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Biocatalysis

# Enantiocomplementary Epoxidation Reactions Catalyzed by an Engineered Cofactor-Independent Non-natural Peroxygenase

*Guangcai Xu, Michele Crotti, Thangavelu Saravanan, Kim M. Kataja, and Gerrit J. Poelarends**

***Abstract:*** *Peroxygenases are heme-dependent enzymes that use peroxide-borne oxygen to catalyze a wide range of oxyfunctionalization reactions. Herein, we report the engineering of an unusual cofactor-independent peroxygenase based on a promiscuous tautomerase that accepts different hydroperoxides (t-BuOOH and $H_2O_2$) to accomplish enantiocomplementary epoxidations of various α,β-unsaturated aldehydes (citral and substituted cinnamaldehydes), providing access to both enantiomers of the corresponding α,β-epoxy-aldehydes. High conversions (up to 98 %), high enantioselectivity (up to 98 % ee), and good product yields (50–80 %) were achieved. The reactions likely proceed via a reactive enzyme-bound iminium ion intermediate, allowing tweaking of the enzyme's activity and selectivity by protein engineering. Our results underscore the potential of catalytic promiscuity for the engineering of new cofactor-independent oxidative enzymes.*

Peroxygenases are oxidative enzymes catalyzing the insertion of an oxygen atom into a variety of substrates using peroxide-borne oxygen instead of dioxygen.[1] Earlier studies have mainly focused on the peroxygenase activity of chloroperoxidases that normally catalyze peroxide-driven halogenation of organic compounds[2] and engineered cytochrome P450 monooxygenases that are able to promote peroxide-mediated hydroxylation reactions.[3] However, since the discovery of the unspecific peroxygenase *Aae*UPO in 2004,[4] which uses hydrogen peroxide to catalyze diverse oxyfunctionalization reactions including oxidations, sulfoxidations, epoxidations and hydroxylations,[5] there has been an increasing interest to discover and develop novel peroxygenases. Note that peroxygenases are cofactor-dependent enzymes, typically relying on heme to react with hydrogen peroxide, forming an oxoferryl-heme as the actual oxygenating species to promote oxyfunctionalization reactions.[1,5a,6]

In this study, we report the engineering of an unusual cofactor-independent peroxygenase based on the promiscuous peroxygenase activity of the enzyme 4-oxalocrotonate tautomerase (4-OT). This engineered non-natural peroxygenase accepts different hydroperoxides (*t*-BuOOH and $H_2O_2$) to achieve enantiocomplementary epoxidations of various α,β-unsaturated aldehydes, providing access to both enantiomers of the resultant α,β-epoxy-aldehydes with high enantiomeric excess (up to 98 %). Our results showcase the potential of catalytic promiscuity for the creation of new cofactor-independent oxidative enzymes.

We have recently demonstrated that 4-OT can catalyze the Michael addition of nitromethane to cinnamaldehyde via an enzyme-bound iminium ion intermediate, which is formed between cinnamaldehyde and the catalytic active site Pro-1 residue.[7] In this study, we examined if this catalytic mechanism of 4-OT could be exploited for other useful synthetic applications, and envisioned that substituting nitromethane with *t*-BuOOH could trigger a C–O bond formation between the reactive enzyme-bound iminium ion species and *t*-BuOOH (Supporting Information, Scheme S1). The resulting enamine intermediate may undergo ring closure to construct the final epoxide moiety. To our delight, when wild-type 4-OT (0.33 mg mL$^{-1}$) was incubated at room temperature with 200 mM *t*-BuOOH (**1**, Figure 1) and 1 mM cinnamaldehyde (**2a**) in 20 mM sodium phosphate buffer (pH 7.3) containing 5 % (v/v) EtOH, 38 % of substrate **2a** was consumed within 48 h and the corresponding α,β-epoxy-aldehyde **3a** was identified by GC-MS analysis. Chiral HPLC analysis of the alcohol derivative of **3a** revealed the product to be (2*R*,3*S*)-**3a** with an e.r. of 62:38 and a d.r. of 92:8 (*syn*/*anti*). The *S* configuration of the C3 carbon of **3a** indicates that *t*-BuOOH selectively attacks cinnamaldehyde from the *re*-face, similar to what was previously observed for the nitromethane addition reaction.[7] These results demonstrated that 4-OT has low-level promiscuous peroxygenase activity and may possibly serve as a template to construct a cofactor-independent peroxygenase.

Motivated by these initial findings, a mutability-landscape-guided protein engineering approach[8] was applied to enhance the promiscuous peroxygenase activity of wild-type 4-OT. For this, a previously constructed collection of 4-OT genes encoding nearly all possible single-mutant variants of 4-OT[9] was screened to identify hotspot positions in the protein where mutations give improved peroxygenase activity. Improved variants were identified by monitoring the depletion of substrate **2a** in a spectrophotometric kinetic assay in multiwell plates (see Supporting Information). Interestingly,

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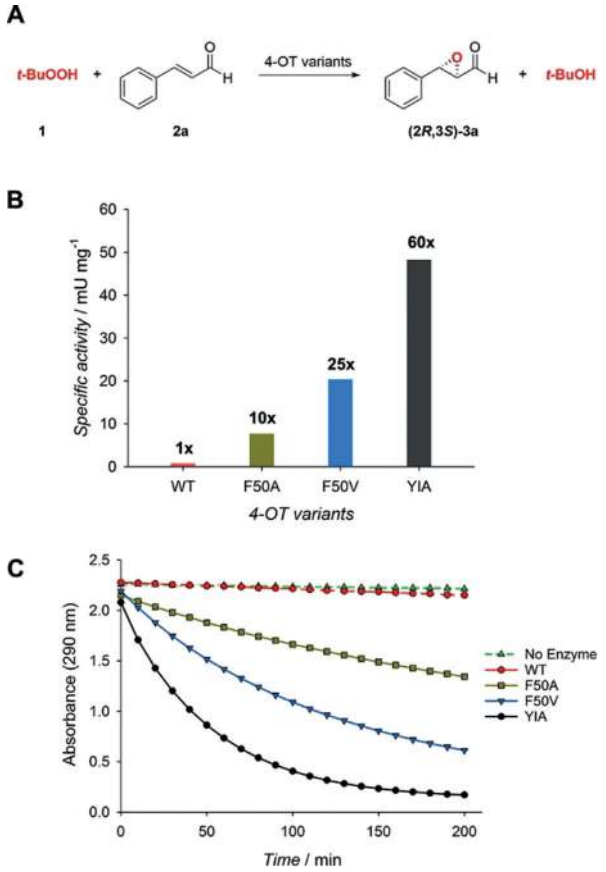


***Figure 1.*** Peroxygenase activity of 4-OT variants. A) Reaction scheme of 4-OT-catalyzed epoxidation of **2a** using *t*-BuOOH as oxidant. B) Comparison of the peroxygenase activity of wild-type 4-OT (WT) and engineered 4-OT variants. C) Reaction progress curves using different 4-OT variants. The initial rate of the 4-OT(YIA)-catalyzed reaction corresponds to 16 μM $min^{-1}$; specific activity is 48 mU per mg of protein.

six mutations at position F50 significantly improved (> 5-fold) the peroxygenase activity, with F50A (10-fold enhanced activity) and F50V (25-fold enhanced activity) being the best mutant enzymes (Figure 1). Several mutations at positions Q4, I41, M45, and H49 also resulted in enzyme variants with 2- to 5-fold enhanced activity compared to wild-type 4-OT, including mutants Q4Y, I41Y, M45I, and H49T. The most beneficial mutations, F50V and F50A, were subsequently combined with mutations at other hotspot positions by using site-saturation mutagenesis (Table S2). Activity screening of the seven mutant libraries resulted in the identification of three double mutants (Q4Y/F50V, Q4F/F50A and M45I/F50A) with around 1.6- to 3.5-fold enhanced activity compared to the parental enzymes. Based on these results, the mutant libraries Q4X/M45I/F50A, Q4Y/M45X/F50A, Q4F/M45X/F50A, and Q4Y/M45I/F50X were constructed (Table S2). Screening of these four libraries led to the discovery of the triple mutant Q4Y/M45I/F50A (YIA) that showed a remarkable 60-fold enhancement in activity for the epoxidation of **2a** compared to the wild-type enzyme (Figure 1). Notably, this mutant enzyme also has enhanced enantioselectivity, allowing the production of (2*R*,3*S*)-**3a** with high enantiopurity (e.r. = 98:2). Replacement of Pro-1 with an alanine in 4-OT(YIA) led to a 210-fold decrease in *t*-BuOOH-dependent epoxidation of **2a** (Figure S31), providing support for a reaction mechanism that proceeds via iminium ion formation between the active site Pro-1 residue and the α,β-unsaturated aldehyde substrate (Scheme S1).

With the improved 4-OT mutant YIA in hand, the substrate scope of this enzyme was explored by testing a set of α,β-unsaturated aldehydes using *t*-BuOOH as oxidant. The results showed that 4-OT(YIA) has a broad substrate scope, accepting *ortho*-, *meta*-, and *para*-substituted cinnamaldehydes (**2a–i**, Figure 2), and catalyzes their epoxidation with good conversions (94–97%) to yield the corresponding products (**3a–i**) with excellent enantiopurity (e.r. up to 99:1). The d.r. values (up to 92:8) obtained indicate that the enzyme has good stereocontrol over both carbons of the double bond of **2**. The enzyme also accepts the aliphatic α,β-unsaturated aldehyde citral (**2j**) with good enantioselectivity, moderate diastereoselectivity, and excellent regioselectivity (Figure 2). All epoxide products have the *syn* configuration with 2*R*,3*S* being the major enantiomer, indicating that *t*-BuOOH exclusively attacked from the *re*-face of the enzyme-bound iminium ion intermediate (Figure 3). Note that while the enzymatic α,β-epoxy-aldehyde products **3** have a 2*R*,3*S* configuration, the corresponding α,β-epoxy-alcohols have a 2*S*,3*S* configuration. This deviant configuration of the α,β-epoxy-alcohols is due to different prioritization of the substituents at the C2 chiral center relative to their α,β-epoxy-aldehyde precursors.

Given that *t*-BuOOH is a relatively large hydroperoxide, we investigated if the use of a small hydroperoxide such as $H_2O_2$ would change the preference of attack to the *si*-face (Figure 3). Again, compound **2a** was used as a substrate for initial testing with $H_2O_2$ as oxidant and mutant 4-OT(YIA) as catalyst. Notably, the epoxidations with $H_2O_2$ showed relatively high non-enzymatic (background) reaction rates when applying the same conditions as used with *t*-BuOOH. To suppress the background reaction, without diminishing the enzymatic activity, the pH of the reaction was lowered from 7.3 to 6.3 (Figure S1). Gratifyingly, the results showed that the use of $H_2O_2$ not only inverted the stereochemistry of the reaction, but product **3a** was also obtained with good e.r. (92:8) and d.r. (84:16) values (Figure 2 and Figure 3). Thus, an enantiocomplementary epoxidation of **2a** was achieved by replacing *t*-BuOOH by $H_2O_2$ as the oxidant. Next, we evaluated the same set of α,β-unsaturated aldehydes **2b–2j** for 4-OT(YIA)-catalyzed epoxidation reactions using $H_2O_2$ (Figure 2). Importantly, the stereo inversions occurred with all aromatic substrates (**2a–2i**), and high conversions and good enantio- and diastereoselectivity were observed. Notably, the epoxidation reaction with the aliphatic substrate citral (**2j**) also showed an inversion of diastereoselectivity when using $H_2O_2$ instead of *t*-BuOOH, resulting in the *anti*-product as the major isomer; the *syn*-product also showed an inverted enantiomer ratio (Figure S30).

Having achieved enantiocomplementary epoxidation reactions, we next investigated semi-preparative scale chemoenzymatic synthesis of α,β-epoxy-alcohols **4a–j** using 4-OT(YIA) as catalyst. With *t*-BuOOH as oxidant, the reactions

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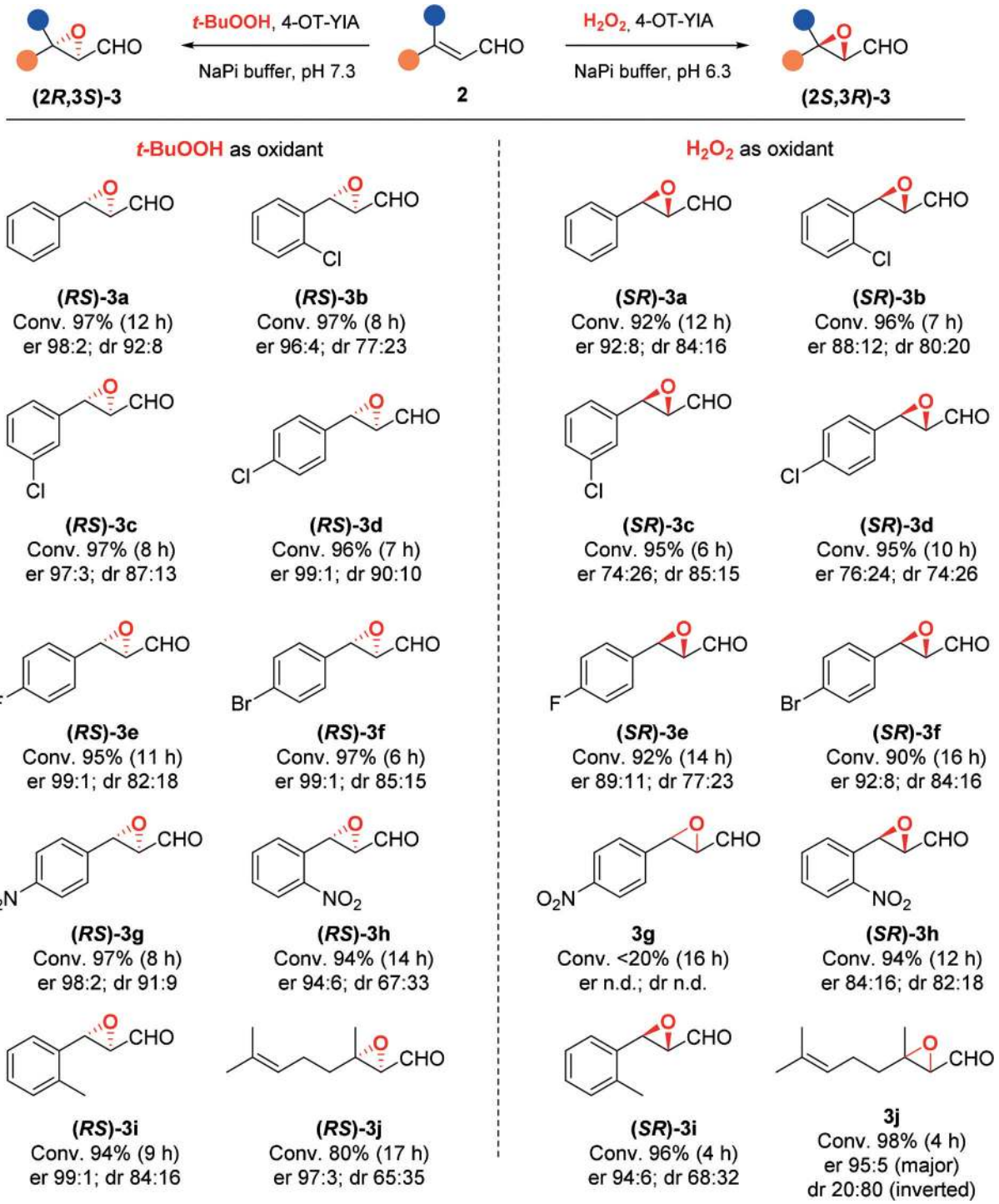


***Figure 2.*** Substrate scope of the epoxidation reactions catalyzed by 4-OT(YIA) (Q4Y/M45I/F50A) using *t*-BuOOH or $H_2O_2$ as the oxidant. Reaction conditions: **2** (1 mM), *t*-BuOOH (100 mM) or $H_2O_2$ (25 mM), 4-OT(YIA) (0.1 mg mL$^{-1}$), NaPi buffer (20 mM), 5% (v/v) EtOH (MeCN for **2f**, **2g** and **2h**). Conversions were calculated based on the depletion of the absorbance corresponding to substrate **2**. Product identifications were performed by GC-MS analysis. The enzymatic α,β-epoxy-aldehyde products **3** were reduced to the corresponding α,β-epoxy-alcohols with $NaBH_4$ for chiral HPLC analysis. The e.r., d.r. and absolute stereochemistry of products were determined by chiral HPLC using authentic standards. The diastereomer ratio (d.r.) is defined as the *syn*/*anti* ratio. **2j**: *E*/*Z* = 3:2; n.d.: not determined.

were performed with higher substrate concentrations (50 mM **2a**, 20 mM **2b**–**g** and **2j**, 5 mM **2i**), maintaining high conversions and stereoselectivity with good isolated product yields (Table 1). Only a minor decrease in enantio- and diastereoselectivity was observed, most likely due to the non-enzymatic background reaction, since somewhat longer reaction times were needed. The semi-preparative scale synthesis using $H_2O_2$ as oxidant was also attempted using

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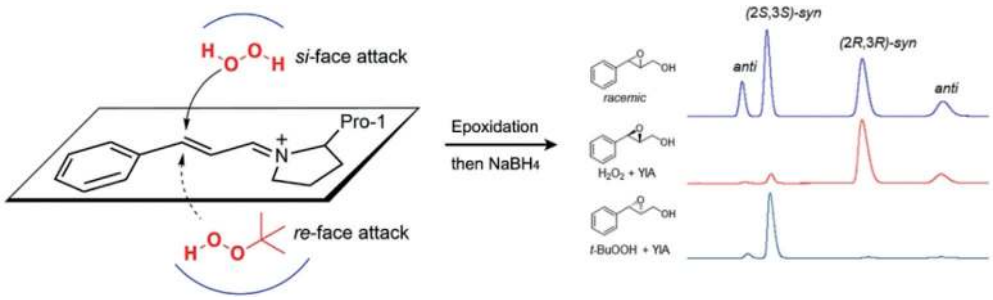
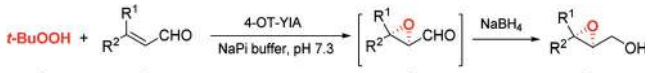



***Figure 3.*** Enantiocomplementary epoxidation of **2a** catalyzed by 4-OT(YIA) using *t*-BuOOH or $H_2O_2$ as oxidant. The predominant enzymatic diastereomer has the *syn* configuration; the minor *anti* diastereomeric product presumably is formed by rotation of the C2-C3 bond after addition of the peroxide, but before ring closure.

20 mM **2a** and 22 mM $H_2O_2$. However, it was observed that the e.r. of the product (isolated as **4a**) substantially decreased to 73:27 (92:8 when using 1 mM **2a**, Figure 2). We further explored this unexpected result by testing different concentrations of **2a** and $H_2O_2$ in analytical-scale reactions (Table S3). The results revealed that an increasing concentration of **2a** has a negative effect on the e.r. of the final product **4a**, while the concentration of $H_2O_2$ has no effect (Table S3). This effect may be caused by the higher concentration of peroxyhydrate (**S4**) formed between the enzymatic aldehyde product (**3a**) and $H_2O_2$ when higher substrate (**2a**) concentrations were used (Scheme S2). This peroxyhydrate can be considered as a large hydroperoxide that, like *t*-BuOOH, attacks the iminium ion on the *re*-face during the reaction, thus lowering the e.r. of the product. In reactions with *t*-BuOOH, the peroxyhydrate (**S5**) of the enzymatic aldehyde product (**3a**) is not a hydroperoxide, and therefore cannot be accepted by the enzyme as an oxidant. Consequently, the negative effect of high substrate concentrations on the enantiopurity of the final product was not observed in 4-OT(YIA)-catalyzed epoxidation reactions with *t*-BuOOH (Scheme S2).

In summary, we have exploited the catalytic promiscuity of 4-OT to engineer an effective peroxygenase (4-OT-YIA) that promotes peroxide-driven asymmetric epoxidations of α,β-unsaturated aldehydes. Unlike the peroxygenases offered by nature, the engineered non-natural peroxygenase 4-OT(YIA) does not require any cofactors, such as heme, for catalysis. While *Candida antarctica* lipase B (CALB) reportedly performs cofactor-independent epoxidation reactions, the described enzymatic products were either racemic[10] or reported without demonstrating any enantioselectivity.[11] Notably, several amino acid and peptide organocatalysts, as well as bovine serum albumin (BSA), have been shown to catalyze asymmetric peroxide-driven epoxidations of α,β-unsaturated aldehydes and ketones, but these systems offer a more general mode of catalysis and, with the exception of BSA, often require the use of organic solvents.[12] In contrast, peroxygenase 4-OT(YIA) uses a distinct active-site process to accomplish highly enantioselective epoxidations of α,β-unsaturated aldehydes, and by using either *t*-BuOOH or $H_2O_2$ to drive the reaction, access to both product enantiomers can be achieved. This may suggest that there are two binding pockets for hydroperoxides in the active site of 4-OT(YIA); one relatively large and hydrophobic pocket at the *re*-face of the enzyme-bound iminium ion that preferably binds *t*-BuOOH and one relatively small and probably more hydrophilic pocket at the *si*-face of the iminium ion that selectively recruits $H_2O_2$ for asymmetric epoxidations (Figure 3). We have initiated structural studies of 4-OT(YIA) aimed at elucidating the mechanistic origin of the enantiocomplementary epoxidation reactions. Furthermore, given that 4-OT(YIA) exhibits a moderate specific activity of 48 mU mg$^{-1}$ for the *t*-BuOOH-driven epoxidation of **2a**, current work in our

***Table 1:*** Preparative-scale 4-OT(YIA)-catalyzed epoxidations of α,β-unsaturated aldehydes using *t*-BuOOH as oxidant.[a]




| Entry | 2 | $R^1$ | $R^2$ | *t* [h] | Conv. (Yield[%])[b] | e.r.[c] | d.r.[d] | Abs. config.[e] |
|---|---|---|---|---|---|---|---|---|
| 1[f] | **a** | H | Ph | 24 | 93 (52) | 98:2 | 92:8 | 2*S*,3*S* |
| 2 | **b** | H | *o*-Cl-Ph | 48 | 85 (55) | 93:7 | 68:32 | 2*S*,3*S* |
| 3 | **c** | H | *m*-Cl-Ph | 66 | 85 (68) | 94:6 | 85:15 | 2*S*,3*S* |
| 4 | **d** | H | *p*-Cl-Ph | 48 | 91 (62) | 97:3 | 89:11 | 2*S*,3*S* |
| 5 | **e** | H | *p*-F-Ph | 24 | 95 (50) | 96:4 | 83:17 | 2*S*,3*S* |
| 6[f] | **f** | H | *p*-Br-Ph | 46 | 98 (80) | 98:2 | 92:8 | 2*S*,3*S* |
| 7 | **g** | H | *p*-$NO_2$-Ph | 48 | 92 (64) | 97:3 | 89:11 | 2*S*,3*S* |
| 8 | **i** | H | *o*-Me-Ph | 45 | 90 (61) | 97:3 | 80:20 | 2*S*,3*S* |
| 9[g] | **j** | Me | $(CH_3)_2CCHCH_2CH_2$ | 96 | 79 (50) | 96:4 | 60:40 | 2*S*,3*S* |

[a] General reaction conditions: **2** (20 mM), *t*-BuOOH (100 mM), 4-OT(YIA) (0.93 mg mL$^{-1}$) in 20 mM NaPi (sodium phosphate, pH 7.3) buffer with 10% (v/v) EtOH (MeCN for **2f** and **2g**), volume = 10 mL (5 mL for **2a**, 40 mL for **2f**), enzymatic product **3** reduced with $NaBH_4$ to **4** after completion of the reaction [b] Conversion determined by GC-MS; yield of the diastereomer mixture. [c] *syn* diastereomer; determined by chiral HPLC. [d] *syn*/*anti*. [e] *syn* diastereomer; determined by comparison with authentic standards on chiral HPLC. [f] conc. **2a** = 50 mM, conc. **2f** = 5 mM, see supporting information for details. [g] **2j**: *E*/*Z* = 3:2; 1.3 mg mL$^{-1}$ 4-OT(YIA).

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group is focused on further improving the peroxygenase activity of this promising cofactor-independent enzyme, making use of a recently developed selective colorimetric "turn-on" probe for efficient engineering of iminium biocatalysis.[13] The non-natural peroxygenase activity of 4-OT, together with its previously reported promiscuous C–C bond-forming Michaelase[7,9,14] and aldolase[15] activities, emphasize the chemical versatility of the 4-OT protein scaffold.

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## Conflict of interest

The authors declare no conflict of interest.

**Keywords:** enzyme engineering · epoxidation · oxidative enzymes · peroxide · peroxygenase

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