

## Expanding the Substrate Scope of Enzymes: Combining Mutations Obtained by CASTing

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**Abstract:** In a previous paper, the combinatorial active-site saturation test (CAST) was introduced as an effective strategy for the directed evolution of enzymes toward broader substrate acceptance. CASTing comprises the systematic design and screening of focused libraries around the complete binding pocket, but it is only the first step of an evolutionary process because only the initial libraries of mutants are considered. In the present study, a simple method is presented for further

optimization of initial hits by combining the mutational changes obtained from two different libraries. Combined lipase mutants were screened for hydrolytic activity against six notoriously difficult substrates (bulky carboxylic acid esters) and improved mutants showing significantly higher activity

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were identified. The enantioselectivity of the mutants in the hydrolytic kinetic resolution of two substrates was also studied, with the best mutant–substrate combination resulting in a selectivity factor of  $E=49$ . Finally, the catalytic profile of the evolved mutants in the hydrolysis of simple nonbranched carboxylic acid esters, ranging from acetate to palmitate, was studied for theoretical reasons.

### Introduction

Directed evolution of proteins is a powerful strategy for enhancing the stability, activity, and/or selectivity of enzymes.<sup>[1]</sup> This process requires the seamless combination of appropriate random-gene mutagenesis, expressions of mutant enzymes, and high-throughput screening<sup>[1,2]</sup> for a given functional property. As neither structural nor mechanistic information is necessary, the process is fundamentally different from so-called rational design, which is based on analysis and uses site-specific mutagenesis.<sup>[3]</sup> We have previously used directed evolution for the creation of enantioselective lipases,<sup>[4]</sup> epoxide hydrolases,<sup>[5]</sup> and monooxygenases,<sup>[6]</sup> and other research groups have contributed to this area as well.<sup>[1,7,8]</sup> These studies utilized error-prone polymerase chain reactions (epPCRs), saturation mutagenesis, and/or DNA shuffling as the gene mutagenesis methods. Moreover, random mutagenesis at defined positions<sup>[9]</sup> in the form of fo-

cused libraries,<sup>[1]</sup> designed on the basis of structural considerations, has been used to enhance ligand binding,<sup>[10]</sup> catalytic activity,<sup>[1,9,11]</sup> and enantioselectivity.<sup>[4c,12]</sup>

The exploitation of enzymes as catalysts in synthetic organic chemistry is often hampered by a limited degree of substrate acceptance.<sup>[13]</sup> This long-standing challenge is related to well-known postulates in modern enzymology,<sup>[14]</sup> such as Fischer's lock-and-key principle<sup>[15]</sup> and the induced-fit model of Koshland.<sup>[16]</sup> Three extreme categories regarding lack of turnover are known: 1) The substrate is too large to enter the binding pocket, or 2) it fits geometrically into the binding pocket but is so small that no reaction occurs, or 3) it binds well but nevertheless fails to react. Some of these problems have been addressed by using directed evolution.<sup>[1,9,11]</sup>

An alternative strategy for expanding substrate acceptance is our recently introduced combinatorial active-site saturation test (CAST).<sup>[17]</sup> Based on the three-dimensional structure of the enzyme, sets of two or three amino acids, whose side chains reside next to the binding pocket, are identified and the respective positions are then randomized simultaneously with the creation of relatively small libraries of mutants. Through the use of this method, it was possible to obtain *Pseudomonas aeruginosa* lipase (PAL) mutants that catalyze the hydrolysis of esters derived from sterically

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demanding acids, which are bulky substrates that are not accepted by the wild-type enzyme (WT-PAL).<sup>[17]</sup>

CASTing thus means the systematic design and screening of focused libraries around the complete binding pocket. However, CASTing is only the first step in an evolutionary process. For further enzyme optimization, iterative CASTing, in which the genes of hits obtained in the first round are used as templates for a further round of CASTing, was introduced.<sup>[18]</sup> Herein we present an alternative strategy for optimizing the catalytic profile of the initial CAST mutants by combining beneficial mutations obtained in the first round of randomization. This procedure is straightforward and requires only a limited number of experiments. In the present study we have used this strategy to broaden the range of substrate acceptance of PAL, and thus, to increase the reaction rates of “difficult” substrates.

## Results and Discussion

**Substrate acceptance of bulky esters:** In a previous study<sup>[17]</sup> we reported our analysis of PAL and the construction of five CAST libraries—A, B, C, D, and E—surrounding the complete binding pocket (Figure 1). Each library comprises two amino acid positions, some being direct neighbors ( $n+1$ ) as in a loop (e.g., positions 16 and 17 in library A), others being further apart (e.g.,  $n+4$ ) as in an  $\alpha$  helix (e.g., positions 131 and 135 in library C). In all cases these positions are close enough to be simultaneously randomized by using

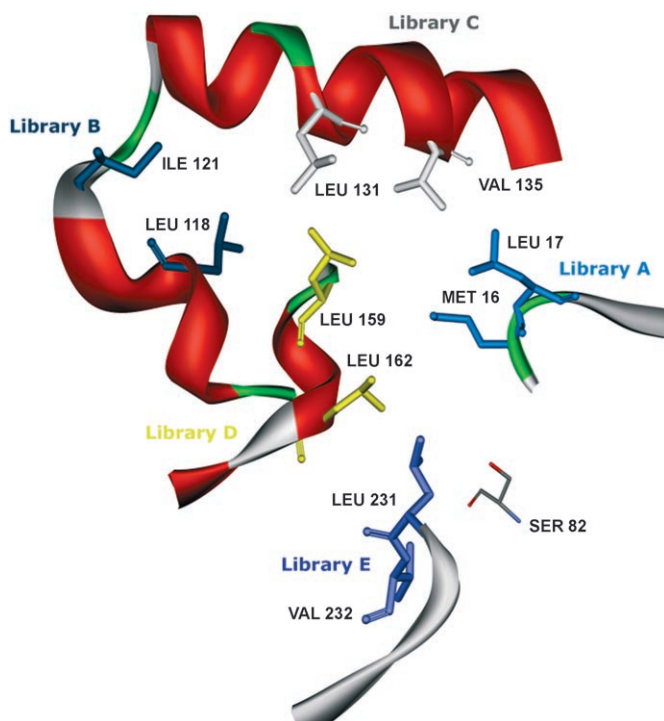


Figure 1. CASTing of PAL leading to the construction of five libraries of mutants (A–E) produced by simultaneous randomization at two amino acid sites.

cassette mutagenesis.<sup>[1]</sup> We have now developed a user-friendly computer program for the design of focused libraries in CAST experiments (CASTER), which can be obtained from the authors.

Libraries A to E were screened for hydrolytic activity against eleven *p*-nitrophenyl (PNP) esters of bulky acids, which react very slowly or are not accepted by the WT lipase.<sup>[17]</sup> This multiple-substrate screening required high-throughput screening, and about 165 000 reactions<sup>[17]</sup> were monitored by using a UV-visible plate reader.<sup>[2,4]</sup>

Although the simultaneous randomization of two amino acids yields  $20^2=400$  unique variants (some being double mutants, others having only a single amino acid exchange plus the original WT combination), the number of variants to be screened depends on the randomization technique used in the construction of a library and on the over-sampling required for 95% coverage of protein-sequence space.<sup>[17,19]</sup>

As a result of CASTing, at least eight variants were identified as hits.<sup>[17]</sup> Most of the hits originate from libraries A and D, the latter showing the highest frequency of improved mutants. The most promising enzyme variant from library A, ACA5, was identified as the double mutant Met16Ala/Leu17Phe. Table 1 lists those CAST mutants used in the present investigation.

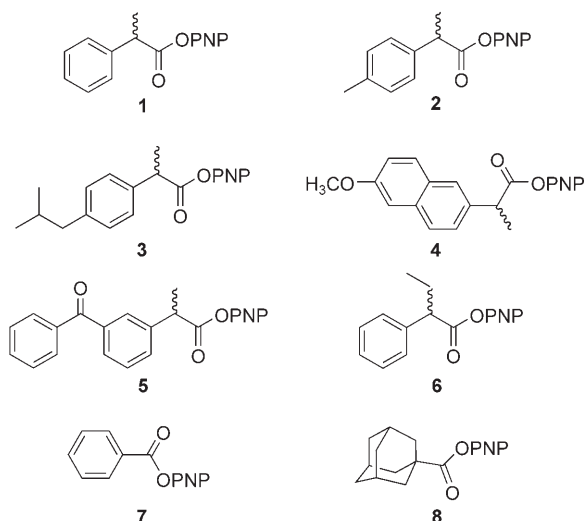
Table 1. Active PAL mutants created by CASTing.<sup>[17]</sup>

Mutant	Library <sup>[a]</sup>	Mutations
ACA5	A	Met16Ala/Leu17Phe
D1A12	D	Leu162Val
D1B10	D	Leu162Thr
D1C4	D	Leu159Trp/Leu162Thr
D1D12	D	Leu162Ile
D1E1	D	Leu162Ala
D1F8	D	Leu162Asn

[a] Library A: (Met16/Leu17); library D: (Leu159/Leu162).

In the present study we focus on eight substrates (1–8), which are hardly accepted by the WT lipase from *P. aeruginosa*. All of them except 5 were considered in our previous report.<sup>[17]</sup>

Using standard molecular biological methods,<sup>[1]</sup> six different A/D combinations were prepared by combining the mutations of ACA5 with those of D1A12, D1B10, D1C4, D1D12, D1E1, and D1F8, respectively. ACA5 was chosen because this variant shows the best catalytic profile.<sup>[17]</sup> The WT and the mutants of PAL are stable in culture media, but in purified form they gradually lose activity.<sup>[17,20]</sup> In the present study, the culture supernatants were therefore used in order to obtain reliable data regarding relative rates. Due to the presence of interfering metabolites in the culture supernatants, immunoassays (dot blots) rather than total protein content were used to compare levels of WT and mutant lipases. The polyclonal antiserum can be expected to react equivalently with functional mutants and WT. These experiments showed that the amounts of lipase in the relevant



series are comparable, which allowed the determination of relative rates. Each substrate was studied separately, that is, the reaction rate of the WT was compared with the reaction rate of the mutants for each substrate. Figure 2 summarizes the results.

The mutants obtained in the original CASTing study are fairly active catalysts for most of the substrates studied.<sup>[17]</sup> As can be seen in Figure 2, the process of combining mutations results in the desired increase in reaction rate in most, but not all, cases. In the hydrolysis of esters **1–4**, each of the

six new mutants is a more active biocatalyst. Rate accelerations of 15- to 25-fold relative to the WT are common. It should be noted that all of the relative rates reported here refer to the background reaction not catalyzed by the lipase. Thus, the rate acceleration with respect to the actual WT-catalyzed reaction can be expected to be much higher.

In the case of the particularly unreactive substrates **5–8**, the effects are even more pronounced. An example is the hydrolysis of benzoic *p*-nitrophenyl ester (**7**), a process which is extremely slow when using the WT lipase. Mutants ACA5, D1A12, and D1E1 show only slight rate increases relative to the WT, in contrast to mutants D1B10, D1C4, and D1F8, which exhibit 105-, 58-, and 82-fold rate accelerations, respectively. The combination ACA5/D1A12 is at least 145-fold more active than the WT and provides the best catalyst for this reaction (Figure 2). Because mutants ACA5 and D1A12 are poor catalysts individually, there must be strong cooperativity operating in ACA5/D1A12. A similar effect emerges when combining ACA5 with D1E1 (84-fold more active).

In contrast to these positive effects, combining the already good mutants D1B10, D1C4, and D1F8 with ACA5 actually *lowers* the activity in the hydrolysis of substrate **7**. This may appear surprising, but in fact there is no theoretical reason to expect additivity or cooperativity for every combination.

The most demanding substrate is the very sterically encumbered adamantane carboxylic acid *p*-nitrophenyl ester (**8**). Among the original noncombined mutants, variant D1F8 is the best catalyst and leads to a 25-fold rate acceleration, which remains unsurpassed even after combining mutants. However, other combinations do in fact lead to positive effects relative to the performance of the original mutants, for example, ACA5/D1A12 and ACA5/D1C4 (Figure 2).

In order to obtain data regarding absolute rates, the percentage conversion of each substrate using the respective best mutant was monitored under a defined set of conditions. For this purpose, the percentage conversion was measured after 90 min at 30°C using the WT, the best respective mutant, and a lipase-negative mutant for ascertaining the background hydrolysis. Table 2 shows that using the WT in-

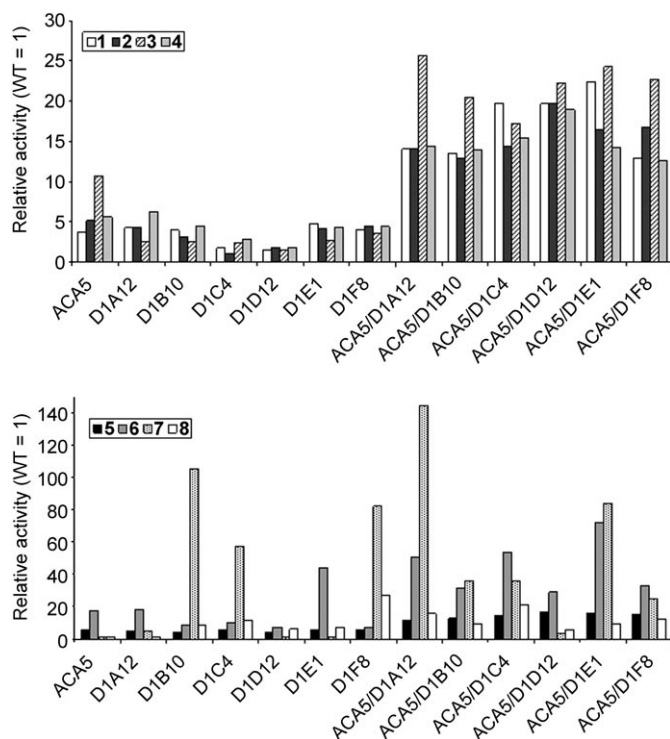


Figure 2. Effects of combining the mutations of variant ACA5 with those of variants D1A12, D1B10, D1C4, D1D12, D1E1, and D1F8 (an average of eight measurements in each case).

Table 2. Activity, indicated by percentage conversion<sup>[a]</sup> after 90 min at 30°C, in the hydrolysis of substrates **1–8** using the host lipase-negative mutant (background), the WT-PAL, and the best variants.<sup>[b,c]</sup>

Substrate	Conversion [%] (background)	Conversion [%] using WT-PAL	Conversion [%] using best variant
<b>1</b>	2	3	61 (ACA5/D1E1)
<b>2</b>	4	5	57 (ACA5/D1D12)
<b>3</b>	4	5	65 (ACA5/D1A12)
<b>4</b>	2	3	50 (ACA5/D1E1)
<b>5</b>	2	2	50 (ACA5/D1E1)
<b>6</b>	2	2	75 (ACA5/D1E1)
<b>7</b>	<2	<2	46 (ACA5/D1A12)
<b>8</b>	<2	<2	15 (D1F8)

[a] Uncertainty in conversion:  $\pm 5\%$ . [b] The expression system<sup>[20]</sup> used yields about  $0.15 \text{ g L}^{-1}$  of enzyme. [c] Conditions: 100  $\mu\text{L}$  of expression culture supernatant, 200  $\mu\text{L}$  of Tris 100 mM pH 7.5 buffer, 20  $\mu\text{L}$  of  $20 \text{ mg mL}^{-1}$  substrate in acetonitrile.

duces essentially no reaction beyond the background reaction, in sharp contrast to the best enzyme variants.

In the absence of a detailed molecular mechanics/quantum mechanics (MM/QM) study, as performed earlier in the case of the directed evolution of enantioselective PAL mutants as catalysts in the hydrolytic kinetic resolution of 2-methyldecanoic *p*-nitrophenyl ester,<sup>[21]</sup> it is difficult to provide a sound explanation of the results. However, preliminary molecular modeling studies using standard molecular dynamics (MD) simulations, taken together with the results of our previous theoretical study,<sup>[21]</sup> shed some light on the molecular basis of the observed substrate acceptance.

It is well known that lipases are serine hydrolases that catalyze the hydrolysis of natural triglycerides.<sup>[13,22]</sup> Structural and mechanistic studies have shown that a catalytic triad composed of aspartate, histidine, and serine is involved, with the activated form of serine undergoing a rate-determining nucleophilic attack at the ester function to form a short-lived oxyanion.<sup>[22]</sup> The acid part of the ester resides in the respective binding pocket (fatty acid moieties in the case of triglycerides as natural substrates), while the alcohol component of the ester is bound elsewhere. All of our studies have focused on the acid binding pocket (Figure 1), including the investigation regarding the enantioselectivity in the kinetic resolution of 2-methyldecanoic *p*-nitrophenyl ester.<sup>[4,21,23]</sup> In that study it was demonstrated that not only the enantioselectivity increases as a result of directed evolution, but also the rate.<sup>[23]</sup> The theoretical analysis based on QM/MM studies pin-pointed one of the crucial mutations as being Leu162Gly, which is next to the binding pocket and close to the active Ser82 (the other mutations being remote).<sup>[21]</sup> It became clear that the mutational change Leu162Gly causes, among other things, the enlargement of the binding pocket to accommodate the methyl group at the stereogenic center, that is, to tolerate branching.<sup>[21]</sup> This result is relevant to the present analysis.

In the present (and previous)<sup>[17]</sup> CASTing study, site D comprises Leu159 and Leu162 (WT). As delineated above, most of the hits originate from this library, and the majority of these have a single mutation at position 162 (also see Table 1). Although glycine is not among the newly introduced amino acids, it is clear that position 162 is a hot spot, in line with previous studies in which very different strategies based on epPCRs, saturation mutagenesis, and DNA shuffling were applied.<sup>[4]</sup>

The data in the present investigation (Figure 2) shows that one of the best mutant combinations is ACA5/D1E1.

The mutational changes in ACA5, namely Met16Ala/Leu17Phe, can be interpreted as follows. The side chain of alanine is sterically smaller than that of the original methionine, thereby providing more space for binding bulky (branched) substrates. The second substitution, Leu17Phe, is more difficult to explain, because a benzyl moiety is usually considered to be more sterically demanding than an isobutyl group. However, the benzyl side chain of phenylalanine can “fold away” if, for example,  $\pi,\pi$  stacking with another aromatic ring in the enzyme occurs, thereby providing more space for the substrate. Such  $\pi,\pi$  stacking is common in many proteins.<sup>[24]</sup> In our system,  $\pi,\pi$  stacking might occur between Phe17 and Phe19. Finally, the mutational change Leu162Ala in the single mutant D1E1 and in the combined mutant ACA5/D1E1 is easy to understand on the basis of size differences. These effects are illustrated in Figure 3.

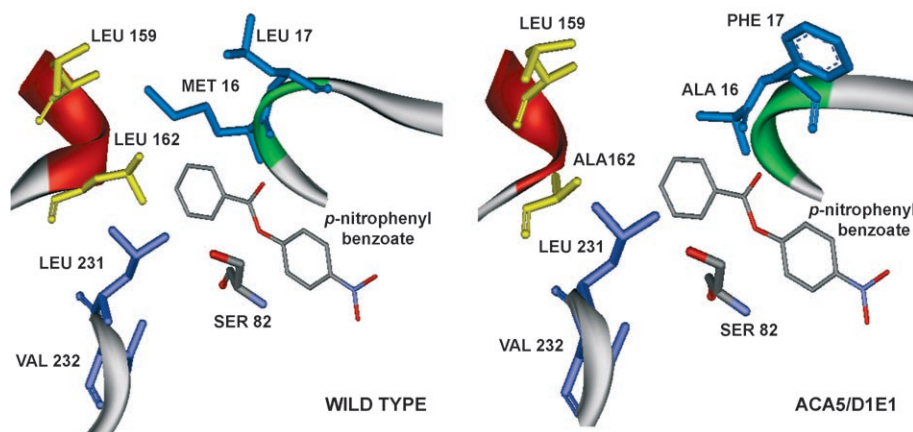


Figure 3. Comparison between PAL-WT (left) and mutant ACA5/D1E1 (Met16Ala, Leu17Phe, Leu162Ala) (right). For illustrative purposes, the binding of substrate **7** is shown.

**Enantioselectivity:** Most of the substrates **1–8** are chiral, so it was therefore of interest to study the enantioselectivity of the various mutants as catalysts in hydrolytic kinetic resolution. Because the screening system was designed to assess activity, and not enantioselectivity, the effect of mutations on the *E* value was unpredictable. We chose substrate **1** and the structurally related, but more sterically demanding, ester **6** for this investigation. The WT-PAL shows almost no activity beyond the background reactions, which meant that it was not possible to obtain reliable *E* values. Nevertheless, some remarkable results regarding the catalytic profiles of the mutants were observed (Table 3). It can be seen that neither the original CAST mutants nor the combined mutants are particularly enantioselective in the hydrolytic kinetic resolution of substrate **1**. Variants ACA5/D1B10 and ACA5/D1E1 provide the highest selectivity factors, but these amount to only *E* = 5. In contrast, the more sterically demanding ester **6**, which has an ethyl instead of a methyl group at the stereogenic center, behaves quite differently. For example, noncombined mutants ACA5, D1A12, and

Table 3. Enantioselectivity in the hydrolytic kinetic resolution of substrates **1** and **6** catalyzed by PAL mutants.

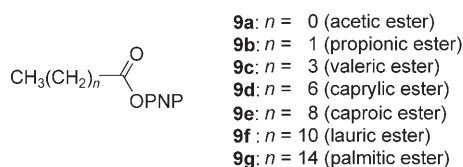
Mutant	$E^{[a]}$ for <b>1</b>	$E^{[a]}$ for <b>6</b>
ACA5	3	20
D1A12	2	18
D1B10	3	11
D1C4	4	6
D1D12	2	9
D1E1	2	1
D1F8	3	17
ACA5/D1A12	3	18
ACA5/D1B10	5	20
ACA5/D1C4	2	19
ACA5/D1D12	3	49
ACA5/D1E1	5	16
ACA5/D1F8	3	16

[a]  $E$  = enantioselectivity of the reaction.

D1F8 result in  $E$  values of 20, 18, and 17, respectively. Mutant D1D12 is less effective ( $E=9$ ), but its combination with ACA5 provides the best variant ACA5/D1D12 ( $E=49$ ). The result of combining the mutations of the two original variants seems to be additive. Apparently, the system responds to the size difference between methyl and ethyl groups quite effectively. In order to increase asymmetric induction for either substrate, further directed evolution is necessary in which the screening process responds to enantioselectivity.<sup>[4]</sup>

#### Substrate acceptance of nonbranched carboxylic acid esters:

In a final series of experiments, the lipase mutants were studied as catalysts in the hydrolysis of simple, nonbranched carboxylic acid *p*-nitrophenyl esters **9a–g**, which are less sterically encumbered than substrates **1–8**.



In view of Fischer's lock-and-key hypothesis<sup>[15]</sup> and the induced-fit model,<sup>[14,16]</sup> it was of theoretical interest to learn whether the mutants, engineered to accept bulky substrates, display higher or lower activity than the WT lipase when catalyzing the sterically less-demanding esters **9a–g**. All mutants turned out to be less active than the WT. In some cases the decrease in activity between the WT and mutant is less than 10%, for example, when catalyzing the hydrolysis of **9a** using ACA5 or ACA5/D1D12, **9c** using D1A12, **9d** using D1A12, D1B10, or ACA5/D1F8, and **9g** using ACA5. In contrast, more than an 80% activity loss is observed when catalyzing the hydrolysis of **9b** using D1E1, ACA5/D1C4, or ACA5/D1F8, **9c** using ACA5/D1C4, and **9g** using ACA5/D1C4. In general, the combined mutants lead to the greatest decrease in activity relative to the WT.

As noted in the Introduction, one category of nonacceptance (i.e., low or no turnover) relates to overly small substrates that fit geometrically into the binding pocket but which nevertheless react only very slowly or not at all (point 2). Such a situation has been created by the generation of the mutants in the present study. The binding pocket of the lipase mutants has been over-enlarged for the slim substrates **9a–g**, and so they are still accommodated, but largely in the form of nonproductive binding.<sup>[14,25]</sup>

## Conclusion

CASTing is a straightforward procedure for probing limited protein-sequence space in the quest to enlarge the scope of substrate acceptance of enzymes.<sup>[17,18]</sup> It involves the systematic preparation and testing of focused libraries around the complete binding pocket by randomizing sets of two or more amino acid positions. If the mutant enzymes arising from such a procedure require further improvement, for example in terms of activity, evolutionary pressure has to be exerted in the form of additional mutagenesis/screening procedures. Here we have shown that combining the mutational changes of positive mutants (hits) discovered in two different CAST libraries is a viable strategy for achieving this goal. Rate increases of up to 145-fold relative to the reactions using the WT were observed in the lipase-catalyzed hydrolysis of bulky esters. All of the relative rates reported in this paper refer to the respective noncatalyzed background reaction, which means that the true values are actually much larger.

Although this directed evolution study was by nature not designed to control enantioselectivity, two of the sterically demanding substrates, which are also chiral, were tested in hydrolytic kinetic resolution reactions. In the case of the bulkier of the two, several of the combined mutants showed appreciable degrees of asymmetric induction, with the respective selectivity factors amounting to  $E=20$ –49. If the specific goal is further improvement of enantioselectivity, a subsequent directed evolution study, for example, iterative CASTing,<sup>[18]</sup> is necessary in which the screening focuses on this property.

Finally, in light of Fischer's lock-and-key hypothesis<sup>[15]</sup> and/or refined models,<sup>[14,16]</sup> the lipase mutants obtained by the first round of CASTing, as well as the combined mutants from this study, were tested as catalysts in the hydrolysis of sterically less-encumbered nonbranched esters. All of the engineered mutants display lower activity than the WT, indicating that the binding pocket has been over-enlarged. This lends support to the often discussed hypothesis that overly small substrates may enter the enzyme, but bind largely in nonproductive forms.<sup>[14]</sup> These and the other data set the stage for detailed theoretical studies.

## Experimental Section

**General:** All the linear *p*-nitrophenyl esters (**9a–g**) were purchased from Sigma-Aldrich.

**Generation of the double mutants:** The genes for the selected PAL mutants from library D were amplified from pUCPL<sup>6</sup>AN<sup>[20]</sup> by using the primers PALNheI (5'-TCCAGGCTAGCACCTACACCCAG-3') and PALA-paI (5'-GAAAGGGCCCGGGCCGAG-3'). The PCR product was cloned into the pCR2.1 vector (Invitrogen) by using the TOPO TA cloning kit. This plasmid served as a template for the introduction of the ACA5 mutations following the Quikchange protocol (Stratagene) with KOD Hot Start polymerase (Novagen). Thereafter, the sequence of the insert was verified by using DNA sequencing (Medigenomix GmbH, Martinsried, Germany) and the PAL gene was re-cloned into the *Pseudomonas* expression vector pUCPL<sup>6</sup>AN by using the restriction enzymes NheI and ApaI.

**Protein expression:** Designed pUCPL<sup>6</sup>AN plasmids encoding improved lipase mutants were used to transform the lipase-negative *P. aeruginosa* host PABST7.1<sup>[20]</sup> competent cells as previously described.<sup>[26]</sup> Single colonies were isolated from Lauria–Bertani (LB) agar plates, supplemented with carbenicillin (100 µg mL<sup>-1</sup>) and tetracycline (50 µg mL<sup>-1</sup>), and were used to inoculate 20 mL of 2×LB media (peptone (20 g), NaCl (10 g), yeast extract (10 g) per liter supplemented with the above-mentioned antibiotics). The cultures were incubated overnight at 30 °C, under shaking at 300 rpm in 100 mL Erlenmeyer flasks. Fresh media was inoculated with 0.5 mL of fresh overnight culture and this was incubated for 5 h as described above. Lipase expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to give a final concentration of 0.1 mM and this was incubated for an additional 5 h. The lipase-containing supernatants were recovered by centrifuging at 8000 rpm for 45 min and were then stored overnight at 4 °C.

**UV/Vis screening assay:** The activity measurement of WT lipase and the mutants was performed by using a Spectramax UV/Vis spectrophotometer from Molecular Devices Corp. The reaction was continuously monitored at λ=405 nm and the maximum slope of each reaction was taken as the experimental parameter to characterize the variants. Reaction mixture: Tris buffer (65 µL, 100 mM, pH 7.5), substrate in acetonitrile (10 µL, 10 mg mL<sup>-1</sup>), and expression culture supernatant (25 µL). Hydrolytic reactions of substrates **1–6** were monitored at λ=405 nm for 10 min and linear esters **9a–g** for 5 min. The substrates were also evaluated by endpoint measurements after 20 min reaction time; the reactions in these cases were developed as described below for the determination of conversion and enantioselectivity.

**Reaction conditions for determining the conversion and enantioselectivity:** The reactions were performed as follows: Tris/HCl buffer (450 µL, 100 mM, pH 7.5) and the substrate in acetonitrile (50 µL, 10 mg mL<sup>-1</sup>) were added to the expression culture supernatant (100 µL). The reaction mixture was shaken at 800 rpm for 90 min at 30 °C. The solution was extracted with dichloromethane (400 µL). Then a second extraction step was performed by adding dichloromethane (400 µL) and HCl 10% (20 µL) to favor acid extraction. The extracted organic phase was transferred to a vial and analyzed by using GC.

**GC analysis:** The analysis of the conversion was performed by using gas chromatography on a Hewlett–Packard 6890N chromatograph with a nonchiral phase column HP-5 (30 m, 0.32 mm, 0.25 µm). Conditions: Carrier (H<sub>2</sub>) flow: 3.1 mL min<sup>-1</sup>; temperature profile: 60 °C, 2 min, 30 °C min<sup>-1</sup>, 300 °C, 15 min. The chiral analyses of substrates **1** and **6** were performed by using a DiMePeBETA-Ivadex-1 chiral column (25 m, 0.25 mm, 0.15 µm) from IVA Analytentechnik, Meerbusch, Germany. Conditions: Carrier (N<sub>2</sub>): 1.4 mL min<sup>-1</sup>; temperature profile: 120 °C, 30 min, 10 °C min<sup>-1</sup>, 200 °C for 40 min. Absolute chirality was determined by comparison with commercial enantiopure products.

**Immunodetection:** Supernatants were manually spotted onto nitrocellulose membranes (porablot NCP, pore size=0.45 µm, Macherey–Nagel, Düren, Germany) and the membranes were processed by using standard procedures. Membranes were blocked with 5% dry milk in TBST (i.e., 25 mM Tris-HCl pH 8.0; 150 mM NaCl; 3 mM KCl; 0.2% Tween 20).

Rabbit-anti-LipA monoclonal antibody, kindly provided by K.-E. Jaeger, and goat-anti-rabbit horseradish peroxidase conjugate (Calbiochem/Merck, Darmstadt, Germany) were diluted in TBST by 1:50000 and 1:10000, respectively. Detection was performed by using stabilized 3,3',5,5'-tetramethylbenzidine (TMB; Pierce, Rockford, IL, USA). The reaction was allowed to proceed for 2 min and the intensity of color development was estimated by using a BioDocII (Biometra, Göttingen, Germany) digital camera system.

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