CHEMICAL REVIEWS

REVIEW

pubs.acs.org/CR

Application of Designed Enzymes in Organic Synthesis

Gernot A. Strohmeier,[†] Harald Pichler,[†,‡] Oliver May,[§] and Mandana Gruber-Khadjawi*[,†]

[†]Austrian Centre of Industrial Biotechnology, Petersgasse 14, A-8010 Graz, Austria

[‡]Institute of Molecular Biotechnology, Graz University of Technology, Petersgasse 14, A-8010 Graz, Austria

[§]DSM—Innovative Synthesis BV, Geleen, P.O. Box 18, 6160 MD Geleen, The Netherlands

CONTENTS

1. INTRODUCTION

Catalysis is a key discipline in chemistry enabling clean and cost-efficient processes for a large variety of different products and, therefore, has a strong economic impact in industry. Its high innovation potential is shown by the number of Noble Prizes devoted to this discipline. The latest prize, in 2010, was awarded jointly to Richard F. Heck, Ei-ichi Negishi, and Akira Suzuki for palladium-catalyzed cross couplings in organic synthesis.

Compared to homogeneous and heterogeneous catalysis, biocatalysis, until the past few decades, received much less attention in chemistry despite its huge potential derived from the exclusive properties of enzymes. High turnover frequencies (up to 10^7 s^{-1}!)[1] and amazing selectivities should be very attractive to synthetic chemists, but the limited number of industrial applications did not reflect this potential for a long period of time. The reason for this situation was not only limited access to enzymes and high enzyme production costs, but also poor performance of "black-box" wild-type strains or extracts of these microorganisms from which the responsible enzyme was not even known.

After the introduction of recombinant DNA technology in the late 1980s, the picture changed significantly in favor of biocatalysis. Enzymes could be identified much easier and produced more cheaply. Recombinant DNA technology also paved the way for enzyme engineering, a tool very much needed to address limitations for synthetic applications of enzymes found in nature.

More than 100 industrial enzymatic processes have been introduced since then,[2] but the perception about inefficient enzymes still persists. There is good reason to believe that this perception will change not only by experiencing a growing number of successful applications, but also by looking at the progress in technologies we have witnessed during the past decade. Truly revolutionary technologies in genome sequencing, bioinformatics, and gene synthesis have provided fast access to a huge biodiversity and at low cost, which nobody would have predicted only 10 years ago. Our synthetic toolbox is, therefore, rapidly filling with thousands of enzymes, providing a much higher chance of success in identifying a useful biocatalyst, enabling a critical step in chemical synthesis, and even if the identified best performing enzymes from nature are still not good enough, there is strong evidence that limitations in stability, activity, or selectivity can be efficiently addressed in our laboratories by increasingly powerful enzyme engineering strategies. In this review, we give a brief overview of different enzyme engineering strategies and highlight successful performance improvements of oxidoreductases, transferases, hydrolases, lyases, and isomerases used in organic synthesis. These examples should give a flavor of our possibilities and the state of the art of using designed enzymes in organic synthesis.

The literature is covered up to early 2010, and 272 references are cited.

2. RECOMBINANT DNA TECHNOLOGY

Before molecular cloning and heterologous protein expression strategies facilitated production of large quantities of recombinant enzymes much cheaper than before, only rather abundant,

Special Issue: 2011 Enzymes in Synthesis

Received: November 13, 2010
Published: May 10, 2011

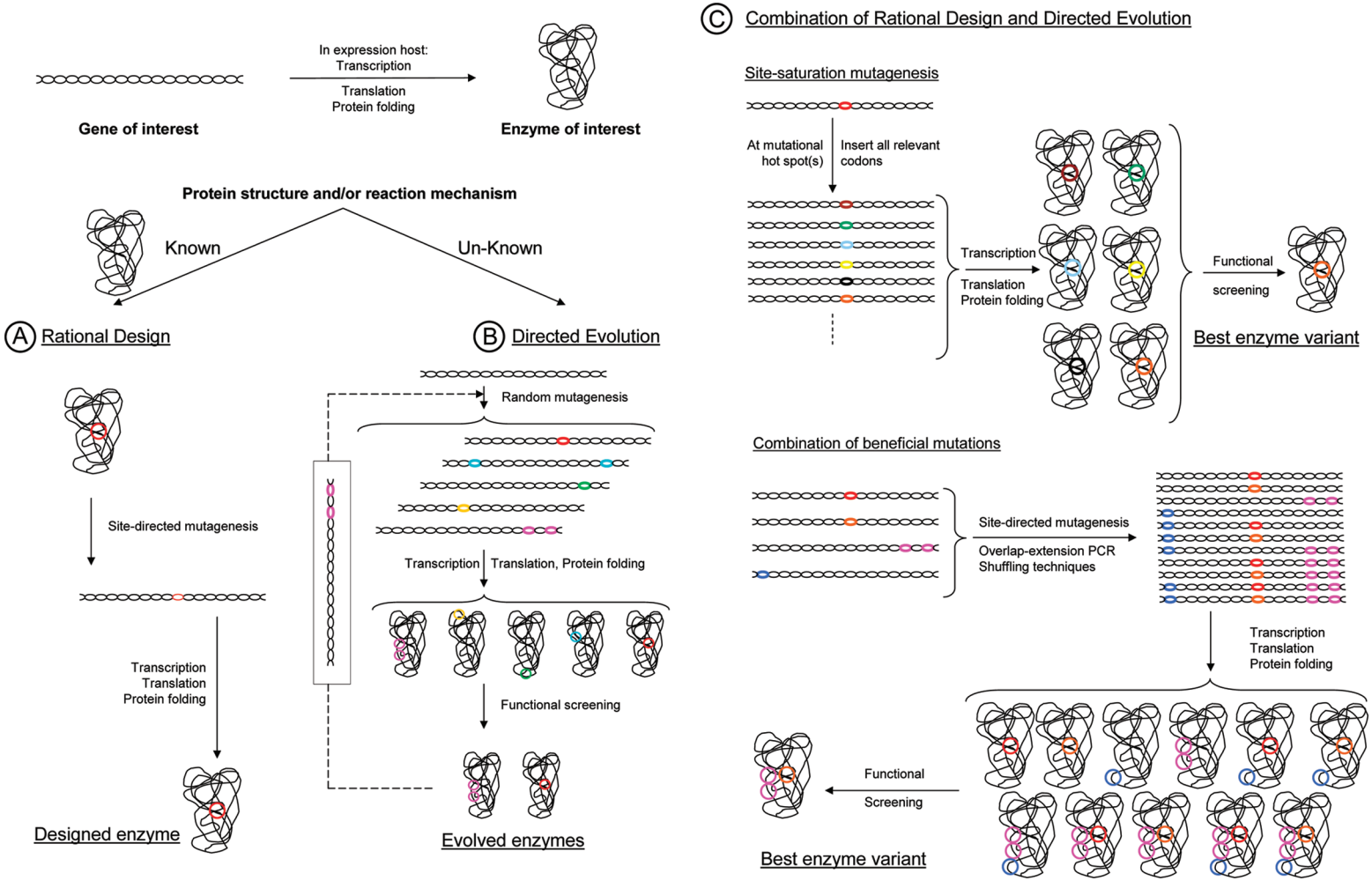


Figure 1. Approaches to designed enzymes. Genes of interest are mutated before or after they are cloned into expression cassettes for the respective expression host. Transcription, translation, and protein folding usually occur in the expression host. If the 3D structure of an enzyme and/or the reaction mechanism is known, specific amino acids can be rationally exchanged by defined base exchanges in the coding sequence, leading to designed enzymes (A). Random mutagenesis followed by functional screening of the resulting enzyme variants leads to evolved enzymes (B). The coding sequence found to be optimal in one round of screening may be the starting point for further mutagenesis/screening cycles. Mutations identified by rational and random approaches are the basis for site-saturation mutagenesis, yielding insertion of all possible amino acids at defined position(s), or for genetically combining two or several mutations in one coding sequence and, therefore, creating enzyme variants with two or multiple amino acid exchanges (C). The best suited novel enzyme variants are identified by functional screenings.

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

natural enzyme sources were applicable to organic synthesis.[3] The initial stages of recombinant enzyme production for biocatalytic applications were based on creation of genomic libraries of organisms that contained the desired enzyme activity.[4] Subsequent activity screens aided in the isolation and identification of the corresponding DNA sequences. At best, high amounts of native enzyme conferring the same chemo-, stereo-, and regioselectivities as the enzyme from the natural source were accessible. Cloning artifacts triggering DNA base exchanges sometimes led to altered selectivities. Point mutations were introduced intentionally into expression constructs by UV radiation,[5] by treatment with mutagenic agents,[6] or by using mutator strains that confer increased mutation rates because of defective DNA repair mechanisms.[7]

Upon the elaboration of the polymerase chain reaction method,[8] amplification of enzyme-encoding DNA sequences not only allowed highly specific base exchanges (site-directed mutagenesis),[9] but also facilitated random mutagenesis[10] and in vitro DNA shuffling approaches.[11] The latter were evolved toward family shuffling techniques combining the features of closely related enzymes.[12] On the other hand, the mutational bias of random mutagenesis methods was largely alleviated by combining two low-fidelity DNA polymerases with opposing bias.[13] Lately, whole genes have been synthesized de novo usually taking into account the codon preferences of the desired protein expression hosts and occasionally also revising undesired DNA sequence elements, e.g., restriction enzyme recognition sites.[14] All of these methods extend the vast DNA sequence space for designing novel enzyme functionalities.

3. APPROACHES TO DESIGNED ENZYMES

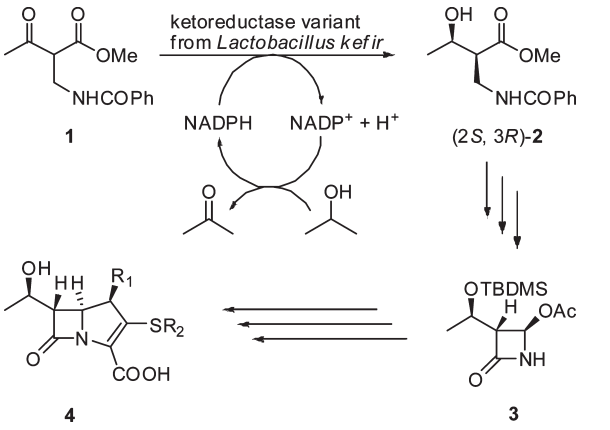
3.1. Rational Design

In the ideal case the enzyme of interest or at least a close homologue thereof has been purified, crystallized, and investigated by X-ray crystallography. Preferentially, this has also been done with cocrystallization of the substrate of interest, the product, or a substrate-like inhibitor, locking the enzyme in a catalytically active state and defining the amino acids (in)directly involved in catalysis. Besides the catalytic residues of the protein, the amino acids delineating the active-site cavity are of importance because they govern whether a non-natural substrate will get access to the active site. This is particularly relevant if the target substrate is bulkier than the natural one(s) or if enantioselective conversions are desired. Guided by crystal structures or homology models, certain amino acids may be exchanged that hinder substrate access to the active site or exit of product, thus improving the functionality of the enzyme (Figure 1A).[15,16] Interestingly, rationally substituting only two amino acids can switch an esterase to a hydroxynitrile lyase.[17] Provided that structural information on the target enzyme is available, rational design may be the easiest and fastest and therefore most straightforward approach of enzyme engineering. However, the chance of success in obtaining the desired results is often still too low and reflects our limited understanding of enzyme function, partially due to the flexibility and dynamics of enzyme catalysis, which cannot be described by the crystal structure.

3.2. Directed Evolution

In most cases, however, structural information on the protein of interest is not sufficiently available to justify a rational design approach. Then a method adopted from nature, enzyme evolution

Scheme 1. Biocatalytic Synthesis of Methyl (2*S*,3*R*)-2-(Benzamidomethyl)-3-hydroxybutyrate Using Ketoreductases



by mutagenesis and selection/screening, is applied. Through one of the random mutagenesis techniques and expression of the resulting variants, a pool of novel enzymes are generated that should be as diverse as possible but still screenable to substitute any amino acid position, potentially influencing enzyme function (Figure 1B). At the same time, concomitant substitution of several amino acids carries the risk of rendering enzymes nonfunctional or even folding-incompetent. Not surprisingly, intrinsic stability of an enzyme to be evolved is beneficial for directed evolution approaches because amino acid substitutions will not destabilize the protein easily.[18] Matsumura and colleagues pointed out that protein engineers should consider the problem of evolvability when choosing starting points for in vitro evolution in the laboratory. Promiscuity and mutational robustness are the variables associated with enzyme evolvability. They recommend exploiting the knowledge of "natural history" of a wild-type enzyme to identify whether it can be evolved.[19] The pool of enzyme variants created, the so-called mutant library, is screened for the desired property. The expression clones showing the best results are selected, and the respective expression construct is sequenced to identify the beneficial mutation(s). Usually, these first-generation mutants are the starting point of further rounds of mutagenesis and screening because there tend to be multiple possibilities of how enzyme performance in terms of stability and processivity can be improved.[20] First-generation libraries may yield an improved enzyme variant already fit for the conversion of the target structure in organic synthesis. More often, they will identify the mutational hot spot(s) for subsequent random or rational approaches.

3.3. Combination of Directed Evolution and Rational Design

Even if structure-guided rational or directed evolution has identified an amino acid that should be substituted to improve enzyme performance, it might be uncertain whether the ideal amino acid exchange has already been identified. Therefore, site-saturation mutagenesis, replacing a certain amino acid by all naturally occurring amino acids, may be performed to identify the best solution by functional screening (Figure 1C).[21] If several mutational hot spots have been identified as interesting for enzyme improvement, an iterative saturation mutagenesis (ISM) will reduce the screening effort.[22] Available structural information on the active-site cavity of enzymes is used to improve substrate

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

acceptance and/or enantioselectivity by combinatorial active-site saturation testing (CASTing).[23] Kazlauskas and co-workers suggested that in many, but not all, cases mutations closer to the active site more effectively influence the catalytic properties of enzymes than distant ones.[24,25]

Independent of whether novel enzyme variants are generated by random mutagenesis only or by involving a rational component as well, the decisive factor for successful enzyme evolution is a stable, functional enzyme assay applicable at high throughput and reflecting the conditions under which the enzyme is intended to be applied, i.e., using the actual target substrate.

4. EXAMPLES OF DESIGNED ENZYMES FOR APPLICATIONS IN ORGANIC SYNTHESIS

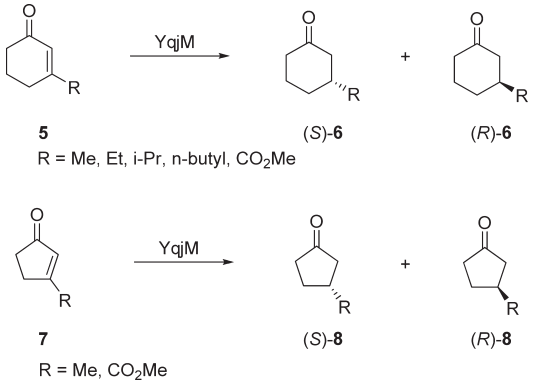
Enzymes are frequently used catalysts, especially for the synthesis of chiral organic compounds. Among the asymmetric catalysts, enzymes often give the most efficient and environmentally benign tools to meet the goal.

4.1. Oxidoreductases E.C.1

4.1.1. Designed Enzymes for Reduction Reactions. *Ketoreductases.* In 2006, Weissman and colleagues reported that five ketoreductase domains from the modular polyketide synthases responsible for biosynthesis of erythromycin were able to reduce a range of simple ketones which were structurally different from polyketide substrates. The activity of two of these reductases, KR1 and KR2, could be improved by directed evolution for the reduction of decalone.[26]

β-Keto ester reductase mutant KER-L54Q from *Penicillium citrinum* was used to produce methyl (*S*)-4-bromo-3-hydroxybutyrate from methyl 4-bromo-3-oxobutyrate in a whole cell system containing a cofactor-regeneration enzyme based on glucose dehydrogenase (GDH). The enzyme variant L54Q exhibited higher enantioselectivity and thermal stability than the wild-type enzyme.[27,28] Researchers from Codexis optimized the biocatalytic synthesis of methyl (2*S*,3*R*)-2-(benzamidomethyl)-3-hydroxybutyrate (**2**), which is an important precursor for the synthesis of carbapenem derivatives **4** via (3*R*,4*R*)-4-acetoxy-3-[(*R*)-1′-((*tert*-butyldimethylsilyl)oxy)ethyl]-2-azetidinon (AOSA; **3**) (Scheme 1). The best mutants from (*R*)-specific *Lactobacillus kefir* produced **2** with excellent selectivity (ee and de > 99%) and high conversion rates in a substrate-coupled approach using 2-propanol for the regeneration of the reduced nicotinamide adenine dinucleotide phosphate (NADPH) cofactor.[29] Mutants of *L. kefir* were also described for the synthesis of protein kinase inhibitor (*R*)-1-[[4-[(4-fluoro-2-methyl-1*H*-indol-5-yl)oxy]-5-methylpyrrolo[2,1-*f*][1,2,4]triazin-6-yl]oxy]propan-2-ol from the corresponding ketone.[30] Variants of *Saccharomyces cerevisiae* ketoreductase were developed for the synthesis of *syn*-3,5-dihydroxyhexanoate esters, which are precursors of statins such as atorvastatin (Lipitor), rosuvastatin (Crestor), or pitavastatin.[31] Alternatively, the synthesis of atorvastatin via enzyme-mediated reduction of ethyl 4-chloro-acetoacetate to ethyl (*S*)-4-chloro-3-hydroxybutyrate with the aid of a designed ketoreductase was described.[32] Using directed evolution, a ketoreductase with superior properties for the asymmetric reduction of (*E*)-methyl 2-(3-(3-(2-(7-chloroquinolin-2-yl)vinyl)phenyl)-3-oxopropyl)benzoate to the corresponding (*S*)-alcohol, a key intermediate in the synthesis of montelukast sodium (Singulair), was developed. The *L. kefir* ketoreductase variant (19 amino acid exchanges from the parent) offers a very high enantioselectivity

Scheme 2. Bioreduction of Cyclic Enones Using YqjM Muteins



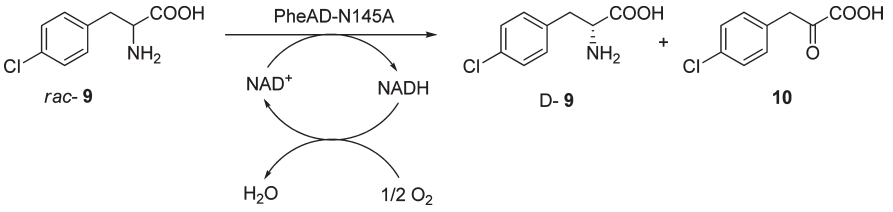
(>99.9% ee) and allows the process to be run at 100 g/L loading in the presence of ~70% organic solvents at 45 °C.[33,34] Other enzyme variants from *L. kefir* were successfully applied for the synthesis of (*R*)-3-hydroxythiolane,[35] a precursor of sulapenem, (*S*)-1-(2,6-dichloro-3-fluorophenyl)ethanol from the corresponding acetophenone precursor,[36] and (4*S*)-3-[(5*S*)-5-(4-fluorophenyl)-5-hydroxypentanoyl]-4-phenyl-1,3-oxazolidin-2-one,[37] an intermediate in the process for making Ezetimibe. The platform of successful applications of ketoreductase variants based on *L. kefir* alcohol dehydrogenase (ADH) was further expanded by the synthesis of (*S*)-*N*,*N*-dimethyl-3-hydroxy-3-thien-2-yl-1-propanamine[38,39] and (*R*)-2-methylpentanol from (*R*)-2-methylpentanal.[40]

Carbonyl reductase from red yeast *Sporobolomyces salmonicolor* AKU4429 (SSCR) and its designed enzymes effectively catalyzed the enantioselective reduction of diaryl ketones to give the corresponding chiral alcohols. Using variant Q245P, the enantiopreference changed to *S* in the case of para-substituted benzophenones as substrates.[41] A stable alcohol dehydrogenase from the hyperthermophilic archaeon *Pyrococcus furiosus* (AdhA) was generated via error-prone polymerase chain reaction (PCR) for the production of (2*S*,5*S*)-hexanediol. The best variant had a 10-fold higher activity compared to the wild-type enzyme.[42]

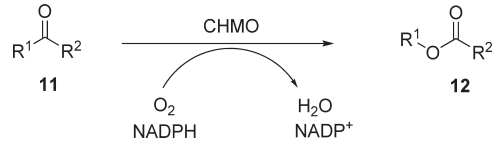
The (*R*)-specific alcohol dehydrogenase from *Lactobacillus brevis* (*Lb*-ADH) catalyzes the enantioselective reduction of prochiral ketones to the corresponding secondary alcohols using NADP(H) (NADP = nicotinamide adenine dinucleotide phosphate) as a cofactor, which is more expensive and labile than NAD(H) (NAD = nicotinamide adenine dinucleotide; NADH = reduced NAD). *Lb*-ADH variants were tested in reduction reactions with acetophenone using NAD(H) or NADP(H) as a cofactor. The mutein R38P showed a 4-fold increased activity with acetophenone and NAD(H) relative to the wild-type.[43]

Phenylethanol dehydrogenase EbN1 from *Azoarcus* sp. was improved by rational design for the reduction of 3-chloro-1-thien-2-ylpropan-1-one to (1*S*)-3-chloro-1-thien-2-ylpropan-1-ol, a precursor of Duloxetin.[44] *Rhodococcus erythropolis* reductase variants were created for the synthesis of optically active (*R*)-1-(3-chlorophenyl)-2-chloroethanol and (*R*)-4-chloro-3-hydroxybutyric acid ester.[45] Carbonyl reductase from *Candida parapsilosis* with *anti*-Prelog stereospecificity was engineered for an inverted cofactor specificity. The S67D/H68D double exchange inside the coenzyme-binding pocket resulted in a nearly 10-fold increase of the k_{cat}/K_M value for NADH and a 20-fold decrease

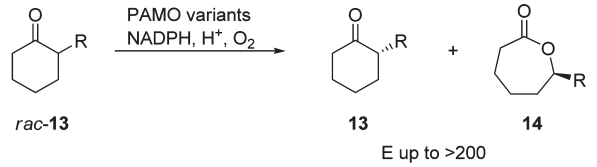
Scheme 3. Resolution of *rac*-4-Chlorophenylalanine with an N145A Variant of L-Phenylalanine Dehydrogenase



Scheme 4. Bayer–Villiger Monooxygenase-Catalyzed Oxidation of a Ketone

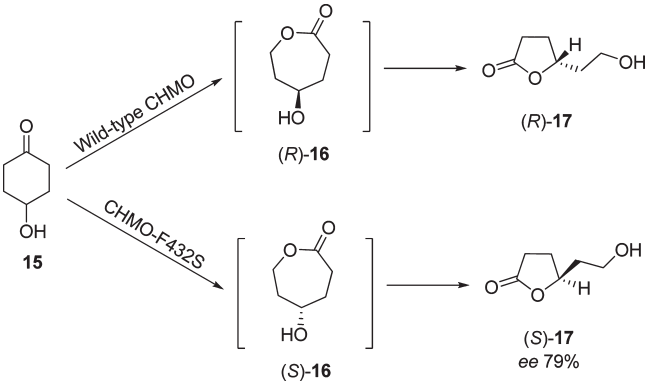


Scheme 5. Bayer–Villiger Monooxygenase-Catalyzed Oxidation of Cyclic Ketones



R = Ph, 4-Me-Ph, Me, Et, n-Pr, n-Bu, Allyl, i-Pr, Cy, Bn, CH_2CH_2CN

Scheme 6. CHMO-Catalyzed Oxidation of 4-Hydroxycyclohexanone



for NADPH.[46] Bioreductions of 2-hydroxyacetophenone using the S67D/H68D variant produced the corresponding (*R*)-alcohol with an ee of 97% instead of the (*S*)-alcohol in the case of the wild-type enzyme.[47] Another successful switching of the cofactor preference of NADPH dependency to NADH was described for dehydrogenase Gre2p variant N9E from *S. cerevisiae*.[48]

A fungal xylose reductase from *Neurospora crassa* with partially reversed selectivity was engineered to prefer D-xylose over L-arabinose.[49]

A new class of enzymes that catalyze the conversion of nitrile-containing compounds to the corresponding amine (such as a primary amine) were described recently. On the basis of the discovery that QueF is a nitrile oxidoreductase, native QueF enzymes can be evolved to obtain nitrile oxidoreductases with an altered specificity for other nitrile substrates.[50]

Enoate Reductases. ISM has been applied to broaden the substrate scope of old yellow enzyme homologue YqjM while controlling the enantioselectivity in the bioreduction of a set of substituted cyclopentenone and cyclohexenone derivatives. Several mutations were identified which yielded improved stereoselectivity and activity compared with those of the wild-type. Additionally, in most of the cases both product enantiomers with high ee's could be obtained using appropriate YqjM variants (Scheme 2).[51] Yeast enone reductases were evolved for the synthesis of (*S*)-carvone and (*R*)-carvone and the reduction of ethyl 2-cyano-3-phenylbut-2-enoate and 8a-methyl-3,4,8,8a-tetrahydronaphthalene-1,6(2*H*,7*H*)-dione.[52]

Among the oxidoreductases, D-amino acid oxidases (DAAOs) are of special interest not only as enantiospecific enzymes for organic synthesis in the laboratory, but also for industrial applications. The flavin adenine dinucleotide (FAD)-containing enzymes have a broad substrate range and act on several D-amino acids with neutral and basic side chains. Site-directed mutagenesis studies and determination of the enzyme crystal structures from various sources have led to a better understanding of structure–function relationships and paved the way for modulating the enzyme properties. Rational design and directed evolution approaches led to DAAO variants with altered substrate specificity or increased thermostability.[53]

The wild-type enzyme L-phenylalanine dehydrogenase from *Bacillus sphaericus* and four active-site muteins were evaluated for their potential applicability in the resolution of racemic non-natural amino acids. The variant N145A was applied in a preparative-scale resolution of *rac*-4-chlorophenylalanine (**9**) with NAD^+ as a cofactor and diaphorase for cofactor regeneration. The pure D-isomer was isolated from the reaction mixture shown in Scheme 3.[54]

Muteins of phenylalanine dehydrogenase (PheDH) from *B. sphaericus* with improved activity and specificity toward propargylglycine, an unsaturated non-natural amino acid of commercial interest, have been obtained by directed evolution and screening of 10000 colonies. By targeting two "hot spots" close to the active site of the enzyme, an evolved enzyme was obtained with a 7.4-fold improved catalytic efficiency toward propargylglycine and a 612-fold improvement of selectivity in comparison between the target substrate and L-phenylalanine.[55]

Aspergillus fumigatus amadoriase II was engineered with the aim to expand its substrate range. After two rounds of directed evolution, a variant (SII-82) was obtained that showed an 8.78-fold increase in the activity toward fructosyl polylysine.[56]

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

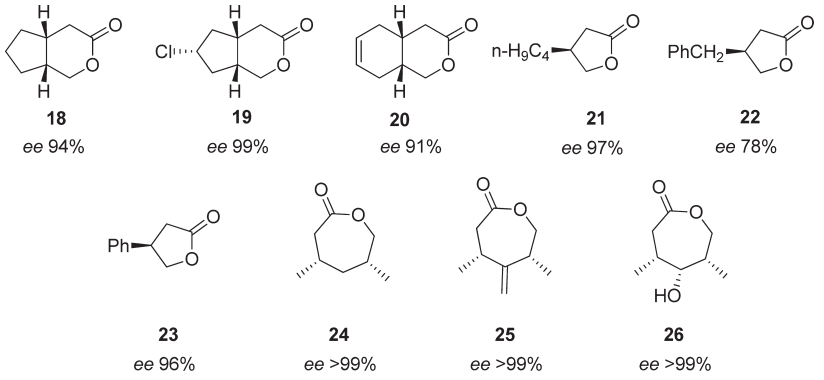
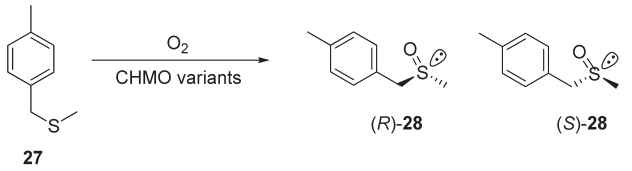


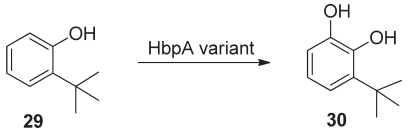
Figure 2. Products of BV oxidation with the CHMO F432 variant.

Scheme 7. Sulfoxidation of Thioethers with CHMO Variants from *Acinetobacter* sp.

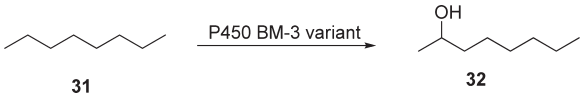


variants	*ee* (*R*)	*ee* (*S*)
D384H	99%	
F432S	99%	
K229I / L248P		98%
Y132C / F246I / V361A / T415A		>99%
F16L / F277S		95%

Scheme 8. 3-*tert*-Butylcatechol by Phenol Oxidation



Scheme 9. P450 BM-3 Variants Were Created with up to 40% ee for Both Enantiomers



4.1.2. Designed Enzymes for Oxidation Reactions. Oxygenases are interesting enzymes in organic synthesis due to their ability to introduce one or two oxygen atoms into organic molecules, leading to enantio-, chemo-, or regioselective product formation. However, practical applications are still limited, as most oxygenases are membrane-associated, are often not stable, and show rather low activity. Most oxygenases require reduction equivalents such as NAD(P)H and also electron-transfer partners such as flavin reductases. To overcome some of the limitations mentioned, protein engineering may serve as a powerful tool.[57]

Monooxygenases. Baeyer–Villiger Monooxygenases. Baeyer–Villiger (BV) oxidation of ketones to esters or lactones is a synthetically important reaction which can be performed chemically as well as enzymatically (Scheme 4). The enzymes are flavin-dependent cyclohexanone (CHMO) and cyclopentanone (CPMO) monooxygenases.

Laboratory evolution to induce allostery by introducing appropriate distal mutations was used to modify the thermostable Baeyer–Villiger monooxygenase, phenylacetone monooxygenase (PAMO) (Scheme 5). A variant, Q93N/P94D, was discovered that catalyzes the asymmetric oxidative kinetic resolution of a set of structurally different 2-substituted cyclohexanone derivatives as well as the desymmetrization of three different 4-substituted cyclohexanones, all with high enantioselectivity.[58] Previously, a set of other PAMO variants for the oxidative kinetic resolution of 2-aryl- and 2-alkylcyclohexanones and bicyclo[3.2.0]hept-2-en-6-one were described.[59] PAMO M446G also shows improved properties compared with the wild-type enzyme.[60,61] Baeyer–Villiger monooxygenase BmoF1 from *Pseudomonas fluorescens* DSM 50106 was engineered for the oxidative kinetic resolution of various 4-hydroxy-2-ketones.[62]

The CHMO from *Acinetobacter* sp. NCIMB 9871 was evolved to invert the selectivity in the oxidation of 4-hydroxycyclohexanone (**15**) by using the directed evolution approach (Scheme 6).[63]

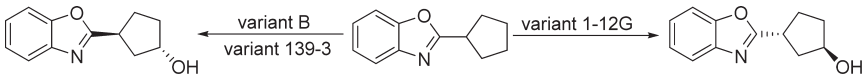
A list of the products synthesized by utilizing variant F432S is given in Figure 2.[64]

To improve the promiscuous activity of CHMO in *Acinetobacter* sp. for enantioselective sulfoxidation of prochiral thioethers, a directed evolution approach was undertaken, leading to variants resulting in >95% ee for substrate **27** (Scheme 7).[65] In this study, the undesired overoxidation to sulfone which accompanied the main reaction could be eliminated. Mutagenesis of toluene *o*-monooxygenase of *Burkholderia cepacia* G4 and the analogous position I100 in toluene 4-monooxygenase of *Pseudomonas mendocina* KR1 improved both the rate and enantioselectivity in the oxidation of methyl phenyl sulfide and methyl *p*-tolyl sulfide to the corresponding sulfoxide.[66]

The Baeyer–Villiger monooxygenase from the thermophilic bacterium *Thermobifida fusca* was evolved toward broader substrate acceptance while preserving the high thermostability.[67] For a Baeyer–Villiger oxidation based resolution of racemic 2-phenylcyclohexanone, the phenylacetone monooxygenase was evolved by a directed evolution approach. Reetz and co-workers describe the use of "drastically" reduced amino acid alphabets at homologous enzyme positions to minimize the effective library size.[68,69] CPMO variants with improved enantioselectivity are reported as well.[70]

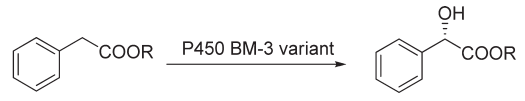
dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 10. Hydroxylation with P450 BM-3 Variants[84]



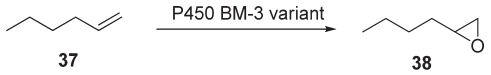
(*S*,*S*) **34** *ee* up to 88% | **33** | (*R*,*R*) **34** *ee* 88%

Scheme 11. Hydroxylation of Arylacetic Acid Esters with P450 BM-3 Variants[85]



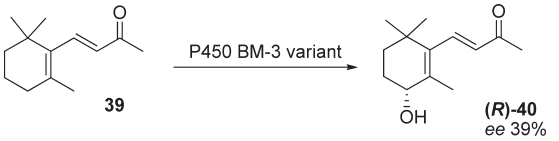
35 R = Me, Et, n-Pr, n-Bu | (*S*)-**36** *ee* 56-95%

Scheme 12. Enantioselective Epoxidation of Terminal Alkenes with Engineered P450 BM-3 Enzyme Variants[86]



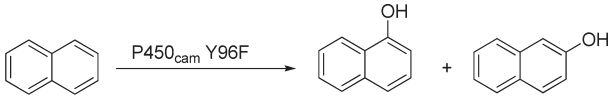
37 | **38** *ee* up to 83%

Scheme 13. Hydroxylation of β-Ionone



39 | (*R*)-**40** *ee* 39%

Scheme 14. Oxidation of Naphthalene by the $P450_{cam}$ Variant



41 | **42** | **43**

Monoamine oxidases (MAOs) are another class of flavin-dependent enzymes that catalyze the stereospecific oxidation of amino acids and are therefore interesting catalysts for synthetic organic chemists. Besides amino acids, the enzymes also accept amines as substrates. As both (*S*)- and (*R*)-selective enzymes are known, enantiopure products can be obtained by using a suitably selective enzyme. (*S*)-Selective *Aspergillus niger* MAO N336S shows a 47-fold increase of activity as well as better enantioselectivity[71] next to a broad range of substrate acceptance[72] upon primary, secondary,[73] and even tertiary[74] amines. More recently, structural data explain the effect of mutations on the enzyme structure.[75]

2-Hydroxybiphenyl 3-monooxygenase (HbpA) from *Pseudomonas azelaica* HBP1 is another nonheme monooxygenase, which was engineered by directed evolution to broaden the substrate range to 2-substituted phenols (Scheme 8).[76,77]

Galactose oxidase has been engineered using the directed evolution approach for the enantioselective oxidation of secondary alcohols. A range of secondary alcohols containing aryl groups were found as preferred substrates. (*R*)-enantiomers were oxidized to the corresponding ketone with up to 50% conversion, and the remaining (*S*)-substrates show up to 99% ee.[78]

Cyctochrome P450s are Fe-heme-dependent enzymes which have been gaining interest as these enzyme find broad applications as biocatalysts in organic synthesis.[79]

Protein engineering of a P450 enzyme from *Bacillus megaterium* was subjected to directed evolution to enhance activity and regioselectivity toward simple substrates such as linear alkanes.[80] The starting point for this evolution study was variants which were evolved to accept alkanes with C3−C8 chain length as the substrate (Scheme 9).[81,82] P450 monooxygenase PikC was modified for the hydroxylation of macrolides.[83]

Examples for transformations with different cyctochrome P450 enzyme variants are given in Schemes 10−12.

The regioselective hydroxylation of highly branched fatty acids by engineered cyctochrome P450 BM-3 (CYP102A1) variants was reported.[87] P450 BM-3 was improved for the synthesis of 4-hydroxy-β-ionone (**40**) by site-directed mutagenesis (Scheme 13). The variant R47L/Y51F/F87V shows a 300-fold increase in activity, while the enantioselectivity for the (*R*)-enantiomer reached 39% optical purity.[88] Cytochrome P450 BM-3 variants were also created for the improved synthesis of hydroxylated indoles[89] and demethylation of protected monosaccharides.[90,91] Other variants were described for the hydroxylation of resveratrol to piceatannol with an up to 18-fold higher formation rate compared to the wild-type.[92]

Schmid and co-workers comment in a review on the preparative use of isolated CYP102 monooxygenases.[93] CYP102A1 (BM-3) mutants were constructed which use polycyclic aromatic hydrocarbons as substrates.[94,95] In a further study, the epoxidation of styrene was chosen as a model reaction for engineered CYP102A1 variants with improved enantioselectivity for the (*R*)-enantiomer. Variants for both enantiomeric styrene oxides were obtained during this study. With CYP102A1 A74E/F87V/P386S an ee value of 58% for (*S*)-styrene oxide was reached. CYP102A1 variant F87G led to (*R*)-styrene oxide with 92% ee.

A CYP102A1 mutein library of 24 variants having two mutated positions close to the center of the heme group was investigated for the hydroxylation reactions of various substrates. Position 87 was shown to mediate substrate specificity and regioselectivity, and position 328 was predicted to interact with all substrates during the oxidation. The library was screened with four terpene substrates, geranylacetone, nerylacetone, (4*R*)-limonene, and (+)-valencene. Eleven variants demonstrated either a strong shift or improved regio- or stereoselectivity in the oxidation of at least one substrate as compared to the CYP102A1 wild-type.[96] Styrene monooxygenase from *Pseudomonas putida* CA-3 was engineered to obtain more than 10-fold increased rates for the

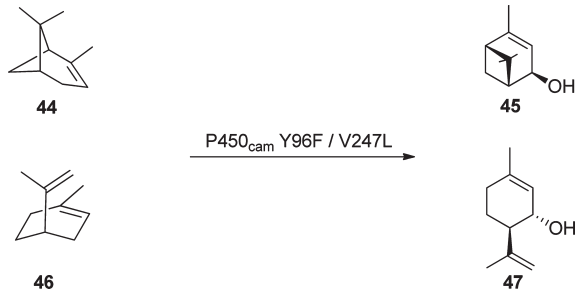
dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

formation of indigo, styrene oxide, and indene oxide compared with the wild-type enzyme.[97]

In a further study, CYP102A1 (P450 BM-3) was subjected to random mutagenesis to improve the activity and expand the substrate range. A variant based on five point mutations, A191T/N239H/I259V/A276T/L353I, displayed about 4-fold higher activity than the wild-type enzyme for the hydroxylation of propylbenzene and almost the same selectivity.[98] The quadruple amino acid exchange in KT5, F87A/A330P/E377A/D425N, yielded 19% desaturation of *p*-cymene and 78% hydroxylation products, whereas the wild-type enzyme gave 84% desaturation and 6% hydroxylation, respectively.[99] Various muteins of CYP102A1 showed improved properties for the oxidation of substrates such as propylbenzene, butylbenzene, naphthalene, and toluene.[100] Muteins of cytochrome P450 monooxygenases from *B. megaterium* (CYP102A1) were used for the oxidation of (−)-α-pinene and (−)-*β*-pinene.[101] A P450 variant shows drastically enhanced activities for propane oxidation and halomethane dehalogenase activities.[102] Application of a panel of *B. megaterium* cytochrome P450 BM-3 variants covers the breadth of reactivity of human P450's by producing 12 of 13 mammalian metabolites for two marketed drugs, verapamil and astemizole.[103] Other examples for the biohydroxylation of diclofenac and chloroxazone were described recently.[104] Ryan and coworkers have shown that P450 BM-3 R966D/W1064S can be used in conjugation with a chemical analogue of NADPH, namely, *N*-benzyl-1,4-dihydronicotinamide, which is a simple compound. This finding could simplify the application of P450 enzymes in preparative synthesis.[105]

Muteins of the heme monooxygenase cytochrome $P450_{cam}$ from *P. putida* in which Y96 has been replaced with hydrophobic residues have been shown to oxidize naphthalene with an oxidation rate 140-fold improved over that of the wild-type (Scheme 14).[106]

Scheme 15. Synthesis of Verbenol (45) and Isopiperitenol (47)



While the wild-type $P450_{cam}$ gives product mixtures by oxidation of monoterpenes (+)-α-pinene (**44**) and (*S*)-limonene (**46**), which are used for fragrances and flavoring, variant Y96F/V247L shows higher activity for the oxidation of both substrates next to high regio- and stereoselectivity (Scheme 15).[107]

Oxidation of the sesquiterpene (+)-valencene by the wild-type and variants of $P450_{cam}$ from *P. putida*, and P450 BM-3 from *B. megaterium*, have been investigated as a potential route toward the fragrance (+)-nootkatone. Wild-type $P450_{cam}$ did not oxidize (+)-valencene, but the recombinant expression system harboring variants showed activities with (+)-*trans*-nootkatol and (+)-nootkatone constituting >85% of the products. Wild-type P450 BM-3 and variants thereof had higher activities than $P450_{cam}$ but were much less selective.[108]

CYP107Z enzyme variants with highly improved regiospecificity were obtained by a combination of random mutagenesis, protein-structure-guided site-directed mutagenesis, and recombination of multiple natural and synthetic CYP107Z gene fragments. These variants were employed for a more economical industrial biocatalytic process to produce semisynthetic insecticide emamectin (Scheme 16). The biocatalytic step in the emamectin manufacturing is the oxidation of 4″-hydroxyavermectin to 4″-oxoavermectin.[109,110]

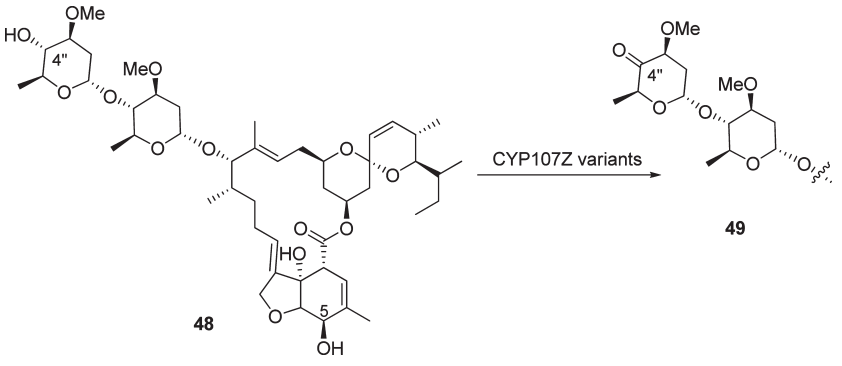
Actinomycete cytochrome P450 from *Nonomuraea recticatena* NBRC 14525 (P450 moxA) catalyzes the hydroxylation of a broad range of substrates, including fatty acids, steroids, and various aromatic compounds. Five amino acid exchanges were identified to have the strongest effect, namely, Q87W/T115A/H132L/R191W/G294D, leading to 20-fold higher activity than that of the wild-type P450.[111]

Horseradish peroxidase variants for the oxidation of tyrosinol and derivatives thereof were developed using a highly efficient selection method for enhanced enzyme enantioselectivity based on yeast surface display and fluorescence-activated cell sorting (FACS). Up to 2 orders of magnitude improved selectivity toward either substrate enantiomer in the case of tyrosinols attached to Alexa Fluor 488 dye, respectively Alexa Fluor 647 dye, was observed. However, in the case of free tyrosinol the selectivity improvement was much lower.[112]

Asparagine oxygenase (AsnO) from *Streptomyces coelicolor* is an Fe^{2+}- and α-ketoglutarate-dependent enzyme that hydroxylates asparagines to L-*threo*-hydroxyasparagine (Scheme 17). Variant D241N, in contrast to the wild-type enzyme, efficiently converts L-aspartic acid into medicinally interesting L-*threo*-hydroxyaspartic acid.[113,114]

Epothilone B hydroxylase (EBH) from *Amycolatopsis orientalis* was improved by mutagenesis to increase the yield of

Scheme 16. Avermectin Oxidation by CYP107Z



dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

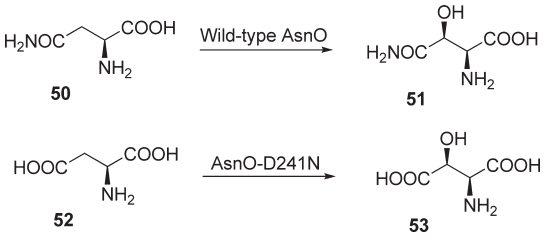
epothilone F through oxidative biotransformation. The yield of epothilone F was increased from 21% to more than 80% utilizing the best EBH mutants.[115]

Streptomyces clavuligerus deacetoxycephalosporin C synthase (ScDAOCS) is an important industrial enzyme for the production of 7-aminodeacetoxycephalosporanic acid, which is a precursor for cephalosporin syntheses. Muteins thereof show improved rates for the conversion of ampicillin, penicillin G, phenethicillin, and carbenicillin to the corresponding cephalosporin derivatives.[116]

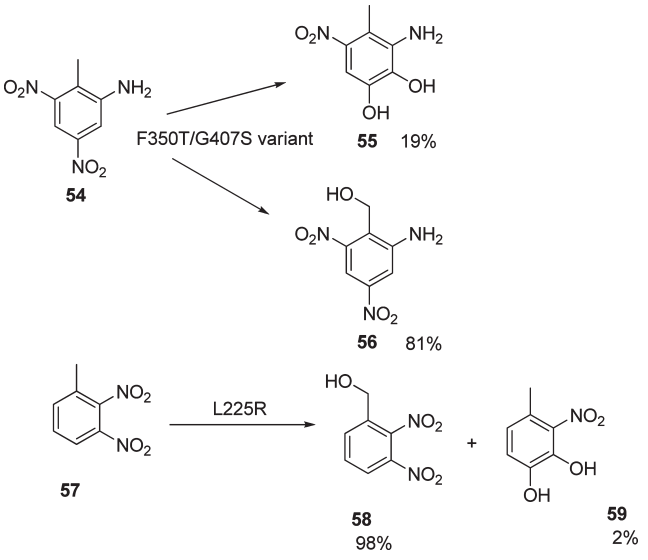
Laccases from *Pleurotus ostreatus* were mutated using molecular evolution techniques. The improved variant 3M7C presents, besides L112F, only one substitution (P494T) responsible both for the significantly increased stability and for the higher activity of this mutant in 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) oxidations.[117]

Dioxygenases are a heterogeneous group of non-heme-iron-containing NAD(P)H-dependent enzymes involved in biosynthesis of secondary metabolites such as flavonoids and alkaloids. They are also key enzymes in the natural degradation of aromatic compounds.[57] Site-directed mutagenesis has been employed to expand the substrate range and activity of 2,4-dinitrotoluene dioxygenase (DDO) from *B. cepacia* R34.[118] Muteins of naphthalene dioxygenase from *Ralstonia* sp. strain U2 were able to build 3-amino-4-methyl-5-nitrocatechol and 2-amino-4,6-dinitrobenzyl alcohol (Scheme 18).[119]

Scheme 17. Hydroxylation of Aspartic Acid Catalyzed by the AsnO Variant



Scheme 18. Oxidation of Dinitrotoluene by Dioxygenase



4.2. Transferases E.C.2

A *glycosyltransferase* capable of catalyzing O-, S-, and N-glycosylation was first reported by Thorson et al. Glycosyltransferase OleD from *Streptomyces antibioticus* with three amino acid exchanges was an efficient catalyst for glycosylation of β-lactam, polyenes, phenols, and the N- and S-analogues, steroids, macrolides, and anthraquinones.[120]

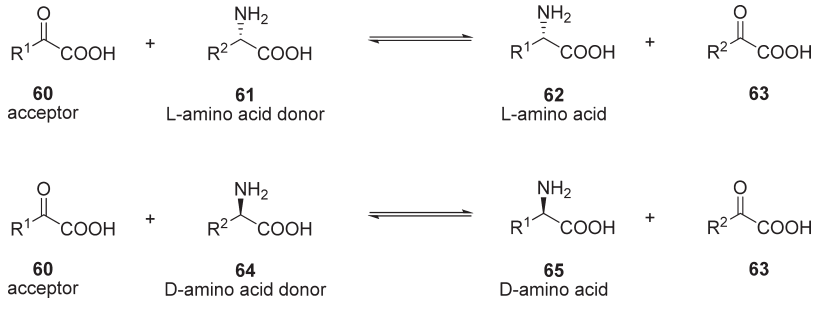
Aminotransferases are important enzymes for industrial applications and have been used for the production of amino acids.[121] Therefore, it is not surprising that they are frequently found among the designed enzymes. Aminotransferases are divided into two classes, namely, α- and ω-transaminases. While α-transaminases catalyze the reaction between an amino acid and an α-keto acid and require the presence of a carboxylic acid moiety in the α-position to the keto and amino groups, ω-transaminases transfer amino groups that are separated from the carbonyl group by at least one methylene group (Scheme 19). As chiral amines are important compounds for diverse applications such as pharmaceuticals, the ω-transaminases are highly desired enzymes and are extensively studied.[122]

Enzymes are known which accept simple amines instead of amino acids as amine donors in the transformation. An aminotransferase could be improved regarding the enantioselectivity in the transformation of β-tetralone into the corresponding (*S*)-amine **67** (Scheme 20).[123]

Researchers from Codexis and Merck engineered the ω-aminotransferase AT-117 for the synthesis of sitagliptin (Scheme 21). After an 11-round mutation strategy inserting 27 mutations, the final catalyst was 27000-fold improved as compared with the wild-type enzyme.[124] To obtain high conversions, the equilibrium-controlled reaction has to be shifted, by applying a large excess of amine donor and/or by removal of the volatile acetone built during the reaction.

Furthermore, the new ω-transaminase is also significantly more active in the synthesis of (*R*)-(+)-1-(2-naphthyl)ethylamine, (*R*)-2-(2-fluorophenyl)pyrrolidine, and several other important

Scheme 19. Principle of Reactions Catalyzed by Aminotransferases



dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

chiral amines from its ketone precursors in the presence of cheap isopropylamine. (*S*)-Selective ω-transaminase from *Arthrobacter citreus* (variant CNB05-01) shows increased enantioselectivity for reactions with (4-fluorophenyl)acetone when possessing a Y331C exchange. A single point mutation yielding variant V328A turns the (*S*)-selective enzyme into an (*R*)-selective one.[125] New muteins of (*S*)-selective ω-transaminase from *Vibrio fluvialis* JS17[126] and (*R*)-selective *Arthrobacter* sp. KNK168[127] were described recently.

Kim and colleagues improved the ω-aminotransferase from *V. fluvialis* JS17 for the synthesis of aliphatic amines (Scheme 22).[128]

The aspartate aminotransferase was evolved to increased enzyme specificity toward amino acids and ketoacids with chain branching in the β-position.[129]

(Iso)eugenol 4-*O*-methyltransferase (IEMT) from *Clarkia breweri* was genetically modified, and a number of muteins were found which catalyzed the methylation reaction much faster than the wild-type using *S*-adenosyl-L-methionine (SAM) as a cofactor.[130]

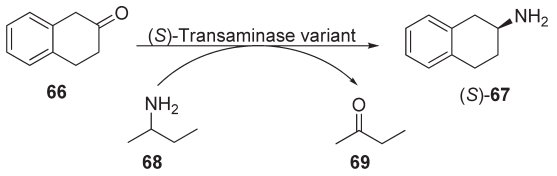
4.3. Hydrolases E.C.3

4.3.1. Designed Enzymes for C−O Bond Formation and Cleavage. *Lipases.* The application of directed evolution to enhance the enantioselectivity was for the first time carried out at the Max-Planck-Institute in Mülheim on *Pseudomonas aeriginosa* lipase (PAL).[70,131] Lipases are the most frequently used enzymes in organic synthesis, performing both the cleavage and the formation of esters. The enantioselectivity of PAL was addressed for the reaction shown in Scheme 23 as a model system.

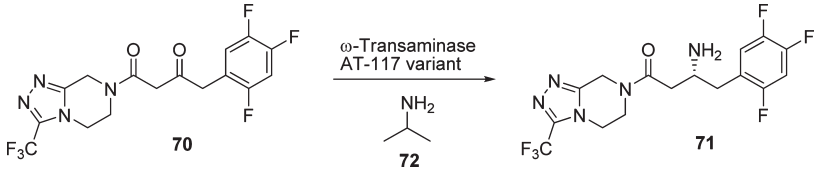
(*R*)- and (*S*)-substrates were studied separately (pairwise) to evaluate the enantioselectivity. After four rounds of low mutation rate error-prone polymerase chain reaction (epPCR), the selectivity factor *E* was increased from 1.1 to 11.3. A further (fifth) cycle led to only slight improvement of the *E* value. The strategy was changed. It was supposed that the monitored sites of amino acid exchange produced by epPCR should be hot spots which are important for the enhancement of enantioselectivity, but the amino acid found by sequencing the variants must not inevitably be the best one. Therefore, several hot spots were exposed to saturation mutagenesis trials.[132] Further variants with enhanced enantioselectivity were obtained by DNA shuffling. For the model reaction also the combined strategy of rational design and random mutagenesis was applied successfully.[133] Finally, a variant with six amino acid exchanges, D20N/S53P/S155M/L162G/T180I/T234S, was observed which not only showed an increased selectivity of *E* = 51 but also displayed higher activity. All exchanges except L162G occurred at positions remote from the active-site center S82. It is interesting that the amino acid exchanges remote from the active site influence the enantioselectivity. Molecular mechanics/quantum mechanics (MM/QM) studies followed to rationalize the findings.[132,134] As a result of theoretical studies, the double mutant S53P/L162G was prepared which exhibited *E* = 64. This finding shows that four out of six mutations in the above-mentioned variant were not necessary for the increased enantioselectivity. PAL was also subjected to studies to broaden the substrate acceptance range. Using ISM, Reetz et al. generated the improved mutein 1B2, M16A/L17F/L162N, of lipase A from *P. aeruginosa*.[22] 1B2 showed highly improved conversion rates and selectivity for (*S*)-2-methyldecanoic acid *p*-nitrophenyl ester and substrates **82**, **83**, and **85** compared with the wild-type enzyme. In the case of 2-methyldecanoic acid *p*-nitrophenyl ester, the enantioselectivity reached more than 500. By employing the CASTing approach,[135] PAL variants for substrates shown in Figure 3 were created. The substrates differ from fatty acid mimicking substrates and are sterically more demanding; some are even nonchiral (compounds **81**, **87**, and **88**).

The lipase from *P. fluorescens* has been engineered for the improved hydrolysis of carboxylic acid esters by performing the combinatorial active-site saturation test (CAST).[136] CASTing resulted in a library of enzymes exhibiting a broad substrate range.

Scheme 20. Conversion of 2-Tetralone to (*S*)-2-Aminotetraline by Designed (*S*)-Transaminase

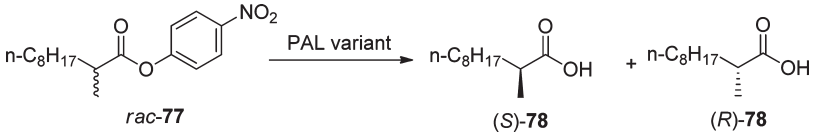


Scheme 21. Synthesis of Sitagliptin



Scheme 22. 2-Aminoalkane Derived from a 2-Ketone Precursor

Scheme 23. Kinetic Resolution of Esters by Lipase Variants



dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

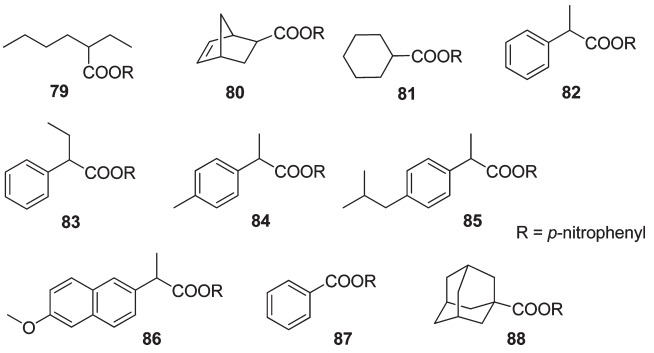


Figure 3. Substrates for kinetic resolution by PAL variants with increased enantioselectivity.[70]

Scheme 24. Lipase-Catalyzed Hydrolysis of *meso*-1,4-Diacetoxycyclopent-2-ene

Lipase A from *Bacillus subtilis* (BSLA) was a further candidate subjected to two directed evolution studies (Scheme 24).[137,138]

While the wild-type enzyme exhibited an ee of 38%, the best variant showed ee values up to 83% with reversed enantioselectivity compared to the wild-type enzyme. The D133G/M134L/I135N variant of the same enzyme was developed for the synthesis of (*S*)-(+)-1,2-*O*-isopropylidene-*sn*-glycerol. Compared with the wild-type enzyme, the variant showed inverted and improved enantioselectivity.[139,140]

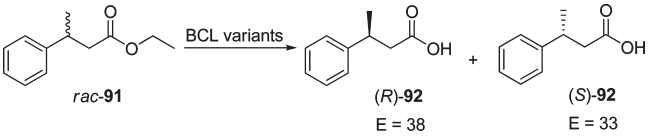
Lipase of *B. cepacia* KWI-56 was investigated in enantioselectivity inversion studies for the hydrolysis of racemic ethyl 3-phenylbutanoate (**91**) (Scheme 25).[141]

In another example, *B. cepacia* lipase mutein L17S/L287I has proven to be a very useful biocatalyst for the resolution of racemic 2-chloro-ethyl-2-bromophenylacetate with an *E* value of 178 compared to 13 of the wild-type.[142] Lip2p, a lipase from *Yarrowia lipolytica*, demonstrates a low (*S*)-enantioselectivity (*E* = 5) during the hydrolytic, kinetic resolution of 2-bromophenylacetic acid octyl ester, which is an important intermediate in the pharmaceutical industry. Using a rational engineering approach, the (*R*)-selective variant D97A/V232F exhibiting an *E* value >200 was discovered.[143]

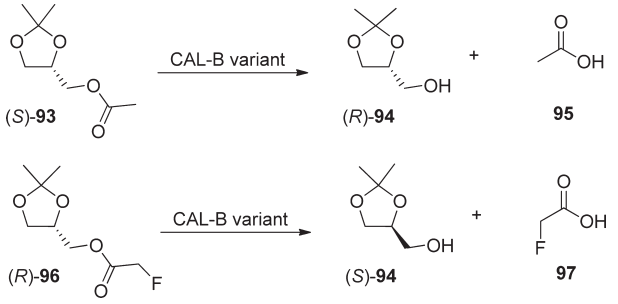
When an isoleucine residue in the active site was replaced by alanine or phenylalanine, the enantioselectivity for the acylation of 2-phenylethanol decreased or increased, respectively, depending on the steric hindrance in the active site.[144]

Lutz and co-workers significantly improved the catalytic activity of *Candida antarctica* lipase B (CALB) by "circular permutation" of the protein structure.[145] A library of CALB variants was constructed linking N- and C-termini of the enzyme. Instead, the protein backbone was genetically cleaved at different regions. Functional permutations were found to be concentrated in three regions of the protein but not tolerated in the catalytic site region. Permutation in helix α17 led to 10-fold higher activity than that of the wild-type enzyme due to increased access to the active site. The redesign of CALB has also been attempted using a rational design approach to obtain an increased acceptance of larger secondary alcohols.[146] The exchange W104A led to an

Scheme 25. Kinetic Resolution of *rac*-3-Phenylbutanoate



Scheme 26. Model System for Genetic Selection Developed by Reetz in 2008[149]



increase in selectivity of CALB for the acylation of 5-nonanol over 2-propanol of about 4×10^5. The variant W104A exhibited (*S*)-selectivity for the acylation of secondary alcohols and is, therefore, enantiocomplementary to the wild-type for the acylation of 1-phenylethanol. The rational redesign of CALB was performed to turn this hydrolase into a lyase for the catalysis of the Michael reaction.[147] The S105A variant catalyzed the addition of several thiols to methyl acrylate.[148]

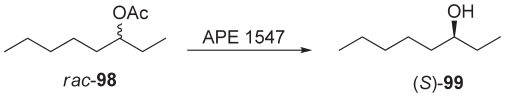
In a method based on genetic selection developed by Reetz and co-workers, a mutein library of CALB expressed in *Pichia pastories* was grown on a mixture of isopropylidene glycerol (IPG) ester,[149] wherein the "pseudoenantiomers" were the acetate ester of (*S*)-IPG and the fluoroacetate ester of (*R*)-IPG (Scheme 26). Wild-type CALB displayed an *E* value of 1.9 in favor of (*R*)-enantiomer hydrolysis. Applying this technique, 8 (*S*)-selective CALB variants with *E* values of up to 8 were obtained from only 10 picked colonies, compared to 25 of 192 colonies shown to display similar improvements using a standard mutagenesis and screening method.[150]

CALB variant cp283 was engineered by circular permutation for an improved hydrolysis of C4−C8 carboxylic esters. Up to 175 times faster hydrolysis of those esters and a 2.6- to 9-fold enhanced *trans*-esterification and interesterification of triglycerides was observed.[151] CALB variants with slightly increased selectivity for the resolution of 2-ethyl-1-hexanol and improved activity for the hydrolysis of *p*-nitrophenyl 2-methylbutyrate were described recently.[152]

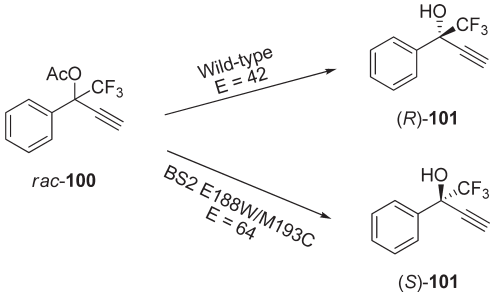
An F149Y/I150N/F233G variant of *C. antarctica* lipase A (CalA) was developed for the hydrolysis of α-substituted *p*-nitrophenyl esters by directed evolution. The *E* values of this variant for seven different esters were up to >200, which is much higher than for the wild-type. Kinetic resolution of some alkyl 2-phenylpropionates could be performed with very high *E* values, thus leading to products with an enantiomeric excess between 95% and 99%. A 30-fold increase in activity was also observed for most substrates. In contrast to most examples using the wild-type, the enzyme variant shows (*R*)-selectivity.[153] Previously, a CAST-based

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 27. Kinetic Resolution of 3-Octyl Acetate 98



Scheme 28. BS2 Variant with Inverted Enantioselectivity



engineering approach led to improved CalA variants with *E* values of 52 (*S*) and 27 (*R*) for the hydrolysis of 4-nitrophenyl 2-methylheptanoate.[154]

LST-03 lipase from organic solvent-tolerant *P. aeruginosa* LST-03 has basically high stability and activity in the presence of various organic solvents. Using directed evolution, the stability of the lipase was further increased. Muteins R65 (S164K/T188F/S211R), R88 (S155L/G157R/G177V/S194R/S202W/D209N), R96 (S155L), and R162 (L145H) show a 2−11 times longer half-life time at pH 8.0 and 30 °C in the presence of 25 vol % DMSO, cyclohexane, *n*-octane, or *n*-decane.[155]

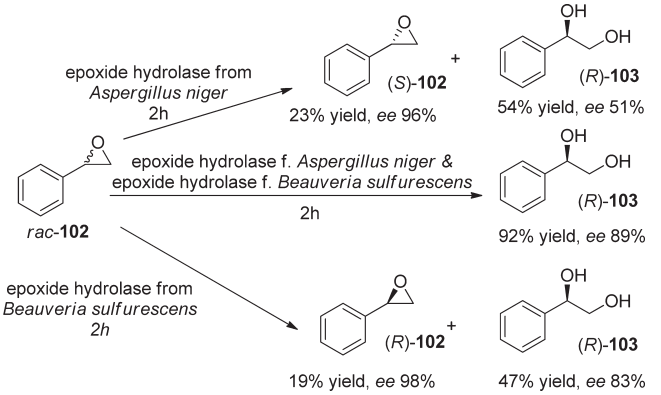
Esterases. In 1996, F. H. Arnold reported the evolution of a *p*-nitrobenzyl esterase for increased stability in aqueous−organic solvents.[156] The *p*-nitrobenzyl residue is frequently used for protection of the acid functionality as an ester in β-lactam antibiotics. The wild-type enzyme has low activity in the presence of the solvent dimethylformamide (DMF), which must be added to solubilize the substrate. A combination of epPCR and DNA shuffling led to the generation of a variant with 150-fold higher activity in 15% DMF compared to the wild-type enzyme. Later, also the thermostability of the enzyme was increased by nearly 14 °C.

P. fluorescens esterase (PFE) variants with slightly increased enantioselectivity as compared to the wild-type enzyme were investigated by Bornscheuer et al. for the kinetic resolution of racemic esters with varying results.[157−159] Later, the selectivity of the kinetic resolution of various 3-phenyl butyrates was improved with muteins M1 (V121I/F198G/V225A) and M2 (V121I/F198C) of an esterase from *P. fluorescens*.[160]

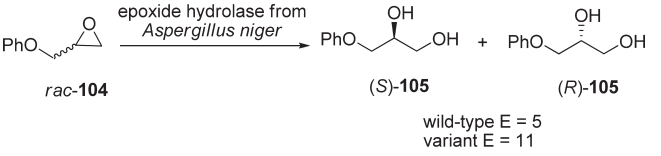
The hyperthermophilic esterase from *Aeropyrum pernix* K1 (APE 1547) was used for the hydrolytic, kinetic resolution with 2.6-fold increased enantioselectivity after a single epPCR cycle with five amino acid exchanges in spatial distance to the active site (Scheme 27).[161]

Among the chiral molecules which are interesting starting materials for the synthesis of natural products and pharmaceuticals are enantiopure tertiary alcohols. In a systematic search, Bornscheuer et al. found hydrolases for the kinetic resolution of tertiary alcohols.[162] During a comprehensive study of sequence−structure−function relationships of 92 microbial serine hydrolases, the motif GGG(A)X was identified as a consensus sequence of

Scheme 29. Enantioconvergent Kinetic Resolution of an Epoxide with Two Enantiocomplementary Hydrolases[171]



Scheme 30. Improved Enantioselectivity of Epoxide Hydrolase from *A. niger*



hydrolases applicable in the kinetic resolution of tertiary alcohols. Protein design led to an improved esterase BS2 mutant from *B. subtilis* toward tertiary alcohols. The mutant showed high selectivity for both enantiomers,[163] but the substrate range was rather limited.[164] A focused directed evolution approach was performed to develop a variant which displayed inverted enantioselectivity toward tertiary alcohols (Scheme 28).[165]

Mutant EstA-AGA from *Paenibacillus barcinonensis* showed improved selectivity in the kinetic resolution of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate and its 4′-fluorophenyl as well as 4′-chlorophenyl derivatives. *E* values larger than 100 were reported.[166] Variant E188W/M193C of esterase BS2 shows an altered selectivity in the hydrolysis reaction of various (*Z*)-glutamate diesters. The variant has a clear preference for the regioselective hydrolysis at the α-position of glutamate diesters, while the wild-type BS2 preferably cleaves the γ-ester.[167]

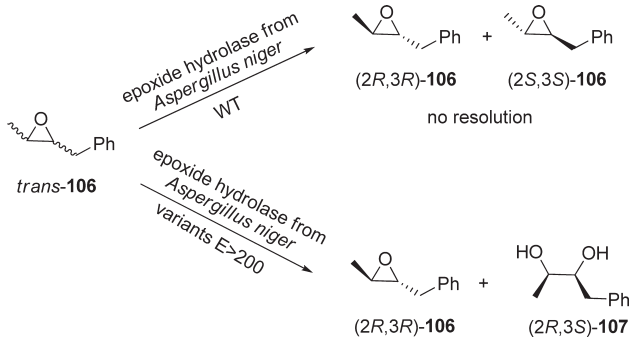
The hydrolysis rate of 7-aminocephalosporanic acid to the 3-desacetyl derivative (HACA) was improved by a factor of 4 when *B. subtilis* MTCC 121 esterase was mutated to variants R231G, Y222H/D43G, and M138V.[168]

Directed evolution was performed to improve the properties of enzyme LovD toward the semisynthesis of simvastatin from monacolin J acid (MJA) and a synthetic α-dimethylbutyryl thioester. Muteins with improved catalytic efficiency, solubility, and thermal stability were obtained, with the best variant displaying an 11-fold increase of performance.[169]

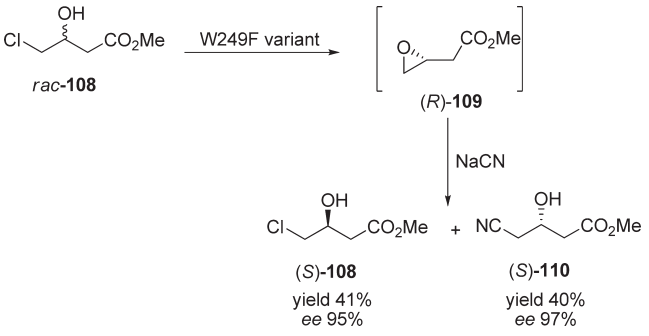
Epoxide Hydrolases. Valuable intermediates in asymmetric synthesis are chiral epoxides and enantiopure vicinal diols. These compounds have been used for the production of high-price products such as anticancer agents.[170] Epoxide hydrolases (EHs) are the enzymes of choice to get access to enantiopure epoxides by kinetic resolution of racemic epoxides and vicinal diols by stereoselective hydrolysis. Epoxide hydrolases with complementary enantioselectivity and regioselectivity lead to the formation

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 31. Kinetic Resolution of *trans*-Epoxide with EH Variants from *A. niger*



Scheme 32. Kinetic Resolution of Halohydrin



of enantiopure vicinal diols from racemic epoxides in high yield. The principle of enantioconvergent hydrolysis is shown in Scheme 29.

For purposes where the wild-type enzymes do not show the desired properties, molecular engineering approaches have been applied.[172] Epoxide hydrolase from *A. niger* (ANEH) has been developed for improved enantioselectivity in epoxide hydrolysis of glycidyl phenyl ether toward the (*S*)-enantiomer, with the best variant having three amino acid exchanges, A217V/K332E/A390E (Scheme 30).[173−175]

This enzyme has been used as a model for the study of relative efficacy when applying different methods of saturation mutagenesis techniques.[176] Via introduction of nine mutations (LW202) into ANEH, the selectivity in the kinetic resolution of *rac*-**104** could be increased to an *E* value of 115 (Scheme 31). Further examples with various aryl derivatives of *rac*-**104**, styrene epoxide, and some aliphatic epoxides highlight the superior properties of the engineered enzyme.[177]

In another approach, an esterase from *P. fluorescens* (PFE) was converted into an epoxide hydrolase. The best variant showed high enantioselectivity (E > 100) for the (*R*)-enantiomer of *p*-nitrostyrene oxide.[178]

The epoxide hydrolase of a marine fish, *Mugil cephalus*, was engineered to improve the selectivity in a kinetic resolution of styrene epoxide. Mutein F193Y/W200L/E378D increased the initial hydrolysis rate significantly, and the reaction time to reach 98% ee of (*S*)-styrene epoxide was 26-fold reduced.[179] Improved variants of epoxide hydrolase from *Agrobacterium radiobacter* AD1 (EchA) were discovered from a site-saturation mutagenesis library at position 108. Mutein F108C converted *cis*-2,3-epoxybutane to (2*R*,3*R*)-2,3-butanediol of >99% ee with a 7-fold improved activity, and mutein F108A hydrolyzed cyclohexene oxide to (1*R*,2*R*)-1,2-cyclohexanediol of >99% ee with a more than 150-fold higher activity than wild-type enzyme.[180]

Halohydrin dehalogenase (HHDH) from *A. radiobacter* is able to open epoxides by addition of nucleophiles such as cyanide. The mutein W249F was used in the sequential kinetic resolution process for the production of 4-cyano-3-hydroxybutanoate methyl ester (Scheme 32).[181] This variant also catalyzed the ring opening of different aryl epoxides by nitrite with excellent enantioselectivities.[182] Further applications of HHDH mutants from *A. radiobacter* for the conversion of epoxides were described recently.[183]

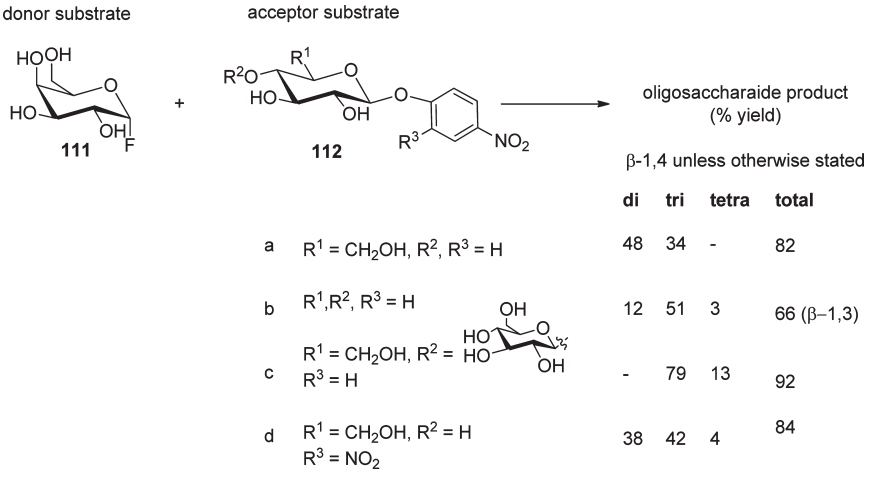
Rational protein engineering was applied to overcome the narrow substrate range of the thermophilic β-glycosidase from *Sulfolobus solfataricus*.[184] Two amino acid exchanges, E342C/W433C, resulted in a variant with reduced specificity for glucose, galactose and fucose, and increased activity toward formation of β-alkylmannosides and β-alkylxylosides.

A huge number of hydrolases belong to the group of sugar-converting enzymes. Shaikh and Withers summarized the work on designed enzymes for carbohydrate conversions in a 2008 review.[185] The mechanism-based design, engineering, and evolution of "glycosynthases" was reviewed in that paper. Glycosynthase is a nucleophile mutant of a glycosidase and, therefore, is able to perform transglycosylation without hydrolyzing the product.[186] The approach was used to expand the repertoire of glycosidic linkages formed, as well as the range of donors and acceptors recognized by the enzymes. The mechanism of the action of glycosidases with net retention of configuration was known.[187,188] On the basis of this knowledge, variants were generated by the exchange of the catalytic nucleophile to a non-nucleophilic residue to suppress the hydrolytic activity. For retaining transglycosylation activity but simultaneously suppressing hydrolysis, glycosyl fluorides with opposite anomeric configuration compare to that of the natural substrate were used for testing the mutant enzymes.[189] β-Glucosidase−galactosidase E358A variant from *Agrobacterium* sp. (Abg) was used for test studies (Scheme 33).

With the Abg variant E358S, a 24-fold improvement of activity was achieved and glycosides could be synthesized which were otherwise not accessible.[190] The Abg variant A19T/Q248R/E358G/M407V shows 1300-fold improvement compared to E358A glycosynthase.[191] While the Abg glycosynthase forms β-(1,4) linkages, a glycosynthase from *Thermus thermophilus* yielded up to 90% products with β-(1,3) glycosidic linkage.[192] Glycosynthase variants obtained from *Thermotoga maritima* were able to catalyze the regioselective synthesis of aryl β-D-Galp-(1→3)-β-D-Glcp and aryl β-D-Glcp-(1→3)-β-D-Glcp in high yields (up to 90%) using aryl β-D-glucosides as acceptors.[193] By rational design and site-directed mutagenesis of the endohexosaminidase EndoA from *Arthrobacter protoformiae*, variant E173Q was created. The catalytic rate of the mutein in the synthesis reaction leading to oligosaccharide was reduced because of the elimination of hydrolytic activity (Q cannot act as a catalytic acid−base), leading to a more productive catalyst in the synthesis direction, acting as a glycosynthase.[194] A glycosynthase derived from the β-glucuronidase from *T. maritima* was able to catalyze the synthesis of β-linked glucuronic and galacturonic acid conjugates.[195] Later, the glycosynthase variant E504G from *Escherichia coli* was created, which catalyzed the formation of β-glucuronides from α-D-glucuronyl fluoride donor and alcohol acceptors such as steroids, benzyl alcohol derivatives,

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 33. Yields of Oligosaccharides Obtained with α-Galactosyl Fluoride and Various Acceptors Catalyzed by Abg Variant E358A[185]



and alicyclic alcohols (yields 37−93%).[196] Xylooligosaccharides were synthesized using an engineered retaining xylanase from *C. fimi*.[197] Another engineered glucosynthase derived from a β-mannosidase from *C. fimi* was able to catalyze the formation of a β-D-mannopyranoside linkage, which is among the most difficult glycosidic bonds to synthesize chemically.[198]

The retaining glycosidase cel5A from *Clostridium cellulolyticum* was engineered to a glycosynthase variant by exchange of the catalytic nucleophile amino acid. Variant E307G efficiently catalyzed the glucosidic bond formation. While the wild-type enzyme displays (1→4)-glycosidase regiospecificity, the mutant shows (1→3) specificity for Glu−Glu ligation.[199] A mutagenesis approach was applied to the β-galactosidase BgaB from *Geobacillus stearothermophilus* KVE39 to improve its enzymatic transglycosylation of lactose into oligosaccharides.[200] 1G5A amylosucrase from *Neisseria polysaccharea* was engineered for the synthesis of disaccharides.[201]

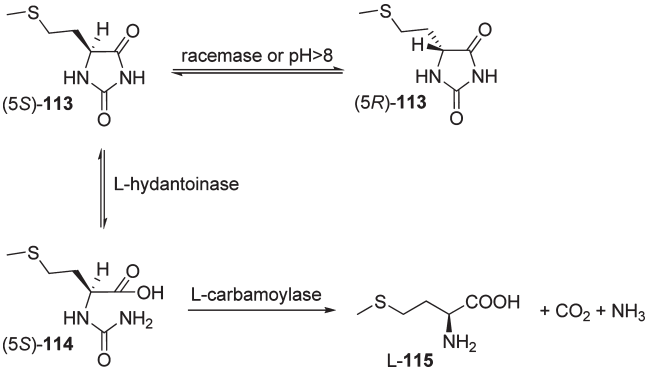
Besides all glycosynthases engineered from retaining β-glucoside hydrolases, one designed enzyme was derived from an inverting glucosidase from *Bacillus halodurans*.[202]

Polysaccharides are not the only compounds that can be synthesized by the use of glycosynthase methodology. Glucosphingolipids were synthesized using variants of endoglucoceramidase from *Rhodococcus* sp., and flavonoid glucosides were synthesized using the E197S variant of *Humicola unsolens* Cel7B.[204] For directed evolution of glycosynthases, new screening methods had to be developed to fulfill the demands of high-throughput analysis.

4.3.2. Designed Enzymes for C−N Bond Formation and Cleavage. *Hydantoinase.* The "hydantoinase process" is currently an established method for the synthesis of chiral α-amino acids with D or L configuration. Applying these enzymes, dynamic kinetic resolution with 100% conversion and complete enantioselectivity is theoretically possible. Hydantoin substrates are easy to racemize either chemically or enzymatically. Directed evolution has been applied in cases of poor asymmetric induction or if the inverted enantioselectivity was needed as for the production of L-methionine with hydantoinase from *Arthrobacter* sp. DSM9771 (Scheme 34).[205]

D-Hydantoinase of *Bacillus stearothermophilus* was engineered for the hydrolysis of (hydroxyphenyl)hydantoin (HPH). As a result, variant M63I/F159S exhibited about 200-fold higher

Scheme 34. Hydantoinase Process for the Synthesis of L-Methionine (115)



specificity for HPH than the wild-type enzyme.[206] Carbamoylase from *Ag. radiobacter* NRRL B11291 cloned in an *E. coli* strain was intensively mutated to improve the thermal stability of the enzyme by three rounds of DNA shuffling. Variant CBL303, with three important amino acid exchanges, including V40A, G75S, and V237A, was found with a 20-fold higher tolerance to heat and hydrogen peroxide.[207]

Brevibacillus agri dihydropyrimidinase (BaDHP) exhibits a substrate preference for D-homophenylalanylhydantoin (D-HPAH). Site-directed mutagenesis of BaDHP was performed, and several variants with increased activity (54−469%) toward L-HPAH were found. The L159V variant was used to convert HPAH to L-homophenylalanine (L-HPA) in the hydantoinase process. As compared with that of the wild-type enzyme, the conversion yield of L-HPA was increased from 39% to 61% by the L159V variant.[208]

Nitrilases catalyze the hydrolysis of the nitrile functionality to the carboxylic acid, which is a valuable synthetic transformation. Very often, naturally occurring nitrilases show limited activity, a narrow substrate range, and low to moderate enantioselectivity. The first nitrilase evolved regarding enantioselectivity was created for the desymmetrization of the prochiral 3-hydroxyglutardinitrile (**116**) to (*R*)-4-cyano-3-hydroxybutyric acid (**117**)

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

(Scheme 35), which is the chiral intermediate of the cholesterol-lowering drug Lipitor.[209]

Nitrilase NIT2 from *Arabidopsis thaliana* was improved for the direct hydrolysis of nitriles to the corresponding carboxylic acids. Variant 7/I2B, I23V/K103I/G109R/I220M/C230S/F238L, showed a 105-fold enhanced activity in the hydrolysis of phenylacetonitrile.[210] A nitrilase derived from *Acidovorax facilis* 72W was engineered to increase the enzyme-specific activity up to 15-fold and the biocatalyst-specific activity up to 125-fold. This improved variant (F168V/L201N) reached the desired volumetric productivity and biocatalyst productivity for the conversion of glycolonitrile to ammonium glycolate.[211]

Acylase. Microbial β-lactam acylases are of great biotechnological interest since they are applicable in the industrial production of semisynthetic antibiotics. These enzymes catalyze the hydrolysis of the amide bond between the β-lactam nucleus and the side-chain carboxylic acid in penicillins and cephalosporins, leaving the cyclic β-lactam amide bond intact. The discovery and use of these enzymes has enabled a green alternative for the production of 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA; **119**), and 7-aminodeacetoxycephalosporanic acid (7-ADCA) on a large scale. Variants obtained by site-directed mutagenesis show new specificities in the hydrolysis or synthesis of β-lactam antibiotics.[212,213] Glutaryl-7-aminocephalosporanic acid acylase was evolved to accept the natural antibiotic cephalosporin C (CephC; **118**) as a substrate.[214−216] Variant E423Y/E442Q/D445N of γ-glutamyltranspeptidase from *B. subtilis* was found to be more than 900 times faster in the hydrolysis reaction of 7-(glutarylamino)cephalosporanic acid (**120**) to 7-ACA (**119**) (Scheme 36).[217] A similar enzyme of *E. coli* K-12 was 50-fold improved by substituting three amino acids, D433N, Y444A, and G484A.[218,219]

The T206G/S213G variant of penicillin amidase from *Alcaligenes faecalis* shows a doubled turnover number for the hydrolysis of penicillin G compared with the wild-type enzyme and a 3-fold improved specificity constant.[220]

The 3G3K variant of the penicillin G acylase (PGA) from *E. coli* obtained by site-directed mutagenesis is characterized by a tag consisting of three lysines alternating with three glycines at the end of the β-chain. The variant shows an improved synthesis/hydrolysis ratio in reactions of (*R*)-mandelic acid methyl ester and cephalosporins.[221] Other muteins of *E. coli* PGA were developed for an improved production of ampicillin. The synthetic rate over the hydrolytic rate was increased 5−15-fold.[222] *Kluyvera citrophila* penicillin G acylase enzymes were generated for the conversion of penicillin G to phenylacetic acid and 6-aminopenicillanic acid using enzyme engineering techniques.[223]

Amidase. In 2003, Asano and co-workers reported the increase of thermal stability and simultaneous increase of catalytic activity for the D-amino acid amidase from *Ochrobactrum authropi* SV3, which facilitates the preparation of optically pure amino acids. The substrate range is broad for amino acid amides, even for those with a bulky side chain. After two rounds of PCR mutagenesis and selection, a variant with 5°C higher denaturation temperature and higher catalytic efficiency was obtained.[224,225]

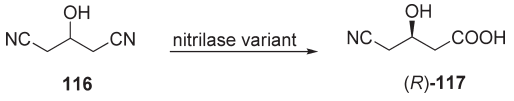
PST-01 protease from *P. aeruginosa* PST-01 was modified at position 114 by substituting tyrosine with phenylalanine for an improved synthesis of *N*-[(benzyloxy)carbonyl]aspartame.[226] Aminopeptidase from *Streptomyces thermocyaneoviolaceus* NBRC14271 was engineered into a transaminopeptidase and used to catalyze an aminolysis reaction to give linear and cyclic dipeptides. The site-directed mutagenesis of catalytic S502 into C abolished aminopeptidase activity and triggered peptide synthesis activity. The S502C variant recognized several ester derivatives of amino acids as acyl donors and acyl acceptors to give diverse peptides.[227]

4.4. Lyases E.C.4

Aldolases are among the most interesting enzymes in organic chemistry as they are able to broaden the asymmetric synthesis tools next to transition-metal catalysis and organocatalysis for the synthesis of complex carbohydrates, often because they are stereocomplementary to the latter (Scheme 37).

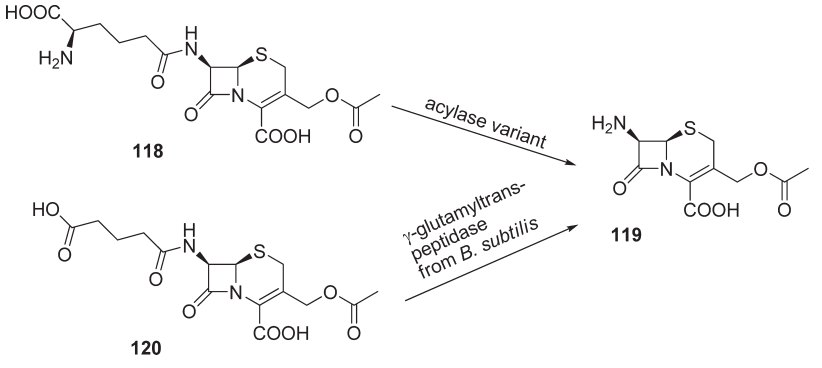
Many aldolases suffer from two main disadvantages, namely, the narrow substrate acceptance and the need for phosphorylated reagents. Therefore, many efforts were undertaken to evolve mutants which accept nonphosphorylated substrates while keeping the high stereoselectivity. Wong and co-workers reported a variant with four amino acid exchanges in KDPG aldolase, T84A/I92F/V118A/E138V, with improved substrate range for nonphosphorylated aldehydes.[228] Next to the ability of the variant to accept a nonphosphorylated substrate, it also catalyzed the condensation of L-glyceraldehyde and pyruvate **76** in addition to the naturally configurated substrate D-glyceraldehyde (Scheme 38).

Scheme 35. Enzymatic Synthesis of 4-Cyano-3-hydroxybutanoic Acid (117)



116 (*R*)-**117**

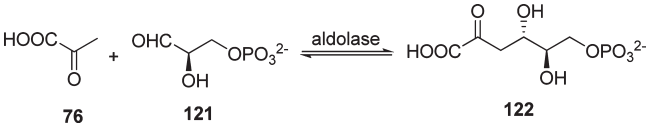
Scheme 36. Enzymatic Hydrolysis of CephC to 7-ACA



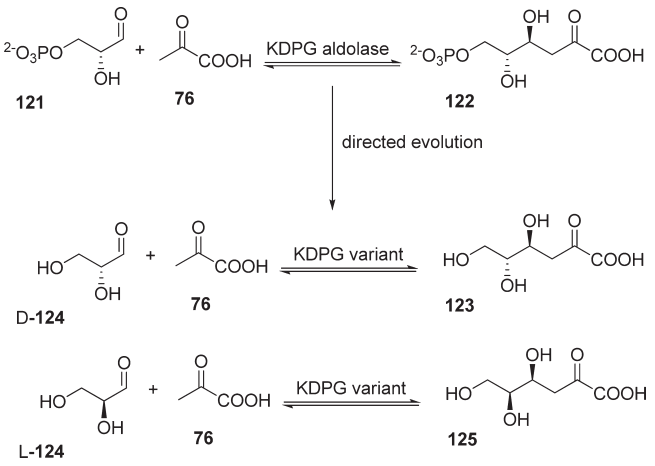
118 **119** **120**

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 37. C−C Bond Formation Catalyzed by D-2-Keto-3-deoxy-6-phosphogluconate (KDPG) Aldolase



Scheme 38. KDPG Variants Accepting Nonphosphorylated D- and L-Glyceraldehyde

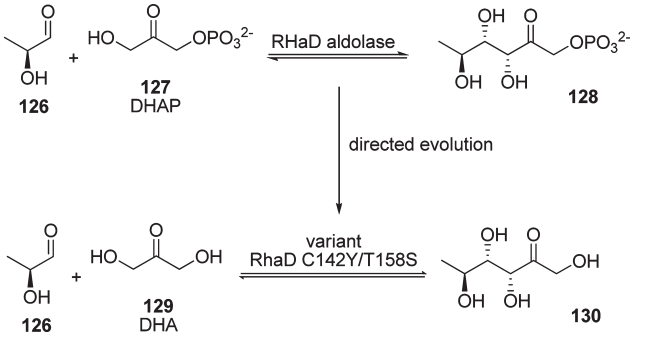


2-Keto-3-deoxygluconate aldolase from the hyperthermophile *S. solfataricus* is a highly thermostable type I aldolase that can catalyze carbon−carbon bond formation using nonphosphorylated substrates. However, it exhibits poor diastereocontrol in many aldol reactions, including the reaction of its natural substrates, pyruvate and D-glyceraldehyde. From the set of muteins tested, some variants that catalyze *re*- or *si*-facial-selective aldol reactions to afford either D-2-keto-3-deoxygluconate (D-KDGlu) or D-2-keto-3-deoxygalactonate (D-KDGal) with good levels of diastereocontrol were identified.[229] Directed evolution approaches were also applied to L-rhamnulose 1-phosphate (RhaD) aldolase to alter the donor substrate specificity of RhaD aldolase from dihydroxyacetone phosphate (DHAP) to dihydroxyacetone (DHA) (Scheme 39). In the presence of borate, RhaD aldolases were identified by an in vivo selection from a genetically engineered *E. coli* strain construct, which accepted nonphosphorylated DHA.[230] This method was used for the one-pot synthesis of L-fructose and the two-step synthesis of L-iminocyclitols.

Another one-pot synthesis was applied with D-fructose-6-phosphate aldolase (FSA) by using DHA, hydroxyacetone, and 1-hydroxy-2-butanone as donor substrates to synthesize several D-iminocyclitols.[231]

N-Acetylneuraminic acid lyase (NAL or Neu5Ac aldolase), which catalyzes the condensation of pyruvate **76** with *N*-acetylmannosamine (**131**) (Scheme 40), is another aldolase evolved from *E. coli* to obtain enhanced catalytic activity and a broader substrate scope.[232] Directed evolution has also been performed to modify the stereochemical properties, next to the expansion of the substrate range.[233] Saturation mutagenesis was used to target residues D191, E192, and S208, which were identified as hot

Scheme 39. RhaD Aldolase Mutant Accepts a Nonphosphorylated Donor Substrate



spots during structural studies.[234] The variant E192N catalyzed the retro-aldol reaction (cleavage) of **134** 5 times more efficiently than the wild-type enzyme in the cleavage of **132**. The substrate scope of the mentioned variant is broad,[235] but unfortunately, the stereoselectivity is poor.[239] However, the variant was used as a starting point for the generation of (4*R*)- and (4*S*)-selective aldolases.

The (4*R*)-selective variant harbors next to E192N also A93V/F109I/N153K/S208G amino acid exchanges, while the (4*S*)-selective variant carries T3M/A10V/T48A/Q61R/E64D/Q173R.[237] Further optimizations led to T167V/E192N and E192N/S208V variants appropriate for the selective cleavage of (4*R*)-**137** and T167G/E192N for (4*S*)-**136** (Scheme 41). The latter showed excellent stereochemical discrimination and >50-fold higher selectivity for (4*S*)-**136**. These two variants possess complementary catalytic properties regarding the stereoselectivity.[233]

Wong and co-workers evolved an NAL variant which accepts L-arabinose instead of *N*-acetylmannosamine (Scheme 42).[232,238]

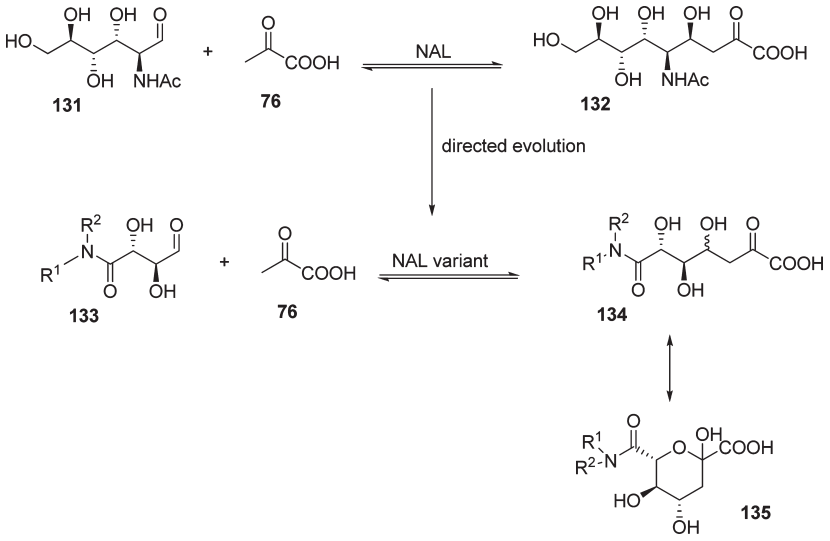
An example for changing the diastereoselectivity of an enzyme was investigated by Berry et al.[239] Tagatose aldolase was transformed into a fructose aldolase (Scheme 43).

2-Deoxyribose-5-phosphate aldolase (DERA) has been evolved for application in the synthesis of statin drug.[240] DERA, which catalyzes the condensation of acetaldehyde and D-glyceraldehyde-3-phosphate (**121**), has been rationally designed to increase the enzyme activity toward nonphosphorylated acceptors. Variant S234D showed 2.5-fold higher activity toward D-glyceraldehyde.[241,242] A library of DERA variants have been prepared using epPCR for the condensation reaction of 3-chloropropanal (**144**) and two acetaldehyde molecules (Scheme 44). Variant F200I/S258T/Y259T was the most efficient catalyst for the synthesis and industrial production of (3*R*,5*S*)-chloro-2,4,6-trideoxyhexapyranoside, which is the key intermediate for the cholesterol-lowering drug Lipitor (atorvastatin).

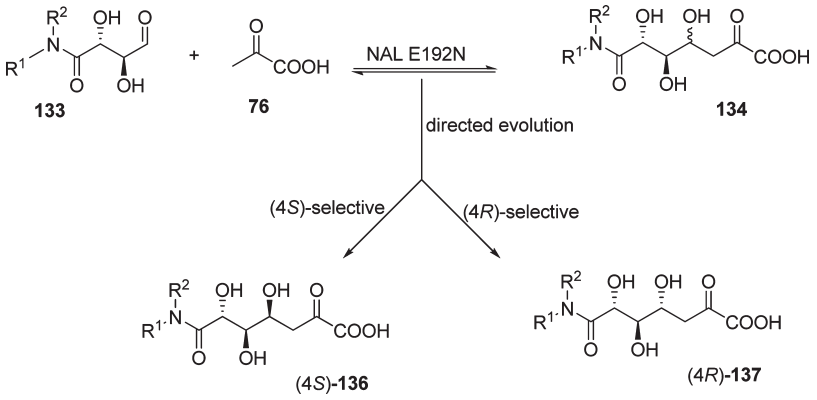
Diastereoselectivity-enhanced variants of L-threonine aldolase (L-TA) for L-*threo*-3,4-dihydroxyphenylserine (L-*threo*-DOPS) synthesis were isolated by error-prone PCR followed by a high-throughput screening. The most improved variant was T3-3mm2, Y34C/Y39C/A48T/Y306C, which showed a 4-fold increase of diastereoselectivity over wild-type L-TA.[243]

Hydroxynitrile Lyases (HNLs). Although many HNLs are well-characterized enzymes and have already made their way to industrial applications, there is still room for improvements. Not all substrates can be converted in sufficient amount and enantiopurity. Enzyme and/or substrate engineering is a widely

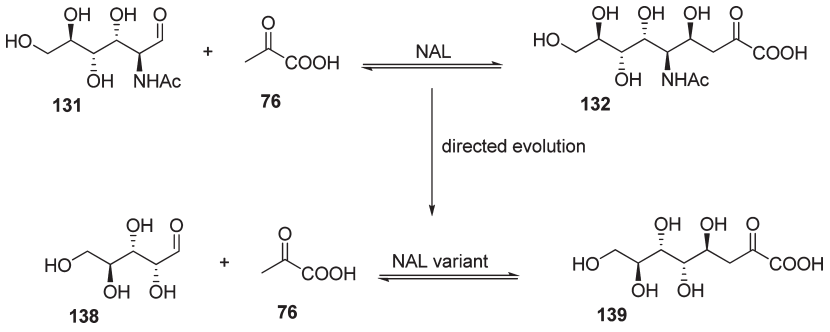
Scheme 40. Directed Evolution of an Aldolase for Application in the Parallel Synthesis of Sialic Acid Mimetics[236]



Scheme 41. NAL Variants with Improved Enantioselectivity toward C-4



Scheme 42. NAL Variant Catalyzes the Condensation of Arabinose and Pyruvate

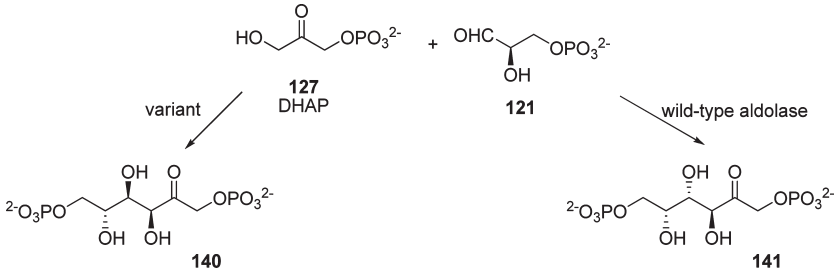


used approach to decrease the shortcomings. Substrate engineering attempts were reported by Griengl and co-workers with HNLs.[244] An example of a coupled approach of substrate and enzyme engineering published recently showed impressive results regarding both activity (10−20 times lower enzyme amount required) and selectivity (ee increased from 10% to about 90%).[245] Designed (*S*)-HNLs of *Manihot esculenta* were generated to improve the synthesis of (*S*)-2-chloromandelonitrile.[246]

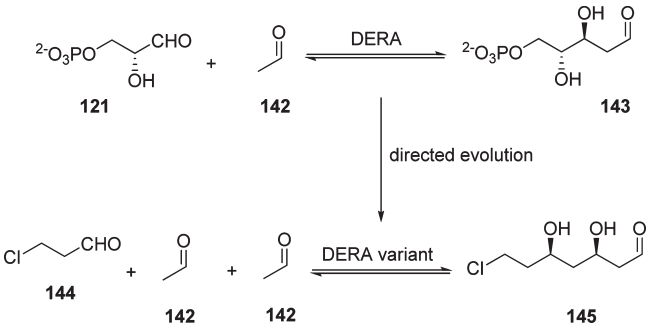
Glieder and co-workers have improved the HNL from *Prunus amygdalus* (*Pa*HNL) starting from (*R*)-HNL isoenzyme 5 for synthesizing (*R*)-pantolactone, which is an intermediate in vitamin B5 synthesis. (*R*)-Pantolactone can be synthesized from

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

Scheme 43. Tagatose Aldolase Engineered to Fructose Aldolase



Scheme 44. (3*R*,5*S*)-7-Chloro-3,5-dihydroxyheptanal Synthesis by the DERA Variant



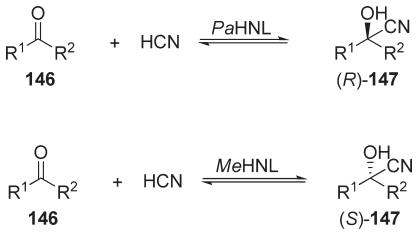
hydroxypivalaldehyde and HCN catalyzed by *Pa*HNL. The ee and the amount of enzyme needed for the reaction were not satisfying. Several preparations of natural and recombinant *Pa*HNL isoenzymes and also other *Rosaceae* HNLs were screened. However, enzymes with improved properties regarding activity and selectivity were not found. At this point, the best enzyme was subjected to saturation mutagenesis at several positions, identified by molecular modeling. The ee could be increased from 89% to 97% thereby.[247,248] Another success story regarding *Pa*HNL improvement is mutein *Pa*HNL5-L1Q-A111G. Large-scale production of (*R*)-2-chloromandelic acid—the chiral building block for the drug Clopidogrel—via (*R*)-2-chlorobenzaldehyde cyanohydrin was hindered by low turnover rates and moderate ee in both the enzymatic and metal-catalyzed reactions. The rationally designed mutation of alanine to glycine at position 111 raised the yield enormously.[249,250]

Another example for improved HNL is the "tunnel variant" W128A of the HNL from *M. esculenta* (*Me*HNL).[251] On the basis of the crystal structure and reaction mechanism of *Me*HNL, a tryptophan residue at the entrance to the active site was supposed to play a crucial role regarding enzyme activity. Replacing the bulky amino acid by the smaller amino acid alanine leads to the enhanced activity.[252]

In two other examples, HNLs were highly improved by single point mutations to convert sterically demanding substrates (Scheme 45)[253] and to obtain improved enantioselectivity.[254] A single amino acid replacement improved the folding and stability of recombinant *Me*HNL expressed in *E. coli*.[255]

Arylmalonate decarboxylase was redesigned by introducing a second cysteine into the active site to yield a racemase. The mutant was active in racemization of α-(2-naphthyl)propionic acid. Five further related substrates could also be racemized.[256] Another engineered arymalonate decarboxylase was created for the inversion of configuration. The decarboxylation takes place

Scheme 45. Synthesis of Chiral Enantiocomplenentary Cyanohydrines by HNLs from Different Sources



through an enolate intermediate and gets finalized by protonation with a cysteine residue, 188, on the *si*-face of the substrate. An enzyme with complementary enantioselectivity was created by the exchange C188S and introduction of a cysteine at the *re*-face of the substrate in the active site.[257] Arylmalonate decarboxylase from *Bordetella bronchiseptica* has been modified, and several variants have been shown to be more effective in the decarboxylation of α-arylmalonates and disubstituted malonates possessing aryl or alkyl residues.[258,259]

Benzoylformate decarboxylase (BFD) is a thiamin diphosphate (ThDP)-dependent enzyme and exhibits carbon−carbon bond formation activity. It can be used for the synthesis of (*S*)-2-hydroxy-1-phenylpropanone from benzaldehyde and acetaldehyde. The carboligase activity of the enzyme from *P. putida* has been increased 5-fold after several rounds of directed evolution.[260] Studies revealed L461 as a hot spot for stereoselectivity in BFD. Exchange to alanine and glycine resulted in variants that catalyze the (*S*)-stereoselective addition of larger acceptor aldehydes, such as propanal with benzaldehyde and its derivatives, which are not catalyzed by the wild-type enzyme.[261] Benzoylformate decarboxylase from *P. putida* was engineered, and some of the variants showed improved decarboxylation activity toward 2-ketohexanoate, 2-ketopentanoate, and 2-ketobutanoate.[262] The formation of (*S*)-2-hydroxy-1-(2-substituted)phenyl propan-1-one (**149**) was performed by BFD variants L476Q and M365L/L461S (Scheme 46).[263]

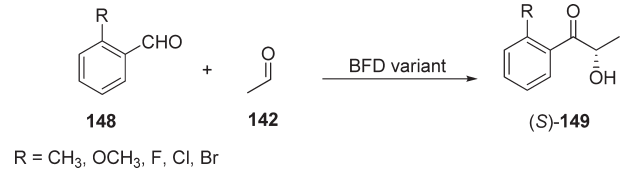
Introducing an A28S mutation into benzaldehyde lyase (BAL) from *P. fluorescens* allows the enzyme to decarboxylate benzoylformate.[264]

Transketolase, another thiamin diphosphate-dependent enzyme of *E. coli*, is involved in the C−C bond formation reaction for the biosynthesis of D-ribose-5-phosphate in nature (Scheme 47). Variants of this enzyme were prepared on the basis of focused mutation within the active site and screened for improved enantioselectivity.

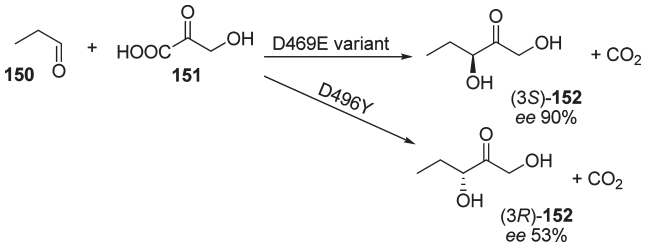
An engineered variant of *E. coli* transketolase (D469T) was used for the initial asymmetric synthesis of (3*S*)-1,3-dihydroxypentan-2-one (**152**) from achiral substrates propanal (**150**) and

dx.doi.org/10.1021/cr100386u |*Chem. Rev.* 2011, 111, 4141–4164

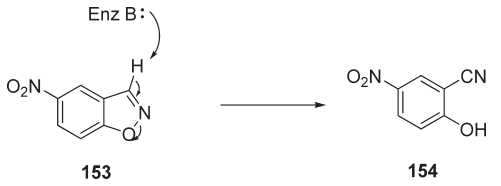
Scheme 46. Enantioselective C−C Bond Formation by BFD



Scheme 47. C−C Bond Formation Catalyzed by Transketolase



Scheme 48. Kemp Elimination



hydroxypyruvate **151**. The variant showed a nearly 5-fold increased specific activity.[265]

Tyrosine phenol-lyase from *Symbiobacterium toebii* was engineered to improve both its stability and its catalytic activity by the application of random mutagenesis and subsequent reassembly of the acquired mutations. Finally, up to 3-fold improved activities and half-inactivation temperatures improved by up to 11.2 °C were obtained. Sequence analysis revealed mutations which were beneficial for the stability or activity improvement.[266] Introduction of selected single point mutations into tyrosine phenol lyase from *Citrobacter freundii* led to enzyme variants which had properties superior to those of the wild-type. The best variant, M379V, transformed, in contrast to the wild-type, *o*-cresol, *o*-methoxyphenol, and *o*-chlorophenol efficiently to the corresponding tyrosine derivatives without any detectable side product.[267]

4.5. Isomerases E.C.5

The alanine racemase from *G. stearothermophilus* was engineered to generate aldolase activity. By introducing a single point mutation in the active site, the Y265A variant was able to catalyze the cleavage of both (2*R*,3*S*) and (2*R*,3*R*)-α-methyl-β-phenylserines to benzaldehyde and alanine with similar rates. The stereoselectivity at the β-position of the substrate was poor, but the α-selectivity was good. Only D-configurated substrates were transformed.[268] Successful examples for the synthesis of β-hydroxy-α-amino acids with a re-engineered alanine racemase were described recently.[269]

Tyrosine 2,3-aminomutase from *Chondromyces crocatus* was engineered, and the selectivity of the E399K variant showed a significantly enhanced selectivity to produce (*R*)-β-tyrosine from L-tyrosine.[270]

4.6. New Enzymes. In Silico Enzyme Design

Baker, Tawfik, and co-workers pioneered the creation of an enzyme catalyzing a reaction for which no natural equivalent is believed to exist. They demonstrated the computational design of an enzyme activity for the Kemp elimination, identifying the requirements for a catalytic process (Scheme 48).

From 59 designs of the catalytic base in 17 different protein folds which were created and evaluated, 8 showed measurable activity. Using a directed evolution approach, the catalytic efficiency could be improved by more than 200-fold.[271,272] This concept can serve as the starting point for the creation of designed enzymes with no natural origin and equivalent for every thinkable chemical reaction.

5. CONCLUSION

The large number of designed enzymes successfully used in organic synthesis convincingly shows that the application of methods and tools of mutagenesis paves the way to improve the efficiency, selectivity, and stability of biocatalysts as required for certain purposes. The development of the methods is progressing rapidly, e.g., by the use of fully synthesized libraries and more efficient analytical methods and increasing knowledge of enzyme function. Our understanding of molecular interactions is still a key limitation and needs more attention. Research should focus not only on designing improved enzymes but also on comprehending the effects we see.

However, even with limited insight into molecular mechanisms, enzymes are having a huge, positive impact on the sustainability parameters of chemical manufacturing processes. Undoubtedly, this impact will grow as the limitations of enzymes will no longer be limitations for these processes. Future enzyme design strategies will enable sustainable processes to be set up within shorter timelines and with lower cost and higher chances of success.

AUTHOR INFORMATION

Corresponding Author

*Phone: +43-316-873 32410. Fax: +43-316-873 1032410. E-mail: mandana.gruber@acib.at.

BIOGRAPHIES

Gernot A. Strohmeier studied chemistry and business economics and obtained his Ph.D. in chemistry from the Karl

Franzens University of Graz in 2003 for research on combinatorial chemistry. In 2005 he joined the Applied Biocatalysis (A-B) Research Centre and started with research on biocatalytic applications for the synthesis of chiral compounds and pharmaceuticals. He is currently a project manager at the Austrian Center of Industrial Biotechnology, the successor of the A-B Research Centre. His main interests focus on the combination of chemical and biocatalytical methods for the generation of chiral molecules, biopolymers, and pharmaceuticals.

Harald Pichler obtained his Ph.D. from Graz University of Technology in 2000 for research on lipid synthesis and transport in yeast under the supervision of Prof. Guenther Daum. Supported by a Federation of European Biochemical Societies long-term fellowship, he undertook postdoctoral studies with Prof. Howard Riezman at both the Biozentrum of the University of Basel and the University of Geneva dealing with the roles of sterols in endocytosis. Upon returning to Graz University of Technology in December 2003, he joined the newly founded Institute of Molecular Biotechnology headed by Prof. Helmut Schwab and extended his research interests to recombinant protein expression and enzyme engineering in *Escherichia coli* and yeasts. In 2005, Harald Pichler became a project manager in the Applied Biocatalysis Research Centre, which developed into the Austrian Center of Industrial Biotechnology in 2010. In 2009, he was offered a tenure-track, assistant professor position at Graz University of Technology and is establishing a research team working on the biotechnology of cellular membranes.

Oliver May is Corporate Scientist Biocatalysis and is responsible for coordinating the biocatalysis-related activities of DSM. Before joining DSM in April 2006 as Competence Manager Biocatalysis of DSM Pharmaceutical Products, he was with Degussa (now Evonik) in various functions, the latest as General Manager of Degussa's Service Center Biocatalysis. He was educated in Germany, receiving a Ph.D. degree in technical biology from the University of Stuttgart, where he worked at the Institute of Biochemical Engineering for Prof. C. Syldatk and at the German Center for Biotechnology in Braunschweig with Prof. D. Schomburg. He joined Caltech as a postdoc in 1998, where he worked until 2000 in the group of Prof. F. H. Arnold on directed evolution of enzymes. Oliver May has authored more than 35 scientific contributions in journals and books and more than 17 patent applications. He and his team were awarded several innovation awards on hydantoinase technology, whole-cell bioredox processes, and the latest on recombinant pig liver esterase in its application for production of a pharma intermediate.

Mandana Gruber-Khadjawi was born in Tehran, Iran. She obtained her Ph.D. in technical chemistry from Graz University of Technology in 1994 for research on asymmetric synthesis by application of chemo- and biocatalysis under the supervision of Prof. Dr. Helmut Hönig. She worked as a scientific assistant and lecturer at Graz University of Technology and Karl-Franzens University before she joined the Austrian Centre of Industrial Biotechnology, formerly the Research Centre of Applied Biocatalysis, in 2004 as a researcher and project manager. Her main research interests include biocatalysis and asymmetric synthesis.

ACKNOWLEDGMENT

We thank Professor Kurt Faber for his thoughtful comments on the manuscript.

REFERENCES

(1) Eigen, M.; Hammes, G. G. *Adv. Enzymol. Relat. Areas Mol. Biol.* **1963**, *25*, 1.
(2) Liese, A.; Seelbach, K.; Wandrey, C. *Industrial Biotransformations*; Wiley-VCH: Weinheim, Germany, 2000.
(3) Turner, M. K. *Trends Biotechnol.* **1995**, *13*, 173.
(4) Maniatis, T.; Hardison, R. C.; Lacy, E.; Lauer, J.; O'Connell, C.; Quon, D.; Sim, G. K.; Efstratiadis, A. *Cell* **1978**, *15*, 687.
(5) Witkin, E. M. *Bacteriol. Rev.* **1976**, *40*, 869.
(6) Singer, B.; Kusmierek, J. T. *Annu. Rev. Biochem.* **1982**, *51*, 655.
(7) Cox, E. C. *Annu. Rev. Genet.* **1976**, *10*, 135.
(8) Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G.; Ehrlich, H. *Cold Spring Harbor Symp. Quant. Biol.* **1986**, *51*, 263.

(9) Ho, S. N.; Hunt, H. D.; Horton, R. M.; Pullen, J. K.; Pease, L. R. *Gene* **1989**, *77*, 51.
(10) Cadwell, R. C.; Joyce, G. F. *PCR Methods Appl.* **1992**, *2*, 28.
(11) Stemmer, W. P. C. *Nature* **1994**, *370*, 389.
(12) Kikuchi, M.; Ohnishi, K.; Harayama, S. *Gene* **1999**, *236*, 159.
(13) Vanhercke, T.; Ampe, C.; Tirry, L.; Denolf, P. *Anal. Biochem.* **2005**, *339*, 9.
(14) Welch, M.; Villalobos, A.; Gustafsson, C.; Minshull, J. *J. R. Soc. Interface* **2009**, *6*, 467.
(15) Cedrone, F.; Menez, A.; Quemeneur, E. *Curr. Opin. Struct. Biol.* **2000**, *10*, 405.
(16) Hult, K.; Berglund, P. *Curr. Opin. Biotechnol.* **2003**, *14*, 395.
(17) Padhi, S. K.; Fujii, R.; Legatt, G. A.; Fossum, S. L.; Berchtold, R.; Kazlauskas, R. J. *Chem. Biol.* **2010**, *17*, 863.
(18) Bloom, J. D.; Labthavikul, S. T.; Otey, C. R.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 5869.
(19) O'Loughlin, T. L.; Patrick, W. M.; Matsumura, I. *Protein Eng., Des. Sel.* **2006**, *19*, 439.
(20) Tracewell, C. A.; Arnold, F. H. *Curr. Opin. Chem. Biol.* **2009**, *13*, 3.
(21) Kretz, K. A.; Richardson, T. H.; Gray, K. A.; Robertson, D. E.; Tan, X.; Short, J. M. *Methods Enzymol.* **2004**, *388*, 3.
(22) Reetz, M. T.; Prasad, S.; Carballeira, J. D.; Gumulya, Y.; Bocola, M. *J. Am. Chem. Soc.* **2010**, *132*, 9144.
(23) Reetz, M. T.; Carballeira, J. D.; Peyralans, J.; Höbenreich, H.; Maichele, A.; Vogel, A. *Chemistry* **2006**, *12*, 6031.
(24) Morley, K. L.; Kazlauskas, R. J. *Trends Biotechnol.* **2005**, *23*, 231.
(25) Kazlauskas, R. J. *Curr. Opin. Chem. Biol.* **2005**, *9*, 195.
(26) Bali, S.; Weissman, K. J. *ChemBioChem* **2006**, *7*, 1935.
(27) Asako, H.; Shimizu, M.; Makino, Y.; Itoh, N. *Tetrahedron Lett.* **2010**, *51*, 2664.
(28) Asako, H.; Shimizu, M.; Itoh, N. *Appl. Microbiol. Biotechnol.* **2008**, *80*, 805.
(29) Campapiano, O.; Mundorff, E.; Borup, B.; Voladri, R. WO 2009046153, April 9, 2009; *Chem. Abstr.* **2009**, *150*, 416448.
(30) Ching, C.; Gruber, J. M.; Huisman, G. W.; Mundorrf, E.; Newman, L. M. WO 2008103248, August 28, 2008; *Chem. Abstr.* **2008**, *149*, 326218.
(31) Giver, L. J.; Newman, L. M.; Mundorff, E. WO 2008042876, April 10, 2008; *Chem. Abstr.* **2008**, *148*, 420222.
(32) Ma, S. K.; Gruber, J.; Davis, C.; Newman, L.; Gray, D.; Wang, A.; Grate, J.; Huisman, G. W.; Sheldon, R. A. *Green Chem.* **2010**, *12*, 81.
(33) Liang, J.; Lalonde, J.; Borup, B.; Mitchell, V.; Mundorff, E.; Trinh, N.; Kochrekar, D. A.; Nair Cherat, R.; Pai, G. G. *Org. Process Res. Dev.* **2010**, *14*, 193.
(34) Liang, J.; Borup, B.; Mitchell, V.; Mundorff, E.; Lalonde, J.; Huisman, G. WO 2009042984, April 2, 2009; *Chem. Abstr.* **2009**, *150*, 347250.
(35) Liang, J.; Jenne, S. J.; Mundorff, E.; Voldri, R.; Lalonde, J.; Huisman, G. WO 2009029554, March 5, 2009; *Chem. Abstr.* **2009**, *150*, 258399.
(36) Liang, J.; Jenne, S. J.; Mundorff, E.; Ching, C.; Gruber, J. M.; Krebber, A.; Huisman, G. W. WO 2009036404, March 19, 2009; *Chem. Abstr.* **2009**, *150*, 347248.
(37) Mundorff, E.; De Vries, E. WO 2010025085, March 4, 2010; *Chem. Abstr.* **2010**, *152*, 329268.
(38) Savile, C.; Gruber, J. M.; Mundorff, E.; Huisman, G. W.; Collier, S. WO 2010025238, March 4, 2010; *Chem. Abstr.* **2010**, *152*, 329266.
(39) Savile, C.; Gruber, J. M.; Mundorff, E.; Huisman, G. W.; Collier, S. WO 2010025287, March 4, 2010; *Chem. Abstr.* **2010**, *152*, 329270.
(40) Voladri, R.; Gooding, O.; Jenne, S.; Mundorff, E. U.S. Patent 20100055751, March 4, 2010; *Chem. Abstr.* **2010**, *152*, 306027.
(41) Li, S.; Chaulagain, M. R.; Knauff, A. R.; Podust, L. M.; Montgomery, J.; Sherman, D. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 18463.
(42) Machielsen, R.; Leferink, N. G. H.; Hendriks, A.; Brouns, S. J. J.; Hennemann, H. G.; Daussmann, T.; Oost, J. *Extremophiles* **2008**, *12*, 587.
(43) Machielsen, R.; Looger, L. L.; Raedts, J.; Dijkhuizen, S.; Hummel, W.; Hennemann, H. G.; Daussmann, T.; van der Oost, J. *Eng. Life Sci.* **2009**, *9*, 38.
(44) Schneider, N.; Höffken, W.WO 2010079068, July 15, 2010; *Chem. Abstr.* **2010**, *153*, 196109.
(45) Tomoko, H.; Ito, S.; Makino, S.; Ori, T. JP 2009225773, Oct 8, 2009; *Chem. Abstr.* **2009**, *151*, 442601.
(46) Zhang, R.; Zhu, G.; Zhang, W.; Cao, S.; Ou, X.; Li, X.; Bartlam, M.; Xu, Y.; Zhang, X. C.; Rao, Z. *Protein Sci.* **2008**, *17*, 1412.
(47) Zhang, R.; Xu, Y.; Sun, Y.; Zhang, W.; Xiao, R. *Appl. Environ. Microbiol.* **2009**, *75*, 2176.
(48) Katzberg, M.; Skorupa-Parachin, N.; Gorwa-Grauslund, M. F.; Bertau, M. *Int. J. Mol. Sci.* **2010**, *11*, 1735.
(49) Nair, N. U.; Zhao, H. *ChemBioChem* **2008**, *9*, 1213.
(50) Iwata-Reuyl, D.; De Crecy-Lagard, V.; Van Lanen, S. U.S. Patent 7364882, April 29, 2008; *Chem. Abstr.* **2008**, *148*, 511878.
(51) Bougioukou, D. J.; Kille, S.; Taglieber, A.; Reetz, M. T. *Adv. Synth. Catal.* **2009**, *351*, 3287.
(52) Savile, C.; Mitchell, V.; Zhang, X.; Huisman, G. WO 2010075574, July 1, 2010; *Chem. Abstr.* **2010**, *153*, 108808.
(53) Pollegioni, L.; Sacchi, S.; Caldinelli, L.; Boselli, A.; Pilone, M.; Piubelli, L.; Molla, G. *Curr. Protein Pept. Sci.* **2007**, *8*, 600.
(54) Paradisi, F.; Conway, P. A.; Maguireb, A. R.; Engel, P. C. *Org. Biomol. Chem.* **2008**, *6*, 611.
(55) Chen, S.; Engel, P. C. *J. Biotechnol.* **2009**, *142*, 127.
(56) Zheng, J.; Guan, H.; Xu, L.; Yang, R.; Lin, Z. *Appl. Microbiol. Biotechnol.* **2010**, *86*, 607.
(57) Urlacher, V. B.; Schmid, R. D. *Curr. Opin. Chem. Biol.* **2006**, *10*, 156.
(58) Wu, S.; Acevedo, J. P.; Reetz, M. T. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 2775.
(59) Reetz, M. T.; Wu, S. *J. Am. Chem. Soc.* **2009**, *131*, 15424.
(60) Torres Pazmiño, D. E.; Riebel, A.; de Lange, J.; Rudroff, F.; Mihovilovic, M. D.; Fraaije, M. W. *ChemBioChem* **2009**, *10*, 2595.
(61) Torres Pazmiño, D. E.; Snajdrova, R.; Rial, D.; Mihovilovic, M.; Fraaije, M. *Adv. Synth. Catal.* **2007**, *349*, 1361.
(62) Kirschner, A.; Bornscheuer, U. T. *Appl. Microbiol. Biotechnol.* **2008**, *81*, 465.
(63) Reetz, M. T.; Brunner, B.; Schneider, T.; Schulz, F.; Clouthier, C. M.; Kayser, M. M. *Angew. Chem., Int. Ed.* **2004**, *43*, 4075.
(64) Mihovilovic, M. D.; Rudroff, F.; Winninger, A.; Schneider, T.; Schulz, F.; Reetz, M. T. *Org. Lett.* **2006**, *8*, 1221.
(65) Reetz, M. T.; Daligault, F.; Brunner, B.; Hinrichs, H.; Deege, A. *Angew. Chem., Int. Ed.* **2004**, *43*, 4078.
(66) Feingersch, R.; Shainsky, J.; Wood, T. K.; Fishman, A. *Appl. Environ. Microbiol.* **2008**, *74*, 1555.
(67) Bocola, M.; Schulz, F.; Leca, F.; Vogel, A.; Fraaije, M. W.; Reetz, M. T. *Adv. Synth. Catal.* **2005**, *347*, 979.
(68) Reetz, M. T.; Wu, S. *Chem. Commun.* **2008**, *49*, 5499.
(69) Reetz, M. T. *Angew. Chem., Int. Ed.* **2011**, *50*, 138.
(70) Reetz, M. T. In *Asymmetric Organic Synthesis with Enzymes*; Gotor, V., Alfonso, I., García-Urdiales, E., Eds.; Wiley-VCH: Weinheim, Germany, 2008; pp 21−63.
(71) Alexeeva, M.; Enright, A.; Dawson, M. J.; Mahmoudian, M.; Turner, N. J. *Angew. Chem., Int. Ed.* **2002**, *41*, 3177.
(72) Carr, R.; Alexeeva, M.; Enright, A.; Eve, T. S. C.; Dawson, M. J.; Turner, N. J. *Angew. Chem., Int. Ed.* **2003**, *42*, 4807.
(73) Carr, R.; Alexeeva, M.; Dawson, M. J.; Gotor-Fernández, V.; Humphrey, C. E.; Turner, N. J. *ChemBioChem* **2005**, *6*, 637.
(74) Dunsmore, C. J.; Carr, R.; Fleming, T.; Turner, N. J. *J. Am. Chem. Soc.* **2006**, *128*, 2224.
(75) Atkin, K. E.; Reiss, R.; Koehler, V.; Bailey, K. R.; Hart, S.; Turkenburg, J. P.; Turner, N. J.; Brzozowski, A. M.; Grogan, G. *J. Mol. Biol.* **2008**, *384*, 1218.
(76) Meyer, A.; Würsten, M.; Schmid, A.; Kohler, H.-P. E.; Witholt, B. *J. Biol. Chem.* **2002**, *277*, 34161.
(77) Meyer, A.; Schmid, A.; Held, M.; Westphal, A. H.; Röthlisberger, M.; Kohler, H.-P. E.; Van Berkel, W. J. H.; Witholt, B. *J. Biol. Chem.* **2002**, *277*, 5575.

(78) Escalettes, F.; Turner, N. J. *ChemBioChem* **2008**, *9*, 857.
(79) Gillam, E. M. J. *Chem. Res. Toxicol.* **2008**, *21*, 220.
(80) Peters, M. W.; Meinhold, P.; Glieder, A.; Arnold, F. H. *J. Am. Chem. Soc.* **2003**, *125*, 13442.
(81) Farinas, E. T.; Schwaneberg, U.; Glieder, A.; Arnold, F. H. *Adv. Synth. Catal.* **2001**, *343*, 601.
(82) Glieder, A.; Farinas, E. T.; Arnold, F. H. *Nat. Biotechnol.* **2002**, *20*, 1135.
(83) Li, S.; Ouellet, H.; Sherman, D. H.; Podust, L. M. *J. Biol. Chem.* **2009**, *284*, 5723.
(84) Münzer, D. F.; Meinhold, P.; Peters, M. W.; Feichtenhofer, S.; Griengl, H.; Arnold, F. H.; Glieder, A.; de Raadt, A. *Chem. Commun.* **2005**, 2597.
(85) Landwehr, M.; Hochrein, L.; Otey, C. R.; Kasrayan, A.; Bäckvall, J.-E.; Arnold, F. H. *J. Am. Chem. Soc.* **2006**, *128*, 6058.
(86) Kubo, T.; Peters, M. W.; Meinhold, P.; Arnold, F. H. *Chem.—Eur. J.* **2006**, *12*, 1216.
(87) Budde, M.; Morr, M.; Schmid, R. D.; Urlacher, V. B. *ChemBioChem* **2006**, *7*, 789.
(88) Urlacher, V. B.; Makhsumkhanov, A.; Schmid, R. D. *Appl. Microbiol. Biotechnol.* **2006**, *70*, 53.
(89) Li, H.-M.; Mei, L.-H.; Urlacher, V. B.; Schmid, R. D. *Appl. Biochem. Biotechnol.* **2008**, *144*, 27.
(90) Lewis, J. C.; Bastian, S.; Bennett, C. S.; Fu, Y.; Mitsuda, Y.; Chen, M. M.; Greenberg, W. A.; Wong, C. H.; Arnold, F. H. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 16550.
(91) Arnold, F. H.; Wong, C.-H.; Mitsuda, Y.; Chen, M. M.; Bennett, C.; Greenberg, W.; Lewis, J. C.; Bastian, S. US 20090124515, May 14, 2009; *Chem. Abstr.* **2009**, *150*, 533112.
(92) Yun, C. H.; Kim, D. H. WO 2010064764, June 10, 2010; *Chem. Abstr.* **2010**, *153*, 35551.
(93) Eiben, S.; Kaysser, L.; Maurer, S.; Kühnel, K.; Urlacher, V. B.; Schmid, R. D. *J. Biotechnol.* **2006**, *124*, 662.
(94) Carmichael, A. B.; Wong, L. L. *Eur. J. Biochem.* **2001**, *268*, 3117.
(95) Li, Q. S.; Ogawa, J.; Schmid, R. D.; Shimizu, S. *Appl. Environ. Microbiol.* **2001**, *67*, 5735.
(96) Seifert, A.; Vomund, S.; Grohmann, K.; Kriening, S.; Urlacher, V. B.; Laschat, S.; Pleiss, J. *ChemBioChem* **2009**, *10*, 853.
(97) Gursky, L. J.; Nikodinovic-Runic, J.; Feenstra, K. A.; O'Connor, K. E. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 995.
(98) Whitehouse, C. J. C.; Bell, S. G.; Tufton, H. G.; Kenny, R. J. P.; Ogilvie, L. C. I.; Wong, L.-L. *Chem. Commun.* **2008**, *8*, 966.
(99) Whitehouse, C. J. C.; Bell, S. G.; Wong, L.-L. *Chem.—Eur. J.* **2008**, *14*, 10905.
(100) Wong, L. L.; Bell, S. G.; Whitehouse, C. WO 2009047498, April 16, 2009; *Chem. Abstr.* **2009**, *150*, 440764.
(101) Branco, R. J. F.; Seifert, A.; Budde, M.; Urlacher, V. B.; Ramos, M. J.; Pleiss, J. *Proteins: Struct., Funct., Bioinf.* **2008**, *73*, 597.
(102) Fasan, R.; Meharenna, Y. T.; Snow, C. D.; Poulos, T. L.; Arnold, F. H. *J. Mol. Biol.* **2008**, *383*, 1069.
(103) Sawayama, A. M.; Chen, M. M. Y.; Kulanthaivel, P.; Kuo, M. S.; Hemmerle, H.; Arnold, F. H. *Chem.—Eur. J.* **2009**, *15*, 11723.
(104) Weis, R.; Winkler, M.; Schittmayer, M.; Kambourakis, S.; Vink, M.; Rozzell, J. D.; Glieder, A. *Adv. Synth. Catal.* **2009**, *351*, 2140.
(105) Ryan, J. D.; Fish, R. H.; Clark, D. S. *ChemBioChem* **2008**, *9*, 2579.
(106) England, P. A.; Harford-Cross, C. F.; Stevenson, J.-A.; Rouch, D. A.; Wong, L.-L. *FEBS Lett.* **1998**, *424*, 271.
(107) Bell, S. G.; Sowden, R. J.; Wong, L.-L. *Chem. Commun.* **2001**, *7*, 635.
(108) Sowden, R. J.; Yasmin, S.; Rees, N. H.; Bell, S. G.; Wong, L.-L. *Org. Biomol. Chem.* **2005**, *3*, 57.
(109) Molnár, I.; Jungmann, V.; Stege, J. T.; Trefzer, A.; Pachlatko, J. P. *Biochem. Soc. Trans.* **2006**, *34*, 1236.
(110) Trefzer, A.; Jungmann, V.; Molnár, I.; Botejue, A.; Buckel, D.; Frey, G.; Hill, D. S.; Jörg, M.; Ligon, J. M.; Mason, D.; Moore, D.; Pachlatko, J. P.; Richardson, T. H.; Spangenberg, P.; Wall, M. A.; Zirkle, R.; Stege, J. T. *Appl. Environ. Microbiol.* **2007**, *73*, 4317.
(111) Kabumoto, H.; Miyazaki, K.; Arisawa, A. *Biosci., Biotechnol., Biochem.* **2009**, *73*, 1922.
(112) Antipov, E.; Cho, A. E.; Wittrup, K. D.; Klibanov, A. M. *Proc. Natl. Acad. Sci. U.S.A.* **2008**, *105*, 17694.
(113) Strieker, M.; Essen, L. O.; Walsh, C. T.; Marahiel, M. A. *ChemBioChem* **2008**, *9*, 374.
(114) Marahiel, M. A.; Strieker, M.; Essen, L. O. DE 102007017861, Oct 16, 2008; *Chem. Abstr.* **2008**, *149*, 469517.
(115) Nayeem, A.; Chiang, S. J.; Liu, S. W.; Sun, Y.; You, L.; Basch, J. *Protein Eng., Des. Sel.* **2009**, *22*, 257.
(116) Goo, K. S.; Chua, C. S.; Sim, T. S. *Appl. Environ. Microbiol.* **2008**, *74*, 1167.
(117) Festa, G.; Autore, F.; Fraternali, F.; Giardina, P.; Sannia, G. *Proteins: Struct., Funct., Bioinf.* **2008**, *72*, 25.
(118) Keenan, B. G.; Leungsakul, T.; Smets, B. F.; Wood, T. K. *Appl. Environ. Microbiol.* **2004**, *70*, 3222.
(119) Keenan, B. G.; Leungsakul, T.; Smets, B. F.; Mori, M.; Henderson, D. E.; Wood, T. K. *J. Bacteriol.* **2005**, *187*, 3302.
(120) Gantt, R. W.; Goff, R. D.; Williams, G. J.; Thorson, J. S. *Angew. Chem., Int. Ed.* **2008**, *47*, 8889.
(121) Rozell, J. D.; Bommarius, A. S. In *Enzyme Catalysis in Organic Synthesis*, 2nd ed.; Drauz, K., Waldmann, H., Eds.; Wiley-VCH: Weinheim, Germany, 2002, Vol. II, pp 873−893.
(122) Höhne, M.; Bornscheuer, U. T. *ChemCatChem* **2009**, *1*, 42.
(123) Matcham, G. W.; Bowen, A. R. S. G. *Chim. Oggi* **1996**, *14*, 20.
(124) Savile, C. K.; Janey, J. M.; Mundorff, E. C.; Moore, J. C.; Tam, S.; Jarvis, W. R.; Colbeck, J. C.; Krebber, A.; Fleitz, F. J.; Brands, J.; Devine, P. N.; Huisman, G. W.; Hughes, G. J. *Science (Washington, DC, U.S.)* **2010**, *329*, 305.
(125) Svedendahl, M.; Branneby, C.; Lindberg, L.; Berglund, P. *ChemCatChem* **2010**, *2*, 976.
(126) Dhawan, I. K.; Miller, G.; Zhang, X. WO 2010081053, July 15, 2010; *Chem. Abstr.* **2010**, *153*, 196108.
(127) Savile, C.; Mundorff, E.; Moore, J. Devine, P. N.; Janey, J. M. WO 2010099501, Sept 2, 2010; *Chem. Abstr.* **2010**, *153*, 357014.
(128) Yun, H.; Hwang, B.-Y; Lee, J.-H.; Kim, B.-G. *Appl. Environ. Microbiol.* **2005**, *71*, 4220.
(129) Yano, T.; Oue, S.; Kagamiyama, H. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 5511.
(130) Bhuiya, M. W.; Liu, C. J. *J. Biol. Chem.* **2010**, *285*, 277.
(131) Reetz, M. T.; Zonta, A.; Schimossek, K.; Liebeton, K.; Jaeger, K.-E. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 2830.
(132) Reetz, M. T.; Puls, M.; Carballeira, J. D.; Vogel, A.; Jaeger, K.-E.; Eggert, T.; Thiel, W.; Bocola, M.; Otte, N. *ChemBioChem* **2007**, *8*, 106.
(133) Reetz, M. T.; Wilensek, S.; Zha, D.; Jaeger, K.-E. *Angew. Chem., Int. Ed.* **2001**, *40*, 3589.
(134) Bocola, M.; Otte, N.; Jaeger, K.-E.; Reetz, M. T.; Thiel, W. *ChemBioChem* **2004**, *5*, 214.
(135) Nardini, M.; Lang, D. A.; Liebeton, K.; Jaeger, K.-E.; Dijkstra, B. W. *J. Biol. Chem.* **2000**, *275*, 31219.
(136) Reetz, M. T.; Bocola, M.; Carballeira, J. D.; Zha, D.; Vogel, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 4192.
(137) Funke, S. A.; Eipper, A.; Reetz, M. T.; Otte, N.; Thiel, W.; van Pouderoyen, G.; Dijkstra, B. W.; Jaeger, K.-E.; Eggert, T. *Biocatal. Biotransform.* **2003**, *21*, 67.
(138) Zha, D.; Eipper, A.; Reetz, M. T. *ChemBioChem* **2003**, *4*, 34.
(139) Boersma, Y. L.; Droege, M. J.; van der Sloot, A. M.; Pijning, T.; Cool, R. H.; Dijkstra, B. W.; Quax, W. J. *ChemBioChem* **2008**, *9*, 1110.
(140) Boersma, Y. L.; Pijning, T.; Bosma, M. S.; van der Sloot, A. M.; Godinho, L. F.; Droege, M. J.; Winter, R. T.; van Pouderoyen, G.; Dijkstra, B. W.; Quax, W. J. *Chem. Biol. (Cambridge, MA, U.S.)* **2008**, *15*, 782.
(141) Koga, Y.; Kato, K.; Nakano, H.; Yamane, T. *J. Mol. Biol.* **2003**, *331*, 585.
(142) Lafaquière, V.; Barbe, S.; Puech-Guenot, S.; Guieysse, D.; Cortés, J.; Monsan, P.; Siméon, T.; André, I.; Remaud-Siméon, M. *ChemBioChem* **2009**, *10*, 2760.

(143) Cambon, E.; Piamtongkam, R.; Bordes, F.; Duquesne, S.; André, I.; Marty, A. *Biotechnol. Bioeng.* **2010**, *106*, 852.
(144) Ema, T.; Fujii, T.; Ozaki, M.; Korenaga, T.; Sakai, T. *Chem. Commun.* **2005**, *37*, 4650.
(145) Qian, Z; Lutz, S. *J. Am. Chem. Soc.* **2005**, *127*, 13466.
(146) Magnusson, A. O.; Rotticci-Mulder, J. C.; Santagostino, A.; Hult, K. *ChemBioChem* **2005**, *6*, 1051.
(147) Svedendahl, M.; Hult, K; Berglund, P. *J. Am. Chem. Soc.* **2005**, *127*, 17988.
(148) Carlqvist, P.; Svedendahl, M.; Branneby, C.; Hult, K; Brinck, T.; Berglund, P. *ChemBioChem* **2005**, *6*, 331.
(149) Reetz, M. T.; Höbenreich, H.; Soni, P.; Fernández, L. *Chem. Commun.* **2008**, *43*, 5502.
(150) Grogan, G. *Annu. Rep. Prog. Chem., Sect. B: Org. Chem.* **2009**, *105*, 206.
(151) Yu, Y.; Lutz, S. *Biotechnol. Bioeng.* **2009**, *105*, 44.
(152) De Maria, L.; Brask, J.; Skjøt, M.; Patkar, S. A.; Borch, K.; Svendsen, A. WO 2008065060, June 5, 2008; *Chem. Abstr.* **2008**, *149*, 47605.
(153) Engström, K.; Nyhlén, J.; Sandström, A. G.; Bäckvall, J. E. J. *Am. Chem. Soc.* **2010**, *132*, 7038.
(154) Sandström, A. G.; Engström, K.; Nyhlén, J.; Kasrayan, A.; Baeckvall, J. E. *Protein Eng., Des. Sel.* **2009**, *22*, 413.
(155) Kawata, T.; Ogino, H. *Biotechnol. Prog.* **2009**, *25*, 1605.
(156) Moore, J. C.; Arnold, F. H. *Nat. Biotechnol.* **1996**, *14*, 458.
(157) Bornscheuer, U. T.; Altenbuchner, J.; Meyer, H. H. *Biotechnol. Bioeng.* **1998**, *58*, 554.
(158) Henke, E.; Bornscheuer, U. T. *Biol. Chem.* **1999**, *380*, 1029.
(159) Schmidt, M.; Hasenpusch, D.; Kähler, M.; Kirchner, U.; Wiggenhorn, K.; Langel, W.; Bornscheuer, U. T. *ChemBioChem* **2006**, *7*, 805.
(160) Jochens, H.; Bornscheuer, U. T. *ChemBioChem* **2010**, *11*, 1861.
(161) Zhang, G.; Gao, R.; Zheng, L.; Zhang, A.; Wang, Y.; Wang, Q.; Feng, Y.; Cao, S. *J. Mol. Catal. B* **2006**, *38*, 148.
(162) Henke, E.; Pleiss, J.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2002**, *41*, 3211.
(163) Heinze, B.; Kourist, R.; Fransson, L.; Hult, K.; Bornscheuer, U. T. *Protein Eng., Des. Sel.* **2007**, *20*, 125.
(164) Kourist, R.; Bartsch, S.; Bornscheuer, U. T. *Adv. Synth. Catal.* **2007**, *349*, 1393.
(165) Bartsch, S.; Kourist, R.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2008**, *47*, 1508.
(166) Bassegoda, A.; Nguyen, G. S.; Schmidt, M.; Kourist, R.; Diaz, P.; Bornscheuer, U. T. *ChemCatChem* **2010**, *2*, 962.
(167) Barbayianni, E.; Kokotos, C. G.; Bartsch, S.; Drakou, C.; Bornscheuer, U. T.; Kokotos, G. *Adv. Synth. Catal.* **2009**, *351*, 2325.
(168) Durairaaj, M.; Vinayagam, V.; Masilamani, T. J.; Thirumoorthy, R.; Krishnan, C. M.; Harit, R. K. WO 2009013611, Jan 29, 2009; *Chem. Abstr.* **2009**, *150*, 185413.
(169) Gao, X.; Xie, X.; Pashkov, I.; Sawaya, M. R.; Laidman, J.; Zhang, W.; Cacho, R.; Yeates, T. O.; Tang, Y. *Chem. Biol. (Cambridge, MA, U.S.)* **2009**, *16*, 1064.
(170) Archelas, A.; Furstoss, R. *Curr. Opin. Chem. Biol.* **2001**, *5*, 112.
(171) Bornscheuer, U. T. *Adv. Biochem. Eng./Biotechnol.* **2005**, *100*, 181.
(172) Lee, E. Y.; Shuler, M. L. *Biotechnol. Bioeng.* **2007**, *98*, 318.
(173) Cedrone, F.; Niel, S.; Roca, S.; Bhatnagar, T.; Ait-abdelkader, N.; Torre, C.; Krumm, H.; Maichele, A.; Reetz, M. T.; Baratti, J. C. *Biocatal. Biotransform.* **2003**, *21*, 357.
(174) Reetz, M. T.; Torre, C.; Eipper, A.; Lohmer, R.; Hermes, M.; Brunner, B.; Maichele, A.; Bocola, M.; Arand, M.; Cronin, A.; Genzel, Y.; Archelas, A.; Furstoss, R. *Org. Lett.* **2004**, *6*, 177.
(175) Reetz, M. T. *Adv. Catal.* **2006**, *49*, 1–69.
(176) Reetz, M. T.; Kahakeaw, D.; Lohmer, R. *ChemBioChem* **2008**, *9*, 1797.
(177) Reetz, M. T.; Bocola, M.; Wang, L.-W.; Sanchis, J.; Cronin, A.; Arand, M.; Zou, J.; Archelas, A.; Bottalla, A.-L.; Naworyta, A.; Mowbray, S. L. *J. Am. Chem. Soc.* **2009**, *131*, 7334.
(178) Jochens, H.; Stiba, K.; Savile, C.; Fujii, R.; Yu, J. G.; Gerassenkov, T.; Kazlauskas, R. J.; Bornscheuer, U. T. *Angew. Chem., Int. Ed.* **2009**, *48*, 3532.
(179) Choi, S. H.; Kim, H. S.; Lee, E. Y. *Biotechnol. Lett.* **2009**, *31*, 1617.
(180) van Loo, B.; Kingma, J.; Heyman, G.; Wittenaar, A.; Lutje Spelberg, J. H.; Sonke, T.; Janssen, D. B. *Enzyme Microb. Technol.* **2009**, *44*, 145.
(181) Elenkov, M. M.; Tang, L.; Hauer, B.; Janssen, D. B. *Org. Lett.* **2006**, *8*, 4227.
(182) Hasnaoui, G.; Spelberg, J. H. L.; de Vries, E.; Tang, L.; Hauer, B.; Janssen, D. B. *Tetrahedron: Asymmetry* **2005**, *16*, 1685.
(183) De Vries, E.; Clark, L.; McVicar, S.; Segraves, E.; Song, S.; Tan, K. L. U.S. Patent 20100173372, July 8, 2010; *Chem. Abstr.* **2010**, *153*, 166226.
(184) Hancock, S. M.; Corbett, K.; Fordham-Skelton, A. P.; Gatehouse, J. A.; Davis, B. G. *ChemBioChem* **2005**, *6*, 866.
(185) Shaikh, F. A.; Withers, S. G. *Biochem. Cell Biol.* **2008**, *86*, 169.
(186) Hancock, S. M.; Vaughan, M. D.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2006**, *10*, 509.
(187) McCarter, J. D.; Withers, S. G. *Curr. Opin. Struct. Biol.* **1994**, *4*, 885.
(188) Zechel, D. L.; Withers, S. G. *Curr. Opin. Chem. Biol.* **2001**, *5*, 643.
(189) Feng, H.-Y.; Drone, J.; Hoffmann, L.; Tran, V.; Tellier, C.; Rabiller, C.; Dion, M. *J. Biol. Chem.* **2005**, *280*, 37088.
(190) Mayer, C.; Zechel, D. L.; Reid, S. P.; Warren, R. A. J.; Withers, S. G. *FEBS Lett.* **2000**, *466*, 40.
(191) Kim, Y.-W.; Lee, S. S.; Warren, R. A. J.; Withers, S. G. *J. Biol. Chem.* **2004**, *279*, 42787.
(192) Drone, J.; Feng, H.-Y.; Tellier, C.; Hoffmann, L.; Tran, V.; Rabiller, C.; Dion, M. *Eur. J. Org. Chem.* **2005**, *10*, 1977.
(193) Marton, Z.; Tran, V.; Tellier, C.; Dion, M.; Drone, J.; Rabiller, C. *Carbohydr. Res.* **2008**, *343*, 2939.
(194) Heidecke, C. D.; Ling, Z.; Bruce, N. C.; Moir, J. W. B.; Parsons, T. B.; Fairbanks, A. J. *ChemBioChem* **2008**, *9*, 2045.
(195) Müllegger, J.; Chen, H.-M.; Chan, W. Y.; Reid, S. P.; Jahn, M.; Warren, R. A. J.; Salleh, H. M.; Withers, S. G. *ChemBioChem* **2006**, *7*, 1028.
(196) Wilkinson, S. M.; Liew, C. W.; Mackay, J. P.; Salleh, H. M.; Withers, S. G.; McLeod, M. D. *Org. Lett.* **2008**, *10*, 1585.
(197) Kim, Y. W.; Fox, D. T.; Hekmat, O.; Kantner, T.; McIntosh, L. P.; Warren, R. A. J.; Withers, S. G. *Org. Biomol. Chem.* **2006**, *4*, 2025.
(198) Nashiru, O.; Zechel, D. L.; Stoll, D.; Mohammadzadeh, T.; Warren, R. A. J.; Withers, S. G. *Angew. Chem., Int. Ed.* **2001**, *40*, 417.
(199) Tao, H.; Peralta-Yahya, P.; Decatur, J.; Cornish, V. W. *ChemBioChem* **2008**, *9*, 681.
(200) Placier, G.; Watzlawick, H.; Rabiller, C.; Mattes, R. *Appl. Environ. Microbiol.* **2009**, *75*, 6312.
(201) Mulard, L.; Andre, I.; Champion, E.; Moulis, C.; Morel, S.; Monsan, P.; Remaud-Simeon, M.; Descroix, K. WO 2009112950, Sept 17, 2009; *Chem. Abstr.* **2009**, *151*, 379465.
(202) Honda, Y.; Kitaoka, M. *J. Biol. Chem.* **2006**, *281*, 1426.
(203) Vaughan, M. D.; Johnson, K.; DeFrees, S.; Tang, X.; Warren, R. A. J.; Withers, S. G. *J. Am. Chem. Soc.* **2006**, *128*, 6300.
(204) Yang, M.; Davies, G. J.; Davis, B. G. *Angew. Chem., Int. Ed.* **2007**, *46*, 3885.
(205) May, O.; Nguyen, P. T.; Arnold, F. H. *Nat. Biotechnol.* **2000**, *18*, 317.
(206) Lee, S. C.; Chang, Y.; Shin, D. M.; Han, J.; Seo, M. H.; Fazelinia, H.; Maranas, C. D.; Kim, H. S. *Enzyme Microb. Technol.* **2009**, *44*, 170.
(207) Chiang, C. J.; Chern, J. T.; Wang, J. Y.; Chao, Y. P. *J. Agric. Food Chem.* **2008**, *56*, 6348.
(208) Lo, C. K.; Kao, C. H.; Wang, W. C.; Wu, H. M.; Hsu, W. H.; Lin, L. L.; Hu, H. Y. *Process Biochem. (Amsterdam, Neth.)* **2009**, *44*, 309.
(209) DeSantis, G.; Wong, K.; Farwell, B.; Chatman, K.; Zhu, Z.; Tomlinson, G.; Huang, H.; Tan, X.; Bibbs, L.; Chen, P.; Kretz, K.; Burk, M. J. *J. Am. Chem. Soc.* **2003**, *125*, 11476.

(210) Schreiner, U.; Steinkellner, G.; Rozzell, J. D.; Glieder, A.; Winkler, M. *ChemCatChem* **2010**, *2*, 263.
(211) Wu, S.; Fogiel, A. J.; Petrillo, K. L.; Hann, E. C.; Mersinger, L. J.; DiCosimo, R.; O'Keefe, D. P.; Ben Bassat, A.; Payne, M. S. *Biotechnol. Bioeng.* **2007**, *97*, 689.
(212) Arroyo, M.; de la Mata, I.; Hormigo, D.; Castillon, M. P.; Acebal, C. In *Microorganisms for Industrial Enzymes and Biocontrol*; Mellado, E., Barredo, J.-L., Eds.; Research Signpost: Trivandrum, India, 2005; pp 129−151.
(213) López-Gallego, F.; Betancor, L.; Sio, C. F.; Reis, C. R.; Jimenez, P. N.; Guisan, J. M.; Quax, W. J.; Fernandez-Lafuente, R. *Adv. Synth. Catal.* **2008**, *350*, 343.
(214) Otten, L. G.; Sio, C. F.; Vrielink, J.; Cool, R. H.; Quax, W. J. *J. Biol. Chem.* **2002**, *277*, 42121.
(215) Pollegioni, L.; Lorenzi, S.; Rosini, E.; Marcone, G. L.; Molla, G.; Verga, R.; Cabri, W.; Pilone, M. S. *Protein Sci.* **2005**, *14*, 3064.
(216) Sylvestre, J.; Chautard, H; Cedrone, F.; Delcourt, M. *Org. Process Res. Dev.* **2006**, *10*, 562.
(217) Suzuki, H.; Yamada, C.; Kijima, K.; Ishihara, S.; Wada, K.; Fukuyama, K.; Kumagai, H. *Biotechnol. J.* **2010**, *5*, 829.
(218) Yamada, C.; Kijima, K.; Ishihara, S.; Miwa, C.; Wada, K.; Okada, T.; Fukuyama, K.; Kumagai, H.; Suzuki, H. *Appl. Environ. Microbiol.* **2008**, *74*, 3400.
(219) Yamada, C.; Kijima, K.; Ishihara, S.; Miwa, C.; Wada, K.; Okada, T.; Fukuyama, K.; Kumagai, H.; Suzuki, H. *Appl. Environ. Microbiol.* **2008**, *74*, 4983.
(220) Yuryev, R.; Kasche, V.; Ignatova, Z.; Galunsky, B. *Protein J.* **2010**, *29*, 181.
(221) Serra, I.; Cecchini, D. A.; Ubiali, D.; Manazza, E. M.; Albertini, A. M.; Terreni, M. *Eur. J. Org. Chem.* **2009**, *9*, 1384.
(222) Jager, S. A. W.; Shapovalova, I. V.; Jekel, P. A.; Alkema, W. B. L.; Svedas, V. K.; Janssen, D. B. *J. Biotechnol.* **2008**, *133*, 18.
(223) Behrouzian, B.; Krebber, A.; Mundorff, E. WO 2010054319, May 14, 2010; *Chem. Abstr.* **2010**, *152*, 543474.
(224) Komeda, H.; Ishikawa, N.; Asano, Y. *J. Mol. Catal. B* **2003**, *21*, 283.
(225) Zagrebelny, S. N. *Russ. Chem. Rev.* **2005**, *74*, 285.
(226) Ogino, H.; Tsuchiyama, S.; Yasuda, M.; Doukyu, N. *Protein Eng., Des. Sel.* **2010**, *23*, 147.
(227) Usuki, H.; Uesugi, Y.; Arima, J.; Yamamoto, Y.; Iwabuchi, M.; Hatanaka, T. *Chem. Commun.* **2010**, *46*, 580.
(228) Fong, S.; Machajewski, T. D.; Mak, C. C.; Wong, C.-H. *Chem. Biol.* **2000**, *7*, 873.
(229) Royer, S. F.; Haslett, L.; Crennell, S. J.; Hough, D. W.; Danson, M. J.; Bull, S. D. *J. Am. Chem. Soc.* **2010**, *132*, 11753.
(230) González-García, E.; Helaine, V.; Klein, G.; Schuermann, M.; Sprenger, G. A.; Fessner, W.-D.; Reymond, J.-L. *Chem.—Eur. J.* **2003**, *9*, 893.
(231) Sugiyama, M.; Greenberg, W.; Wong, C.-H. *Yuki Gosei Kagaku Kyokaishi* **2008**, *66*, 605.
(232) Wada, M.; Hsu, C.-C.; Franke, D.; Mitchell, M.; Heine, A.; Wilson, I.; Wong, C.-H. *Bioorg. Med. Chem.* **2003**, *11*, 2091.
(233) Bolt, A.; Berry, A.; Nelson, A. *Arch. Biochem. Biophys.* **2008**, *474*, 318.
(234) Barbosa, J. A. R. G.; Smith, B. J.; DeGori, R.; Ooi, H. C.; Marcuccio, S. M.; Campi, E. M.; Jackson, W. R.; Brossmer, R.; Sommer, M.; Lawrence, M. C. *J. Mol. Biol.* **2000**, *303*, 405.
(235) Smith, P. W.; Sollis, S. L.; Howes, P. D.; Cherry, P. C.; Starkey, I. D.; Cobley, K. N.; Weston, H.; Scicinski, J.; Merritt, A.; Whittington, A.; Wyatt, P.; Taylor, N.; Green, D.; Bethell, R.; Madar, S.; Fenton, R. J.; Morley, P. J.; Pateman, T.; Beresford, A. *J. Med. Chem.* **1998**, *41*, 787.
(236) Woodhall, T.; Williams, G. J.; Berry, A.; Nelson, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 2109.
(237) Williams, G. J.; Woodhall, T.; Farnsworth, L. M.; Nelson, A.; Berry, A. *J. Am. Chem. Soc.* **2006**, *128*, 16238.
(238) Hsu, C.-C.; Hong, Z.; Wada, M.; Franke, D.; Wong, C.-H. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 9122.
(239) Williams, G. J.; Domann, S.; Nelson, A.; Berry, A. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 3143.
(240) Jennewein, S.; Schürmann, M.; Wolberg, M.; Hilker, I.; Luiten, R.; Wubbolts, M.; Mink, D. *Biotechnol. J.* **2006**, *1*, 537.
(241) DeSantis, G.; Liu, J.; Clark, D. P.; Heine, A.; Wilson, I. A.; Wong, C.-H. *Bioorg. Med. Chem.* **2003**, *11*, 43.
(242) Liu, J.; Hsu, C.-C.; Wong, C.-H. *Tetrahedron Lett.* **2004**, *45*, 2439.
(243) Gwon, H.; Baik, S. *Biotechnol. Lett.* **2010**, *32*, 143.
(244) Fechter, M. H.; Gruber, K.; Avi, M.; Skranc, W.; Schuster, C.; Pöchlauer, P.; Klepp, K. O.; Griengl, H. *Chem.—Eur. J.* **2007**, *13*, 3369.
(245) Avi, M.; Wiedner, R. M.; Griengl, H.; Schwab, H. *Chem.—Eur. J.* **2008**, *14*, 11415.
(246) Semba, H.; Ichige, E. WO 2008041272, April 10, 2008; *Chem. Abstr.* **2008**, *148*, 420215.
(247) Glieder, A. WO 2008071695, June 19, 2008; *Chem. Abstr.* **2008**, *149*, 72401.
(248) Pscheidt, B.; Avi, M.; Gaisberger, R.; Hartner, F. S.; Skranc, W.; Glieder, A. *J. Mol. Catal. B* **2008**, *52*−*53*, 183.
(249) Gaisberger, R.; Weis, R.; Luiten, R.; Skranc, W.; Wubbolts, M.; Griengl, H.; Glieder, A. *J. Biotechnol.* **2007**, *129*, 30.
(250) Liu, Z.; Pscheidt, B.; Avi, M.; Gaisberger, R.; Hartner, F. S.; Schuster, C.; Skranc, W.; Gruber, K.; Glieder, A. *ChemBioChem* **2008**, *9*, 58.
(251) Lauble, H.; Miehlich, B.; Förster, S.; Kobler, C.; Wajant, H.; Effenberger, F. *Protein Sci.* **2002**, *11*, 65.
(252) Bühler, H.; Effenberger, F.; Förster, S.; Roos, J.; Wajant, H. *ChemBioChem* **2003**, *4*, 211.
(253) Glieder, A.; Weis, R.; Skranc, W.; Pöchlauer, P.; Dreveny, I.; Majer, S.; Wubbolts, M.; Schwab, H.; Gruber, K. *Angew. Chem., Int. Ed.* **2003**, *42*, 4815.
(254) Weis, R.; Gaisberger, R.; Skranc, W.; Gruber, K.; Glieder, A. *Angew. Chem., Int. Ed.* **2005**, *44*, 4700.
(255) Yan, G.; Cheng, S.; Zhao, G.; Wu, S.; Liu, Y.; Sun, W. *Biotechnol. Lett.* **2003**, *25*, 1041.
(256) Terao, Y.; Miyamoto, K.; Ohta, H. *Chem. Commun.* **2006**, *34*, 3600.
(257) Ijima, Y.; Matoishi, K.; Terao, Y.; Doi, N.; Yanagawa, H.; Ohta, H. *Chem. Commun.* **2005**, *7*, 877.
(258) Okrasa, K.; Levy, C.; Wilding, M.; Goodall, M.; Baudendistel, N.; Hauer, B.; Leys, D.; Micklefield, J. *Angew. Chem., Int. Ed.* **2009**, *48*, 7691.
(259) Hauer, B.; Baudendistel, N.; Okrasa, K.; Micklefield, J.; Leys, D.; Levy, C. WO 2009068308, June 4, 2009; *Chem. Abstr.* **2009**, *151*, 26602.
(260) Lingen, B.; Groetzinger, J.; Kolter, D.; Kula, M.-R.; Pohl, M. *Protein Eng.* **2002**, *15*, 585.
(261) Gocke, D.; Walter, L.; Gauchenova, E.; Kolter, G.; Knoll, M.; Berthold, C. L.; Schneider, G.; Pleiss, J.; Müller, M.; Pohl, M. *ChemBioChem* **2008**, *9*, 406.
(262) Yep, A.; McLeish, M. J. *Biochemistry* **2009**, *48*, 8387.
(263) Kataoka, M.; Yamamoto, K.; Kawabata, H.; Wada, M.; Kita, K.; Yanase, H.; Shimizu, S. *Appl. Microbiol. Biotechnol.* **1999**, *51*, 486.
(264) Brandt, G. S.; Kneen, M. M.; Petsko, G. A.; Ringe, D.; McLeish, M. J. *J. Am. Chem. Soc.* **2010**, *132*, 438.
(265) Smith, M. E. B.; Chen, B. H.; Hibbert, E. G.; Kaulmann, U.; Smithies, K.; Galman, J. L.; Baganz, F.; Dalby, P. A.; Hailes, H. C.; Lye, G. J.; Ward, J. M.; Woodley, J. M.; Micheletti, M. *Org. Process Res. Dev.* **2010**, *14*, 99.
(266) Rha, E.; Kim, S.; Choi, S. L.; Hong, S. P.; Sung, M. H.; Song, J. J.; Lee, S. G. *FEBS J.* **2009**, *276*, 6187.
(267) Seisser, B.; Zinkl, R.; Gruber, K.; Kaufmann, F.; Hafner, A.; Kroutil, W. *Adv. Synth. Catal.* **2010**, *352*, 731.
(268) Seebeck, F. P.; Guainazzi, A.; Amoreira, C.; Baldridge, K. K.; Hilvert, D. *Angew. Chem., Int. Ed.* **2006**, *45*, 6824.
(269) Fesko, K.; Giger, L.; Hilvert, D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5987.
(270) Krug, D.; Mueller, R. *ChemBioChem* **2009**, *10*, 741.
(271) Röthlisberger, D.; Khersonsky, O.; Wollacott, A. M.; Jiang, L.; DeChancie, J.; Betker, J.; Gallaher, J. L.; Althoff, E. A.; Zanghellini, A.; Dym, O.; Albeck, S.; Houk, K. N.; Tawfik, D. S.; Baker, D. *Nature (London, U.K.)* **2008**, *453*, 190.
(272) Khersonsky, O.; Röthlisberger, D.; Dym, O.; Albeck, S.; Jackson, C. J.; Baker, D.; Tawfik, D. S. *J. Mol. Biol.* **2010**, *396*, 1025.