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Ultrahigh-Throughput Screening Enables Efficient Single-Round Oxidase Remodeling

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Biocatalysis provides a potentially sustainable means of chemical manufacturing. However, the tailoring of enzymes to industrial processes is often laborious and time consuming, limiting broad implementation of this approach. High throughput screening methods can expedite the search for suitable catalysts but are often constrained by the need for labeled substrates. Generalization of such techniques would therefore significantly expand their impact. Here we have established a versatile ultrahigh-throughput microfluidic assay that enables isolation of functional oxidases from libraries containing up to 10^7 members. The increased throughput over prevalent methods led to complete active-site remodeling of cyclohexylamine oxidase in one round of directed evolution. A 960-fold increase in catalytic efficiency afforded an enzyme with wild-type levels of activity for a non-natural substrate, allowing biocatalytic synthesis of a sterically demanding pharmaceutical intermediate with complete stereocontrol. The coupled enzyme assay is label free and can be easily adapted to reengineer any oxidase.

Chiral amines play an indispensable role in the production of high-value chemicals. Approximately forty percent of active pharmaceutical ingredients (APIs) depend on optically active amines as intermediates during production¹, and consequently asymmetric amine synthesis has been identified as one of the key challenges towards a greener and more sustainable chemical industry². In recent years, biocatalysis has emerged as an attractive and competitive technology for chiral amine synthesis^{3–6}, as enzymes display high levels of activity and stereocontrol, function under ambient conditions, and can in principle be readily adapted for industrial purposes using laboratory evolution. Indeed, engineered enzymes have been applied in the large-scale manufacture of APIs and key chiral pharmaceutical building blocks^{7,8}. Adopting these biocatalysts has decreased the number of synthetic steps required, circumvented the need for costly transition metal catalysts, reduced the demand for organic solvents, and delivered products with increased (optical) purity, thus alleviating the need for expensive downstream purification steps.

Amine oxidases have proven to be especially effective for the production of optically pure amines through deracemization, a process combining oxidation catalyzed by an enantioselective enzyme and a non-selective chemical

reducing agent⁹. Cycles of selective oxidation and non-selective reduction lead to the accumulation of a single enantiomer in high yield. For example, monoamine oxidase from *Aspergillus niger* (MAO-N) and cyclohexylamine oxidase from *Brevibacterium oxydans* (CHAO) have been engineered to deracemize chiral auxiliaries⁹ as well as secondary¹⁰ and tertiary¹¹ amine-containing APIs and pharmaceutical building blocks¹². Engineered amine oxidases have also been exploited in oxidative desymmetrization processes, most notably in the large scale synthesis of a key chiral pyrrolidine derivative used in the production of the hepatitis C virus protease inhibitor Boceprevir⁸.

Despite these notable successes, adapting oxidases to perform new tasks remains a challenging, time-consuming endeavor that restricts the potential impact of biocatalysis on many industrial processes. While powerful, directed evolution experiments are generally time consuming (months to years) and cost intensive (man hours, consumables, equipment)¹³, rendering them incompatible with the fast pace of business and preventing broader implementation of biocatalysis¹⁴. To increase the viability of biocatalysis for green chemistry, methods that accelerate directed evolution are urgently needed^{15–18}.

Amine oxidases can be assayed by coupling the universal hydrogen peroxide by-product formed during cofactor recycling to a reaction that generates a dye (Figure 1a), providing a versatile method of screening in microtiter plates¹⁹, on agar plates⁹, and in cells²⁰. Nevertheless, engineering MAO-N for the Boceprevir manufacturing process required the introduction of 65 mutations. Even with dedicated infrastructure for automated and high-throughput enzyme engineering, biocatalyst optimization was arduous with most rounds contributing only single digit improvements in activity.

Enzymatic assays using monodisperse emulsions generated on microfluidics chips have emerged as a powerful ultrahigh-throughput method in the search for novel biocatalysts¹⁷. This approach utilizes pL to nL volume aqueous droplets, stabilised by surfactant, in an inert, fluorinated oil, as independent microreactors that contain single cells (Figure 1b)²¹ akin to wells in a microtiter plate. On-chip microfluidic modules allow multiple operations, including droplet production, splitting, fusion, reagent addition, incubation, fluorescence detection, and fluorescence-activated droplet sorting (FADS), all at kHz frequencies^{22,23}. To screen for novel enzymes, single cells that express individual variants are typically encapsulated in droplets containing a fluorogenic substrate. Active enzymes can be displayed on the cell surface¹³, secreted^{24,25}, or released by cell lysis²⁶. Compartmentalization thus links genotype and the fluorescent product, enabling enrichment of rare but active variants from large libraries by sorting according to activity (Figure 1c). FADS increases the screening power dramatically compared to low- or medium-throughput techniques. Approximately 10⁷ variants can be assayed per experiment in a sensitive manner, reducing the expenditure of time and cost in consumables by several orders of magnitude¹³.

Here, we describe a label-free, ultrahigh-throughput, FADS-based coupled assay for oxidase engineering and its application to rapid remodeling of the CHAO active site for an industrially relevant substrate for which the original enzyme showed only low activity. We were able to generate an enzyme with a 3-order of magnitude increase in catalytic efficiency and wild-type levels of activity on the non-natural substrate in a single round of directed evolution.

Results

Fluorescence-activated droplet sorting of cyclohexylamine oxidase. Although CHAO oxidizes a range of primary (*S*)-configured amines, it is reportedly inactive with 1-phenyl-1,2,3,4-tetrahydroisoquinoline (PheTIQ, **7**)²⁷, whose (*S*)-enantiomer is a key precursor of the blockbuster drug Solifenacin. Kinetic characterization of the wild-type enzyme using high catalyst concentration revealed that it shows no detectable activity on the (*S*)-enantiomer and possesses a k_{cat}/K_M of $10 \pm 1 \text{ M}^{-1}\text{s}^{-1}$ for the (*R*)-enantiomer, which is three orders of magnitude lower than the value reported for the native cyclohexylamine substrate²⁸.

To remodel the active site of CHAO to accept PheTIQ, we targeted eight residues for mutagenesis: seven of the eleven residues that are within 5.5 Å of bound cyclohexanone in the crystal structure of wild-type CHAO (Tyr321, Phe368, and Pro422 plus Thr198, Leu199, Phe351 and Leu353) plus Met226, which is located a little farther away in the substrate diffusion channel (Supplementary Figure 1). With the exception of Phe351 and Pro422, which are absolutely conserved, phylogenetic analysis shows that most of these residues have been subject to conservative mutation in natural CHAO homologs (Supplementary Table 1). All eight residues were randomized simultaneously using primers with either DYT codons, which encode six amino acids (A, S, T, V, I and F), or the BYT codon for Pro422 (encoding A, S, P, V, L and F). The codons were designed to incorporate hydrophobic side-chains in a range of sizes and alcohols for hydrogen-bonding interactions with the substrate, thus maintaining the overall polarity of the native binding pocket but allowing variation in shape. The resulting library contained 1.7×10^6 theoretical members, with no diversity lost to stop codons and, since each amino acid is specified by a single codon, no frequency bias for one amino acid over another (Supplementary Table 2).

The gene library was expressed in *Escherichia coli* and the cells were injected into a microfluidic chip, where they were mixed with lysis agents, *rac*-PheTIQ, and a reporter cascade consisting of HRP and the fluorogenic dye Amplex UltraRed, and compartmentalized in 15 pL droplets with, on average, 0.3 cells per droplet to ensure that few droplets (<4 %) contained more than one cell. To exert evolutionary pressure on k_{cat} , screening was performed at a substrate concentration higher than the Michaelis constant for wild-type CHAO (4 mM versus ~1 mM, Supplementary Table 4). The library was sorted three times, collecting the 0.1% most active droplets in each population. The starting population consisted of $>10^7$ transformants, representing a greater than three-fold oversampling of all possible variants. After sorting, plasmid DNA was purified from the collected droplets. The genes were amplified by PCR, subcloned, and transformed back into *E. coli* cells. The resulting transformants were used for the next sort. In the first two sorts, the emulsion was incubated for 2 h off-chip prior to screening. For the final sort, the emulsion was only incubated for 15 minutes on-chip to increase screening stringency. The purified plasmids from this sort were used directly to transform *E. coli* for further analysis of the enriched population.

Individual variants from each cycle were analyzed and compared to the starting point using a colorimetric lysate-based activity assay in microtiter plates. The fraction of clones with improved activity increased from 3% before sorting to 43%

after the third round of sorting and the mean activity of the sorted population increased 78-fold compared to the unsorted library. The most active clones were chosen for sequencing (Supplementary Figure 2, Supplementary Table 3). Remarkably, the entire workflow, including library creation, three rounds of FADS, microtiter plate assay, isolation and sequencing of hits, was performed in under two weeks (Supplementary Figure 3).

Isolation and characterization of PT.1. The three most active clones from the third sort had identical amino acid sequences, differing only in silent mutations that arose spontaneously during PCR, indicating that they originated from independent sorting events. This variant, referred to as PT.1, has five amino acid changes compared to wild-type CHAO: L199V, M226T, Y321S, L353I, and P422S (Figure 2a, Supplementary Figure 4). PT.1 was purified and kinetically characterized with both enantiomers of PheTIQ (Supplementary Figure 5). It has a k_{cat}/K_M of $9400 \text{ M}^{-1} \text{ s}^{-1}$ with (*R*)-PheTIQ, which constitutes a 960-fold improvement compared to wild-type CHAO with the same substrate (Figure 2b) and is similar to the reported catalytic efficiency of the wild-type enzyme with its native substrate cyclohexylamine ($k_{\text{cat}}/K_M = 10,630 \text{ M}^{-1} \text{ s}^{-1}$)²⁸. The improvement is mainly due to a 340-fold larger turnover number ($k_{\text{cat}} = 3.2 \text{ s}^{-1}$ vs. 0.0095 s^{-1}). Furthermore, PT.1 is highly stereoselective for the (*R*)-enantiomer, with a selectivity factor $S = (k_{\text{cat}}/K_M)_R / (k_{\text{cat}}/K_M)_S = 4200$ (Supplementary Table 4).

Analysis of the substrate scope of PT.1 revealed a dramatic change in substrate acceptance (Figure 2d). Whereas the wild-type enzyme prefers primary amines like cyclohexylamine and the (*S*)-configured α -benzyl amines **2** and **3**, PT.1 is almost inactive towards these substrates. Instead, it converts (*R*)-configured α -benzyl amines with sterically demanding minor substituents (e.g. **4** and **8**) and bulky secondary amines like **6** and **7**.

Biocatalytic transformations. Purified PT.1 and wild-type CHAO were compared for their efficacy in producing the optically pure (*S*)-PheTIQ Solifenacin precursor by deracemization of (*rac*)-PheTIQ (Figure 2c). The processes were initiated by adding purified oxidase to a buffered solution of the racemic substrate and 4 eq. of ammonia borane complex as reducing agent. Once the optical purity converged, the product was extracted using organic solvent. At 0.05% catalyst loading, PT.1 achieved a 99% e.e. for the desired (*S*)-enantiomer within 9 h, whereas the wild-type enzyme did not enrich either enantiomer over two days under the same conditions (Figure 2c, Supplementary Figure 6d).

Analogous results were obtained when the reaction with (*rac*)-**7** was carried out with whole cells producing the PT.1 variant. The latter conditions were therefore used in deracemization experiments with the other tetrahydroisoquinoline derivatives shown in Figure 2 (Table 1). Compound **6**, which has an ethyl rather than a phenyl substituent, afforded (*S*)-EthylTIQ with an e.e. of 98% after 36 h. In contrast, little enantiomeric enrichment (e.e. 5 %) was observed for compound **5** with the even smaller methyl substituent, reflecting both its modest steric demands and substantially lower activity with the enzyme.

Attempts to deracemize primary amines like **3**, **4** and **8** were complicated by the competitive hydrolysis of the iminium ion product of the enzymatic reaction before it can be reduced by the ammonium borane, leading to accumulation of

unwanted ketone and/or alcohol side products. Nevertheless, compound **4**, which has a phenyl and a propyl group that are readily distinguishable and sufficiently large to bind productively to the enlarged PT.1 pocket, gave (*S*)-1-phenylbutylamine in 35% yield and 50% e.e. after 48 h reaction. In contrast, no enantiomeric enrichment was observed for **3** or **8**. Although kinetic measurements with the individual enantiomers of **3** indicate an *S* value of 17 (Figure 2d), this compound is a relatively poor substrate for the enzyme, making preparative deracemization impractical over a reasonable time frame. Although **8** is a good substrate for the enzyme, the remodeled PT.1 binding pocket is apparently unable to distinguish between the phenyl and *p*-chlorophenyl substituents.

Molecular modeling. To gain insight into the molecular basis of the switch in substrate specificity, we preformed docking experiments on wild-type CHAO (PDB ID: 4i59) and an in silico model of the optimized PT.1 catalyst with both (*R*)- and (*S*)-PheTIQ. Although the starting enzyme cannot accommodate either substrate enantiomer without substantial steric clashes, the active site substitutions remodeled the binding pocket for shape complementary recognition of the larger PheTIQ substrate. In particular, the Y321S and P422S mutations carved out additional space for the tetrahydroisoquinoline ring, allowing the experimentally preferred (*R*)-PheTIQ to dock in a catalytically competent orientation proximal to the flavin cofactor (Supplementary Figure 7a). Although (*S*)-PheTIQ also fits in the enlarged pocket, it adopts an orientation that precludes hydride transfer to the cofactor because the relevant C-H bond points away. A hydrogen-bonding interaction between the (*S*)-PheTIQ amine and the side chain of Ser422, which was introduced during optimization, and the backbone carbonyl of Thr198 favors this unproductive pose (Supplementary Figure 7b). Mutation of Met226 in the substrate diffusion channel to threonine matches previous reports showing that smaller residues at this site boost activity for a multitude of substrates²⁸, likely improving substrate uptake and/or product release.

Discussion

Enzymes have the potential to play a major role in reducing the environmental impact of chemical manufacture. The dramatically enhanced screening power of droplet microfluidics compared to robotic, microtiter plate-based techniques is facilitating both their discovery and optimization. To date, screening large libraries from bacteria^{23,26,29}, yeast^{13,25} and filamentous fungi²⁴ and large (> 1 million genes) metagenomic libraries comprising environmental DNA from microorganisms³⁰ has yielded active hydrolases^{30,31}, peroxidases¹³, cellulases³², and aldolases²⁹. Screening pairs of enantiomeric substrates with different colored fluorescent leaving groups even enables discovery of enantioselective catalysts³¹. Non-fluorescent screening of enzymatic reactions in droplets using absorbance-activated droplet sorting (AADS) and Raman-activated droplet sorting (RADS) promises to further expand these capabilities^{33,34}.

If biocatalysis is to be competitive with conventional synthesis, it has been suggested that directed evolution efforts to adapt natural and designed enzymes for specific tasks needs to become at least an order of magnitude faster¹⁴. Droplet

microfluidics is poised to make this goal a reality. It has already proven particularly efficacious in cases where directed evolution using microtiter plate-based approaches fail as a result of reaching an apparent local fitness plateau, from which it is only possible to escape by screening a larger number of variants. For instance, directed evolution of an artificial aldolase using droplet microfluidics enabled the best enzyme from a stalled microtiter-plate selection to be improved 30-fold to give a $>10^9$ rate enhancement that rivals the efficiency of class I aldolases²⁹.

Nevertheless, most examples of enzyme evolution using droplet microfluidics to date have focused on improving enzymes that already show important levels of activity over multiple rounds of evolution and use fluorogenic model substrates of little industrial interest. As highlighted here, coupled enzyme assays have the potential to generalize this approach. Implementation of a simple means to link hydrogen peroxide formation with a fluorescent readout enabled time-efficient isolation of active amine oxidases from large libraries (here 1.7×10^6 permutations of 8 residues) by FADS. In a single round of evolution, a nearly three order of magnitude leap in catalytic efficiency and a radically reshaped substrate profile was achieved. This approach outperformed conventional evolutionary optimization of MAO-N for PheTIQ, which entailed separate screening of six different libraries and a second round of mutagenesis to produce a catalyst with a k_{cat}/K_M an order of magnitude lower than PT.1³⁵, underscoring the advantage of simultaneously mutating multiple residues to facilitate the discovery of novel active site configurations.

In conclusion, our work highlights FADS as a powerful and versatile technology for rapidly engineering oxidases to perform new and valuable functions. It enabled engineering of a tailor-made, synthetically useful enzyme for an important chiral API precursor in a timeframe compatible with today's product plant development. FADS has the flexibility of a typical microtiter plate assay, making it easy to adapt established screening approaches. In contrast to *in cellulo* assays²⁰, it is not limited by poor uptake of substrates or product diffusion. Moreover, the coupled assay used here is label free, obviating the need for surrogate substrates through detection of a common by-product, and should be readily transferrable to any other oxidase. These capabilities promise to facilitate rapid tailoring of substrate specificity for a whole class of industrially important enzymes for the production of chiral amines, secondary thiols³⁶, and alcohols³⁷. This approach should be easily extendable to other enzyme families that produce detectable (by-)products during catalytic turnover.

Methods

Materials. Chemicals were purchased from Sigma, ABCR, Acros, Fluorochem, Thermo Fisher Scientific, and Dow Corning. Chemical standards for (*R*)- and (*S*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline (*R*-**7** and *S*-**7**) were bought from Toronto Research Chemicals and TCI Chemicals, respectively. Compounds (*R*)-**7** and (*S*)-**7** were further purified by recrystallizing the HCl salt in ethanol and diethyl ether, filtering, and drying *in vacuo*. Amplex UltraRed was purchased from Thermo Fisher Scientific.

General Methods. NMR spectra were recorded on an AVIII 400 (^1H 400 MHz, ^{13}C 100 MHz) spectrometer. Small molecules were further analyzed by LC-MS (Waters H-class UPLC/SQD-2) using an Acquity UPLC BEH C-18 column (50 x 2.1 mm, 1.7 μM), 1 μL injection, monitoring ESI+, solvent A = H_2O + 0.1% TFA, solvent B = MeCN + 0.1% TFA, flowrate = 1 mL/min, initial conditions = 5% B, 0-1.5 min ramp to 80% B, 1.5-2 min ramp to 100% B, 2-2.2 min = 100% B, 2.2-2.3 min ramp to 5% B, 2.3-3 min re-equilibration = 5% B.

Construction of pKNTET-CHAO_Kan. The plasmid pACYC-6His-CHAO³⁸ was used to amplify the wild-type CHAO gene with primers *chao* fw and *chao* rv (Supplementary Table 5), which introduced 5' NdeI and 3' SpeI restriction sites. The insert was purified by agarose gel electrophoresis. The gene and the plasmid pKNTET-0³⁹ were both digested with NdeI and SpeI-HF (New England Biolabs) overnight at 37 °C and purified by agarose gel electrophoresis. The fragments were ligated using T4 DNA ligase (New England Biolabs) overnight at 16 °C and purified using Clean and Concentrator 5 Kit (Zymo Research). The resulting construct pKNTET-CHAO_Amp was transformed into XL10-Gold (Agilent) by electroporation. pKNTET-6His-CHAO_Amp and pET-29b(+) (Novagen) were digested with BspHI, excising both their antibiotic marker, and the relevant fragments purified using agarose gel electrophoresis. The kanamycin resistance cassette of pET-29b(+) was ligated into the pKNTET-CHAO vector for 1h at room temperature, used to transform XL1-Blue cells which were plated on selective medium. The resulting plasmid pKNTET-6His-CHAO_Kan was verified by sequencing (Microsynth).

Library generation. Cloning primers and primers for cassette mutagenesis were purchased from Microsynth AG. Six pairs of primers were designed to introduce seven DYT and one BYT degenerate codons into the wild-type gene (Supplementary Table 6). Seven fragments were generated in individual PCR reactions, purified by agarose gel electrophoresis, and assembled by overlap extension PCR.⁴⁰ The resulting full-length library amplicon and pKNTET-6His-CHAO_Kan plasmid were digested with NdeI and SpeI-HF endonucleases overnight at 37 °C. The digested gene fragment was purified using Clean and Concentrator 5 Kit, whereas the plasmid was purified by agarose gel electrophoresis and further desalted using Clean and Concentrator 5 Kit (Zymo Research). The fragments were ligated with T4 DNA ligase at 16 °C overnight and desalted prior to electroporation into XL1-Blue cells.

Microfluidic setup. A description of the optical setup used in this study can be found in ref. ²⁹. In brief, the setup consists of a laser combiner (Omicron Laserage GmbH) for illumination with a 375 nm diode laser and a 561 nm SPPS laser. An inverted fluorescence microscope is used to detect fluorescence using photomultipliers (H10722-30, Hamamatsu Photonics) and corresponding single band pass filters, FF01-488/561/635 and FF01-609/57-25 (Semrock) for detection at 448 nm and 609 nm, respectively. A high voltage amplifier (632B, Trek) was used to induce the dielectrophoretic sorting pulses. The PMT's and high voltage controller were connected to a field-programmable gate array (FPGA) card (NI USB-7856R - R Series Multifunction RIO with Kintex-7 160T FPGA, National Instruments) and connected to a computer requiring a run time

engine (National Instruments). The gain of the PMTs and the high voltage amplifier were controlled and the signals recorded using a custom-made LabView software.

Microfluidic chip production. The design of the chips used and production of the photomasks were described previously^{29,41}. A droplet maker with two aqueous inlets and an oil inlet was used to collect droplets off-chip. These emulsions were sorted on a separate device with two inlets for emulsions, two inlets for spacing oil, and two outlets. For on-chip incubation a 15-minute delay line, designed to keep an equal time distribution of the droplets⁴², connected a T-junction droplet maker with three aqueous inlets and one oil inlet with a sorting device. Sylgard 184 base and curing agent (Dow Corning) were mixed in a 10:1 ratio and casted into silicon-SU8 molds (Wunderlichips). A desiccator was used to remove bubbles from the liquid PDMS. The degassed chips were cured at 90 °C for 30 min. The resulting PDMS slab was separated from the mold. Inlets, outlets, and connections for the electrodes were punched using surgical punchers (Shoney Scientific) with a 0.3 mm diameter. The PDMS slabs and glass slides (Corning) were cleaned using water and isopropanol and dried at 90 °C. The PDMS slabs and glass slides were bonded together using a plasma cleaner (PDC-32G, Harrick Plasma) at 0.5 mbar for 30 s and incubated at 90 °C overnight. Chips containing electrodes were placed on a hotplate heated to 90 °C, 51In 32.5Bi 16.5Sn alloy (Indium Corporation of America) was melted into the electrode channels, and copper cables were inserted into the inlets⁴³. The finished chips were treated with trichloro(1H, 1H, 2H, 2H-perfluorooctyl)silane (Sigma-Aldrich) *in vacuo* overnight.

Microfluidic assay. The library was transformed by electroporation into XL1-Blue *E. coli* cells and recovered in 50 mL Super Optimal broth with Catabolite repression (SOC) at 37 °C shaking at 230 rpm for one hour before adding kanamycin to a concentration of 25 µg/mL. After three hours 15 mL culture were used to inoculate a 50 mL LB culture (25 µg/mL kanamycin) which was grown at 37 °C shaking at 230 rpm. After reaching an OD₆₀₀ of 0.3 the temperature was lowered to 20 °C and gene expression induced by supplementing tetracycline to a final concentration of 2 µg/mL. After overnight expression 2 mL of the expression culture were harvested by centrifugation at 3500 xg and 4 °C and were washed three times with 1 mL supplemented M9 medium (47.6 mM Na₂HPO₄, 22 mM KH₂PO₄, 8.5 mM NaCl, 18.6 mM NH₄Cl, 0.1 mM CaCl₂, 1 mM MgSO₄, 0.4% glucose, 5 µg/mL thiamine, 0.08% yeast ForMedium Complete Supplement Mixture (ForMedium), 1x US* trace element mix⁴⁴, pH 7.5). Cells were resuspended, filtered through a 5 µm syringe filter (Millipore), supplemented with 30% Percoll (equilibrated with 10x M9 salts) and 2 U/mL DNase I (New England Biolabs) and diluted to an OD₆₀₀ of 0.04 or 0.06 for off-chip storage or on-chip storage, respectively.

Off-chip storage: The emulsion was generated with a flow focusing nozzle⁴⁵ at a 300 µL/h flow for the oil phase (Novec HFE-7500 fluorinated oil (3M) containing 2% (w/w) 008-FluoroSurfactant from RAN Biotechnologies) and 100 µL/h flow for the two aqueous phases, generating 15 pL droplets at a frequency of 3 kHz. The cell suspension was co-encapsulated with a

solution containing 4 U HRP, 11 μ M Esculin, 2 mg/mL lysozyme, 4 mg/mL polymyxin B, 0.4 wt. % Pluronic F127, 0.4 mM Amplex UltraRed, 10 μ M EGTA, 20 μ M EDTA, 8 mM substrate and 4% DMSO in 50 mM sodium phosphate buffer at pH 7.0. The emulsion, which contains an average λ of 0.3 cells per droplet, was collected in a 1 mL syringe purged with HFE-7500. After an incubation of approximately 2 h at room temperature droplets were reinjected into the sorting device at a flowrate of 40 μ L/h and spaced by injecting Novec HFE 7100 oil (3M) at a flowrate of 650-750 μ L/h. The emulsion was sorted with an electric pulse frequency 15 kHz, at a voltage of 620 kV and pulse length ranging from 0.5 – 0.8 ms. The droplets were sorted at a frequency of ca. 1000 Hz.

On-chip incubation: Droplets were generated at a T-junction⁴⁶ with the oil phase (Novec HFE-7500 fluorinated oil (3M) containing 2% (w/w) 008-FluoroSurfactant from RAN Biotechnologies) flowing at 30 μ L/h and all the aqueous phases at 20 μ L/h. A lysis mixture containing 6 U HRP, 15 μ M Esculin, 3 mg/mL lysozyme, 6 mg/mL polymyxin B, 0.6 wt. % Pluronic F127, 15 μ M EGTA, 30 μ M EDTA, in 50 mM sodium phosphate buffer pH 7.0 was injected alongside the detection and substrate mixture containing 0.6 mM Amplex UltraRed, 3 μ M Esculin, 12 mM substrate, and 3% (v/v) DMSO in 50 mM sodium phosphate buffer at pH 7.0. The emulsion, which contains an average λ of 0.3 cells per droplet, was spaced with Novec HFE-7100 oil (3M) at a flowrate of 650-750 μ L/h. Sorting parameters were identical to the ones described above.

Generally, the droplet size was determined using the fluorescence of Esculin excited at 375 nm and measured at 448 nm. The CHAO activity is proportional to the fluorescence, exciting at 561 nm and detecting at 609 nm, resulting from the HRP catalyzed oxidation of Amplex UltraRed through hydrogen peroxide. Although the total number of active droplets is unknown at the outset of a sorting experiment, the distribution of measured activities usually converges within seconds and changes only slowly over time. Based on the initial distribution, the sorting gate was set to collect the top 0.1% droplets that displayed the highest fluorescence. For off-chip incubations, the active population shifts over time, requiring periodic adjustment of the threshold, whereas incubation times are uniform for on-chip incubation, so readjusting the sorting gate is seldom necessary. While complete coverage of a library is not a necessity for successful directed evolution, the chances of seeing every variant of the 10^6 -library members should be ~99%, according to Bosley and Ostermeier⁴⁷, assuming an occupancy of 22% singly encapsulated cells, an average sorting frequency of 1 kHz, and ~9 h sorting runs. The sorted emulsion was broken by adding 75 μ L 1H,1H,2H,2H-perfluorooctanol and 15 μ L 10 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM NaCl, 1% Triton X-100, 1 mg/mL proteinase K. Following one hour incubation at room temperature, DNA was recovered using the DNA Clean and Concentrator – 5 kit and amplified by PCR using JumpStart Taq DNA Polymerase (Sigma Aldrich) (30 s 94 °C, 34 x (30 s 94 °C, 30 s 55 °C, 2 min 72 °C), 10 min 72 °C, final hold 4 °C) using *chao fwd* and *chao rv* primers. The PCR product was purified by agarose gel electrophoresis and subcloned into pKNTET_Kan vector and used to transform electro competent XL1-Blue cells. After the third round of sorting the extracted plasmids were directly used to transformed electro competent XL1-Blue cells. Single clones from each round were further analyzed in a microtiter plate assay.

Microtiter plate assay¹⁹. After each round of microfluidics sorting the subcloned library or extracted plasmids were transformed by electroporation into XL1-Blue cells and plated on LB Agar containing 25 µg/mL kanamycin. Single colonies from the transformations alongside three wild-type control colonies were used to inoculate pre-cultures on a microtiter plate containing 150 µL LB medium, supplemented with 25 µg/mL kanamycin and the plates were sealed with a gas-permeable membrane (Breathe Easy, Diversified Biotech). After overnight incubation at 30 °C, 700 rpm, and 100% humidity, 30 µL were used to inoculate a deep-well plate containing 1.8 mL LB medium, 25 µg/mL kanamycin per well and plates were sealed with a gas-permeable membrane (Breathe Easier, Diversified Biotech). Cultures were grown at 37 °C and after reaching OD₆₀₀ of 0.3, gene expression was induced by adding Tetracycline to a final concentration of 2 µg/mL. Subsequently, the plates were incubated at 20 °C and 240 rpm overnight. Cells were harvested by centrifugation at 4000 rpm for 20 min at 4 °C, the supernatant discarded, and the pellets stored at -20 °C. Lysis was carried out by four freeze thaw cycles and resuspension of the pellets in 50 mM sodium phosphate, 0.2 mg/mL lysozyme, 1 mg/mL polymyxin B, 10 µg/mL DNAaseI pH 7.0 and 3 h incubation at room temperature. The lysates were cleared by centrifugation at 4000 rpm for 20 min at 4 °C.

In a flat bottom UV-Vis microtiter plate (Nunc MicroWell with Nunclon Delta Surface, Thermo Scientific) 150 µL assay solution containing 3 mM substrate, 5 U/mL HRP, 0.15 mg/mL 4-aminoantipyrine, 1 mM vanillic acid, and 2% DMSO in 50 mM sodium phosphate buffer, pH 7.0 was distributed to each well. The reaction was initiated by adding 50 µL cleared lysate. Activity values were determined by measuring the change of absorption at 498 nm in a microtiter plate reader (Varioskan, Thermo Scientific) and normalizing the slope to the average of three internal wild-type controls was performed in R (version 3.3.2).

Enzyme purification. Purified plasmids of single variants identified on microtiter plates were used to transform BL21 (DE3) pLysS *E. coli* cells (Agilent). Cultures containing 900 mL LB medium supplemented with 25 mg/mL kanamycin and 30 mg/mL chloramphenicol were inoculated from a starter culture and grown at 37 °C and 240 rpm until reaching OD₆₀₀ of 0.3 and induced by addition of 0.5 mM IPTG. The enzyme was produced overnight at 25 °C followed by harvesting the cells by centrifugation at 4500 *xg* for 20 min at 4 °C. After discarding the supernatant, the pellets were frozen at -20 °C. The entire following purification was carried out at 4 °C. Pellets were resuspended in 5 mL 50 mM sodium phosphate buffer (pH 7.0) containing 0.2 mg/mL lysozyme, 1 mg/mL polymyxin B, 2 mM β-mercaptoethanol and incubated for 3 h. The suspension subsequently sonicated in the presence of DNase I (NEB). The soluble fraction was recovered by centrifugation at 14000 *xg* for 30 min and CHAO purified by Ni-NTA (Qiagen) affinity chromatography, washing with 10 column volumes of 25 mM imidazole in 50 mM sodium phosphate buffer (pH 7.0), repeated washing with the same amount of 50 mM imidazole 50 mM sodium phosphate buffer pH 7.0 and finally eluting with 250 mM imidazole in 50 mM sodium phosphate buffer (pH 7.0). Samples were analyzed by 20% SDS-PAGE (GE Healthcare). Fractions containing CHAO were pooled and dialyzed two times against 2 L 50 mM sodium phosphate (pH 7.0) 2 mM DTT and concentrated using 30 kDa MWCO centrifugation filters

(Amicon, Millipore), spinning at 4000 xg and 4 °C. Concentration was determined by absorption of FAD at 450 nm using a extinction coefficient⁴⁸ of 11,300 M⁻¹ cm⁻¹ on a NanoDrop 2000 (Thermo Fisher). Typical yields were 10-20 mg/L culture.

Enzyme kinetics. All kinetic characterizations were performed with three batches of independently produced enzyme. Purified variants were characterized by monitoring the coupled reaction between hydrogen peroxide, 4-aminoantipyrine, and vanillic acid, catalyzed by HRP, at 498 nm ($\epsilon = 6234 \text{ M}^{-1}\text{cm}^{-1}$)¹⁹. Reactions contained 0.23 mg/mL 4-aminoantipyrine, 1 mM vanillic acid, 5 U HRP, and 2% DMSO in 50 mM sodium phosphate buffer (pH 7.0). All reactions were carried out at 30 °C and were initiated by adding enzyme to a final concentration of either 1 μM or 10 nM for wild-type and PT.1, respectively. For (*R*)-1-phenyl-1,2,3,4-tetrahydroisoquinoline a full Michaelis-Menten kinetic was recorded, whereas for other enzyme-substrate combinations $k_{\text{cat}}/K_{\text{M}}$ was determined from a single point measurement at the lowest substrate concentration where the reaction could still be observed. Kinetic parameters were determined using Prism 8 (GraphPad Software, Inc.) (Supplementary Figure 5).

Substrate scope. The substrate scope was determined in measurements analogous to the kinetic characterizations. Three individually purified enzyme batches of each variant were characterized by monitoring the coupled reaction between hydrogen peroxide, 4-aminoantipyrine, and vanillic acid, catalyzed by HRP at 498 nm ($\epsilon = 6234 \text{ M}^{-1}\text{cm}^{-1}$)¹⁹. Reactions contained 2 mM substrate, 0.23 mg/mL 4-aminoantipyrine, 1 mM vanillic acid, 5 U HRP, and 2% DMSO in 50 mM sodium phosphate buffer (pH 7.0). All reactions were carried out at 30 °C and were initiated by addition of the enzyme. The enzyme concentration was varied in the nM to μM range in order for v_0 to be linear within the first 10 % of substrate consumption.

Chiral analysis. Enantiomeric excess was determined via HPLC on a chiral stationary phase using a Waters 717plus Autosampler (Waters Corporation) equipped with two Waters 515 HPLC Pumps (Waters Corporation) and a Waters 996 Photodiode Array Detector (Waters Corporation).

Deracemization of 1-Phenyl-1,2,3,4-tetrahydroisoquinoline. The deracemization procedure was adapted from Ghislieri *et al.*¹². All deracemizations were carried out with 10 mM 1-phenyl-1,2,3,4-tetrahydroisoquinoline (**7**, 54 mg), 5 μM purified CHAO wild-type or PT.1, 40 mM borane-ammonia complex in 25 mL 1M sodium phosphate buffer at pH 7.0, and catalytic amounts of catalase (Sigma Aldrich). The reaction was initiated by adding the oxidase and incubated at 30 °C. HPLC samples were prepared by adding 20 μL 10 M NaOH to 250 μL sample and extracting the quenched reaction with 1 mL tert-butyl methyl ether (TBME). The organic phase was dried over a MgSO_4 plug and filtered through a 0.2 μm AcroPrep 96 filter plate (Pall) and separated on a Chiralcel OD-H (Chiral Technologies Europe) column (150 x 4.6 mm, particles diameter = 5 μM) at room temperature, eluting isocratically with 70:30 n-hexane:isopropanol at a flow rate of 1 mL/min (Supplementary Figure

6d). The reaction was worked up by adding 200 μ L 10 M NaOH and extracting with 50 mL TBME. The organic phase was dried over MgSO_4 and the solvent removed *in vacuo* to yield (S)-**7** as a white solid (38 mg, 71% yield, 99% e.e.). HRMS: Calculated $[\text{M}+\text{H}]^+$ 210.1277, found 210.1275. ^1H NMR, 400 MHz, CDCl_3 δ ppm: 7.4 – 7.25 (m, 5H), 7.21–7.14 (m, 2H), 7.10 – 7.03 (m, 1H), 6.78 (d, J = 7.76 Hz, 1H), 5.14 (s, 1H), 3.30 (m, 7.1 Hz, 1H), 3.18 – 3.01 (m, 2H), 2.92 – 2.80 (m, 1H), 1.82 (s, 1H). ^{13}C NMR, 100 MHz, CDCl_3 δ ppm: 144.7, 138.15, 135.39, 129.0, 128.9, 128.4, 128.1, 127.4, 126.3, 125.7, 62.1, 42.2, 29.7.

Preparative deracemization of compounds 3-6 & 8. The deracemization reactions were set up as whole cell processes. The respective amine (15 mM), and 60 mM borane-ammonia complex were dissolved in 25 mL 1 M sodium phosphate buffer at pH 7.0. The reaction was initiated by addition of 2 g of a wet cell pellet of BL21 (DE3) pLysS *E. coli* cells expressing PT.1 CHAO. Aliquots were periodically analyzed by quenching 250 μ L of the reaction mixture with 20 μ L 10 M NaOH, followed by extraction of the amine with 1 mL TBME. The organic phase was dried over MgSO_4 and filtered through a 0.2 μ m AcroPrep 96 filter plate (Pall) and analyzed by HPLC on a chiral stationary phase. When the e.e. of the product converged, the reaction was quenched by raising the pH to 12 with 10 M NaOH and extracted three times with 25 mL TBME. The organic phase was combined, dried over MgSO_4 , and solvent removed *in vacuo*.

Compound **3** was analyzed on a Chiralcel OD-H (Chiral Technologies Europe) column (150 x 4.6 mm, particles diameter = 5 μ m) eluted with a mixture of n-hexane and ethanol (95:5) containing 0.05% diethylamine at 1 mL/min at room temperature. No enantiomeric enrichment was detected over 48 h. The product was not further purified or analyzed.

Deracemization of **4** yielded 19 mg of a colorless oil (35% yield, e.e. 50%). The product enantiomers were separated on a Chiralcel OD-H (Chiral Technologies Europe) column (150 x 4.6 mm, particles diameter = 5 μ m) eluted with a mobile phase of n-hexane and ethanol (95:5) containing 0.05% diethylamine at 1 mL/min at room. The reaction was quenched by raising the pH to 12 with 10 M NaOH when the e.e. converged. After extraction with 3x25 mL TBME, the organic phase was combined, 75 mL water added, and the pH lowered to 1 by addition of 1 M HCl. The phases were separated and the aqueous phase was again titrated to pH 12 with 10 M NaOH and extracted with 75 mL TBME, the organic phase was collected, dried over MgSO_4 and removed *in vacuo* and the product analyzed again by HPLC on a chiral stationary phase. The retention times were assigned according to the literature⁴⁹. HRMS: Calculated $[\text{M}+\text{H}]^+$ 148.1121, found 148.1122. ^1H NMR, 400 MHz, CDCl_3 δ ppm: 7.46 – 7.20 (m, 5H), 3.94 (td, J = 7.1, 2.1 Hz, 1H), 1.79 – 1.65 (m, 2H), 1.42 – 1.19 (m, 2H), 0.92 (t, J = 7.3 Hz, 3H); ^{13}C NMR, 100 MHz, CDCl_3 δ ppm: 128.62, 128.50, 127.16, 127.10, 126.46, 126.19, 56.06, 41.20, 19.66, 13.97

Deracemization of **5** yielded 41 mg yellow oil (75% yield, e.e. 5%). An authentic standard of the racemic compound and the isolated product was separated using a Chiralpak AD-H column (4.6 mm x 250 mm, Daicel) with a mixture of n-hexane and ethanol (98:2) containing 0.05% diethylamine at 1 mL/min at room temperature (Supplementary Figure 6b). The e.e. was determined to be 5% (S). The enantiomers assigned by comparing to literature values of the retention time³⁵. HRMS:

Calculated $[M+H]^+$ 148.1121, found 148.1120. ^1H NMR, 400 MHz, CDCl_3 δ ppm: 7.34 – 7.07 (m, 4H), 4.64 (d, J = 7.3 Hz, 1H), 3.65 – 2.97 (m, 4H), 1.87 (d, J = 6.8 Hz, 3H); ^{13}C NMR, 100 MHz, CDCl_3 δ ppm: 133.11, 131.15, 129.16, 127.93, 127.32, 126.05, 51.04, 39.12, 25.71, 20.17

Deracemization of **6** yielded 49 mg product as a yellow oil (81% yield, e.e. 98%). The racemic standard and the reaction product were analyzed on a Chiralcel OD-H (Chiral Technologies Europe) column (150 x 4.6 mm, particles diameter = 5 μM) at room temperature using n-hexane and ethanol (99:1) containing 0.05% diethylamine as an eluent at a flow rate of 1 mL/min (Supplementary Figure 6c). The enantiomers were assigned by comparison to literature retention times³⁵. The e.e. was determined to be 98% (*S*)-**6**. HRMS: Calculated $[M+H]^+$ 162.1277, found 162.1281. ^1H NMR, 400 MHz, CDCl_3 δ ppm: 7.33 – 7.13 (m, 4H), 4.50 (t, J = 5.6 Hz, 1H), 3.67 (d, J = 11.9 Hz, 1H), 3.42 – 3.25 (m, 2H), 3.17 – 3.03 (m, 1H), 2.26 – 2.17 (m, 2H), 1.24 (t, 3H); ^{13}C NMR, 100 MHz, CDCl_3 δ ppm: 131.99, 131.66, 129.21, 127.86, 127.14, 126.40, 56.49, 39.92, 27.37, 25.76, 10.19

Compound **8** was analyzed on a Chiralpak AD-H column (4.6 mm x 250 mm, Daicel) eluted a mixture of n-hexane and ethanol (95:5) containing 0.05% diethylamine at 1 mL/min at room temperature. No enantiomeric enrichment was detected over 48 h. The product was not further purified or analyzed.

Modeling. The crystal structure of cyclohexylamine oxidase from *Brevibacterium oxydans* IH-35A (PDB ID: 4i59) was used as reference structure for the docking studies. The five mutations in PT.1 (L199V, M226T, Y321S, L353I and P422S) were manually introduced using the ProteinBuilder module in MOE2016.08 (Molecular Operating Environment, Chemical Computing Group). To prepare the structure for docking, the following steps were performed with the MOE2016.08 StructurePreparation module: the structure was protonated at pH 7.4, structural issues were addressed (adding hydrogens, correct partial charges and hybridization). The resulting structure as well as all ligand complexes were minimized using the AMBER10:EHT (Extended Hückel Theory) forcefield (termination: root mean square gradient < 0.1 kcal mol⁻¹ Å⁻²).

Ligand docking. Potential binding sites were calculated by Site Finder in MOE2016.08. The active site identified next to the co-crystallized ligand was selected as the binding pocket for docking. Docking was executed in MOE2016.08 using the placement method Triangle Matcher and London dG as scoring method. 1000 poses were returned and 100 poses were passed to the refinement step. “Induced Fit” was chosen as the post-placement refinement method with default parameters using GBVI/WSA dG as final scoring method. 10 poses were finally retained. To confirm the identified poses, the docking was repeated with GOLD 5.5 (CCDC Software) as integrated in MOE2016.08. This re-docking was performed with default parameters (efficiency = 100) using the ChemPLP scoring function and “Induced Fit” as refinement method with GBVI/WSA dG as final scoring function. 100 poses were passed to the refinement step, and 10 poses were saved.

Code availability. No custom code was used to generate data presented in this study.

Data availability. The data that support the plots and other findings of this study are available from the corresponding author upon reasonable request.

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Figure 1. Detection strategy and fluorescence-activated droplet sorting (FADS) sorting of CHAO. **a**, CHAO converts amines to the corresponding imine, reducing one equivalent of flavin adenine dinucleotide (FAD). Oxygen-dependent cofactor recycling produces equimolar amounts of hydrogen peroxide, which is detected by downstream oxidation of a fluorogenic substrate by horseradish peroxidase (HRP). **b**, Surfactant stabilized droplets act as a diffusion barrier for catalyst-encoding DNA and the fluorescent reporter generated in the coupled enzymatic reactions catalyzed by CHAO and HRP. **c**, Schematic representation of a typical microfluidic chip used in this study²⁹. Single cells are co-encapsulated with lysis agents, substrate, and the detection cascade in droplets dispersed in fluorinated oil. The cells are lysed in the droplets, releasing the enzyme, and, after incubation, droplet fluorescence is analyzed. Droplets with fluorescence exceeding a defined threshold are sorted via dielectrophoresis and collected. The DNA of active clones is pooled, recovered by PCR, and subcloned for the next round of sorting and analysis.

Figure 2. Characterization of the engineered PT.1 variant and wild-type CHAO. **a**, Active site model of PT.1 with the FAD cofactor in green, (R)-PheTIQ in yellow, and the five mutated amino acids in orange. **b**, The catalytic efficiency of PT.1 towards (R)-PheTIQ is improved by almost three orders of magnitude compared to the wild-type enzyme. Furthermore, PT.1 displays an impressive specificity constant (S) of 4200. **c**, The isolated variant, PT.1, was used to deracemize PheTIQ in combination with ammonia borane. Deracemizations mediated by PT.1 resulted in accumulation of (S)-PheTIQ (>99% e.e.) over the course of several hours, whereas analogous reactions with wild-type CHAO gave no enrichment in optical purity. **d**, Comparison of the substrate scope of PT.1 and wild-type CHAO. Initial reaction velocities with 2 mM of substrates **1-8** and nM to μ M enzyme concentrations were determined by monitoring the coupled reaction between the hydrogen peroxide byproduct, 4-aminoantipyrine, and vanillic acid catalyzed by HRP at 498 nm. The evolved variant preferentially oxidizes bulky amines **6-8**, while wild-type CHAO prefers smaller primary amines such as the native substrate **1** and α -benzyl amines **2** and **3**. Interestingly, with larger α -substituents as in **3** and **4**, PT.1 exhibits switched stereospecificity compared to wild-type. All error bars indicate the s.d. of three measurements using independent batches of purified enzyme; N.A. stands for not active.

552 **Table 1. Preparative deracemization reactions^a**
 553

entry	compound	% e.e.	% yield	Time (h)
1	3	0	n.d. [†]	48
2	4	50 (S)	35	48
3	5	5 (S)	75	36
4	6	98 (S)	81	36
5	7	99 (S)	71	9
6	8	0	n.d.	48

554 ^a For compound **7**, deracemization was carried out with 5 μM
 555 purified enzyme and 4 eq. NH₃BH₃ in 1M sodium phosphate buffer at
 556 pH 7.0 at 30 °C. For the other substrates, the reactions were
 557 performed with whole cells producing PT.1 (see Methods). The e.e.
 558 values were determined by HPLC with a chiral stationary phase.

559 [†] n.d. denominates not determined