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Biocatalysis in Organic Chemistry and Biotechnology: Past, Present, and Future

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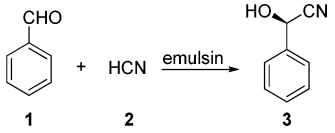
ABSTRACT: Enzymes as catalysts in synthetic organic chemistry gained importance in the latter half of the 20th century, but nevertheless suffered from two major limitations. First, many enzymes were not accessible in large enough quantities for practical applications. The advent of recombinant DNA technology changed this dramatically in the late 1970s. Second, many enzymes showed a narrow substrate scope, often poor stereo- and/or regioselectivity and/or insufficient stability under operating conditions. With the development of directed evolution beginning in the 1990s and continuing to the present day, all of these problems can be addressed and generally solved. The present Perspective focuses on these and other developments which have popularized enzymes as part of the toolkit of synthetic organic chemists and biotechnologists. Included is a discussion of the scope and limitation of cascade reactions using enzyme mixtures in vitro and of metabolic engineering of pathways in cells as factories for the production of simple compounds such as biofuels and complex natural products. Future trends and problems are also highlighted, as is the discussion concerning biocatalysis versus nonbiological catalysis in synthetic organic chemistry. This Perspective does not constitute a comprehensive review, and therefore the author apologizes to those researchers whose work is not specifically treated here.

1. SELECTED MILESTONES OF THE PAST

Humans have utilized enzymes for thousands of years in the form of fermentation as a means to produce and preserve foodstuffs such as cheese, beer, vinegar, and wine. In more modern times it was Louis Pasteur who in 1858 placed a milestone in biocatalysis by treating an aqueous solution of racemic tartaric acid ammonium salt with a culture of the mold *Penicillium glaucum*, leading to the consumption of (+)-tartaric acid and concomitant enrichment of the (−)-enantiomer.[1] This can be considered to be a forerunner of enzyme-catalyzed kinetic resolution as it is widely practiced today in academia and industry.[2] Pasteur's publication was followed by Emil Fischer's monumental carbohydrate research which culminated in the lock-and-key hypothesis of stereoselective enzyme catalysis in 1894.[3a] One hundred years later Albert Eschenmoser[3b] and Friedrich Lichtenthaler[3c] wrote enlightening essays which illuminated the impact of Fischer's hypothesis on modern chemistry, biochemistry, and immunology. Linus Pauling's hypothesis regarding the stabilization of transition states of enzyme-catalyzed reactions[4a] is an extension of Fischer's idea, as is Daniel Koshland's induced fit model.[4b]

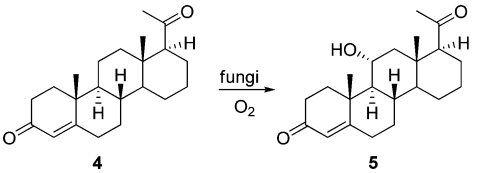
Another cornerstone of biocatalysis was laid by Eduard Buchner who in 1897 reported the successful fermentation of sugar by cell-free yeast extracts, which constitutes indisputable proof that biological transformations do not necessarily require living cells.[5a] This paradigm shift opened the door to modern biocatalysis including fermentation technology for the production of a vast array of achiral and chiral products.[2] As noted by Lothar Jaenicke,[5b] Buchner's contribution was recognized by another master of organic chemistry at the time, Adolf von Baeyer, who stated that "*This will make him famous, even if he doesn't have any talent for chemistry.*"

In the first half of the 20th century scientists in academia and industry learned how to use whole cells, cell extracts, or partially purified enzymes in various biocatalytic processes. In what can be called the beginning of modern enzyme-mediated asymmetric catalysis, Ludwig Rosenthaler described in 1913 the preparation of (*R*)-mandelonitrile (**3**) by treating benzaldehyde



(**1**) with HCN (**2**) in the presence of emulsin extracted from bitter almonds (a mixture of enzymes including oxynitrilase).[6a] Today both *R*- and *S*-selective oxynitrilases are commercially available which catalyze the asymmetric cyanohydrin formation of a variety of structurally different aldehydes and ketones (enantiomeric excess (ee) > 95%).[6b,c] These are useful starting materials in the synthesis of numerous chiral products as demonstrated by Franz Effenberger and others.[2,6b,c]

Yet another landmark that likewise set the stage for present day research and technology concerns the use of microbial strains that catalyze the regio- and stereoselective oxidative hydroxylation of steroids, as first demonstrated in the early 1950s by such pharmaceutical companies as Upjohn, Schering, Pfizer, and Merck. A key example is the oxidative hydroxylation of readily available progesterone (**4**) to the 11α-product **5** by



Rhizopus arrhizus or *Aspergillus niger* strains,[7] a compound that was easily transformed into such important commercial

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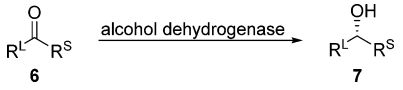
products as cortisone and hydrocortisone by chemical means (the latter with inversion of configuration at C11). Later it was shown that oxidations of this kind involve selective catalysis by cytochrome P450 enzymes.[2,7b] The combination of biocatalytic and chemical synthetic steps offered a practical alternative to Robert B. Woodward's 1952 landmark 40-step synthesis of hydrocortisone.[8]

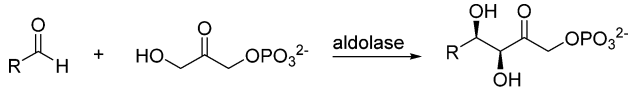
In the mid-1950s Vladimir Prelog began his seminal studies on the use of microbial strains as catalysts for the stereoselective reduction of ketones **6** (L = large; S = small), which



6 **7**

ultimately led to the Prelog rule, a mnemonic device for predicting the stereochemical outcome of such reactions.[9a] Subsequently the relevant enzymes, alcohol dehydrogenases, were isolated, structurally characterized, and used in vitro as catalysts in asymmetric reduction of prochiral ketones.[2,9b] Today a number of Prelog and anti-Prelog alcohol dehydrogenases are commercially available. Due to the high price of NAD(P)H, cofactor regeneration systems had to be developed for practical applications.[2]

Techniques for isolating and purifying enzymes were optimized in the middle of the 20th century, which were then employed as biocatalysts in stereoselective transformations of unnatural substrates. Nevertheless, these early examples were more or less isolated cases which were ignored by most organic chemists. The pace of methodology development regarding biocatalysts in synthetic organic chemistry gradually increased in the 1960s, 1970s, and 1980s, parallel to advances in uncovering the mechanisms of various classes of enzymes as well as elucidating biosynthetic pathways. Only a few of many prominent pioneers are named here, and the reader is referred to full accounts elsewhere.[2] For example, the groups of Brian Jones, Klaus Kieslich, Maria-Regina Kula, and George Whitesides, among others, published seminal work which helped to popularize enzyme catalysis, although at the time many synthetic organic chemists remained skeptical. An impressive example was provided by the Whitesides group describing the use of an aldolase as the catalyst in stereoselective aldol addition of ketone **9** to



8 **9** **10**

aldehydes **8** with stereoselective formation of adducts **10**.[10] Synthetic nonbiological chiral catalysts rivaling aldolases as catalysts in these kinds of aldol reactions have not been developed to date.

Another important contribution was made by Alexander Klibanov, who showed that enzymes such as lipases retain some (but not all) of their activity in organic solvents.[11] This proved to be particularly important in those transformations which are problematic in aqueous media, e.g., lipase-catalyzed stereoselective formation of chiral esters, the stereogenic center being either in the alcohol or the acid portion of the esterification products.[2]

2. THE ADVENT OF RECOMBINANT DNA METHODS

A serious problem in enzyme catalysis persisted until the end of the 1970s, namely how to obtain proteins in sufficient quantities for practical applications in a general manner. Traditionally, enzymes were isolated from their respective sources, these being microorganisms, fungi, insects, plants, or mammalian species. However, this was often tedious, and in many cases reasonable amounts were not accessible. *This long-standing limitation constituted one of the major obstacles to generalizing biocatalysis in synthetic organic chemistry and biotechnology.* The breakthrough, catalyzed by the work of Paul Berg, Herbert Boyer, and Stanley Cohen, came with the development of recombinant DNA methododology, according to which a given enzyme occurring in one organism can be overexpressed in another host organism.[12] *Escherichia coli* and *Bacillus subtilis* are typical host organisms.

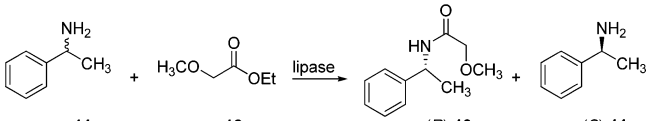
In this way large quantities of enzymes became accessible, sometimes in ton amounts, an example being protein-based detergents produced by such companies as Henkel (Germany) or Novozyme (Denmark). In some cases enzymes cannot be expressed in the usual bacterial hosts, therefore requiring the development of alternative expression systems.[2] Biotechnological engineering of reactors, enzyme immobilization, and methods for the optimization of downstream workup contributed heavily to making biocatalysis practical and industrially viable.[2,13]

The polymerase chain reaction (PCR) developed by Kary Mullis in 1983 rapidly emerged as an indispensable tool in molecular biology,[12,14] its use in protein engineering being one of many areas of application.

3. SELECTED EXAMPLES OF INDUSTRIAL PROCESSES BASED ON ENZYMES

By the 1990s many enzymes were commercialized, and a number of industrial processes were operating, most often for producing fine chemicals such as chiral compounds required in the synthesis of pharmaceuticals, plant protecting agents, and fragrances.[2,13] Numerous fermentation processes for obtaining chiral compounds in enantiomerically pure form had also been established, e.g., in the large-scale production of L-amino acids.[2,13]

The monograph by A. Liese, K. Seelbach, and C. Wandrey illustrates numerous industrial applications of enzymes up to 2006 by including informative reaction flow-sheets and the applied reactor technology.[13a] Other reviews likewise dispel myths regarding biocatalysis.[13b−g] One of numerous examples is the BASF process for the production of chiral amines in enantiomerically pure form.[15] Racemic amines such as *rac*-**11**



rac-**11** **12** (*R*)-**13** (*S*)-**11**

are acylated with methoxyacetic acid ethyl ester (**12**), an appropriate lipase serving as the catalyst in kinetic resolutions characterized by very high selectivity factors (E > 200), depending upon the particular structure of the starting amine.[15] More recently a system for performing dynamic kinetic resolution of chiral amines using a racemizing Ru catalyst in combination with a lipase was developed by Bäckvall et al. which has the important advantage of enabling full conversion and high enantioselectivity,[16a] similar to the prior development of dynamic kinetic resolution of chiral alcohols and diols.[16b,c] The advantages of dynamic kinetic resolution using other enzymes have been reported by Faber et al. in a number of studies which greatly enrich synthetic organic chemistry.[16d] The same holds true for biocatalytic deracemization procedures.[16d−f]

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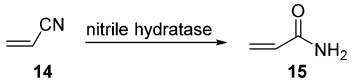
Other examples of industrial uses of enzymes as catalysts in medium- and large-scale production of fine and specialty chemicals include the preparation of semisynthetic β-lactam antibiotics such as Cephalexin, of (*S*)-*tert*-leucine, and of ephedrine.[2,13] An example of a bulk chemical produced by enzymatic catalysis concerns the partial hydrolysis of acrylonitrile (**14**) with formation of ultrapure acrylamide (**15**) catalyzed by a



14 **15**

nitrile hydratase as developed by Nitto Chemical Industry Company. The Japanese plant operates at a capacity of 4×10^5 tons/year.[2a,17a] Lonza (Switzerland) uses a nitrile hydratase to convert 3-cyanopyridine into nicotinamide (6×10^3 tons/year).[2,17b]

4. TRADITIONAL LIMITATIONS OF ENZYMES AS CATALYSTS IN SYNTHETIC ORGANIC CHEMISTRY AND BIOTECHNOLOGY

Despite these successes, enzymes as catalysts in synthetic organic chemistry and biotechnology continued to suffer from the following traditional limitations, namely the often observed

- insufficient stereoselectivity
- poor regioselectivity
- narrow substrate scope (or low rate)
- insufficient stability under operating conditions
- product inhibition

These long-standing drawbacks not only limited the application of enzymes in a practical way but also resulted in a (psychological) barrier in the minds of many organic chemists regarding biocatalysis in general.

5. EARLY ATTEMPTS TO EXPAND THE UTILITY OF ENZYMES

Many attempts at solving at least some of the above problems were undertaken in the 1980s. Rational design based on site-specific mutagenesis, a genetic method introduced in 1978 by Michael Smith for replacing a given amino acid in a protein by one of the other 19 canonical representatives,[18] paved the way for manipulating the structure and catalytic properties of enzymes.[19] Rational design proved to be (partially) successful in studies aimed at enhancing the thermostability of proteins.[19,20a,b] Amino acid substitutions were designed which led to new intramolecular H-bonds on the protein surface, thereby helping to prevent undesired unfolding upon heat treatment. Site-specific mutagenesis for improving stereoselectivity, a more challenging task, was shown to be successful in some cases,[19,20c,d] but due to the complexity of enzymes, generalization has not been accomplished to date. Some researchers in the 1980s generated libraries of mutants using mutating chemical agents, mutator strains, or even genetic methods and screened these for enhanced thermostability or activity.[21] However, a second mutagenesis/screening cycle, which would have exerted "evolutionary pressure", was not considered (with one exception).[21d]

In the mid-1980s the first examples of catalytic antibodies were published independently by Richard Lerner and Peter Schultz, which sparked a flurry of activities toward a possible alternative to enzymes.[22] Many fascinating studies of a variety of reaction types have appeared.[22d] Nevertheless, generalization to the point of real practical applications of these protein-based catalysts has not been achieved. Most of them are quite sensitive. Moreover, the mechanism of an immune system in which the antibodies are produced involves binding properties, not catalysis. Optimal binding requires shape complementarity but not necessarily activating effects in the respective transition states necessary for low-energy reaction pathways in catalysis. Therefore, the activity of catalytic antibodies is generally low.

6. THE ADVENT OF DIRECTED EVOLUTION

Today all of the problems listed in section 4 can be addressed by applying a different protein engineering technique called directed evolution (or laboratory evolution).[19,23] It involves repeating cycles of gene mutagenesis, expression, and screening (or selection) of mutant enzyme libraries in an overall process which simulates natural evolution. The most common gene mutagenesis methods for generating mutant libraries are

- error-prone polymerase chain reaction (epPCR)
- saturation mutagenesis
- DNA shuffling

Since epPCR targets the whole gene and therefore the entire protein, it is a kind of shotgun method, although amino acid bias exists.[19,24a] It is the most often used technique and does not require structural information about the enzyme. In a seminal study published by Frances Arnold in 1993, several rounds of epPCR were applied in order to increase the robustness of the protease subtilisin E toward the hostile solvent dimethylformamide.[24b] Later this approach was

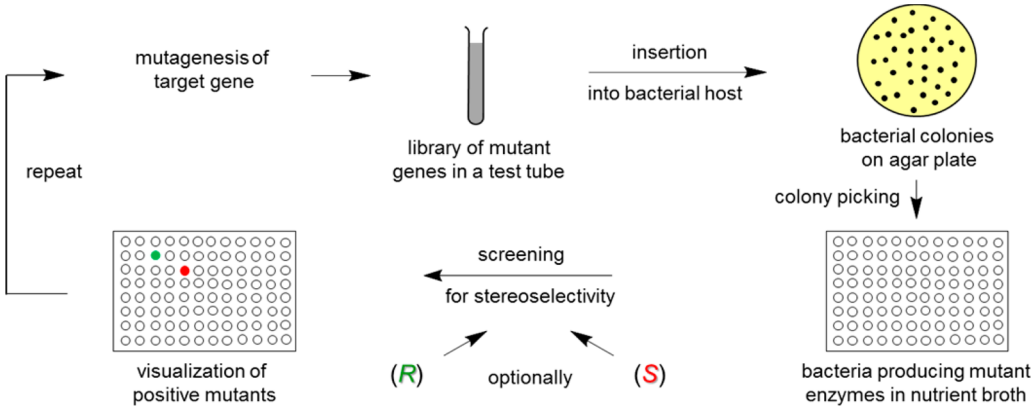


Figure 1. Scheme illustrating laboratory evolution of stereoselective enzymes,[26a] adapted from ref 23a. The green and red dots on the bottom left 96-well microtiter plate indicate an *R*- and *S*-selective mutant, respectively. Yeasts can also serve as hosts.

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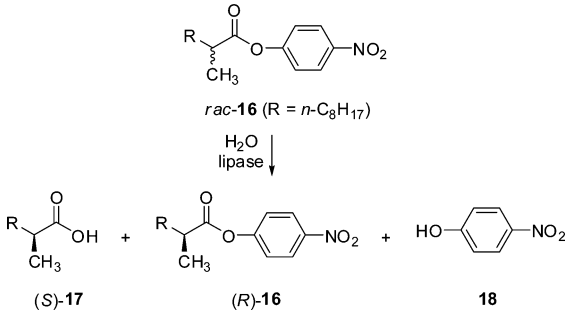
extended by many groups to include the enhancement of thermostability using epPCR and other genetic techniques.[19,25]

As synthetic organic chemists, my group became interested in laboratory evolution back in 1995 because we wanted to control a catalytic parameter which is at the heart of modern organic synthesis, namely stereoselectivity. As schematized in Figure 1, it is a logical strategy for generating enzyme mutants as catalysts in asymmetric transformations.[26] Each cycle exerts evolutionary pressure on enantioselectivity, the experimenter targeting either the desired *R*- or the *S*-product on an optional basis. At the outset it was not clear whether directed evolution would be sensitive enough to be successful in this endeavor. Moreover, assaying thousands (or more!) of mutants for enantioselectivity posed yet another problem, because methods for determining so many ee values did not exist at the time. Several medium- and high-throughput ee-screening systems were developed, examples being UV/vis-based systems using plate readers for 96-well microtiter plates (~700 samples/day)[26a] or the more expensive multiplexed MS-based assay utilizing isotope-labeled substrates (up to 10 000 exact ee values/day).[27a] No single screening system is universal, but fortunately today a variety of different ee assays are available, including automated GC and HPLC.[27]

In 1997 proof-of-principle of this new approach to asymmetric catalysis was provided for the first time,[26a] the lipase from *Pseudomonas aeruginosa* serving as the catalyst in the hydrolytic kinetic resolution of the chiral ester *rac*-**16** with preferential formation of (*S*)-**17**. Wild-type (WT) lipase is only marginally *S*-selective, the selectivity factor amounting to only $E = 1.2$.



Four rounds of epPCR at low mutation rate with the introduction of a single point mutation in each generation improved enantioselectivity to $E = 11$ (*S*) (Figure 2). Shortly thereafter other genetic methods were applied, resulting in notably improved mutants (see below).[26b]

This Darwinian approach to creating catalysts for asymmetric transformations in organic chemistry is fundamentally different from techniques traditionally used in designing and synthesizing chiral transition metal catalysts or organocatalysts. As delineated in section 7, this initial example set the stage for numerous subsequent studies employing essentially all major types of enzymes: hydrolases, reductases, oxidases, transferases, lyases, isomerases, and ligases (formation of C–C, C–O, C–S, or C–N bonds), progress up to 2004 being summarized elsewhere.[19,23,26c]

In contrast to epPCR, oligonucleotide-based saturation mutagenesis involves combinatorial randomization at predetermined sites, a given site comprising one or more residues in the enzyme.[19,23,26b,d] Traditionally, this entails the introduction of all of the 20 canonical amino acids at an appropriately

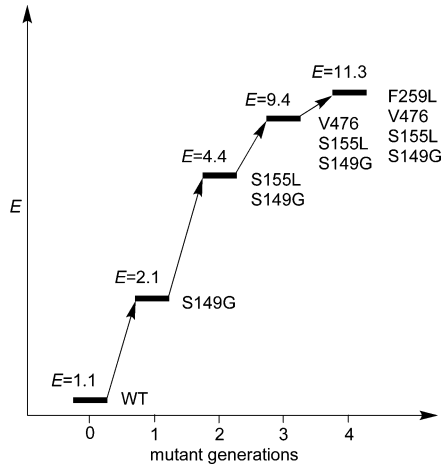


Figure 2. First example of directed evolution of an enantioselective enzyme:[26a] *P. aeruginosa* lipase as the catalyst in the hydrolytic kinetic resolution of *rac*-16 and epPCR at low mutation rate as the gene mutagenesis method, adapted from ref 26c.

chosen site with formation of focused mutant libraries. Today the so-called QuikChange protocol from Stratagene is routinely used which is based on numerous previous studies (Figure 3).[28]

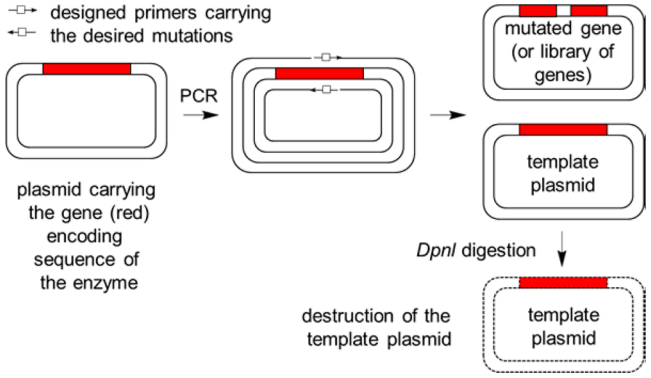


Figure 3. Steps involved in saturation mutagenesis according to the QuikChange protocol, adapted from ref 28.

Simply speaking, the genetic information encoding the mutational changes is registered in the designed primers (appropriate oligonucleotides) which are prepared (commercially) and subsequently introduced into a plasmid.

When applying saturation mutagenesis, a choice regarding the optimal randomization sites has to be made. When targeting stereo- or regioselectivity, substrate scope, and/or rate, sites around the enzyme's binding pocket constitute logical choices. The first example of an enantioselective mutant enzyme generated by saturation mutagenesis at a site lining the binding pocket concerns the aforementioned lipase-catalyzed kinetic resolution of *rac*-**16**, in which a four-residue site in the lipase PAL was randomized followed by the screening of 5000 transformants.[26b] This resulted in the discovery of an *S*-selective mutant ($E = 30$). Later a convenient acronym was introduced to describe such a procedure, CAST ("combinatorial active-site saturation test").[29] Sites comprising one or more amino acid positions are identified on the basis of the X-ray structure or homology model (Figure 4). It is a way to reshape the "lock" in Emil Fischer's lock-and-key hypothesis.[3] Guidance by bioinformatics such as the consensus approach which had

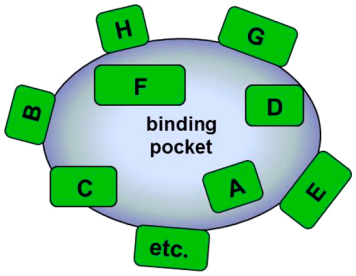


Figure 4. General scheme for CASTing in the quest to enhance stereoselectivity,[29] adapted from ref 23a. The sites A, B, C, etc. comprise one or more amino acid positions.[29]

previously been used for protein thermostabilization[30a] can be helpful.[30b,c] As delineated in section 7, the real power of this strategy begins when using it in the form of iterative saturation mutagenesis (ISM).

Saturation mutagenesis can also be applied when aiming to enhance protein thermostability or resistance to denaturing organic solvents, in this case *B*-factors from X-ray data being necessary. Those residues having the highest average *B*-factors, reflecting maximum flexibility, are chosen for saturation mutagenesis iteratively.[31] The overall process, called B-FIT, is an alternative to conventional approaches based on epPCR, DNA shuffling, or rational design.[19,25]

A final gene mutagenesis method featured here is DNA shuffling, introduced in 1994 by the late Pim Stemmer, who used a β-lactamase as the model enzyme in the successful attempt to increase its activity.[32a] Several versions including family shuffling are possible.[32b] Homologous genes encoding the respective enzymes are first digested with a DNase to yield double-stranded oligonucleotide fragments generally defined by 10−50 base pairs, which are amplified by PCR. Repeated cycles of strand separation and reannealing by an appropriate DNA polymerase followed by final PCR amplification ensure the reassembly of full-length mutant genes. The case of shuffling mutants prepared previously by some procedure is illustrated in Figure 5. Synthetic shuffling was also developed, in which

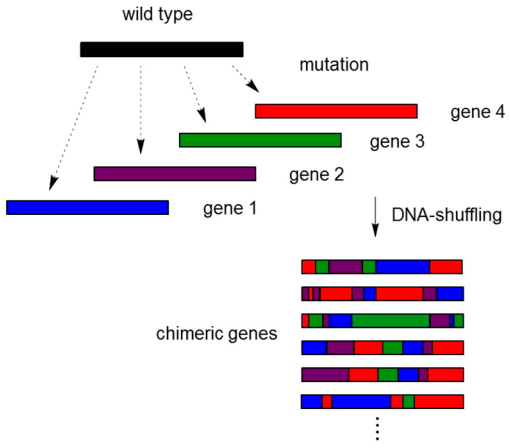
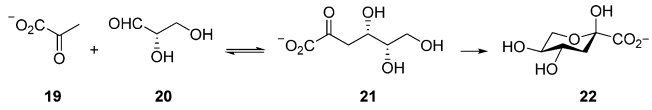


Figure 5. Scheme illustrating DNA shuffling,[32] the example featuring the case of shuffling mutants of a given enzyme.

designed DNA fragments are first prepared separately and subsequently assembled into full length genes.[19]

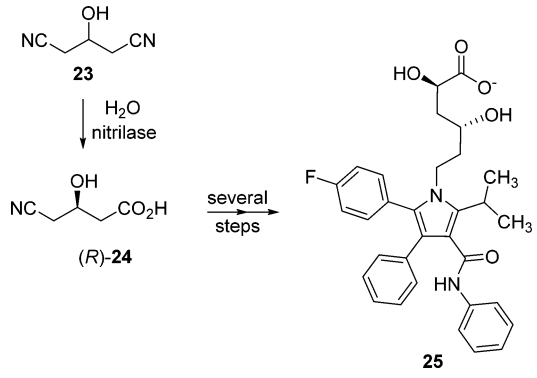
By 2004, directed evolution had emerged as a general method for optimizing the stereoselectivity of enzymes.[26c] In most cases epPCR and DNA shuffling were the mutagenesis methods of choice, saturation mutagenesis playing only a minor role. The lipase-catalyzed kinetic resolution of *rac*-**16** served as a platform for comparing different approaches. At that time the combination of high error rate epPCR, DNA shuffling, and selected saturation mutagenesis led to a mutant of *P. aeruginosa* lipase having a selectivity factor of E = 51 (S) in the kinetic resolution of *rac*-**16**, a procedure that required the screening of about 50 000 transformants.[26b] Reversal of enantioselectivity in favor of (R)-**2** was also achieved (E = 30).[26c]

Other prominent examples include the directed evolution of stereoselective aldolases,[33] Baeyer−Villiger monooxygenases,[34] nitrilases,[35] and monoamine oxidases.[36] In an early study regarding the aldolase KDPG as a catalyst in the reaction of **19** with **20**, several rounds of epPCR and DNA shuffling provided



a mutant characterized by four point mutations (T84A, I92F, V118A, and E138V) showing perfect control of diastereoselectivity in the formation of **21**, which is a precursor of the sugar **22**.[33a] Directed evolution has since pervaded the entire area of aldolases in a synthetically intriguing manner.[33c] The reaction shown here and numerous other examples demonstrate the complementarity of biocatalysis and catalysis using transition metal complexes or organocatalysts.

Two industrial examples of laboratory evolution are also highlighted here, further examples covering the period up to 2004 and 2008, respectively, being reviewed elsewhere.[26c,27c] The first case concerns the directed evolution of a nitrilase as a catalyst for the desymmetrization of the prochiral dinitrile **23** with formation of (R)-**24** as reported by scientists at Diversa



(Verenium).[35] The product is an intermediate in the synthesis of the cholesterol-lowering pharmaceutical Lipitor (**25**). The patents for this multi-blockbuster (>$12 billion/year) have recently expired, which means that various companies are devising cheap productions of **25** for the generic market. Genomic libraries from environmental samples were first screened for this transformation, and on a small scale a nitrilase showing 94.5% ee in favor of (R)-**24** was discovered. However, when the reaction was performed at a concentration of 2.25 M necessary in a practical process, activity was strongly reduced, indicating product inhibition. Saturation mutagenesis was applied at all 330 amino acid positions individually, the respective 330 mutant libraries being screened with an MS assay[27a] using ^{15}N-labeled substrate.[35] After screening about 32

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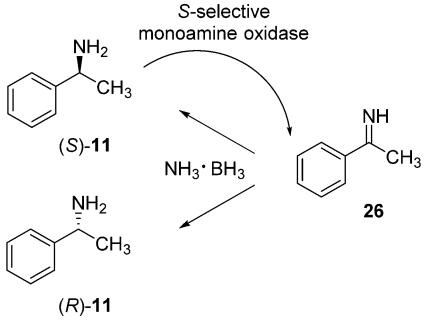
000 transformants, more than a dozen hits were identified. The best one leads at high 3 M concentration to 96% conversion and 98.5% ee. *This is a nice example of solving the problem of product inhibition*, while simultaneously enhancing enantioselectivity.

The second example featured here concerns the industrial production of chiral amines based on directed evolution of a monoamine oxidase, a clever de-racemization process developed by Turner et al. and commercialized by Ingenza.[36] Either *R*- or *S*-configured amines such as **11** are accessible from the



respective racemate in high enantiomeric purity, depending upon the stereoselectivity of the applied monoamine oxidase (*S*- or *R*-selectivity). Interestingly, a mutator strain was used as the mutagenesis system, although the company also utilizes genetic techniques such as saturation mutagenesis. This elegant de-racemization strategy is an alternative to the BASF lipase-based process for obtaining enantiomerically pure or enriched amines[15] (section 3). Moreover, the procedure works very well even for chiral secondary and tertiary amines, which is an impressive achievement not readily possible using other technologies.[36c,d]

7. CHALLENGES IN MAXIMIZING THE EFFICACY AND SPEED OF DIRECTED EVOLUTION

Some degree of catalyst improvement can always be expected from directed evolution, depending upon how much lab work the experimenter is willing to invest. To this day screening is the bottleneck of directed evolution.[19,23,27] Unfortunately, few studies focus on systematic comparisons, which means that it often not clear which mutagenesis strategy is in fact optimal.[37] In an early example concerning the transformation of a β-galactosidase into a β-fucosidase, Matsumura et al. demonstrated that saturation mutagenesis is more efficient than DNA shuffling, but the authors were careful not to generalize.[37a] In the case of the lipase-catalyzed kinetic resolution of *rac*-**16** (section 6), the best strategy available in 2001 utilized mainly epPCR and DNA shuffling,[26c] but a subsequent QM/MM study predicted that of the six point mutations of the best mutant showing E = 51, four are superfluous.[38] Indeed, the double mutant was generated and shown to be even more selective (E = 63). While this was a triumph of theory, it indicated inefficiency of the mutagenesis procedure which had been applied. Extensive deconvolution of all point mutations of a variant in a given enzyme system is necessary to uncover any superfluous mutations, but this is rarely done.[23a]

Efficacy would not be so crucial if selection instead of screening systems could be used in a general way in which the host organism experiences a growth advantage because it houses improved mutant enzymes of interest to the experimenter, as pointed out by Hilvert,[39a] Cornish,[39b] Tawfik,[39c] and others.[19] However, developing experimental platforms for dealing with stereo- and/or regioselectivity is exceedingly difficult, and thus far only two attempts have been reported. They proved to be moderately succcessful.[40]

In more recent times, several groups have addressed the crucial question of efficacy by dedicating research to the *development of methods which ensure higher-quality mutant libraries, these being smaller and requiring less screening*.[23] Ideally, the size of individual mutant libraries should not exceed 2000−3000 transformants, because such numbers can be handled within a reasonable time by modern automated GC or HPLC, making more expensive instrumentation superfluous.[27a] The challenge in reaching this goal is the numbers problem in directed evolution, which relates to the vastness of protein sequence space. Several approaches have been published as summarized elsewhere.[19,23] However, in most of these endeavors comparisons with alternative strategies were not made, and in the majority of proposals the respective methods have not been tested by other groups.

Impressive industrial studies have appeared in the academic literature reporting active and selective mutants,[19,23k] but sequence data characterizing the mutants and even the mutagenesis procedure are often not revealed.[23l] A number of bio-companies offer enzyme kits which contain mutants generated by directed evolution, but information as to the sequence or structure of the biocatalysts is generally not provided. For practical purposes this may not be important, but such information would be essential for interpreting the results upon employing such enzyme mutants.

Following its systematization in 2006,[41a] ISM has emerged as a particularly efficient method in directed evolution which can be applied for improving different types of catalytic parameters.[23a,41b] When considering rate, substrate scope, and stereo- and/or regioselectivity, it constitutes a rational extension of the original CASTing approach for enantioselectivity which relies on structural biology[29] (Figure 4, section 6). The chosen randomization sites A, B, C, etc. around the binding pocket are subjected to saturation mutagenesis with the creation of first generation focused libraries. A hit from one library is then used as a template for randomization at the other sites, and the process is continued until all sites have been visited once in a given upward pathway. The simplest case is a two-site ISM system with two pathways A → B and B → A, respectively, but three- and four-site systems featuring 6 and 24 different upward pathways, respectively, have also been used (Figure 6). Thus, CASTing in

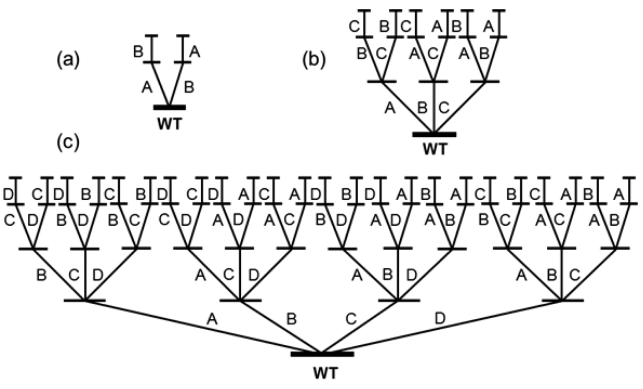


Figure 6. Schemes of two-site (a), three-site (b), and four-site (c) ISM systems featuring 2, 6, and 24 upward pathways, respectively, the vertical axis symbolizing an improved enzyme property such as stereoselectivity or thermostability, adapted from refs 23a and 41.

the form of ISM is a systematic approach to reshaping the binding pocket of an enzyme, in contrast to earlier studies in

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which saturation mutagenesis was repeated in a nonsystematic manner, sometimes alternating with epPCR or combined with DNA shuffling.[26b,d]

Different choices can be made regarding the number of residues in a site, how to group them, and which pathway to choose. Thus far, arbitrary choices were made in more than two dozen successful studies, suggesting that such decisions are not crucial. Nevertheless, these questions are the focus of current research, and one trend is apparent: It is more efficient to choose sites comprising more than one amino acid position, which raises the question of oversampling in the screening process.[23a] This statistical issue has been addressed[23a,41b,42] using the Patrick–Firth statistical algorithms[42a] as incorporated in the computer aid CASTER which is useful in the design of saturation mutagenesis libraries (available free of charge on the author's homepage, www.kofo.mpg.de/de/forschung/organische-synthese). The degree of oversampling for covering 95% of a given library depends upon the number of residues in a site and upon the chosen codon degeneracy, the latter defining the (reduced) amino acid alphabet.[43] When using NNK codon degeneracy (N = adenine/cytosine/guanine/thymine; K = guanine/thymine) encoding all 20 canonical amino acids, it can be seen that the number of transformants that need to be screened for essentially full library coverage increases dramatically as the number of residues in a site increases (Table 1). In contrast, when using a reduced amino acid alphabet such as NDT codon degeneracy (D = adenine/guanine/thymine; T = thymine) encoding only 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly) as building blocks in the randomization process, the screening effort is reduced notably, truly drastic effects occurring when using even smaller alphabets.[23a,30b,41b]

Table 1. Oversampling Necessary for 95% Coverage as a Function of NNK and NDT Codon Degeneracy Calculated on the Basis of Conventional Statistics[23a,41b,42]

	NNK		NDT	
no. of amino acid positions at one site	codons	transformants needed	codons	transformants needed
1	32	94	12	34
2	1028	3066	144	430
3	32 768	98 163	1728	5175
4	1.05×10^6	3.14×10^6	20 736	62 118
5	3.36×10^7	1×10^8	2.49×10^5	7.45×10^5
6	$>1 \times 10^9$	$>3.2 \times 10^9$	$>2.9 \times 10^6$	$>8.9 \times 10^6$
7	3.4×10^{10}	1×10^{11}	3.5×10^7	1.1×10^8
8	1×10^{12}	3.3×10^{12}	4.2×10^8	1.3×10^9

In experiments using an enantioselective epoxide hydrolase designed for testing the virtues of NNK versus NDT codon degeneracy, the identical number of transformants were screened in both cases.[43] The use of NDT codon degeneracy led to notably higher library quality, meaning a higher frequency of hits and superior catalyst performance in terms of evolved enantioselectivity. Following this study, the use of reduced amino acid alphabets has been generalized.[23a] Most recently, Yuval Nov has proposed a different statistical analysis for estimating the degree of oversampling which has significant practical ramifications.[44] When focusing the search in the restricted protein sequence space on the second or third best mutant, the number of transformants needed for successful navigation in the fitness landscape is much lower.

Several other recent improvements have increased the efficacy of saturation mutagenesis further:

- bioinformatics as a guide in choosing optimal reduced amino acid alphabets[30b,c]
- pooling techniques in the screening process[45]
- improved saturation mutagenesis method for difficult-to-amplify templates[46]
- methods for reducing amino acid bias[47]
- Quick Quality Control (QQC) of saturation mutagenesis libraries[45b]
- in silico guidance at each evolutionary stage[48]

The underlying reason for the efficacy of ISM was uncovered in several studies by performing extensive deconvolution experiments coupled with the construction of fitness landscapes.[23a,49] These showed that none of the mutations are superfluous while also revealing strong cooperative effects operating between point mutations and sets of point mutations. *This means that the influence of mutations in ISM-based directed evolution is notably more than additive, i.e., nonadditive in a synergistic manner.* The importance of cooperative and antagonistic nonadditivity as opposed to "classical" mathematical additivity in directed evolution and in other areas of protein engineering has been analyzed in a recent review.[49]

A universal problem in all of directed evolution occurs whenever a mutant library along a given evolutionary pathway fails to harbor an improved enzyme variant, irrespective of the mutagenesis method.[19,23] In such situations the experimenter can screen more transformants (mutants), turn to a different mutagenesis technique, or give up. In a recent ISM study focusing on the enantioselectivity of an epoxide hydrolase in which all 24 pathways of a four-site system were explored, intriguing discoveries were made.[50] It was found that 16 of the 24 pathways are energetically favored in the fitness landscape with no local minima (Figure 7). In the other eight cases, local minima were encountered; i.e., the respective libraries did not contain any mutants showing enhanced enantioselectivity. In these cases a counterintuitive decision was made by choosing an inferior mutant in that library as the template in the subsequent ISM step.[50] In all cases this procedure proved to be a successful way to escape from local minima! It is reminiscent of the neutral drift theory[51a] as propagated by Dan Tawfik and others and/or the quasi species hypothesis of Manfred Eigen

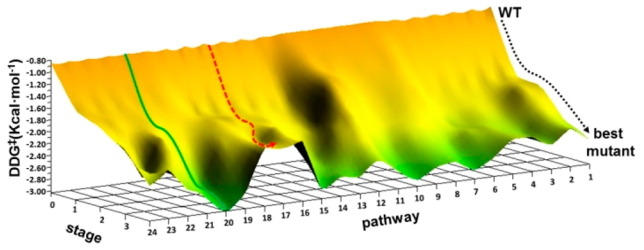


Figure 7. Experimental fitness-pathway landscape featuring all 24 pathways of a four-site ISM system using an epoxide hydrolase as catalyst in the kinetic resolution of a racemic epoxide, adapted from ref 50. Green line: Typical trajectory in which every mutant library in the four-step sequence contains an improved mutant (no local minima). Red line: Typical trajectory in which at least one library along the four-step sequence is devoid of an improved mutant (local minimum). Of the 24 pathways, only 8 have local minima, escapes being possible.

and Peter Schuster for explaining natural evolution,[51b] which have been invoked by Bengt Mannervik in protein engineering studies of glutathione transferases.[51c]

Since the lipase-catalyzed hydrolytic kinetic resolution of *rac*-**16** is the most systematically studied transformation in directed evolution (section 3), it was essential to test ISM in this model system.[52a] With the help of the X-ray structure of the *P. aeruginosa* lipase,[52b] six residues were identified for saturation mutagenesis, these being grouped into three sites: A (Met16/Leu17), B (Leu159/Leu162), and C (Leu231/Val232) (Figure 8).

Figure 8. Schematic representation of amino acid residues considered for saturation mutagenesis,[52a] based on the X-ray structure of WT *P. aeruginosa* lipase:[52b] sites A (Met16/Leu17, green), B (Leu159/Leu162, blue), and C (Leu231/Val232, yellow) around the active site Ser82 (stick representation in gray and red) in the acid-binding pocket (white circle). The red circle marks the alcohol binding pocket, in the case at hand harboring the *p*-nitrophenyl moiety of *rac*-**16**. At the top of the picture, helix and loop in pink (right, Asp113-Leu156) and purple (left, Pro203-Asn228) represent lid 1 and lid 2, respectively.

Pathway B → A proved to be most prolific (Figure 9), providing a triple mutant Leu162Asn/Met16Ala/Leu17Phe

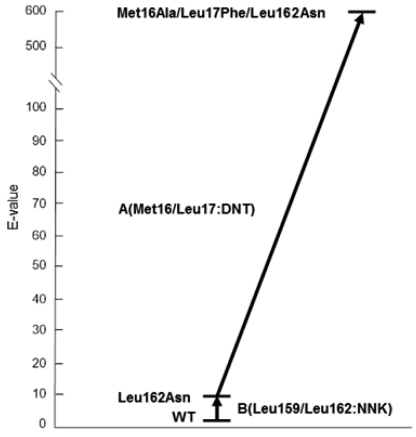


Figure 9. Real-scale scheme of the best pathway, WT→B→A, leading to the best mutant Leu162Asn/Met16Ala/Leu17Phe with a selectivity factor of E = 594 (S) in the hydrolytic kinetic resolution of *rac*-**16**, adapted from ref 52a.

with a selectivity factor of E = 594 (S). Site C was not visited because enantioselectivity and activity as shown by enzyme kinetics were already so high.[52a] The initial libraries in two other pathways contained no improved mutants, but attempts to escape from the local minimum were not undertaken due to the success of pathway B → A. This study allowed the rigorous comparison of ISM with previously applied gene mutagenesis strategies. The triple mutant is clearly superior to the previous best mutant with six point mutations obtained mainly by epPCR and DNA shuffling, while requiring significantly less screening (10 000 versus 50 000 transformants).

Deconvolution of the triple mutant uncovered strong cooperative effects acting between the first mutation Leu162Asn (E = 8) from library B and the accumulated second set Met16Ala/Leu17Phe from library A which alone shows poor *S*-enantioselectivity (E = 2.6).[52a] Thus, when they act in concert, cooperativity amounts to 8 kJ/mol! The reader is referred to the original study which includes the elucidation of the mechanism of this dramatic effect on a molecular level.[52a] Cooperative interaction between point mutations and sets of mutations is characteristic of ISM and explains its efficacy.[23a,52a]

Table 2 summarizes recent ISM studies. The procedure is useful not only when generating improved enzymes for a defined transformation, but also when manipulating binding properties or metabolic pathways.

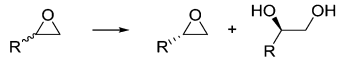
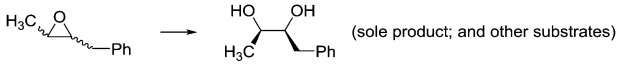
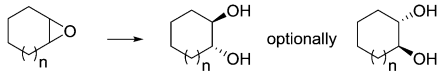
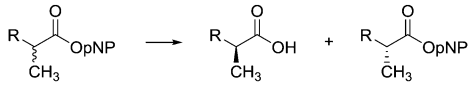
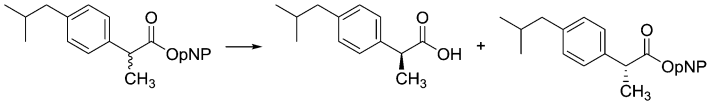
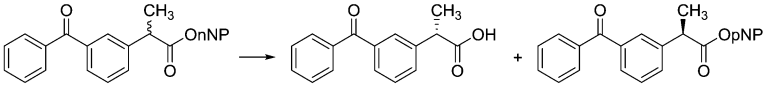
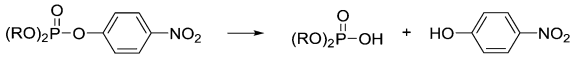
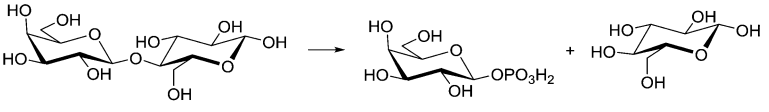
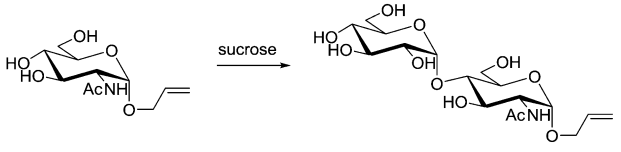
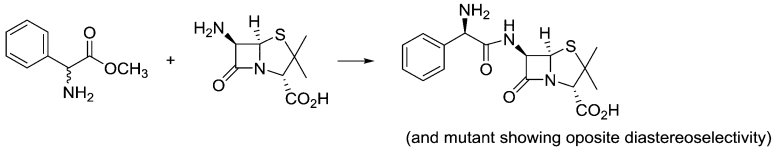
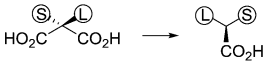
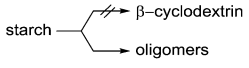
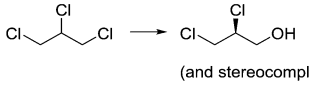
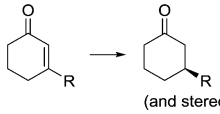
A strategy combining extensive saturation mutagenesis with random mutagenesis and rational design was reported by scientists at Codexis in the directed evolution of a stereoselective transaminase as the catalyst in the industrial preparation of the anti-diabetic compound sitagliptin[69b] (Table 2). ISM has also been used to enhance protein robustness, e.g., by applying the B-FIT method or, when multimeric enzymes are involved, by performing surface engineering as demonstrated by Matthias Bechtold et al.[75f] (Table 2). A number of other important advancements in developing efficient mutagenesis methods and strategies in laboratory evolution have been reported, including such procedures as circular permutation, domain swapping, and synthetic DNA shuffling.[19,23,37] Thus far most of these have not been applied extensively by groups other than the original scientists who developed them. Comparisons with other methods or strategies were generally not made, which makes critical assessments difficult.

Rather than striving for smaller, higher-quality mutant libraries, some researchers have chosen to go the opposite way by generating very large libraries of up to 10^8 members, thereby covering more protein sequence space. Since the conventional screening assays cannot be applied in these cases, various display systems need to be used, including phage display, bacterial surface display, and yeast display.[19,27b] In some cases the mutants were identified directly from a suspension of the display species, while in other systems analytical methods such as fluorescence-activated cell sorting (FACS) had to be applied.[76] In vitro compartmentalization by water-in-oil emulsions has also been developed as a method in directed evolution, often in combination with FACS.[77]

Excellent results have been reported using these methods; nevertheless, general use of such techniques awaits further development. For example, phage display is designed mainly for binding properties, not so much for catalysis, and indeed attempts to use this technique for evolving stereoselective enzymes have not been very successful.[78]

An important practical aspect of laboratory evolution concerns the question of whether a mutant, evolved for the selective transformation of a specific substrate, can also function as an efficient catalyst in the reaction of other compounds. No catalyst can ever be universal, but organic chemists need catalysts that are active and selective for a reasonable number of

Table 2. Recent Studies Employing Iterative Saturation Mutagenesis

Enzyme (process)	Reactions (comments)	Refs.
Epoxide hydrolases (kinetic resolution)		41[a],53
Epoxide hydrolases (stereoconvergency)	 (sole product; and other substrates)	54
Limonene epoxide hydrolase (desymmetrization)	 optionally	55
Lipase *Ps. aeruginosa* (kinetic resolution)	 ($R = n\text{-}C_8H_{17}$)	52[a]
Lipase *Candida antarctica* A (kinetic resolution)	 (and other substrates)	56
Lipase *Candida antarctica* B (kinetic resolution)	 (and other substrates)	57
Amidohydrolase Dr-OPH (promiscuous reaction)		58
Cellobiose phosphorylase (promiscuous reaction)		59
Amylosucrase (glycosylation)		60
Penicillin G acylase (kinetic resolution)	 (and mutant showing oposite diastereoselectivity)	61
Aryl malonate decarboxylase (enantioselective decarboxylation)	 S = CH_3, OH, NH_2; L = aryl, alkenyl (and stereocomplementary mutant)	62
Cyclodextrin glucanotransferase (CGTase) (promiscuous reaction possible by turning a CGTase into an α-amylase)		63
Haloalkane dehalogenase DhaH 31 (desymmetrization)	 (and stereocomplementary mutant)	64
Enoate reductase YqjM (conjugate reduction)	 (and stereocomplementary mutant)	45[b]

dx.doi.org/10.1021/ja405051f | J. Am. Chem. Soc. 2013, 135, 12480−12496

Table 2. continued

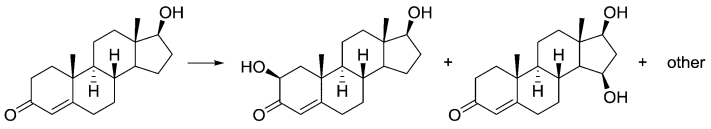
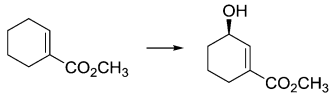
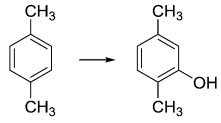
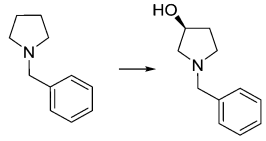
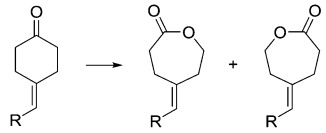
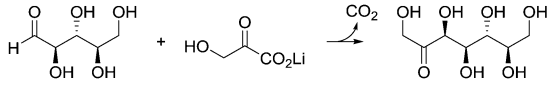
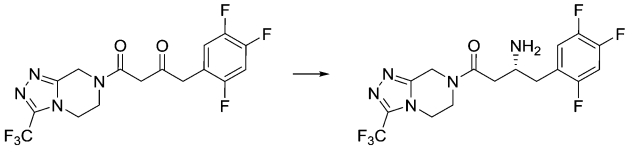
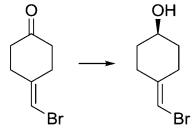
Enzyme (process)	Reactions (comments)	Refs.
P450-BM3 (regio- and stereoselective oxidative hydroxylation)	 2β - mutant 97 : 3 : 0; 15β - mutant 3 : 96 : 1 (and other steroids)	65[a]
P450-BM3 (regio- and enantioselective oxidative hydroxylation)	 (and stereocomplementary mutant and other substrates)	65[b]
P450-BM3 (regioselective oxidative hydroxylation)		65[c]
P450-HXN-200 (regio- and enantioselective oxidative hydroxylation)	 (and stereocomplementary mutant)	66
Baeyer-Villiger monoxygenase PAMO (diastereoselective oxidation)	 *E* *Z* (*E*- and *Z*-selective mutants)	67
Transketolase (stereoselective C-C bond formation)	 (and other substrates)	68
Leucine dehydrogenase (stereoselective reductive amination)		69[a]
Transaminase (stereoselective reductive amination)		69[b]
Alcohol dehydrogenase (enantioselective reduction)	 (and stereocomplementary mutant)	70
Isoeugenol 4-O-methyltransferase IEMT	Modulating lignin biosynthesis for better utilization of plants in paper making and biofuel production	71
Xylose reductase PsXR	Changing NADPH to NADH specificity	72

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Table 2. continued

Enzyme (process)	Reactions (comments)	Refs.
Pyrrolysyl-tRNA synthetase (substrate acceptance)	Modification of proteins by unnatural amino acids for click chemistry	73
Streptavidin harboring an achiral Rh-diphosphine complex (enantioselectivity)	Enhancing enantioselectivity of a Rh-containing hybrid catalyst	74
Lipases	Enhancing stability by applying B-FIT	31, 75[a,b]
Esterase PFE	Enhancing stability by applying B-FIT	75[c]
Uridine phosphorylase	Enhancing robustness by applying B-FIT	75[d]
α-Amino ester hydrolase	Enhancing stability by applying B-FIT and disulfide bond formation	75[e]
D-Tagatose epimerase	Enhancing stability by surface engineering of a multimeric enzyme	75[f]

compounds. This has in fact been demonstrated in numerous cases of evolved stereoselective enzyme mutants.[19,23]

8. LESSONS LEARNED FROM DIRECTED EVOLUTION

Two types of lessons are possible when performing laboratory evolution, provided thorough theoretical analyses flank the experimental work. One kind of insight results when studying the origin of enhanced activity, stereoselectivity, or thermostability. The analyses using biophysical methods, ideally in conjunction with QM/MM, not only unveil the factors responsible for improved catalytic performance but also help researchers to understand the intricacies of enzyme mechanisms in general. A rare example in which X-ray structural data of a stereoselective mutant were included concerns the protein engineering of an enantioselective epoxide hydrolase evolved in five ISM steps.[79] Using this information in combination with enzyme kinetics, inhibition experiments, molecular dynamics simulations, and docking experiments, light was shed on the molecular change occurring in the binding pocket at each evolutionary stage. In the case of applying ISM to the limonene epoxide hydrolase as a catalyst in the desymmetrization of cyclopentane oxide,[55] Fahmi Himo applied a QM-based cluster approach to model enantioselectivity at all mutational stages of the respective *R*- and *S*-selective pathways, which proved to display high predictive power.[55b]

Another case concerns the QM/MM study by Walter Thiel et al. regarding the origin of stereoselectivity of a Baeyer−Villiger monooxygenase and mutants thereof produced by epPCR.[80a] Whereas Chris Walsh and others had previously uncovered the general mechanism of these enzymes[81a] and synthetic organic chemists had used them for decades,[81b,c] only mnemonic devices for predicting the outcome of stereoselectivity were available. The Thiel study shows for the first time how the enzyme cyclohexanone monooxygenase as a standard Baeyer−Villiger monooxygenase works in detail. In the enantioselective desymmetrization of 4-methylcyclohexanone with formation of the respective *S*-lactone (>95% ee), the substrate is trapped in the binding pocket in a way that the Criegee intermediate is smoothly formed in the rate-determing step, followed by the usual σ-bond migration by one of the two enantiotopic groups. The computations show that, in the case of a chair conformation in which the 4-methyl group is in the equatorial position, only one of the two possible σ-bond migrations fulfills the traditional stereoelectronic requirement of anti-periplanar bond arrangement.[80a] It is indeed the one that leads to the observed *S*-lactone. When the methyl group occupies the axial position, the other enantiotopic σ-bond migrates preferentially, leading to the enantiomeric product, which means that the difference in energy between the two chair conformers (∼2.3 kcal/mol) determines the degree of stereoselectivity! Recently the study has been extended to mutants catalyzing the desymmetrization of 4-hydroxycyclohexanone.[80b]

A second type of lesson that can be learned from directed evolution concerns insights arising from deconvolution experiments, especially if performed systematically to include the construction of fitness landscapes as reported in some ISM studies.[23a,49,50] Such data allow the following questions to be answered:

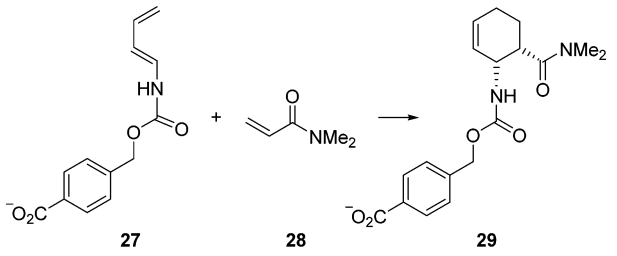
- How many evolutionary pathways lead to fitter enzymes in a given case?
  Answer: This depends upon the type of mutagenesis technique and strategy. See references to support this conclusion,[23a,50] but also a contrary opinion.[82]
- How can the experimenter escape from local minima?
  Answer: Choose an inferior mutant in the respective library as a template in the subsequent mutagenesis cycle.[50]
- Do additive or nonadditive mutational effects dominate in laboratory evolution?
  Answer: Not enough experimental data is currently available, but cooperative effects were revealed whenever deconvolution was performed on mutants evolved by saturation mutagenesis.[23a] *See a recent review outlining the importance of nonadditive effects in protein engineering,*[49] *including pioneering contributions by A. Fersht, J. Wells, A. Horovitz, D. Shortle, S. J. Benkovic, and A. S. Mildvan.*
- Do cooperative nonadditive effects (more than additivity) occur in natural Darwinian evolution?
  Answer: Sufficient data are not currently available to answer this intriguing question in a general way, but it is likely.[49]
- Can data from laboratory evolution be used to draw conclusions regarding natural Darwinian evolution?
  Answer: Generally not, but carefully designed and critically analyzed experiments may be helpful.

9. DEVISING ENZYME PROMISCUITY

Since enzymes cannot catalyze the multitude of reaction types that nonbiological catalysts such as transition metal complexes make possible, numerous attempts have been made to devise "promiscuous enzymes".[83] Thus far the respective protein-based

catalysts fail to display the high activity characteristic of enzymes catalyzing their respective natural reaction types. An interesting mechanism-based concept was developed by Per Berglund et al. in which lipases were used as biocatalysts in aldol and Michael reactions.[83c] Another approach is to anchor site-specifically a transition metal complex covalently or non-covalently to an appropriate host protein with formation of a hybrid catalyst as originally demonstrated by Whitesides in 1978, which has since been generalized.[83d,e] This provides in each case a single catalyst which may or may not be efficient. Unfortunately, catalyst activity proved to be uniformly low. Thus far only one study has appeared describing the application of directed evolution for improving the catalytic profile of such hybrid catalysts, specifically in the quest to enhance enantioselectivity, but the issue of activity was not addressed.[74] The introduction of unnatural amino acids[84] bearing appropriate ligands for binding transition metals should be tested in future studies combined with directed evolution. In yet another approach, the Fe-heme moiety in P450 enzymes was cleverly exploited as a carbene transfer catalyst.[85] Thus far none of these and other "synthetic" biocatalysts can compete with conventional transition metal catalysts.[83]

A very different and impressive concept concerns computation-based de novo design as originally proposed by David Baker et al. and extensively developed in collaboration with Ken Houk, Don Hilvert, Justin Siegel, Daniela Röthlisberger, Dan Tawfik, and others.[83f] Using the Rosetta algorithm, they designed biocatalysts for such transformations as the Kemp elimination, Diels–Alder cycloaddition, and Morita–Baylis–Hillman reaction. The computational process involves ab initio protein structure prediction, QM energy refinement, and sequence design. Significant rates in the sense of practical applications have not resulted from these fascinating studies, but directed evolution provides a way to remedy the situation. In the case of the Diels–Alder reaction **27** + **28** → **29**, the



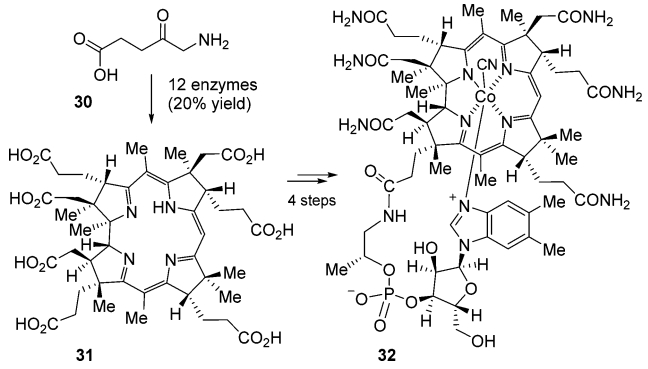
27 28 29

endo-(3*S*,4*R*)-configuration was aimed for in the design step.[86a] Out of 84 computed and in *E. coli* expressed enzymes, two showed Diels–Alder activity. The best hit showed the predicted stereoselectivity in laboratory experiments, which is a remarkable result. The question why the results in the case of the Morita–Baylis–Hillman reaction[86b] proved to be less successful needs to be addressed (no enantioselectivity reported in this asymmetric transformation).

10. MIXTURES OF ENZYMES IN CASCADE REACTIONS

The benefits of synthetic strategies based on one-pot sequential organic transformations without isolating intermediate compounds have been documented in numerous studies, these processes being termed "cascade", "domino", or "tandem" reactions.[87] Enzymes or nonbiological catalysts can be used or combinations thereof. This intuitively attractive concept has limitations for several reasons. Chemical compatibility between the catalysts but also chemo-, regio-, and stereoselectivity of the individual reaction steps are required, in addition to easy downstream workup. Many synthetic catalysts are not likely to be compatible, e.g., oxidants and reductants, in contrast to enzymes,[87c] e.g., oxidases and reductases.

When targeting complex natural products, the idea of using enzyme mixtures guided by knowledge of biosynthetic pathways appears particularly attractive.[88] A classic example was reported in 1994 by Ian Scott et al. describing the cascade synthesis of a vitamin B12 precursor starting from a simple compound.[88a] Based on the biosynthetic pathway, a mixture of 12 enzymes was employed in a one-pot 17-step synthesis of hydrogenobyrinic acid (**31**) from 5-aminolevulinic acid (**30**). Subsequent nonbiological steps provide vitamin B12 (**32**), which is an alternative to the classical purely nonbiological synthesis by R. B. Woodward, A. Eschenmoser, and co-workers. Of course, the goals and spinoffs of the two studies are different.



Recent examples of in vitro synthesis of complex natural products using enzyme mixtures include the preparation of polyketides enterocin and wailupemycin by Bradley Moore et al.,[88c] terrequinone A by Chris Walsh et al.,[88d] and defucogilvocarcin M by Jürgen Rohr et al. (15 enzymes!).[88e]

11. METABOLIC PATHWAY ENGINEERING AS A MEANS TO ACCESS SIMPLE AND COMPLEX COMPOUNDS

An alternative to the use of enzyme mixtures in vitro is metabolic pathway engineering of in vivo systems based on cells as factories for the production of simple and complex compounds.[89] The technique involves the manipulation of cellular properties by appropriate engineering methods. In most cases complex natural products are the targets, but simple compounds such as ethanol and other biofuels are also the focus of current interest. Metabolic engineering is not new. For example, in the 1980s scientists induced *E. coli* to produce such important compounds as erythropoitin, insulin, and therapeutic antibodies,[89] targets outside the realm of synthetic organic chemistry. Bulk chemicals can also be produced; 1,3-propanediol according to the Dupont process is a notable example.[89] During the past 10–15 years new techniques arising from basic research in synthetic biology, systems biology such as in silico genome-scale modeling, and the omics technologies (genomics, proteomics, transcriptomics, metabolomics, and fluxomics) have provided invaluable tools needed in making further progress.

From a synthetic organic viewpoint, the production of complex natural compounds serving as therapeutic drugs is of particular interest. Several examples have been reported, which can then be assessed relative to modern total synthesis. However, the natural product itself is seldom the actual

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therapeutic drug. Researchers in the field of metabolic engineering have therefore learned to manipulate synthetic pathways so that an intermediate compound and not the final natural compound becomes the main product. Once the structurally complex and generally chiral scaffold (not necessarily the final natural product) have been assembled by the biological machinery, subsequent synthetic steps can be designed to afford any number of derivatives (or the natural product itself). This requires collaborative efforts between metabolic engineers and synthetic organic chemists (*are they also engineers?*).

An impressive example of metabolic engineering of complex natural products was recently reported by Jay Keasling and collaborators.[90] In the endeavor to make the anti-malaria therapeutic drug artemisinin available on a large and practical scale, they targeted the production of artemisinic acid, an intermediate which can be transformed into artemisinin in just a few synthetic steps. Following earlier efforts,[89b] an improved version, also based on the orchestration of metabolic engineering and synthetic organic chemistry, was claimed to be more practical (Figure 10).[90] A likewise practical access to

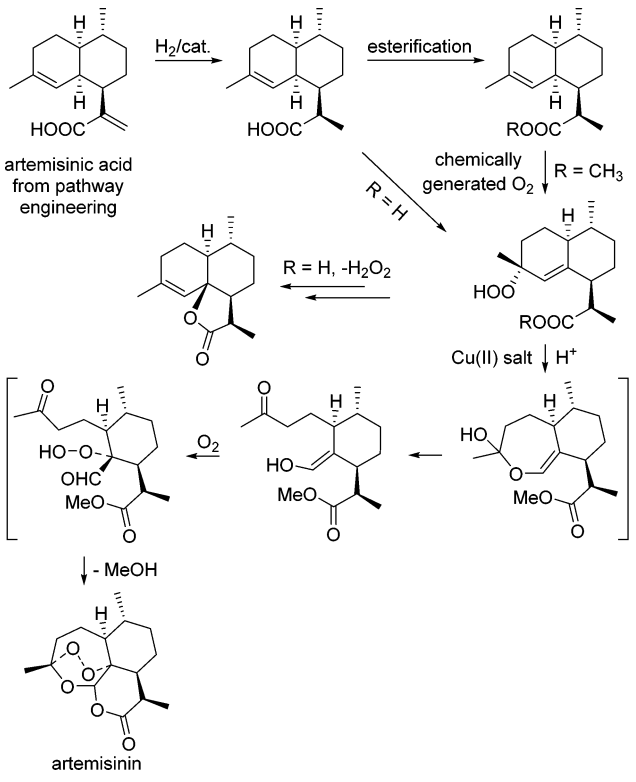

Figure 10. Scheme outlining the semisynthetic route to artemisinin based on pathway engineering leading to arteminisinic acid followed by synthetic organic steps.[90]

artemisinin utilizing total synthesis was recently reported by the group of Silas Cook.[91] It will be interesting to see which option (or both?) will be applied on an industrial scale.

Metabolic engineering offers many opportunities for the production of useful chemicals,[89] including natural products and biofuels, and the future will reveal whether efficient industrial processes can be developed in a general way. Continued basic research is needed. Synthetic organic chemistry is likewise experiencing enormous progress in developing economically and ecologically viable methods and novel strategies, which will continue for some time to come. Present-day metabolic engineering cannot replace synthetic organic chemistry, but it already contributes in a complementary manner. As indicated above, productive constellations can be anticipated in which the enormous power of the two fields will interact synergistically. In a constructive debate, Jay Keasling and Phil Baran have recently exchanged their personal views regarding synthetic biology versus synthetic organic chemistry.[92] Surely, both sciences will continue to thrive.

12. CONCLUDING REMARKS

For a long time the use of enzymes as catalysts in synthetic organic chemistry and biotechnology has suffered mainly from two types of limitations. The unfortunate fact that many in principle useful enzymes could not be expressed easily in large amounts limited the whole field, but this changed dramatically in the late 1970s with the development of recombinant DNA technology.[12] The second major traditional limitation was the often observed poor stereo- and/or regioselectivity, narrow substrate scope, and sometimes insufficient stability under operating conditions.[2,13] With the advent of directed evolution, these problems can now be addressed and generally solved, as demonstrated by numerous academic studies and a rapidly increasing number of industrial applications.[19,23,35,36c,d,69b] This form of protein engineering can also be applied in the elimination of product inhibition[35] or reduction of undesired side products.[34c] It is likely that the already available advanced directed evolution techniques using the most efficient genetic methods and strategies will continue to be applied to all of the enzymatic reaction types. The introduction of unnatural amino acids in conjunction with directed evolution techniques is also an interesting perspective.[84] *Of particular synthetic importance are those enzyme-catalyzed transformations which are difficult or impossible using the best nonbiological catalysts.*[23,70] Systematic use of directed evolution of computationally designed enzymes for promiscuous transformations is likewise an area for present and future research,[83f] specifically in the quest to generalize the concept and to boost activity so that such biocatalysts can begin to compete with well-established nonbiological catalysts.

Engineering of bioreactor systems, bioprocess design, enzyme immobilization, and improved downstream workup for ecologically and economically viable processes have made many industrial applications of biocatalysis possible,[13e−g] and this is likely to continue in the future. Methodologies based on cell-free protein expression are also likely to gain in importance.[13]

All of these advancements now allow for *biocatalytic retrosynthesis in synthetic organic chemistry*, which in the distant past was problematic, especially when attempting to combine enzymes with synthetic catalysts and reagents in cascade processes. Today a new situation has emerged. Turner and O'Reilly have recently issued guidelines for retrosynthetic analyses incorporating enzymes and synthetic catalysts which promise to be useful for synthetic organic chemists.[93]

Metabolic engineering has clearly entered the stage in a different sense, catalysis occurring in cells in which pathways have been engineered leading to the production of complex natural products or simple compounds such as biofuels.[89] This is not a trivial undertaking, and time will tell how far it can be generalized. Microbial genome mining for new natural products and biosynthetic pathways followed by metabolic pathway engineering can be expected to provide access to novel therapeutic drugs. The marriage of metabolic engineering and synthetic organic chemistry is also likely to be rewarding. Laboratory

evolution will continue to be an indispensible tool in pathway engineering, as it is when using mixtures of isolated WT enzymes or mutants in cascade reactions or when engineering a single enzyme for superior performance. A particular challenge for the future is the general and practical realization of any given artificial multistep pathway that a synthetic organic chemist might envision involving solely unnatural substrates in designer cells serving as de novo "chemical factories".[94] This goal is different from metabolic engineering of secondary metabolites as it is practiced today.

Further improvements in the efficacy of directed evolution are desirable. For example, developing general selection methods replacing screening assays for application in laboratory evolution of stereo- and regioselective enzymes remains a challenge. The dynamics of enzymes also need to be considered more so than in the past,[95] an area in which protein engineering can help. Directed evolution is also a useful tool in other research fields,[19] the study of the all-important phenomenon of protein−protein interactions being a prime example.[96]

Increased efforts in interpreting the results of mutations on a molecular level are likewise needed, especially when they prove to be cooperative (more than additive),[49,52a] as revealed by systematic deconvolution studies.[23a,50] Since cooperativity as a form of nonadditivity of mutations appears to be more general than previously thought, enzymes seem to be part of nonlinear systems which are difficult to handle theoretically.[49] Nevertheless, advanced theoretical techniques may someday lead to a general theory of enzymes which could enable the reliable prediction of mutations necessary for improving any given catalytic parameter of interest to the experimenter.

Since methodology developments in biocatalysis and in modern synthetic organic chemistry are flourishing research fields serving society, *both areas deserve fair and balanced financial support by governmental and private agencies*. Finally, it can be hoped that the chemical curricula of undergraduate and graduate education will be modified in the future to treat at least the basics of biocatalysis as well as the handling of representative enzymes and whole cells in the laboratory.

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