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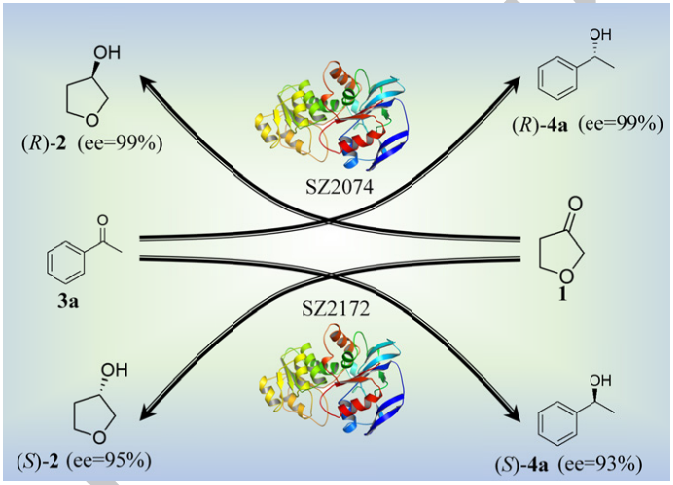

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Exploring the substrate scope of mutants derived from the robust alcohol dehydrogenase TbSADH

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ABSTRACT

Directed evolution of an enzyme as catalyst for a given stereoselective transformation provides a mutant for that particular reaction, but organic chemists need catalysts that are characterized by broad substrate acceptance. In a previous study we succeeded in evolving a set of variants of the thermally robust alcohol dehydrogenase TbSADH from *Thermoanaerobacter brockii* as catalysts in the (*R*)- and (*S*)-selective reduction of tetrahydrofuran-3-one, this difficult-to-reduce compound being a sterically small substrate. These mutants were now tested in the asymmetric reduction of seven structurally unrelated and sterically more demanding substrates, including acetophenone, benzyl methyl ketone, 4-phenyl-2-butanone and 2-oxo-4-phenyl-butanoic acid ethyl ester. The variants clearly out-perform WT TbSADH, but overly bulky substituted benzophenone derivatives are not accepted by WT or mutants.

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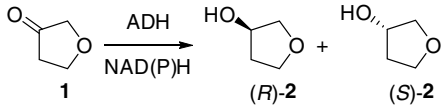
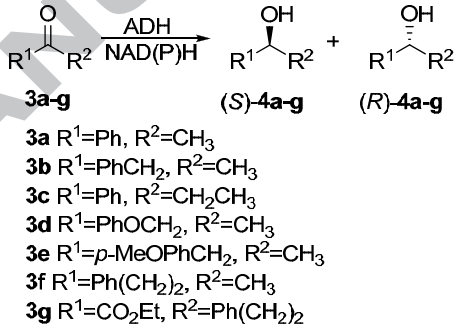
The asymmetric reduction of prochiral ketones with formation of chiral alcohols can be performed either with chiral transition metal catalysts[1] or enzymes of the type alcohol dehydrogenases (ADHs),[2] often in a complementary manner. Numerous ADHs are commercially available, but not all are robust enough for industrial applications. We were attracted by the pronounced thermostability of the ADH from *Thermoanaerobacter brockii* (TbSADH),[3,4] while realizing that its substrate scope with high stereoselectivity is not as broad as organic chemists would like it to be. For example, the asymmetric reduction of tetrahydrofuran-3-one (**1**) catalyzed by wildtype (WT) TbSADH leads to the formation of (*R*)-**2** with low enantioselectivity (23% ee) (Scheme 1),[5] which is unfortunate because its enantiomer (*S*)-**2** is a building block in the synthesis of the HIV inhibitors amprenavir and fosamprenavir.[6] Indeed, ketone **1** is a challenging substrate because the α- and α'-substituents flanking the carbonyl function are isosteric. Whenever prochiral ketones are characterized by sterically and electronically similar α- and α'-substituents, WT TbSADH and many other ADHs[2] as well as modern Ru-based synthetic catalysts[1] fail to deliver sufficiently high enantioselectivity.



Scheme 1. ADH-catalyzed asymmetric reduction of prochiral ketone **1**.

Protein engineering based on rational site-specific mutagenesis[7] or directed evolution[5,8] has been applied for enhancing and sometimes inverting the enantioselectivity of ADH-catalyzed ketone reductions.[9] For example, Phillips has engineered several active and enantioselective mutants of TbSADH which catalyze the asymmetric reduction of several structurally different ketones.[9a] Since screening is the bottleneck of directed evolution,[8] methods and strategies for evolving small but "smart" mutant libraries requiring a minimum of screening are needed. In this endeavor we recently proposed triple code saturation mutagenesis (TCSM) as a particularly efficient approach, and applied it successfully to limonene epoxide hydrolase[10] and to the alcohol dehydrogenase TbSADH, in the latter case as catalyst in the reduction of ketone **1**.[5] It was possible to enhance (*R*)-selectivity to 99% ee, and to invert stereoselectivity up to 95% ee (*S*) without significant tradeoff in thermostability (see also Table 2).[5] The best variants were also successful in the asymmetric reduction of three structurally related ketones.[5]

In the present study we report the performance of some of the previously evolved TbSADH variants[5] as catalysts in the asymmetric reduction of prochiral ketones which are not structurally related to **1**, namely **3a-g** (Scheme 2). Rather than screening the previous libraries or resorting to additional genetic optimization, a select set of mutants (Table S1) was assayed. The results are summarized in Table 1.



Scheme 2. Asymmetric reduction of ketones **3a-g** catalyzed by TbSADH mutants previously evolved as catalysts for the reduction of substrate **1**.

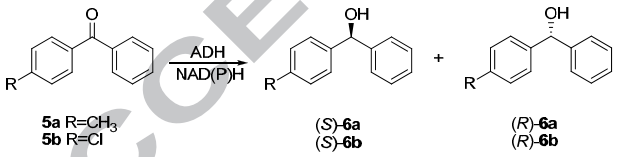
Table 1. Biocatalytic reduction of ketones **3a-g** by TbSADH mutants evolved for substrate **1**.

Entry	ADH[a] code	Reactions													
		3a→4a		**3b→4b**		**3c→4c**		**3d→4d**		**3e→4e**		**3f→4f**		**3g→4g**[b]	
		ee%	c%	ee%	c%	ee%	c%	ee%	c%	ee%	c%	ee%	c%	ee%	c%
1	WT	18(*S*)	12	78(*S*)	46	45(*S*)	36	>99(*S*)	>99	90(*S*)	37	>99(*S*)	>99	92(*R*)	92
2	SZ2006	91(*S*)	25	51(*S*)	99	95(*S*)	16	97(*S*)	>99	99(*S*)	94	71(*S*)	>99	27(*R*)	77
3	SZ2007	>99(S)	98	91(S)	>99	98(*S*)	82	>99(*S*)	>99	99(*S*)	94	>99(*S*)	>99	71(*R*)	76
4	SZ2012	>99(S)	97	96(S)	>99	97(*S*)	65	>99(*S*)	>99	99(*S*)	95	>99(*S*)	>99	9(*S*)	55
5	SZ2052	97(*R*)	90	63(*S*)	8	93(*R*)	86	>99(*S*)	75	nd	<5	94(*S*)	34	74(*R*)	81
6	SZ2055	72(*R*)	<5	81(*S*)	7	nd	<5	95(*S*)	15	nd	<5	>99(*S*)	29	44(*R*)	50
7	SZ2056	nd	<5	89(*S*)	7	nd	<5	>99(*S*)	8	nd	<5	nd	<5	5(*R*)	21
8	SZ2057	50(*R*)	<5	87(*S*)	7	nd	<5	92(*S*)	7	nd	<5	>99(*S*)	13	39(*R*)	41
9	SZ2058	28(*R*)	<5	24(*S*)	10	36(*S*)	7	>99(*S*)	84	nd	<5	>99(*S*)	24	64(*R*)	59
10	SZ2063	99(*R*)	96	nd	<5	98(*R*)	97	>99(*S*)	34	nd	<5	>99(*S*)	18	96(*R*)	99
11	SZ2074	99(*R*)	97	41(*S*)	9	91(*S*)	77	>99(*S*)	>99	99(*S*)	92	>99(*S*)	36	22(*R*)	28
12	SZ2110	92(*S*)	26	87(*S*)	>99	98(*S*)	20	>99(*S*)	>99	99(*S*)	95	90(*S*)	99	8(*R*)	69
13	SZ2111	>99(*S*)	99	>99(*S*)	99	>99(S)	98	>99(*S*)	>99	99(*S*)	95	>99(*S*)	>99	21(*R*)	43
14	SZ2114	98(*R*)	98	96(*R*)	95	97(*R*)	98	>99(*S*)	99	92(*S*)	8	>99(*S*)	97	97(*R*)	99
15	SZ2172	93(*S*)	71	95(*S*)	99	97(*R*)	97	>99(*S*)	64	nd	<5	>99(*S*)	84	>99(*R*)	99
16	SZ2176	96(*R*)	97	54(*R*)	13	97(*R*)	97	>99(*S*)	94	87(*S*)	<5	>99(*S*)	79	91(*R*)	95
17	SZ2205	99(*R*)	98	77(*R*)	41	98(*R*)	99	>99(*S*)	98	93(*S*)	7	>99(*S*)	98	>99(*R*)	>99
18	SZ2218	56(*R*)	6	>99(S)	99	51(*R*)	8	>99(*S*)	91	93(*S*)	9	>99(*S*)	98	50(*R*)	55
19	SZ2219	96(*S*)	95	>99(S)	>99	98(*S*)	98	>99(*S*)	99	99(*S*)	97	>99(*S*)	99	86(*R*)	97
20	SZ2224	5(*R*)	<5	73(*S*)	19	90(*R*)	59	>99(*S*)	97	95(*S*)	13	>99(*S*)	22	17(*S*)	25
21	SZ2225	10(*R*)	11	85(*S*)	19	15(*S*)	24	>99(*S*)	69	86(*S*)	<5	>99(*S*)	32	59(*R*)	59
22	SZ2257	69(*S*)	<5	>99(S)	97	82(*S*)	10	>99(*S*)	98	99(*S*)	64	>99(*S*)	96	4(*S*)	32

[a]The mutations of the ADH codes are summarized in Table S1, [b]Reaction time is 2h. The others are 14~16h; nd: not determined.

Several results are noteworthy, beginning with the performance of WT TbSADH. It proved to be a poor catalyst in the reduction of ketones **3a-c**, both enantioselectivity and conversion under the reaction conditions being unacceptable. In contrast, several mutants are excellent catalysts. For example, the poor (*S*)-selectivity (18% ee) and low conversion (12%) of WT TbSADH in the reaction of acetophenone (**3a**) was boosted by several variants to >99% ee at 97-98% conversion. Moreover, it was possible to invert stereoselectivity with preferential formation of (*R*)-**4** using several mutants (99% ee; 97-98% conversion). Similar results were observed for substrates **3b-c**. It is interesting to note that single mutants SZ2006 (W110A) and SZ1114 (I86A) have been prepared previously by directed evolution based on a different strategy.[9a] W110A is a poor catalyst for substrates **3a-c**, and I86A provides only to (*R*)-enantiomeric products.[9a] The advantage of the present new mutants is the possibility of obtaining both (*R*)- and (*S*)-products (see also Table S1).[5]

In the case of substrates **3d-f**, WT TbSADH shows good to excellent enantioselectivity in the range 90-99% ee in favor of the (*S*)-alcohols, although conversion in the reaction of **3e** turned out to be moderate (37% with 90% ee). In this case several mutants led to values of 99% ee and 94-95% conversion. However, for the keto-ester **3g**, WT TbSADH leads to (*R*)- **4g** (92% ee), which is the key precursor for the production of angiotensin-converting enzyme (ACE) inhibitors.[11] Fortunately, several mutants show improved enantioselectivity reaching 99% ee with full conversion. Although all of the ketones are already structurally very different from the original substrate **1**, we decided to go one step further by subjecting the very bulky benzophenone derivatives **5a-b** to reduction using the same TbSADH variants (Scheme 3). None of the mutants accepted these ketones, probably due to steric reasons. In a previous directed evolution study of TbSADH reported by Phillips and coworkers, mutants for the stereoselective reduction of structurally similar benzophenone substrates were in fact evolved.[9a] Thus, a toolbox of different robust TbSADH mutants now exists for a variety of different substrates.



Scheme 3. Attempted asymmetric reduction of ketones **5a-b** catalyzed by TbSADH mutants previously evolved for the reduction of substrate **1**.

WT TbSADH and the best (*R*)- and (*S*)-selective mutants SZ2074 and SZ2172, respectively, were characterized by kinetic experiments (Table 2). Relative to WT, both mutants show notably higher catalytic efficiency as reflected by the respective k_{cat}/K_m values. There is some tradeoff in thermostability (Table 2), but the mutants are still robust and likely to be acceptable candidates of industrial applications. One of the best mutants, SZ2074, was tested under preparative scale conditions using 30 mM (129 mg) of ketone **3a**. The conversion reached 94% within 6 h, enantioselectivity remaining at 99% ee with an isolated yield of 75%.

Table 2. Kinetic parameters of WT TbSADH and best mutants in the reaction of substrate **3a**

Enzymes	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m ($min^{-1}M^{-1}$)	Thermostability[5] T_{50}^{15}(°C)
WT	19.01±1.68	1.80±0.07	94.69	86
SZ2074	15.82±0.81	2.72±0.06	171.92	70
SZ2172	18.53±1.18	2.68±0.08	144.63	75

In order to explain the effect of mutations on stereoselectivity at the molecular level, induced fit docking calculations using substrate **3a** were performed. The configuration of reduced product is determined by attack of the nicotinamide hydride of NADPH from either the *Si*- or *Re*-face of the ketone, which leads to the (*R*)- or (*S*)-alcohol, respectively. Upon docking substrate **3a** into WT TbSADH, both *Si*- and *Re*-face additions of hydride to the carbonyl group appeared to be possible in the least-energy conformations (Fig. S1). This model explains the low enantioselectivity (18% *S*) observed for the WT-catalyzed reduction of **3a**. The situation in the case of the best mutants is quite different. According to the docking results using variant SZ2074, the nicotinamide ring of cofactor NADPH is located at the *Si*-face of **3a** (Fig. 1a), consistent with the observed (*R*)-selectivity. In the case of the (*S*)-selective variant SZ2172, the *Re*-face of **3a** is exposed to hydride attack by NADPH (Figure 1b). Additionally, the distances between NADPH cofactor and the carbonyl C-atom of the substrate are shorter in mutants SZ2074 and SZ2172 than WT TbSADH, in line with the observation that both mutants show higher catalytic efficiency relative to WT TbSADH.

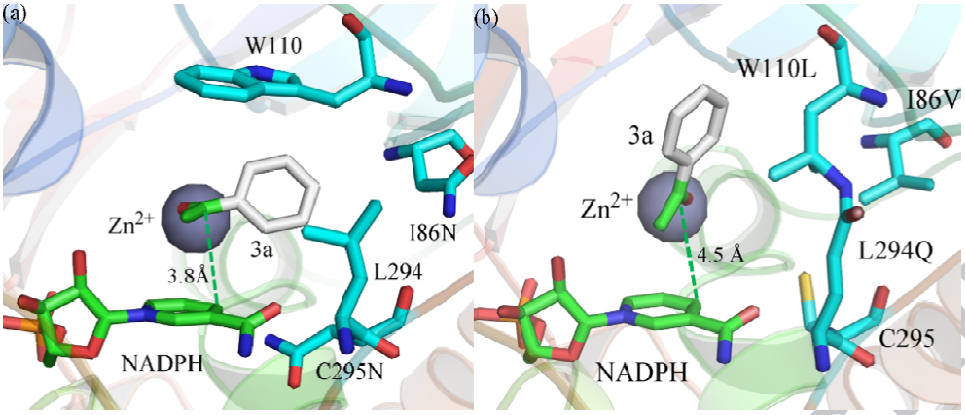


Figure 1. Docking poses of **3a** in (a) SZ2074 mutant of TbSADH and in (b) SZ2172 mutant. The distance between NADPH cofactor and the carbonyl C-atom of the substrate are highlighted by green dashed lines.

Conclusion

In conclusion, several robust mutants of TbSADH, previously evolved for the asymmetric reduction of the sterically small prochiral ketone **1** with little tradeoff in thermostability,[5] proved to be active and highly stereoselective in the reduction of a set of structurally unrelated and sterically more demanding ketones **3a-g**, but not of the extremely bulky benzophenone derivatives **5a-b**. In most cases these variants clearly out-performed WT TbSADH in terms of conversion and enantioselectivity. Thus, the substrate scope of this robust ADH has been expanded, while extending the list of useful TbSADH mutants.[5,9a,9b,9h] If stereoselectivity of the present mutants utilizing other substrates in future studies should prove to be insufficient, they can be employed as templates for further genetic optimization.

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These authors contributed equally.

Supplementary Material

Supplementary data associated with this article can be found, in the online version, at www.elsevier.com.

References and notes

1. (a) Noyori, R. *Angew. Chem.Int. Ed.* **2002**, *41*, 2008-2022; (b) Sandoval, C. A.; Li, Y.; Ding, K.; Noyori, R. *Chem. Asian J.* **2008**, *3*, 1801-1810; (c) Corey, E. J.; Helal, C. J. *Angew. Chem. Int. Ed.* **1998**, *37*, 1986–2012; (d) Morris, R. H. *Chem. Soc. Rev.* **2009**, *38*, 2282-2291.
2. Alcohol dehydrogenases (ADHs) as catalysts in asymmetric ketone reduction reviews: (a) Gröger, H.; Hummel, W.; Borchert, S.; Kraußer, M. in *Enzyme Catalysis in Organic Synthesis*, *3rd Edition*; Drauz, K.; Gröger, H.; May, O., Ed.; Wiley–VCH, Weinheim, 2012, pp 1035–1110; (b) Götz, K.; Hilterhaus, L.; Liese, A. in *Enzyme Catalysis in Organic Synthesis*, *3rd Edition*; Drauz, K.; Gröger, H.; May, O., Ed.; Wiley–VCH, Weinheim, 2012, pp. 1205–1223; (c) Woodley, J. M. *Trends Biotechnol.* **2008**, *26*, 321-327; (d) Matsuda, T.; Yamanaka, R.; Nakamura, K. *Tetrahedron: Asymmetry* **2009**, *20*, 513-557; (e) Musa, M. M.; Phillips, R. S. *Catal. Sci. Technol.* **2011**, *1*, 1311-1323; (f) Itoh, N. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 3889-3904.
3. (a) Keinan, E.; Hafeli, E. K.; Seth, K. K.; Lamed, R. *J. Am. Chem. Soc.* **1986**, *108*, 162-169; (b) Lamed, R. J.; Keinan, E.; Zeikus, J. G. *Enzyme Microb. Techol.* **1981**, *3*, 144-148; (c) Burdette, D. S.; Vieille, C.; Zeikus, J. G. *Biochem. J.* **1996**, *316*, 115-122; (d) Burdette, D. S.; Secundo, F.; Phillips, R. S.; Dong, J.; Scott, R. A.; Zeikus, J. G. *Biochem. J.* **1997**, *326*, 717-724.
4. Korkhin, Y.; Kalb, A. J.; Peretz, M.; Bogin, O.; Burstein, Y.; Frolow, F. J. *J. Mol. Biol.* **1998**, *278*, 967–981.
5. Sun, Z.; Lonsdale, R.; Ilie, A.; Li, G.; Zhou, J.; Reetz, M. T. *ACS. Catal.* **2016**, *6*, 1598-1605.
6. (a) Tandon, V. K.; Vanleusen, A. M.; Wynberg, H. *J. Org. Chem.* **1983**, *48*, 2767-2769; (b) Nobili, A.; Gall, M. G.; Pavlidis, I. V.; Thompson, M. L.; Schmidt, M.; Bornscheuer, U. T. *Febs. J.* **2013**, *280*, 3084-3093.
7. Review of rational design in protein engineering: Pleiss, J. in *Enzyme Catalysis in Organic Synthesis*; Drauz, K.; Gröger, H.; May, O., Ed.; Wiley–VCH, Weinheim, 2012, *Vol. 1*, pp. 89-117.
8. Recent reviews of directed evolution: (a) Bommarius, A. S. *Annu. Rev. Chem. Biomol. Eng.* **2015**, *6*, 319-345; (b) Currin, A.; Swainston, N.; Day, P. J.; Kell, D. B. *Chem. Soc. Rev.* **2015**, *44*, 1172-1239; (c) Denard, C. A.; Ren, H.; Zhao, H. *Curr. Opin. Chem. Biol.* **2015**, *25*, 55-64; (d) Gillam, E. M. J.; Copp, J. N.; F. D. Ackerley, in *Methods in Molecular Biology*, *Vol 1179*, Humana Press, Totowa, NJ, 2014; (e) Goldsmith, M.; Tawfik, D. S. *Methods Enzymol.* **2013**, *523*, 257-283; (f) Brustad, E. M.; Arnold, F. H. *Curr. Opin. Chem. Biol.* **2011**, *15*, 201-210; (g) Reetz, M. T. *Angew. Chem. Int. Ed.* **2011**, *50*, 138-174; (h) Jäckel, C.; Hilvert, D. *Curr. Opin. Biotechnol.* **2010**, *21*, 753-759; (i) Turner, N. J. *Nat. Chem. Biol.* **2009**, *5*, 568-574; (j) Lutz, S.; Bornscheuer, U. T. *Protein Engineering Handbook*, Wiley–VCH, Weinheim, 2009.
9. Examples of protein engineering of ADHs for enhanced stereoselectivity: (a) Nealon, C. M.; Musa, M. M.; Patel, J. M.; Phillips, R. S. *ACS Catal.* **2015**, *5*, 2100-2114; (b) Xu, G.; Shang, Y.; Yu, H.; Xu, J. *Chem. Commun.* **2015**, *51*, 15728-15731; (c) Zhang, D.; Chen, X.; Chi, J.; Feng, J.; Wu, Q.; Zhu, D. *ACS Catal.* **2015**, *5*, 2452-2457; (d) Liang, J.; Lalonde, J.; Borup, B.; Mitchell, V.; Mundorff, E.; Trinh, N.; Kochrekar, D. A.; Cherat, R. N.; Pai, G. G. *Org. Process Res. Dev.* **2010**, *14*, 193-198; (e) Spickermann, D.; Hausmann, S.; Degering, C.; Schwaneberg, U.; Leggewie, C. *ChemBioChem.* **2014**, *15*, 2050-2052; (f) Loderer, C.; Dhoke, G. V.; Davari, M. D.; Kroutil, W.; Schwaneberg, U.; Bocola, M.; Ansorge-Schumacher, M. B. *ChemBioChem* **2015**, *16*, 1512-1519; (g) Asako, H.; Shimizu, M.; Itoh, N. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 805-812; (h) Agudo, R.; Roiban, G. D.; Reetz, M. T. *J. Am. Chem. Soc.* **2013**, *135*, 1665-1668; (i) Patrikainen, P.; Niiranen, L.; Thapa, K.; Paananen, P.; Tähtinen, P.; Mäntsälä, P.; Niemi, J.; Metsä-Ketelä, M. *Chem. Biol.* **2014**, *21*, 2100-2114; (j) Li, H.; Yang, Y.; Zhu, D.; Hua, L.; Kantardjieff, K. *J. Org. Chem.* **2010**, *75*, 7559-7564.
10. Sun, Z.; Lonsdale, R.; Wu, L.; Li, G.; Li, A.; Wang, J.; Zhou, J.; Reetz, M. T. *ACS Catal.* **2016**, *6*, 1590-1597.

11. Xu, G.-C.; Ni, Y. *Bioresour. Bioprocess.* **2015**, *2*, 1-11.

Highlights

- Wide substrate scope of alcohol dehydrogenase mutants in asymmetric ketone reduction.
- Thermostable alcohol dehydrogenase mutants in enantioselective ketone reduction.
- Mild stereoselective biocatalysts as an alternative to chiral man-made Ru-catalysts.