ASYMMETRIC SYNTHESIS OF PHENYL-RING-CONTAINING ALCOHOLS USING *THERMOANAEROBACTER ETHANOLICUS* W110A SECONDARY ALCOHOL DEHYDROGENASE

by

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(Under the Direction of ROBERT S. PHILLIPS)

ABSTRACT

This dissertation includes five chapters. Chapter 1 includes introduction and literature review. Chapter 2 and chapter 3 are reprinted from published articles. Chapter 4 is submitted for publication. Chapter 5 includes conclusions.

Chapter 2 of this dissertation describes an enantioselective asymmetric reduction of phenyl-ring-containing ketones to yield the corresponding optically active secondary alcohols by using W110A secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (W110A TeSADH) in Tris-HCl buffer solution using 2-propanol (30%, v/v) as the cosolvent and cosubstrate. The resulting alcohols have *S*-configuration, in agreement with Prelog's rule, in which the nicotinamide-adenine dinucleotide phosphate (NADPH) cofactor transfers its *pro*-R hydride to the *re* face of the ketone. (*R*)-Alcohols, the *anti*-Prelog products, were obtained by enantiospecific oxidation of (*S*)-alcohols through oxidative kinetic resolution of the *rac*-alcohols using W110A TeSADH in Tris-HCl buffer solution/acetone (90:10, v/v).

Chapter 3 of this dissertation describes the aforementioned asymmetric reductions of hydrophobic ketones by using xerogel-immobilized W110A TeSADH in organic solvents, which

were achieved in comparable yields to those obtained using the free enzyme, and, in some cases, with higher enantioselectivities. The use of xerogel-encapsulated ADH is a facile method as it allows the reuse of the enzyme, it makes it more stable, and it can affect its enantioselectivity by switching to organic solvents.

Chapter 4 of this dissertation describes the results when these transformations were performed in mono- and biphasic systems containing either organic solvents or ionic liquids. Both yield and enantioselectivity for these transformations can be controlled by changing the reaction medium. The enzyme showed high tolerance to both water-miscible and -immiscible organic solvents and ionic liquids, which allows biotransformations to be conducted at high substrate concentrations.

INDEX WORDS: Alcohol dehydrogenase, Asymmetric synthesis, Biocatalysis, Enantioselective reduction, Ionic liquids, Oxidoreductases, Sol-gel process, *Thermoanaerobacter ethanolicus*, Xerogel.

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DEDICATION

To my parents, brothers and sisters for their love, patience and support over the years

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shaping my life in many ways. Thanks to all my friends in Athens for their continuous encouragement.

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LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
ADPR	Adenosine Diphosphate Ribose
[bmim][BF_4]	1-Butyl-3-methylimidazolium tetrafluoroborate
[bmim][NTf_2]	1-Butyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)imide
brs	broad singlet
Calcd.	Calculated
Conv.	Conversion
d	doublet
Da	Dalton
de	diastereomeric excess
dd	doublet of doublet
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DIPE	Diisopropyl Ether
DKR	Dynamic Kinetic Resolution
DMF	Dimethylformamide
dt	doublet of triplet
E. coli	*Escherichia coli*
ee	enantiomeric excess
Et	Ethyl
FDH	Formate Dehydrogenase

GC	Gas Chromatography
GDH	Glucose Dehydrogenase
HLADH	Horse Liver Alcohol Dehydrogenase
HRMS	High Resolution Mass Spectrometry
i.d.	internal diameter
IL	Ionic Liquid
KR	Kinetic Resolution
LbADH	*Lactobacillus brevis* Alcohol dehydrogenase
m	multiplet
Me	Methyl
mg	milligram
mM	millimolar
mmol	millimole
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Nicotinamide Adenine Dinucleotide, Reduced
$NADP^+$	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, Reduced
nm	nanometer
NMR	Nuclear Magnetic Resonance
qd	quartet of doublet
rac	racemic
Ph	Phenyl
RT	Room Temperature

TbADH	*Thermoanaerobium brockii* Alcohol Dehydrogenase
TBME	*tert*-Butyl Methyl Ether
TeSADH	*Thermoanaerobacter ethanolicus* Secondary Alcohol Dehydrogenase
TMOS	Tetramethyl Orthosilicate
TMS	Tetramethyl Silane
Tris-HCl	Tris(hydroxymethyl)aminomethane Hydrochloride
YADH	Yeast Alcohol Dehydrogenase

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

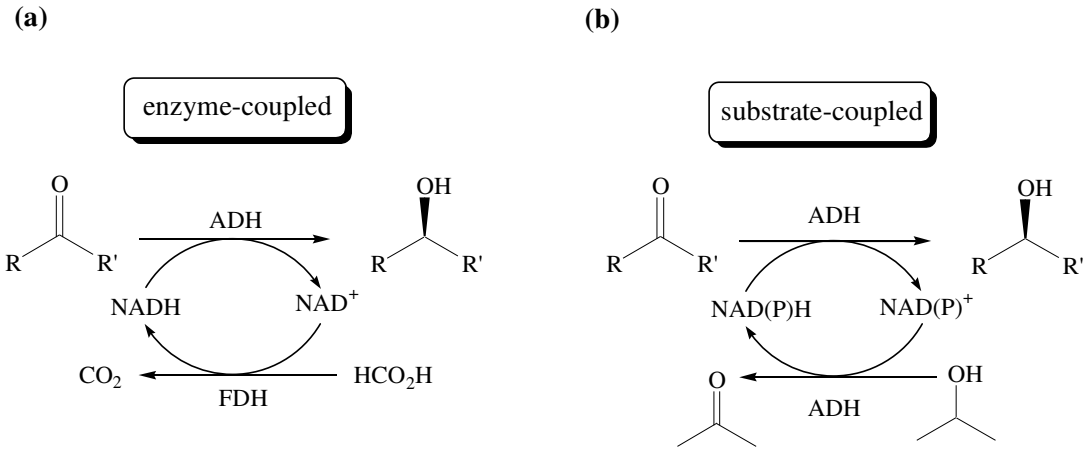
Biocatalysis

Development of new methods for asymmetric synthesis is of great interest to satisfy the huge demand for enantiomerically pure compounds.[1] Development of such methods can be done using chemical catalysts or biocatalysts. Biocatalysis is defined as the utilization of enzymes to perform chemical transformations. Biocatalysts are preferable to chemical catalysts for several reasons. First, biocatalysts have high chemo-, regio- and enantioselectivities. Second, biocatalytic reactions are safer because they do not require dangerous reagents or solvents. Third, they are environmentally benign because they are natural catalysts. Fourth, the reaction conditions are mild, which minimize side products by preventing isomerization, racemization, epimerization, and rearrangement reactions. Even with these advantages, organic chemists still hesitate to consider a biocatalytic method for a synthetic problem for several reasons.[1a] Organic chemists have difficulty handling biological systems, and the sensitivity of enzymes as well as their high cost is daunting. However, recent advances in the biocatalysis field have increased the stability of a large number of enzymes and therefore simplified their handling. Thus, a lot of enzymes are now just as commercially available as any other chemical. One of the major disadvantages of using biocatalysts is the difficulty encountered in large scale synthesis because the natural environment of enzymes is water. Recently, it has been shown that some biotransformations can be done in organic media.[2] Although the activity of most enzymes is

usually lowered in such environments, the many advantages of using organic media and the overall increase in efficiency for many processes lessen the effect of this disadvantage.

Alcohol dehydrogenases

Alcohol dehydrogenases (EC 1.1.1.X, X=1 or 2, ADHs) are enzymes that catalyze the reversible reduction of ketones and aldehydes to their corresponding alcohols.[3] ADHs require a coenzyme such as nicotinamide-adenine dinucleotide (NAD^+) or its phosphate ($NADP^+$). Because these coenzymes are costly, a successful regeneration of the coenzyme is crucial to make ADH-catalyzed transformations catalytic.[1,3b] This can be done by using a coupled enzyme approach, which requires two different enzymes (Scheme 1.1).[3b] A more simple approach is the use of a coupled substrate approach. In this method, a cosubstrate such as 2-propanol or glucose in the reduction pathway or acetone in the oxidation pathway, is required (Scheme 1.1).[1,3b]



Scheme 1.1. Examples of cofactor regeneration in ADH-catalyzed reactions: (a) enzyme coupled using formate dehydrogenase (FDH), (b) substrate coupled approach using 2-propanol.

There are four possible pathways to deliver the hydride from NAD(P)H to a prochiral substrate, as shown in Figure 1.1.[3c] The hydride can attack from the *si* face of a prochiral ketone,

as in E1 and E2, to produce the corresponding (*R*)-alcohol. It can also attack from the *re* face of a ketone, as in E3 and E4, to produce the corresponding (*S*)-alcohol. Sometimes the products have opposite assignments because the small alkyl group has a higher Cahn-Ingold-Prelog priority than that for the large one. The majority of commercially available ADHs, like yeast ADH (YADH), horse liver ADH (HLADH), and *Thermoanaerobium brockii* ADH (TbADH), fall in the second category (i.e. they deliver the hydride from the *re* face of a prochiral ketone). This is known as Prelog's rule.[4] Some ADHs are known to be *anti*-Prelog, however only few of them are commercially available, such as *Lactobacillus kefir* ADH.[3b]

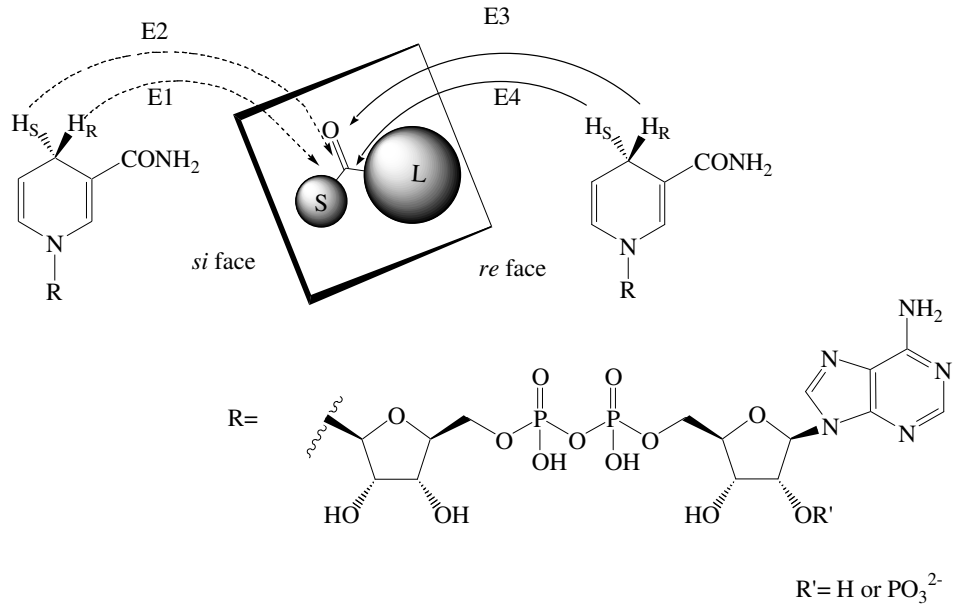


Figure 1.1. Stereochemistry of the hydride transfer from NAD(P)H to the carbonyl carbon on a substrate (S is a small group and L is a large group).

Thermoanaerobacter ethanolicus secondary ADH (TeSADH, EC 1.1.1.2), an $NADP^+$-dependent, thermostable oxidoreductase, has been isolated and characterized.[5] Since it is obtained from a thermotolerant microorganism, it is remarkably thermostable (up to 85 ºC).[6] It

also tolerates the presence of organic solvents and exhibits high activity towards cyclic and acyclic secondary alcohols and ketoesters.[7] This enzyme is very similar to the commercially available TbADH.[8] Keinan *et al.* proposed a model for the active site of TbADH suggesting both large and small hydrophobic binding pockets with different affinities toward the alkyl groups of ketone substrates, the small site having higher binding affinity (Figure 1.2).[9] (*R*)-Alcohols were produced from the asymmetric reductions of ketones with small alkyl groups attached to the carbonyl (e.g. methyl ethyl, methyl isopropyl, or methyl cyclopropyl). However, (*S*)-alcohols were produced with methyl ketones containing alkyl substituents larger than propyl (i.e. the enzyme follows Prelog's rule).[4] It was noticed that the enantioselectivity increased significantly with larger ketones like 2-hexanone and 2-heptanone because they can only fit in one mode within the active site.

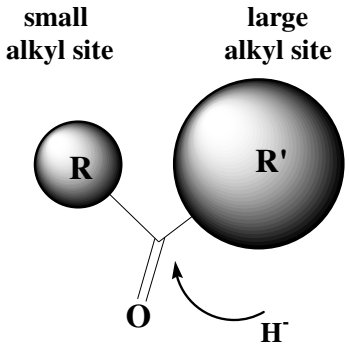


Figure 1.2. Pocket model for TbADH.

The crystal structure of TbADH has been determined to be a tetramer of 37,652 Da subunits.[10] Each unit is composed of 352 amino acids, and contains a Zn^{2+} ion. Its binary complex with 2-butanol as a substrate has also been determined.[11] It has been shown that the enzyme has a preference for secondary over primary alcohols because of Van der Waals

interactions of the C1 atom of 2-butanol with atoms in His59, Ala85, Trp110, Asp150, and Leu294 (Figure 1.3).[11] The crystal structure also shows a crevice between the surface and the active site, which allows the substrates and products to move in and out. This crevice contains the hydrophobic residues Ile49, Leu107, Trp110, Tyr267 and Cys283 as well as Met285 from another polypeptide. Li *et al.* reported that these hydrophobic residues might explain the high tolerance of this enzyme to organics solvents.[11] The two residues His59 and Asp150 have been shown to be essential for catalysis because the hydroxyl group of the substrates make hydrogen bonds with them.[11] An active site model of wild-type TeSADH with isopropyl 4-oxo-5-hexynoate substrate based on the X-ray crystal structure of TbADH was proposed by Heiss *et al.* (Figure 1.4),[12] which is similar to those proposed for TbADH.[13]

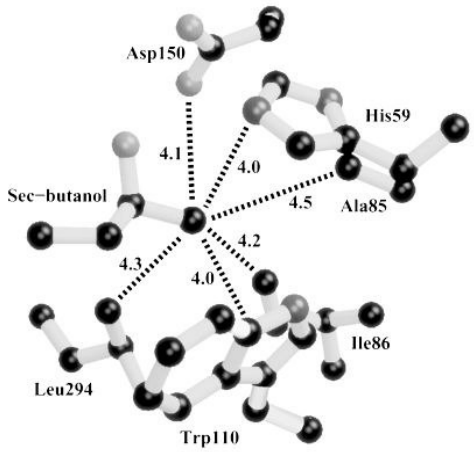


Figure 1.3. Van der Waals interactions of 2-butanol with the active site residue of TbADH. Reprinted with permission from [Li, C. *et al. Proteins* **1999**, *37*, 619-627] © 1999 John Wiley & Sons, Inc.

Recently, we have reported a new mutant of TeSADH, where tryptophan-110 was replaced with alanine (W110A TeSADH).[14] This replacement makes the large pocket in the active site bigger, and it is therefore able to accommodate large substituents like phenyl rings.[15]

This modification makes this mutant able to recognize aromatic ketones and alcohols, which are not substrates for wild–type TeSADH.

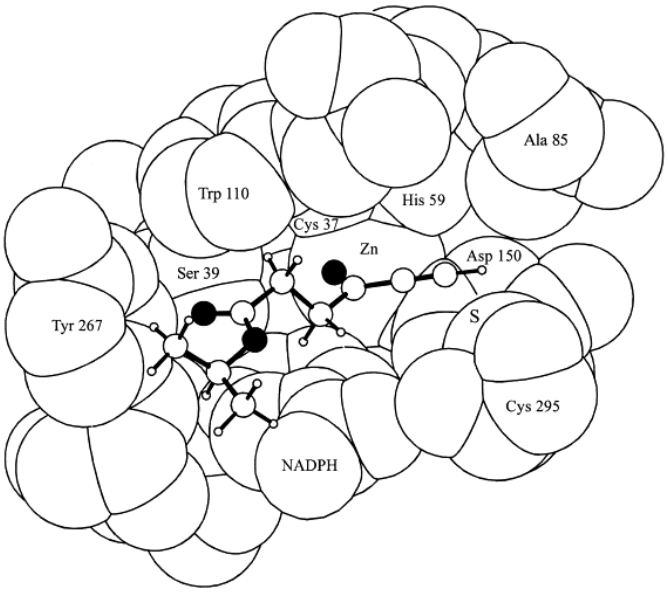


Figure 1.4. Active site model for wild-type TeSADH with isopropyl 4-oxo-5-hexynoate substrate. Reprinted with permission from [Heiss, C. *et al. Bioorg. Med. Chem.* **2001**, *9*, 1659-1666] © 2001 Elsevier Science Ltd.

Sol-gel immobilization of enzymes

Immobilization of an enzyme is its attachment to an inert and insoluble material. There are several ways in which an enzyme can be immobilized, such as absorption in glass or alginate beads, covalent binding, or entrapment in silica gel. Immobilization of enzymes has several advantages including convenient handling of enzymes, enhanced stability, ease of recovery and reuse.[16]

An interesting mild method of enzyme immobilization is the so-called sol-gel method.[16,17] This method allows the synthesis of silica glass at room temperature. It is suitable to large scale operation because the starting materials are inexpensive and non-toxic. In the last few

decades, it has been shown that it is possible to entrap enzymes within these silica glasses without losing biological activity. In 1992, Ellerby *et al.* reported a novel technique for enzyme-immobilization using the sol-gel method that is suitable for encapsulation of proteins because it is mild.[18] This method is initiated by hydrolysis of a tetraalkyl orthosilicate ($Si(OR)_4$) catalyzed by a weak acid or base (Equation 1.1). The most frequently used alkoxide for protein encapsulation is tetramethyl orthosilicate (TMOS). Hydrolysis of TMOS forms silicic acid, $Si(OH)_4$, and methanol, and after subsequent condensation, silica $(SiO_2)_n$ is formed as shown in Equation 1.2. The overall reaction is as shown in Equation 1.3. The condensation is followed by gelation then aging upon addition of the protein to be encapsulated, in buffer solution, to the silica solution. The process of condensation continues during the aging step. The sol-gel formed, known as hydrogel, contains methanol, formed as a side product, and water. Careful drying of the hydrogel leads to the formation of dried gel $(SiO_2)_n$. xH_2O, the so-called xerogel. The xerogel pore diameters range from 1 to 10 nm, which allow small molecules, but not the larger enzymes, to diffuse into and out of the xerogel matrix.

$$Si(OMe)_4 + 4H_2O \xrightarrow{H^+ \text{ or } OH^-} Si(OH)_4 + 4MeOH \qquad (1.1)$$

$$Si(OH)_4 \longrightarrow SiO_2 + 2H_2O \qquad (1.2)$$

$$Si(OMe)_4 + 2H_2O \xrightarrow{H^+ \text{ or } OH^-} SiO_2 + 4MeOH \qquad (1.3)$$

Xerogels retain the water necessary for enzyme activity, which therefore makes this encapsulation process suitable for enzymes. Several reports have shown that enzymes

encapsulated by sol-gel method retain their chemical and biological functionality.[19] However, there are no reports which describe the use of sol-gel-encapsulated ADH in organic solvents for synthetic applications.

Ionic liquids

Ionic liquids (ILs) are liquids that contain only ions.[20] Those that are liquid at room temperature are called room-temperature ionic liquids. They have extremely low vapor pressure, and they are nonflammable. They can be recycled and reused. Due to the aforementioned reasons, room temperature ILs can be a "green" replacement for environmentally harmful organic solvents. Performing enzyme-catalyzed reactions, which are also environmentally friendly, in ILs is of great interest from environmental aspects.

The most popular ILs for biocatalysts are imidazolium-based, like1-butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]), 1-butyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)imide ([bmim][NTf_2]), and 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim][PF_6]) (Figure 1.4). Among these, [bmim][BF_4] is water-miscible, and [bmim][NTf_2] as well as [bmim][PF_6] are water-immiscible.[20b] Several research groups have recently reported that enzyme-catalyzed transformations, including oxidoreduction ones, can be conducted in ILs. It was also reported that an enantioselectivity enhancement was noticed in lipase-catalyzed transesterification reactions in ILs.[21]

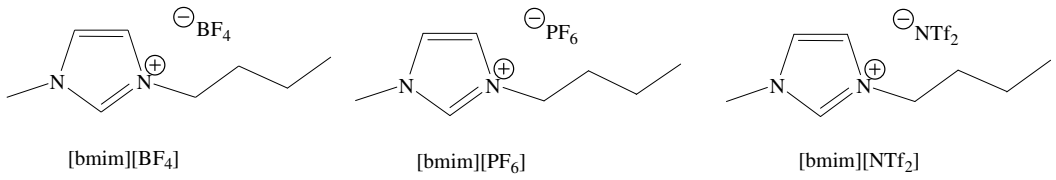


Figure 1.4. Common ionic liquids in enzyme-catalyzed reactions.

In 2004, Eckstein *et al.* reported the first example of asymmetric reduction using ADH in a biphasic system containing [bmim][NTf_2].[22] They also reported that, taking advantage of the partition coefficients of 2-propanol and acetone, 2-propanol preferably remained in the aqueous phase and improved ADH-catalyzed reduction yields were obtained.

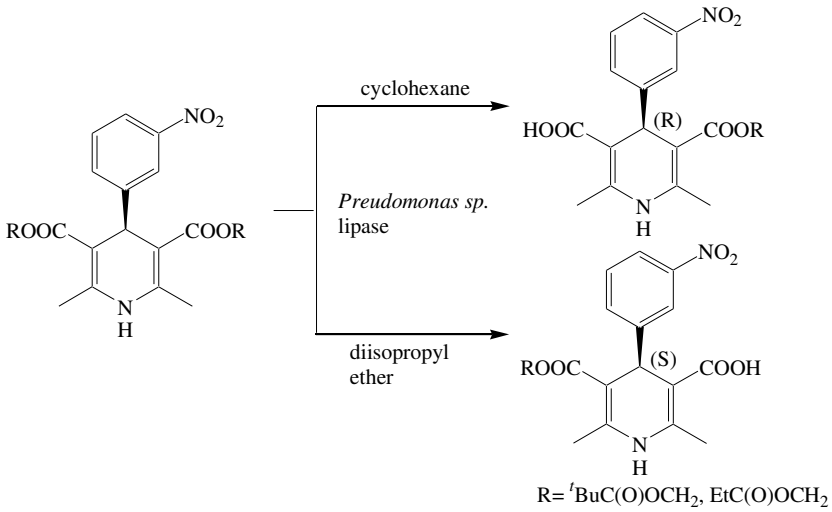
Medium engineering of enzymatic reactions

The switch from water as a solvent to organic solvents in enzyme-catalyzed transformations is necessary in preparative organic chemistry not only because water is a poor solvent for nearly all organic compounds, but also because it has a relatively high boiling point, which makes its removal tedious. The isolated yield can also be improved when using organic solvents because of eliminating an extraction step during the work-up procedure, which may also cause formation of emulsions.

Medium engineering is the possibility of influencing enzyme properties by changing the reaction medium.[23] It can be a substitute to protein engineering.[2] Both yield and enantioselectivity of an enzyme-catalyzed transformation can be affected by changing the reaction medium.[23] Several reports have shown that this is possible for lipases.[2,23,24] In some cases, an enhancement in enantioselectivity was noticed.[21] In other cases, a switch in the stereochemical preference was noticed, like the one reported by Hirose *et al.* for the desymmetrization of prochiral dihydropyridine dicarboxylate catalyzed by *Pseudomonas sp.* lipase (Scheme 1.2).[25]

Cowan and co-workers reported that the enantioselectivity of *Thermoanaerobium sp.* Ket4B1 secondary ADH-catalyzed reductions can be controlled by changing the concentration of the cosolvent used.[26] They used 2-butanone as a substrate and 2-propanol as a cosubstrate and 0-40% (v/v) of acetonitrile, methanol, dimethylformamide, or dimethyl sulfoxide . They have

shown that it is possible to control the enantioselectivity of ADH-catalyzed transformations to a limited extent.[26]



Scheme 1.2. Asymmetric hydrolysis of dihydropyridine: influence of solvent on stereochemical preference.

Various explanations have been proposed for the medium engineering phenomenon.[23] One explanation depends on differences in solvation of enzyme active sites when different solvents with different physicochemical properties are used.[27] This will alter the molecular recognition between substrate and enzyme. Another explanation is that solvent molecules could bind to the enzyme's active site and therefore alter its shape.[23] This will lead to differences in enzyme recognition of one enantiomer and the other. Klibanov and co-workers were the first to report that selectivity of lipase-catalyzed transesterifications can be modified by changing the reaction medium.[28] They also reported that the enantioselectivity correlates inversely with Log *P* (*P* is the partition coefficient between 1-octanol and water, what is called hydrophobicity).[28a] Recently, Filho *et al.* reported that a single physicochemical property is not enough to explain

the biocompatibility of organic solvents in ADH-catalyzed reductions but rather the solvent functionality would be of great significance.[29]

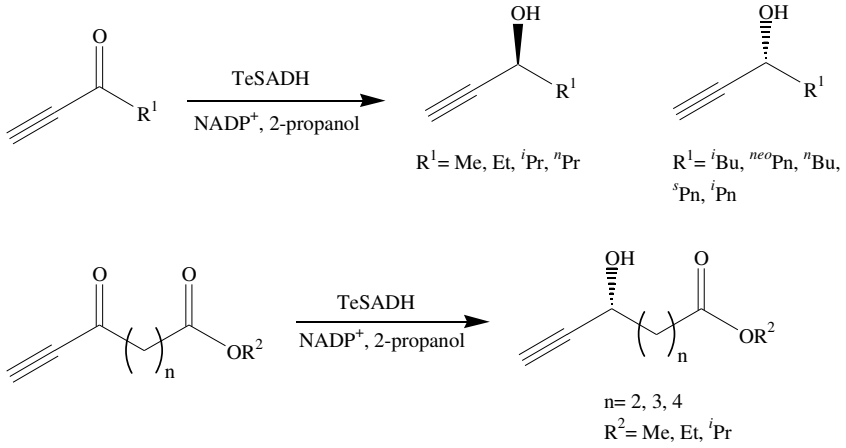
Synthetic applications

It is well-accepted that lipases can function in nonaqueous media containing little water,[2] which makes them an excellent choice for organic chemists to produce optically active alcohols. However, with the exception of dynamic kinetic resolution (DKR), in which the slow reacting enantiomer is racemized *in situ*, lipase-catalyzed reactions suffer from the drawback of being limited to 50% yield with high enantioselectivity. The use of ADHs in asymmetric reduction, as alternatives for lipases, is of great interest because up to 100% conversion of prochiral ketones to the corresponding optically active alcohols with high enantioselectivities can be achieved. This means finding a solution to the problem of poor solubility of hydrophobic substrates in ADH-catalyzed transformations is critical. Various methods have been proposed to overcome this limitation. The most popular method is the use of organic solvents, water-miscible or –immiscible.

Klibanov and co-workers demonstrated the first example of asymmetric oxidoreduction catalyzed by ADH in an organic solvent.[30] They deposited HLADH and its cofactor NAD^+ onto the surface of glass beads, which were then used for asymmetric oxidoreductions in isopropyl ether presaturated with Tris-HCl buffer solution using ethanol in the reduction pathway and isobutyraldehyde in the oxidation pathway to regenerate NADH and NAD^+, respectively. The drawback of this method is the low percent conversion obtained (about 20% after 6 days).

A series of ethynyl ketones and ethynyl ketoesters were reduced enantioselectively to the corresponding non-racemic propargyl alcohols using wild-type TeSADH (Scheme 1.3).[31] These reactions were conducted in aqueous media containing 2-propanol (15% v/v), which acts as a

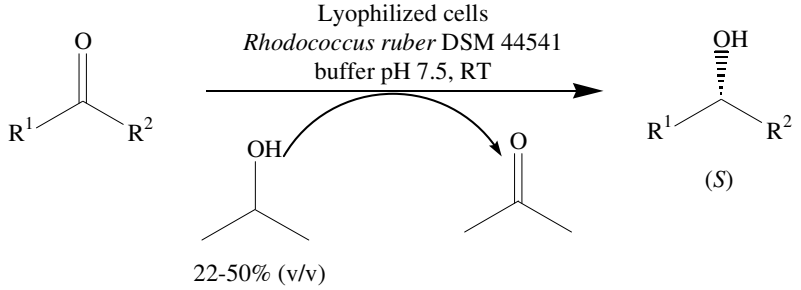
cosolvent and a cosubstrate at the same time. High concentrations of TeSADH were needed because these substrates cause irreversible inactivation of the enzyme. Ethynyl ketones with small alkyl substituents like methyl, ethyl, isopropyl, and propyl were reduced to (*S*)-alcohols, the *anti*-Prelog products. (*R*)-Alcohols were produced from ethynyl ketones with larger alkyl groups. (*R*)-Ethynylhydroxyesters were produced from the asymmetric reduction of ethynylketoesters with excellent optical purities. These results are in agreement with those obtained by Keinan *et al.* in the asymmetric reduction of aliphatic ketones using TbADH.[9]



Scheme 1.3. Asymmetric reduction of ethynyl ketones and ketoesters using TeSADH.

Kroutil and co-workers isolated a secondary ADH from *Rhodococcus ruber* DSM 44541, which is exceptionally stable toward organic solvents.[32] They were able to achieve asymmetric reductions of prochiral ketones by using 2-propanol as the cosubstrate and cosolvent with concentrations up to 50% (v/v) producing (*S*)-configured alcohols (Scheme 1.4). They were also able to produce the (*R*)-configured alcohols through enantiospecific kinetic resolution of the corresponding racemic alcohols. Beside this example, few other ADHs including TeSADH showed high tolerance to organic cosolvents.[3b] Recently, Gonzalo *et al.* reported a method for

enzymatic reduction of ketones catalyzed by *Rhodococcus ruber* ADH-A in micro-aqueous media, monophasic solvent systems composed of ≤1 % water and ≥99 % organic solvent.[33] This method allowed stereoselective enzymatic asymmetric reductions at substrate concentrations as high as 2.0 M.

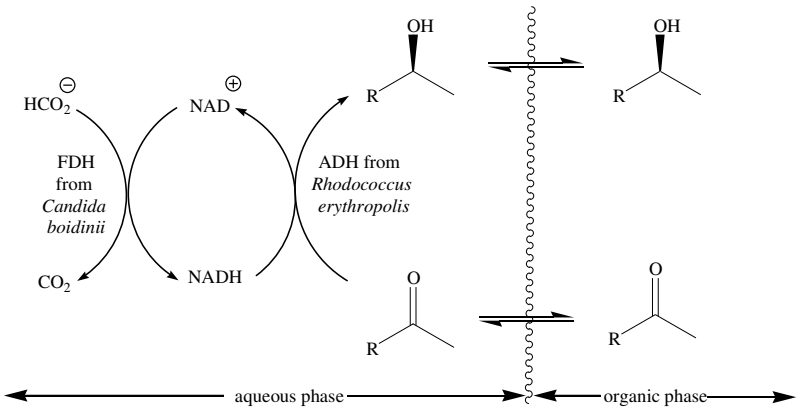


R^1	R^2	% conv.	ee (%)
Me	*n*-C_8H_{17}	94	>99
Me	*n*-C_7H_{15}	94	>99
Me	*n*-C_6H_{13}	92	>99
Me	*n*-C_5H_{11}	92	>99
Et	*n*-C_5H_{11}	79	97
Me	Ph	81	>99
Me	*p*-$MeOC_6H_4$	61	>99
Me	2-naphthyl	82	>99
Me	(*E*)-Ph-HC=CH	52	>99

Scheme 1.4

Gröger *et al*. reported a practical method for asymmetric reductions of poorly water-soluble ketones using *Rhodococcus erythropolis* ADH, an NAD^+-dependent ADH, in water/n-heptane (4:1, v/v) biphasic systems with satisfactory conversions.[34] They used formate dehydrogenase (FDH) from *Candida boidinii* to regenerate the cofactor NADH. They used this method to reduce poorly water-soluble ketones with concentrations up to 200 mM to produce their corresponding (*S*)-alcohols with moderate to good conversions and high ee (Scheme 1.5). The use of biphasic systems in ADH-catalyzed transformations is of great interest because the

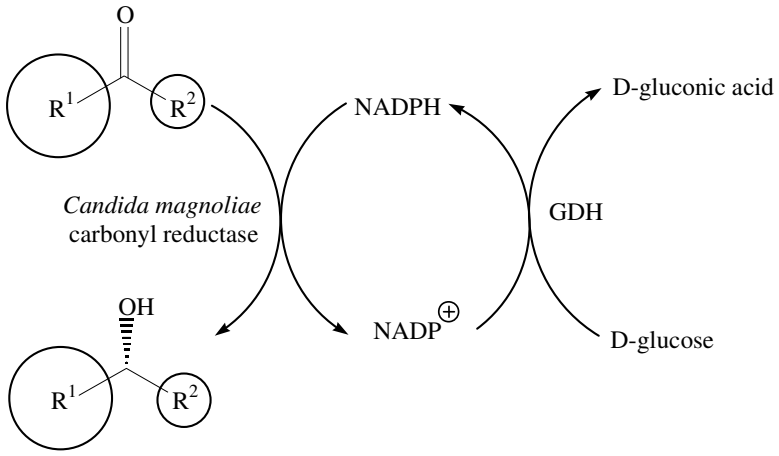
enzyme and its cofactor are dissolved in the aqueous phase, where the reaction takes place, while the reactant, product, cosubstrate, and coproduct are all distributed in the two phases, in most cases preferentially in the nonaqueous phase. This distribution reduces the possibility of enzyme inhibition. Another advantage of using biphasic systems for enzymatic reactions is the ability to recycle the enzyme.

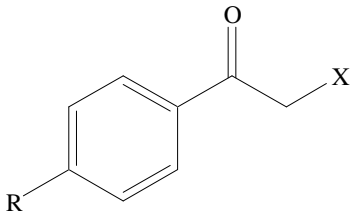


R	conv. (%)	ee (%)
p-ClC_6H_4	69	>99
p-BrC_6H_4	65	97
$PhOCH_2$	95	>99

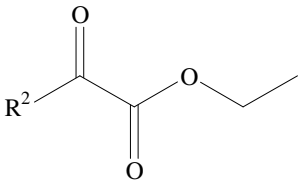
Scheme 1.5

Hua and co-workers reported a stereoselective enzymatic synthesis of optically active alcohols using a carbonyl reductase from *Candida magnoliae*, an $NADP^+$-dependent oxidoreductase.[35] They were able to reduce a diversity of ketones with high enantioselectivity to produce the *anti*-Prelog alcohols (Scheme 1.6). They used D-glucose dehydrogenase (GDH) and D-glucose to regenerate NADPH. This example is one of the few oxidoreductases that showed *anti*-Prelog enantioselectivity, which works as a complement to the others with Prelog enantioselectivity.

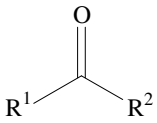




X	R	yield (%)	ee (%)
H	F	89	>99
H	CF_3	94	>99
Cl	H	92	>99



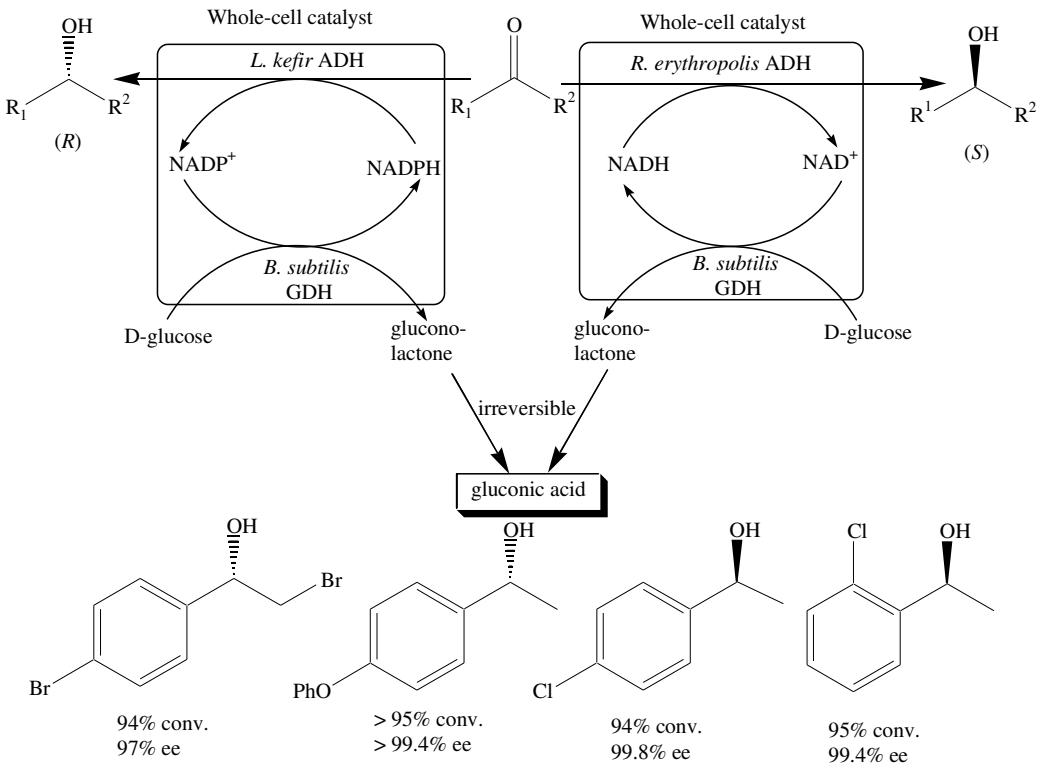
R^2	yield (%)	ee (%)
$CH(CH_3)_2$	87	>99
$C(CH_3)_3$	91	99



R^2	R^1	yield (%)	ee (%)
Me	$(CH_2)_5CH_3$	92	>99
Et	$(CH_2)_4CH_3$	90	>99

Scheme 1.6

Gröger *et al.* reported a practical biocatalytic reduction that proceeds at high substrate concentrations (>100 gL^{-1}) without adding an external cofactor.[36] They used "designer cells" containing either *Rhodococcus erythropolis* ADH, an (*S*)-selective ADH, or *Lactobacillus kefir* ADH, an (*R*)-selective ADH, as well as *Bacillus subtilis* GDH, which regenerates the cofactor NAD(P)H in situ. This method was applied to produce the desired optically active alcohol with high conversions and excellent enantioselectivities in pure aqueous media using D-glucose as the reducing agent (scheme 1.7).

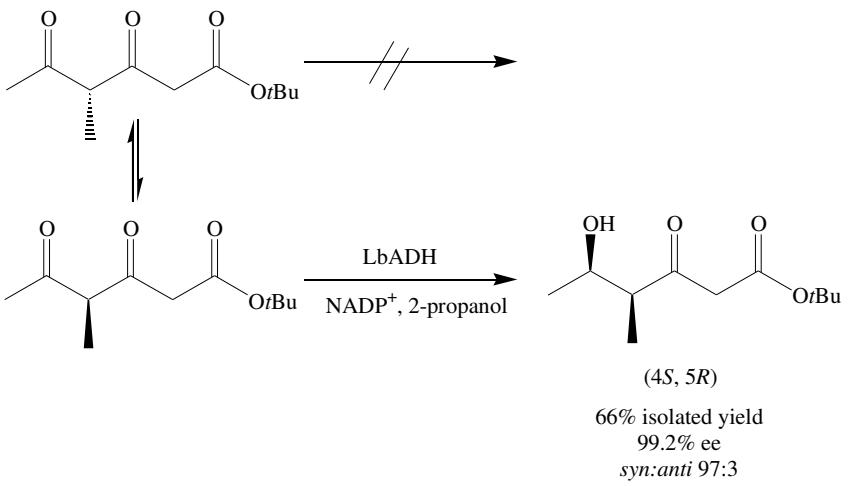


Scheme 1.7

It has also been shown that intact cells from cut plants, vegetables and fruits can be used in asymmetric reduction of prochiral ketones.[2a] A major drawback for this method is the slow rate of growth. On the other hand, the whole cells ensure the recycling of the oxidized cofactor. One of the most successful applications found in literature is the use of *Daucus carota* carrot.[37] A recent emerging area article by Blanchard and Weghe was devoted to explain the recent results obtained in *Daucus carota* mediated bioreduction of prochiral ketones.[38]

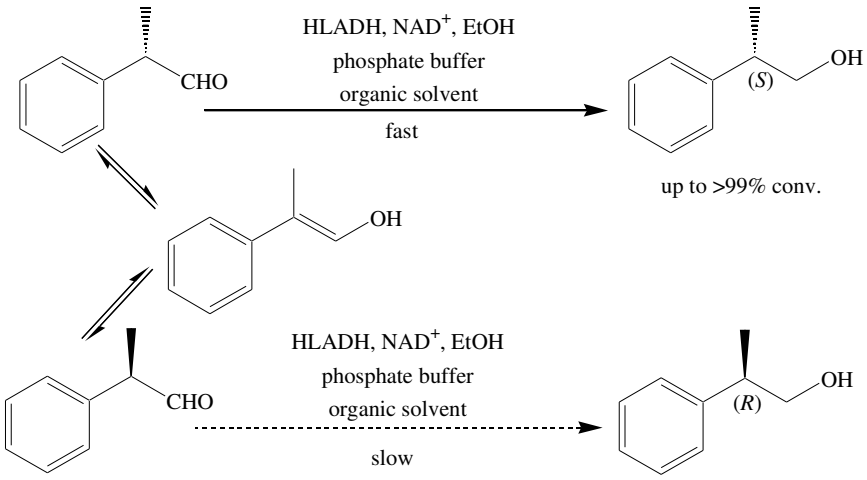
Recently, several examples of DKR through enzymatic reductions have been reported. Müller and co-workers reported a regio- and enantioselective reduction of racemic *tert*-butyl 4-methyl-3,5-dioxohexanate by using *Lactobacillus brevis* ADH (LbADH), an $NADP^+$-dependent ADH (Scheme 1.8).[39] They noticed that the reduction proceeded via DKR with regioselective monoreduction of the keto group at C-5 to give almost enantiomerically and diastereomerically

pure compound with 66% conversion, which indicates the process involves in situ racemization for the slow reacting enantiomer.



Scheme 1.8

Giacomini *et al.* reported a highly efficient reduction of arylpropionic aldehydes by using HLADH through DKR.[40] They were able to produce (2*S*)-2-phenylpropanol and (2*S*)-2-(4-*iso*-butylphenyl)propanol ((*S*)-Ibuprofenol) with good yields and enantiomeric ratios (Scheme 1.9).



Scheme 1.9

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CHAPTER 2

ASYMMETRIC REDUCTION AND OXIDATION OF AROMATIC KETONES AND ALCOHOLS USING W110A SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS*[1]

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[1] Musa, M. M.; Ziegelmann-Fjeld, K. I.; Vieille, C.; Zeikus, J. G.; Phillips, R. S. *J. Org. Chem.*, **2007**, *72*, 30-34.

Abstract

An enantioselective asymmetric reduction of phenyl ring-containing prochiral ketones to yield the corresponding optically active secondary alcohols was achieved with W110A secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (W110A TeSADH) in Tris-HCl buffer using 2-propanol (30%, v/v) as cosolvent and cosubstrate. This concentration of 2-propanol was crucial not only to enhance the solubility of hydrophobic phenyl ring-containing substrates in the aqueous reaction medium, but also to shift the equilibrium in the reduction direction. The resulting alcohols have *S*-configuration, in agreement with Prelog's rule, in which the nicotinamide-adenine dinucleotide phosphate (NADPH) cofactor transfers its *pro*-R hydride to the *re* face of the ketone. A series of phenyl ring-containing ketones, such as 4-phenyl-2-butanone (**1a**) and 1-phenyl-1,3-butadione (**2a**), were reduced with good to excellent yields and high enantioselectivities. On the other hand, 1-phenyl-2-propanone (**7a**) was reduced with lower ee than 2-butanone derivatives. (*R*)-Alcohols, the *anti*-Prelog products, were obtained by enantiospecific oxidation of (*S*)-alcohols through oxidative kinetic resolution of the *rac*-alcohols using W110A TeSADH in Tris-HCl buffer/acetone (90:10, v/v).

Introduction

Tremendous efforts have been made in recent years to establish enantioselective routes to enantiomerically pure compounds, due to their importance in pharmaceutical, agricultural, and food industries.[1] Recent developments in medicine have shown that a single enantiomer is biologically active in most chiral drugs.[2] Optically active alcohols are one of the most important synthons. They can be produced from their corresponding prochiral ketones via asymmetric reduction, or from their racemic alcohols via enantiospecific kinetic resolution (KR).[3,4] Chiral metal complexes have been used as catalysts for these purposes,[5] however, these methods

produce toxic residual metals that create environmental problems. Enzymes are recognized to be among the most effective catalysts for producing optically active alcohols. Among their advantages are their chemo-, regio-, and stereoselectivities due to the strict recognition of a particular substrate by a given enzyme. Biocatalytic processes also are less hazardous and energy consuming than conventional chemistry methodologies. They are normally carried out under mild conditions, which minimize problems of product isomerization, racemization, or epimerization. Biocatalysts are easily produced at low cost and with minimum waste, and they can be decomposed in the environment after use. Unfortunately, they do have some disadvantages. For example, many enzymes are thermally unstable. Another disadvantage is the limited solubility of most organic substrates in water; this leads to larger reaction volumes, a need for cosolvents, and complicated product recovery.[6]

Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X=1 or 2) are enzymes that catalyze the reversible reduction of ketones and aldehydes to the corresponding alcohols. The asymmetric reduction of ketones using the commercially available yeast ADH and horse liver ADH is limited not only due to their temperature sensitivity, but also due to their sensitivity towards organic solvents and their loss of activity upon immobilization. An additional disadvantage of horse liver ADH is its low affinity for acyclic ketones.[7,1b] Secondary ADH from *Thermoanaerobacter ethanolicus* (TeSADH, EC 1.1.1.2), a highly thermostable enzyme, has been isolated and characterized.[8] NADPH is required by this enzyme, from which the hydride is transferred to the carbonyl carbon. Since NADPH is a costly cofactor, alcohols like 2-propanol or ketones like acetone are used as hydrogen source or hydrogen sink to regenerate the cofactor and therefore make both processes catalytic. This enzyme is stable at temperatures up to 80 ºC and it exhibits high activity in the asymmetric reduction of ketones.[9] Because of its thermostabilty, resistance to

organic solvents, and reactivity for a wide variety of substrates, it is a useful biocatalyst for synthetic applications.[10]

A series of ethynyl ketones and ethynylketoesters were reduced enantioselectively to the corresponding non-racemic propargyl alcohols using wild-type TeSADH.[10a] The behavior of TeSADH has been shown to be similar to results obtained from reductions with a very highly homologous (99% identity),[8b] NADPH-dependent, *Thermoanaerobium brockii* ADH (TbADH).[11] For TbADH, Keinan *et al.* suggested that the two alkyl groups of substrates occupy two hydrophobic sites which differ from one another in volume and also in their affinities toward the alkyl groups (Figure 2.1).[11] It was also shown that the small site, which has higher affinity toward the alkyl groups of the ketone, can accommodate up to three carbon substituents, like the isopropyl group.[10a,b,11]

We have recently reported a new mutant of TeSADH, where tryptophan-110 was substituted by alanine, (W110A TeSADH).[12] This replacement makes the large pocket able to accommodate phenyl ring-containing substrates that are not substrates for wild-type TeSADH.[10b] Its modified substrate range makes this mutant enzyme useful for the enantioselective reduction of phenyl ring-containing ketones such as 4-phenyl-2-butanone (**1a**) and, in the reverse direction, for the enantiospecific oxidation via KR of racemic phenyl ring-containing secondary alcohols.

Results and Discussion

A series of phenyl ring-containing ketones, which could not be reduced by wild-type TeSADH, were reduced by W110A TeSADH to produce the corresponding non-racemic alcohols with good yields and high optical purities (Table 2.1). The reductions were carried out in Tris-HCl buffer containing 30% (v/v) 2-propanol, which serves as both cosolvent and hydride

source to reduce the oxidized coenzyme. The use of such a high percentage of 2-propanol was crucial not only to enhance the solubility of the hydrophobic phenyl ring-containing ketone substrates in aqueous media, but also to shift the equilibrium into the reduction direction. The produced alcohols had *S* configuration, in agreement with Prelog's rule, in which the NADPH cofactor transfers its *pro*-R hydride to the *re* face of the ketone (Figure 2.1).[13,1b,c]

Phenyl ring-containing 2-butanone derivatives were reduced to the corresponding (*S*)-alcohols with excellent stereoselectivities and moderate to excellent yields (Table 2.1). 4-Phenyl-2-butanone (**1a**) was reduced stereoselectively to produce (*S*)-4-phenyl-2-butanol ((*S*)-**1b**) with excellent chemical and optical yields. The *β*–diketone 1-phenyl-1,3-butadione (**2a**) was reduced regio- and stereoselectively to furnish the monohydroxy ketone (*S*)-3-hydroxy-1-phenyl-1-butanone ((*S*)-**2b**) with excellent yield and ee, leaving the other keto group at C-1 intact. (*E*)-4-Phenyl-3-butene-2-one (**3a**) was reduced with moderate yield and excellent optical purity to produce the allylic alcohol (*S*)-4-phenyl-3-butene-2-ol ((*S*)-**3b**). The presence of the methoxy group at the para position of the phenyl ring in 4-(4′-methoxyphenyl)-2-butanone (**4a**) affected the ee of the produced (*S*)-4-(4′-methoxyphenyl)-2-butanol ((*S*)-**4b**) (91% ee), which is lower than for (*S*)-**1b**. Phenoxy-2-propanone (**5a**) was reduced with very high yield and optical purity to produce the corresponding (*S*)-phenoxy-2-propanol ((*S*)-**5b**). When the α-chloroketone, 3-Chloro-4-(4′-chlorophenyl)-2-butanone (**6a**), was reduced with W110A TeSADH, (+)-(2*S*,3*R*)-3-chloro-4-(4′-chlorophenyl)-2-butanol ((+)-(2*S*,3*R*)-**6b**) was produced with high enantioselectivity (>99% ee) and diastereoselectivity (92:8 mixture of *anti* and *syn*-α-chlorohydrins). The absolute configuration of (+)-(2*S*,3*R*)-**6b** was confirmed by comparing the sign of the optical rotation with that reported previously for the very similar compound, (+)-(2*S*,3*R*)-4-phenyl-3-bromo-2-butanol) ($[\alpha]^{20}_{D}$ +29.2, *c* 2.08, $CHCl_3$; lit.[14] $[\alpha]^{25}_{J}$ +37, *c* 0.06 , $CHCl_3$, 95% ee). In a separate

experiment, reduction of **6a** with $NaBH_4$, which is expected to give mainly the *syn* product,[15] afforded a mixture of four diastereomers ((±)-**6b**) (88:12 mixture of *syn*- and *anti*-α-chlorohydrins), in which the *syn*-**6b** had a different retention time than (+)-(2*S*,3*R*)-**6b** by injection in a chiral column GC (Figure 2.2a,b). Reduction of **6a** to almost a single stereoisomer, (+)-(2*S*,3*R*)-**6b**, using W110A TeSADH indicated that the process involves a KR, and this should be combined with isolation of (*S*)-**6a** as unreacted enantiomer and a maximum yield of 50% of the produced α-chlorohydrin. We have noticed that the yield is higher than 50%, and the isolated unreacted **6a** is a racemic mixture. This indicates that the reduction of **6a** with W110A TeSADH proceeds by dynamic kinetic resolution via a facile buffer-catalyzed enolization, which enables the unreacted enantiomer (*S*)-**6a** to racemize after the depletion of (*R*)-**6a** starts.[16] The α-chlorohydrin (+)-(2*S*,3*R*)-**6b** was then converted quantitatively to the corresponding epoxide, (-)-(2*S*,3*S*)-4-(4′-chlorophenyl)-2,3-epoxybutane ((-)-(2*S*,3*S*)-**6c**), without racemization using 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (Scheme 2.1, Figure 2.2c).[3d] The absolute configuration of (-)-(2*S*,3*S*)-**6c** was confirmed by the comparison of the sign of optical rotation with that reported for the very similar compound (-)-(2*S*,3*S*)-4-phenyl-2,3-epoxybutane ($[\alpha]^{20}_{D}$ -26.2, *c* 2.32, $CHCl_3$; lit.[14] $[\alpha]^{25}_{J}$ -27, *c* 0.04 , $CHCl_3$, >98% ee).

Unexpectedly, 1-phenyl-2-propanone (**7a**) was reduced to produce (*S*)-1-phenyl-2-propanol ((*S*)-**7b**) with poor enantioselectivity, indicating that **7a** can fit in alternative modes in the active site within the large pocket allowing the NADPH cofactor to deliver its *pro*-R hydride from either *re* or *si* faces. 1-(4′-Methoxyphenyl)-2-propanone (**8a**) was reduced to produce (*S*)-1-(4′-methoxyphenyl)-2-propanol ((*S*)-**8b**) with excellent chemical yield and ee, which means that the sterically bulky para methoxy substituent in **8a** restricts the substrate to only a single binding mode within the active site. The cyclic ketone 2-tetralone (**9a**) was reduced with high yield and

moderate stereopreference to produce (*S*)-2-tetralol ((*S*)-**9b**). Enzymatic asymmetric reduction of substrates with sterically hindered groups on both sides of the carbonyl, like **9a**, is of great interest because these substrates are typically either poor or non-substrates for ADHs, therefore very few ADHs are able to achieve such asymmetric reductions.[1b,3c]

Oxidation via KR of phenyl ring-containing *rac*-alcohols was used to produce their (*R*)-alcohols, the *anti*-Prelog configurated alcohols, as unreacted enantiomers with moderate to high enantiomeric ratios using W110A TeSADH. The reactions were carried out in Tris-HCl buffer containing 10% (v/v) acetone. The amount of acetone needed was less than the amount of 2-propanol used in the reduction pathway simply because alcohols are more soluble than their corresponding hydrophobic ketones in aqueous media. As with all KRs, these reactions suffer from the limitation that the maximum theoretical yield with high enantiomeric ratio of a single enantiomer, (*R*) in this case, is 50% (Table 2.2). As expected, the substrates reduced with high ee showed high stereospecificities in the oxidation pathway and *vice versa*.

The enantiospecific oxidation via KR using W110A TeSADH exclusively oxidized the *S* enantiomers of *rac*-**1b** and *rac*-**3b** to the corresponding ketones **1a** and **3a,** respectively, leaving their (*R*)-alcohols as unreacted enantiomers with excellent enantiomeric ratios (Table 2.2). The production of optically active **1b** is important as it is a precursor for antihypertensive agents, such as bufeniode and labetalol.[3b,17] For *rac*-**4b**, it was resolved by oxidative KR to furnish (*R*)-**4b** with moderate stereopreference (77% ee at 75% conversion). Under the same conditions, KR of *rac*-**5b** furnished (*R*)-**5b** with 25% ee at only 19% conversion, indicating that the KR of this alcohol takes place with high enantiomeric discrimination. Even with addition of more enzyme and acetone, we were not able to push the reaction to higher yield. The racemic 1-phenyl-2-propanol (*rac*-**7a**) was resolved, as expected, with low enantiospecificity because it was reduced

with low ee. (*S*)-1-(4-Methoxyphenyl)-2-propanol ((*S*)-**8b**) was oxidized with excellent enantiospecificity to its corresponding ketone **8a** leaving (*R*)-**8b** as enantiomerically pure unreacted enantiomer. Although **9a** was reduced with high yield and moderate ee, *rac*-**9b** was not oxidized by W110A TeSADH. The same results for *rac*-**9b** were obtained by Stampfer *et al.* using *Rhodococcus ruber* DSM 44541.[3b]

Resistance of TeSADH to organic cosolvents allowed the redox reactions in both directions to be carried out at relatively high substrate concentration (35 mM in the reduction pathway and 70 mM in the oxidation pathway). The design of new TeSADH mutants such as W110A TeSADH in addition to TeSADH's resistance to organic solvents and high concentrations of substrates make this enzyme useful for synthetic applications.

Experimental Section

General procedures

Capillary gas chromatographic measurements were performed on a GC equipped with a flame ionization detector and a Supelco β–Dex 120 chiral column (30 m, 0.25 mm [i.d.], 0.25 μm film thickness) using Helium as the carrier gas. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz spectrometer at room temperature in $CDCl_3$ using either solvent peak or tetramethylsilane as internal standard. Column chromatographies were carried out on standard grade silica gel (60Å, 32-63μm) with ethyl acetate in hexane as eluent.

Materials

Commercial grade solvents were used without further purification. $NADP^+$, Novozyme 435, and $NaBH_4$ were used as purchased from commercial sources. Substrates **1a-6a**, **9a**, *rac*-**1b,** *rac*-**7b,** (*R*)-**7b**, and (*S*)-**7b** were used as purchased from commercial suppliers. **7a** and **8a** were

prepared as described previously.[18] *rac*-**3b,** *rac*-**4b,** *rac*-**5b**, *rac*-**8b,** and *rac*-**9b** were prepared by reducing the corresponding ketones with $NaBH_4$.[19]

Gene expression and purification of W110A TeSADH. W110A TeSADH was expressed in recombinant *E. coli* HB101(DE3) cells and purified as described.[12]

General procedure for asymmetric reduction of phenyl-ring-containing ketones with W110A TeSADH: Reactions were conducted with 0.34 mmol of substrate, 2 mg $NADP^+$, and 0.75 mg of W110A TeSADH in 10.0 mL of 50 mM Tris-HCl buffer (pH 8.0)/2-propanol (70:30, v/v). The reaction mixture was stirred at 50 ºC for 10 h then it was extracted with 3 × 5 mL CH_2Cl_2. The combined organic layers were dried with Na_2SO_4 and concentrated under vacuum. The remaining residue was analyzed by chiral column GC to determine the percent conversion and ee of the produced alcohols then purified with silica gel using hexane/ethyl acetate (85/15) (95/5 for **6b**).

(*S*)-4-Phenyl-2-butanol ((*S*)-1b): $[\alpha]^{20}_D$ +16.5 (*c* 1.81, $CHCl_3$) >99% ee, lit.[20] $[\alpha]_D$ +17.4 *c* 1.80, $CHCl_3$) 99% ee. Spectral data were consistent with those reported.[21]

(*S*)-3-Hydroxy-1-phenyl-1-butanone ((*S*)-2b): $[\alpha]^{20}_D$ +61.5 (*c* 1.65, $CHCl_3$) >99% ee, lit.[22] $[\alpha]^{25}_D$ +48.5 (*c* 2.8, $CHCl_3$) 77% ee. Spectral data were consistent with those reported.[23]

(*S*)-4-Phenyl-3-butene-2-ol ((*S*)-3b): $[\alpha]^{20}_D$ -33.1 (*c* 1.02, $CHCl_3$) >99% ee, (*R*)-**3b** lit.[24] $[\alpha]^{20}_D$ +27.0 (*c* 0.5, $CHCl_3$) 84% ee. Spectral data were consistent with those reported.[25]

(*S*)-4-(4'-Methoxyphenyl)-2-butanol ((*S*)-4b): $[\alpha]^{20}_D$ +12.8 (*c* 2.41, $CHCl_3$) 91% ee, lit.[26] $[\alpha]^{20}_D$ +30.9 (*c* 1.0, $CHCl_3$) 94% ee. Spectral data were consistent with those reported.[26]

(*S*)-Phenoxy-2-propanol ((*S*)-5b): $[\alpha]^{20}_D$ +30.7 (*c* 1.32, $CHCl_3$) >99% ee, lit.[27] $[\alpha]^{20}_D$ +28.9 (*c* 1.10, $CHCl_3$) 99% ee. Spectral data were consistent with those reported.[28]

(-)-(2*S*,3*R*)-3-Chloro-4-(4′-chlorophenyl)-2-butanol ((-)-(2*S*,3*R*)-6b): $[\alpha]^{20}_{D}$ +29.2 (*c* 2.08 , $CHCl_3$) >99% ee, 84% de; ^{1}H NMR, δ: 1.33 (d, 3H, *J*= 6.4 Hz), 1.91 (brs, 1H), 2.91 (dd, 1H, *J*= 14.6 Hz, *J*= 9.8 Hz), 3.10 (dd, 1H, *J*=14.6 Hz, *J*= 4.2 Hz), 3.96 (qd, 1H, J= 6.4, J= 4.0), 4.14 (dt, 1H, *J*= 9.6, *J*= 4.0), 7.17 (d, 2H, *J*= 8.0), 7.29 (d, 2H, *J*= 8.0); ^{13}C NMR, δ: 18.8, 39.2, 69.4, 70.3, 128.9, 130.8, 132.9, 136.3; HRMS calcd for $C_{10}H_{12}OCl_2$ $[M + H]^+$, 219.0343; found, 219.0347.

(*S*)-1-Phenyl-2-propanol ((*S*)-7b): $[\alpha]^{20}_{D}$ +14.5 (*c* 1.04, $CHCl_3$) 37% ee, lit.[29] $[\alpha]^{25}_{D}$ +42.2 (*c* 1.0, $CHCl_3$) >99% ee. Spectral data were consistent with those reported.[30]

(*S*)-4-(4′-Methoxyphenyl)-2-propanol ((*S*)-8b): $[\alpha]^{20}_{D}$ +16.3 (*c* 1.86, $CHCl_3$) >99% ee, lit.[31] $[\alpha]^{20}_{D}$ +27.0 (*c* 4.40, $CHCl_3$) 95% ee). Spectral data were consistent with those reported.[29]

(*S*)-2-Tetralol ((*S*)-9b): $[\alpha]^{20}_{D}$ -43.77 (*c* 0.911, $CHCl_3$) 71% ee, lit.[3b] $[\alpha]^{20}_{D}$ -29.6 (*c* 0.50, $CHCl_3$) 85% ee). Spectral data were consistent with those reported.[32]

General procedure for kinetic resolution of phenyl-ring-containing racemic alcohols with W110A TeSADH: Reactions were conducted with 0.34 mmol of substrate, 1 mg $NADP^+$, and 0.38 mg of W110A TeSADH in 5.0 mL of 50 mM Tris-HCl buffer/acetone (90:10) (v/v). The reaction mixture was stirred at 50 ºC for 12 h then it was extracted with 3 × 5 mL CH_2Cl_2. The combined organic layers were dried with Na_2SO_4 and concentrated under vacuum. The remaining residue was analyzed by chiral stationary phase GC to determine the percent conversion to ketone and ee of the unreacted (*R*)-alcohol.

Synthesis of (-)-(2*S*,3*S*)-4-(4′-Chlorophenyl)-2,3-epoxybutane ((-)-(2*S*,3*S*)-6c): It was prepared from (2*S*,3*R*)-**6b** using a previously reported procedure for epoxidation.[3d] $[\alpha]^{20}_{D}$ -26.2 (*c* 2.32, $CHCl_3$) >99% ee, 84% de; ^{1}H NMR, δ: 1.23 (d, 3H, *J*= 5.2 Hz), 2.71-2.80 (m, 4H), 7.10 (d, 2H,

J= 8.4), 7.20 (d, 2H, *J*= 8.8); ^{13}C NMR, δ: 17.1, 37.9, 54.6, 59.6, 128.8, 130.5, 132.6, 136.1; HRMS calcd for $C_{10}H_{11}OCl$ $[M + H]^+$, 183.0576; found, 183.0571.

Determination of absolute configuration

The absolute configurations of the following compounds were determined by comparing of the sign of the optical rotation with that reported in the literature: (*S*)-**1b**,[20] (*S*)-**2b**,[22] (*S*)-**3b**,[24] (*S*)-**4b**,[26] (*S*)-**5b**,[27] (*S*)-**7b**,[29] (*S*)-**8b**,[31] and (*S*)-**9b**.[3b] The absolute configuration of (*S*)-**7b** was also demonstrated by coinjection on a chiral column GC with commercially available (*R*)-**7b** and (*S*)-**7b**. The absolute configuration of (*S*)-**1b** was confirmed by coinjection on a chiral column GC with (*R*)-**1b,** which was prepared by KR of *rac*-**1b** using Novozyme 435.[33] The absolute configurations of (*R*)-**1b**, (*R*)-**3b**, (*R*)-**4b**, (*R*)-**5b,** (*R*)-**7b**, and (*R*)-**8b** were elucidated by coinjection on GC using a chiral stationary phase with their *S* enantiomers prepared from asymmetric reduction of the corresponding ketones using W110A TeSADH.

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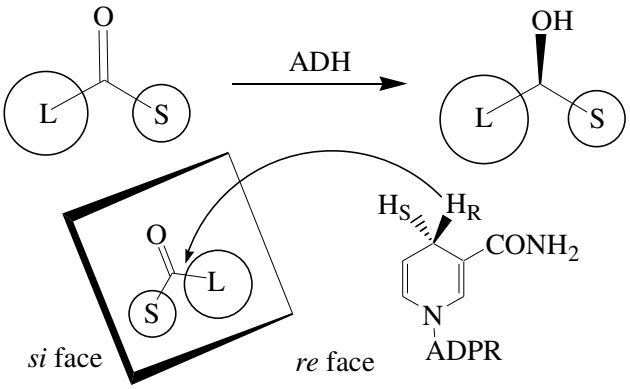


Figure 2.1. Prelog's rule for predicting the stereochemistry of alcohols formed from their corresponding ketones by asymmetric reduction with ADHs.

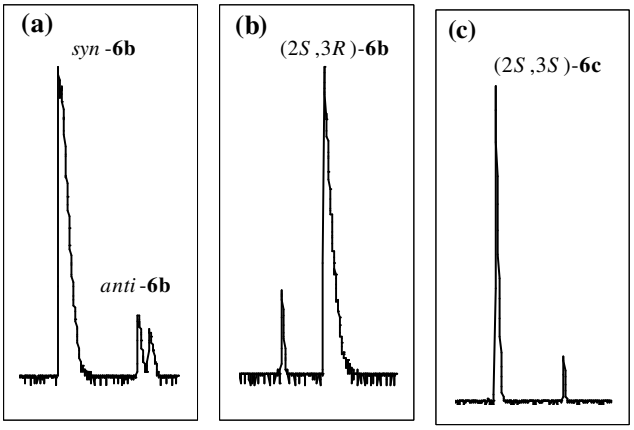
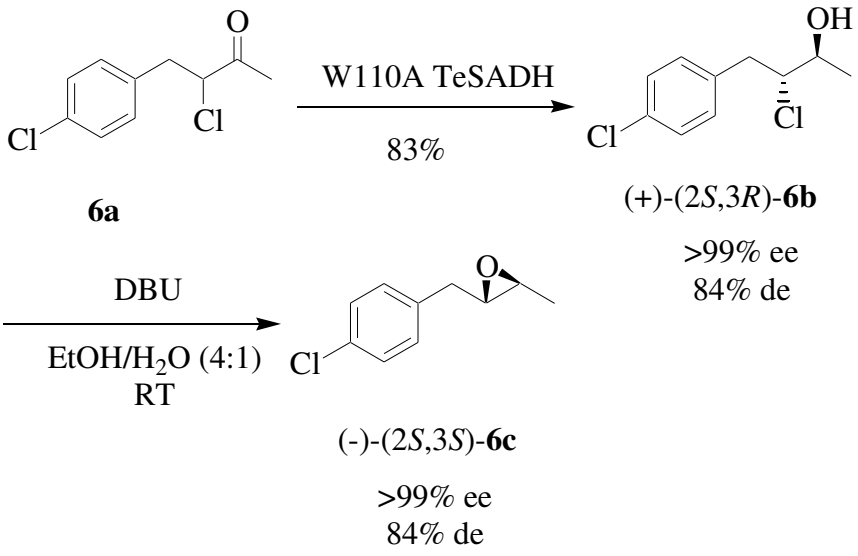
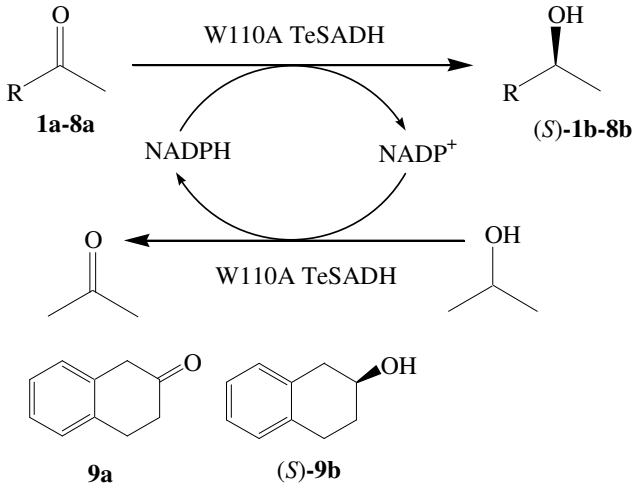


Figure 2.2. GC chromatograms illustrating**:** a: the products of $NaBH_4$ reduction of **6a**. b: the products of W110A TeSADH reduction of **6a**. c: (-)-(2*S*,3*S*)-**6c** produced from (+)-(2*S*,3*R*)-**6b**.



Scheme 2.1. Conversion of (+)-(2*S*,3*R*)-**6b** into (-)-(2*S*,3*S*)-**6c.**

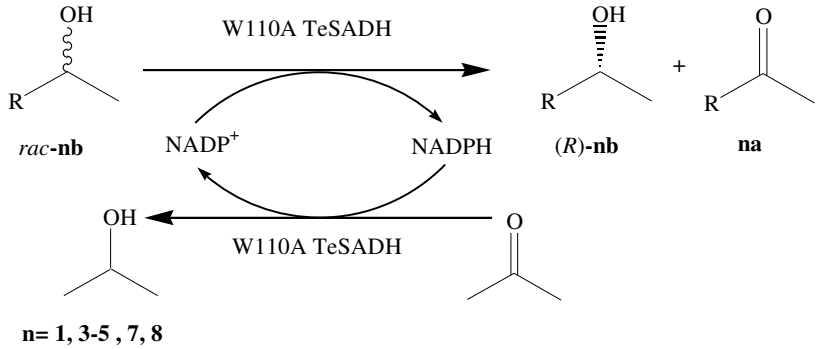
Table 2.1. Asymmetric reduction of phenyl ring-containing ketones using W110A TeSADH.



substrate	R	product[a]	conv. (%)[b]	ee (%)[d]
1a	$PhCH_2CH_2$	(*S*)-**1b**	99	>99
2a	$Ph(C{=}O)CH_2$	(*S*)-**2b**	98	>99
3a	(*E*)-Ph-HC=CH	(*S*)-**3b**	64	>99
4a	*p*-$MeOC_6H_4(CH_2)_2$	(*S*)-**4b**	87	91
5a	$PhOCH_2$	(*S*)-**5b**	>99	>99[e]
6a	*p*-$ClC_6H_4CH_2CHCl$	(2*S*,3*R*)-**6b**	83[c]	>99
7a	$PhCH_2$	(*S*)-**7b**	95	37[e]
8a	*p*-$MeOC_6H_4CH_2$	(*S*)-**8b**	97	>99[e]
9a		(*S*)-**9b**	>99	71[e]

[a] The absolute configurations of the products were determined by comparison of the signs of the optical rotation with those reported previously. [b] % conversion was determined by GC. [c] Isolated yield. [d] Unless otherwise mentioned, ee was determined by chiral stationary phase GC for the produced alcohol. [e] ee was determined for the corresponding acetate derivative.[25]

Table 2.2. Enantiospecific kinetic resolution of phenyl-ring-containing *rac*-alcohols using W110A TeSADH.



substrate	R	product[a]	conv. (%)[b]	ee (%)[c]
rac-**1b**	$PhCH_2CH_2$	(*R*)-**1b**	50	>99
rac-**3b**	(*E*)-Ph-HC=CH	(*R*)-**3b**	50	>99
rac-**4b**	*p*-$MeOC_6H_4(CH_2)_2$	(*R*)-**4b**	75	77[d]
rac-**5b**	$PhOCH_2$	(*R*)-**5b**	19	25
rac-**7b**	$PhCH_2$	(*R*)-**7b**	49	39[d]
rac-**8b**	*p*-$MeOC_6H_4CH_2$	(*R*)-**8b**	48	92[d]

[a] The absolute configurations of the unreacted alcohols were confirmed by coinjection in a chiral column GC with their *S* enantiomers prepared by the asymmetric reduction of the corresponding ketones employing W110A TeSADH (Table 2.1). [b] % conversion was determined by GC. [c] Unless otherwise mentioned, ee was determined by a chiral stationary phase GC for the alcohols. [d] ee was determined for the corresponding acetate derivative.[25]

CHAPTER 3

XEROGEL-ENCAPSULATED W110A SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS* PERFORMS ASYMMETRIC REDUCTION OF HYDROPHOBIC KETONES IN ORGANIC SOLVENTS[1]

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[1]Musa, M. M.; Ziegelmann-Fjeld, K. I.; Vieille, C.; Zeikus, J. G.; Phillips, R. S. *Angew. Chem. Int. Ed.*, **2007**, *46*, 3091-3094 .

Abstract

The asymmetric reduction of hydrophobic ketones by xerogel immobilized W110A secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* in organic solvents afforded their (*S*)-alcohols in comparable yields to those achieved using the free enzyme, and, in some cases, with higher enantioselectivities. The use of xerogel-encapsulated ADH is a facile method as it allows the reuse of the enzyme, it makes it more stable, and it can affect its enantioselectivity by switching to organic solvents.

Introduction

The use of biocatalysts in organic synthesis has become an effective and sometimes preferable alternative to normal chemical methodologies for the production of optically active compounds.[1,2] The asymmetric reduction of ketones and the kinetic resolution (KR) of racemic alcohols are the most important reactions for producing optically active alcohols that then can be used to synthesize industrially important compounds like natural products.

A practical technique to improve enzyme performance is enzyme immobilization.[3] Most enzyme-immobilization methods involve covalent attachment of the enzyme to an activated group on a solid or gel support, which may result in significant loss of activity. A simple and efficient non-covalent immobilization method is enzyme encapsulation in transparent porous silicate glasses prepared by the sol-gel method.[4] The resulting glasses allow the transport of small molecules, but not enzyme molecules, into and out of the glasses pores.[5] The sol-gel encapsulation of enzymes has a lot of advantages, such as ease of recycling, broad applicability, cost effectiveness, and safety.[3]

Alcohol dehydrogenases (ADHs) are enzymes that catalyze the reversible reduction of aldehydes and ketones to the corresponding alcohols.[6] However, ADHs have not been widely

used for synthetic purposes in organic chemistry laboratories in part because they require aqueous media, in which many ketone and alcohol substrates are poorly or not soluble; this leads to large reaction volumes and complicated product recovery.[2c,e] An obvious solution for this problem, using organic solvents,[7] was first demonstrated by Klibanov and co-workers.[8]

Secondary ADH (EC 1.1.1.2) from *Thermoanaerobacter ethanolicus* (TeSADH), a nicotinamide-adenine dinucleotide phosphate ($NADP^+$)-dependent thermostable enzyme,[9,10] is a useful biocatalyst for synthetic applications because it tolerates organic solvents, and it accepts ketones and alcohols as substrates with high activities.[11,12] TeSADH obeys Prelog's rule, in which the coenzyme NADPH delivers its *pro*-R hydride from the *re* face of ketone substrates.[13] Recently, we have reported a new mutant of TeSADH, in which tryptophan-110 was replaced with alanine, W110A TeSADH.[14] Although this mutant is able to reduce phenyl-ring-containing ketones at concentrations of 35 mM to produce their corresponding *S*-configured alcohols in Tris-HCl buffer solution/2-propanol (70:30, v/v), higher substrate concentrations are required for practical production of optically active alcohols.

Herein, we report the use of encapsulated W110A TeSADH in sol-gel glasses to overcome the aforementioned limitation. In 2003, Gröger *et al*. reported a practical asymmetric enzymatic reduction of poorly water-soluble ketones using an ADH-compatible biphasic reaction medium.[15] One problem associated with using mixed aqueous and organic solvents, water-miscible or -immiscible, for enzymatic reactions is the tendency of these solutions to form emulsions in the workup, which causes problems of product separation. If the water, necessary for enzyme activity, is entrapped with the enzyme within the sol gel, the workup procedure can be simplified by using water-immiscible organic solvents, and therefore emulsion formation can be avoided.

Results and Discussion

Sol-gel-encapsulated W110A TeSADH was prepared as previously reported,[5,16] although with some modifications. The sol gel was kept in Tris-HCl buffer solution medium until it was used as wet sol gel (hydrogel). The asymmetric reduction of 4-phenyl-2-butanone (**1a**) to (*S*)-4-phenyl-2-butanol ((*S*)-**1b**), a precursor for the synthesis of bufeniode and labetalol (antihypertensive agents),[17] was used as a model in the screening reactions in this study. The hydrogel-encapsulated W110A TeSADH was used to reduce **1a** to (*S*)-**1b** in several different solvent systems (Table 3.1). The reduction carried out in aqueous buffer solution gave almost the same yield as with the free enzyme.[14a] However, the same sol gel was reused three more times to give 56%, 30%, and 10% conversion, respectively. It was necessary to add 2.0 mg of $NADP^+$ for every new reaction because $NADP^+$ molecules either escape from the pores of the sol-gel glasses or become inactivated during turnover.[18a] The asymmetric reduction of **1a** was also carried out in Tris-HCl buffer solution/acetonitrile/2-propanol (41:41:18, v/v) to produce (*S*)-**1b** in good yield (81%). When the same sol gel was reused, the yield was lower (43%). This indicates that W110A TeSADH is not inactivated by polar solvents. In all cases (*S*)-**1b** was produced with high enantioselectivity (>96% *ee*).

The asymmetric reduction of **1a** to (*S*)-**1b** was also performed in hexane and diisopropyl ether to give good to moderate conversions (80% and 40%, respectively) by using hydrogel-encapsulated W110A TeSADH (Table 3.1). Although **1a** was reduced with higher yield in aqueous medium using sol-gel-encapsulated W110A TeSADH, the use organic solvents makes the process more efficient by allowing the use of high concentrations of substrates (≈140 mM). It also makes this asymmetric reduction accessible to hydrophobic substrates.

The W110A TeSADH hydrogel was dried in air for 24 h to form a xerogel (SiO_2. n H_2O). When this xerogel was used for asymmetric reduction of **1a** in Tris-HCl buffer/2-propanol (70:30, v/v), it gave the same conversion as that achieved by the hydrogel (Table 3.1). Asymmetric reduction of **1a** using the xerogel-encapsulated W110A TeSADH in hexane gave 74% conversion, compared to 80% with the hydrogel form. These results indicate that the xerogel retains the essential water molecules required for enzyme activity.[18b] Use of the xerogel instead of hydrogel is preferable as it simplifies the work up procedure.[18c]

The lower yield for the asymmetric reduction using sol-gel-encapsulated enzyme, compared to the reduction using free enzyme,[14a] could be due to the slow diffusion of substrate, product, and co-substrate into and out of the sol-gel glasses. Regardless, the use of sol-gel-encapsulated ADHs is of great advantage for several reasons beside the ease of the workup procedure. First, it makes these enzymes more stable than the free form, which makes them more attractive to organic chemists. Second, it allows the reuse of the enzyme. Third, it might allow these redox reactions to be mixed in situ with other organic reactions.

A series of phenyl-ring-containing ketones were reduced using xerogel-encapsulated W110A TeSADH in hexane as a solvent and 2-propanol as a co-substrate to produce their corresponding (*S*)-alcohols with good yields and high enantioselectivities (Table 3.2). All reactions were performed at 140 mM substrate concentrations. 1-Phenoxy-2-propanone (**2a**) was reduced with very high yield and enantioselectivity to produce (*S*)-1-phenoxy-2-propanol, (*S*)-**2b**. (*S*)-4-(4'-Methoxyphenyl)-2-butanol, (*S*)-**3b**, was obtained from the enantioselective reduction of 4-(4'-methoxyphenyl)-2-butanone (**3a**) with a moderate yield and a higher enantioselectivity, compared to the same alcohol produced by asymmetric reduction using free W110A TeSADH in Tris-HCl buffer (Table 3.2).[14a] Although 1-phenyl-2-propanone (**4a**) was

reduced to (*S*)-1-phenyl-2-propanol, (*S*)-**4b**, with high yield but a rather low *ee* (37%) in aqueous media,[14a] we were pleased to obtain good yield and significantly improved enantioselectivity (69% *ee*) by using xerogel W110A TeSADH in hexane. The asymmetric reduction of **4a** was also performed by using xerogel W110A TeSADH in toluene, *tert*-butyl alcohol, and diisopropyl ether to produce (*S*)-**4b** with 55, 63, and 73% *ee*, respectively. This indicates that the solvent can affect the enzyme enantioselectivity.[19] The lower yield in toluene (Table 3.2) may be due to competitive inhibition of aromatic ketone binding by toluene. The enantioselectivity of the reduction of **4a** by W110A TeSADH correlates neither with the hydrophobicity nor with the dipole moment of the solvent. This is consistent with the recent study of ADH-catalyzed reactions in biphasic systems by Filho *et al.*,[20] who reported that a single physicochemical parameter does not predict the biocompatibility of organic solvents but rather the solvent functionality would be of great significance. 1-(4'-Methoxyphenyl)-2-propanone (**5a**) was reduced by using the xerogel W110A TeSADH with a lower yield but the same *ee* value when compared with that using the free enzyme, producing (*S*)-1-(4'-methoxyphenyl)-2-propanol ((*S*)-**5b**). The cyclic ketone, 2-tetralone (**6a**), was reduced to the corresponding (*S*)-2-tetralol ((*S*)-**6b**) by the xerogel with yields comparable to that produced by using free W110A TeSADH in aqueous medium. However, the *ee* of (*S*)-**6b** was improved in hexane by using the xerogel (Table 3.2).

The low enantioselectivity observed in the reduction of **4a** and **6a** is a result of binding of these substrates in alternative ways within the large pocket of the active site,[14a] allowing NADPH to deliver its *pro*-R hydride to either the *re* face or the *si* face of the substrate. The improvement in enantioselectivity observed when these substrates are reduced by the xerogel W110A TeSADH in organic solvents is likely due to differences in solvation of the enzyme active site.[21]

In an aqueous environment, the binding of a large substrate must displace solvent water from the active site. The binding of the substrate in the "wrong" orientation may actually displace more water, making it favorable entropically.[22] In a non-aqueous medium, this entropic advantage would be diminished. We have previously proposed that active site solvation plays a significant role in the stereospecificity of aliphatic secondary alcohols by TeSADH.[23]

To our knowledge, this is the first report of a preparative scale asymmetric reduction using xerogel-encapsulated ADH in pure organic solvent media. This study clearly demonstrates that the misconception that practical non-aqueous enzymology is limited to hydrolases is false.

In summary, the tolerance of TeSADH to high concentrations of organic solvents allows asymmetric reduction of phenyl-ring-containing hydrophobic ketones by using xerogel-encapsulated W110A TeSADH. Sol-gel immobilization is a convenient method not only for reusing the enzyme but also for making the enzyme accessible to a wide variety of water-insoluble substrates by switching the traditional aqueous medium to organic media. This new method allows for the use of high concentrations of substrates that are crucial for large-scale synthetic applications. Reusable catalysts for chemo-, regio-, and enantioselective asymmetric reduction may be of industrial interest.

Experimental Section

Commercial grade solvents were used without further purification. $NADP^+$, tetramethyl orthosilicate (TMOS), **1a**-**3a**, and **6a** were used as purchased from commercial suppliers. Compounds **4a** and **5a** were prepared as described previously.[25]

Gene expression and purification of W110A TeSADH: W110A TeSADH was expressed in recombinant *Escherichia coli* HB101(DE3) cells and purified as described.[14b]

Preparation of sol-gel encapsulated W110A TeSADH: The silica sol was prepared by mixing TMOS (2.10 g), distilled water (0.47 g) and HCl (0.04 M, 3 drops). The mixture was then sonicated until one layer was formed. The gels were prepared by mixing 1.0 mL of the above sol with 1.0 mL of enzyme stock in a 10 mL round-bottomed flask. The enzyme stock was prepared in 50 mM Tris-HCl buffer solution (pH 8.0) such that the concentration of the enzyme was 0.43 mg/mL and that of $NADP^+$ was 3.0 mg/mL. The sol gel was then left in the same flask closed with Parafilm at RT for 48 h to allow gel aging. It was then used as is in the case of hydrogel. The hydrogel was dried at RT in air for 24 h to give hydrated silica $SiO_2.nH_2O$, the so-called xerogel.

Asymmetric reduction using xerogel-encapsulated W110A TeSADH in organic solvents: Unless otherwise mentioned, all reactions were performed using W110A TeSADH (0.43 mg) and $NADP^+$ (3.0 mg) encapsulated in sol gel, substrate (0.34 mmol), 2-propanol (600 μL), and organic solvent (2.0 mL) in a 10 mL round-bottomed flask equipped with a magnetic stirrer. The reaction mixture was stirred at 50˚C for 12 h. The sol gel was then removed by filtration and washed with ethyl acetate (2×2 mL). The combined organic solvent was then concentrated under vacuum, and the remaining residue was analyzed by a chiral-column GC to determine the yield. The residue was then converted to the corresponding acetate ester derivative to determine the *ee* of the product alcohol by GC.[23]

Capillary GC measurements were performed on a Varian 3300 GC equipped with a flame ionization detector and a Supelco β–Dex 120 chiral column (30 m, 0.25 mm (internal diameter), 0.25 μm film thickness) by using He as the carrier gas. All products were isolated and characterized as described previously.[14a] Their absolute configurations were determined by coinjection on a chiral-column GC with their (*S*)- or (*R*)-configured alcohols, which were

prepared by asymmetric reduction of the corresponding ketones or KR for the corresponding racemates by using free W110A TeSADH.[14a]

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18. (a) When the same sol-gel sample was reused without adding $NADP^+$, only 10% conversion was achieved in the second reaction compared to 56% when new $NADP^+$ was added. (b) About 65% by weight of the xerogel is water after 24 h drying in air. (c) It was noticed that the xerogel loses its activity after drying for longer time (i.e. more than four days). However, the hydrogel is stable at room temperature for longer times as this enzyme has long-term stability.

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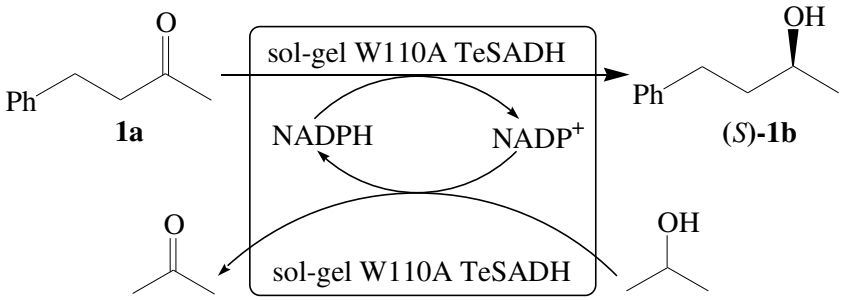
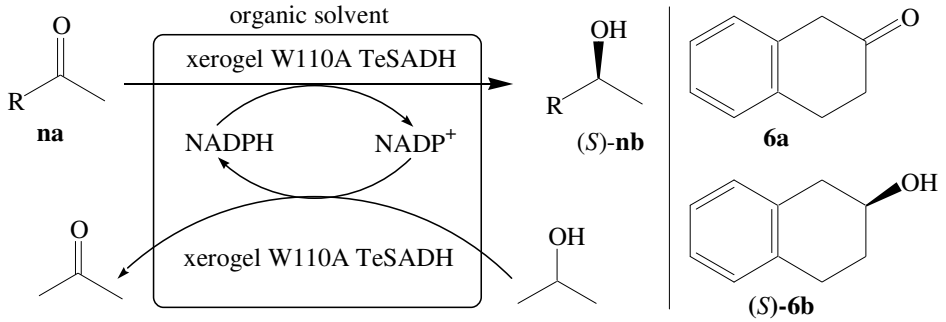
Table 3.1. Asymmetric reduction of **1a** using sol-gel encapsulated W110A TeSADH in different media.[a]



Solvent	Hydrogel		Xerogel	
	Conv. (%)[d]	ee (%)[e]	Conv. (%)[d]	ee (%)[e]
50 mM Tris-HCl buffer pH 8.0[b]	93 (1st) 56 (2nd) 30 (3rd) 10 (4th)	98 98 98 98	92	98
50 mM Tris-HCl buffer/ CH_3CN (1:1)[c]	81 (1st) 43 (2nd)	97 97	-	-
Hexane	80	96	74	97
diisopropyl ether	40	97	-	-

[a] Unless otherwise stated, all reactions were performed at 50˚C using sol-gel samples containing W110A TeSADH (0.43 mg) and $NADP^+$ (3.0 mg), **1a** (0.34 mmol), 2-propanol (600 µL), and 2.0 mL solvent. [b] 50 mM Tris-HCl buffer pH 8.0 (3.5 mL) and 2-propanol (1.5 mL). [c] 50 mM Tris-HCl buffer pH 8.0 (1.5 mL), CH_3CN (1.5 mL) and 2-propanol (600 µL). [d] % conversion was determined by GC. [e] ee was determined by chiral stationary phase GC for the corresponding acetate derivative.[24]

Table 3.2. Asymmetric reduction of phenyl-ring-containing ketones using xerogel W110A TeSADH in organic solvents.[a,b]



n	R, (solvent)	Conv. (%)[c,d]	ee (%)[c,e]
1	$Ph(CH_2)_2$	74 (99)	97 (>99)
2	$PhOCH_2$	>99 (>99)	>99 (>99)
3	*p*-$MeOC_6H_4(CH_2)_2$	61 (87)	94 (91)
4	$PhCH_2$, (hexane)	80 (95)	69 (37)
	$PhCH_2$, (toluene)	24	55
	$PhCH_2$, (diisopropyl ether)	37	73
	$PhCH_2$, (*tert*-butyl alcohol)	38	63
5	*p*-$MeOC_6H_4CH_2$	67 (97)	>99 (>99)
6		94 (>99)	76 (71)

[a] All reactions were performed at 50˚C using xerogel samples containing W110A TeSADH (0.43 mg), and $NADP^+$ (3.0 mg), substrate (0.34 mmol), 2-propanol (600 μL), and 2.0 mL hexane. [b] The absolute configuration was determined as described previously.[14a] [c] Results of reduction with free W110A TeSADH in 50 mM Tris-HCl buffer (pH 8.0)/2-propanol (70:30, v/v) are given in parentheses.[14a] [d] % conversion was determined by GC. [e] *ee* was determined by chiral stationary phase GC for the corresponding acetate derivative.[24]

CHAPTER 4

ACTIVITY AND SELECTIVITY OF W110A SECONDARY ALCOHOL DEHYDROGENASE FROM *THERMOANAEROBACTER ETHANOLICUS* IN ORGANIC SOLVENTS AND IONIC LIQUIDS: MONO- AND BIPHASIC MEDIA[1]

[1] Musa, M. M.; Ziegelmann-Fjeld, K. I.; Vieille, C.; Phillips, R. S.. Submitted to *Org. Biomol. Chem.*, 11/05/2007

Abstract

The asymmetric reduction of hydrophobic phenyl-ring-containing ketones and the enantiospecific kinetic resolution of the corresponding racemic alcohols catalyzed by *Thermoanaerobacter ethanolicus* W110A secondary alcohol dehydrogenase were performed in mono- and biphasic systems containing either organic solvents or ionic liquids. Both yield and enantioselectivity for these transformations can be controlled by changing the reaction medium. The enzyme showed high tolerance to both water-miscible and -immiscible solvents, which allows biotransformations to be conducted at high substrate concentrations.

Introduction

Using biocatalysts in organic synthesis has become an effective methodology for the production of optically active compounds due to the high chemo-, regio-, and enantioselectivities of enzymes.[1] The natural environment for biocatalysts is an aqueous medium, which, in most cases, does not satisfy organic chemists, because most interesting substrates and products are either insoluble or only sparingly soluble in aqueous media. One solution for this limitation is the use of organic solvents.[2] A similar, recently developed solution is the use of room temperature ionic liquids (ILs) as solvents, which are known as environmentally friendly because they are nonvolatile and nonflammable.[3] Both organic solvents and ILs can be used as cosolvents with aqueous media as either monophasic or biphasic systems to enhance the solubility of hydrophobic substrates in the biocatalytic transformations.

Alcohol dehydrogenases (ADHs, EC 1.1.1.X, X= 1 or 2) are enzymes that catalyze the reversible reduction of ketones and aldehydes to the corresponding alcohols.[4] There has been great interest in the use of ADHs in asymmetric synthesis to produce enantiomerically pure alcohols, which are important building blocks in pharmaceutical and agricultural compounds.[5]

Gröger *et al*. reported a practical method for asymmetric reductions of poorly water-soluble ketones using *Rhodococcus erythropolis* ADH in water/n-heptane (4:1, v/v) biphasic systems with satisfactory conversions.[1] Recently, Gonzalo *et al.* reported a method for enzymatic reduction of ketones catalyzed by *Rhodococcus ruber* ADH-A in micro-aqueous media.[7] This method allowed substrate concentrations as high as 2.0 M.

Most enzymatic reactions that have been used in organic synthesis involve lipases because of their availability, thermal stability and high tolerance to organic solvents.[1,2] Several reports have shown that activity and enantioselectivity of lipases can be controlled by changing the reaction medium.[2c,8] It is well-known, though, that lipase-catalyzed resolutions suffer from the drawback of 50% maximum yields with high enantiomeric purity. In contrast, ADH-catalyzed asymmetric reductions can convert prochiral ketones to their corresponding optically active alcohols with up to 100% theoretical yields. It is thus of great interest to enhance the performance of ADHs in nonaqueous media to replace lipases in the production of optically active alcohols.

We have been studying *Thermoanaerobacter ethanolicus* secondary ADH (TeSADH, EC 1.1.1.2), a nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent enzyme.[9] This enzyme is thermally stable, it accepts ketones and alcohols as substrates with high activities, and it resists denaturation in organic solvents.[10] For the above-mentioned reasons, TeSADH is a useful biocatalyst for synthetic applications.[11] 2-Propanol and acetone can be used as cosubstrates in the reduction and oxidation pathways, respectively, to regenerate the coenzyme. This therefore makes the process catalytic as shown in Figure 1. Recently, we designed a new TeSADH mutant, where tryptophan-110 was replaced with alanine (W110A TeSADH).[12] This mutant accepts phenyl-ring-containing ketones and their corresponding alcohols as substrates

with high enantioselectivities and enantiospecificities.[13] The enzyme TeSADH and its mutant W110A TeSADH obey Prelog's rule, in which NADPH delivers its *pro*-R hydride from the *re* face of the ketone (Figure 1).[14] We have also shown that xerogel-encapsulated W110A TeSADH can be used in organic solvents as a solution to the solubility problem for hydrophobic phenyl-ring-containing ketones and their corresponding alcohols.[15] In the same report, we noticed that the reaction's enantioselectivity was higher when substrates such as phenylacetone were reduced in organic solvents than in aqueous media.[15]

In this paper, we report the results of asymmetric reductions and oxidations using W110A TeSADH in ILs as environmentally friendly solvents representing a solution for the problem of poor solubility of the substrates and products. 1-Butyl-3-methylimidazolium tetrafluoroborate ([bmim][BF_4]) was used as a cosolvent with Tris-HCl buffer in monophasic systems. 1-Butyl-3-methylimidazolium bis((trifluoromethyl)sulfonyl)imide ([bmim][NTf_2]), which is water-immiscible, was used as the nonaqueous phase in biphasic systems. Those transformations were also conducted in water-miscible and water-immiscible organic solvents for comparison. Yields and stereoselectivities for each W110A TeSADH-catalyzed biotransformation were compared in the different solvent systems to show that both could be affected by changing the reaction medium.

Results and Discussion

Asymmetric reduction using W110A TeSADH in monophasic systems.

The asymmetric reductions of phenyl-ring-containing ketones catalyzed by W110A TeSADH were conducted in media containing Tris-HCl buffer and water-miscible nonaqueous solvents to enhance the solubility of hydrophobic substrates. 4-Phenyl-2-butanone (**1a**) was reduced in high yield and high enantioselectivity to produce (*S*)-4-phenyl-2-butanol ((*S*)-**1b**) in a

monophasic medium containing [bmim][BF_4], 50 mM Tris-HCl buffer (pH 8.0), and 2-propanol (38:38:24 [v/v/v]). 2-Propanol was used as a cosubstrate to regenerate NADPH from the oxidized $NADP^+$ and therefore make the process catalytic. Although an IL cosolvent was used, 2-propanol had to be used in excess to shift the equilibrium to the reduction direction. The same results were obtained when water-miscible organic solvents such as dimethyl formamide (DMF), acetonitrile, or *tert*-butanol were used as cosolvents (Table 1). This means that W110A TeSADH remains active in water-miscible organic solvents or ILs in high concentrations (>60% by volume), which is remarkable for an ADH. With only a few exceptions in the literature,[16] ADHs are observed to be unstable under these conditions.[4c,d] Under every condition, (*S*)-**1b** was produced with high yield and enantioselectivity.

Impressed with the activity of W110A TeSADH in media containing such a high percentage of nonaqueous water-miscible solvents, we investigated the asymmetric reduction of other phenyl-ring-containing ketones using [bmim][BF_4] as the cosolvent and 2-propanol as the cosubstrate. Under these conditions, phenoxy-2-propanone (**2a**) was reduced to (*S*)-phenoxy-2-propanol ((*S*)-**2b**) with high yield and enantioselectivity. The asymmetric reduction of 4-(4′-methoxyphenyl)-2-butanone (**3a**) afforded 4-(4′-methoxyphenyl)-2-butanol ((*S*)-**3b**) in 40% yield and 87% ee. These values are comparable to those reported previously using Tris-HCl buffer and 2-propanol as both the cosolvent and cosubstrate.[13] 1-Phenyl-2-propanol ((*S*)-**4b**) was obtained from the asymmetric reduction of 1-phenyl-2-propanone (**4a**) in high yield and 38 % ee, in agreement with the results obtained previously for the asymmetric reduction of **4a** using W110A TeSADH in monophasic medium using 2-propanol as both the cosolvent and cosubstrate. The results in Table 1 show that the enantioselectivities of the asymmetric reduction reactions catalyzed by W110A TeSADH in media containing Tris-HCl buffer, 2-propanol as the

cosubstrate, and either water-miscible organic solvents or ILs as cosolvents are similar to those in Tris-HCl buffer and 2-propanol as both the cosolvent and cosubstrate.[13]

Asymmetric reduction using W110A TeSADH in biphasic systems.

The asymmetric reductions of hydrophobic ketones catalyzed by W110A TeSADH were conducted in biphasic media with either ILs or organic solvents as the nonaqueous phase. The W110A TeSADH-catalyzed asymmetric reduction of **1a** to (*S*)-**1b** was investigated in a biphasic system containing [bmim][NTf_2], a water-immiscible IL, 50 mM Tris-HCl buffer (pH 8.0), and 2-propanol as the cosubstrate (Table 2). This reaction was also investigated in a series of water-immiscible organic solvents (Table 2). (*S*)-**1b** was produced with high enantioselectivities in all cases; however the percent conversions were different from one solvent to the other. The asymmetric reduction in the biphasic system containing [bmim][NTf_2] as the nonaqueous phase had a lower yield than in the biphasic systems containing cyclohexane, hexane, heptane, or di*iso*propyl ether (DIPE), but it had a higher yield than in the biphasic systems containing toluene and *tert*-butyl methyl ether (TBME). Eckstein *et al.* reported the first example of asymmetric reduction using ADH in a biphasic system containing [bmim][NTf_2].[17] They also reported that taking advantage of the partition coefficients of 2-propanol and acetone, 2-propanol preferably remains in the aqueous phase and improved ADH-catalyzed reduction yields are obtained. We believe that the partition coefficients of 2-propanol and acetone between aqueous medium and [bmim][NTf_2] are not the only factor that controls the percent conversion in these biphasic systems because the partition coefficients of ketone substrates and their corresponding alcohols also play an important role. It is always good to consider environmentally friendly IL solvents as substitutes to organic solvents but they are not always the best in terms of percent yield. Reducing the 2-propanol concentration when hexane was used as the organic solvent

resulted in decreases in both percent conversion and ee, which can be explained due to time-dependent racemization as the possibility of reversibility increases.

The asymmetric reductions of **2a** and **3a** catalyzed by W110A TeSADH were conducted in a biphasic system containing [bmim][NTf_2] to produce (*S*)-**2b** and (*S*)-**3b** with yields and enantioselectivities comparable to those achieved previously in aqueous media with 2-propanol as the cosolvent.[13] Under the same conditions, (*S*)-**4b** was produced from the asymmetric reduction of **4a** with high yield and higher ee than obtained in monophasic systems with 2-propanol or [bmim][BF_4] as the cosolvent (Table 1 and Table 2).[13] The same enantioselectivity enhancement was noticed when the asymmetric reduction of **4a** was conducted in water-immiscible organic solvents such as hexane, toluene, or DIPE using xerogel-encapsulated W110A TeSADH.[15] This enantioselectivity enhancement can be explained by differences in solvation of the enzyme active site as proposed previously.[15,18] It can also be explained as the result of the substrate concentration in the aqueous phase in biphasic systems being lower than in the monophasic systems containing 2-propanol, water-miscible IL, or organic solvent as the cosolvent with 2-propanol as the cosubstrate.

The use of biphasic systems in enzymatic transformations is of great interest not only because of the ease of work-up, but also because the enzyme and its cofactor are dissolved in the aqueous phase, where the reaction takes place, while the reactant, product, cosubstrate, and coproduct are all distributed in the two phases, in most cases preferentially in the nonaqueous phase. This distribution reduces the possibility of enzyme inhibition by the substrate, product or solvent. Another advantage of using biphasic systems for enzymatic reactions is the ability to recycle the enzyme easily.

Enantiospecific kinetic resolution using W110A TeSADH.

It is of great interest to generate both enantiomers of chiral alcohols with high optical purities. Because most ADHs follow Prelog's rule,[4d] producing (*S*)-enantiomers in most cases, it is important to develop methods that produce the *anti*-Prelog enantiomers using ADHs. One way to do this is to use an (*S*)-selective ADH in the oxidation direction (i.e. kinetic resolution [KR]). If, instead of 2-propanol, acetone is used as the cosubstrate in ADH-catalyzed biotransformations, KR will be achieved, thus stereospecifically converting the (*S*)-enantiomer to the corresponding ketone and leaving the (*R*)-enantiomer with a maximum theoretical yield of 50% with high ee. We have shown that W110A TeSADH can be used to catalyze the stereospecific KR of a series of phenyl-ring-containing alcohols to produce their (*R*)-enantiomers with moderate to high ees in aqueous media containing acetone as both the cosolvent and cosubstrate.[13] Because acetone is known to inhibit ADHs and it cannot be used as a cosolvent at high concentrations, substrate and product solubilities remain major issues. For this reason, using an alternative cosolvent that can minimize the amount of acetone needed for enantiospecific KR might be advantageous.

The enantiospecific KR of *rac*-**1b** catalyzed by W110A TeSADH was investigated in a biphasic system containing [bmim][NTf_2] as the nonaqueous phase with acetone as the cosubstrate. (*S*)-**1b** was converted enantiospecifically to **1a**, leaving (*R*)-**1b** as the unreacted enantiomer with a high *E*-value (Table 3). Acetone was used as a cosubstrate in relatively low concentrations (4 eq.) instead of being used as a cosolvent as previously.[13] The same asymmetric transformation was conducted in several water-immiscible organic solvents (Table 3) to produce (*R*)-**1b** with high *E*-value. In agreement with the results obtained in Table 2 for asymmetric reduction of **1a**, enantiospecific KR of *rac*-**1b** gave higher yields when either [bmim][NTf_2],

hexane, or DIPE were used as the nonaqueous phase than when either TBME or toluene were used. This result is a clear indication that the partition coefficients of 2-propanol and acetone are not the only factors that control the equilibrium in asymmetric redox reactions catalyzed by ADHs. In all cases the *E*-value was higher than 17, which indicates that this reaction is a very selective KR.[19]

We decided to carry out the enantiospecific KR of *rac*-**3b** under the previous conditions to clarify the observed change of the enantioselectivity upon reaction medium used for these biotransformations. Since **3a** was reduced to (*S*)-**3b** with lower enantioselectivity than **1a** using W110A TeSADH, it is expected that the *E*-value for enantiospecific KR of *rac*-**3b** will be less than that for *rac*-**1b**. In agreement with the results obtained for enantiospecific KR of *rac*-**1b** in biphasic systems, enantiospecific KR of *rac*-**3b** in a biphasic system gave a higher percent conversion with either [bmim][NTf_2], DIPE, or hexane as the non-aqueous phase than with toluene or TBME. The lowest *E*–value was obtained with hexane but this *E*-value increased dramatically when the acetone concentration was increased, which can be explained as the result of reducing the possibility of reversibility by increasing the amount of acetone used. Although toluene showed the worst percent conversion in both the reduction and oxidation directions, it exhibited the highest stereospecificity (i.e., the highest *E*-value for substrates *rac*-**1b** and *rac*-**3b**).

It is not easy to conclude which solvent is the best for a specific ADH-catalyzed biotransformation where a cosubstrate (here, either 2-propanol or acetone) is needed to regenerate the coenzyme. At this time, we can conclude that the selectivity of ADH-catalyzed transformations can be controlled by changing the reaction medium.

Experimental Section

General

Capillary gas chromatographic measurements were performed on a GC equipped with a flame ionization detector and a Supelco β–Dex 120 chiral column (30 m, 0.25 mm [i.d.], 0.25 μm film thickness) using Helium as the carrier gas. ^{1}H NMR and ^{13}C NMR spectra were recorded on 400 MHz spectrometer at room temperature in $CDCl_3$ using either solvent peak or tetramethylsilane as internal standard. Commercial grade solvents were used without further purification. $NADP^+$, *Candida antarctica* Lipase B (Novozyme 435), phenylacetic acid, acetic anhydride, and $NaBH_4$ were used as purchased from commercial sources. Ketones **1a-3a** and *rac*-**1b** were used as purchased from commercial suppliers. The ketone **4a** was prepared as described.[20] *rac*-**3b** was prepared by reducing **3a** with $NaBH_4$ as described.[21]

Gene expression and purification of W110A TeSADH. W110A TeSADH was expressed in recombinant *Escherichia coli* HB101(DE3) cells and purified as described.[12]

Determination of absolute configuration. The absolute configurations of both alcohol enantiomers were determined by co-injection on a chiral GC column with samples prepared previously either by asymmetric reduction or oxidation using TeSADH or by KR using Novozyme 435 as described.[13]

Preparation of [bmim][BF_4] and [bmim][NTf_2]. [bmim][BF_4] was prepared as described.[22] To prepare [bmim][NTf_2], [bmim][BF_4] (3.94 g, 17.4 mmol) and bis(trifluoromethane)sulfonamide lithium salt (5.0 g, 17.4 mmol) were mixed in distilled water (7.0 mL) in a 50-mL round bottomed flask. The mixture was stirred at room temperature for 2 h to form two layers. After removing the water under vacuum, methylene chloride (20 mL) was added to the residue, which contains [bmim][NTf_2] and $LiBF_4$. The solution was then filtered and dried with $NaSO_4$. The

solvent was then removed under vacuum to produce the ionic liquid [bmim][NTf_2] as a colorless oil. Spectral data were consistent with those reported.[23]

General procedure for asymmetric reduction using W110A TeSADH in monophasic systems. A mixture of substrate (0.244 mmol), $NADP^+$ (1.33 mg), water-miscible organic solvent or IL (1.0 mL), Tris-HCl (1.0 mL, 50 mM, pH adjusted to 8.0 at 25 °C) containing W110A TeSADH (0.48 mg), and 2-propanol (quantities described in Table 1) was stirred at 50 ºC for 24 h. The reaction mixture was extracted with diethyl ether (3×2 mL). The combined organic layers were combined and dried with Na_2SO_4. A sample was injected in a GC to determine the percent conversion. The solvent then was removed under vacuum and the remaining residue was treated with pyridine and acetic anhydride to convert the product alcohol to the corresponding acetate as reported,[21] which was analyzed by a chiral column GC to determine the percent ee.

General procedure for asymmetric reduction using W110A TeSADH in biphasic systems. A mixture of substrate **na** (0.244 mmol), $NADP^+$ (1.33 mg), solvent (1.0 mL), Tris-HCl (1.0 mL, 50 mM, pH adjusted to 8.0 at 25 °C) containing W110A TeSADH (0.48 mg), and 2-propanol (quantities described in Table 2) was stirred as two layers at maximum speed to keep a suspension at 50 ºC for 24 h. The two layers were then separated and the aqueous layer was extracted with diethyl ether (3×2 mL). The combined organic layers were combined with the original organic layer and dried with Na_2SO_4 (In the case of [bmim][NTf_2], the IL layer was extracted with hexane (6×2 mL), then the combined hexane layers were combined with the organic layers from the aqueous layer extraction). The product was then analyzed as described above.

General procedure for asymmetric kinetic resolution using W110A TeSADH in monophasic and biphasic systems. A mixture of substrate (*rac*)-**nb** (0.17 mmol), $NADP^+$ (1.0 mg), solvent (750 μL), Tris-HCl (750 μL, 50 mM, pH adjusted to 8.0 at 25 °C) containing W110A TeSADH (0.24 mg), and acetone (quantities described in Table 3) was stirred at 50 ºC for 24 h. The reaction mixture was then worked up and its contents were analyzed as described above for the asymmetric reduction.

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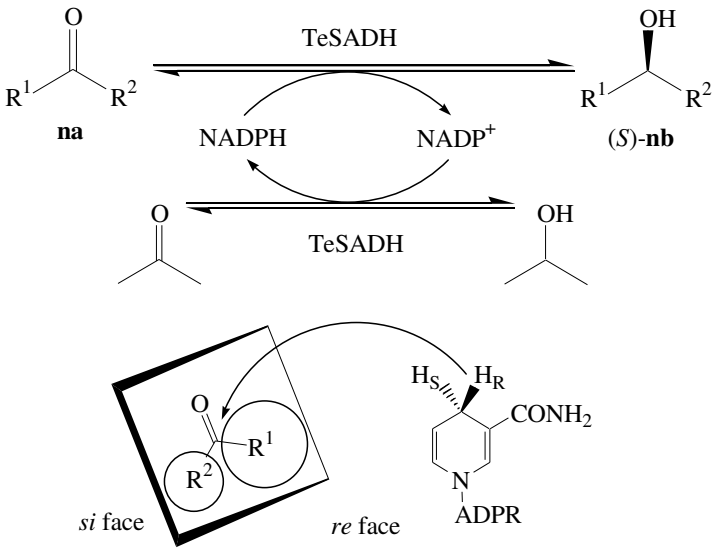
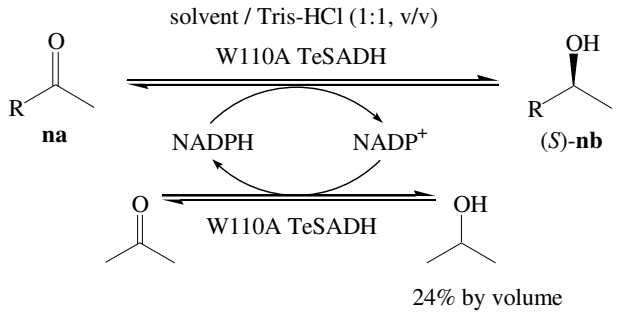


R^1 is more sterically hindered and has higher Cahn-Ingold-Prelog priority than R^2

Figure 4.1. Prelog's rule for predicting the stereochemical outcome for ADH-catalyzed asymmetric reduction.

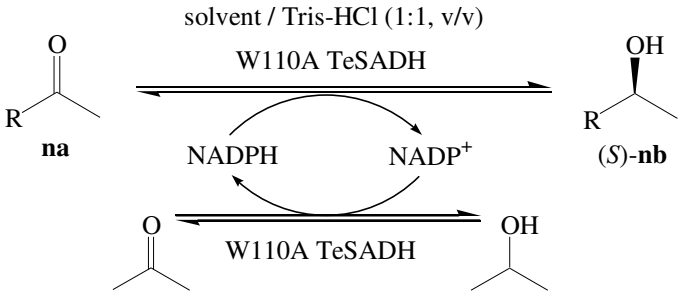
Table 4.1. Asymmetric reduction of phenyl-ring-containing ketones by W110A TeSADH in water-miscible solvents (monophasic).[a]



substrate	R	product[b]	solvent	conv. (%)[c]	ee (%)[d]
1a	$Ph(CH_2)_2$	(*S*)-**1b**	[bmim][BF_4]	86	98
			DMF	95	95
			acetonitrile	94	97
			tert-butanol	85	97
2a	$PhOCH_2$	(*S*)-**2b**	[bmim][BF_4]	96	97
3a	4-$MeOC_6H_4(CH_2)_2$	(*S*)-**3b**	[bmim][BF_4]	40	87
4a	$PhCH_2$	(*S*)-**4b**	[bmim][BF_4]	88	38

[a] Conditions: substrate (0.244 mmol), $NADP^+$ (1.33 mg), solvent (1.0 mL), W110A TeSADH (0.48 mg), Tris-HCl (1.0 mL, 50 mM, pH 8.0), and 2-propanol (600 μL). [b] The absolute configuration was confirmed by coinjection of their acetate derivatives with both (*S*) and (*R*)-acetates made before either by enantioselective reduction or enantiospecific oxidation using W110A TeSADH.[13] [c] % Conversion was determined by GC. [d] % ee values were determined on the corresponding acetate by a GC equipped with a chiral column as described.[13]

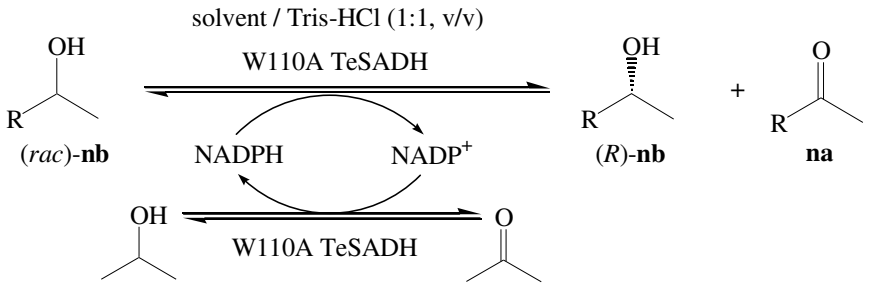
Table 4.2. Asymmetric reduction of phenyl-ring-containing ketones by W110A TeSADH in biphasic systems.[a]



substrate	R	product[b]	solvent	2-propanol (eq.)	conv. (%)[c]	ee (%)[d]
1a	$Ph(CH_2)_2$	(*S*)-**1b**	[bmim][NTf_2]	16	65	97
			DIPE	16	94	99
			TBME	16	59	96
			toluene	16	36	97
			cyclohexane	16	83	97
			hexane	16	95	96
			hexane	8	87	94
			hexane	4	69	81
			heptane	16	92	96
2a	$PhOCH_2$	(*S*)-**2b**	[bmim][NTf_2]	16	96	>99
3a	4-$MeOC_6H_4(CH_2)_2$	(*S*)-**3b**	[bmim][NTf_2]	16	52	88
4a	$PhCH_2$	(*S*)-**4b**	[bmim][NTf_2]	16	>99	60

[a] Conditions: substrate (0.244 mmol), $NADP^+$ (1.33 mg), water-immiscible solvent (1.0 mL), W110A TeSADH (0.48 mg), Tris-HCl (1.0 mL, 50 mM, pH 8.0), and 2-propanol. [b] The absolute configuration was confirmed by coinjection with both (*S*)- and (*R*)-alcohols made before.[13] [c] % Conversion was determined by GC. [d] % ee values were determined on the corresponding acetate by a GC equipped with a chiral column as described.[13]

Table 4.3. Enantiospecific oxidation of phenyl-ring-containing alcohols by W110A TeSADH in biphasic media.[a]



Substrate	R	Solvent	Product[b]	Acetone (eq.)	*c* (%)[c]	ee_s (%)[d]	*E*-value[e]
rac-**1b**	$Ph(CH_2)_2$	[bmim][NTf_2]	(*R*)-**1b**	4	44	80	>100
		TBME		4	31	38	17
		DIPE		4	44	75	97
		toluene		4	18	22	>100
		cyclohexane		4	48	83	49
		Hexane		4	48	78	28
rac-**3b**	4-$MeOC_6H_4(CH_2)_2$	[bmim][NTf_2]	(*R*)-**3b**	4	52	61	7
		TBME		4	19	22	39
		DIPE		4	41	52	12
		toluene		4	20	25	>100
		hexane		4	58	9	1.3
		hexane		8	66	51	2.7
		Hexane		16	63	80	6.4

[a] Conditions: substrate (0.17 mmol), $NADP^+$ (1.0 mg), solvent (750 μL), Tris-HCl (750 μL, 50 mM, pH 8.0) containing W110A TeSADH (0.24 mg), and acetone. [b] The absolute configuration was confirmed by comparing the retention time with that for the *S* enantiomer. [c] % Conversion was determined by GC. [d] % ee values were determined on the corresponding acetate by a GC equipped with a chiral column as described.[13] [e] *E*-value was calculated from the formula $E=\ln[(1-c)(1-ee_s)]/\ln[(1-c)(1+ee_s)]$, where c is percent conversion of alcohol to ketone, and ee_s is enantiomeric excess of the unreacted (*R*)-alcohol.

CHAPTER 5

CONCLUSIONS

We have been able to produce both enantiomers of a series of phenyl-ring-containing secondary alcohols by asymmetric reduction and enantiospecific oxidation via KR using W110A TeSADH. (*S*)-Alcohols were produced via asymmetric reduction with high chemical and moderate to high optical yields using 2-propanol as a cosubstrate for coenzyme regeneration and as a cosolvent. A number of racemic phenyl-ring-containing alcohols were resolved with W110A TeSADH using acetone as a hydrogen acceptor and a cosolvent. These reactions produced a mixture of (*R*)-alcohols as unreacted enantiomer with good enantiomeric ratios and the corresponding ketones, which could be recycled. The use of 2-propanol (30%, v/v) and acetone (10% v/v) in high concentration in the reduction and oxidation pathways was crucial not only to enhance the solubility of hydrophobic phenyl-ring-containing substrates, but also to shift the equilibrium to the desired direction. It is of great interest to produce optically active alcohols of both enantiomers using the same enzyme because the two enantiomers are often of equal importance and only a few *anti*-Prelog enzymes are available. W110A TeSADH will be of great interest to organic chemists for the preparation of optically active phenyl-ring-containing alcohols because of its thermal stability and high tolerance to organic cosolvents.

The tolerance of TeSADH to high concentrations of organic solvents allows asymmetric reduction of phenyl-ring-containing hydrophobic ketones using xerogel-encapsulated W110A TeSADH. Sol-gel immobilization is a convenient method not only for reusing the enzyme but also for making the enzyme accessible to a wide variety of water-insoluble substrates by switching the traditional aqueous medium to organic media. This new method allows for the use

of high concentrations of substrates that are crucial for large-scale synthetic applications. Reusable catalysts for chemo-, regio-, and enantioselective asymmetric reduction may be of industrial interest.

The high tolerance of W110A TeSADH to elevated concentrations of both organic solvents and ILs allows asymmetric redox reactions to be conducted in both directions by choosing the appropriate cosubstrate (i.e., 2-propanol or acetone) and by using high concentrations of hydrophobic substrates. The enantioselectivity and yield of the reactions catalyzed by W110A TeSADH can be controlled by changing the reaction medium. The partition coefficients of 2-propanol and acetone in a biphasic system containing an organic solvent or IL are not the only factors that control the equilibrium in the asymmetric transformations catalyzed by ADHs. This study also shows that ADH selectivity can be tuned by changing the reaction medium. The efficient production of both enantiomers of optically active alcohols is of great interest as they are building blocks for the synthesis of pharmaceutically important molecules.