Supporting Information

Directed Evolution of an Enantioselective Lipase with Broad Substrate Scope for Hydrolysis of α-Substituted Esters

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I. General

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. 2-Phenylpentanoic acid was synthesized according to literature procedure.[1] ^{1}H and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz respectively. Chemical shifts (δ) are reported in ppm, using the residual solvent peak in $CDCl_3$ (δ_H 7.26 and δ_C 77.0) as internal standard. GC analyses were performed using an IVADEX-1 chiral column.

II. Growth media recipes

LB medium (10 mg/mL tryptone, 5 mg/mL yeast extract, 5 mg/mL NaCl, pH 7.0) was used for bacterial cultivation. YPD medium (10 mg/mL yeast extract, 20 mg/mL peptone, 20 mg/mL dextrose) was used for cultivation and expression in *P. pastoris*. YPDS (10 mg/mL yeast extract, 20 mg/mL peptone, 20 mg/mL dextrose, 1 M sorbitol) was used for cultivation of recently transformed *P. pastoris*.

III. Mutagenic primers

The following primers were used for the mutagenesis (5′-3′):

Library FG (F233NDT/G237NDT)
LibFG_fw GACCCTTCGCCGGCNDTGCCCTGGCGNDTGTTTCGGGTC
LibFG_rv GAGAGACCCGAAACAHNCGCCAGGGCAHNGCCGGCGAAG

Lib FI (F149NDT/I150NDT)
FI2_fw GGCTTCAAAGCCGCCNDTNDTGCTGGCTACGAAG
FI2_rv CTCTTCGTAGCCAGCAHNAHNGGCGGCTTTGAAG

IV. Preparation of *p*-nitrophenyl esters 1-3, 5-7.

The corresponding acids of esters **1-3** and **5-7** was purchased from commercial sources. Acid (6.42 mmol), DMAP (78 mg, 0.64 mmol) and Et_3N (0.94 mL, 6.74 mmol) were dissolved in dry DCM (10 mL) under argon and stirred at 0 ºC for 15 min. *p*-Nitrophenyl chloroformate

(1.28 g, 6.36 mmol) dissolved in DCM (2 mL) was added to the reaction mixture that was then stirred at 0 ºC for 2 h. The reaction mixture was diluted with DCM and extracted with HCl (0.1 M), $NaHCO_3$ (1 M) and finally with brine. The organic phase was dried over $MgSO_4$ and concentrated. Purification by flash column chromatography (Pentane/EtOAc) gave esters **1-3**, **5-7,** which were characterized by ^{1}H NMR spectroscopy, in yields between 49-92%.

V. Preparation of *p*-nitrophenyl 2-phenylpentanoate (4).

2-Phenylpentanoic acid was prepared by alkylation of the α-position of ethyl 2-phenylacetate and subsequent hydrolysis to the acid, according to literature procedure.[1] The *p*-nitrophenyl ester **4** was then prepared from the acid in 78 % yield as described above. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.00 (t, *J* = 7.4 Hz, 3H), 1.33-1.50 (m, 2H), 1.85-1.97 (m, 1H), 2.15-2.26 (m, 1H), 3.84 (t, *J* = 7.8 Hz, 1H), 7.17-7.23 (m, 2H), 7.31-7.44 (m, 5H), 8.25 (m, 2H) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): δ = 13.8, 20.7, 35.4, 51.5, 122.4, 125.1, 127.7, 127.9, 128.9, 138.0, 145.3, 155.6, 171.8 ppm.

VI. Preparation of ethyl 2-phenylpropanate (8).

2-Phenylpropionic acid (0.50 g, 3.33 mmol) was dissolved in EtOH under argon in flame-dried glassware and was cooled to 0 ºC. Thionyl chloride (0.73 mL, 10.0 mmol) was added dropwise, and the reaction mixture was refluxed at 95 ºC for 3 h. The solution was allowed to cool to ambient temperature and the remaining thionyl chloride was quenched by addition of saturated aqueous $NaHCO_3$. The resulting solution was extracted twice with EtOAc. The combined organic phases were washed with brine, dried over $MgSO_4$ and concentrated. Purification by flash column chromatography (Pentane/EtOAc) afforded 516 mg of the product (87% yield).

VII. Preparation of nonyl 2-phenylpropanate (9).

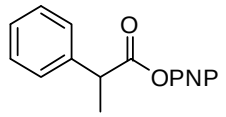
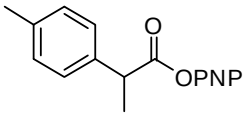
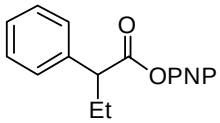
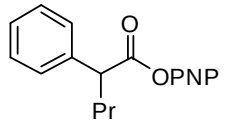
2-Phenylpropionic acid (100 mg, 0.66 mmol), 1-nonanol (0.46 mL, 2.66 mmol) and DMAP

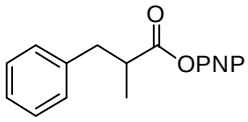
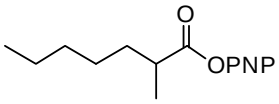
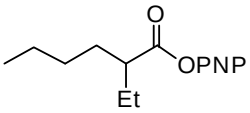
(6.7 mg, 0.066 mmol) were dissolved in DCM (5 mL) and stirred at 0 ºC for 30 min. DCC (150 mg, 0.73 mmol) was added while still at 0 ºC, thereafter the reaction mixture was allowed to reach room temperature, stirred over night and then concentrated. The crude product was purified by flash column chromatography (Pentane/EtOAc 9.6:0.4) to give 122 mg of the product (67% yield).

VIII. Preparation of phenyl 2-phenylpropanate (10).

2-Phenylpropionic acid (200 mg, 1.32 mmol), phenol (493 mg, 5.24 mmol) and DMAP (13.4 mg, 0.13 mmol) were dissolved in DCM (10 mL) and stirred at 0 ºC for 30 min. DCC (300 mg, 1.46 mmol) was added while still at 0 ºC, thereafter the reaction mixture was allowed to reach room temperature, stirred over night and then concentrated. The crude product was purified by flash column chromatography (Pentane/EtOAc 9:1) to give 174 mg of the product (59% yield).

IX. Experimental data for *E* value determinations

Substrate	Enzyme	Time (min)	Conv. (%)	ee_p (%)	*E*	Mean *E*
 **1**	wt	120	32	85.0	18	20 ± 2 (*S*)
		120	31	86.9	20	
		120	47	82.9	23	
		120	42	84.0	21	
	F233G	2	22	98.8	218	259 ± 40 (*R*)
		2	17	99.2	303	
		4	28	98.8	235	
		4	31	98.9	280	
	YNG	3	31	98.9	280	276 ± 24 (*R*)
		3	28	98.8	241	
		4	33	98.9	294	
		4	31	98.9	288	
 **2**	wt	240	22	57.3	4	4 ± 1 (*S*)
		240	24	54.7	4	
		240	25	55.7	4	
		240	21	54.7	4	
	F233G	2	30	94.8	56	32 ± 16 (*R*)
		2	28	90.5	28	
		3	29	87.8	22	
		3	28	87.4	21	
	YNG	4	38	94.3	61	63 ± 8 (*R*)
		4	33	94.1	52	
		6	40	94.5	68	
		6	39	94.8	70	
 **3**	wt	240	7	30	2	2 ± 1 (*R*)
		240	14	3	1	
	F233G	0.5	21	97.0	84	57 ± 20 (*R*)
		0.5	15	94.7	43	
		0.5	21	94.1	42	
		0.5	21	95.8	60	
	YNG	1	11	96.6	64	79 ± 14 (*R*)
		2	19	97.2	88	
		2	20	97.1	86	
 **4**	wt	270	11	89.2	20	18 ± 2 (*R*)
		270	11	88.1	18	
		270	10	87.6	17	
		270	12	87.5	17	
	F233G	2	25	98.0	133	88 ± 46 (*R*)
		2	27	97.6	116	
		2	16	97.4	91	
		4	37	95.2	71	
	YNG	4	12	98.1	119	109 ± 13 (*R*)
		6	15	97.9	112	
		6	15	97.5	95	

Substrate	Enzyme	Time (min)	Conv. (%)	ee_p (%)	*E*	Mean *E*
 **5**	wt	240	8	84.1	12	10 ± 3 (*S*)
		240	6	76.4	8	
	F233G	4	5	53.0	3	3 ± 1 (*R*)
		4	7	37.3	2	
		6	7	43.3	3	
		6	7	45.0	3	
	YNG	15	27	97.0	93	84 ± 11 (*R*)
		15	25	96.9	87	
		15	30	96.6	87	
		15	25	96.1	69	
 **6**	wt	3	15	80.7	11	11 ± 1 (*S*)
		3	13	80.4	10	
		5	26	80.9	12	
	F233G	2	27	83.9	15	17 ± 3 (*R*)
		2	24	87.1	19	
		3	32	85.7	19	
		3	27	83.4	15	
	YNG	2	12	96.6	66	104 ± 36 (*R*)
		4	39	97.3	138	
		4	41	96.3	107	
 **7**	wt	120	27	87.8	21	19 ± 3 (*S*)
		120	20	86.9	18	
	F233G	3	9	96.2	56	54 ± 7 (*R*)
		3	7	95.5	47	
		4	12	96.3	60	
	YNG	3	6	95.4	45	45 ± 5 (*R*)
		3	5	95.5	46	
		4	7	95.7	50	
		4	7	94.8	40	

X. Sequencing of DNA from interesting enzyme variants

Interesting enzyme variants from the screening were sequenced. Pelleted cells from master plates were used to inoculate YPD containing zeocin (100 μg/mL) and carbenicillin (100 μg/mL), and the cultures were shaken at 30 ºC for 48 h. The plasmid was then extracted using a yeast plasmid kit, and subsequently transformed into *E. coli* DH5α in order to obtain a higher plasmid yield. The bacterial cells were cultivated and the plasmid preparations were produced and sequenced using appropriate primers.

XI. Preparation of active site pictures of the CalA variants

Models of the enzyme variants with bound (*R*)-*p*-nitrophenyl 2-phenylpropanoate were based on the crystal structure (PDB ID: 2VEO).[2] The model was allowed to equilibrate for 1 ns by a molecular dynamics simulation using the MAB force field[3] implemented in the Moloc

computational package. An appropriate frame from the simulation was used for generating the pictures.[4]

XII. References

[1] Terao, Y.; Miyamoto, K.; Ohta, H. *Chem Commun.* **2006**, *34*, 3600-3602.

[2] Ericsson, D. J.; Kasrayan, A.; Johansson, P.; Bergfors, T.; Sandström, A. G.; Bäckvall, J.-E.; Mowbray, S. L. *J. Mol. Biol.* **2008**, *376*, 109-119.

[3] Gerber, P. R. *J. Comput.-Aided Mol. Des.* **1998**, *12*, 37-51.

[4] Pictures were generated using the PyMOL software. (DeLano WL (**2002**) The PyMOL molecular graphics system http://www.pymol.org.