

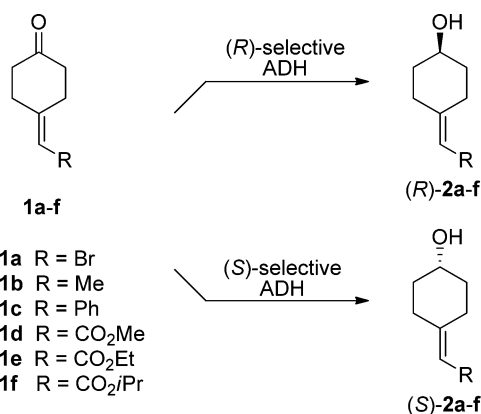
Induced Axial Chirality in Biocatalytic Asymmetric Ketone Reduction

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S Supporting Information

ABSTRACT: Catalytic asymmetric reduction of prochiral ketones of type 4-alkylidene cyclohexanone with formation of the corresponding axially chiral *R*-configured alcohols (up to 99% ee) was achieved using alcohol dehydrogenases, whereas chiral transition-metal catalysts fail. Reversal of enantioselectivity proved to be possible by directed evolution based on saturation mutagenesis (up to 98% ee (*S*)). Utilization of ketone with a vinyl bromide moiety allows respective *R*- and *S*-alcohols to be exploited as key compounds in Pd-catalyzed cascade reactions.

Type 2 axially chiral molecules¹ characterized by a substituted 4-alkylidene cyclohexane structure are of interest in circular dichroism exciton chirality studies^{2,3} and as precursors to enantiomerically pure liquid crystals.⁴ Several synthetic routes to this class of chiral compounds have been described, all involving traditional antipode separation or utilization of stoichiometric amounts of nonracemic chiral reagents or auxiliaries.^{3,5} An attractive synthetic approach is the catalytic enantioselective reduction of corresponding prochiral ketones **1** using chiral transition-metal catalysts. Unfortunately, using one of the most efficient chiral Ru-based catalysts ((TsDPEN)(*p*-cymene)RuCl₂)⁶ in ketone **1b** reduction, formation of alcohol **2b** as a racemate was observed. This is not surprising because high stereoselectivity in ketone reductions can be achieved only when α - and α' -positions flanking the carbonyl function differ sterically to a notable degree.^{6,7} So we turned to biocatalytic reduction using alcohol dehydrogenases (ADHs).⁸



In exploratory experiments, we first tested two commercially available ADH kits, one with 35 enzymes⁹ and the other 12

members,¹⁰ as catalysts in ketones **1a–f** reduction. Stereoselective reduction of ketone **1a** appeared particularly attractive because *R*- and *S*-**2a** could subsequently serve as pivotal compounds in transition-metal catalyzed C–C bond-forming reactions with formation of a variety of structurally different axially chiral compounds. Since companies do not reveal the ADHs identity (i.e., some kit members may be identical), in all cases we used one standard protocol (glucose dehydrogenase/glucose as the cofactor regeneration system with either NAD⁺ or NADP⁺, pH 7, 30 °C, 16 h; see Supporting Information, SI). Typical data are summarized in Table 1 (full data in Table S1), including the most promising results. High enantioselectivity amounting to $\geq 96\%$ ee resulted in the reduction of all ketones except **1b** (maximally 86% ee). In all high enantioselectivity cases, except alcohol **2c**, *R*-selectivity was observed, showing that the preferred direction of hydride attack by NADPH occurs consistently from the *Re* side of the carbonyl function. Enantioselectivity reversal proved to be possible in some cases but not with high ee values (SI).

Absolute configuration assignment of *R/S*-**2d** was made by comparison with the known absolute configuration of *S*-**2d** determined by Walborsky.³ For the other axially chiral compounds, chemical correlation was performed, e.g., by Pd-catalyzed carbonylation and Suzuki coupling using the vinyl bromide **2a** (SI).

We also tested *Thermoethanolicus brockii* (TbSADH*) ADH,^{11,12} which is of industrial interest because it is unusually thermostable, tolerates organic solvents, and exhibits high activity and stereoselectivity toward many structurally different prochiral ketones. It is a zinc-dependent ADH in which the substrate carbonyl moiety binds to the metal, thereby positioning and activating the carbonyl function for hydride attack to occur from NADPH. Wild-type TbSADH generally obeys Prelog's rule in the normal case of ketones leading to central chirality,^{13,14} although anti-Prelog products were observed in some cases, leading Keinan to propose a model based on small and large hydrophobic pockets assessment.¹³ Subsequently, several crystal structures were determined,¹⁵ one a binary complex with cofactor NADP⁺ bound to the zinc^{15a} and a different one with *sec*-butanol as guest in the binding pocket bound to the zinc.^{15b} Unfortunately for ketone **1a**, this robust ADH is only moderately *R*-selective (66% ee).

To obtain highly *R*- and *S*-selective mutants of the robust TbSADH*, we turned to directed evolution¹⁶ using the combinatorial active-site saturation test (CAST)^{16g} which

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Table 1. Reduction of Model Ketones 1a–f with Commercial ADHs^{a,b}

entry	ADH	cofactor ^c	substrate											
			1a→2a		1b→2b ^d		1c→2c		1d→2d		1e→2e		1f→2f	
			conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee
1	A1	NADP ⁺	39	46(S)	>99	47(S)	>99	97(S)	>99	60(S)	>99	27(S)	>99	39(R)
2	A2	NADP ⁺	≤20	nd ^e	>99	23(S)	>99	96(S)	>99	58(S)	>99	27(S)	>99	38(R)
3	A7	NAD ⁺	>99	64(R)	>99	1(S)	>99	97(R)	>99	98(R)	>99	99(R)	>99	96(R)
4	A8	NAD ⁺	>99	96(R)	>99	62(R)	87	98(R)	>99	95(R)	>99	>99(R)	>99	99(R)
5	A10	NAD ⁺	>99	96(R)	>99	73(R)	>99	98(R)	>99	95(R)	>99	>99(R)	>99	99(R)
6	A13	NAD ⁺	>99	95(R)	>99	67(R)	97	98(R)	>99	95(R)	>99	>99(R)	>99	99(R)
7	A14	NAD ⁺	>99	74(R)	>99	27(R)	96	98(R)	>99	97(R)	>99	99(R)	>99	96(R)
8	A15	NAD ⁺	54	91(R)	60	68(R)	81	96(R)	29	92(R)	82	98(R)	>99	>99(R)
9	A18	NAD ⁺	≤20	nd ^e	>99	2(R)	97	97(R)	>99	97(R)	>99	99(R)	>99	96(R)
10	A21	NAD ⁺	>99	93(R)	>99	74(R)	95	96(R)	>99	96(R)	>99	>99(R)	>99	>99(R)
11	A22	NAD ⁺	41	73(R)	≤20	nd ^e	60	93(R)	≤20	nd ^e	27	90(R)	>99	99(R)
12	A25	NAD ⁺	>99	61(R)	>99	0	88	93(R)	>99	97(R)	>99	95(R)	>99	96(R)
13	A29	NAD ⁺	41	80(R)	≤20	nd ^e	67	87(R)	≤20	nd ^e	61	96(R)	>99	99(R)
14	A30	NAD ⁺	>99	87(R)	>99	72(R)	88	84(R)	>99	91(R)	>99	96(R)	>99	98(R)
15	A32	NADP ⁺	>99	81(S)	>99	42(S)	>99	96(S)	>99	59(S)	>99	27(S)	>99	40 (R)
16	A33	NAD ⁺	>99	65(R)	>99	11(R)	94	96(R)	>99	98(R)	>99	98(R)	>99	95(R)
17	A34	NADP ⁺	>99	82(S)	>99	46(S)	97	96(S)	>99	59(S)	>99	27(S)	>99	40 (R)
18	1.1.020	NAD ⁺	>99	91(R)	90	86(R)	96	89(R)	>99	95(R)	>99	97(R)	>99	97(R)
19	1.1.030	NAD ⁺	>99	83(R)	>99	5(R)	94	95(R)	>99	96(R)	>99	96(R)	>99	93(R)
20	1.1.210	NAD ⁺	>99	11(R)	>99	0	>99	97(R)	>99	94(R)	>99	98(R)	>99	92(R)
21	1.1.260	NADP ⁺	>99	51(R)	>99	50(R)	91	94(R)	>99	97(R)	>99	97(R)	>99	92(R)

^aAverage of at least two independent measurements. ^bDeviation in values obtained did not exceed 10% of average ee value shown. Entries 1–13 are ADHs from X-zymes kit;⁹ entries 14–17 are ADHs from Evocat kit.¹⁰ Experimental conditions are detailed in SI. ^cCofactor used upon recommendation of ADH supplier. ^dIn 1b→2b, none of commercial ADHs led to ee values >86%. ^eNot determined (nd) due to low conversion.

constitutes a systematization of the first example of randomization at the active site of an enzyme to control stereo-selectivity.¹⁷ Accordingly, sites composed of one or more amino acids positioned around the binding pocket are subjected to saturation mutagenesis, if necessary in an iterative manner.^{16g} Guided by the crystal structure of TbSADH¹⁵ and site-directed mutagenesis, reported by Phillips,¹⁸ six amino acid positions were chosen for saturation mutagenesis: S39, A85, I86, W110, Y267, and C295 (Figure 1), using ketone 1a as the model substrate.

To minimize screening effort (oversampling) while ensuring full library coverage,^{16g} we generated six CAST libraries at the defined residues using a highly reduced amino acid alphabet defined by RNG codon degeneracy (R: adenine/guanine; N: adenine/cytosine/guanine/thymine; G: guanine). It encodes eight amino acids of different steric “size” irrespective of electronic properties, viz., Ala, Arg, Glu, Gly, Lys, Met, Thr, and Val, and requires oversampling of only 31 transformants for 98% library coverage.^{16g,19} This choice was inspired by Prelog’s diamond lattice mnemonic device for predicting the stereo-selectivity of ADH-mediated ketone reduction following consideration of relative size of the groups flanking the carbonyl moiety;²⁰ a useful guide that was also employed by Phillips in interpreting certain mutations introduced in TbSADH by site-directed mutagenesis.¹⁸ It should be noted that in the present case of ketone 1, the steric properties of the respective first three C atoms flanking the carbonyl function are identical. Therefore, application of Prelog’s rule goes beyond conventional considerations. The best results originated from libraries generated at single-residue sites 85, 86, 110, and 295 (Table 2). Remarkably, both *R*- and *S*-selective mutants were identified having ee values >97% in both stereochemical

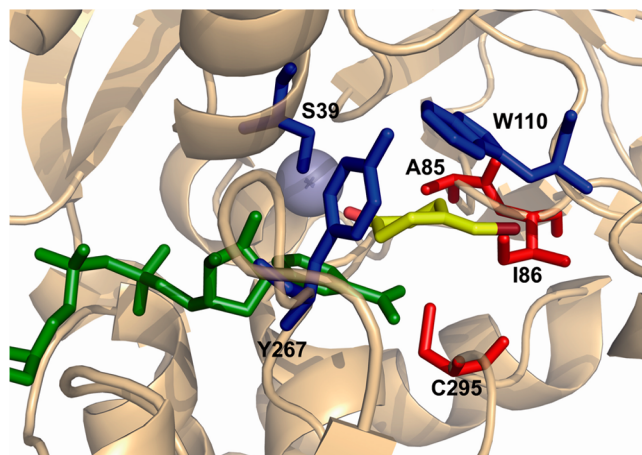


Figure 1. TbSADH active site model harboring compound 1a (yellow) as a rough guide for choosing six CAST sites. 1a coordinates were generated using MAESTRO program and subsequently manually fitted in the active site of TbSADH based on PDB 1BXZ.^{15b} NADP⁺ (green) and zinc (gray) were modeled by superimposition using PyMOL software (SI). Randomization sites Y267, S39, and W110 (blue) align the large part of the binding pocket, while A85, I86, and C295 (red) are next to the small part.

regimes. Thus, iterative rounds of CASTing were not necessary for obtaining useful mutants. Interestingly, mutants I86A and W110A (Table 2, entries 3 and 8, respectively) were prepared previously by site-specific mutagenesis as catalysts in asymmetric reduction of structurally completely different ketones.^{14f} RNG-limited randomization libraries generated by at positions 39 and 267 failed to harbor mutants with notable

Table 2. Performance of Best TbSADH* Mutants Specifically Evolved for **1a** as Catalysts in Asymmetric Reduction of Ketones **1a–f**^{a,b}

entry	site	mutation	substrate											
			1a→2a		1b→2b		1c→2c		1d→2d		1e→2e		1f→2f	
			conv. (%) ^c	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee	conv. (%)	% ee
1	-	TbSADH*	≥95	66(R)	92	91(R)	38	77(R)	26	89(R)	33	87(R)	42	92(R)
2	85	A8SV	≥95	95(R)	90	96(R)	28	63(R)	18	94(R)	22	91(R)	26	93(R)
3	86	I86A	≥95	98(S)	≥99	74(S)	33	65(R)	13	75(R)	18	85(R)	29	95(R)
4	86	I86G	≥95	98(S)	≥99	82(S)	30	66(R)	24	24(S)	25	8(R)	24	93(R)
5	86	I86E	≥95	95(S)	≥99	84(S)	22	88(R)	18	58(R)	30	89(R)	31	92(R)
6	86	I86M	≥95	92(S)	≥99	25(S)	27	73(R)	35	84(R)	52	86(R)	46	90(R)
7	86	I86T	≥95	92(S)	89	61(S)	15	74(R)	21	40(R)	23	57(R)	27	93(R)
8	110	W110A	≥99	82(R)	≥99	93(R)	≥99	99(R)	≥99	98(R)	≥99	99(R)	≥99	99(R)
9	110	W110E	≥99	91(R)	≥99	92(R)	≥99	89(R)	≥99	91(R)	≥99	92(R)	≥99	91(R)
10	110	W110M	≥99	97(R)	≥99	95(R)	≥99	99(R)	≥99	98(R)	≥99	99(R)	≥99	99(R)
11	110	W110T	≥99	97(R)	≥99	92(R)	≥99	97(R)	≥99	98(R)	≥99	99(R)	≥99	99(R)
12	295	C295E	16	84(R)	27	79(R)	10	88(R)	8	65(R)	10	64(R)	14	72(R)

^aAverage of two independent measurements. Deviation in values obtained did not exceed 3% of average ee value shown. ^bOnly hits from plate screening confirmed in medium scale reactions (60 mg of **1a**, 63 mM final concn) are presented here. See SI for exptl details. ^c≤5% side products in all cases.

enantioselectivity (ee ≥ 80%). However, noteworthy is mutant S39T which induces enantioselectivity reversal (38% ee in favor of **2a**).

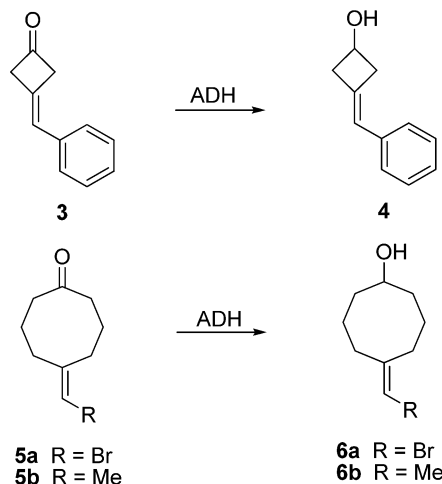
Reaction **1a**→**2a** was upscaled employing two different mutants: *R*-selective variant W110T using 500 mg (2.64 mmol) of **1a** at 67 mM concn and *S*-selective variant I86A using 630 mg (3.33 mmol) of **1a** at 67 mM concn. Isolated yields were 81% and 84% for (*R*)- and (*S*)-**2a**, respectively (SI), which underscores practical utility of the mutants. Indeed, with access to both enantiomers, many different kinds of transition-metal catalyzed transformations can be envisioned.

We then tested the best hits specifically evolved for ketone **1a** as catalysts in asymmetric reduction of the other substrates **1b–f**. Table 2 shows mostly excellent enantioselectivity and high conversion to *R*-configured alcohols are possible, but asymmetric induction reversal with formation of respective *S*-alcohols was not achieved.

Most results summarized in Table 2 are in accord with Prelog's diamond lattice model,²⁰ extended in the present system. All hits arising from saturation mutagenesis at position I86 using RNG codon degeneracy showed a preference for *S*-**2a**, although the side chains of some of the introduced amino acids are not substantially smaller than that of isoleucine (e.g., glutamic acid or methionine; Table 2, entries 5 and 6, respectively). To study the residue 86 influence on reaction **1a**→**2a** stereoselectivity, we prepared by site-directed mutagenesis (QuikChange)²¹ the rest of the theoretically possible mutants. Surprisingly, we observed *S*-selectivity in all new mutants regardless of size or electronic nature of respective amino acid side chains, e.g., ≥98% ee for I86D, I86C, I86Y, and I86P (see Table S2 for mutants with lower *S*-selectivity).

Finally, we wanted to see if asymmetric reduction of analogous four- and especially eight-membered cyclic ketones is also possible, so ketones **3** and **5a–b** were prepared and subjected to reduction using some of the previously obtained TbSADH* mutants without performing additional mutagenesis. TbSADH* itself essentially does not accept these substrates, which means that enantioselectivity could not be determined reliably. For ketone **3**, all mutants characterized by position 110 point mutations led to high activity and complete

enantioselectivity (≥99% ee); absolute configuration is presently unknown (Table S3). While testing ketones **5a** and **5b** reduction, the best mutant, W110T, showed respectable enantioselectivity, 79 and 62% ee, respectively (see SI for other mutants performance). This mutant is therefore a good starting point for future genetic optimization.



In addition to these substrates, we tested the performance of some mutants as catalysts in reduction of a "normal" ketone, specifically acetophenone leading to phenyl ethanol. WT TbSADH* does not accept this compound. Mutant I86N proved to be active and *R*-selective (99% ee), while mutant W110M led to inversion of enantioselectivity (98% ee (*S*)), (Table S5).

In conclusion, ketones of type **1a–f** can be reduced enantioselectively with formation of axially chiral alcohols **2a–f**, being possible with appropriate alcohol dehydrogenases but not with transition-metal catalysts. Various ADHs from two commercial kits provide respective *R*-alcohols with essentially complete stereoselectivity (≥98% ee), while robust *T. brockii* (TbSADH*)^{11,12} proved to be only moderately *R*-selective (66% ee). To solve this problem, directed evolution was applied with generation of highly *R*- and *S*-selective TbSADH*

mutants. In the alcohol **2a** case with a vinyl bromide moiety, a variety of transition-metal catalyzed C–C bond forming substitution reactions are possible, as demonstrated in many Pd-catalyzed carbonylation and Suzuki coupling (SI) cases. Moreover, alcohol function (or the respective tosylate) reactions of can be envisioned with stereospecific introduction of fluorine or alkyl groups using standard methods, which means that a wide variety of structurally different axially chiral compounds is now readily accessible in *R*- or *S*-form. Initial work using four- and eight-membered cyclic ketones **3** and **5** shows that asymmetric reduction is likewise possible. Future efforts will focus on biophysical characterization of key mutants with emphasis on QM/MM-based elucidation of the stereo-selectivity source on a molecular level.

■ ASSOCIATED CONTENT

■ Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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