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Biocatalysis

International Edition: DOI: 10.1002/anie.201713189
German Edition: DOI: 10.1002/ange.201713189Kinetic Resolution of *sec*-Thiols by Enantioselective Oxidation with Rationally Engineered 5-(Hydroxymethyl)furfural Oxidase

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Abstract: Various flavoprotein oxidases were recently shown to oxidize primary thiols. Herein, this reactivity is extended to *sec*-thiols by using structure-guided engineering of 5-(hydroxymethyl)furfural oxidase (HMFO). The variants obtained were employed for the oxidative kinetic resolution of racemic *sec*-thiols, thus yielding the corresponding thioketones and nonreacted *R*-configured thiols with excellent enantioselectivities ($E \geq 200$). The engineering strategy applied went beyond the classic approach of replacing bulky amino acid residues with smaller ones, as the active site was additionally enlarged by a newly introduced Thr residue. This residue established a hydrogen-bonding interaction with the substrates, as verified in the crystal structure of the variant. These strategies unlocked HMFO variants for the enantioselective oxidation of a range of *sec*-thiols.

The demand for chiral building blocks for pharmaceutical applications is a driving force for the development of new methodologies in modern synthetic chemistry. In this context, biocatalysis, which excels in producing chiral molecules, represents an increasingly important tool for synthetic chemists.^[1] Organosulfur moieties constitute highly relevant structural elements with a broad range of bioactivities, and efficient strategies for their selective production are sought. Examples include the acyl-coA: cholesterol acyltransferase inhibitor Eflucimibe, the leukotriene receptor antagonist Montelukast, or the calcium channel blocker Diltiazem (Figure 1).^[2] Recent approaches to the synthesis of non-racemic *sec*-thiols include the ring-opening of chiral thiazol-4-ones produced by organocatalytic Michael addition^[3] and S–H bond insertion cooperatively catalyzed by a rhodium

Figure 1. Examples of active pharmaceutical ingredients containing *sec*-thiol moieties.

catalyst and a chiral spirophosphoric acid,^[2f] and the formation of chiral *vic*-hydroxythiols from *cis*-epoxides.^[4] However, these systems suffer from the requirement of complex starting materials and/or high loadings of sophisticated organocatalysts. For the kinetic resolution of racemic thiols by esterification, organo- and biocatalytic methods are known. Cinchona-alkaloid-based organocatalysts allowed the resolution of various benzylic *sec*-thiols, though excellent enantioselectivities were achieved only with a single substrate at high catalyst loadings (Scheme 1a).^[5] Biocatalytic methods, such as the kinetic resolution of thiols with baker's yeast were only moderately successful.^[6] The prominent lipase B from *Candida antarctica* catalyzes the enantioselective hydrolysis of thioesters (Scheme 1a). In contrast, the kinetic resolution by esterification of thiols failed.^[7]

Flavoprotein oxidases, which are well established for the dioxygen-dependent oxidation of alcohols to carbonyl moieties,^[8] were recently shown to catalyze the transformation of primary thiols into the corresponding thiocarbonyl analogues.^[9] One of the enzymes exhibiting this activity is 5-(hydroxymethyl)furfural oxidase (HMFO; EC 1.1.3.47).^[10] In addition to its ability to produce the polymer building block 2,5-furandicarboxylic acid from 5-(hydroxymethyl)furfural,^[11] HFMO is able to oxidize a broad range of electronically activated benzylic and cinnamic primary alcohols,^[10] and its variants transform *sec*-alcohols in a stereo-preferred fashion.^[12] The enzyme oxidizes the alcohol (or the hydrated aldehyde) to the corresponding carbonyl/carboxy product by

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Scheme 2. Biocatalytic enantioselective oxidation of the (S)-1-phenylethanethiols **1a–h** with HMFO variants.

first-order rate constants for anaerobic flavin reduction under saturation conditions (k_{red}) were determined using a stopped-flow apparatus. This data allowed us to estimate the overall activity because hydride transfer in the reductive half-reaction was demonstrated to be the rate-limiting step.^[12b,c] Furthermore, the effect of mutations on substrate binding was simultaneously studied by determining the apparent macroscopic dissociation constant for binding of the substrate to the enzyme (K_d). For all stopped-flow measurements, the model substrate **rac-1a** was used (Scheme 2). Whereas wild-type HMFO, Trp466His HMFO, and Trp466Ala HMFO did not show any detectable activity with **rac-1a**, all other variants exhibited significant flavin reduction (Table 1). Remarkably,

Table 1: Pre-steady-state kinetic parameters of HMFO variants on **rac-1a** phenylethanethiol (**1a**).^[a]

HMFO	k_{red} [s ⁻¹]	K_d [mM]
Wild-type	< 0.001	n.d.
Trp466His	< 0.001	n.d.
Trp466Ala	< 0.001	n.d.
Trp466Phe	0.034 ± 0.007	1.3 ± 0.5
Trp466Tyr	0.010 ± 0.001	1.1 ± 0.3
Val465Ser	0.020 ± 0.002	0.6 ± 0.1
Val465Thr	0.021 ± 0.003	0.6 ± 0.2
Val465Thr/Trp466Phe	0.009 ± 0.003	1.3 ± 0.9

[a] Experiments were performed at 30 °C in phosphate buffer (100 mM, pH 7.0) under anaerobic conditions. Reduction was measured at A_{max} of HMFO-variant (see Table S2). n.d. = not detectable.

the activities and K_d values were in a similar range. An attempt to combine both space-creating effects in a double mutant Trp466Phe/Val465Thr failed: no additional improvement over the corresponding single mutants could be generated. Overall, the strategy to introduce activity on *sec*-thiols was successful, as the K_d value of **rac-1a** for all active variants was comparable to that of benzyl alcohol with wild-type HMFO ($K_d = 1.7$ mM).^[12b,c]

Motivated by the promising kinetic properties observed for the above HMFO variants, their biocatalytic applicability was investigated with substrate **rac-1a**. DL-dithiothreitol (DTT) was added as an anti-oxidant to the reaction mixture (see Figure S1 in the Supporting Information).^[13] This addi-

tion suppressed the formation of disulfide adducts and traces of the elimination product styrene for all tested substrates. Wild-type HMFO was almost unreactive with **rac-1a** (ca. 3 % conversion), thus confirming the kinetic data. In contrast, all variants gave good conversions in the oxidation of **rac-1a** (up to 42 %, Figure 3) and an enantiomeric excess of substrate

Figure 3. Conversion of **rac-1a** by HMFO variants. Reactions were performed in phosphate buffer (100 mM, pH 7.0) containing isopropanol (1%, v/v), DTT (50 mM), 5 mM substrate, and HMFO variants (3.5 μ M), at 30 °C for 24 h in air-saturated solutions (235 μ M dioxygen). Disulfide formation less than 3 %.

(ee_s) of up to 91 % (Table 2). The degrees of conversion correlate well with the determined kinetic parameters (Figure 3 and Table 1). The highest conversions were obtained with the two Val465 mutants which also displayed the lowest K_d values. No conversion of the substrate was observed in control reactions without the enzyme. Overall, Val465Ser was identified as the most potent variant and was used for follow-up experiments.

To prove the general applicability of this method, the tolerance of electron-donating and electron-withdrawing substituents on the aromatic ring were evaluated since the electron density on the aromatic ring may influence the rate

Table 2: Enantioselective oxidation of 1-phenylethanethiol derivatives by Val465Ser HMFO variant.^[a]

<i>rac</i> -Substrate	Conversion [%] ^[b]	<i>ee_s</i> (<i>R</i>) [%] ^[c]	<i>E</i> ^[d]
1a	48	91	> 200
1b	50	88	45
1c	47	87	> 200
1d	44 (50) ^[e]	78 (> 97) ^[e]	> 200
1e	30 (45) ^[e]	42 (80) ^[e]	150
1f	50	> 97	> 200
1g	49	> 97	> 200
1h	44 (49) ^[e]	78 (93) ^[e]	> 200

[a] Reactions were performed in phosphate buffer (100 mM, pH 7.0) containing isopropanol (1 % v/v), DTT (50 mM), 5 mM substrate, and Val465Ser HMFO variant (3.5 μ M), at 30 °C for 24 h in air-saturated solutions. [b] Conversion was measured by GC-MS after extraction with dichloromethane. [c] Enantiomeric excess of the *R*-configured enantiomer was measured by HPLC after derivatization. [d] *E*-value determined from *ee_s* value and conversion.^[14] [e] Doubled concentration of HMFO variant (7 μ M).

of hydride transfer in HMFO.^[15] The *m*- and *p*-substituted substrates *rac*-**1b–g** and the naphthyl analogue **1h** were tested (Scheme 2). The latter were prepared from the corresponding alcohols by mesylation through a Mitsunobu protocol, with subsequent nucleophilic substitution with thioacetate and thioester reduction over lithium aluminium hydride (see the Supporting Information).^[5]

HMFO-Val465Ser accepted all 1-phenylethanethiol analogues (Table 2). *Rac*-**1f** and *rac*-**1g**, bearing activating electron-donating substituents were converted in up to 50 % with *ee_s* values of greater than 97 % for *R*-configured enantiomers, and corresponds to perfect *E* values of greater than 200. On the contrary, the conversions of the bromo-substituted substrates *rac*-**1d** and *rac*-**1e** were slower, albeit with excellent enantioselectivities. Overall, unreacted *R*-configured enantiomers were recovered for all substrates in yields of 15–61 % after column chromatography. The absolute configurations of **1a**, **1c**, **1g**, and **1h** were determined to be *R* by comparison of optical rotation values with those in the literature.^[5,16] The absolute configurations of **1b** and **1d–f** were concluded to be *R* because of the contiguous (+) sense of optical rotation of **1a–h**, as it is in line with an identical elution order on chiral-phase HPLC [(*R*) first, (*S*) second; see the Supporting Information]. The absolute configuration for **1e** was additionally confirmed by CD spectroscopy (see Figure S8). Overall, the Val465Ser HMFO variant proved to be a reliable biocatalyst for the kinetic resolution of benzylic *sec*-thiols.

The formation of thioacetophenone (**2b**) as an oxidation product of *rac*-**1b** was proven by GC-analysis using the reference material **2b**, which was independently synthesized using Lawesson's reagent (see Figure S4),^[17] thus confirming previous observations.^[9] However, the latter are rapidly hydrolyzed, via monothioketals, in aqueous medium to yield the corresponding carbonyl analogues along with the formation of hydrogen sulfide. Consequently, the corresponding acetophenone derivatives **3a–h** were predominantly found in the reaction mixture after 24 hours (see Figure S5).^[18]

Attempts to convert the kinetic resolution into a cyclic deracemization^[19] through (chemical) in situ reduction of the thioketone, formed in analogy to the cyclic deracemization of amines employing amino acid^[20] or amine oxidases^[21] and boron-based reducing agents, unfortunately failed. Neither borohydrides nor borane complexes were reactive enough to compete with the fast spontaneous hydrolysis of the thioketone intermediate (see the Supporting Information).

In summary, rationally designed variants of HMFO were employed for the enantioselective oxidation of 1-phenylethanethiols, and allowed their kinetic resolution with unprecedented selectivities (*E* up to > 200). Variants were created by not only replacing bulky amino acid residues with smaller ones, but also by a space-creating rearrangement of amino acids within the active site enforced by additional hydrogen bonding, which was predicted by molecular modeling and confirmed by crystal structure analysis. This method provides a broadly applicable tool for the preparation of nonracemic *sec*-thiol building blocks.

General procedure for the kinetic resolution of thiols: Purified HMFO variants (3.5 μ M) and DTT (50 mM) were dissolved in sodium phosphate buffer (500 μ L, pH 7.0, 100 mM) containing isopropanol (1 % v/v). Then the substrate (**1a–h**) was added (5 mM). The vials were shaken at 30 °C with 750 rpm for 24 h in a horizontal position on a Heidolph Titramax 1000 plate shaker. The reaction was stopped by extraction with dichloromethane (2 $\times$ 0.5 mL). The combined organic phases were dried over anhydrous Na₂SO₄ and the conversion was determined on GC-MS. Ketone and thiol were separated by column chromatography on silica gel and the *ee* value of the *R*-configured thiols was measured by chiral-phase HPLC after derivatization (see the Supporting Information).

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Conflict of interest

The authors declare no conflict of interest.

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