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Asymmetric Carbonyl Reductions with Microbial Ketoreductases

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Abstract: The biocatalytic reduction of β-keto esters and some aromatic ketones in the presence of a variety of ketoreductases from different microbial origins was investigated. The prochiral selectivity was generally high and both product enantiomers could be obtained by a proper choice of enzyme. Aromatic ketones reacted slower than the esters but the prochiral selectivity was often high. The organic cosolvent tolerance of these enzymes was rather variable but useful activity could be maintained in a number of cases. Reduction of the oxidized cofactors NAD and NADP, employing 2-propanol as a sacrificial reductant, was catalyzed by the ketoreductases from *Rhodococcus erythropolis* and *Lactobacillus kefir*, respectively.

Keywords: enantioselectivity; β-keto esters; ketones; ketoreductase; reduction

Introduction

Biocatalytic reduction of carbonyl compounds is a very attractive synthetic route towards enantiomerically pure secondary alcohols.[1,2] The chemo-, regio- and enantioselectivities are often very high due to the often strict substrate recognition of reducing enzymes. The biocatalytic reduction of carbonyl compounds using whole cells of *Saccharomyces cerevisiae* has been known and employed widely since the pioneering work in 1914.[3] This latter organism is commercially available, inexpensive and simple to apply but the enantioselectivity is generally poor, which is a major drawback. This is ascribed to the presence of multiple reductases with conflicting enantiomeric preferences.[4] Approaches to overcome this latter problem have been modestly successful.[5–8]

Isolated ketoreductases do not suffer from such problems but require an efficient NAD(P)H recyling strategy.[2] Two-enzyme,[9,10] as well as one-enzyme[11,12] recycling methodologies have been demonstrated from the early 1980s onwards.

The interest in synthetic strategies for the production of homochiral β-hydroxy esters and β,δ-dihydroxy esters has provoked a thorough investigation of the reduction of the corresponding keto esters with whole cells of baker's yeast and a number of its purified enzymes.[4,13–15] Additional to the enzymes from baker's yeast, numerous ketoreductases from a wide variety of other organisms have been studied in the reduction of these substrates.[16,17] Recently, ketoreductases have been isolated from their parent organism and overexpressed in a suitable host, using the advanced biochemical tools that are available nowadays. An overview of the latest achievements in the field of asymmetric biocatalytic carbonyl reductions can be found in recent reviews.[18,19]

We here report the performance of microbial ketoreductases in the reduction of a number of keto esters and aromatic ketones. Furthermore we have investigated the effects of organic co-solvents as well as *in-situ* cofactor regeneration using 2-propanol as a sacrificial reductant.

Results and Discussion

The ketoreductases originated from six different micro-organisms and were expressed in a recombinant *E. coli* strain. These included a *Candida magnoliae* ketoreductase S1 mutant (CmKr10) that had originally been evolved for the production of the industrial intermediate ethyl (*S*)-4-chloro-3-hydroxybutanoate.[20] We investigated, furthermore, a ketoreductase from *Rhodococcus erythropolis* as well as a number of mutants. Also included in our study were wild-type ketoreductases from *Saccharomyces cerevisiae*, *Sporobolomyces salmonicolor*, *Streptomyces coe-*

Table 1. The studied ketoreductases, their source and cofactor preference.

Source organism	Identifier	Required cofactor
Candida magnoliae mutant	CmKr10	NADPH
Saccharomyces cerevisiae	YdlC	NADPH
Saccharomyces cerevisiae	YglC	NADH
Saccharomyces cerevisiae	YprC	NADPH
Saccharomyces cerevisiae	GreC	NADH, NADPH
Rhodococcus erythropolis	RhoC, Rhhoxx[a]	NADH
Sporobolomyces salmonicolor	SpbC	NADH
Streptomyces coelicolor	StaC	NADH
Lactobacillus kefir	KefC	NADPH

[a] x indicates an identification number of a variant from directed evolution; numbers do not indicate a generation.

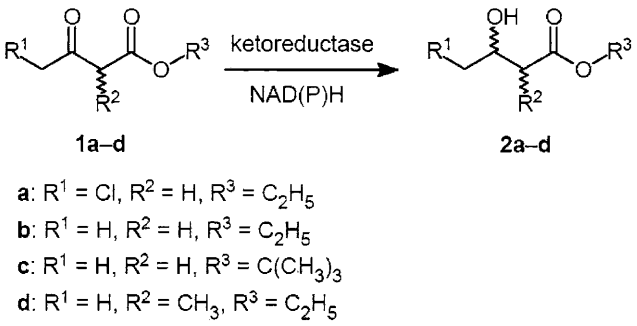


Figure 1. Reduction of β-keto esters in the presence of microbial ketoreductases.

licolor and *Lactobacillus kefir*. These enzymes and their cofactor preferences are listed in Table 1.

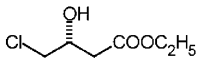
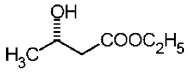
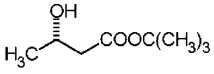
Reduction of β-Keto Esters

Non-branched β-keto esters (**1a–c**, see Figure 1) were reduced in the presence of the microbial ketoreductase panel; the results, with regard to initial rate and enantiomeric preference, are listed in Table 2.

Keto ester **1a** was reduced slowly by all of the ketoreductases, with the exception of CmKr10 which had been evolved for this particular reactant.[20] Keto ester **1b**, in which the chlorine atom is lacking, was converted much faster than **1a** in the presence of the *Rh. erythropolis* and *L. kefir* enzymes as well as YprC from *S. cerevisiae*, which showed the best activity with this latter substrate. The other enzymes from *S. cerevisiae* as well as the ones from *S. salmonicolor* and *S. coelicolor* remained low in activity. The same trend was observed with **1c**, albeit with activities that were in general lower than with **1b**, which we ascribe to steric hindrance by the bulky *tert*-butyl ester. Our preparation of *S. salmonicolor* ketoreductase converted **1a** approx. 25 times slower than reported in the literature.[21] It would seem that a different protein was used by these authors, as their enzyme produced (*S*)-**2a**, whereas the enantiomeric preference of our *S. salmonicolor* ketoreductase was (*R*).

The ketoreductase mediated reduction of **1a–1c** generally occurred with high enantioselectivity

Table 2. Asymmetric reduction of non-branched β-keto esters **1a–c** catalyzed by microbial ketoreductases.[a]

	 (*R*)-**2a**		 (*S*)-**2b**		 (*S*)-**2c**	
KRED	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]
CmKr10	7763	>99 (*S*)	214	>99 (*R*)	41	>99 (*R*)
RhoC	60	>99 (*R*)	587	>99 (*S*)	122	>99 (*S*)
Rhh001[b]	51	>99 (*R*)	341	>99 (*S*)	135	>99 (*S*)
Rhh004[b]	55	>99 (*R*)	314	>99 (*S*)	126	>99 (*S*)
Rhh014[b]	45	>99 (*R*)	599	>99 (*S*)	120	>99 (*S*)
YdlC	48	>99 (*S*)	17	>99 (*R*)	5	36 (*R*)
YglC	62	77 (*R*)	60	>99 (*S*)	42	>99 (*S*)
YprC	53	>99 (*R*)	969	>99 (*S*)	240	>99 (*S*)
GreC	30	>99 (*R*)	76	>99 (*S*)	21	>99 (*S*)
StaC	29	>99 (*R*)	47	50 (*S*)	26	>99 (*S*)
SpbC	56	>99 (*R*)	66	19 (*S*)	37	>99 (*S*)
KefC	46	>99 (*S*)	590	>99 (*R*)	105	>99 (*R*)

[a] *Activity measurement conditions:* 2 mL Tris-HCl buffer (50 mM, pH 7.6), 0.16 mM NAD(P)H, 10 mM substrate, 0.1 mg enzyme. *Enantioselectivity measurement conditions:* 0.2 mL Tris-HCl buffer (50 mM, pH 7.6), 11 mM NAD(P)H, 10 mM substrate, 0.2 mg enzyme.

[b] Numbers do not indicate the generation in evolution.

(Table 2). Most of the enzymes obeyed Prelog's rule, which predicts that (*R*)-**2a**, (*S*)-**2b** and (*S*)-**2c** will be preferred.[22,23] Notable exceptions were the ketoreductases from *C. magnoliae, L. kefir* and YdlC from *S. cerevisiae*. KefC from *L. kefir* and YprC from *S. cerevisiae* both exhibited good reaction rates and absolute but opposite enantioselectivity. Hence, these enzymes afford full access to both pure enantiomers of these substrates.

Reduction of α-Substituted Keto Esters

The sensitivity of the ketoreductases with regard to branching on the C-2 position rather diverged. YprC and KefC, which showed a relatively high activity with the non-branched keto ester **1b** (Table 2), reduced **1d** at a comparable rate. CmKr10 even converted **1d** twice as fast as **1b**. The other enzymes had a moderately lower activity for **1d** compared to **1b**.

The reduction of the α-substituted keto ester ethyl 2-methylacetoacetate (**1d**) involves a kinetic resolution (2*R vs.* 2*S*) as well as prochiral selectivity. Most enzymes preferentially reduced (2*R*)-**1d** (Table 3). CmKr10 and KefC, in contrast, showed a modest kinetic bias towards (2*S*)-**1d** and the enantiomeric preference of *S. cerevisiae* YdlC and YglC was low.

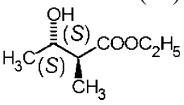
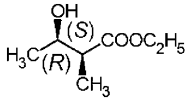
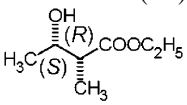
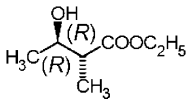
Most of the enzymes reduced **1d** with the same prochiral selectivity as shown in the reduction of **1b** (Table 2 and Table 3). The ketoreducctases from *Rh. erythropolis*, for example, reduced the ketone function with complete (*S*)-selectivity, similar to **1a**–**c**. There were some notable exceptions, however. An improved prochiral selectivity became apparent with StaC and SpbC, which reduced **1b** with, respectively, 50 and 19% prochiral selectivity but showed a near absolute selectivity with **1d**.

The stereochemical selectivity of CmKr10 in the reduction of **1d** was rather complex and merits a more detailed discussion. This latter enzyme acted with pro-(*R*) prochiral selectivity in the reduction of **1b** as well as (2*R*)-**1d**. The kinetically preferred (2*S*)-**1d**, in contrast, was reduced with high prochiral selectivity into (2*S*,3*S*)-**2d**. It would seem that **1b** and (2*R*)-**1d** bind in the active site in a similar way, whereas (2*S*)-**1d** binds in a inverted configuration, presumably due to steric interactions of the methyl group at C-2. A very similar effect was apparent with YdlC, which formed approximately equal amounts of (2*S*,3*R*)-**2d** and (2*R*,3*S*)-**2d**, indicating that (2*S*)-**1d** binds in the same configuration as **1b**, whereas (2*R*)-**1d** binds in an inverted configuration.

Reduction of Aromatic Carbonyl Compounds

The reduction of some aromatic ketones using the ketoreductases mentioned above was studied. The model substrates studied were: acetophenone (**3**), α-tetralone (**4**), β-tetralone (**5**) and 2-phenylacetophenone. This latter ketone proved to be completely unreactive with all of the ketoreductases studied. The tetralones reacted very sluggishly; the reduction rate of **5** was in many cases even too low to quantify and

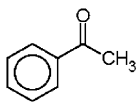
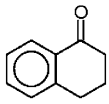
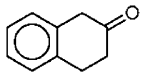
Table 3. Asymmetric reduction of the branched β-keto ester **1d** catalyzed by microbial ketoreductases.[a]

KRED	V_{ini} [μmol·min^{-1}g^{-1}]	(2*S*)-Products		(2*R*)-Products	
					
		[%]	[%]	[%]	[%]
CmKr10	469	78.3	2.4	1.6	17.8
RhoC	123	19.9	0	80.1	0
Rhh001[b]	68	6.0	0	94.0	0
Rhh004[b]	117	20.0	0	80.0	0
Rhh014[b]	222	14.2	0	85.8	0
YdlC	8	7.3	46.4	46.3	0
YglC	72	54.7	0	45.3	0
YprC	962	0	0	100	0
GreC	25	13.5	0.2	86.0	0.2
StaC	27	0.7	0	99.3	0
SpbC	42	0	0	100	0
KefC	675	36.1	27.4	0	36.5

[a] *Activity measurement conditions:* 2 mL Tris-HCl buffer (50 mM, pH 7.6), 0.16 mM NADPH, 10 mM substrate, 0.1 mg enzyme. *Enantioselectivity measurement conditions:* 0.2 mL Tris-HCl buffer (50 mM, pH 7.6), 11 mM NADPH, 10 mM substrate, 0.2 mg enzyme.

[b] Numbers do not indicate the generation in evolution.

Table 4. Asymmetric reduction of aromatic carbonyl compounds catalyzed by various microbial ketoreductases.[a]

	 3		 4		 5	
KRED	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]	V_{ini} [μmol·min^{-1}g^{-1}]	*ee* [%]
CmKr10	9	>99 (*R*)	3	91 (*S*)	<0.5	n.d.[b]
RhoC	80	>99 (*S*)	3	93 (*S*)	<0.5	n.d.
Rhh001[c]	35	>99 (*S*)	2	94 (*S*)	<0.5	n.d.
Rhh004[c]	9	>99 (*S*)	3	93 (*S*)	<0.5	n.d.
Rhh014[c]	66	>99 (*S*)	3	95 (*S*)	<0.5	n.d.
YdlC	19	10 (*S*)	9	53 (*R*)	1.0	n.d.
YglC	12	>99 (*S*)	3	97 (*S*)	<0.5	n.d.
YprC	14	97 (*S*)	4	97 (*S*)	<0.5	n.d.
GreC	4	>99 (*S*)	4	95 (*S*)	<0.5	n.d.
StaC	2	>99 (*S*)	4	95 (*S*)	<0.5	n.d.
SpbC	2	31 (*R*)	3	17 (*R*)	0.5	n.d.
KefC	256	>99 (*R*)	3	62 (*S*)	2.0	n.d.

[a] *Reaction conditions:* 0.2 mL Tris-HCl buffer (50 mM pH 7.6), 11 mM NAD(P)H, 10 mM substrate, 0.1 mg enzyme. Activity determined over first hour of reaction.
[b] n.d.: not determined.
[c] Numbers do not indicate the generation in evolution.

the enantioselectivity could not be determined reliably (see Table 4). Acetophenone (**3**) was the best-accepted aromatic ketone by the ketoreductases used in these studies. Scant literature data are available for comparison. Whereas we found that KefC reduced **3** 85 times as fast as **4**, a ratio of 1.7 has been reported with alcohol dehydrogenase from *L. kefir*.[24] The authors may have used an enzyme preparation with different properties, or diffusion limitation resulting from immobilization may have slowed down the conversion of 3.

Most of the ketoreductases that showed high (*S*) prochiral selectivity with **1b**, also were (*S*)-selective in the reduction of **3** and **4**. This latter outcome would be expected on the basis of Prelog's predictive model[22] and indicates that the prochiral selectivity of these enzymes is dominated by steric interactions. The same reasoning holds for CmKr10, which was highly (*R*)-selective in the reduction of **1b** as well as **3** and **4**. KefC behaved in a similar way although its enantioselectivity in the reduction of **4** was more modest.

StaC, which reduced **1b** with modest bias towards (*S*)-**2b**, converted **3** and **4**, as well as **1c**, with high pro-(S) selectivity. The enzyme possibly requires a bulky group for high prochiral selectivity. SpbC, which reduced **1b** with little enantiomeric bias, likewise showed a low enantioselectivity in the conversion of **3** and **4**.

Tolerance towards Organic Solvents

Many reactants are only sparingly soluble in aqueous medium. Adding a water-miscible organic solvent is one way to perform reactions at practical concentrations while maintaining a monophasic reaction medium, which is often desirable. Hence, organic co-solvent tolerance is an important enzyme characteristic. We performed reductions of **1b** using our panel of ketoreductases in a range of aqueous-organic media; the results are compiled in Table 5.

2-Propanol caused, at 20% concentration, a severe deactivation, with YglC and GreC as notable exceptions. In 80% 2-propanol deactivation was nearly complete, except with CmKr10. 20% *tert*-Butyl alcohol was tolerated rather well by KefC and to some extent (35% residual activity) by YglC but not by the other ketoreductases. In 80% *tert*-butyl alcohol, in contrast, many enzymes showed a somewhat higher activity. Hyperactivation to >153% of the original activity even became apparent with YglC. Except with KefC and YprC, which tolerated low concentrations of 1,2-dimethoxyethane quite well, this latter solvent was highly deactivating.

Taking it all in all, these enzymes, with a few exceptions, are prone to severe activity loss in such media. Presumably, precipitation takes place at 80% cosolvent, although it could not be visually observed owing to the minute (0.05 mg mL^{-1}) enzyme concentrations but this will not cause deactivation *per se*.[25] Stabilization as a cross-linked enzyme aggregate (CLEA) could improve their solvent tolerance.

Table 5. Residual activities (%) of the microbial ketoreductases using various amounts of water-miscible organic solvents.[a]

KRED	2-Propanol		*tert*-Butyl alcohol		1,2-Dimethoxyethane	
	20% (v/v)	80% (v/v)	20% (v/v)	80% (v/v)	20% (v/v)	80% (v/v)
CmKr10	19.5	26.7	11.6	10.1	0.6	0
Rhoc	16.0	7.5	9.0	3.7	1.4	0
Rhho01	6.6	0.1	4.1	4.9	0	2.5
Rhho04	6.3	0	0	0.3	0	4.2
Rhho14	12.6	0	7.5	2.4	0	17.5
YdlC	6.9	1.1	0	40.3	0	2.4
YglC	45.5	5.5	34.6	152.8	9.7	6.4
YprC	11.7	0.2	18.3	0.1	52.9	13.2
GreC	98.3	0	0.9	11.9	2.3	0
StaC	1.8	1.8	0.6	28.4	0	0
SpbC	2.0	0.8	0.8	19.1	0	0
KefC	1.3	0.2	57.7	2.7	77.9	0

[a] *Reaction conditions:* Tris-HCl buffer (50 mM, pH 7.6)/organic solvent total 2 mL reaction volume, 0.16 mM NAD(P)H, 10 mM substrate **1b**, 0.1 mg enzyme; activities were compared with those in pure Tris-HCl buffer (50 mM, pH 7.6) as solvent.

Cofactor Regeneration

Besides acting as a cosolvent for hydrophobic reactants, 2-propanol can be used as a sacrificial reductant to regenerate the cofactor, obviating the need for a regeneration cycle. We measured the rate of cofactor (NAD, NADP) reduction in 5% (v/v) aqueous 2-propanol in the presence of our ketoreductases (see Table 6). Unfortunately, the rates generally were low or even below the detection threshold. Exceptions were the enzymes from *Rh. erythropolis* and *L. kefir*, which exhibited good reduction rates. Therefore cofactor regeneration by the addition of 2-propanol to the reaction medium seems feasible with these enzymes.

Deactivation by 5% 2-propanol does not seem to cause the low activities as there is no correlation with the residual activities in 20% 2-propanol. Thus, the ketoreductases from *Rh. erythropolis* and *L. kefir*, which reduced NAD and NADP, respectively, at a useful rate, were scantly active in 20% 2-propanol (Table 5). In contrast, YglC and GreC, which had 46 and 98% residual activity, respectively, in 20% 2-propanol were hardly active in the NAD(P) reduction test.

Table 6. Reduction rate with various microbial ketoreductases of the oxidized cofactors (NAD^+, $NADP^+$) in the presence of 5% (v/v) 2-propanol.

KRED	Rate [μmol·min^{-1}g^{-1}]	KRED	Rate [μmol·min^{-1}g^{-1}]
CmKr10	0	YglC	0
RhoC	194	YprC	2
Rhh001	59	GreC	3
Rhh004	100	StaC	3
Rhh014	185	SpbC	5
YdlC	3	KefC	585

Ketoreductases Compared

Our library contained a number of WT enzymes as well as ones that have been subjected to directed evolution. The most highly evolved one, CmKr10, was outstandingly active with its evolution substrate, but not with any other ketone. Hence, it would seem that mutagenesis may afford ketoreductases with high specificity even for structurally simple reactants. This notion will be further discussed in a forthcoming paper.

The ketoreductases from *Rh. erythropolis*, as well as YprC, generally showed a high activity with the β-keto ester reactants combined with high (Prelog) enantioselectivity. These compounds were also rapidly reduced in the presence of KefC but with high anti-Prelog enantioselectivity. This latter enzyme was the only one that converted **3** at >200 μmol min^{-1}g^{-1}. The ketoreductases from *Rh. erythropolis* showed promise with 2-propanol as a sacrificial reductant for cofactor recycling.

Conclusions

Enantiomerically pure, unbranched β-hydroxy esters have been obtained *via* reduction of the corresponding keto ester in the presence of a range of microbial ketoreductases. Both enantiomers are accessible by a proper choice of the enzyme. Aromatic ketones reacted slower than the esters but the prochiral selectivity was often high.

The organic cosolvent tolerance of these enzymes was rather variable but useful activity could be maintained in a number of cases. Reduction of the oxidized cofactors NAD and NADP, employing 2-propanol as a sacrificial reductant, was catalyzed by the ketoreductases from *Rhodococcus erythropolis* and *Lactobacillus kefir*, respectively.

Experimental Section

Materials

Ethyl 4-chloroacetoacetate (**1a**), ethyl acetoacetate (**1b**), *tert*-butyl acetoacetate (**1c**), ethyl 2-methylacetoacetate (**1d**),

acetophenone (**3**), α-tetralone (**4**) and β-tetralone (**5**) were purchased from Sigma–Aldrich. The corresponding alcohols were obtained by reduction with sodium borohydride in methanol. The cofactors NADH and NADPH were bought from Jülich Fine Chemicals. Tris(hydroxymethyl)aminoethane (99.8%) was purchased from Fluka. All commercial reagents were used without additional purification.

Codexis, Inc. made a panel of ketoreductases from various microbial organisms (Codex™ KRED) available.

Analytical Procedures

UV activity measurements were carried out on a Varian Cary 3 Bio UV-visible spectrophotometer at a wavelength of 340 nm.

HPLC analysis of aromatic substances was performed on two 4.6×50 mm Chromolith SpeedROD® RP-18e columns placed in series, eluent MeOH-H_2O (25:75, v/v), flow rate 1 mL min^{-1} with UV detection (Shimadzu SPD-6 A) at 215 nm. Trimethoxybenzene was used as internal standard.

Chiral HPLC analyses of **2d** were performed on a 4.6× 250 mm 5μ Chiralcel AD-H column, eluent hexane-2-propanol (90:10, v/v), flow rate 0.6 mL min^{-1} with UV detection (Waters 486) at 215 nm. Chiral HPLC analyses of the reduction products of **3–5** were performed on a 4.6×250 mm 5μ Chiralcel OD-H column, eluent hexane-2-propanol (98:2, v/v), flow rate 0.6 mL min^{-1}.

Chiral GC analyses of **2a–c** were performed using a 0.32 mm×25 m Varian Chirasil Dex CB, d_f 0.25 μ column with helium as the carrier gas and flame ionization detection.

Activity Assay

Enzyme stock solutions were prepared by dissolving approx. 10 mg mL^{-1} of enzyme powder in Tris buffer pH 7.6.

Keto esters: To Tris buffer (1.78 mL, 50 mM, pH 7.6) in a 2-mL cuvette were added a solution of NAD(P)H (10 μL, 32 mM) and a solution of keto ester (200 μL, 100 mM). The assay was started by the addition 10 μL of enzyme stock solution. The substrates used were ethyl 4-chloroacetoacetate (**1a**), ethyl acetoacetate (**1b**), *tert*-butyl acetoacetate (**1c**), ethyl 2-methylacetoacetate (**1d**). The mixture was maintained at 20 °C and the UV absorption at 340 nm was monitored until a steady slope was observed. The molar absorption coefficient used to quantify the NAD(P)H consumption was 6.22 L mmol^{-1} cm^{-1}.

Aromatic ketones: To Tris-buffer (103 μL, 50 mM, pH 7.6) in a 1-mL flask were added a solution of NAD(P)H (67 μL, 32 mM) and a solution of ketone in 2-propanol (20 μL, 100 mM). The assay was started by the addition of 10 μL of enzyme stock solution. The substrates used were: acetophenone (**3**), α-tetralone (**4**) and β-tetralone (**5**) and deoxybenzoin (**6**). After 1 h the reaction was stopped by quenching with acetone (400 μL). After centrifugation and membrane filtration over Millipore centripreps with a 10 kD cut-off to remove all dissolved protein, the filtrate was analyzed by HPLC as described above.

Assignment of Absolute Configurations

The absolute configurations of **2a–c** were assigned by correlation to published results obtained with reduction in the presence of *Candida magnoliae* S1[26] and *Saccharomyces cerevisiae*.[27] The enantiomeric preference was assessed with chiral GC (0.32 mm×25 m Varian Chirasil Dex CB, d_f 0.25 μ column, carrier gas helium, flame ionization detection); at 95 °C (**2a**) or 85 °C (**2b**, **2c**).

Samples of stereoisomer mixtures of **2d** were prepared as described for **2a–c**, using the same ketoreductases. Stereoisomer structures were assigned by correlating the retention times of 3,5-dinitrobenzoylated derivatives on chiral HPLC – as described above – with literature data,[26,27] using an identical system. Both sources support the following elution order of the isomers: (2*S*,3*S*), (2*R*,3*S*), (2*S*,3*R*), (2*R*,3*R*).

The absolute configurations of the reduction product of **3–5** were determined by comparing the HPLC retention times on an OD-H column with the results of *Burkholderia cepacia* lipase-catalyzed esterification with vinyl acetate, which is known to be (*R*)-selective.[28,29]

Determination of Enantioselectivity

Reactions were performed for each enzyme and each substrate under the following conditions. To Tris-buffer (63 μL, 50 mM, pH 7.6) in a 1-mL vial were added a solution of NAD(P)H (67 μL, 32 mM) and a solution of substrate (20 μL, 100 mM). 50 μL of a 10 mg mL^{-1} stock solution of each enzyme were added to start the reaction. The reaction was left shaking overnight. In the case of substrates **1a–1c** the reaction products were extracted from the reaction mixture with ethyl acetate (500 μL). The organic layer was dried and injected in the chiral GC. With substrate **1d** extraction was performed with toluene (2×1 mL); to the combined organic layers 3,5-dinitribenzoyl chloride (20 mg) and pyridine (2 mL) were added. After standing for 1 h at room temperature the solvent was evaporated, the residue was dissolved in hexane-2-propanol (95:5, v/v) analyzed by chiral HPLC using the AD-H column.

Reactions with the aromatic substrates **3–5** were performed under similar conditions, only with 10% (v/v) 2-propanol as cosolvent present. Samples were taken by extraction with hexane (500 μL) and subjected to chiral HPLC using the OD-H column.

Stability towards Organic Solvents

The stability towards organic solvents was studied according to the procedure for the activity assay in which the amount of buffer is reduced and the various amounts of organic solvents are substituting this to add up to a total reaction volume of 2 mL. The model substrate used for the comparison was **1b**.

Cofactor Regeneration

The ability of the various enzymes to use 2-propanol to regenerate their cofactor was measured by following the production of NAD(P)H from NAD(P)$^+$ by means of its UV absorption. To Tris-buffer (1.58 mL, 50 mM, pH 7.6) in a 2-mL cuvette were added: 2-propanol (200 μL) and a solution of NAD(P)$^+$ (10 μL, 32 mM). The assay was started by adding 10 μL of a diluted sample containing approx. 10 mg mL^{-1} enzyme and monitoring the UV absorption at 340 nm and 20 °C. The molar absorption coefficient used for the consumed NAD(P)H was 6.22 L mmol^{-1} cm^{-1}.

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