 mM were obtained. The synthesized chiral compounds are valuable building blocks for the synthesis of pharmaceuticals, agrochemicals and natural products.

The attention of organic synthesis has been focused on homochiral compounds since it became clear that biological systems can distinguish between enantiomers. One enantiomer of a chiral compound may be biologically active while the other enantiomer is inactive at best, but could also exhibit undesirable activities. Thus, the need to produce optically pure compounds from chiral intermediates is obvious. Besides other methods, enzyme-catalyzed reactions provide an attractive route for the production of homochiral intermediates[1]. Oxidoreductases offer great potential for the stereoselective reduction of prochiral compounds as demonstrated in several publications[1,2,3,4,5,6,7,8].

The practical use of the two commercially available NADH dependent alcohol dehydrogenases from yeast and horse liver is limited, however, due to temperature sensitivity, loss of activity upon immobilization and sensitivity to organic solvents[2,6]. Besides these well known enzymes other NADPH dependent alcohol dehydrogenases, carbonyl reductases, ketopantoyl reductases and aldehyde reductases have been isolated from other sources and in some cases applied in the synthesis of chiral intermediates[4,5,6,9,10,11]. NADH is preferred as a coenzyme because of lower cost and well established regeneration schemes[6].

Recently, we have isolated and characterized two novel NADH-dependent carbonyl reductases from *Candida parapsilosis* (*CP*CR) and *Rhodococcus erythropolis* (*RE*CR), respectively[12,13]. These enzymes exhibit an extremely broad substrate range including aliphatic, cyclic and aromatic ketones, keto esters of different substitution patterns, ketoacetals, diketones and aldehydes. We present here some applications of these novel carbonyl reductases.

A number of representative carbonyl compounds have been reduced on a preparative scale employing two different systems for the regeneration of the coenzyme. In Figure 1 the reaction schemes are shown.

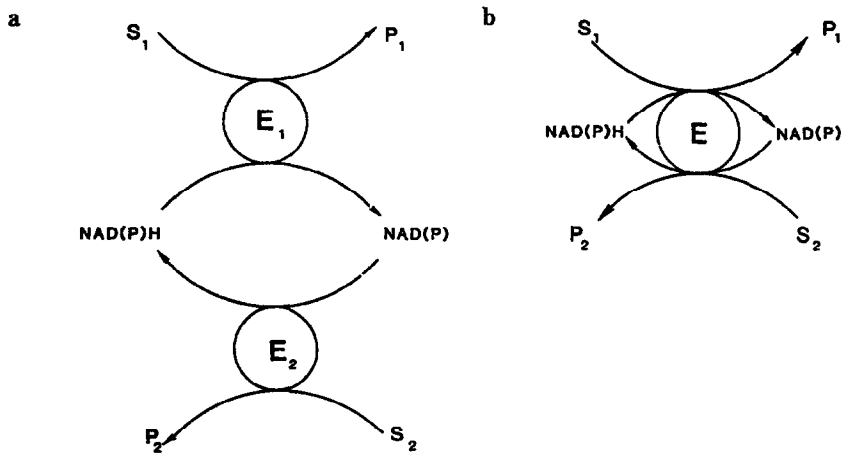
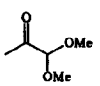
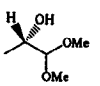
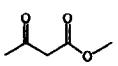
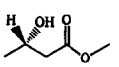
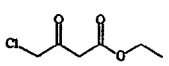
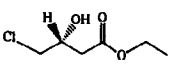
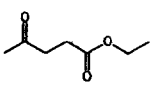
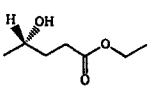
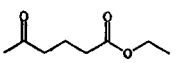
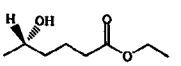
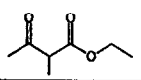
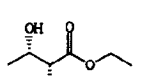


Figure 1 Approaches for the in situ regeneration of coenzymes (a) enzyme-coupled system (b) substrate-coupled system. S: substrate, P: product, E: enzyme

In the enzyme-coupled system for coenzyme-regeneration the formate dehydrogenase from *Candida boidinii* was used[14]. In the substrate-coupled system the carbonyl reduction and NADH-regeneration was achieved by using only one enzyme (*CP*CR) and isopropanol as hydride donor. As shown in Table 1 and Table 2 the conversion rates of the enzyme-coupled system are higher compared to the conversion rates in the substrate-coupled system. One important advantage of the formate dehydrogenase system is that the equilibrium is far on the carbon dioxide/NADH side, pushing the equilibrium of the coupled system towards the product side if an excess of formate is employed in the reaction mixture. In contrast, the equilibrium of the substrate-coupled system reached a steady-state after 20 hours with a conversion of 61%.

Table 1 Products prepared from *CP*CR and *RE*CR catalyzed reductions with enzyme-coupled coenzyme regeneration (10 mmol scale)

Substrate	Product	ee or de (%)	Conversion (%)	enzyme
		>99[a]	100[b]	*CP*CR
		99/>99[c]	95/90[b]	*CP*CR/*RE*CR
		>99[c]	100[e]	*RE*CR
		>99[c]	95[b]	*CP*CR
		>99[c]	96[b]	*CP*CR
		98/95[d]	83/49[b]	*CP*CR/*RE*CR

[a] Enantiomeric excess was determined by enzymatic analysis (see experimental section). [b] Yield was determined by HPLC or GC analysis. [c] Enantiomeric excess and absolute configuration was determined by gas chromatography on a chiral cyclodextrin phase (Lipodex E) by comparison to authentic enantiomers or racemic compounds. The (*D*)-enantiomers eluted before the (*L*)-enantiomers. [d] Diastereomeric excess was determined by gas chromatography on Lipodex E and by ^{1}H-NMR analysis. [e] Yield was determined by TLC.

Keto esters of different carbon chain length and position of the carbonyl group are reduced in excellent enantiomeric excess and high yield, to the corresponding (*S*) configured hydroxy compounds. Even for pyruvic aldehyde dimethyl acetal the enantiomeric excess is very high demonstrating that the *CP*CR is able to distinguish between methyl and acetal side chains efficiently. The secondary alcohol dehydrogenase from *Thermoanaerobium brockii*[5] and the PED alcohol dehydrogenase recently isolated from *Pseudomonas* sp.[4] show low enantioselectivities for 2- and 3-ketones with carbon chains between C-4 and C-8. Thus, a major advantage of the carbonyl reductase from *C. parapsilosis* is high enantioselectivity for carbonyl compounds with closely related side chains. The product of the reduction of pyruvic aldehyde dimethyl acetal, L-lactaldehyde dimethyl acetal, has been used already as chiral starting material for the synthesis of 6-deoxy-L-sorbose and 2O-6-deoxy-α-L-sorbofuranosyl-D-glucose[15].

Enzymes reducing β-keto esters on the expense of NADPH have been isolated from baker's yeast[16]. These enzymes have rather low activities with 4- and 5-oxoesters[17]. The *CP*CR exhibits very good activities with a broad range of keto esters including 3-, 4- and 5-oxoesters with different substitution[13]. To our knowledge this is the first report on the enantioselective enzymatic reduction of 4- and 5-oxoesters on the preparative scale. Keto esters were reduced within 24 to 96 hours in high yield and enantiomeric excess of greater than 99%. The remaining activity of the carbonyl reductases depends on the substrate/product and was recovered by ultrafiltration (95% yield). Thus, the carbonyl reductase and the formate dehydrogenase may be used repeatedly. Only ethyl 4-chloro 3-oxobutanoate and pyruvic aldehyde dimethyl acetal influenced the activity of the formate dehydrogenase. Ethyl 4-chloro 3-oxobutanoate slowly desactivated the FDH irreversibly presumably by alkylation of an essential cysteine side chain. Thus, the FDH had to be added at times during the reaction. Pyruvic aldehyde dimethyl acetal was found to be a competitive inhibitor of the FDH. Thus despite the good solubility of the educt the concentration of pyruvic aldehyde dimethyl acetal was limited to 200 mM. Under these conditions the remaining activity of the FDH was about 85% and increased during the course of the reaction.

The chiral hydroxy acid esters synthesized are valuable building blocks for the synthesis of natural products containing chiral carbinol centers. For example, 3-(*S*)-hydroxybutyric acid esters have been used in synthesis of several pheromones, antiobiotics and macrolides[18].

Ethyl 4-chloro 3-(*R*)-hydroxybutanoate is a starting material for the synthesis of L-carnitine[19].

There have been several attempts to reduce α-alkylated keto esters diastereoselectively. The rapid racemization at C_2 of these compounds in water is a prerequisite to obtain only one of four possible diastereomers by enzymatic reduction of the carbonyl group[18]. In Figure 2 the reactions are compiled.

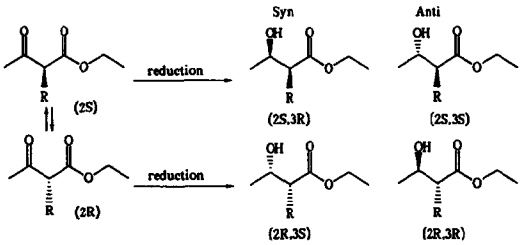


Figure 2 Reactions leading to four possible diastereomers of 2-alkylated β-hydroxyacid esters

To our knowledge this is the first report on the diastereoselective reduction of α-alkylated β-keto esters using NADH-dependent enzymes. Both carbonyl reductases may be used for the diastereoselective reduction of 2-alkylated keto esters. As shown in Table 1 very high diastereoselectivities of 98% were obtained. Thus, two chiral centers may be formed simultaneously by the action of the NADH-dependent carbonyl reductases from *C. parapsilosis* or *R. erythropolis*.

Table 2 shows the results employing the substrate-coupled enzyme systems. The *CP*CR is stable in up to 10% (v/v) isopropanol which can be used as a hydride donor for the regeneration of NADH. Besides the function as hydride donor the presence of isopropanol in the reaction mixture improves the solubility of hydrophobic compounds such as acetophenone or phenylethanol. With ethanol/acetaldehyde the regeneration was poor compared to the isopropanol/acetone system and the conversion was only 21%. The steady-states of the substrate-coupled systems were very stable. No decrease in conversion rate and in the enantiomeric excess was observed over a period of 100 hours. The stability of the *CP*CR during the reaction time was confirmed by assaying for enzyme activity.

Table 2 Products prepared from *Cp*CR catalyzed reductions with substrate-coupled coenzyme regeneration (5 mmol scale)

Substrate	Product	Enantiomeric Excess (%)	Conversion (%)	Remaining Activity (%)	Reaction Time (h)
isopropanol-system **:**					
		94[a]	61[b]	100	24
ethanol-system **:**					
		94[a]	21[b]	100	100

[a] Enantiomeric excess and absolute configuration was determined by gas chromatography on a chiral cyclodextrin phase (Lipodex E) by comparison to authentic enantiomers or racemic compounds. The (*R*)-enantiomers eluted before the (*S*)-enantiomers. [b] Yield was determined by HPLC analysis.

As demonstrated in this paper, the two recently isolated oxidoreductases from *C. parapsilosis* and *R. erythropolis* are very versatile biocatalysts for the enantio- and diastereoselective reduction of carbonyl compounds on the preparative scale. Both, enzyme- and substrate-coupled systems can be employed for the regeneration of NADH.

Synthetic applications of optically active γ- and δ-hydroxyacid esters are few due to their limited availability. Other chiral compounds with 1,4- and 1,5-functionalities have already been used in the synthesis of pheromones, juvenilhormone, inhibitors of the steroid biosynthetic pathway, sesquiterpene antibiotics and natural products[18]. Consequently, there are interesting applications of chiral γ- and δ-hydroxyacid esters once they become readily available.

EXPERIMENTAL

NMR spectra were obtained on a Varian VXR 300 or a Bruker AM-200 spectrometer in $CDCl_3$. Shifts are reported as ppm relative to TMS as an internal reference. Abbreviations: m = multiplet, d = doublet, d x q = doublet of a quartet, t = triplet. $[\alpha]_D$ was measured on a Perkin-Elmer model 241 MC polarimeter using a 10-cm path-length cell.

Enzyme assays. The assay mixture contained in a total volume of 1 ml 35 mM carbonyl compound, 0.2 mM NADH in 100 mM triethanolamine-NaOH buffer pH 7.5 (CPCR) or glycyl-glycine-piperazine-NaOH buffer pH 5.5 (RECR) and limiting amounts of enzyme. The consumption of the reduced coenzyme was followed in a Shimadzu UV-160 spectrophotometer (Düsseldorf, FRG) at 334 nm and 30°C using a molar absorption coefficient of 6180 $M^{-1}cm^{-1}$ for calcuation. One unit (U) of carbonyl reductase is defined as the amount of enzyme which catalyzes the reduction of 1 μmol of NADH per min under the conditions specified.

Batch conversion. The enzymatic transformations were carried out at room temperature with stirring (120 rpm) in a total volume of 100 ml. The following conditions were used for the substrate-coupled system: 50 mM acetophenone, 650 mM 2-propanol (5% v/v), 10 U of *CP*CR, 0.5 mM NAD^+, 100 mM triethanolamine (TEA)-NaOH buffer, pH 7.5. The enzyme-coupled systems consisted of the following compounds: 1 M sodium formate, 0.1 mM NAD^+, 100 mM carbonyl compound (200 mM in the case of pyruvic aldehyde dimethyl acetal), 10 U carbonyl reductase, 40 U FDH and 100 mM TEA-NaOH buffer, pH 7.5. At times samples were removed, products isolated by extraction with acetonitrile or chloroform and analyzed by high performance liquid chromatography or chiral gas chromatography.

Racemates of various hydroxy compounds. 12 mmol $NaBH_4$ was dissolved in 50 ml ethanol with cooling in an ice bath. Subsequently, 30 mmol carbonyl compound were added dropwise and the solution was stirred over night at room temperature. The reaction was stopped by adding 75 ml distilled water and 3 ml HCl (6 N). The product was extracted with diethyl ether, dried over $MgSO_4$ and the solvent was removed under reduced pressure. The resulting oil was further purified by flash chromatography on silica gel 60 (50 ml gel volume) using diethyl ether and $benzene_{60\text{-}80}$ (2:1 v/v) as solvent. Fractions containing hydroxy compound were pooled and the solvent was evaporated under reduced pressure. The retention times and R_f values are given below.

Sample preparation. After separation of the organic phase (chloroform) the sample was derivatized with trifluoroacetic anhydride according to König and coworkers[20] and an ali-

quot (1 µl) applied directly for gas chromatographic analysis.

Identification of product by gas chromatography. Samples (1 µl) were applied on a chiral Lipodex-E γ-cyclodextrin column (25 m x 0.25 mm ID, Macherey and Nagel, Düren, FRG). The temperature of the injector and detector of the chromatograph (GC 9A, Shimadzu, Düsseldorf, FRG) was set at 260 °C, detection was carried out with a flame ionization detector (FID). Helium was used as carrier gas. Separation was performed isothermically at the temperatures indicated in parenthesis and retention times of the enantiomers are as follows: (*R*)-phenylethanol: 20 min, (*S*)-phenylethanol: 21 min (60°C); methyl 3-(*R*)-hydroxybutanoate, 7.9 min, methyl 3-(*S*)-hydroxybutanoate, 9.9 min (70°C); ethyl 4-(*R*)-hydroxypentanoate, 8.5 min, ethyl 4-(*S*)-hydroxypentanoate, 9.4 min (85°C); ethyl 5-(*R*)-hydroxyhexanoate, 16.6 min; ethyl 5-(*S*)-hydroxyhexanoate, 17.5 min (85°C); ethyl 4-chloro 3-(*R*)-hydroxybutanoate, 48.9 min; ethyl 4-chloro 3-(*S*)-hydroxybutanoate, 50.7 min (80°C); Ethyl 2-methyl-3-hydroxybutanoate (4 isomers), 13.1 min, 13.5 min, 13.9 min, 15.6 min (60°C).

High performance liquid chromatography. Aliquots of the reaction mixture were extracted by the addition of an equal volume of acetonitrile and 1 g ammonium sulfate. After separation of the organic phase, 400 µl of the upper phase were mixed with 600 µl of NaH_2PO_4-H_3PO_4 buffer, 25 mM, pH 2.1. Samples (20 µl) were analyzed by HPLC on a RP-18 column (250 × 0.4 mm, 5mm), acetonitrile/NaH_2PO_4, 25 mM, pH 2.1 (H_3PO_4), 40:60 (v/v), with a flow rate of 0.8 ml/min. The retention times were as follows : phenylethanol, 6.7 min; acetophenone, 10.1 min; methyl 3-oxobutanoate, 4.5 min; methyl 3-hydroxybutanoate, 3.9 min; ethyl 4-oxopentanoate, 6.5 min; ethyl 4-hydroxypentanoate, 4.9 min; ethyl 5-oxohexanoate, 7.9 min; ethyl 5-hydroxyhexanoate, 6.3 min. Pyruvic aldehyde dimethyl acetal and L-lactaldehyde were determined by HPLC analysis on a RP-4 column, acetonitrile/water 90:10, with a flow rate of 0.4 ml/min. The retention times were 7.74 min for pyruvic aldehyde dimethyl acetal and 7.12 min for L-lactaldehyde.

Enzymatic determination of the optical purity of lactaldehyde. The optical purity of lactaldehyde dimethyl acetal was determined after hydrolyzation of a sample with Dowex 50

(H^+ form) to lactaldehyde and aliquotes of the solution were oxidized to lactic acid with aldehyde dehydrogenase as catalyst and NAD^+ as cofactor. Subsequently, the concentrations of D- and L-lactic acid were determined enzymatically with D- and L-lactic acid dehydrogenase according to Bergmeyer (1983)[21]. Instead of glutamate-pyruvate transaminase the pyruvate decarboxylase from *Zymomonas mobilis* was used in order to draw the equilibrium. Pyruvate decarboxylase was kindly provided by Dipl. Chem. H. Bruhn (institute of enzyme technology, Jülich). Authentic enantiomers of D- and L-lactic acid were used as standard material. Determination of the enantiomeric excess of lactaldehyde indicated that the lactaldehyde obtained had the L-configuration with ≥ 99% ee.

Thin layer chromatography (TLC). Substrate and product could be separated due to their different solubility in the mobile phase. Samples (up to 5 µl) were applied on a silica plate 60 F_{254} (Merck, Darmstadt, FRG) and developed with diethyl ether/benzene$_{60-80}$ (2:1). After drying in a warm air current the plates were dipped in 10% ethanolic solution of molybdatophosphoric acid for two seconds. Green spots were visualized by heating the plates in a hot air current. Carbonyl compounds have higher R_f-values than the corresponding hydroxy compounds: methyl 3-oxobutanoat, 0.64; methyl 3-hydroxybutanoate, 0.33; ethyl 4-chloro 3-oxobutanoate, 0.69; ethyl 4-chloro 3-hydroxybutanoate, 0.46; ethyl 2-methyl 3-oxobutanoate, 0.74; ethyl 2-methyl 3-hydroxybutanoate, 0.53; ethyl 4-oxopentanoate, 0.57; ethyl 4-hydroxypentanoate, 0.37; ethyl 5-oxohexanoate, 0.57; ethyl 5-hydroxyhexanoate, 0.38; acetophenone, 0.79; phenylethanol, 0.55; pyruvic aldehyde dimethyl acetal, 0.63; lactaldehyde dimethyl acetal, 0.25.

^{1}H-NMR-data and specific rotations. Ethyl 4-chloro 3-(*R*)-hydroxybutanoate (200 MHz): δ 3.59 (dd, 2H-C_4), 1.28 (t, $OCH_2\underline{CH_3}$), 2.61 (dd, 2H-C_2), 3.21 (br s, OH), 4.16 (q, $O\underline{CH_2}CH_3$), 4.17-4.28 (m, H-C_3) ppm. The ^{1}H-NMR data are consistent with data from the literature[4]. $[\alpha]_D^{22}$: +31.8 (c = 0.5, $CHCl_3$).

Ethyl 2-(R)-methyl-3-(S)-hydroxybutanoate (300 MHz): δ 4.17 (q, $O\underline{CH_2}CH_3$), 4.09-4.03 (m, H-C_3), 2.87 (br d, OH), 2.54-2.45 (d x q, H-C_2, $J_{2,3}$ = 4.2 Hz), 1.28 (t, $OCH_2\underline{CH_3}$), 1.19 (d, 3H-$C_{1'}$, $J_{1',2}$ = 7.2 Hz), 1.18 (d, 3H-C_4, $J_{3,4}$ = 6.4 Hz) ppm. The ^{1}H-NMR data are consistent with data from the literature[22,23]. $[\alpha]_D^{22}$: +7.0 (c = 1, $CHCl_3$).

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