Enzyme Catalysis | *Hot Paper*

A Photoclick-Based High-Throughput Screening for the Directed Evolution of Decarboxylase OleT

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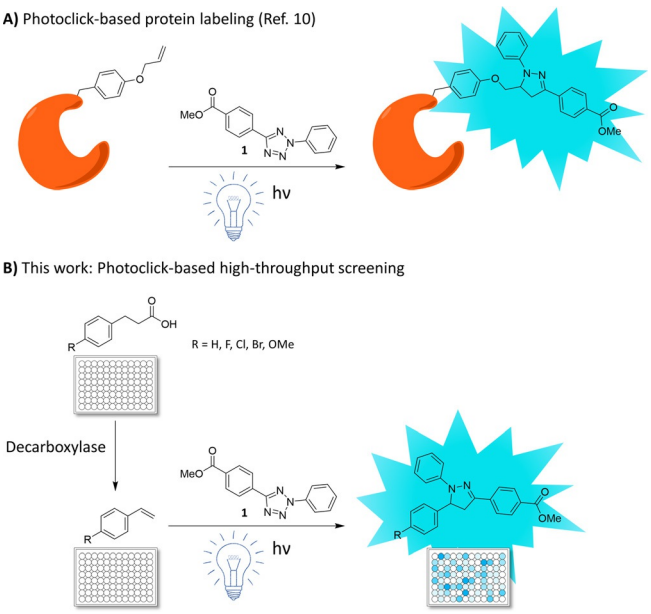
Abstract: Enzymatic oxidative decarboxylation is an up-and-coming reaction yet lacking efficient screening methods for the directed evolution of decarboxylases. Here, we describe a simple photoclick assay for the detection of decarboxylation products and its application in a proof-of-principle directed evolution study on the decarboxylase OleT. The assay was compatible with two frequently used OleT operation modes (directly using hydrogen peroxide as the enzyme's co-substrate or using a reductase partner) and the screening of saturation mutagenesis libraries identified two enzyme variants shifting the enzyme's substrate preference from long chain fatty acids toward styrene derivatives. Overall, this photoclick assay holds promise to speed-up the directed evolution of OleT and other decarboxylases.

Incorporation of biobased resources into the value chain is of great importance in the context of a more sustainable society. In the recent past, synthetic routes have been explored to produce molecules of industrial relevance from renewable feedstocks. Carboxylic acids are ubiquitous in nature and their oxidative decarboxylation gives access to olefins, which are fundamental in chemical synthesis, e.g., as precursors in natural compound synthesis or as starting material for polymerization reactions. However, producing olefins from natural carboxylic acids is difficult by chemical means. Most of the reactions involve radical formation (e.g., Hunsdiecker reaction[1] or Kolbe electrolysis)[2] and therefore produce side products. Another disadvantage is the use of toxic or expensive metalorganic species for decarboxylation reactions (e.g., Kochi reaction[3] or Barton decarboxylation).[4] Thus, mild and catalytic oxidative decarboxylations are sought-after reactions.

OleT from *Jeotgalicoccus* sp. ATCC 8456 is a P450 peroxygenase of the CYP152 family.[5] It catalyzes the oxidative decarboxylation of long chain fatty acids using hydrogen peroxide as oxidant,[6] which can either be added directly to the reaction or produced in situ.[7] Alternatively, OleT was successfully combined with surrogate redox partners[7c,8] or genetically fused to reductase domains.[8b]

Previously, protein engineering was used to widen the substrate scope of OleT toward aromatic carboxylic acids. However, due to low protein expression levels and the lack of a high-throughput assay, OleT variants had to be produced in a bigger scale and screened using chromatographic methods.[8c] In search of a simple plate reader assay for the detection of small terminal alkenes, a study by Song et al. drew our interest. They fluorescently labeled a non-canonical amino acid containing a terminal olefin moiety (*O*-allyl-tyrosine)[9] using diaryltetrazoles by forming a fluorescent cycloadduct[10] (Scheme 1 A). Inspired by that work, we set out to develop a photoclick-chemistry-based high-throughput screening assay to interrogate mutagenesis libraries of the decarboxylase OleT for



Scheme 1. Diaryltetrazole **1** as photoclick reagent.

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Supporting information and the ORCID identification numbers for the authors of this article can be found under: https://doi.org/10.1002/chem.202003637.

enzyme variants with improved selectivity for aromatic substrates (Scheme 1 B). As a first step, we investigated the use of diaryltetrazole **1** as a photoclickable detector of various potential OleT decarboxylation products. Among the compounds investigated, styrene derivatives **2 b**–**6 b** produced the highest fluorescence signal after reacting with tetrazole **1**, exhibiting excitation and emission maxima at 385 nm and 504 nm, respectively (Figure S1 A and B, Supporting Information). Non-styrenyl substrates were detected with lower sensitivity. After initiating the reaction by irradiation on a conventional UV light transilluminator, the photoclick reaction was completed approximately within one hour, forming a stable fluorescent product (Figure S1 C, Supporting Information). Gratifyingly, an excellent linear correlation of fluorescence signal and olefin concentration was found in the micromolar concentration range (Figure S1 D, Supporting Information).

Next, we set out to investigate whether tetrazole **1** can be used to assay OleT decarboxylase activity in a high-throughput screening set-up using cell lysates. As a model decarboxylase, we chose OleT-BM3R. OleT-BM3R was recently described by Lu et al.[11] as a fusion protein of the P450 decarboxylase OleT and the *Bacillus megaterium* P450-BM3 reductase domain (termed BM3R; P450-BM3 residues 451–1048; Figure 1 A).[12] We considered OleT-BM3R to be the ideal candidate as it can be run in two different operational modes, both of which are frequently used in OleT studies:[7,8] i) the hydrogen peroxide-driven mode ("peroxide mode"), and ii) the reductase-driven mode ("reductase mode"; Figure 1 B). In the peroxide mode, hydrogen peroxide is added and initiates Compound I formation in the OleT active site.[6b,c,13] In the reductase mode, a reducing equivalent (here: NADPH) is used and a reductase (here: BM3R) shuttles electrons from the reducing equivalent toward OleT leading to its activation.[8a,b,11]

We prepared two identical 96-well plates. One half of the wells of each plate were inoculated with *E. coli* cells harboring Ole-BM3R; the other half contained an empty vector control. After cell lysis and centrifugation, the cleared lysates were applied for the decarboxylation of 3-(4-bromophenyl)propanoic acid (**5 a**) using the peroxide mode (one plate) and the reductase mode (the other plate), respectively. In the latter case, the reaction was combined with a cofactor recycling system using an engineered variant of phosphite dehydrogenase (PTDH; Figure 1 B).[14] After overnight incubation, the plates were subjected to the photoclick assay (detailed procedures in Supporting Information). In both reaction modes the wells containing OleT-BM3R exhibited higher fluorescence than the empty vector control with a coefficient of variance (CV) of 15% and 14% for the peroxide mode and the reductase mode, respectively, and a strictly standardized mean difference (SSMD) of 4.2 and 5.3, respectively (Figure S2, Supporting information). A CV $\leq$ 15% is frequently and successfully used in directed evolution experiments[15] and a cutoff criterion of SSMD $>$ 3 is often used to evaluate high-throughput assay quality.[16]

With conditions for a microplate photoclick assay in hand, we set out to perform a proof-of-principle directed evolution study on OleT-BM3R to improve the enzyme's selectivity for substrate **5 a**. We started by computationally docking **5 a** into the active site of the OleT crystal structure (details in Supporting Information) to identify key amino acids involved in the binding of **5 a**. The molecular docking study revealed that the substrate binds close to the heme cofactor with a binding energy of -7.66 kcal mol^{-1} and forms two hydrogen bonds with R245 (Figure S3, Supporting Information), much like the hydrogen bonds formed with the natural fatty acid substrates.[6a,17] Among the residues in the vicinity of **5 a** (Figure S3, Supporting Information), residue L78 of OleT-BM3R was subjected to saturation mutagenesis. The mutant library was screened for the conversion of **5 a** using the established photoclick assay.[18] Unfortunately, most of the generated variants were inactive as determined by the photoclick assay and the

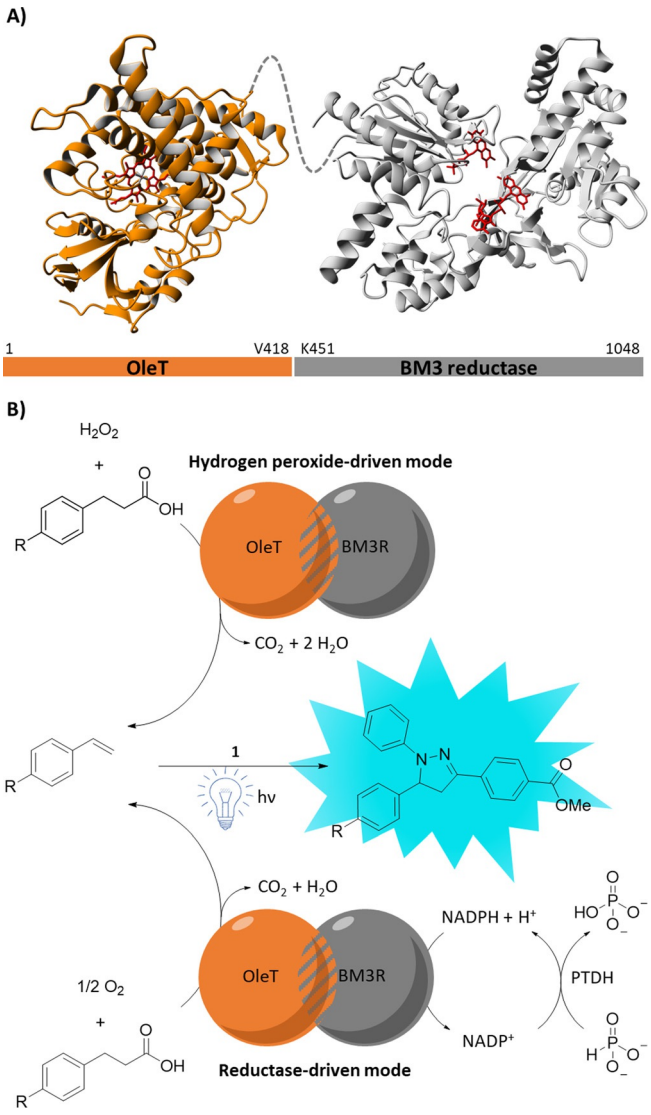


Figure 1. Graphical representation and operation modes of OleT-BM3R. A) The P450-BM3 reductase domain (BM3R) was genetically fused to OleT (cofactors are shown in red; details on the graphical model can be found in the Supporting Information). B) OleT-BM3R can decarboxylate carboxylic acids either by using hydrogen peroxide as cosubstrate (upper half) or through BM3R-mediated electron transfer from NADPH (lower half). In this study, NADPH was recycled by means of a variant of phosphite dehydrogenase (PTDH).[14a,c]

active variants did not show an improvement over wildtype (Figure S4, Supporting Information). We proceeded by saturating F79 of OleT-BM3R. According to our docking model, F79 is in close contact with **5a** (5.6 Å between aromatic rings of F79 and **5a**) and previous studies suggested the importance of this position for substrate selectivity.[8c,17] Two potential "hits" revealed the F79L and F79V substitutions, respectively, and both variants were found to have improved decarboxylation activity of **5a** in a confirmatory validation experiment at larger scale (shaking flask expression) using cleared lysates (Figure 2).

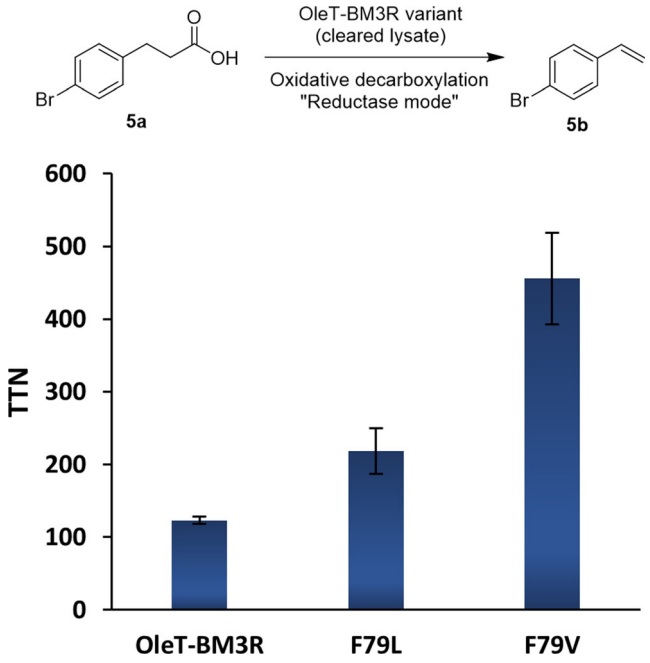


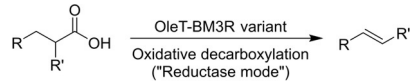
Figure 2. Validation of potential hits of the OleT-BM3R saturation mutagenesis library at position 79. Reaction conditions (reductase mode): 50 μL cleared P450 lysate, 1 mM **5a**, 200 μM NADPH, 10 mM sodium phosphite, 5 μM PTDH, 5% DMSO as cosolvent (total reaction volume: 500 μL), 16 h, room temperature (RT).

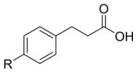
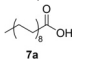
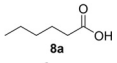
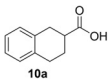
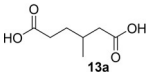
The F79L substitution improved the total turnover number 1.8-fold (219 TTN) with respect to the OleT-BM3R parent (123 TTN; Figure 2). This substitution was previously reported by Wang et al.[8c] to improve the conversion of aromatic substrates. In their study, OleT was combined with the CamAB reductase system[19] (i.e., a reductase-driven reaction mode) and the authors identified the F79L substitution after gas chromatography-based screening of a library with reduced amino acid alphabet. Indeed, our photoclick assay was able to discover the same amino acid substitution from a saturation mutagenesis library in a microplate-based high-throughput screening set-up. Moreover, with the F79V substitution our assay identified a previously not reported variant, outperforming the parent enzyme by a factor of 3.7 (456 TTN) in cleared lysates (Figure 2).

Finally, we examined the performance of purified OleT-BM3R and both purified variants (F79L and F79V), (Figure S5, Supporting Information). In a previous study, members of the OleT CYP152 family were combined with a reductase system and reduced product formation was reported upon addition of catalase.[20] Indeed, OleT-BM3R lost >90% of its activity toward substrate **5a** in the presence of 10 U mL^{-1} catalase (Figure S6, Supporting Information). No activity was detected when 100 U mL^{-1} catalase were present. Recently Wise et al. performed a detailed mechanistic study of OleT in combination with different redox donor systems revealing that the electron transfer to the active site was inefficient.[21] Their results indicate that the electron transfer causes superoxide formation followed by disproportionation to hydrogen peroxide. Accordingly, Compound I formation and turnover are attributed to hydrogen peroxide rather than the direct reduction of dioxygen at the heme. Our study corroborates these findings, suggesting that the in situ formation of hydrogen peroxide plays an important role also in the activation of OleT-BM3R in "reductase mode" and that, unlike in P450-BM3, the electron transfer between reductase domain and OleT is not optimally tuned. In order to maximize total turnovers, however, omitting catalase and thus deliberately allowing in situ hydrogen peroxide formation renders OleT-BM3R a much more productive decarboxylation system. To study the substrate scope of OleT-BM3R and the F79L and F79V variants, reactions were carried out in the absence of catalase (Table 1).

In general, the purified enzymes performed better compared to the set-up in cleared lysate (compare Table 1, entry 1 and Figure 2), which we attribute to the presence of host cell catalases in the lysates. Notably, the purified F79V variant exhibited 1,3-fold improved activity compared to the OleT-BM3R parent using substrate **5a** (3.7-fold for the reaction set-up in cleared lysates), whereas the F79L variant was less active than OleT-BM3R (vs. 1.8-fold improvement in lysates). Differences in performance in whole cells/cell lysates vs. purified enzymes are not uncommon,[22] which is why validation of promising hits in vitro is often necessary. They are often governed by the stabilizing effects present in cells and lysates.[23] Among the 3-phenylpropanoic acid derivatives (**2a**–**6a**, entry 1–5) the different OleT-BM3R variants exhibited different preferences: Unsurprisingly, F79V preferred the bromo-substituted substrate which was used in the microplate screening. F79L was the most efficient producer of the fluoro-substituted styrene derivative **3b**. Overall, OleT-BM3R and the two variants have a broad substrate scope ranging from its native long chain fatty acid substrate (**7a**, entry 6) to shorter chain lengths (**8a**, entry 7) or even small cyclic compounds (**9a**, entry 8). Notably, the F79V variant lost its preference for fatty acid substrates (**7a**; 616 TTN) in favor of substrate **5a** (1452 TTN). For the decarboxylation of the tetrahydronaphthalene derivative **10a** we observed that the **10b**:**10c** selectivity was tunable upon substituting F79 by either L or V (Table 1, entry 9 and footnote b). Looking at the OleT active site, it is conceivable that π-stacking interactions between F79 and substrate **10a** orient the substrate in a way that favors hydrogen abstraction from the C-β that leads to the formation of **10b**.[6b,c] Replacing F79 with the non-aromatic L or V could facilitate the movement of substrate **10a** in the active site, allowing hydrogen abstraction at the alternative C-β and leading to increased formation of **10c**. In the reaction of the β-methyl-substituted substrate **11a**, however, only

Table 1. Comparative substrate scope of OleT-BM3R and its variants (F79L and F79V).



Entry	Substrate	TTN OleT-BM3R	TTN F79L	TTN F79V
				
1[a]	**5a** (R = Br)	1084	886	1452
2	**2a** (R = H)	1200	758	530
3	**3a** (R = F)	750	965	635
4[a]	**4a** (R = Cl)	765	704	849
5	**6a** (R = OMe)	269	239	243
6[a]	 **7a**	2290	1402	616
7	 **8a**	151	94	59
8	 **9a**	116	56	14
9	 **10a**	690[b,c]	486[b,c]	412[b,c]
10	 **11a**	52[c,d]	37[c,d]	29[c,d]
11	 **12a**	0	0	0
12	 **13a**	0[e]	0[e]	0[e]

Reaction conditions (reductase mode): 1 mM carboxylic acid substrate, 0.5 μM purified OleT-BM3R (or variants thereof), 200 μM NADPH, 10 mM sodium phosphite, 5 μM PTDH, 5% DMSO as cosolvent, 16 h, 23 °C. No oxo-transfer side products (e.g., hydroxylation of substrate or epoxidation of styrenyl product) were observed. [a] 0.05 μM purified OleT-BM3R (or variants thereof) were used. [b] 1,2-dihydronaphthalene (**10b**) and 1,4-dihydronaphthalene (**10c**) were formed, respectively. **10b**:**10c** ratios were: OleT-BM3R = 99:1; F79L = 93:7; F79*V* = 87:13. [c] Kinetic resolution of the substrate was not observed. [d] 2-pentene (**11b**) was formed as *trans*- and *cis*-isomer. *trans*:*cis* ratios were: OleT-BM3R = 98:2; F79L = 98:2; F79*V* = 97:3. 1-pentene was not observed. [e] No single- or double-decarboxylation products were detected.

minor changes in the *trans*:*cis* selectivity were observed (Table 1, entry 10 and footnote d). Moreover, 1-pentene was not detected. The branched chain substrate **12a** and the dicarboxylic acid **13a** were not converted by any of the three enzyme variants (entries 11 and 12) although double-decarboxylation has been previously reported for other substrates.[11]

In summary, we developed a robust 96-well photoclick assay for the quantitative detection of styrene derivatives in the micromolar range and potentially other alkene-products resulting from decarboxylation reactions (e.g., derivatives of the *O*-allyltyrosine initially used by Song et al.).[10] The simple handling and convenient fluorescent read-out enable high-throughput screening of comprehensive decarboxylase variant libraries within hours. Moreover, the assay can be performed with minimal instrumentation, as most laboratories active in the field of directed evolution are already equipped with UV transilluminators. In a proof-of-principle directed evolution study, we applied our assay to change the substrate specificity of the long chain fatty acid preferring OleT-BM3R fusion enzyme toward the conversion of a non-natural aromatic substrate. This study has potential implications for the directed evolution of OleT and other decarboxylases,[20,24] in particular, for changing the enzyme's substrate selectivity, its resistance toward hydrogen peroxide, or for fine-tuning the hitherto suboptimal electron transfer of reductase partner systems to OleT.

Acknowledgements

This work was supported by the Bundesministerium für Bildung und Forschung (BMBF) (FKZ 031B0297). Open access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare no conflict of interest.

Keywords: decarboxylase · directed evolution · high-throughput screening · P450 · photoclick chemistry

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Manuscript received: August 5, 2020
Revised manuscript received: September 15, 2020
Accepted manuscript online: September 21, 2020
Version of record online: December 9, 2020