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Ketoreductases: stereoselective catalysts for the facile synthesis of chiral alcohols

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Abstract—The results of a reduction of a wide range of ketones using 31 commercially available isolated ketoreductases (KREDs) are presented. All enzymes accepted a wide substrate range. The stereoselectivity of each enzyme was measured for the reduction of benzoyl-hydroxyacetone and ethyl-3-oxobutanoate, and in each case, enzymes which produce enantiomerically pure (*R*)- and (*S*)-alcohols were found. The preparative scale reactions were investigated using two ketones with different hydrophobicities (benzoyl-hydroxyacetone and α-tetralone) and using enzymes with varying specific activities for their reduction. Regardless of the hydrophobicity of the substrate, high titers of ketone (0.75–1.4 M) were reduced in high yield using catalytic amounts of enzyme (1–7% g/g relative to substrate) and cofactor (0.1–0.2% equiv. relative to ketone) within 4–24 h. The cofactor was efficiently regenerated in situ via the oxidation of glucose by glucose dehydrogenase, an enzyme that has also been cloned and over-expressed. These results show that isolated ketoreductases can be quickly and easily screened against target ketones, and the reactions can be scaled to produce preparative amounts of chiral alcohols. Ketoreductase enzymes should become a standard addition to the organic chemists toolbox of asymmetric catalysts for stereoselective ketone reduction.

1. Introduction

Stereoselective reduction of ketones to form chiral alcohols is one of the most useful reactions in organic synthesis.[1,2] Chiral alcohols are present in a variety of pharmaceutical and other high value compounds and they are frequently used as intermediates for multi-step chiral syntheses. It is therefore not surprising that significant effort has been devoted to the development of chemical methods for the reduction of ketones to produce alcohols of high enantiomeric purity.[2] Ketoreductases (KREDs) offer important advantages as catalysts in ketone reduction processes. Recent enzyme discovery efforts have expanded dramatically upon the number of KREDs available for use[3,4] and cost-effective methods for recycling of the nicotinamide cofactors have been developed.[4] Reactions using ketoreductases typically require no special conditions (20–45 °C, atmospheric pressure), and waste disposal problems are minimal since enzymes are completely biodegradable and reactions can be performed in aqueous media. High yields and optical purities (>99%) are frequently achieved, and KREDs that can catalyze the synthesis of either the (*R*)- or (*S*)-alcohol from a starting ketone are usually available.[3–5] The cost of KREDs also compares favorably to that of other chiral catalysts. KRED enzymes can be produced economically in large quantities scale using microbial fermentations. Enzymatic reductions of ketones are thus an important addition to the synthetic chemist's toolbox, complementing and expanding the established stereoselective chemical reductions.

Identification of the best enzyme for a desired application is the key step in developing a KRED-catalyzed route to a chiral alcohol. Although whole cell systems have been frequently reported,[4,6] screening of isolated enzymes is a more rapid and convenient approach for chemists. In contrast to whole cell systems, which require labor-intensive culturing of viable cells, KRED enzymes can be added directly to the reaction mixture using standard laboratory equipment. Rate and purity is another advantage: whole cells typically display low reaction rates and multiple competing ketoreductase activities, while isolated KRED enzymes can convert high titers of substrate quickly using catalytic amounts of enzyme. Importantly, no competing reactions by other ketoreductases occur since each isolated enzyme contains a single, specific ketoreductase activity.

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Herein, we report the results of the screening of 31 cloned ketoreductase enzymes[5] for the stereoselective reduction of a set of 10 structurally different ketones. The substrate range of these reductive enzymes, the stereoselectivity thus obtained and the potential scale-up of such enzymatic processes, are discussed using some selected examples.

2. Results and discussion

Until recently, very few ketoreductases were commercially available with the potential for scale-up to commercial production.[7] With the recent introduction of ketoreductases from a variety of sources as individual, isolated enzyme activities,[3–5] the rapid screening of libraries of these enzymes for reduction of a target ketone has become possible for the first time. A number of such enzymes have been cloned and characterized,[3,4] however, using easily accessible, commercially available ketoreductase screening sets offers a convenient and rapid method to screen a group of diverse enzymes against the reduction of any target ketone. In Table 1, we present an example of the range of substrates that can be reduced using such a commercial set consisting of 31 NADPH-dependent ketoreductases.[5] Every enzyme was found to act on multiple ketone substrates with activities ranging from 0.01 to 59 U/mg (Table 1). Twelve enzymes showed activity on almost every ketone assayed, while the other 19 enzymes were active on four or more ketones. High activities, in the range of 10–59 U/mg, were frequently observed, and more importantly, for every ketone there was at least one enzyme identified, which showed good to excellent catalytic activity. Even with an activity of 0.1 U/mg or less, complete product formation was achieved if the reaction was allowed to progress for 24–30 h. Details about the titers and yields of product that were achieved are discussed later on. The activity range of these enzymes as shown in Table 1 is especially impressive, given the structural diversity of the assayed substrates. The ketones are depicted in Figure 1 and include various substituted β-keto esters **1**–**4**, one α-keto ester **9**, one α-keto acid **8** one aromatic hydroxy ketone **5**, benzyl protected hydroxyacetone **10**, a bicyclic ketone **6**, and an aliphatic ketone **7**.

In addition to the substrates shown in Table 1, the same set of commercially available enzymes has recently been utilized by our laboratory and others for the reduction

Table 1. Specific activities (μmol/min × mg^{-1}) on assays performed with 10 substrates (Fig. 1) and 31 ketoreductases

KRED	Enzyme activities (U/mg)[a] for substrates **1**–**10**									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
101	***2.00***	**0.96**	**0.32**	***1.18***	***2.19***	***1.48***	**0.120**	0.014	***27.0***	***1.48***
102	***4.27***	***1.26***	0.083	***1.98***	**0.94**		0.076	***1.18***	**0.64**	***4.74***
103	**0.94**	0.056		0.084	**0.18**		0.074	**0.168**		**0.18**
104	0.022						0.013			
105	**0.148**	0.038		0.022	0.040					**0.204**
106	**0.384**	0.074		**0.178**	0.029		0.020	**0.105**	0.072	**0.112**
107	***18.0***	0.022	0.054	0.073	***13.8***		**0.137**	**0.134**	0.014	***1.03***
108	***9.60***	**0.54**	**0.29**	***2.17***	0.035		0.028	**0.834**	***2.61***	***30.12***
109	**0.582**	**0.12**			0.011			0.053	0.027	0.049
110	0.053	0.060			0.021			**0.127**	0.051	
111	**0.564**	***4.26***	**0.146**	0.074	0.092	**0.270**	0.020	0.046	***1.02***	0.075
112	***1.19***	**0.94**	**0.138**	**0.678**	**0.678**	**0.516**	**0.210**	**0.864**	***7.86***	**0.336**
113	***2.70***	***1.07***	0.064	**0.127**	**0.44**	**0.300**	0.024	0.068	***14.46***	**0.20**
114	**0.288**	***11.88***	***3.24***	**0.168**	**0.23**	**0.492**	**0.110**	0.023	***3.24***	**0.142**
115	**0.22**	***2.52***	0.088	0.084	0.072	**0.144**	0.008	**0.168**	**0.630**	0.026
116	***26.64***	***3.84***	**0.348**	***4.32***	**0.122**		**0.10**	***1.11***	***7.44***	***36.9***
117	***26.58***	**0.91**	**0.216**	***3.66***	**0.33**		0.061	***1.63***	***8.10***	***32.1***
118	***15.9***	***5.82***	***1.92***	***8.94***	**0.510**	0.034	**0.324**	0.088	***22.38***	***45.2***
119	***23.52***	***15.60***	***5.16***	***20.52***	**0.708**	0.084	**0.486**	**0.282**	***47.04***	***58.9***
120	***1.94***	**0.318**	0.089	**0.930**	**0.234**		0.030	**0.474**	**0.774**	***3.60***
121	**0.246**	***4.26***	**0.306**	0.058	0.052	**0.124**	0.040	0.046	0.060	0.060
123	**0.396**	0.085	0.063	**0.198**	0.055	**0.112**	0.020	0.042	***1.38***	**0.342**
124	**0.954**		0.049		0.056		0.021		**0.173**	***1.82***
125	0.068	0.028	0.021	0.060	0.016				0.018	0.055
126	**0.29**	0.046	0.046	0.022	0.024		0.046		0.053	***1.32***
127	***1.81***			0.08	0.024		0.081	0.036	**0.607**	***1.33***
128	**0.23**		0.097	**0.142**	0.038	0.013	**0.183**		**0.314**	0.045
129	0.085			**0.156**	0.038		0.058	0.045	***1.59***	
130	***1.11***	**0.100**	**0.60**	**0.157**	0.034		0.074	**0.146**	***1.04***	0.015
131	**0.318**	***1.20***	**0.122**	***1.15***	**0.15**				**0.936**	0.042
132	***7.20***	**0.652**	***2.09***	***3.10***	***2.88***	***2.20***	***5.09***			**0.94**

Activity was measured in spectrophotometric cuvette assays by the loss of absorbance at 340 nm resulting from the decrease of NADPH concentration in enzyme–substrate mixtures. Details in experimental.

Bold: Activities between 0.1 and 1 U/mg; bold italics: activities > 1 U/mg.

[a] Unit definition: 1 U/mg is the conversion of 1 μmol ketone per minute per mg of enzyme.

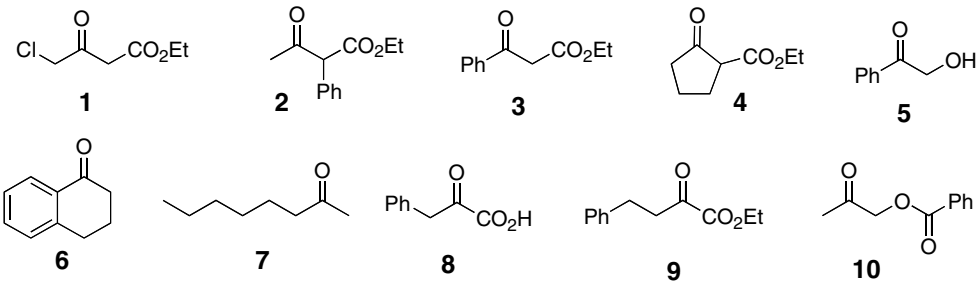


Figure 1. Substrates shown in Table 1.

of a variety of other ketones with high efficiency and stereoselectivity. Substrates that were utilized for reduction included α-alkyl substituted 1,3-diketones,[8] α- and β-alkyl substituted β-keto ketoesters,[9] a wide range of substituted acetophenones[10] and substituted diethyl 2-alkyl ketoglutarates.[11] The latter compounds have been used in the synthesis of various amino alcohols and hydroxyl-amino acids.[11]

The substrates that are presented in Table 1 include several industrially useful ketones. For example (*S*)-ethyl-4-chloro 3-hydroxy butanoate (produced by the reduction of ketone **1**) is a key intermediate in the synthesis of the cholesterol lowering drugs Lipitor and Zocor, while (*R*)-ethyl-4-phenyl-2-hydroxy-butanoate (produced by the reduction of ketone **9**) was utilized in the synthesis of hypertension drugs Lisinopril and Enalaprilat. Aryl substituted diols (from ketone **5**) can be used for the synthesis of various pharmaceuticals.[12] Analogues of the alcohol derived from α-tetralone (ketone **6**) have been used in the synthesis of antagonists of serotonin 5-HT7 receptors for the treatment of schizophrenia[13a] and in the synthesis of inhibitors of β-secretase in the treatment of Alzheimers disease.[13b] All other ketones presented in Figure 1 and in the references[8–11] also represent ketone precursors for commercially useful chiral alcohols.

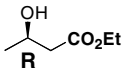
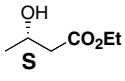
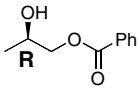
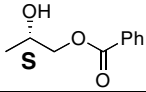
High stereoselectivity of reduction is another advantage of using isolated ketoreductases as catalysts for reduction.[2,3,8–11] This is clearly illustrated in Table 2, where the stereoselectivity of the thirty one ketoreductases towards the reduction of two structurally similar ketones is presented. The enzymes that gave more than 95% formation of one enantiomer are indicated in bold. The enantiomeric ratios were determined by chiral GC analysis of crude extracts from 5 mL overnight reactions containing 20 mM substrate and 2–5 mg of each enzyme (details in experimental). Two observations can be made immediately by looking at this table. First, about half of the enzymes gave reductions with more than 95% enantioselectivity for both substrates. Secondly, both enantiomers of each chiral alcohol can be synthesized in excellent enantiomeric excess once the appropriate enzyme is identified. Although in most cases, the absolute stereochemistry of the alcohol formed by an enzyme was the same for both substrates, there are two cases, namely KRED104 and KRED123, where enzymes gave the opposite enantiomer as the major product in each substrate. In a few cases, the stereoselectivity was marginally different between the two substrates as shown by the product ratios of KRED110, KRED113 and KRED125. For example, KRED110 gave close to a racemic mixture upon reduction of benzyl-hydroxyacetone, while 97% of the (*S*)-enantiomer was produced from the reduction of ethyl-3-oxobutyrate. Such an unpredictable preference of reduction using the same KRED screening set has previously been observed in the reduction of other classes of compounds.[8–11]

These results indicate that the activity and stereoselectivity of the reduction of an unknown ketone often correlates with the activity and stereoselectivity toward other similar substrates, but predictions cannot be made with absolute certainty. It is, therefore, advisable to screen the set of enzymes for activity and stereoselectivity for each new ketone to ensure that the best enzyme is not missed; and this can be very easily done using the methods described herein. It should be noted that in some cases even when no activity was detected in spectrophotometric assays (Table 1), product was observed after overnight incubation with the enzyme.

In Table 3, preparative scale (5–25 g) reactions of two substrates with varying hydrophobicities using enzymes with varying activities towards their reductions are presented. Although the reactions shown in this table have not been optimized, the results presented here clearly show that high titers and yields of product can be achieved, even when a hydrophobic substrate or an enzyme with relatively low specific activity for reduction is utilized.

The amount of enzyme that was necessary to complete every reduction depended on its specific activity for the substrate, its stability under the reaction conditions over time, and the concentration of the dissolved substrate in the reaction media. The effect of the specific activity and enzymatic stability on the outcome of the enzymatic reaction is clearly shown by the reduction of benzoyl-hydroxyacetone using KRED107 and KRED117 (Table 3, entries 2 and 3). The much higher specific activity of KRED117 towards benzoyl-hydroxyacetone (Table 3, entry 3) resulted in the need for significantly less enzyme to complete the reaction in the same time compared to KRED107 (Table 3, entry 2). Multiple additions of KRED117 during the course of the reaction were more effective in carrying the reaction to completion compared to a single addition. On the other hand, using a highly stable enzyme such as KRED107, a single addition of

Table 2. Stereoselectivity[a] of reduction using the ketoreductases of Table 1

KRED			U/mg			U/mg
101	23%	77%	*0.49*	33%	67%	*1.48*
102	<1%	>**99%**	*1.13*	1%	**99%**	*4.74*
103	<1%	>**99%**	*0.242*	<1%	>**99%**	*0.18*
104	7%	93%	ND	76%	24%	*ND*
105	8%	92%	*ND*	1%	**99%**	*0.204*
106	<2%	>**98%**	*0.142*	2%	**98%**	*0.112*
107	>**99%**	<1%	*2.00*	>**99%**	<1%	*1.03*
108	<1%	>**99%**	*3.18*	2%	**98%**	*30.12*
109	12%	88%	*0.012*	16%	84%	*0.049*
110	3%	**97%**	*0.020*	45%	55%	*ND*
111	33%	67%	*0.360*	29%	71%	*0.075*
112	11%	89%	*0.822*	42%	58%	*0.336*
113	<1%	>**99%**	*0.312*	31%	69%	*0.20*
114	32%	68%	*0.058*	39%	61%	*0.142*
115	33%	67%	*0.016*	29%	71%	*0.026*
116	<1%	>**99%**	*6.12*	<1%	>**99%**	*36.9*
117	<1%	>**99%**	*5.34*	<1%	>**99%**	*32.1*
118	<1%	>**99%**	*13.14*	9%	91%	*45.2*
119	<1%	>**99%**	*23.4*	<1%	>**99%**	*58.9*
120	<1%	>**99%**	*1.20*	<1%	>**99%**	*3.60*
121	65%	35%	*0.033*	65%	35%	*0.060*
123	62%	38%	*0.030*	31%	69%	*0.342*
124	<1%	>**99%**	*0.050*	1%	**99%**	*1.82*
125	40%	60%	*ND*	4%	**96%**	*0.055*
126	<1%	>**99%**	*0.649*	<1%	>**99%**	*1.32*
127	<1%	>**99%**	*0.710*	4%	**96%**	*1.33*
128	<1%	>**99%**	*0.404*	4%	**96%**	*0.045*
129	72%	28%	*0.014*	68%	32%	*ND*
130	14%	86%	*0.084*	22%	78%	*0.015*
131	75%	25%	*0.059*	64%	36%	*0.042*
132	>**99%**	ND	*4.44*	>**99%**	ND	*0.94*

[a] The enantiomeric ratio was measured by chiral GC analysis of crude organic extracts from 5 mL reactions containing 20 mM ketone and 2–5 mg of each enzyme (details in experimental).

Table 3. Preparative-scale (5–25 g) synthesis of selected substrates and ketoreductases with varying activities

Entry	Substrate, Figure 1	KRED	U/mg	Substrate concentration (M)	Substrate titer (g/L)	Enzyme concentration (g/L)	DMSO % (v/v)	NADPH concentration (mM)	Rxn time (h)	Isolated yield
1	**10**	111	0.075	0.140	25	1.0	3.5	0.20	7	75%[c]
2	**10**	107	1.03	0.140	25	0.5	3.5	1.25	4	91%[b]
3	**10**	117	32.1	0.140	25	0.25[a]	3.5	0.80	4	89%[b]
4	**10**	117	32.1	0.750	134	3.0[a]	5.0	1.5[e]	5	89%[b]
5	**10**	117	32.1	1.40	249	5.0[a]	5.0	2.0[e]	13	92%[b]
6	**6**	101	1.48	0.125	18	1.2	8.0	0.34	10	87%[d]
7	**6**	101	1.48	0.750	110	7.5	10.0	1.5[e]	24	86%[c,d]

[a] Because KRED117 was less stable under these reaction conditions it was added in batches (usually every 1–2 h) giving the combined final concentration shown in the table.

[b] >98% ee was observed in the product of these reactions in agreement with the small-scale reactions (20 mM, Table 2).

[c] The isolated product contained a small amount of starting ketone due to incomplete reaction. The reported yield only refers to the alcohol produced and isolated.

[d] The major enantiomer was *R* (92% ee).

[e] 1 mM NADPH was added at the beginning of each reaction, and at later time points NADP+ was added giving the final concentrations of cofactor shown in the table.

enzyme at the beginning of the reaction was sufficient to complete the reaction in 4 h. The ketoreductase concentrations shown in Table 3 represent the total amount of enzyme that was added during the course of the reaction. Even when the specific activity of a KRED towards a substrate was relatively low, as in the case of KRED111 towards benzyl-hydroxyacetone (Table 3, entry 1), high yields of conversion to product were achieved by increasing the enzyme concentration and the reaction time. Such an enzymatic process might even be economical if the enzyme is active over long reaction times. It can be produced on large scale by fermentation of recombinant bacteria, especially if the substrate that it is reducing is a high value intermediate.

High titers of substrate can be effectively reduced using catalytic amounts of enzyme and cofactor even when ketones with low aqueous solubility are utilized as shown by the results in Table 3. The ketone does not need to be completely dissolved in the reaction mixture for the reaction to reach completion. Both reactions with α-tetralone (Table 3, entries 6 and 7) and the high titer reactions with benzoyl-hydroxyacetone (Table 3, entries 4 and 5) gave heterogeneous mixtures after the addition of each substrate to the enzyme aqueous medium. However, a small increase of the co-solvent added and the KRED concentration were necessary to complete these high substrate titer reactions in a timely fashion. In the case of benzoyl-hydroxyacetone, 1% (g/g relative to the substrate) of KRED117 was enough to complete the reaction containing 0.14 M of substrate in 4 h (Table 3, entry 3). A small increase of the enzyme loading to 2% (g/g relative to the substrate) was only required to complete the same reduction when the substrate concentration increased 5- or 10-fold (Table 3, entries 4 and 5, respectively) albeit a longer reaction time was required for the reaction containing 1.4 M of substrate (Table 3, entry 5). In the case of the more hydrophobic α-tetralone, the same relative concentration of KRED101 (6.7% g/g relative to ketone) completed both the low and high titer reactions (Table 3, entries 6 and 7) requiring, as before, longer incubation time in the high titer reaction. The co-solvent concentration, which is required in every case has to be carefully examined since it can affect the stability of some KREDs. A concentration of 10% (v/v) of DMSO is, in most cases, enough to partially dissolve any ketone in the aqueous enzymatic media and facilitate its reduction without significantly affecting the stability and catalytic activity of most KREDs. Higher concentrations of this (or other water miscible) co-solvent can be utilized; however, the stability of the desired KRED in each case must be evaluated.

In all the reactions presented in Table 3, NADPH was added in catalytic amounts and was efficiently recycled using D-glucose and glucose dehydrogenase (GDH), an enzyme that regenerates NADPH from NADP+ with the concomitant oxidation of D-glucose. In the high titer reductions (0.75 M–1.4 M, entries 4, 5 and 7 Table 3), the concentration of NADPH (added as NADP+ which was immediately converted to NADPH with the glucose DH/glucose system) was kept at 1.5–2.0 mM (or 0.14–0.2% equiv relative to substrate), which was enough to give quantitative yields using the NADPH regeneration system.

The ease of using KREDs for stereoselective ketone reduction is another important feature. The reactions described herein were performed using equipment available in any organic chemistry lab. The enzymes were added in the reaction media as lyophilized powders, and the substrates were dissolved in DMSO prior to their addition to the reaction media. Product isolation involved an aqueous organic extraction where all enzymes, cofactors and buffers remained in the aqueous layer and only the organic products were extracted. The product purity was very high and depended only on the purity of the starting material. If larger quantities of the product are required, the reaction methods described herein can be directly scaled-up using stirred tanks with mixing and pH and temperature control.

3. Conclusion

The substrate range and stereoselectivity of 31 cloned, overexpressed and partially purified ketoreductases were investigated in this report. With a few exceptions, most enzymes showed a broad substrate range (Table 1) reducing α- and β-ketoesters **1**–**4** and **9**,[9] an α-ketoacid **8**, hydroxy acetophenone **5** as well as various alkyl-substituted acetophenones **10** aliphatic ketone **7**, a benzyl-substituted hydroxy acetone **10** and a bicyclic ketone such as α-tetralone **6**. Using two structurally similar ketones, ethyl-3-oxobutyrate and benzoyl-hydroxyacetone, the stereoselectivity of reduction for every enzyme was identified. Both the (*R*)- and (*S*)-enantiomers were produced in high enantiomeric excess (>99%) by one or more enzymes.

The potential for its use in preparative-scale (5–25 g) reactions was also investigated for two substrates. We showed that large titers of product can be effectively reduced even when hydrophobic substrates are utilized, and that it is not necessary for a ketone substrate to be completely dissolved in order for its reduction to reach completion (Table 3). In the heterogeneous mixtures that were produced when high titers of ketone substrate were prepared (0.75–1.40 M), complete product formation was achieved by slightly increasing the amount of DMSO co-solvent and the reaction time when compared with the reductions on lower substrate concentrations (0.125–0.14 M). Depending on the reaction, enzymatic amounts of 1–7% (w/w relative to substrate) were enough to complete the reactions within 4–24 h. Product isolation was accomplished in a straightforward fashion by organic extraction of the aqueous reaction mixture. The required NADPH cofactor was added in catalytic amounts (0.1–0.2% equiv relative to substrate) and was efficiently recycled using glucose and glucose dehydrogenase (GDH). Considering the low cost of D-glucose and the fact that GDH has been cloned, over-expressed and can be produced in large quantities, the additional cost of the cofactor recycling to the overall process is low.

The results presented herein clearly show that isolated cofactor-dependent ketoreductases are important and versatile catalysts for the production of chiral alcohols. Besides the ketoreductases that were investigated herein, many more similar enzymes have been cloned and characterized;[3,4] this number will increase in the following years. For example, our group has recently cloned and characterized another set of more than 30 ketoreductases with wide substrate and stereoselectivity range. In this report, we sought to demonstrate the ease of using and handling ketoreductase enzymes as catalysts for organic synthesis and to show that any organic chemist with minimal training in their handling can benefit from their tremendous potential.

4. Experimental

4.1. General method for the spectrophotometric assays (Table 1 data)

The specific activities in units/mg (1 unit/mg is the conversion of 1 μmol substrate/mg enzyme × min^{-1}) were calculated by cuvette assays by measuring the rate of NADPH consumption from the decrease of the absorbance at 340 nm. In a typical assay, 940 μL of a potassium phosphate buffer (Kpi, 50 mM, pH 6.5) was mixed with 10 μL of an NADPH solution (giving an absorbance between 1.2 and 1.5 absorbance units), 30 μL DMSO containing every ketone substrate (0.5 M substrate concentration in DMSO) and 20 μL of every enzyme solution that was made by dissolving 10 mg of every enzyme lyophilized powder in 1 mL of potassium phosphate buffer (50 mM pH 7.0). In many assays, dilutions of 1/10 to 1/100 were required in order to obtain good absorbance slopes.

Essentially, the same assays can be (and were) performed in 96-well plates by mixing 150 μL of a reaction buffer (containing 50 mM Kpi, 3.5% v/v DMSO, 2–10 mM substrate and NADPH) with 5 μL of enzyme lysate. Simultaneous measurement of all the slopes can be measured using a UV-plate reader (SpectraMax Plus, Molecular Devices Inc). Using this method, 96 assays can be performed at the same time allowing for a rapid identification of active enzymes towards a substrate as well as the comparison and the simultaneous calculation of enzymatic activities.

4.2. Small-scale reductions of benzoyl-hydroxyacetone and ethyl-3-oxobutanoate (Table 2 data)

In test tubes carrying a stirring bar, 5 mL of phosphate buffer (200 mM Kpi pH 6.9) 2.5 mM NADPH and 50 mM D-glucose were added, followed by the addition of DMSO-substrate solution giving a final concentration of 4% v/v DMSO and 20 mM ketone substrate. In this mixture, 2 mg glucose dehydrogenase (GDH) and 2–5 mg ketoreductase were dissolved and the reactions were left stirring at 34 °C for 12–16 h. The product was isolated by extraction of the crude reaction mixtures with EtOAc (3 mL). After drying with Na_2SO_4 and evaporation of the organic solvent under reduced pressure, the oily product was dissolved in CH_2Cl_2/Ac_2O/TMSOTf[14] solution in order to acetylate the alcohol. The crude mixture was then analyzed by chiral GC chromatography as described in the following paragraphs.

4.3. Large-scale reduction of benzoyl-hydroxyacetone and α-tetralone and product identification (Table 3 data)

Every reduction described in Table 3 is discussed in detail in the following paragraphs. Product analysis was based on chiral GC analysis for ethyl-3-hydroxybutanoate and 1-benzoyl-1,2 dihydroxypropane, and chiral HPLC analysis for the reduction products of α-tetralone. Chiral GC utilized Chirasil Dex CB column (P7502, Varian Inc.). Both alcohols were first derivitized to the corresponding acetyl-esters using Ac_2O/TMSOTf[14] prior to injection. A temperature gradient of 95–130 °C (1 °C/min) was used to separate the two acetylated enantiomers of ethyl-3-hydroxybutanoate and a gradient of 145–165 °C (1 °C/min) for the acetylated enantiomers 1-benzoyl-2-acetyl-1,2 dihydroxypropane. The assignments of each enantiomer for ethyl-hydroxybutanoate were based on pure enantiomers purchased by Aldrich. The (*R*)- and (*S*)-assignments for 1-benzoyl-1,2 dihydroxypropane were based on the literature reports where the (*R*)-enantiomer was synthesized using whole cell reactions of *Saccharomyces cerivisiae*.[15] We utilized these published methods to synthesize each enantiomer and used it as a standard. The enantiomeric alcohols of the reduction of α-tetralone were separated using Chiral HPLC analysis utilized (*S*,*S*)-Whelk-01 column (Regis Technologies) elution conditions (1 mL/min, hexane/isopropanol, 99:1, v/v). No derivatization of the alcohol was required. The absolute configuration was determined using enantiomerically pure (*R*)-enantiomer and racemic 1,2,3,4 tetrahydro-1-naphthol both purchased by Aldrich.

All large-scale reaction products were analyzed by GC ^{1}H NMR and ^{13}C NMR:

(i) 1-Benzoyl-1,2-dihydroxy-propane:

^{1}H NMR (400 MHz, CD_2Cl_2): $\delta = 1.24$ (d, $J = 6.4$ Hz, 3H), 4.14 (m, 2H), 4.27 (m, 1H), 7.44+7.57+8.02 (m+m+m, 5H). ^{13}C NMR (100 MHz, CD_2Cl_2): $\delta = 19.5$, 53.8 (m, CD_2Cl_2), 66.4, 70.4, 128.8, 129.8, 130.4, 133.4, 166.8.

(ii) 1,2,3,4-Tetrahydro-1-naphthol (reduction product of α-tetralone):

^{1}H NMR (400 MHz, CD_2Cl_2): 1.75+1.86–2.0 (m+m, 4H), 2.72+2.80 (m+m, 2H), 4.76 (t, broad 1H), 7.1+7.2+7.42 (m+m+m, 4H). ^{13}C NMR (100 MHz, CD_2Cl_2): 18.8, 29.2, 23.3, 53.8 (q, CD_2Cl_2), 68.1, 126.1, 127.5, 128.6, 129.0, 130.1, 138.8.

4.3.1. Synthesis of (*R*)-(1-benzyl)-1,2-dihydroxy propane using KRED107

4.3.1.1. KRED107 25 g (0.14 M) substrate, 1.25 mM NADPH (entry 2 Table 3). In 950 mL of a potassium phosphate buffer (Kpi, 200 mM, pH 6.9), a mixture of DMSO-substrate (35 mL DMSO and 25 g substrate) was added giving a final concentration of 3.5% v/v DMSO and 140 mM substrate. In this solution, 45 g glucose (250 mM), 1.10 g NADPH (1.25 mM), 0.25 g glucose dehydrogenase (GDH 102) and 0.5 g KRED107 were dissolved. The reaction was stirred at 34 °C using a pH-stat that was keeping the pH at 6.85–6.90 with the addition of NaOH (3 M solution, ~50 mL added during the reaction).

The reaction was followed by TLC and GC analysis. After 2 h, GC analysis showed 80% conversion and after 4 h traces of starting material were detected. At this point, the reaction mixture was extracted with EtOAc (200 mL, 3×). The combined organic extracts were back-extracted with brine, dried over Na_2SO_4, and evaporated to dryness

giving 24 g of a cream-colored viscous liquid that was crystallized after overnight incubation at 4 °C.

GC analysis of this oil revealed 96% of alcohol present in the extracted product along with traces of the (*S*)-enantiomer and 4% of another unidentified product. No starting ketone was detected.

Final isolated yield of pure alcohol: 91%; Enantiomeric excess: >98% ee.

4.3.2. Synthesis of (*S*)-(1-benzyl)-1,2-dihydroxy propane using KRED117 and KRED111

4.3.2.1. KRED117 25 g (0.14 M) substrate 0.8 mM NADPH (Table 3, entry 3). A reaction similar to the one described earlier is prepared as follows:

In 950 mL Kpi 200 mM pH 6.9 add DMSO-substrate: 35 mL–25 g (3.5% v/v DMSO, 140 mM substrate), glucose: 45 g (250 mM), NADPH: 0.7 g (0.8 mM), GDH: 0.2 g and KRED117: 0.15 g. The reaction was stirred at 34 °C with a pH-stat that kept the pH at 6.8–6.9 by the addition of 3 M NaOH.

After 3 h, TLC analysis of a reaction aliquot showed ~50% product formation. However, at this point no decrease of the pH was observed over time (~10–15 min) and no more product formation was observed by GC analysis. This meant that the reaction had stopped, probably because of KRED117 inactivation or GDH inactivation or NADPH decomposition. GDH and NADPH should be stable over at least 4 h since no problem was observed in the reduction using KRED107. As a result, another 0.1 g of KRED117 were added to the reaction (bringing the total to 0.25 g) resulting in an immediate decrease of pH from 6.9 to 6.8 within 5 min. After 1 h from the second KRED117 addition (4 h from the beginning of the reaction) analysis of an aliquot showed complete conversion to the alcohol. It is clear therefore that KRED117 is not stable for more than 3 h under the reaction conditions and should be added at incremental amounts.

At this point, the reaction mixture was extracted with EtOAc and the product was isolated using the method described earlier for the isolation of the (*R*)-enantiomer. A cream viscous liquid (23 g) was isolated. GC analysis showed 98% of the (*S*)-enantiomer, traces of the (*R*)-enantiomer and 2% of an unidentified product. No starting ketone was detected.

Final isolated yield of pure alcohol: 89% Optical purity: >98% ee.

4.3.2.2. KRED117 5.3 g (0.75 M) and 10 g (1.4 M) substrate 1.5 mM and 2.0 mM NADPH, respectively (Table 3, entries 4 and 5)

4.3.2.2.1. 0.75 M benzoyl-hydroxyacetone. The concentrated reactions were prepared as follows. In 38 mL of potassium phosphate buffer (Kpi, 0.25 M), 7.2 g D-glucose (1 M), 5.3 g (0.03 mol, 0.75 M) of benzoyl-hydroxyacetone that was dissolved in 2 mL of DMSO (5% v/v), 35 mg NADPH (1 mM), 50 mg GDH and 50 mg KRED117 were added to the medium. The heterogeneous mixture was stirred at 34 °C using a pH-stat that was used to keep the pH at 6.9 by the addition of 3 M NaOH. The reaction progress was monitored by GC analysis of crude samples every one hour. Product formation stopped after the first hour since a yield of 40% was measured at the 1 h and the 2 h time points due to enzyme inactivation as shown earlier. From this point on, KRED117 was added every hour: 30 mg at 2 h (40% yield), 20 mg at 3 h (70% yield) and 20 mg at 4 h (87% yield) bringing the total amount of KR117 added to 0.12 g. At the 3 h time point, another 20 mg of GDH and 20 mg NADP+ (0.5 mM) were added to the reaction. These additions kept the reaction rate at an almost constant rate giving a 98% yield after 5 h of reaction time.

At this point, the reaction mixture was extracted with EtOAc (50 mL, 2×), the combined organic extracts were back extracted with brine, dried using sodium sulfate and evaporated to dryness giving 4.8 g of a clear oil containing >98% of the alcohol and traces of starting ketone. The total isolated yield was therefore calculated to be 89%.

4.3.2.2.2. 1.4 M benzoyl hydroxyacetone. The reaction mixture was prepared exactly the same way as described earlier. The only difference was the amount of benzoyl-hydroxyacetone where 10 g were dissolved in 2 mL DMSO prior to the addition in the reaction mixture (38 mL) containing GDH (50 mg), KRED117 (50 mg), NADPH (35 mg, 1 mM) and D-glucose (14.4 g, 2 M). Additional KRED117 was added at increment amounts as follows. 1 h: 20 mg (20% yield), 2 h: 20 mg (32% yield), 3 h: 20 mg (38% yield), 4 h: 20 mg (47% yield), 5 h: 15 mg, 6 h:15 mg (61% yield), 8 h: 20 mg (72% yield), 10 h: 20 mg. The reaction stopped at 13 h where >99% product yield was detected by GC analysis of a crude reaction extract. In addition to KRED117 glucose dehydrogenase (GDH) and NADP+ were added at 3 h (20 mg GDH, 0.5 mM NADP+) and at 6 h (15 mg GDH and 0.5 mM NADP+), bringing the total concentrations of enzymes and cofactor to 0.2 g for KRED117 (or 5 g/L), 0.085 g for GDH (or 2.12 g/L) and 2 mM for the cofactor (1 mM NADPH and 1 mM NADP+). The final product (9.2 g) was isolated by EtOAc extraction as described earlier giving a clear oil containing only the alcohol product at 92% isolated yield.

4.3.2.3. KRED111 10 g (0.14 M) substrate 0.20 mM NADPH (Table 1, entry 1). The reaction mixture was prepared as described earlier for the reduction of 10 g with KRED107. The only difference was that 0.5 g of enzyme KRED111 and 0.2 mM NADPH were used to reduce 10 g of benzyl-hydroxy acetone (140 mM). The reaction mixture was stirred at 34 °C and the progress was followed using GC analysis of crude mixtures: 2 h—31% yield; 3 h—47% yield; 4 h—55% yield; 7 h—94 %.

After 7 h of reaction, the reaction mixture was extracted twice with EtOAc, combined organic extracts washed with brine, and evaporated to dryness giving 8.0 g of a clear oily product that contained 6% of the starting

ketone and 94% of the alcohol product. Final yield of alcohol: 75%.

4.3.3. Large-scale reduction of α-tetralone

4.3.3.1. KRED101 9.1 g (0.125 M) substrate 0.34 M NADPH (Table 3, entry 6). In 460 mL of a potassium phosphate buffered solution (200 mM, pH 6.9), 9.1 g of α-tetralone dissolved in 40 mL of DMSO were added giving final concentrations of 0.125 M for tetralone and 8.0% (v/v) for DMSO. In this mixture, 22.5 g of glucose (0.25 M final concentration), 100 mg NADPH (0.11 mM), 0.25 g of glucose dehydrogenase (GDH) and 0.35 g of KRED101 were added. The reaction was stirred at 34 °C using a pH-stat that kept the pH at 6.9 with the addition of 3 M NaOH solution. After 4 h and while TLC analysis showed about 50% conversion, another 250 mg of KRED101, 50 mg of GDH and 50 mg of NADPH were added to the reaction mixture. The reaction progress was followed by TLC and after 10 h only product was detected by TLC analysis. At this point, the reaction mixture was extracted twice with EtOAc (180 mL each time), dried over $MgSO_4$ and the solvent was evaporated to dryness giving 7.9 g of a light orange oil in 87% isolated yield. NMR analysis confirmed the product structure and purity along with HPLC analysis. Chiral HPLC using (*S*,*S*)-Whelk-01 column separated the two enantiomers giving the (*R*)-isomer as the major product (92% ee).

4.3.3.2. KRED101 4.4 g (0.75 M) substrate 1.5 M NADPH (Table 3, entry 7). The reaction mixture (40 mL total volume, 0.25 M Kpi) was prepared as described earlier for the synthesis of benzoyl-hydroxyacetone under the same substrate concentration. The only difference was the initial concentration of KRED101, which was 0.2 g, while the rest of the reactants were 0.75 M or 4.4 g for α-tetralone dissolved in 4 mL DMSO, 1 M or 7.2 g for D-glucose, 1 mM or 35 mg for NADPH and 50 mg for GDH. The reaction mixture was stirred at 34 °C using a pH-stat to keep the pH at 6.9 and after 8 h of reaction an additional 25 mg of NADP+, 0.1 g KERD101 and 25 mg GDH were added to the heterogeneous mixture. The reaction was almost complete after 24 h (>95% yield). At this point, the mixture was extracted with EtOAc (50 mL 2×), the combined organic layers were back extracted with brine, dried using Na_2SO_4 and evaporated to dryness giving 3.97 g as an oily compound. TLC and HPLC analysis revealed a small amount of residual ketone (<5%) giving a final isolated yield of 89% for the alcohol. Chiral HPLC analysis showed formation of the same enantiomeric mixture as before.

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