# Enantioselective Enzymes for Organic Synthesis Created by Directed Evolution

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**Abstract:** A new concept for the creation of enzymes displaying improved enantioselectivity in a given reaction is described; it is based on "evolution in the test tube". Accordingly, proper molecular biological methods for random mutagenesis, gene expression, and high-throughput screening systems for the rapid assay of enantioselectivity are combined. Several rounds of mutagenesis and screening are generally necessary in order to create mutant enzymes that show high degrees of enantioselectivity, as in the case of the lipase-catalyzed hydrolytic kinetic resolution of a chiral ester in which the original enantioselectivity of 2 % *ee* ($E = 1$) increases to > 90 % *ee* ($E = 25$).

**Keywords:** asymmetric catalysis · directed evolution · enzyme catalysis · high-throughput screening · mutagenesis

## Introduction

The world market for chiral fine chemicals, such as pharmaceuticals or plant protecting agents, is expanding rapidly.[1] Among the various ways to prepare enantiopure compounds, the use of asymmetric catalysts constitutes the most elegant and efficient strategy. When choosing this approach organic chemists have two options: transition metal catalysis[2] or biocatalysis.[3] The decision as to which option is best depends on a number of factors that include the cost of catalysts, degree of catalyst stability, activity and enantioselectivity, type of solvent, and ease of workup.[4] Most organic chemists tend to try transition metal catalysis first. If the available transition metal catalysts fail to provide high activity and enantioselectivity for a given transformation of interest, A → B, a new catalyst needs to be developed. In doing so, several aspects are crucial to success, namely intuition, experience, knowledge of the reaction mechanism, ability to assess steric and electronic factors in ligand tuning, and a great deal of trial and error.[2] It is therefore of no surprise that among the 2000 chiral, chelating phosphorus-containing ligands, that have been prepared and reported, only a handful are really effective in enantioselective reactions.[2] Moreover, for many substrates and reaction types truly useful catalysts are not yet available.

Among the various options in biocatalysis, enzymes are generally chosen owing to their high activity.[3] Indeed, a wide variety of enzyme kits are commercially available. An important innovation in the field was the discovery that many enzymes perform well in organic solvents.[3, 5] Although a trend in industry to consider biocatalysis more so than in the past is clearly emerging,[6] one of the problems with using enzymes is the fact that they are substrate-specific. For a given reaction of interest enantioselectivity may be unacceptably low. In principle, a type of "ligand tuning" should be possible, namely the exchange of a specific amino acid in the enzyme by one of the remaining 19 natural amino acids by using site-directed mutagenesis, a standard technique in molecular biology.[7] Unfortunately, due to the complexity of enzymes, this method has not proven to be a straightforward and generally successful tool in the difficult endeavor of increasing the *ee* value (*ee* = enantiomeric excess) of a given reaction in which a prochiral substrate is transformed into a chiral product. The same applies to the problem of increasing the selectivity factor $E$ in kinetic resolutions of racemic substrates.

We have recently introduced a different approach to the development of enantioselective catalysts for use in organic synthesis that is based on directed evolution of enzymes.[8] Accordingly, the combination of proper molecular biological methods for random mutagenesis and gene expression coupled with high-throughput screening systems for the rapid identification of enantioselective mutant enzymes forms the basis of the concept. The idea is to start with a natural (wild-type) enzyme that has an unacceptable *ee* or $E$ value for a given transformation of interest, A → B, to create a library of

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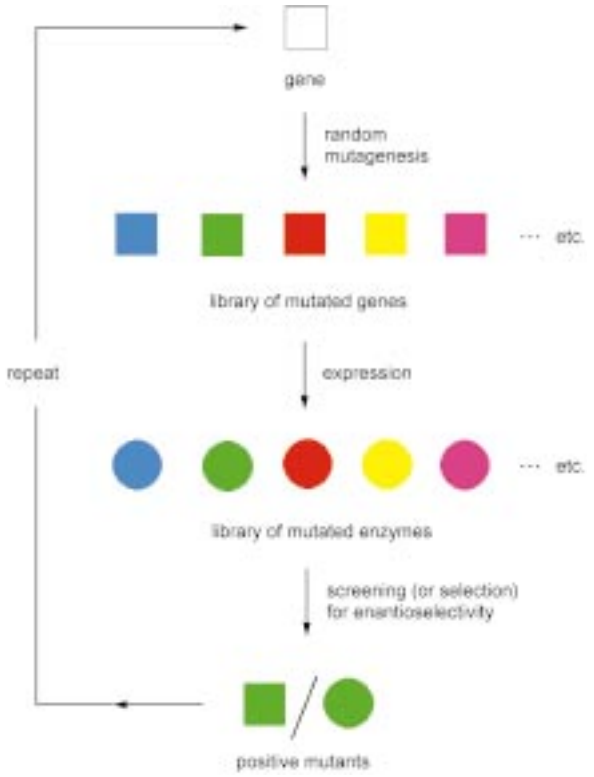
mutants from which a more enantioselective variant is identified, and to repeat the process as often as necessary by using, in each case, an improved mutant for the next round of mutagenesis. Since the inferior mutants are discarded, the evolutionary character of the overall process becomes apparent. In doing so, random mutagenesis is not performed on the enzyme itself, but on the gene (DNA segment) which encodes the protein (Scheme 1).



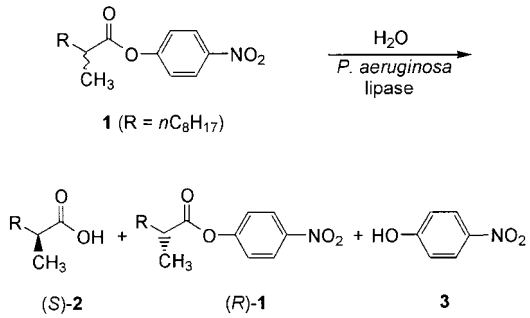

Scheme 1. Strategy for directed evolution of an enantioselective enzyme.

The concept summarized in Scheme 1 breaks with the usual forms of so-called rational or de novo design traditionally practiced (or attempted) in the area of transition metal catalysis[2] or biocatalysis[3, 9] because structural and mechanistic aspects are not part of the strategy! This means that the preoccupation with steric and electronic effects, which organic chemists have such a fondness for inspite of the necessity of trial and error, is absent. Nevertheless, the concept we describe here is rational. Moreover, the challenges that the new approach entails are equally intriguing, albeit in a completely different manner. Among the major problems to be solved is the development of high-throughput screening systems for enantioselectivity.

## Discussion

In the late 1980s and early 1990s molecular biologists began to develop new and practical techniques for random mutagenesis. One of the landmarks was a report by Leung, Chen, and Goedell, who described the technique of "error prone polymerase chain reactions" (epPCRs), in which the conditions of the classical PCR were varied empirically (e.g., the $MgCl_2$ concentration) so as to attain the desired mutation rate.[10] This procedure of inducing point mutations was followed in 1994 by Stemmer's method of DNA shuffling[11] and in 1998 and 1999 by Arnold's staggered-extension process[12] and random priming recombination method,[13] respectively; these are all recombinative processes that result in a high diversity of mutant genes. Since then these and other methods such as saturation mutagenesis (in which the substitution or insertion of codons is performed that leads to all possible 20 amino acids at any predetermined position in the gene) have been applied in the quest to obtain mutant enzymes with improved stability and activity.[11–15] However, enantioselectivity is a particularly difficult parameter to deal with, and at the outset of our efforts it was not clear whether the technique of directed evolution, or in vitro evolution as it is sometimes called,[11–15] would work for this particular purpose.

We decided to test our concept by applying epPCR in the development of an enantioselective enzyme as a catalyst in the hydrolytic kinetic resolution of the chiral ester **1**.[8, 15] Hydrolysis generates *p*-nitrophenol **3** which can be detected by UV/Vis-spectroscopy as a function of time (Scheme 2).




Scheme 2. Scheme illustrating the test reaction.

Thus, we envisioned as a screening system the use of a commercially available 96 well microtiter plate on which the (*R*)- and (*S*)-esters **1** are allowed to react separately in each of 48 wells. Indeed, it was possible to screen 48 mutants on a 96 well microtiter plate within 6–8 minutes.[8] The enzyme that we chose for this model reaction was the bacterial lipase from *Pseudomonas aeruginosa*.[16] It consists of 285 amino acids and leads to an *ee* value of only 2 % in slight favor of the (*S*)-product **2**. This corresponds to the lowest possible selectivity factor of about $E = 1$, which reflects the relative reaction rates of (*S*)-**1** and (*R*)-**1**. We also had to develop an efficient expression system, which was accomplished by first ligating the mutated genes into a suitable expression vector, amplifying in *E. coli*, and then transforming into *P. aeruginosa*.[8] This particular system ensures secretion of the mutant enzymes into the medium so that the supernatants can be used directly in the screening.

When performing random mutagenesis, the problem of exploring protein sequence space needs to be considered first. In the present case complete randomization allowing for all possible permutations would theoretically result in $20^{285}$ different mutant enzymes, the masses of which would greatly



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exceed the mass of the universe, even if only one molecule of each enzyme were to be produced.[15] The other end of the scale entails the minimum amount of structural change, namely the substitution of just one amino acid per molecule of enzyme. On the basis of the algorithm $N = 19^M \times 285!/[(285 - M)! \times M!]$, in which $M$ = number of amino acid substitutions per enzyme molecule (here $M = 1$), the library of mutants would theoretically contain 5415 members.[8, 15] However, due to the degeneracy of the genetic code, inter alia, it is impossible to generate by epPCR a library which in fact contains all of the 5415 variants. If the mutation rate is increased to such an extent that an average of two amino acids are exchanged per enzyme molecule ($M = 2$), then the number of mutant *P. aeruginosa* lipases predicted by the above algorithm increases dramatically to about 15 million, which would be very difficult to assay even when applying the best high-throughput screening systems currently available.

The strategy that we therefore initially used was to apply a relatively low mutation frequency and to rely on step-wise improvements in enantioselectivity.[8, 15] When creating thousands of lipase mutants (or of any other enzyme), the challenge of deconvolution might appear to be an insurmountable task. However, this problem never arises because subsequent to mutagenesis and expression, bacterial colonies on agar plates are obtained, each originating from a single cell (Scheme 3). This means that each bacterial colony produces only one mutant enzyme (although some may occur more than once on the agar plate). The bacterial colonies are then collected either manually by using toothpicks or automatically by a robot colony picker and are placed in the wells of microtiter plates that contain nutrient broth. In this way the supernatant of each well contains one mutant enzyme that is ready to be screened as a catalyst in the model reaction.

Upon generating a library of only 1000 mutants in the first generation, about 12 improved mutants were identified, the best one resulting in an *ee* value of 31 % ($E = 2.1$) in the test reaction. The process was repeated as outlined in Scheme 1 with formation of slightly larger libraries of mutants (2000–3000), an endeavor that led to an *ee* of 81 % in the fourth generation; this corresponds to a selectivity factor of $E = 11.3$ (Figure 1).[8]

Following these remarkable observations a larger library of mutant enzymes was created in the fifth generation, which led to further improvements.[17] However, we decided to develop more efficient ways to explore protein sequence space with respect to enantioselectivity in the given test reaction.[15] The basic problem relates to the fact that upon passing from one mutant generation to the next, many different "pathways" in protein sequence space are possible.

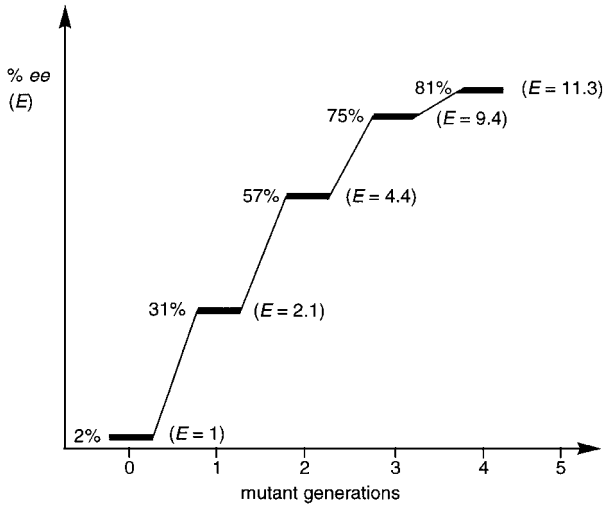
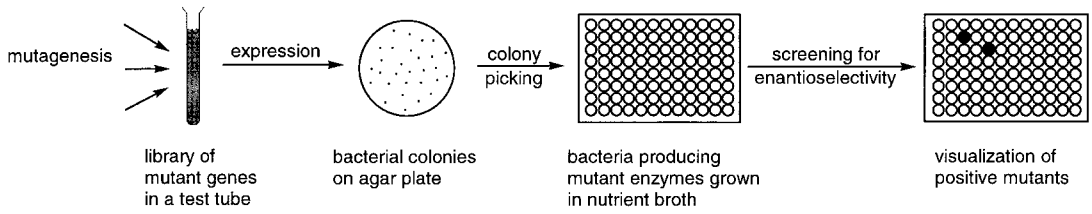



Figure 1. Increasing the *ee* and *E* values of the lipase-catalyzed hydrolysis of the chiral ester **1**.

Thus, as in natural evolution itself,[18] the analogy with a tree having many branches is useful (Figure 2). The challenge is to find the shortest possible route in climbing up the "*ee* tree" (or "*E* tree"). Parenthetically, the tree also has roots (not shown in Figure 2), symbolizing the evolution of mutants which catalyze the formation of the product that has the opposite absolute configuration. Although the cartoon in Figure 2 indicates considerable complexity, it also suggests that the solution to the problem of creating and finding a highly enantioselective catalyst for a given reaction is not unique. This means that it should be possible to obtain a set of different mutants, all with high degrees of enantioselectivity for a given reaction.

We therefore developed a strategy that not only works well in the present situation,[15, 17] but which may turn out to be useful in the case of other substrates and enzymes as well. Accordingly, DNA sequencing was first performed on the best members of the various mutant generations in order to define the position and nature of amino acid substitutions responsible for the increase in enantioselectivity. Typical data for the present case are presented in Scheme 4.

At this point the actual three-dimensional structure of the wild-type or mutant lipases was of no concern. Indeed, we did not even consider the enzyme mechanism! Rather, a logical way to proceed was to conclude that we had identified sensitive positions ("hot spots") in the protein that are instrumental in improving the enantioselectivity. Moreover, owing to the limitations of epPCR, it was reasonable to




Scheme 3. The experimental stages of directed evolution of enantioselective enzymes.

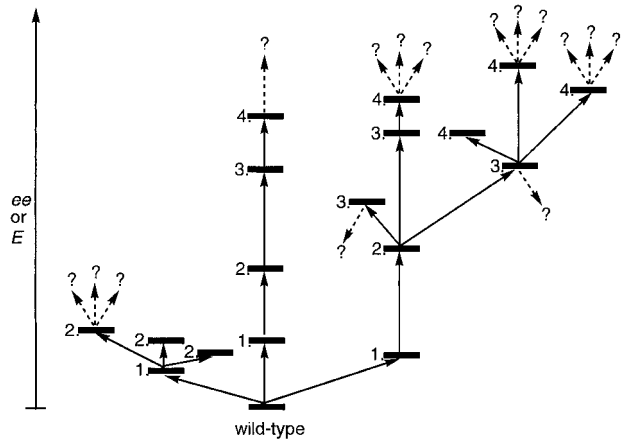



Figure 2. An "evolutionary tree" illustrating the complexity of protein sequence space with respect to enantioselectivity. The numbers denote positive mutants obtained from repetitive cycles of mutagenesis and screening in the respective generations. The arrows pointing down symbolize inferior variants which of course outnumber the few positive mutants.

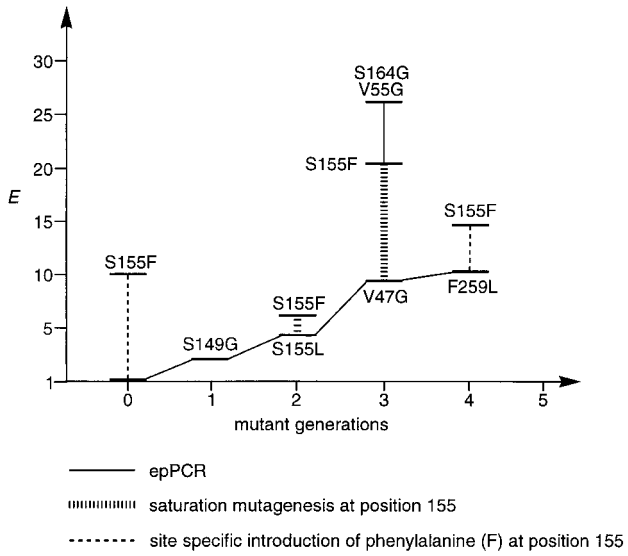



Figure 3. Further improvements in enantioselectivity of the lipase-catalyzed model reaction of **1** (S = serine; F = phenylalanine; G = glycine; L = leucine; V = valine). Details are outlined in ref. [17].

Mutant 01E4 (*ee* = 31 %): $Ser_{149} \longrightarrow Gly_{149}$

Mutant 08H3 (*ee* = 57 %): $Ser_{149} \longrightarrow Gly_{149}$; $Ser_{155} \longrightarrow Leu_{155}$

Mutant 13D10 (*ee* = 75 %): $Ser_{149} \longrightarrow Gly_{149}$; $Ser_{155} \longrightarrow Leu_{155}$; $Val_{47} \longrightarrow Gly_{47}$

Mutant 04H3 (*ee* = 81 %): $Ser_{149} \longrightarrow Gly_{149}$; $Ser_{155} \longrightarrow Leu_{155}$; $Val_{47} \longrightarrow Gly_{47}$; $Phe_{259} \longrightarrow Leu_{259}$

Scheme 4. Data of amino acid exchanges in the best mutants of the first four generations.

assume that the observed amino acid exchanges imply the correct position, but not necessarily the optimal amino acid. Thus, it seemed worthwhile to apply saturation mutagenesis[15] at one of these "hot spots", for example, at position 155, this being possible in any of the mutant generations or even the wild-type lipase. We began by applying saturation mutagenesis on the mutant gene by encoding the best variant with $E = 4.7$ in the second generation in which serine (S) has been substituted by leucine (L), that is, with O8H3. The best mutant enzyme in this newly formed library showed a slightly improved selectivity factor ($E = 5.3$) (Figure 3). The new amino acid at position 155 of this particular mutant turned out to be phenylalanine (F). Saturation mutagenesis at position 155 with the gene that encoded the most selective enzyme (with V47G) in the third generation led to the identification of an even better mutant ($E = 21$). In this case phenylalanine was again identified as the new amino acid at position 155. This seemed to indicate that position 155 is indeed a sensitive spot and that out of all the 20 natural amino acids phenylalanine has the greatest positive influence on enantioselectivity at this position (Figure 3). Thus, in order to minimize further screening efforts, we decided to introduce phenylalanine in the fourth generation and in the wild-type lipase by site specific mutagenesis. Indeed, significant improvements were observed (Figure 3). It then appeared logical to utilize any one of these mutants as the starting point for further rounds of epPCR. For the sake of clarity and illustration we show only part of the data that is typical for this type of strategy, namely the result of epPCR in the third generation leading to a mutant with an $E$ value of 25 ($ee = 90\,\%$).[17]

Although optimization of enantioselectivity for the model reaction has not been finalized,[17] we conclude that the *combination* of epPCR and saturation mutagenesis constitutes an efficient way to explore protein sequence space with respect to enantioselectivity. Indeed, this strategy led to the creation of several other highly *S*-selective mutant lipases ($ee = 90-93\,\%$; $E > 20$),[15, 17] all of them being the descendents of the parent wild-type lipase, which has an *ee* of only 2 % ($E = 1$). An equally challenging task is to start the process over again and to try to evolve *R*-selective mutants. Indeed, preliminary efforts prove that inversion of enantioselectivity is in fact possible, although optimization has not yet been carried out.[15] Several *R*-selective mutants have been obtained with *ee* values of 10–28%.

Our results clearly demonstrate that the use of directed evolution in the creation of an enantioselective enzyme does not result in a single unique mutant, but in an array of structurally related mutants that have high enantioselectivities. This corresponds to the expectations indicated in the top part of Figure 2. As preliminary results demonstrate, the combination of epPCR and DNA shuffling is likewise successful in generating families of enantioselective enzymes,[15] and in fact may turn out to be especially efficient. We therefore re-emphasize our previously made suggestion that various combinations of different types of mutagenesis constitute practical methods of exploring protein sequence space with respect to enantioselectivity.[15] When striving for



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industrial viability in which the optimization of several different parameters such as enantioselectivity, activity, and stability (e.g., with respect to temperature, pH and type of solvent) is required, additional challenges arise. In such cases it may be necessary to devise an evolutionary strategy in which these parameters are not optimized simultaneously in each generation of mutants, but alternately upon passing from one round of mutagenesis to the next. Molecular breeding, such as DNA family shuffling,[19] should also be tested when attempting to maximize enantioselectivity, activity, and stability all in one system.

The screening system that we used in the model reaction is evidently restricted to the kinetic resolution of chiral esters. In order to be able to study other substrates and other enzymes, we have started to develop further screening systems. One of them makes use of black-body radiation as a function of chirality, a process in which an appropriate IR-thermographic camera detects differences in heat evolution in reactions of *R* and *S* substrates.[20] Another novel approach is based on the use of isotopically labeled substrates in the form of pseudo enantiomers or pseudo prochiral compounds.[21] Generally, deuterium labels are employed. The course of the enzyme-catalyzed asymmetric transformation is then detected by electrospray ionization spectrometry (ESI-MS), a process which enables the exact determination of enantioselectivity in about 1000 reactions per day. Two basically different stereochemical processes can be monitored by this method: kinetic resolution of racemates and asymmetric transformation of substrates that are prochiral due to the presence of enantiotopic groups.[21]

Finally, research in this area has another facet that is just as important and fascinating as the practical goal of creating enantioselective enzymes for use in organic synthesis, namely the question of structure–enantioselectivity relationships in the general area of enzyme catalysis. Since the X-ray structural analysis of the lipase from *P. aeruginosa* was recently completed, we were put into the fortunate position of being able to see just where in the protein the *ee*-improving substitutions have occurred. It turned out that the "hot spots" are located on the surface of the enzyme,[17] far removed from the catalytical triad[16] that consists of the residues serine, histidine, and aspartate and is buried inside the complex three-dimensional structure.[22] Moreover, the sensitive positions occur in loops, which are flexible regions of the amino acid chain. We also note that among the newly introduced amino acids glycine appears quite often. These conspicuous observations point to an interesting aspect that was not obvious at the outset of our studies. Since the presence of glycine generally increases the flexibility of an enzyme, it may be that such a conformational change, if induced at the proper position in the amino acid chain, results in an increase in enantioselectivity.[17] Currently, we do not know the exact three-dimensional structure of the mutants that have improved enantioselectivity. Hopefully, X-ray analyses or perhaps even molecular modeling and/or molecular dynamics calculations will shed light on this intriguing question. The stepwise "evolution" of a stereorandom enzyme towards a truly enantioselective variant will then be visible on a structural level.

## Conclusions

In summary, we have presented proof of principle with respect to the use of directed evolution in the creation of enantioselective enzymes for application in organic synthesis.[8, 15–17, 23] The particular example that we have described here pertains to the kinetic resolution of a racemic substrate. However, the general principle is not restricted to kinetic resolution, since reactions involving the transformation of prochiral substrates into chiral products are just as relevant. Our approach is independent of structural or mechanistic thinking. Owing to the evolutionary nature of the concept, it goes far beyond combinatorial catalysis.[24] A number of challenges remain in this new field of endeavor; these include the development of further high-throughput screening systems for enantioselectivity[25] (e.g., by applying phage display),[26] the use of other substrates, the study of different types of enzymes[27] (e.g., oxidases, reductases, aldolases, etc.), the development of even more efficient ways to explore protein sequence space (e.g., by the use of genetic algorithms), and the systematic creation of enzyme libraries.[23] We anticipate that it should be possible to turn one enzyme type in another and to use the newly evolved enzymes in enantioselective reactions, or to create mutant enzymes by directed evolution that catalyze asymmetric reactions not found in nature (e.g., Diels–Alder reactions).[28] The idea of applying selection instead of screening in the creation of enantioselective enzymes also needs to be pursued.[15] It will be interesting to compare such upcoming developments with the possibility of utilizing evolutionary techniques in the fabrication of enantioselective catalysts based on nucleic acid structures,[29] as well as with the perspectives that catalytic antibodies offer.[9c] Finally, we expect that subsequent to obtaining highly enantioselective enzymes, studies directed towards an understanding of the structure–stereoselectivity relationship of mutants will enrich our knowledge of how enzymes function.

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