Supplementary Material (ESI) for Chemical Communications

Directed Evolution and Axial Chirality: Optimization of the Enantioselectivity of *Pseudomonas aeruginosa* Lipase towards the Kinetic Resolution of a Racemic Allene

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Bacterial Strains and Growth Conditions

Plasmids were transformed into *Pseudomonas aeruginosa* PABST7.1 competent cells as previously described.[i] Single colonies were picked from Luria-Bertani (LB) agar plates (supplemented with the appropriate amount of antibiotics: 100 μg/mL and tetracycline 50 μg/mL) and used to inoculate 15 mL of LB 2x liquid media in a 100 mL Erlenmeyer flask as preculture. The preculture was grown in an orbital shaker at 30ºC and 300 rpm overnight.

1 mL of this preculture was used to inoculate a new erlenmeyer flask with 15 mL of LB 2x media. After 5h at 30ºC and 300 rpm, lipase expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.1 mM, being incubated for 5h more. The lipase-containing supernatants were recovered by centrifuging at 8000 rpm (= 8228 x g) for 45 min and stored overnight at 4ºC. (An Eppendorf Centrifuge 5804 provided with a 6x85 mL High Speed Fixed Angle Rotor, radius 11.5 cm was used).

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Reaction Conditions

100 μL of culture supernatant, 1 mL of 100mM Tris/HCl buffer pH 7.5 and 25 μL of substrate solution 10 mg/mL in acetonitrile were used as reaction mixture. The reactions were developed until a conversion between 15-45% had been reached. The reactions with commercial enzymes were performed in similar conditions but using 100 μL of enzyme solution 5 mg/mL in buffer 100mM Tris/HCl buffer pH 7.5.

Extraction: 400 μL of DCM were added to the reaction mixture and vortex for 30 s. Then 20 μL of HCl 10% were added and stirred for additional 30 s. The solvent was transferred to a 96-glass plate for analysis by GC.

Analytical Conditions

The chiral analyses of the hydrolysis of the *p*-nitrophenyl 4-cyclohexyl-2-methylbuta-2,3-dienoate (**1**) used as substrate, were performed by GC using an IVADEX-1 chiral column (IVA analysentechnik, Meerbusch, Germany). 25m, 0,15 μm, 0,25 mm. 160ºC (20min); 20ºC/min to 200ºC for 50 min (total time 72 min). Carrier: Nitrogen. Press: 1.2 bar; Flow: 1.3 mL/min; 39 cm/s. Retention times: (+)-4-cyclohexyl-2-methylbuta-2,3-dienoic acid: 13.8 min, (-)-4-cyclohexyl-2-methylbuta-2,3-dienoic acid: 16.1 min, *p*-nitrophenol: 23.8 min, *p*-nitrophenyl 4-cyclohexyl-2-methylbuta-2,3-dienoate (**1**): 67 min. HPLC: Column 150 mm Chiralpak AD-RH, 4,6 mm i.D. Acetonitrile/0,1% TFA 35:65 after 40 min Gradient to 90:10 in 5 min. Retention times: (+)-4-cyclohexyl-2-methylbuta-2,3-dienoic acid: 30.0 min; (-)-4-cyclohexyl-2-methylbuta-2,3-dienoic acid: 38.1 min; *p*-nitrophenol: 9.6 min; *p*-nitrophenyl 4-cyclohexyl-2-methylbuta-2,3-dienoate (**1**): 51.8 min. The enantiomers elute in the same order as by GC.

Other lipases tested

Bacillus subtilis lipase A (expressed in *E.coli* BL21(DE3) cells using the pET22 vector as previously described,[ii] *Candida antarctica* lipase A (Roche), *Candida antarctica* lipase B (Roche), *Candida lipolytica* (Fluka), *Candida rugosa* (Sigma), Hog Pancreas Lipase (Fluka),

Penicillium roqueforti (Fluka), Porcine Pancreas (Fluka), *Pseudomonas cepacia* (Roche), *Pseudomonas sp* (Roche), *Rhizomucor miehei* (Roche), Wheat germ lipase (Fluka).

The mutant Leu162Phe was superior to all other lipases tested for the kinetic resolution of **1**. The following observations for other lipases are interesting to note: (i) *Bacillus subtilis* lipase A wild-type reached an E value of 33 (ee = 89% (+) at 42% conversion), (ii) *Candida antarctica* lipase A gave an E value of 7.5 (ee = 62% (+) at 48 % conversion), and (iii) *Candida rugosa* lipase was the only commercial enzyme tested that showed reverse enantioselectivity in the kinetic resolution of **1** (ee =18% (–) at 43% conversion, E = 1.6).

CAST libraries

CAST Combinatorial Active Site Saturation Test is a directed evolution strategy based on the systematic creation of focused saturation mutagenesis libraries around the substrate binding site, following specific criteria to select the amino acid residues involved.[iii]

During the development of CASTing using *Pseudomonas aeruginosa* lipase (PAL), five different saturation mutagenesis libraries (NNK degeneracy)[iv] were selected according to the combinatorial active site saturation test (CAST) criteria (Fig. 1). These libraries were tested towards a collection of bulky *p*-nitrophenyl esters with the objective to expand the substrate scope, and in parallel to enhance the enantioselectivity in the kinetic resolution of *p*-nitrophenyl 2-methyldecanoate. The wild-type enzyme gives a very low enantioselectivity (E = 1.1 (*S*)) with the latter model substrate. From the enantioselectivity point of view enzyme variants from libraries A, C and E (the only library where variants with reverse enantioselectivity were found) presented some interesting results but the mutants showing a real improvement were all from library D.

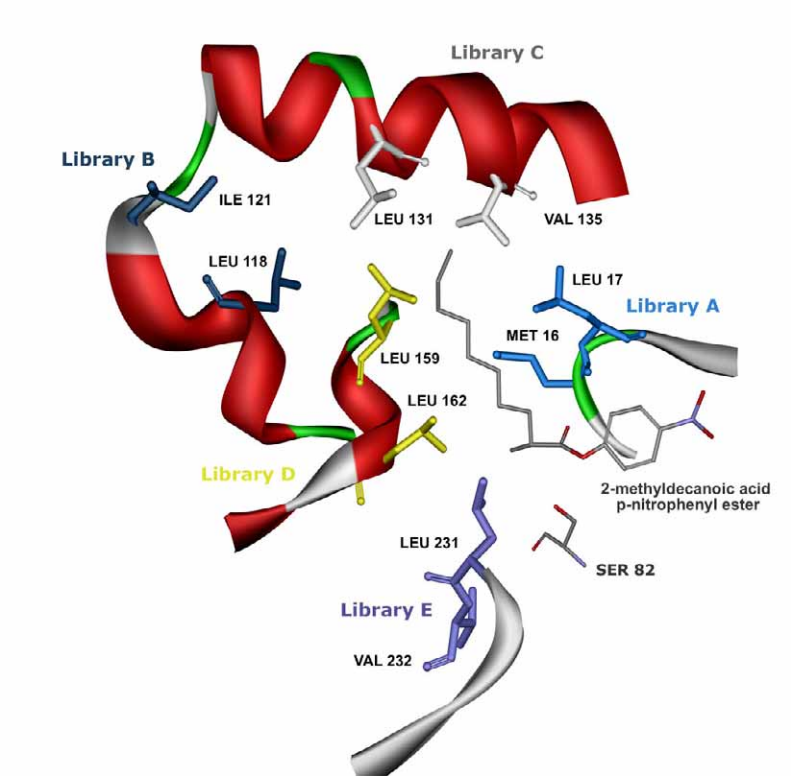

Figure 1. CAST libraries

Synthesis of 4-nitrophenyl 4-cyclohexyl-2-methyl-2,3-butadienoate (1). This substrate was prepared from the allenic acid using an esterification procedure described in ref v. The allenic acid was obtained according to ref vi.

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[ii] S.A. Funke, A. Eipper, M.T. Reetz, N. Otte, W. Thiel, G. Van Pouderoyen, B.W. Dijkstra, K.-E. Jaeger, T. Eggert. Directed Evolution of an enantioselective *Bacillus subtilis* Lipase. *Biocatalysis and Biotransformation* **2003**, *21*, 67-73.

[iii] (a) M. T. Reetz, M. Bocola, J. D. Carballeira, D. Zha and A. Vogel. *Angew. Chem. Int. Ed.* **2005**, *44*, 27, 4192 – 4196. (b) M. T. Reetz, J. D. Carballeira, J. Peyralans, H. Hoebenreich, A. Maichele and A. Vogel, *Chem. Eur. J.* **2006**, *12*, 6031 – 6038.

[iv] The cost of the mutagenic primers can be reduced by means of the DNA degenerate code from the International Union of Biochemistry (IUB) (K = G/T, M = A/C, N = A/C/G/T, R = A/G, S = G/C, W = A/T, Y = C/T). Therefore, apart from the standard saturation mutagenesis approaches based on the use of NNK as degeneracy, the degree of saturation can be adjusted to reduce the screening effort. CASTER a small informatic tool developed in our group to help in the design of such libraries. This tool is available at http://www.mpi-muelheim.mpg.de/reetz.html.

[v] S. Kim, J. I. Lee, Y. C. Kim, *J. Org. Chem*., **1985**, *50*, 560-565.

[vi] J. A. Marshall, M. A. Wolf, E. M. Wallace, *J. Org. Chem*., **1997**, *62*, 367-371