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Racemization of enantiopure secondary alcohols by *Thermoanaerobacter ethanolicus* secondary alcohol dehydrogenase†

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Controlled racemization of enantiopure phenyl-ring-containing secondary alcohols is achieved in this study using W110A secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (W110A TeSADH) and in the presence of the reduced and oxidized forms of its cofactor nicotinamide-adenine dinucleotide. Racemization of both enantiomers of alcohols accepted by W110A TeSADH, not only with low, but also with reasonably high, enantiomeric discrimination is achieved by this method. Furthermore, the high tolerance of TeSADH to organic solvents allows TeSADH-catalyzed racemization to be conducted in media containing up to 50% (v/v) of organic solvents.

Introduction

There is a growing interest in developing racemization methods for enantiomerically pure secondary alcohols.[1] Racemization is the key step for successful dynamic kinetic resolution (DKR), a method that gained significant interest in the last two decades to produce optically active alcohols, in addition to other interesting enantiopure compounds.[2] Racemization can be used not only *in situ* with kinetic resolution (KR) to generate DKR, but also in a succession of KR and racemization rounds to recycle the unreactive enantiomer in KR reactions. With the exclusion of a few examples, like cyanohydrins and α-hydroxy carbonyl compounds, alcohols are non-stereolabile, and therefore racemization of their enantiopure forms is not an easy task.[3] A very well studied method for racemization of enantiopure secondary alcohols utilizes a transition metal-catalyzed redox pathway.[4] It has been shown that those organometallic catalysts are compatible with KR reactions catalyzed by either lipase or subtilisin, and therefore they have been employed successfully in DKR.[5]

New developments in biotechnology have made biocatalysis a practical and environmentally benign alternative to conventional organic and organometallic catalysis for a wide variety of reactions.[6] Biocatalysts are also known for their high chemo- and regioselectivity, which minimizes the formation of undesired by-products. A pair of alcohol dehydrogenases (ADHs) with opposite stereopreferences has been employed in racemization of enantiopure alcohols by a group guided by Faber and Kroutil.[7] They were also able to achieve the same task by employing *Pseudomonas fluorescens* ADH, a non-stereoselective ADH. However, such non-selective ADHs are uncommonly found.[7]

We have been working with the secondary alcohol dehydrogenase from *Thermoanaerobacter ethanolicus* (TeSADH, EC 1.1.1.2),[8] a nicotinamide-adenine dinucleotide phosphate (NADPH)-dependent enzyme, to produce enantiomerically pure alcohols. We have also applied site-directed mutagenesis to expand its substrate specificity.[9] With their exceptional characteristics, like high thermal stability and high tolerance to organic solvents,[10] TeSADH and its mutants have been successfully employed in asymmetric transformations to produce enantiomerically pure alcohols from their corresponding ketones *via* asymmetric reduction or from their racemates through KR.[9]

One of the more interesting mutants is W110A TeSADH, a Prelog stereoselective ADH that recognizes phenyl-ring-containing alcohols and their corresponding ketones with good activity.[11] We noticed in our previous studies that phenylacetone was reduced to (*S*)-1-phenyl-2-propanol ((*S*)-**1a**) by

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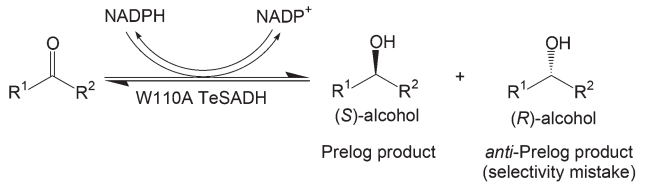
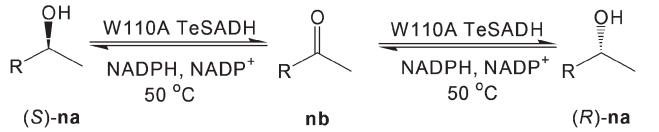


Scheme 1 Asymmetric reduction of phenyl-ring-containing ketones by W110A TeSADH.

W110A TeSADH with low enantioselectivity (37% ee).[11] Such low enantioselectivity can be explained by the reverse fit of the substrate in the active site of the enzyme that leads to delivering the hydride from the undesired face of the ketone, *i.e.* the *Si* face rather than the *Re* face, leading to what is called "selectivity mistake", as shown in Scheme 1. This low enantioselectivity stimulated us to study the W110A TeSADH-catalyzed racemization of enantiopure secondary alcohols.

In this paper, W110A TeSADH was used to racemize phenyl-ring-containing enantiopure alcohols in an attempt to recycle the slow reacting alcohol enantiomer in KR either *in situ* to achieve DKR or separately by sequential KR and racemization cycles. We found that W110A TeSADH can racemize not only a substrate for which it shows low stereospecificity (*e.g.*, 1-phenyl-2-propanol), but also substrates for which it shows moderately high stereospecificity.

Table 1 W110A TeSADH-catalyzed racemization of enantiopure phenyl-ring-containing secondary alcohols[a]



Entry	R	Substrate	*E* value (*S*/*R*)[b]	ee[c] (%) Before	After
1	$PhCH_2$	(*S*)-**1a**	3.4	>99	6.3 (*S*)
2	$PhCH_2$	(*S*)-**1a**	3.4	>99	4.3 (*S*)[d]
3	$PhCH_2$	(*R*)-**1a**	3.4	>99	10.3 (*R*)
4	$PhCH_2$	(*R*)-**1a**	3.4	>99	15.4 (*R*)[d]
5	*p*-$MeOC_6H_4(CH_2)_2$	(*S*)-**2a**	12[e]	91	44.0 (*S*)
6	$PhCH_2CH_2$	(*R*)-**3a**	>100	>99	34.5 (*R*)
7	$PhCH_2CH_2$	(*S*)-**3a**	>100	99	82.4 (*S*)
8	$PhCH_2CH_2$	(*S*)-**3a**	>100	72.7	51.8 (*S*)
9	$PhOCH_2$	(*S*)-**4a**	40	>99	>99 (*S*)

[a] Unless otherwise mentioned, reactions were performed at 50 °C using enantiopure **na** (3 μL), W110A TeSADH (0.3 mg), NADPH (1.0 mg), $NADP^+$ (0.5 mg), CH_3CN (200 μL), and Tris-HCl buffer solution (800 μL, 50 mM, pH 8.0). [b] *E*-values were calculated as described in the Experimental section for KR reactions in Tris-buffer solution containing 10% (v/v) acetone, reported in ref. 11. [c] Determined by GC equipped with a chiral column for the corresponding acetate derivative. [d] DMF was used instead of CH_3CN as a cosolvent. [e] This *E*-value is for a KR reaction conducted in biphasic system containing diisopropyl ether as a cosolvent and 4 eq. of acetone as a cosubstrate, reported in ref. 12.

Results and discussion

We describe W110A TeSADH-catalyzed racemization of phenyl-ring-containing enantiopure alcohols. In this report, W110A TeSADH was used as a racemizing agent, instead of being used to generate optically active alcohols. This racemization was accomplished by manipulating the reversible interconversion between ketone and alcohol substrates in W110A TeSADH-catalyzed redox transformations. We started our racemization efforts on (*S*)-**1a** and (*R*)-**1a** substrates due to their poor enantiomeric discrimination by W110A TeSADH. The racemization was conducted in Tris-HCl buffer solution containing 20% (v/v) acetonitrile as a cosolvent to enhance the solubility of the substrate. Both NADPH and $NADP^+$ (2 : 1, w/w) were included in the reaction medium to allow the racemization to take place by facilitating the equilibrium in the redox reaction. The ratio between NADPH and $NADP^+$ was chosen to allow racemization to take place with minimal formation of the prochiral ketone. We were able to reduce the ee of (*S*)-**1a** from >99% to 6.3% in 48 h at 50 °C, as reported in Table 1. The same procedure was employed to racemize (*R*)-**1a**, and we were able to reduce its ee from >99% to 10.3%, as shown in Table 1. Similar results were obtained when dimethyl formamide (DMF) was used rather than acetonitrile as a cosolvent, as shown in the second and fourth entries of Table 1. (*S*)-4-(4′-Methoxyphenyl)-2-butanol ((*S*)-**2a**) was exposed to the same racemization conditions, and its ee was decreased from 91% to 44%. As expected, (*S*)-**2a** was racemized to a lesser extent than (*S*)-**1a**, which is simply explained by the difference in enantiomeric ratio, *i.e.* *E*-value, for the W110A TeSADH-catalyzed enantiospecific KR for their racemates.[12]

The racemization method elucidated above depends on the *E*-value; the lower the *E*-value, the better the racemization efficiency. We have reported that this value for W110A TeSADH-catalyzed enantiospecific KR reactions varies by changing the organic cosolvent used.[12] For example, for the KR reaction with *rac*-**2a**, the *E*-value varies from one to 100 by switching the cosolvent from hexane to toluene.[12] Such solvent-dependent selectivity might serve as a guide for choosing the proper solvent in conducting racemization.

In theory, we should be able to racemize enantiopure alcohols that are recognized by W110A TeSADH with relatively high enantiospecificity, as long as there is a selectivity mistake, because racemization is a thermodynamically downhill process owing to the entropy increase associated with forming the two enantiomers from one.[13] In most ADH-catalyzed asymmetric transformations, a large excess of a cosubstrate is normally used to enhance the solubility of substrates and to drive the equilibrium toward the reduction or oxidation direction, a common approach for cofactor recycling called "coupled-substrate".[14] In ADH-catalyzed racemization, no cosubstrate is needed for cofactor recycling, because the net redox balance is zero,[7] and therefore the interconversion between alcohol and ketone substrates is facilitated by having NADPH and $NADP^+$ in the reaction medium, leading to more selectivity mistakes. We have reported that benzylacetone (**3b**)

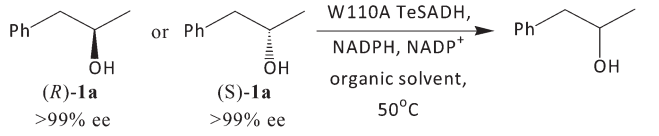
was reduced to (*S*)-4-phenyl-2-butanol ((*S*)-**3a**) with high enantioselectivity (99% ee).[11] Using the same racemization procedure as used for (*S*)-**1a** and (*R*)-**1a**, we were able to decrease the ee of (*R*)-**3a** from >99% to 34.5% in 48 h (Table 1). However, the ee of (*S*)-**3a** was reduced from 99% to 82.4% in 48 h. In a separate experiment, we were able to reduce the ee of (*S*)-**3a** from 72.7% to 51.8% in 48 h, which indicates that (*S*)-**3a** can be racemized by W110A TeSADH to a lesser extent than (*R*)-**3a**, but that it could be racemized if adequate time is allowed. These results indicate that even alcohols accepted by W110A TeSADH with high enantiomeric discrimination (*e.g.*, (*S*)-**3a** and its *anti*-Prelog analog, (*R*)-**3a**) can still be racemized by this method. As shown in Table 1, the *E*-value for stereospecific KR of (*rac*)-**3a** is >100.

However, using the above-mentioned racemization procedure, the ee value for (*S*)-phenoxy-2-propanol ((*S*)-**4a**) remained >99% after 48 h. We have previously noticed that (*S*)-**4a** was produced from phenoxy-2-propanone (**4b**) in high yield and high enantioselectivity (both >99%), but in the stereospecific oxidation of *rac*-**4a**, we were not able to drive the equilibrium to more than 19% conversion of (*S*)-**4a** to the corresponding ketone.[11] These results indicate that the equilibrium favors the reduction direction in this case, probably due to the formation of an intramolecular hydrogen bond between the alcoholic hydrogen and the oxygen of the ether, and therefore restricts the interconversion between **4b** and its alcohol. Furthermore, the electronegative phenoxy substituent probably makes the equilibrium favor the alcohol more than for the other aromatic substrates.

It is worth mentioning that the percentage of ketone formed as a result of equilibrium with alcohols did not exceed 3% in the cases described, except for **2a** which was 7% (see Fig. S4, ESI†). Such percentage could become even less if this racemization method is combined with a KR reaction that will deplete one enantiomer of the alcohols and thus pull the equilibrium in the reduction direction.

In order to be able to utilize this racemization method in combination with interesting reactions such as KR and with useful scales, we sought to carry out W110A TeSADH-catalyzed racemization in non-aqueous media. We reported previously that W110A TeSADH can perform redox reactions in high concentrations of water-miscible and -immiscible organic solvents with more than 50% (v/v) and with reasonable concentrations of substrate (up to 120 mM).[12] However, racemization of (*S*)-**1a** and (*R*)-**1a** in monophasic media containing acetonitrile (50%, v/v) as the organic cosolvent and Tris-HCl buffer solution was not successful. This is because W110A TeSADH tolerates acetonitrile at high concentration as a cosolvent for a restricted amount of time that is not enough to conduct racemization as evident by the observation that ee for (*R*)-**1a** and (*S*)-**1a** was reduced from >99% to about 94% (Table 2, entries 1 and 2). It should be noted that we have reported that W110A TeSADH can perform asymmetric redox reactions in high yield using acetonitrile, Tris-HCl buffer solution, and 2-propanol [38 : 38 : 24 (v/v/v)] as the reaction medium.[12]

Table 2 Racemization of (*S*)-**1a** and (*R*)-**1a** by using W110A TeSADH in organic solvent[a]



Entry	Substrate	Solvent	ee[b] (%)
1	(*S*)-**1a**	CH_3CN[c]	94 (*S*)
2	(*R*)-**1a**	CH_3CN[c]	95 (*R*)
3	(*S*)-**1a**	Hexane	81 (*S*)
4	(*S*)-**1a**	Hexane[d]	86 (*S*)
5	(*S*)-**1a**	MTBE	41 (*S*)
6	(*R*)-**1a**	MTBE	52 (*R*)

[a] Unless otherwise mentioned, reactions were performed at 50 °C using enantiopure **1a** (0.22 mmol), W110A TeSADH (1.4 mg), NADPH (2.0 mg), $NADP^+$ (2.0 mg), organic solvent (700 μL), and Tris-HCl buffer solution (900 μL, 50 mM, pH 8.0). [b] Determined by GC equipped with a chiral column for the corresponding acetate derivative. [c] 1.5 mL of Tris-HCl buffer solution (50 mM, pH 8.0) and 1.5 mL of organic solvent were used. [d] 200 μL of CH_3CN and 700 μL of hexane were added.

We were discouraged by the racemization efficiency in monophasic media containing CH_3CN (50%, v/v). We decided therefore to conduct the W110A TeSADH-catalyzed racemization in biphasic media that consisted of organic water-immiscible solvents as the non-aqueous phase and Tris-HCl buffer solution as the aqueous phase. When hexane was used as the organic solvent, we were able to reduce the ee of (*S*)-**1a** from >99% to 81%. This low racemization efficiency could be explained by the low solubility of the corresponding ketone in aqueous solution, which could drive it to reside in hexane and therefore to be retarded from the redox reaction. The addition of 12% (v/v) of acetonitrile did not facilitate the racemization of (*S*)-**1a**, and the ee of the isolated alcohol was 86% after 48 h (Table 2, entry 4). When methyl *tert*-butyl ether (MTBE) was used as the organic solvent, the ee of (*S*)-**1a** was reduced from >99% to 41%. We attributed this to the difference in the hydrophobicity of hexane and MTBE, with the latter being less. Under the same conditions, we observed that racemization of (*R*)-**1a** took place to a similar extent as (*S*)-**1a**; and its ee was reduced from >99% to 52%.

When W110A TeSADH-catalyzed racemization of enantiopure **1a** was conducted on the scale described in Table 2, the corresponding ketone was either not detected or hardly observed (<1%). This is due to the negligible ratio of the cofactor with respect to the alcohol when compared with the small scale described in Table 1. With the exception of the negligible amount of the ketone formed by W110A TeSADH-catalyzed redox reaction, we did not notice the formation of any other by-products. This is due to the high enzyme selectivity and the mild reaction conditions employed. For example, the percent recovery for the reaction that is explained by entry 6 in Table 2 was 89% of pure alcohol. The purity of the isolated product was confirmed by GC and ^1H NMR.

The high tolerance of TeSADH to organic solvents together with its high thermal stability might allow this environmentally benign mild racemization method to be incorporated with other organic reactions such as enzyme-catalyzed KR.[15] This racemization protocol seems to be slower than what one expects for an efficient DKR. However, the latter can be achieved with a racemization method that has a rate constant (k_{rac}) less than the more reactive enantiomer in KR (k_{fast}) if k_{rac} is much greater than the rate constant for the slow reacting enantiomer in KR (k_{slow}) (*i.e.* $k_{fast} > k_{rac} \gg k_{slow}$).[13a] This enzyme-catalyzed racemization is an attractive approach due to the high chemo- and regioselectivity of enzymes. More site-directed mutagenesis is needed to construct non-selective mutants of TeSADH to increase the efficiency of this racemization approach. Further developments will be reported in due course.

Conclusions

In conclusion, W110A TeSADH has shown to be active in racemization of phenyl-ring-containing enantiopure alcohols. We demonstrated that W110A TeSADH-catalyzed racemization is valid for both enantiomers of alcohols accepted by this enzyme with not only low, but also high enantiomeric discrimination. We have also shown that this racemization protocol can be conducted with useful scales in organic solvents. The capability of TeSADH to perform racemization under mild conditions in addition to its high thermal stability and high tolerance to organic solvents is of great interest. It also represents an attractive approach especially if it proves successful *in situ* with other interesting reactions such as KR.

Experimental

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 He as the carrier gas. Commercial grade solvents were used without further purification. $NADP^+$, NADPH, (*rac*)-**1a**, (*rac*)-**3a**, (*R*)-**1a** (>99% ee), (*S*)-**1a** (>99% ee), (*S*)-4-(4′-methoxyphenyl)-2-butanol (**2b**), benzyl acetone (**3b**) and **4b** were used as purchased from commercial sources. (*R*)-**3a** (>99% ee) and (*S*)-**3a** (72.7% ee) were prepared by lipase-catalyzed KR of their racemates as reported.[16] (*S*)-**3a** (99% ee), (*S*)-**4a** (>99% ee) and (*S*)-**2a** (91% ee) were prepared by W110A TeSADH-catalyzed reduction of their corresponding ketones as reported.[11] All Tris-HCl buffer solutions were adjusted to pH 8.0 at room temperature.

Gene expression and purification of W110A TeSADH

W110A TeSADH, used in the small scale experiments, was expressed in recombinant *Escherichia coli* HB101(DE3) cells and purified as reported.[16] W110A TeSADH, used in racemization in organic solvents, was expressed in recombinant *Escherichia coli* BL21(DE3) cells and purified as reported[16] with modifications. The resuspended cells in lysis buffer were lysed by sonication (40% amplitude for 5 min), and the lysate was centrifuged (70 000 *g* for 30 min) to remove cell debris. The heat-treated crude extract was spun down (70 000 *g* for 30 min). The cleared crude extract was loaded on a 5 ml-HisTrap FF (GE Healthcare) column. The eluted proteins were collected and diluted to adjust imidazole concentration to be less than 20 mM and loaded on 5 ml-HisTrap and eluted by imidazole gradient.

General procedure for small scale W110A TeSADH-catalyzed racemization

A mixture of enantiopure alcohol (3 μL), $NADP^+$ (0.5 mg), NADPH (1.0 mg), W110A TeSADH (0.3 mg), Tris-HCl buffer solution (800 μL, 50 mM, pH 8.0), and acetonitrile (200 μL) were placed in a 1.5 mL plastic tube. The reaction mixture was shaken at 50 °C at 200 rpm for 48 h then extracted with ethyl acetate (2 × 500 μL). The combined organic layer was dried with sodium sulfate and concentrated to dryness. The remaining residue was treated with pyridine and acetic anhydride to convert the alcohols to their corresponding acetates as described.[17] The acetate products were analyzed by GC equipped with a chiral column to determine their ee.

W110A TeSADH-catalyzed racemization in monophasic systems

A mixture of $NADP^+$ (2.0 mg), NADPH (2.0 mg), Tris-HCl buffer solution (1.3 mL, 50 mM, pH 8.0), W110A TeSADH [1.4 mg in 200 μL of 50 mM Tris-HCl buffer (pH 8.0)], CH_3CN (1.5 mL), and enantiopure alcohol (0.22 mmol) was added in the same sequence to a round-bottomed flask equipped with a magnetic stirrer. The reaction mixture was stirred at 50 °C for 48 h. It was then extracted with ethyl acetate (2 × 3 mL). The organic layers were combined and extracted with brine solution (3.0 mL) then dried with sodium sulfate then concentrated under vacuum. The remaining residue was treated with pyridine and acetic anhydride to convert alcohols to the corresponding esters.[17] The acetate products were analyzed by GC equipped with a chiral column.

W110A TeSADH-catalyzed racemization in biphasic systems

A mixture of $NADP^+$ (2.0 mg), NADPH (2.0 mg), and W110A TeSADH (1.4 mg in 900 μL of Tris-HCl buffer solution, 50 mM, pH 8.0) was added to water-immiscible organic solvent (700 μL) containing enantiopure alcohol (0.22 mmol) in a round-bottomed flask equipped with a magnetic stirrer. The mixture was stirred at maximum speed at 50 °C for 48 h. The two layers were then separated and the aqueous layer was extracted with ethyl acetate (2 × 3 mL). The combined organic layers were mixed with the original organic layer and extracted with brine solution (3.0 mL) then dried with sodium sulfate. Volatile components were evaporated under vacuum. The mass of the remaining residue was reported to calculate percent recovery of alcohol. Alcohols obtained were converted

to the corresponding acetate esters prior to their analysis by GC equipped with a chiral column.

Determination of absolute configuration of alcohols

The absolute configurations of the produced alcohols were elucidated by comparing the retention time of their acetate derivatives with either their *S*- or *R*-acetate enantiomer after injection on a GC equipped with a chiral stationary phase.

Calculation of *E*-values

E-values were calculated from the formula $E = \ln[(1 - c)(1 - ee_s)]/\ln[(1 - c)(1 + ee_s)]$, where c is percentage conversion of alcohol to ketone, and ee_s is enantiomeric excess of the slow reacting (*R*)-alcohol in W110A TeSADH-catalyzed KR.[11]

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