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Article

Selective Colorimetric "Turn-On" Probe for Efficient Engineering of Iminium Biocatalysis

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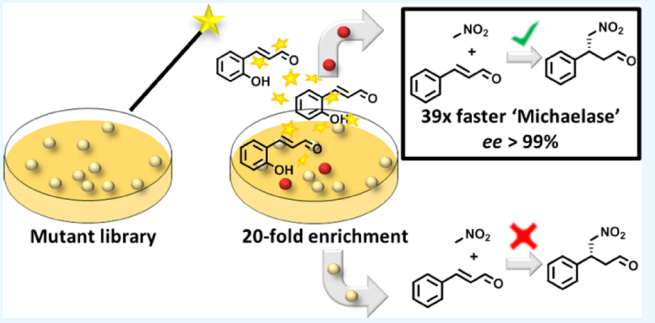
ABSTRACT: The efficient engineering of iminium biocatalysis has drawn considerable attention, with many applications in pharmaceutical synthesis. Here, we report a tailor-made iminium-activated colorimetric "turn-on" probe, specifically designed as a prescreening tool to facilitate engineering of iminium biocatalysis. Upon complexation of the probe with the catalytic Pro-1 residue of the model enzyme 4-oxalocrotonate tautomerase (4-OT), a brightly colored merocyanine-dye-type structure is formed. 4-OT mutants that formed this brightly colored species upon incubation with the probe proved to have a substantial activity for the iminium-based Michael-type addition of nitromethane to cinnamaldehyde, whereas mutants that showed no staining by the probe exhibited no or very low-level "Michaelase" activity. This system was exploited in a solid-phase prescreening assay termed as activated iminium colony staining (AICS) to enrich libraries for active mutants. AICS prescreening reduced the screening effort up to 20-fold. After two rounds of directed evolution, two artificial Michaelases were identified with up to 39-fold improvement in the activity for the addition of nitromethane to cinnamaldehyde, yielding the target γ-nitroaldehyde product with excellent isolated yield (up to 95%) and enantiopurity (up to >99% ee). The colorimetric activation of the turn-on probe could be extended to the class I aldolase 2-deoxy-D-ribose 5-phosphate aldolase, implicating a broader application of AICS in engineering iminium biocatalysis.



INTRODUCTION

Iminium-based catalysts are among the most versatile catalysts, finding applications in a myriad of C−C bond-forming transformations.[1] Many of these transformations lead to the formation of one or more asymmetric carbon atoms. Hence, researchers have focused on the discovery and engineering of iminium-based enzymes that could be applied as biocatalysts.[2−4] Unfortunately, nature's repertoire of iminium-based carboligases is limited. This has motivated researchers to supplement natural iminium-based biocatalysts with artificial biocatalysts, either obtained by computational design[5,6] or inspired by natural scaffolds.[7,8] Regardless of the source of the biocatalyst, enzyme engineering is often required to improve the catalytic rate, enantioselectivity, or stability. In the Hilvert laboratory, the computationally designed retroaldolase RA95.0 was engineered toward Michael-type additions,[9] aldol reactions,[10,11] Knoevenagel condensations,[12] and Henry condensations.[13] In our group, we have focused on the engineering of a natural tautomerase, 4-oxalocrotonate tautomerase (4-OT). By exploiting the nucleophilic character of the key catalytic residue Pro-1, we have engineered 4-OT towards several enamine or iminium-mediated C−C bond-forming reactions, including Michael-type additions and aldol reactions.[14−18]

Despite large steps that have been made in techniques to construct small smart libraries, typically only a small fraction of the mutants within a library display the desired improved characteristics.[19−21] Especially improving the catalytic rate is challenging, as it is difficult to predict how mutations influence the catalytic machinery of the enzyme. As a consequence, the screening of mutants is still considered to be the bottleneck in enzyme engineering.[20]

Ultrahigh-throughput screening techniques, such as fluorescence-activated cell sorting (FACS)[22] and fluorescence-activated droplet sorting (FADS),[23,24] have enabled the screening of very large mutant libraries to enrich these libraries for active mutants. The resulting enriched libraries are small enough that they can be screened using lower throughput screening techniques such as 96-well assays, or high-performance liquid chromatography/gas chromatography (HPLC/GC)-based assays to identify individual mutants with the desired characteristic. The effectiveness of this approach was elegantly demonstrated by the FADS-based directed evolution of retroaldolase RA95.5-8, improving the catalytic efficiency >20-fold.[11,25] However, these techniques require specialized equipment and nontrivial reaction setup.

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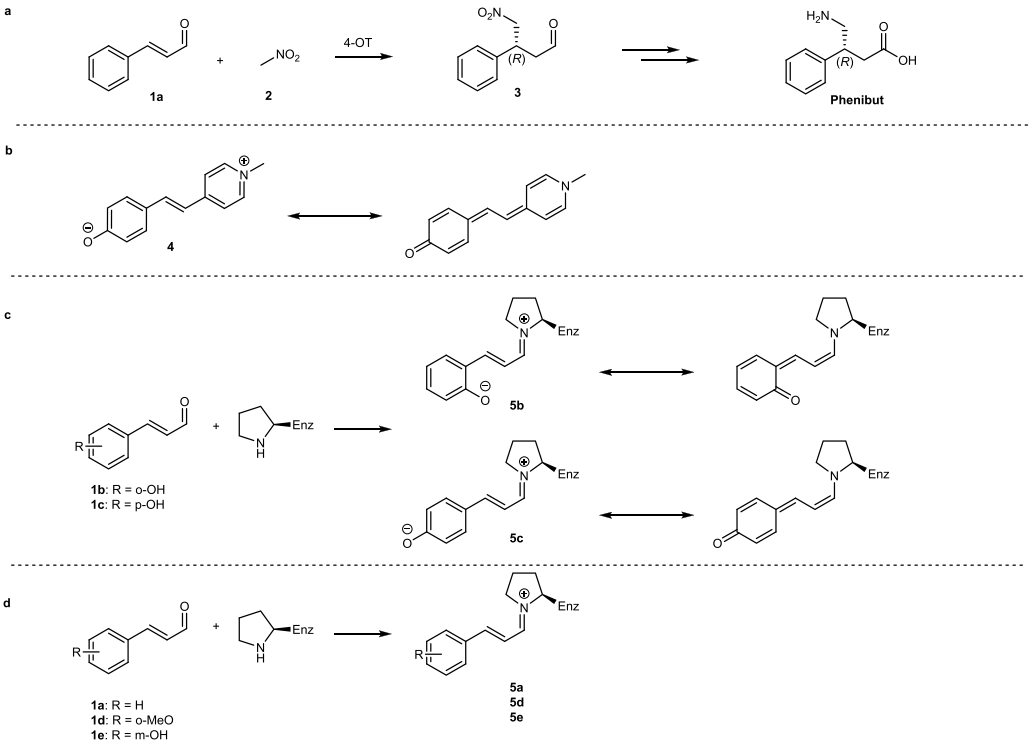

Figure 1. 4-OT catalyzed Michael-type addition, Brooker's merocyanine, and the reaction between Pro-1 of 4-OT and cinnamaldehydes. (a) 4-OT catalyzed Michael-type addition of **2** to **1a**. Product *R*-**3** is a precursor for Phenibut. (b) Resonance forms of Brooker's merocyanine. (c) Turn-on probes **1b** and **1c** reacting with Pro-1 of 4-OT, forming a zwitterionic resonating species. (d) Compounds **1a**, **1d**, and **1e** reacting with Pro-1 of 4-OT, forming a species without zwitterionic resonance.

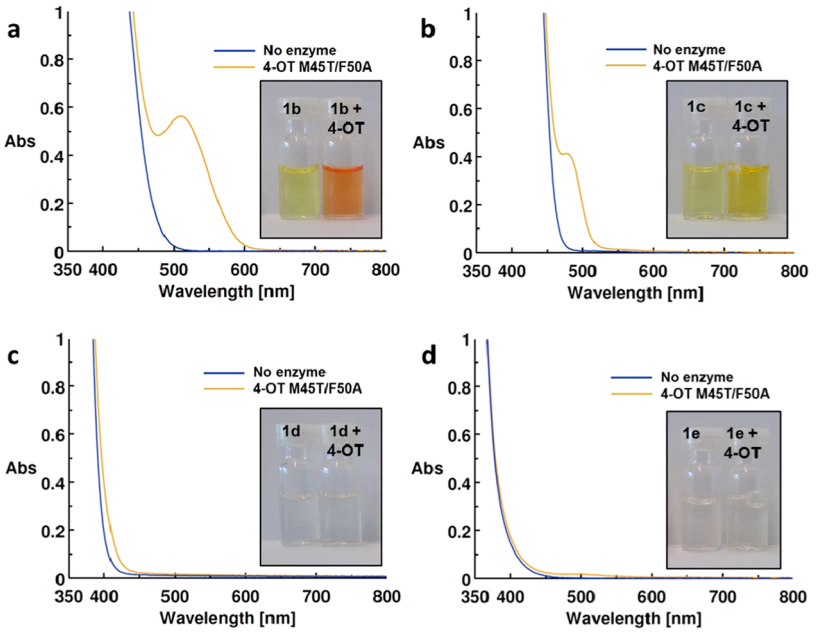

Figure 2. Absorption spectra of **1b**−**1e** in the presence and absence of 4-OT M45T/F50A. (a) Absorption spectrum of **1b** with and without 4-OT M45T/F50A. (b) Absorption spectrum of **1c** with and without 4-OT M45T/F50A. (c) Absorption spectrum of **1d** with and without 4-OT M45T/F50A. (d) Absorption spectra of **1e** with and without 4-OT M45T/F50A. Conditions: 2.5% dimethyl sulfoxide (DMSO), 20 mM sodium phosphate (pH 7.3), 1 mM **1b**−**1e**, 50 μM 4-OT M45T/F50A, 1 cm quartz cuvettes.

Here, we present a simple prescreening technique based on the iminium-activated colorimetric turn-on probe **1b** to prescreen large 4-OT mutant libraries for improved activity toward the iminium-mediated Michael-type addition of **2** to **1a** (Figure 1). The principle behind the prescreening technique is based on the iminium species that is selectively formed between **1b** and Pro-1 of 4-OT (Figure 1). This structure resembles a merocyanine dye and results in a new relatively bathochromic absorption peak that can easily be observed by the naked eye. This staining is not, or to a much lesser extent, observed in many inactive mutants, which allows for those mutants to be excluded from subsequent screenings. We

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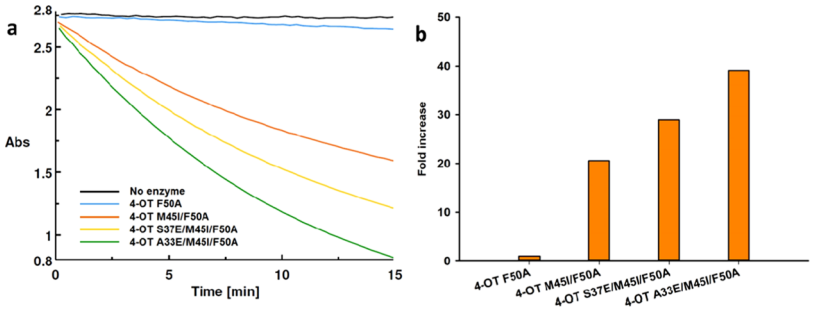

Figure 3. Activity for the Michael-type addition of **2** to **1a** catalyzed by different 4-OT mutants. (a) Progress curve of the Michael-type addition of **2** to **1a** catalyzed by different 4-OT mutants. (b) Initial rate of the Michael-type addition of **2** to **1a** catalyzed by different 4-OT mutants. The data are presented as fold increase compared to the initial rate of starting mutant 4-OT F50A. Assay conditions: 5% ethanol, 20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-ethanesulfonic acid (HEPES) (pH 6.5), 1 mM **1a**, 100 mM **2**, 50 mM sodium formate.

applied this technique in a solid-phase preselection assay that was termed as activated iminium colony staining (AICS). AICS prescreening reduced screening efforts up to 20-fold and allowed for the screening of large mutant libraries. After two rounds of directed evolution, we could improve the Michaelase activity of 4-OT for the addition of **2** to **1a** up to 39-fold. We show that the principle behind AICS can be extended to other iminium-based biocatalysts like the class I aldolase 2-deoxy-D-ribose 5-phosphate aldolase (DERA), which potentially paves the way for the engineering of novel biocatalysts for iminium-mediated C−C bond-forming reactions.

RESULTS

Identification of a Selective Turn-On Probe for 4-OT. Recently, it was shown that Pro-1 of 4-OT can react with **1a** to form complex **5a**, a catalytic intermediate in the Michael-type addition of **2** to **1a** yielding **3**.[18] Analogously, we hypothesized that **1b** and **1c** could react with Pro-1 of 4-OT to form **5b** and **5c**, respectively, structures that bear chemical resemblance to Brooker's merocyanine (**4**) (Figure 1). Compound **4** is a member of the diverse class of merocyanine dyes, which are characterized by an electron-donating N-atom connected via a streptopolymethine chain to an electron-withdrawing carbonyl group.[26,27] As a consequence, merocyanine dyes possess a neutral and zwitterionic resonance form and have a relatively high λ_{max}. As compound **1b** nor **1c** nor free 4-OT bear this characteristic chemical scaffold, incubation of **1b** and **1c** with 4-OT is expected to induce the formation of complexes **5b** and **5c**, respectively, each having a new relatively bathochromic absorption peak.

To test this hypothesis, **1b** and **1c** were chemically synthesized (Figure S1). As a control, we synthesized compounds **1d** and **1e** that, like substrate **1a**, should not be able to form merocyanine dye-type structures upon condensation with Pro-1 of 4-OT because they lack an *o*-OH or *p*-OH group (Figure 1d). For testing, we used 4-OT M45T/F50A, a well-studied 4-OT mutant that was constructed for the synthesis of **1a** via the aldol condensation of acetaldehyde with benzaldehyde.[15] Gratifyingly, upon incubation of **1b** or **1c** with 4-OT M45T/F50A, the color of the solution changed within a few seconds (Figure 2). Spectroscopic analysis revealed the appearance of a novel absorption peak at 516 nm for **1b** and 478 nm for **1c**, whereas incubation of 4-OT M45T/F50A with **1d** and **1e** did not reveal any new absorption peaks (Figure 2). Especially, the bright red species that was formed upon incubation of **1b** with 4-OT M45T/F50A could clearly be seen by the naked eye, which made **1b** a powerful turn-on probe for 4-OT. A single-site binding relationship between **1b** and 4-OT M45T/F50A was observed, with an estimated K_D of 0.119 ± 0.0055 mM, which indicates that binding of **1b** to 4-OT is site-specific (Figure S2).

To gain further evidence for the formation of complex **5b**, 4-OT M45T/F50A was incubated with **1b** for 15 min, followed by the addition of $NaCNBH_3$ to reduce any iminium species and covalently trap the interaction of the probe and the protein. Subsequent analysis by electrospray ionization mass spectrometry (ESI-MS) showed a mass consistent with that of 4-OT M45T/F50A modified with a single molecule of **1b** (Figure S3). Control reactions with 4-OT M45T/F50A incubated with only **1b** or only $NaCNBH_3$ did not show any modification. The labeled 4-OT was digested with the endoproteinase Glu-C and the resulting peptides were analyzed by ESI-MS, revealing the labeling of **1b** on the peptide fragment Pro-1 to Glu-9 (Figure S4). As Pro-1 is the only likely candidate on this peptide to form an iminium species with **1b**, Pro-1 was reckoned as the site of labeling.

Interestingly, incubation of **1b** with the enzyme 2-deoxy-D-ribose 5-phosphate aldolase (DERA), a well-studied class I aldolase,[4,28] also resulted in an increase in absorbance at 516 nm (Figure S5), implicating broader applicability of probe **1b**.

Mutants with Improved Michaelase Activity Show Strong Staining with 1b. Having developed a selective turn-on probe for 4-OT, we were interested in applying this system to aid our directed evolution efforts. Therefore, the effect of mutations of 4-OT on staining with **1b** was investigated. Cells producing a defined set of 53 single mutants, covering nearly all single mutants at positions 6, 45, and 50 were grown in a deep 96-well plate. These positions were selected because mutations at these positions have shown to influence the activity of 4-OT for several promiscuous reactions.[14−16,18] Incubation of cells producing the 53 single mutants with **1b** demonstrated that single mutations can significantly affect the staining with **1b** (Figure S6). Most notably, single mutants 4-OT F50A, F50I, and F50V showed strong staining upon incubation with **1b**. Interestingly, these three mutants were previously found in a screen for mutants with enhanced activity for the Michael-type addition of **2** to **1a**, forming **3**.[18] These results prompted us to investigate if **1b** could be used as a prescreening tool to enrich libraries for mutants with activity towards the Michael-type addition of **2** to **1a** yielding **3**, a precursor for phenibut, an important γ-aminobutyric acid (GABA) analogue.

Activated Iminium Colony Staining-Based Directed Evolution of 4-OT. To improve the Michaelase activity of 4-

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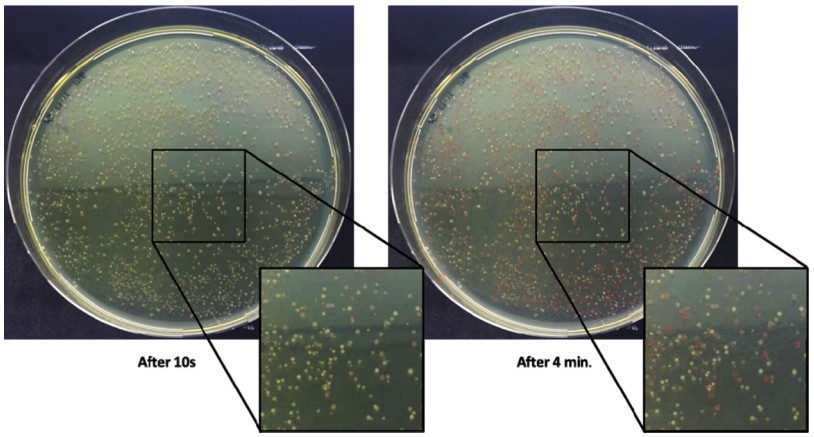

Figure 4. Agar plates 10 s (left) and 4 min (right) after performing the AICS assay. Transformants of library 21−40_IA were stained with the AICS assay as described in the Experimental Section.

OT for the addition of **2** to **1a**, we started by constructing a triple-site NNK library. We randomized the active site residues Gln-4, Leu-8, and Met-45 in the context of 4-OT F50A, the reported best mutant for this reaction,[18] yielding library Q4X/L8X/M45X/F50A. The addition of 0.2% lactose to agar plates induced the expression of 4-OT in freshly transformed cells, without any effect on the transformation efficiency. A solution of 0.75% agarose containing **1b** was poured on the agar plate and was allowed to solidify. Within 1 min, ∼5% of the colonies stained bright red and could be distinguished from other colonies by the naked eye.

A total of 1472 stained colonies were picked and screened for an improved activity for the addition of **2** to **1a**. Several mutants with improved activity were identified. Almost all mutants harbored the M45I mutation, without any obvious additive effect of mutations at positions 4 and 8. Hence, double mutant 4-OT M45I/F50A was constructed, which showed a 20-fold improvement in activity compared to 4-OT F50A, using optimized reaction conditions (Figure 3).

In the second round of directed evolution, two libraries, 2−20_IA and 21−40_IA, were constructed containing a single random mutation from positions 2−20 and 21−40, respectively in the context of 4-OT M45I/F50A. No mutations were introduced in the C-terminal part of 4-OT (positions 41−62) as this region is highly important for stabilization of the hexameric structure.[29] The library was transformed into *Escherichia coli* cells, and colonies were stained using the AICS assay (Figure 4). Comparing the activity of 92 stained colonies with 92 unstained colonies of library 21−40_IA demonstrated the high sensitivity of AICS prescreening, as 32% of the stained mutants showed activity comparable or higher than the parental mutant 4-OT M45I/F50A, whereas all of the unstained mutants showed no activity or less than 50% of the activity of the parental mutant 4-OT M45I/F50A (Figure 5).

From each library, 368 stained colonies were picked and screened for an improved activity for the addition of **2** to **1a**. Although library 2−20_IA yielded many active mutants, unfortunately, none were significantly more active than the parental mutant 4-OT M45I/F50A. Gratifyingly, library 21−40_IA yielded two improved triple mutants: 4-OT S37E/M45I/F50A and 4-OT A33E/M45I/F50A with 29- and 39-fold improved activity compared to 4-OT F50A (Figure 3). The synthetic usefulness of the newly engineered mutants 4-

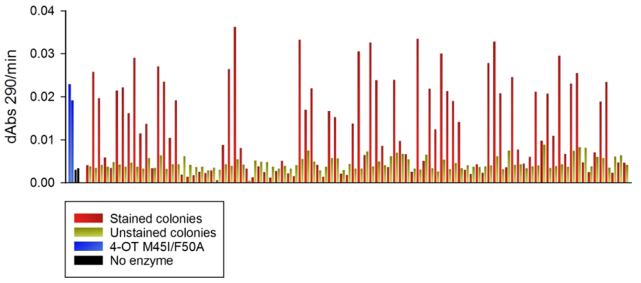

Figure 5. Activity for the Michael-type addition of **2** to **1a** catalyzed by CFEs of 92 stained and 92 unstained colonies. Colonies from cells that were freshly transformed with library 21−40_IA were stained using the AICS procedure. In total, 92 stained colonies and 92 unstained colonies were picked, grown overnight, and the CFE was assayed for the Michaelase activity using substrates **2** and **1a**. The Michaelase activity was measured in a plate reader by following the depletion of **1a** at 290 nm. Assay conditions: 5% ethanol, 30% CFE, 0.5 mM **1a**, 100 mM **2**, 50 mM sodium formate. Analysis of CFEs of unstained colonies by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) showed that eight out of 16 show significant 4-OT expression, indicating that the AICS procedure can efficiently be used to eliminate colonies not producing enzyme or producing enzyme with low-level activity.

OT S37E/M45I/F50A and 4-OT A33E/M45I/F50A was demonstrated in semipreparative scale Michael-type addition reactions using substrates **1a** and **2**. Target product *R*-**3** could be obtained in good to excellent isolated yield (up to 95%) and with excellent enantiopurity (up to >99% enantiomeric excess, ee) (Table S1 and Figures S7−S11). Given that only a small number of colonies were assayed, these results demonstrate the power of using the AICS prescreening tool for the rapid engineering of iminium biocatalysis.

DISCUSSION

The use and development of novel, higher throughput screening systems have drawn considerable attention.[30] As the screening of large mutant libraries is often limiting enzyme engineering efforts, systems that reduce screening efforts or allow for higher throughput of screening have significantly attributed to the success of enzyme engineering studies.[25,31,32] The design of novel smart (pre)screening techniques is therefore expected to have a strong impact. We wanted to contribute to this field by designing a smart iminium-based

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turn-on probe that could be used to remove a considerable part of 4-OT mutants with no or low activity from large mutant libraries.

Although there are a few reports on turn-on probes that make use of an activated lysine in the acceptor protein,[33−35] these studies focused on noncatalytic proteins. Instead, we aimed to design the first iminium-activated turn-on probe for an enzyme, exploiting the characteristic nucleophilic N-terminal proline of 4-OT. In the past, we showed that **1a** binds specifically to Pro-1 of 4-OT, forming **5a** (Figure 1d).[18] Hence, we designed turn-on probes **1b** and **1c**, which are close mimics of substrate **1a**. Incubation of **1b** and **1c** with 4-OT M45T/F50A resulted in the formation of a novel relatively bathochromic absorption peak characteristic of merocyanine dyes. Especially, incubation of turn-on probe **1b** with 4-OT M45T/F50A induced a remarkable spectroscopic shift that could easily be observed by the naked eye. Labeling studies further supported our hypothesis that Pro-1 of 4-OT reacts with **1b** to form merocyanine dye **5b**. Note that substrate **1a** and analogues **1d** and **1e**, which were prepared as controls, cannot form merocyanine dye-type structures upon condensation with Pro-1 of 4-OT because they lack an *o*-OH or *p*-OH group (Figure 1d).

Merocyanine dye **5b** is very similar to **5a**, a key catalytic intermediate for the Michael-type addition of **2** to **1a**. Therefore, we investigated if there is a correlation between 4-OT single mutants that upon incubation with probe **1b** show strong staining and 4-OT single mutants with improved Michaelase activity. Indeed, we found that, from a subset of 4-OT single mutants, specifically mutants with enhanced Michaelase activity showed strong staining upon incubation with **1b**. This inspired us to use turn-on probe **1b** as a prescreening tool in the engineering of improved artificial Michaelases. We developed the AICS technique, a colorimetric solid-phase prescreening procedure based on selective staining of colonies producing an active 4-OT mutant with **1b**, and used it to enrich large libraries for active 4-OT mutants. The developed AICS prescreening technique proved to be highly effective, reducing the screening effort for a single round of directed evolution up to 20-fold. In addition, the sensitivity of AICS prescreening proved to be very high, as from a subset of 92 unstained colonies all showed less than 50% activity compared to the parental mutant.

After two rounds of directed evolution, two triple mutants, 4-OT S37E/M45I/F50A and 4-OT A33E/M45I/F50A, were identified with a 29- and 39-fold improvement in activity compared to starting mutant 4-OT F50A. Semipreparative scale reactions of the Michael-type addition of **2** to **1a** catalyzed by 4-OT S37E/M45I/F50A and 4-OT A33E/M45I/F50A resulted in the isolation of target product *R*-**3** with excellent yield (up to 95%) and enantiopurity (ee up to >99%).

The developed AICS prescreening technique provides a novel application of turn-on probes, adding to the usage of turn-on probes as tools for noninvasive tissue imaging or colorimetric pH sensors.[36−38] Contrary to many reported colorimetric assays (e.g., refs 39−42), AICS does not rely on a (coupled) chemical reaction producing a chromogenic product, but instead solely relies on a conjugate formed between the probe and the enzyme. As such, AICS is very quick and nicely complements available reported colorimetric assays.

It would be interesting to investigate the application of AICS to a broader range of iminium biocatalysts, as preliminary results indicate that turn-on probe **1b** was also activated by the lysine-dependent aldolase DERA. The further development of turn-on probes as tools for enzyme engineering potentially opens up new possibilities for the efficient engineering of novel classes of iminium-based biocatalysts.

EXPERIMENTAL SECTION

Spectrophotometric Analysis of 1b−1e Incubated with Purified 4-OT. A stock solution of 40 mM **1b−1e** was prepared in 100% DMSO. A total of 25 μL was added to a solution of 20 mM sodium phosphate buffer (pH 7.3) with and without 50 μM 4-OT M45T/F50A; the final volume was 1 mL. The mixture was transferred to a 1 cm quartz cuvette and a spectrum from 350 to 800 nm was recorded. To analyze the binding behavior of **1b** to 4-OT M45T/F50A, a binding curve was constructed. **1b** (4−0 mM, 2-fold dilution series in 20 mM sodium phosphate pH 7.3, 5% DMSO) was incubated with 4-OT M45T/F50A (50 μM) for 15 min (300 μL final volume). The mixtures were transferred to 1 mm quartz cuvettes and the absorbance at 516 nm was recorded and plotted against the concentration of **1b**. The experiment was performed in triplicate. SigmaPlot was used to fit the data against the one-site saturation model and to calculate the apparent K_D value (Figure S2).

Covalent Labeling of 1b to 4-OT. To gain further evidence for the formation of **5b**, 4 mM **1b** was incubated with 150 μM 4-OT M45T/F50A in 10 mM sodium phosphate (pH 7.3), 5% DMSO at room temperature; the total volume was 500 μL. After 15 min, 167 μL of 100 mM $NaCNBH_3$ was added in H_2O to reduce any iminium species. The reaction was incubated at room temperature for 30 min. The buffer was exchanged to 10 mM ammonium formate in H_2O using a prepacked PD-10 sephadex G-25 gelfiltration column. A sample of the collected enzyme was analyzed by ESI-MS (Figure S3). From the remaining fraction, 36 μL was digested with GluC (New England BioLabs, 4 μL from a 100 ng/μL stock solution) in a total volume of 80 μL for 24 h at 37 °C using the supplied buffer. A sample from the digested mixture was analyzed by ESI-MS. As controls, reaction without **1b** and reaction without the addition of $NaCNBH_3$ (no reduction) were included. In both cases, no modified 4-OT could be detected by ESI-MS (Figures S3 and S4).

Staining of 4-OT Single Mutants with 1b. From a reported set of 4-OT single mutants,[16] BL21 DE3 glycerol stocks of all single mutants with a mutation at positions 6, 45, or 50 were used to inoculate 1 mL of lysogeny broth (LB) medium supplemented with ampicillin (100 μg/mL) and IPTG (100 μM) and grown overnight at 37 °C in 96-deep well plates (Greiner Bio-one, 96-well Masterblock), covered with sterile gas-permeable seals (Greiner Bio-one, BREATHseal). The cells were harvested by centrifugation and the supernatant was removed. The cell pellets were resuspended in 200 μL of 20 mM sodium phosphate buffer (pH 7.3) and 50 μL was transferred to a round bottom 96-well plate (96-well U-bottom, Greiner Bio-One). To this, 2.5 μL of 40 mM **1b** in 100% DMSO was added. The cells were allowed to settle on the bottom of the 96-well plate.

Cloning and Mutagenesis. Triple-site library 4-OT Q4X/L8X/M45X/F50A was constructed by polymerase chain reaction (PCR) with degenerate primers. The first part of the 4-OT gene was amplified using the following primers:

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5′-GGAGATATACATATGCCTATTGCC**NNK**ATCCACATC**NNK**GAAGGCCGCAGCGACG-3′ and 5′-CAGGGAGCGCGAGATGGCC-3′. The mutated codons Gln-4 and Leu-8 are indicated in bold. Met-45 was randomized using primers 5′-GTGATTATCACGGAG**NNK**GCCAAGGGCCAC-3′ and 5′-GTGGCCCTTGGC**MNN**CTCCGTGATAATCAC-3′ by means of QuikChange technology. DNA of 4-OT F50A was used as a template. The mutated codon Met-45 is indicated in bold. The second part of 4-OT was amplified from the QuikChange product using the following primers: 5′-GGCCATCTCGCGCTCCCTG-3′ and 5′-GCTAGGGGGATCCTCAGCGTCTGACCTTGCTGGC-CAGTTCGCCGCC-3′, using the QuikChange product as template. The amplified parts of the 4-OT gene were combined by overlap extension PCR and further amplified using primers 5′-TTGAAGGAGATATACATATGCCT-3′ and 5′-GCTAGGGGGATCCTCAGCG-3′. An empty pET20b backbone was generated by amplification of a pET20b plasmid using the following primers: 5′-TGGGCAATAGGCATATGTATATCTCCTTCTTAAAGTTAAAC-3′ and 5′-AAGGTCAGACGCTGAGGATCCGAATTCGAGCTCCG-3′. The assembled 4-OT gene and the empty pET20b backbone were purified and digested with restriction enzymes NdeI, BamHI, and DpnI. The empty pET20b backbone was dephosphorylated using alkaline phosphatase. The digested DNA products were purified and ligated by T4 DNA ligase. The ligation mixture was purified and used to electroporate electrocompetent *E. coli* DH5α cells. After 1 h of outgrowth with SOC medium, a small sample of the transformed DH5α cells was spread on an agar plate to determine the transformation efficiency. The remainder of the transformation mixture was added to 5 mL LB medium, supplemented with ampicillin (100 μg/mL), and grown overnight. The overnight culture was harvested and the plasmids were isolated and used to transform BL21 DE3 cells.

For the construction of library 2−20_IA, a previously reported set of nearly all single mutants was used.[16] From a glycerol stock, each DH10B culture that carried a plasmid harboring a 4-OT gene with a mutation at positions 2−20 was separately grown overnight in 300 μL of LB medium supplemented with ampicillin (100 μg/mL). From all individual cultures, 100 μL of each was mixed together. From a sample of this mixed culture, plasmid DNA was isolated to obtain a library of 4-OT genes with a random single mutation at positions 2−20. The second part of the 4-OT gene was amplified using the following primers: 5′-GATTATCACGGAG**ATT**GCCAAGGGCCAC**GCC**GGCATCGGCGGCG-3′ and 5′-GCTAGGGGGATCCTCAGCG-3′. The mutated residues Ile-45 and Ala-50 are indicated in bold. Using the library of 4-OT genes with a random single mutation at positions 2−20 as a template, the first part of the 4-OT gene was amplified using the following primers: 5′-TTGAAGGAGATATACATATGCCT-3′ and 5′-CGCCGCCGATGCC**GGC**GTGGCCCTTGGC**AAT**CTCCGTGATAATC-3′. Using overlap extension PCR, a full-length 4-OT gene was obtained containing a random mutation at positions 2−20, combined with the M45I and F50A mutation. The full-length 4-OT gene was digested, ligated, and cloned into the pET20b plasmid according to the procedure described for the construction of the Q4X/L8X/M45X/F50A library to obtain library 2−20_IA. The same procedure was followed for the construction of library 21−40_IA, using the single mutants with a mutation at positions 21−40.

Expression and Purification of Enzymes. All 4-OT mutants were expressed and purified according to a reported procedure.[7] DERA with C-terminal His-tag was expressed and purified from *E. coli* using the pET20b(+) expression system. A colony of freshly transformed BL21 DE3 cells was used to inoculate 5 mL of LB medium supplemented with ampicillin (100 μg/mL) and grown for 8 h at 37 °C. This starter culture was used to innoculate 100 mL of LB medium supplemented with ampicillin (100 μg/mL) and lactose (0.2% w/v). The cells were incubated at 30 °C overnight at 200 rpm in a shaker incubator. Cells were harvested by centrifugation, resuspended in 30 mL of 20 mM potassium phosphate buffer (pH 7.0), and lysed by sonication. The lysate was cleared by centrifugation and transferred to a clean tube. Ni-sepharose (0.5 mL) was added to the lysate and incubated at 4 °C under slow rotation for 30 min. The lysate with Ni-sepharose was loaded on a column, washed with 8 mL of 20 mM potassium phosphate buffer (pH 7.0), followed by 8 mL of 20 mM potassium phosphate buffer (pH 7.0) containing 30 mM imidazole. Final elution was performed with 2.5 mL of 20 mM potassium phosphate buffer (pH 7.0) containing 250 mM imidazole (>95% purity as assessed by SDS-PAGE). The buffer was exchanged to 20 mM potassium phosphate buffer (pH 7.0) using a prepacked PD-10 sephadex G-25 gelfiltration column. The concentration of the purified enzyme was determined by the Waddell method.[43] DERA K167L was purified according to the same protocol. After purification, the purified DERA and DERA K167L were immediately used in subsequent experiments.

Activated Iminium Colony Staining (AICS). BL21 DE3 cells were transformed with library DNA and plated on LB agar plates supplemented with ampicillin (100 μg/mL) and lactose (0.2% w/v). Colonies were grown for ∼16 h at 37 °C, after which the agar plate was stored at room temperature. A solution of 1.5% agarose in H_2O was prepared by heating in a microwave and the solution was kept at 60 °C. A second solution of 1 mM **1b** dissolved in 20 mM sodium phosphate (pH 7.3) was filter sterilized and stored at room temperature. Seven milliliters of the 1.5% agarose solution were mixed with 7 mL of the solution containing 1 mM **1b** in 20 mM sodium phosphate and immediately after mixing poured on the agar plate. Typically, staining of the first colonies was observed after 10 s. The plate was incubated at room temperature for 5 min to complete the staining and the agarose was allowed to solidify. The staining is stable for at least several hours. For screening of libraries 2−20_IA and 21−40_IA, the concentration of **1b** in the second solution was reduced to 0.5 mM to increase the stringency of selection.

Library Screening. Colonies that showed clear staining after the AICS assay were picked with sterile toothpicks and were used to inoculate 1 mL of LB medium supplemented with ampicillin (100 μg/mL) and lactose (0.2% w/v) in 96-deep well plates (Greiner Bio-one, 96-well Masterblock). Two wells in the 96-deep well plate were used to inoculate the parental mutant (4-OT F50A or 4-OT M45I/F50A). The plates were sealed with sterile gas-permeable seals (Greiner Bio-one, BREATHseal) and incubated at 37 °C, with overnight shaking at 250 rpm. Eighty microliters of the culture were mixed with 20 μL of a sterile solution of 80% glycerol in H_2O and stored at −80 °C for later reference. The remainder of the culture was centrifuged. The supernatant was discarded and the pellets

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were resuspended with 200 μL of BugBuster (Novagen) supplemented with 15 U/mL benzonase (Novagen). After 20 min of incubation at room temperature, the lysates were cleared by centrifugation after which the CFE was obtained. For the screening of libraries 2−20_IA and 21−40_IA, prior to centrifugation, the lysed cells were diluted with 200 μL of sterile H_2O. For the screening of library Q4X/L8X/M45X/F50A, the final reaction mixture for monitoring the addition of **2** to **1a** consisted of 40% CFE, 25 mM **2**, 250 μM **1a**, 5% ethanol in 20 mM HEPES, pH 6.5; the final volume was 100 μL. For the screening of libraries 2-20_IA and 21−40_IA, the final reaction mixture for monitoring the addition of **2** to **1a** consisted of 30% CFE, 100 mM **2**, 1 mM **1a**, 50 mM sodium formate, 5% ethanol in 20 mM HEPES pH 6.5; the final volume was 100 μL. Sodium formate was added to the reaction mixture, as this was found to positively affect the catalytic rate of 4-OT M45I/F50A. The reaction was performed in a 96-well plate (Greiner Bio-one, UV-star F-bottom microplate) and reaction progress was followed in a plate reader by following the depletion in absorbance at 290 nm. For the screening of libraries 2−20_IA and 21−40_IA, the initial absorbance was too high for the plate reader, so the time point at which the absorbance went below the absorbance threshold of the plate reader was used as a reference point for activity. For the comparison of the activity of 92 stained mutants with 92 unstained mutants, the same procedure as described for library 21−40_IA was followed. However, the reaction mixtures consisted of 0.5 mM **1a** instead of 1 mM **1a**.

Progress Curves of the Enzymatic Reactions. For monitoring the progress of the enzymatic addition of **2** to **1a** the following reaction setup was used. To a solution of **1a** in ethanol, **2** in 20 mM HEPES (pH 6.5) and purified 4-OT F50A, 4-OT M45I/F50A, 4-OT S37E/M45I/F50A or 4-OT A33E/M45I/F50A were added. The final reaction mixture consisted of the following: 5% ethanol, 20 mM HEPES (pH 6.5), 1 mM **1a**, 100 mM **2**, 50 mM sodium formate, 20 μM 4-OT. The reaction mixtures were transferred to 1 mm quartz cuvettes and the absorption at 290 nm was recorded every 20 s.

Semipreparative Scale Reactions. Semipreparative scale experiments were performed using 4-OT S37E/M45I/F50A and 4-OT A33E/M45I/F50A as biocatalysts. The reaction mixtures consisted of the following: 5 mL ethanol (5% v/v), 20 mg **1a**, 25 mM **2**, 50 mM sodium formate, 20 mM HEPES (pH 6.5), 7.5 μM enzyme; the final volume was 100 mL. At timely intervals, a sample was withdrawn from the reaction mixture and the reaction progress was monitored by following the depletion in absorbance at 290 nm. After 10.5 h for 4-OT S37E/M45I/F50A and 7.5 h for 4-OT A33E/M45I/F50A, the reaction was finished and the reaction mixture was extracted 3× with 20 mL ethyl acetate. The combined organic layers were washed with brine and dried over anhydrous Na_2SO_4. For the reaction catalyzed by S37E/M45I/F50A, the organic layer was concentrated in vacuo, yielding **3** (27.8 mg, 95% isolated yield). For the 4-OT A33E/M45I/F50A catalyzed reaction, the organic layer was concentrated in vacuo and the product was purified by silica gel column chromatography (hexane/ethyl acetate 4:1) to obtain **3** (16.0 mg, 55% yield).

Condensation of 1b with DERA and 4-OT. The condensation of **1b** with DERA and 4-OT was monitored in time by following the absorbance at 516 nm. For the condensation with DERA and DERA K167L, a 2× stock solution was prepared consisting of the following: 20 mM potassium phosphate (pH 7.0), 10% DMSO, and 8 mM **1b**. The stock solution (500 μL) was mixed in a 1 cm quartz cuvette with 500 μL of 200 μM DERA or DERA K167L (5.54 mg/mL) in 20 mM potassium phosphate (pH 7.0). Immediately after mixing, the absorbance at 516 nm was measured every 0.5 s for 190 s. For 4-OT, a 2× stock solution was prepared consisting of the following: 20 mM HEPES (pH 6.5), 10% ethanol, and 0.6 mM **1b**. The stock solution (500 μL) was mixed in a 1 cm quartz cuvette with 500 μL of 100 μM 4-OT in 20 mM HEPES (pH 6.5). Immediately after mixing, the absorbance at 516 nm was measured every 0.1 s for 190 s.

Derivatization of 3 for Enantiomeric Excess Determination. The aldehyde functionality of **3** was derivatized to a cyclic acetal according to a literature procedure.[44,45] The enantiopurity of derivatized **3** was analyzed by reverse-phase HPLC using a column with a chiral stationary phase (chiralpak AD-RH, 150 mm × 4.6 mm, Daicel); detection at 220 nm, retention time for *R*-**3** was 8.8 min and for *S*-**3**, it was 12.2 min. The absolute configuration was determined by literature comparison.[16]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03849.

Synthesis of starting materials and reference compounds; additional tables and figures including ESI-MS, NMR, HPLC, and UV−vis spectra (PDF)

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Author Contributions

L.B. performed 4-OT labeling experiments, developed the AICS screening assay, and performed experiments with DERA. L.B. and M.C. performed the screening of mutant libraries. M.S. synthesized cinnamaldehyde derivatives. G.J.P. supervised scientific work. All authors contributed to the writing of the manuscript.

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Notes

The authors declare no competing financial interest.

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