Biocatalysis

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

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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 (2R,3S)-**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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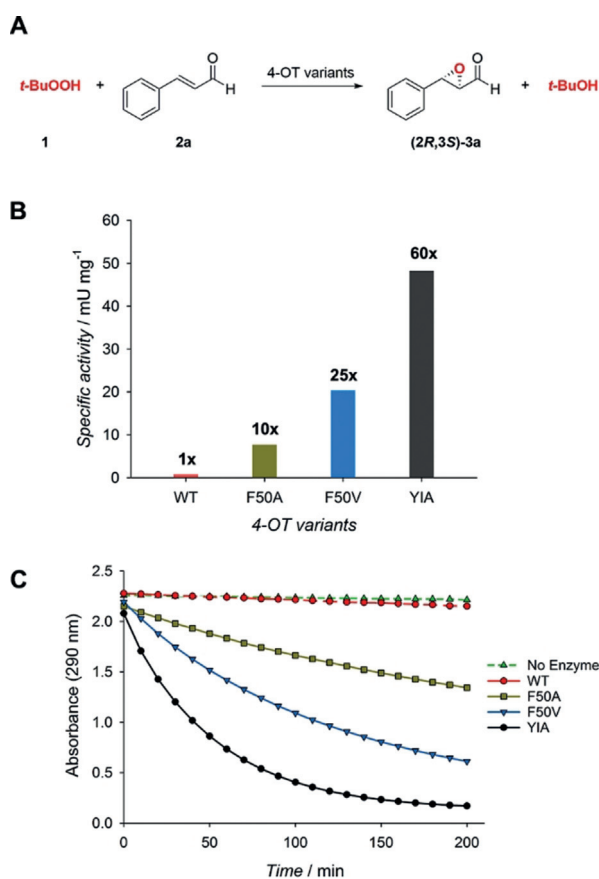P.O. Central University, Gachibowli, Hyderabad 500046 (India)

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$_2$O$_2$ 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$_2$O$_2$ as oxidant and mutant 4-OT(YIA) as catalyst. Notably, the epoxidations with H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ (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$_2$O$_2$ 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 chemo-enzymatic synthesis of α,β-epoxy-alcohols **4a–j** using 4-OT-(YIA) as catalyst. With *t*-BuOOH as oxidant, the reactions
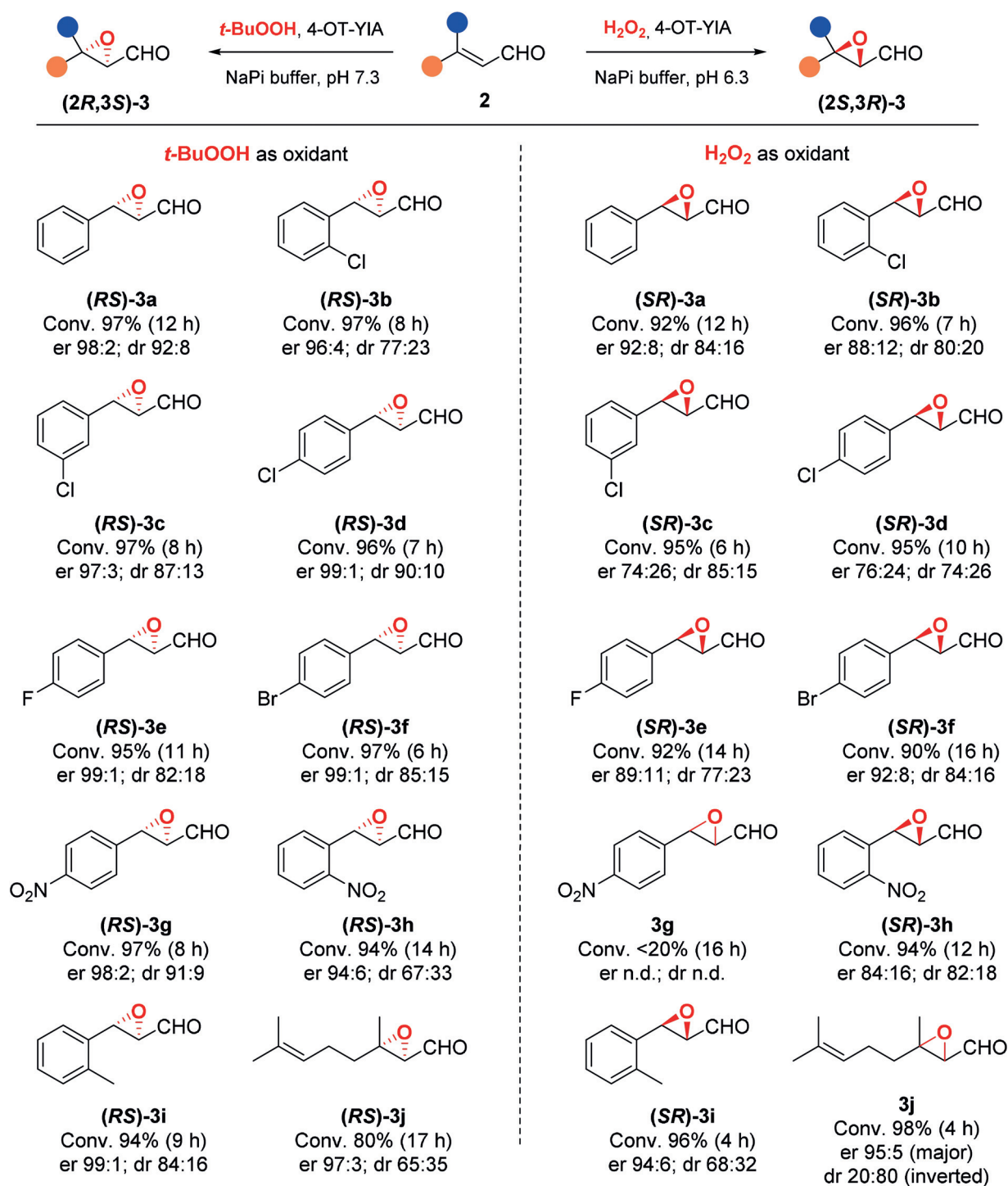
Figure 2. Substrate scope of the epoxidation reactions catalyzed by 4-OT(YIA) (Q4Y/M45I/F50A) using *t*-BuOOH or H$_2$O$_2$ as the oxidant. Reaction conditions: **2** (1 mM), *t*-BuOOH (100 mM) or H$_2$O$_2$ (25 mM), 4-OT(YIA) (0.1 mg mL^{-1}), NaPi buffer (20 mM), 5% (v/v) EtOH (MeCN for **2f**, **2g** 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$_2$O$_2$ as oxidant was also attempted using
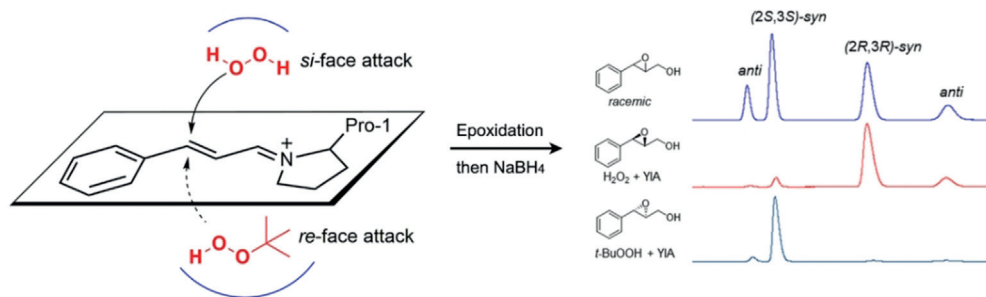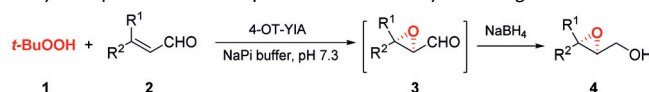
Figure 3. Enantiocomplementary epoxidation of **2a** catalyzed by 4-OT(YIA) using *t*-BuOOH or H$_2$O$_2$ as oxidant. The predominant enzymatic diastereomer has the *syn* configuration; the minor *anti* diastereomeric product presumably is formed by rotation of the C2-C3 bond after addition of the peroxide, but before ring closure.

20 mᴍ **2a** and 22 mᴍ H$_2$O$_2$. However, it was observed that the e.r. of the product (isolated as **4a**) substantially decreased to 73:27 (92:8 when using 1 mᴍ **2a**, Figure 2). We further explored this unexpected result by testing different concentrations of **2a** and H$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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$_2$O$_2$ 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	2S,3S
2	b	H	*o*-Cl-Ph	48	85 (55)	93:7	68:32	2S,3S
3	c	H	*m*-Cl-Ph	66	85 (68)	94:6	85:15	2S,3S
4	d	H	*p*-Cl-Ph	48	91 (62)	97:3	89:11	2S,3S
5	e	H	*p*-F-Ph	24	95 (50)	96:4	83:17	2S,3S
6[f]	f	H	*p*-Br-Ph	46	98 (80)	98:2	92:8	2S,3S
7	g	H	*p*-NO$_2$-Ph	48	92 (64)	97:3	89:11	2S,3S
8	i	H	*o*-Me-Ph	45	90 (61)	97:3	80:20	2S,3S
9[g]	j	Me	(CH$_3$)$_2$CCHCH$_2$CH$_2$	96	79 (50)	96:4	60:40	2S,3S

[a] General reaction conditions: **2** (20 mᴍ), *t*-BuOOH (100 mᴍ), 4-OT(YIA) (0.93 mg mL^{-1}) in 20 mᴍ 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 mᴍ, conc. **2f** = 5 mᴍ, see supporting information for details. [g] **2j**: *E/Z* = 3:2; 1.3 mg mL^{-1} 4-OT(YIA).

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.

Conflict of interest

The authors declare no conflict of interest.

[1] Y. Wang, D. Lan, R. Durrani, F. Hollmann, *Curr. Opin. Chem. Biol.* **2017**, *37*, 1–9.

[2] a) D. R. Morris, L. P. Hager, *J. Biol. Chem.* **1966**, *241*, 1763–1768; b) A. Zaks, D. R. Dodds, *J. Am. Chem. Soc.* **1995**, *117*, 10419–10424; c) J. Littlechild, *Curr. Opin. Chem. Biol.* **1999**, *3*, 28–34.

[3] a) H. Joo, Z. Lin, F. H. Arnold, *Nature* **1999**, *399*, 670–673; b) P. C. Cirino, F. H. Arnold, *Angew. Chem. Int. Ed.* **2003**, *42*, 3299–3301; *Angew. Chem.* **2003**, *115*, 3421–3423; c) J. Chen, F. Kong, N. Ma, P. Zhao, C. Liu, X. Wang, Z. Cong, *ACS Catal.* **2019**, *9*, 7350–7355.

[4] R. Ullrich, J. Nueske, K. Scheibner, J. Spantzel, M. Hofrichter, *Appl. Environ. Microbiol.* **2004**, *70*, 4575–4581.

[5] a) M. Hofrichter, R. Ullrich, *Curr. Opin. Chem. Biol.* **2014**, *19*, 116–125; b) I. Bassanini, E. E. Ferrandi, M. Vanoni, G. Ottolina, S. Riva, M. Crotti, E. Brenna, D. Monti, *Eur. J. Org. Chem.* **2017**, 7186–7189.

[6] a) C. K. Prier, F. H. Arnold, *J. Am. Chem. Soc.* **2015**, *137*, 13992–14006; b) F. H. Arnold, *Angew. Chem. Int. Ed.* **2018**, *57*, 4143–4148; *Angew. Chem.* **2018**, *130*, 4212–4218.

[7] C. Guo, M. Saifuddin, T. Saravanan, G. J. Poelarends, *ACS Catal.* **2019**, *9*, 4369–4373.

[8] J. Y. van der Meer, L. Biewenga, G. J. Poelarends, *ChemBioChem* **2016**, *17*, 1792–1799.

[9] J. Y. Van Der Meer, H. Poddar, B. J. Baas, Y. Miao, M. Rahimi, A. Kunzendorf, R. Van Merkerk, P. G. Tepper, E. M. Geertsema, A. M. W. H. Thunnissen, et al., *Nat. Commun.* **2016**, *7*, 1–16.

[10] a) P. Zhou, X. Wang, B. Yang, F. Hollmann, Y. Wang, *RSC Adv.* **2017**, *7*, 12518–12523; b) S. Ranganathan, S. Zeitlhofer, V. Sieber, *Green Chem.* **2017**, *19*, 2576–2586.

[11] M. Svedendahl, P. Carlqvist, C. Branneby, O. Allnér, A. Frise, K. Hult, P. Berglund, T. Brinck, *ChemBioChem* **2008**, *9*, 2443–2451.

[12] a) S. Juliá, J. Masana, J. C. Vega, *Angew. Chem. Int. Ed. Engl.* **1980**, *19*, 929–931; *Angew. Chem.* **1980**, *92*, 968–969; b) S. Colonna, A. Manfredi, *Tetrahedron Lett.* **1986**, *27*, 387–390; c) S. Colonna, A. Manfredi, R. Annunziata, M. Spadoni, *Tetrahedron* **1987**, *43*, 2157–2164; d) S. Baars, K.-H. Drauz, H.-P. Krimmer, S. M. Roberts, J. Sander, J. Skidmore, G. Zanardi, *Org. Process Res. Dev.* **2003**, *7*, 509–513; e) H. Sundén, I. Ibrahem, A. Córdova, *Tetrahedron Lett.* **2006**, *47*, 99–103.

[13] L. Biewenga, M. Crotti, M. Saifuddin, G. J. Poelarends, *ACS Omega* **2020**, *5*, 2397–2405.

[14] a) E. Zandvoort, E. M. Geertsema, B. J. Baas, W. J. Quax, G. J. Poelarends, *Angew. Chem. Int. Ed.* **2012**, *51*, 1240–1243; *Angew. Chem.* **2012**, *124*, 1266–1269; b) Y. Miao, E. M. Geertsema, P. G. Tepper, E. Zandvoort, G. J. Poelarends, *ChemBioChem* **2013**, *14*, 191–194; c) E. M. Geertsema, Y. Miao, P. G. Tepper, P. Dehaan, E. Zandvoort, G. J. Poelarends, *Chem. Eur. J.* **2013**, *19*, 14407–14410; d) Y. Miao, P. G. Tepper, E. M. Geertsema, G. J. Poelarends, *Eur. J. Org. Chem.* **2016**, 5350–5354; e) L. Biewenga, T. Saravanan, A. Kunzendorf, J. Van Der Meer, T. Pijning, P. G. Tepper, R. Van Merkerk, S. J. Charnock, A. W. H. Thunnissen, G. J. Poelarends, *ACS Catal.* **2019**, *9*, 1503–1513.

[15] a) E. Zandvoort, B. J. Baas, W. J. Quax, G. J. Poelarends, *ChemBioChem* **2011**, *12*, 602–609; b) M. Rahimi, J. Y. van der Meer, E. M. Geertsema, H. Poddar, B. J. Baas, G. J. Poelarends, *ChemBioChem* **2016**, *17*, 1225–1228; c) M. Rahimi, E. M. Geertsema, Y. Miao, J.-Y. van der Meer, T. van den Bosch, P. de Haan, E. Zandvoort, G. J. Poelarends, *Org. Biomol. Chem.* **2017**, *15*, 2809–2816.