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

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Abstract: A variant of *Candida antarctica* lipase A (CalA) was developed for the hydrolysis of α-substituted *p*-nitrophenyl esters by directed evolution. The *E* values of this variant for 7 different esters was 45−276, which is a large improvement compared to 2−20 for the wild type. The broad substrate scope of this enzyme variant is of synthetic use, and hydrolysis of the tested substrates proceeded with an enantiomeric excess between 95−99%. A 30-fold increase in activity was also observed for most substrates. The developed enzyme variant shows (*R*)-selectivity, which is reversed compared to the wild type that is (*S*)-selective for most substrates.

Introduction

2-Arylpropionic acids are an important class of pharmaceuticals that belong to the so-called "profens", a group of nonsteroidal anti-inflammatory drugs (NSAIDs).[1] These drugs are used to treat pain, fever, inflammation, and stiffness, and examples of substances from this class are Naproxen, Ibuprofen, and Flurbiprofen.[2] Methods for the preparation of enantiomerically pure "profens" are highly desirable since FDA requires that most chiral drugs are marketed as single enantiomers today.[3]

Enzymes are nature's catalysts working with great efficiency and selectivity. Unfortunately, the substrate scope of enzymes is usually quite narrow. However, by applying the tools of protein engineering, enzymes can be modified to accept unnatural substrates and to evolve enantioselectivity. Directed evolution is a strategy which mimics nature's evolution in the laboratory.[4] This is achieved by performing iterative rounds of (i) generation of a mutated gene library, (ii) expression of the library of mutated proteins, and (iii) screening of the mutated proteins for the desired property. The best protein variant found in the first round is used as a template in the next round of mutagenesis. Directed evolution has successfully been used to improve specific qualities of enzymes.[5−7]

We have previously demonstrated the advantage of using the episomally replicating pBGP1[8] vector in *Pichia pastoris* for heterologous expression in directed evolution experiments.[9] The latter technique was used to improve the enantioselectivity of *Candida antarctica* lipase A (CalA) toward *p*-nitrophenyl 2-methylheptanoate. The enzyme variants obtained from this screening showed a very narrow substrate scope, and we therefore decided to screen for more interesting analogues such as 2-arylpropionic acids (profens). In this paper we report on a triple mutant with a remarkably broad substrate scope[10] and excellent enantioselectivity.

In the present study the aim was to improve the enantioselectivity of CalA toward the α-substituted esters. CalA not only shows a modest enantioselectivity toward these substrates (*E* values 2−20) but also has a very low activity, and therefore the enzyme is of limited use in synthetic applications. The first objective was to improve the activity of the enzyme using *p*-nitrophenyl 2-phenylpropanoate (**1**) as a model substrate. Successful improvement of the activity of *Pseudomonas aeruginosa* lipase toward α-methyl substituted carboxylic acids by the use of combinatorial active-site saturation test (CAST) was

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(2) (a) Davies, N. M.; Anderson, K. E. *Clin. Pharmacokinet.* **1997**, *32*, 268–293. (b) Davies, N. M. *Clin. Pharmacokinet.* **1995**, *28*, 100–114. (c) Davies, N. M. *Clin. Pharmacokinet.* **1998**, *34*, 101–154.
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(6) (a) Reetz, M. T.; Wu, S. *J. Am. Chem. Soc.* **2009**, *131*, 15424–15432. (b) Lewis, J. C.; Bastian, S.; Bennett, C. S.; Fu, Y.; Mitsuda, Y.; Chen, M. M.; Greenberg, W. A.; Wong, C.-H.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 16550–16555.
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(8) Lee, C. C.; Williams, T. G.; Wong, D. W.; Robertson, G. H. *Plasmid* **2005**, *54*, 80–85.
(9) Sandström, A. G.; Engström, K.; Nyhlén, J.; Kasrayan, A.; Bäckvall, J.-E. *Protein Eng., Des. Sel.* **2009**, *22*, 413–420.
(10) For previous examples of extended substrate scope, see: (a) Wada, M.; Hsu, C.-C.; Franke, D.; Mitchell, M.; Heine, A.; Wilson, I.; Wong, C.-H. *Bioorg. Med. Chem.* **2003**, *11*, 2091–2098. (b) Mihovilovic, M. D.; Rudroff, F.; Winninger, A.; Schneider, T.; Schulz, F.; Reetz, M. T. *Org. Lett.* **2006**, *8*, 1221–1224. (c) Reetz, M. T.; Bocola, M.; Wang, L.-W.; Sanchis, J.; Cronin, A.; Arand, M.; Zou, J.; Archelas, A.; Bottala, A.-L.; Navoryta, A.; Mowbray, S. L. *J. Am. Chem. Soc.* **2009**, *131*, 7334–7343.

10.1021/ja100593j

reported by Reetz et al.[11] In this approach amino acids surrounding the active site are varied pairwise which accounts for synergistic effects. The CAST method has also been successfully used to improve enantioselectivity.[12−14]

Results and Discussion

Library Design. Based on the crystal structure of CalA,[15] residues situated close to the active site with side chains pointing toward the substrate cavity were selected for the CASTing libraries. Since the enzyme was crystallized in a closed conformation we first had to create a model of the enzyme in an open conformation; this was done by using Moloc computational software.[16] A molecular dynamics equilibration of the enzyme revealed that the amino acid region 425−440 (active site flap) was moving quite substantially, and an equilibrium conformation was reached after approximately 5 ns. The equilibrium conformation has a substantially more open entry to the active site. For the selection of amino acid residues the covalently bound *p*-nitrophenyl 2-phenylpropanoate (**1**) had been modeled into the structure. The carboxylic acid part of the substrate was positioned in the model guided by the tetraethylene glycol already available in the X-ray structure.

For the mutagenic libraries, amino acids surrounding the nucleophilic serine (Ser184) with the side chains pointing toward the substrate were chosen, since it is believed that these residues have a large impact on the enantioselectivity. Furthermore, according to the CASTing methodology, an approach of targeting two amino acid positions at the same time was used. The amino acids at these two positions were randomized simultaneously. Two libraries were considered to be the most relevant: (i) library FI (Phe149 and Ile150) with side chains directed toward the α-methyl group of the substrate and (ii) library FG (Phe233 and Gly237) with side chains pointing toward the acyl pocket of the active site (Figure 1).

Reduced libraries were employed to decrease the theoretical number of enzyme variants. NDT degeneracy, which codes for 12 amino acids (CDFHGILNRSVY), was chosen for the created libraries. This decreased the colony sampling requirements significantly. The number of picked colonies was enough to obtain at least 95% theoretical codon coverage; 600 colonies were picked for each of the libraries. Mutated variants of CalA were expressed in *P. pastoris* and screened using a spectrophotometric assay, where the release of *p*-nitrophenol by the hydrolysis with CalA was followed.

Library Screening. Since a previous study showed that a library containing Phe233 and Gly237 had a large impact on the enantioselectivity toward *p*-nitrophenyl 2-methylheptanoate,[9] a library containing these residues was the first to be evaluated (library FG). The main focus of the initial screening was to improve the activity of the enzyme since the wild type has very low activity toward this substrate; screening could therefore be performed with *rac*-**1**. Several enzyme variants from this library showed a large improvement in activity. Highly active variants were subsequently used in a kinetic resolution of *rac*-**1** to determine their enantioselectivity, and several of the variants showed a great improvement of the enantioselectivity. The best enzyme variant found, with the mutation Phe233Gly (F233G), had an *E* value of 259 (Table 1, entry 2). The F233G variant showed (*R*)-selectivity, which is reversed compared to wild type CalA.

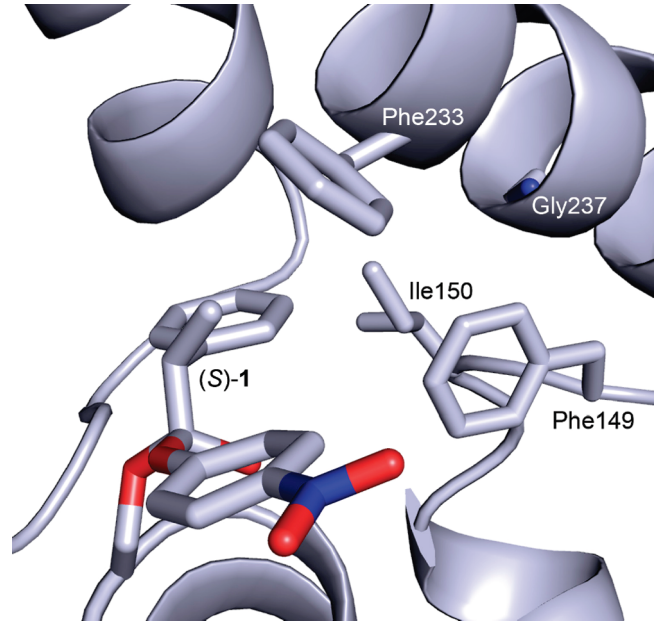

Figure 1. Amino acids selected for the CASTing libraries: library FI (Phe149 and Ile150) and library FG (Phe233 and Gly237).

Table 1. Results of the Directed Evolution for Increased Enantioselectivity of CalA toward 2-Phenylpropanoate **1**

Entry	Enzyme	Library origin	*E* value
1	wild type	−	20 (*S*)
2	F233G	Lib FG	>200 (259) (*R*)
3	YNG	Lib FG/Lib FI	>200 (276) (*R*)

To elucidate if it was possible to improve this enzyme variant further, a new pair of amino acids in the vicinity of the active site, Phe149 and Ile150 (library FI), was targeted using the F233G variant as a template. In this round the focus was on improving the enantioselectivity, not the activity. Therefore the screening was performed with single enantiomers of the substrate separately, and the ratio between the rates of hydrolysis of the two enantiomers was used to estimate the enantioselectivity (E_{app}).[17] The enzyme variants with the best ratio were subsequently used in a kinetic resolution of the *p*-nitrophenyl ester that was analyzed by chiral GC to determine the *E* value. With the model substrate, this library gave only a slight improvement in enantioselectivity, the triple mutant Phe149Tyr/Ile150Asn/Phe233Gly (YNG) had an *E* value of 276, with preference for the (*R*)-enantiomer (Table 1, entry 3).

Substrate Scope. To determine the substrate scope, similar substrates were used in the kinetic resolution with the two enzyme variants F233G and YNG. For the 2-phenylpropanoate **1** the two extra mutations in YNG (Phe149Tyr and Ile150Asn)

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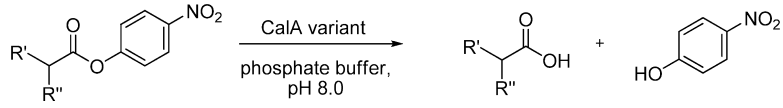
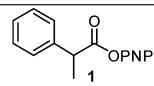
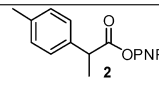
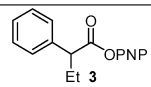
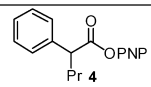
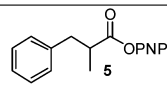
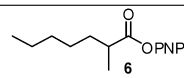
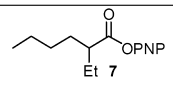
(12) (a) Reetz, M. T.; Sanchis, J. *ChemBioChem* **2008**, *9*, 2260–2267. (b) See also ref 9 and ref 10c.
(13) Bartsch, S.; Kourist, R.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 1508–1511.
(14) Liang, L.; Zhang, J.; Lin, Z. *Microb. Cell Fact.* **2007**, *6*, 36.
(15) Ericsson, D. J.; Kasrayan, A.; Johansson, P.; Bergfors, T.; Sandström, A. G.; Bäckvall, J.-E.; Mowbray, S. L. *J. Mol. Biol.* **2008**, *376*, 109–119.
(16) Gerber, P. R. *J. Comput.-Aided Mol. Des.* **1998**, *12*, 37–51.
(17) Janes, E.; Kazlauskas, R. J. *J. Org. Chem.* **1997**, *62*, 4560–4561.

Table 2. Results of the Hydrolytic Kinetic Resolution of *p*-Nitrophenyl Esters of α-Substituted Carboxylic Acids with Wild Type CalA (wt), Single Mutant Phe233Gly (F233G), and Triple Mutant Phe149Tyr/Ile150Asn/Phe233Gly (YNG)[a]



Entry	Substrate	Enzyme	Time[b] (min)	Conv.[b,c] (%)	ee$_p$[b,d] (%)	E[b]
1		wt	150	38	84.7	20 (*S*)
2		F233G	3	25	98.9	>200 (259) (*R*)
3		YNG	3.5	31	98.9	>200 (276) (*R*)
4		wt	240	23	55.6	4 (*S*)
5		F233G	2.5	29	90.1	32 (*R*)
6		YNG	5	38	94.4	63 (*R*)
7		wt	240	11	17.0	2 (*R*)
8		F233G	0.5	20	95.4	57 (*R*)
9		YNG	1.7	17	97.0	79 (*R*)
10		wt	270	11	88.1	18 (*R*)
11		F233G	2.5	26	97.1	88 (*R*)
12		YNG	5.3	14	97.8	109 (*R*)
13		wt	240	7	80.3	10 (*S*)
14		F233G	5	7	44.7	3 (*R*)
15		YNG	15	27	96.7	84 (*R*)
16		wt	3.7	18	80.7	11 (*S*)
17		F233G	2.5	28	85.0	17 (*R*)
18		YNG	3.3	31	96.7	104 (*R*)
19		wt	60	14	75.3	19 (*S*)
20		F233G	3.3	9	96.0	54 (*R*)
21		YNG	3.5	6	95.4	45 (*R*)

[a] Reaction conditions: *p*-nitrophenyl ester (1.25 mL, 2 mg/mL in acetonitrile), enzyme solution (20 μL, 10 μg/μL), potassium phosphate buffer (8.5 mL, 100 mM, pH 8.0). [b] Mean value of 2−4 reactions; results for the separate reactions are available in the Supporting Information. [c] Determined by ^{1}H NMR. [d] Determined by chiral GC.

seemed to play a minor role since the single mutation (Phe233Gly) gave, within experimental error, the same enantioselectivity. However, the two extra mutations were of crucial importance for a broad substrate selectivity. Thus, the YNG variant gave high to excellent *E* values for all substrates in Table 2, whereas the F233G variant completely failed for the 2-benzylpropanoate **5** (entry 14) and gave a moderate *E* value of 17 for the 2-methylheptanoate **6** (entry 17). For the 2-ethylhexanoate **7** the F233G variant was slightly better than the YNG variant (entries 20 and 21).

A methyl group in the *para*-position of the phenyl (**2**) was accepted with a high enantioselectivity (Table 2, entry 6). However, with a larger substituent, such as isobutyl (ibuprofen), both the F233G and the YNG variant showed low reactivity and gave an unselective reaction. Larger substituents in the α-position, ethyl and propyl, were accepted with good enantioselectivity (entries 9 and 12). An aliphatic chain in exchange for the 2-phenyl substituent was also accepted (entry 18). The broad substrate scope of the YNG variant is summarized in Table 2.

The reaction rates for the YNG variant were increased dramatically compared to wild type CalA. An increase of more than 30-fold was observed for most substrates. This increase in reaction rate improves the synthetic utility of the enzyme significantly, as shorter reaction times and lower enzyme loadings can be used.

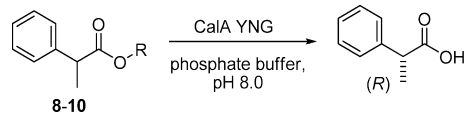
Less Reactive Esters as Substrates. For synthetic purposes it is of interest to use other esters than the *p*-nitrophenyl esters. The latter esters are highly reactive, and changing to an alkyl ester or a simple phenyl ester will lead to lower reaction rates. In particular, CalA is known to be very slow in catalyzing hydrolysis of alkyl esters.[18] Three different esters **8**, **9**, and **10**, the ethyl, nonyl, and phenyl ester analogues, respectively, of the *p*-nitrophenyl ester **1**, were used as substrates in the kinetic resolution catalyzed by the YNG variant. The results of the kinetic resolution show that the enantioselectivity is maintained for all three esters (Table 3), and for the nonyl and phenyl ester the *E* values were even higher than those for the *p*-nitrophenyl ester (Table 3, entries 2 and 3 vs Table 2, entry 3). As expected the hydrolysis of the ethyl and nonyl ester was slower than that of the phenyl ester but much faster than the corresponding hydrolysis of alkyl esters by wild type CalA.[18,19]

Active Site Models. Active site models of the enzyme variants F233G and YNG were created and compared with a model of the wild type enzyme to rationalize the improved rate and selectivity (Figure 2). By examining the models it seems that substitution of the large phenylalanine (Phe233) for a small glycine creates more space in the active site. The increased space in the active site can be used to accommodate the substrate, and this would explain the observed increase in activity. Furthermore, the modeling indicates that the space created is

(18) Barbayianni, E.; Fotakopoulou, I.; Schmidt, M.; Constantinou-Kokotou, V.; Bornscheuer, U. T.; Kokotos, G. *J. Org. Chem.* **2005**, *70*, 8730–8733.

(19) The hydrolysis of related alkyl esters catalyzed by CalA in ref 18 employed a much higher concentration of enzyme (50×) and a higher concentration of substrate (10×), and still the reactions typically took 24−48 h.

Table 3. Results of Kinetic Resolution of Esters with Different Alcohol Side Chains by YNG, a Triple-Mutated Variant of CalA (Phe149Tyr/Ile150Asn/Phe233Gly)[a]



8-10

Entry	R	Time (h)	Conv.[b] (%)	ee_p[c] (%)	*E*
1	Et	3	14	98.9	>200 (211)
2	Nonyl	3	21	99.6	>200 (650)
3	Ph	0.5	22	99.6	>200 (657)

[a] Reaction conditions: Ester (1.25 mL, 2 mg/mL in acetonitrile), enzyme solution (100 μL (entries 1 and 2) or 20 μL (entry 3), 10 μg/μL), potassium phosphate buffer (8.5 mL, 100 mM, pH 8.0). [b] Determined by ^{1}H NMR. [c] Determined by chiral GC.

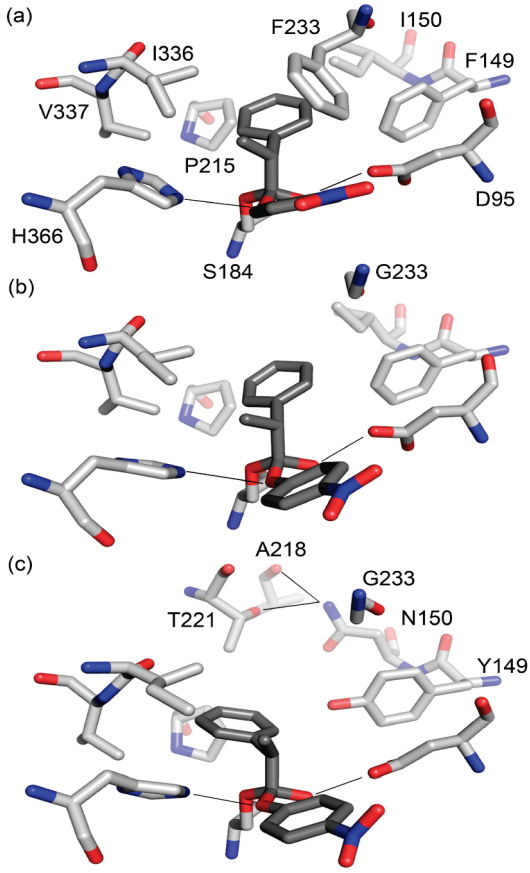


Figure 2. Models of the active site for (a) wild type CalA, (b) Phe233Gly (F233G) variant, and (c) Phe149Tyr/Ile150Asn/Phe233Gly (YNG) variant. In all cases (*R*)-*p*-nitrophenyl 2-phenyl propanoate ((*R*)-**1**) is covalently bound to the enzyme. Hydrogen bonds are indicated with black lines.

only of advantage for the (*R*)-enantiomer, which rationalizes the large effect on the enantioselectivity. The increased enantioselectivity for the triple mutant (YNG) could be explained by the increase in steric bulk introduced; the elongation of the Phe149 side chain by a hydroxyl group (Phe149Tyr) creates congestion with the α-methyl group, which disfavors the (*S*) configuration. In addition, two new hydrogen bonds are found in the YNG variant.

Conclusions

In conclusion, we have used a directed evolution approach to develop a triple-mutated variant of CalA with a broad substrate scope that can be used to produce α-substituted acids in 95−99% enantiomeric excess from ester hydrolysis. Furthermore, a 30-fold increase in activity was observed for most substrates. The developed enzyme variant shows (*R*)-selectivity, which is reversed compared to the wild type enzyme that is (*S*)-selective for most substrates.

Experimental Section

Unless otherwise noted, all materials were obtained from commercial suppliers and used without further purification. GC analyses were performed using an IVADEX-1 chiral column.

Plasmid Library Construction. The construction of the pBGP1-CalA vector has been described previously.[9] Mutagenic libraries were created by site-directed mutagenesis using degenerate primers. The PCRs were performed with the following protocol; a preincubation was performed at 95 °C for 10 min, after which a high-fidelity DNA polymerase was added. The cycle of denaturation at 98 °C for 10 s, annealing at 58−68 °C for 30 s, and elongation at 72 °C for 3 min was repeated 30 times. A 10 min elongation at 72 °C ended the reaction. The PCR products were purified using a Cycle-pure kit followed by DpnI treatment to remove the wild type template. The nicked plasmid was transformed into *E. coli* DH5α, and a large fraction of the transformed cells were grown overnight for a plasmid preparation. In parallel, a fraction of the transformed cells were plated to prepare for a sequencing assay. All pBGP1-carrying DH5α were selected using carbenicillin (50 μg/mL) in LB and LB-agar plates.

Library Expression in *P. pastoris*. *P. pastoris* X33 was made electrocompetent, and until employed, the cells were stored in 40 mL aliquots at −80 °C without any treatment. Library plasmid preparation was mixed with thawed cells and electroporated. Cells were incubated at 30 °C with YPDS (1 mL) for 2 h followed by plating on YPDS-agar plates containing zeocin (100 μg/mL) and carbenicillin (100 μg/mL). Plates were incubated at 30 °C for 3 days.

Single colonies were picked and inoculated in conical deep 96-well plates. Each well contained YPD (800 μL), zeocin (100 μg/mL), and carbenicillin (100 μg/mL). The deep well plates were shaken at 250 rpm for 5 days at 29 °C, leaning approximately 30°. The shaking amplitude was 2.5 cm. After the overexpression, the yeast was pelleted by centrifugation. The supernatant was harvested by aspiration and used directly for optical screening or stored at 4 °C. The protein concentration of the harvested supernatants was on average 0.2 mg/mL. Master plates containing the pelleted cells were stored at −80 °C until further analysis or cultivation.

Optical Screening for Improved Activity. Screening buffer (165 μL, 100 mM potassium phosphate, 10% v/v acetone, 4% v/v Triton X-100, 0.2% w/v gum arabic, pH 8.0)[20] and rac-**1** (10 μL, 2 mg/mL in acetonitrile) were premixed before dispension. Yeast library supernatant (25 μL) and the premixed buffer and substrate solution (175 μL) were dispensed into a microtiter plate, and absorbance was measured at 410 nm for 15 min. Wild type CalA was used as reference. Variants that showed high activity were selected for a kinetic resolution experiment to determine the enantioselectivity (*E*).

Optical Screening for Improved Enantioselectivity. Separate enantiomers of the substrate were hydrolyzed in parallel reactions. Screening buffer (165 μL, 100 mM potassium phosphate, 10% v/v acetone, 4% v/v Triton X-100, 0.2% w/v gum arabic, pH 8.0)[20] and (*R* or *S*)-**1** (10 μL, 2 mg/mL in acetonitrile) were premixed before dispension. Yeast library supernatant (25 μL) and the premixed buffer and substrate solution (175 μL) were dispensed into a microtiter plate, and absorbance was measured at 410 nm for 15 min. The ratio between the initial rates of hydrolysis of the (*S*)- and the (*R*)-enantiomer was calculated. Variants that showed

(20) Schulz, N.; Hobley, T. J.; Syldatk, C. *Biotechnol. Lett.* **2007**, *29*, 365–371.

high ratio and acceptable activity were selected for a kinetic resolution experiment to determine the enantioselectivity (*E*). All samples were measured in duplicates.

Cultivation of Enzyme Variants. The most interesting enzyme variants were produced in larger batches to yield more enzyme that could be used for further analysis. Master plates were used to inoculate shaking flasks containing YPD (50 mL), zeocin (100 μg/mL), and carbenicillin (100 μg/mL), which were incubated at 250 rpm for 96 h at 29 °C. Supernatant containing the enzyme was harvested after centrifugation. The enzyme solution was concentrated; at the same time the buffer was exchanged to Tris-HCl (25 mL, 20 mM, pH 7.8), to a final enzyme concentration of 10 μg/μL.

Kinetic Resolution. Potassium phosphate buffer (8.5 mL, 100 mM, pH 8.0), the racemic *p*-nitrophenyl ester (**1**−**7**) (1.25 mL, 2 mg/mL in acetonitrile), and enzyme solution (20 μL, 10 μg/μL in 20 mM Tris-buffer, pH 7.8) were shaken at room temperature until the reaction had reached 10−40% conversion. The reaction mixture was acidified by addition of HCl (1 mL, 1 M), extracted twice with DCM, and concentrated. Conversion was determined by ^{1}H NMR, and the enantiomeric excess was determined by chiral GC. Optical rotation was measured, and absolute stereochemistry was determined by comparison with literature values.

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Supporting Information Available: Experimental procedures and characterization data of all new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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