Biocatalysis

DOI: 10.1002/anie.201302195

New Generation of Biocatalysts for Organic Synthesis**

*Bettina M. Nestl, Stephan C. Hammer, Bernd A. Nebel, and Bernhard Hauer**

Keywords:
biocatalysis · bioorganic chemistry · enzyme catalysis · synthetic methods

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

The use of enzymes as catalysts for the preparation of novel compounds has received steadily increasing attention over the past few years. High demands are placed on the identification of new biocatalysts for organic synthesis. The catalysis of more ambitious reactions reflects the high expectations of this field of research. Enzymes play an increasingly important role as biocatalysts in the synthesis of key intermediates for the pharmaceutical and chemical industry, and new enzymatic technologies and processes have been established. Enzymes are an important part of the spectrum of catalysts available for synthetic chemistry. The advantages and applications of the most recent and attractive biocatalysts—reductases, transaminases, ammonia lyases, epoxide hydrolases, and dehalogenases—will be discussed herein and exemplified by the syntheses of interesting compounds.

From the Contents

1. Introduction

Catalysis—in particular acid, base, and metal catalysis—was and is still important to the success of organic synthesis. An emerging group of catalysts are the biocatalysts, which facilitate the syntheses of complex organic molecules by chemo-, regio-, and stereoselective bond-forming and -breaking reactions. The synthetic capacity of some enzymes, which can be made in their native hosts in high yields and low costs, has already been adapted for the industrial production of commodities and fine-chemicals as well as pharmaceuticals. However, the full potential of enzymes as alternative catalysts to their chemical counterparts for the creation of new routes to targets in organic syntheses has yet to be fully exploited. The growth in interest and application of enzymes and microorganisms as catalysts in synthetic strategies demonstrates that we are a considerable step closer to the goal. Biocatalysis has now advanced to the point where we can seriously think about harnessing these biological catalysts for individual chemical reaction steps. The high selectivity and specificity is one reason for considering enzymes as catalysts for specific problems in synthetic chemistry. Biocatalysts can be used not only for the introduction of a chiral center in a molecule but also for syntheses under mild conditions, where chemo- and regioselectivity are often a key issue. The active sites of enzymes are chiral and, therefore, enzymes are readily accepted as catalysts for reactions that generate the (often enantiomerically pure) intermediates and products demanded by organic chemists. In this respect, numerous books and reviews describe the advantages that biocatalysis can bring to complex organic syntheses.[1] This also highlights that enzymes can offer significant advantages over the use of other catalysts.

Biocatalysis is becoming more and more the method of choice for the preparation of some chiral molecules in the chemical and pharmaceutical industry. Enantiomerically pure amino acids, amino alcohols, amines, alcohols, and epoxides are particularly important classes of compounds for the synthesis of many active pharmaceutical and agrochemical products.[2] Certain well-known and already established biocatalysts that are able to perform a range of chemical reactions will be highlighted in Section 2. Hydrolases and ketoreductases are the most frequently used biocatalysts in industrial organic synthesis. In the last few years the formation of valuable compounds through hydrolysis reactions was a dominating element mainly because of the availability and ease of use of a wide variety of esterases, lipases, and (to a lesser extent) acylases. The lack of sensitive cofactors that would have to be recycled as well as the relaxed substrate specificities and stability in solvents make this class of enzymes attractive.[3] A further newly established field where lipases have been applied is polymerization. This allows access to a large variety of polymers by polycondensation and ring-opening polymerization of cyclic monomers.[4] Chemical esterifications are traditionally carried out under metal or acid catalysis at elevated temperatures. This is, however, impossible with acrylate derivatives, since they polymerize spontaneously under such conditions. Unlike chemical catalysts, lipases can efficiently catalyze esterification reactions of acrylates at ambient temperatures,[5,6] thus highlighting the industrial interest.[7] Focus has also turned towards the use of ketoreductases.[8] The nicotinamide coenzyme dependent ketoreductases that catalyze the regio- and stereoselective reduction of a broad structural range of ketones to their corresponding alcohols is one of the most common redox reactions in organic chemistry.[9] It has been pointed out by the Merck Process Group that the application of ketoreductases and alcohol dehydrogenases has great potential for an increased process chemistry culture at Merck.[10] Ketoreductases along with the Baeyer–Villiger monooxygenases have been frequently employed, even

[*] B. M. Nestl, S. C. Hammer, B. A. Nebel, Prof. Dr. B. Hauer
Technische Biochemie, Universität Stuttgart
Stuttgart (Germany)
E-mail: Bernhard.Hauer@itb.uni-stuttgart.de

[**] The title picture "Guiding" was painted by the young Austrian artist Claudia Nebel. The painting illustrates how the artist interprets the topic of biocatalysis. The picture shows the interaction of a substrate with the active site of an enzyme.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

though in both cases the method is more complex and involves regeneration and, thus, retention of the cofactor. Furthermore, Baeyer–Villiger monooxygenases have been used in the past for the oxidation of various linear and cyclic ketones to provide enantiomerically pure intermediates.[11] Besides oxidoreductases, novel C–C, C–N, and C–O bond-forming biocatalysts with broad substrate acceptance belong to the class of enzymes with the most need for biotransformation options.[12,13] Among them, aldolases, hydroxynitrile lyases, and thiamine diphosphate dependent lyases, such as benzaldehyde lyase or pyruvate decarboxylase, have been used as powerful biocatalysts for various applications. These enzymes allow the preparation of diverse sugars and carbocycles as well as the synthesis of acyloins and cyanohydrins.[13]

Currently there are a number of well-established biocatalytic strategies for introducing chirality into a molecule. These biocatalysts can be used together, in sequences or cooperatively, to accomplish multistep reaction sequences.[14] There are also a few examples where biocatalysts have been combined with chemical catalysts in a single reaction vessel. The potential of diverse enzymes in parallel approaches is especially pronounced because of their inert chemical behavior. However, this potential has been hardly explored as a result of the limited variety of chemical reactions that are catalyzed by enzymes.

Many catalysts (mostly hydrolases, lyases, and oxidoreductases) have been found for the syntheses of various organic chemical compounds; however, there is still a growing need for novel biocatalysts to cover a wide field of chemical reactions. A number of enabling techniques are currently available to identify and develop novel biocatalysts: 1) screening of new strains and novel enzyme activities, 2) enzyme evolution to engineer an enzyme with the desired function, 3) development of alternative reaction media and unconventional solvent systems, as well as 4) bioinformatics to study biological systems with in silico modeling studies and experimental rational design.[5,15] All these powerful methods for screening microorganisms, including enrichment selection and metagenomics, computational enzyme design, and de novo engineering of designer proteins, as well as the exploration of the catalytic flexibility of existing biocatalysts for various unexpected chemical transformations offer new approaches for the exploration of enzymes as catalysts.

This Review will highlight examples of biocatalysts for the selective synthesis of enantiomerically pure molecules which are difficult to access by conventional chemical methods. Recent advancements in biocatalysis with respect to epoxides, amines, amino acids, and reduced alkenes are summarized. A summary of some types of biocatalysts that are useful in organic synthesis and their broad applicability is also

Bernhard Hauer (2nd from left) studied biology at the University of Hohenheim (Germany), where he completed his PhD with Franz Lingens in 1982. After postdoctoral research at the University of Chicago with James Shapiro, in 1983 he joined BASF SE, where he was scientific director for the research area biocatalysis. He completed his habilitation in 1996 at the University of Heidelberg. Since 2009 he has been the head of the Institute of Technical Biochemistry at the University of Stuttgart. His research interest is the provision of novel biocatalysts to further expand the spectrum of accessible biocatalytic reactions.

Bettina M. Nestl (2nd from right) studied chemistry at the University of Graz (Austria) and completed her PhD in 2007 with Kurt Faber. After postdoctoral research at the University of Manchester (UK) with Nicholas J. Turner, in 2010 she joined the Institute of Technical Biochemistry as a group leader. Her interests concern the development and optimization of proteins with novel catalytic properties and their application in enzymatic synthesis.

Stephan C. Hammer (right) studied chemistry at the University of Marburg (Germany) and Cambridge (UK), after completing professional training and qualification as a technician. He then joined the Institute of Technical Biochemistry at the University of Stuttgart for diploma studies. He is currently carrying out PhD research with Bernhard Hauer on the development of biocatalysts for chiral Brønsted acid catalysis.

Bernd A. Nebel (left) studied environmental system science, specializing in chemistry and chemical engineering, at the University of Graz (Austria). He completed his PhD under the guidance of Martin Mittelbach in 2008. After postdoctoral research with Nicholas J. Turner at the research center CoEBio3 in Manchester, UK, he moved to the Institute of Technical Biochemistry in 2010. He currently coordinates the group for Applied Catalysis and researches unconventional reaction media for the modification of the renewable raw materials and their subsequent industrial application.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

included. Grouping together relevant information concerning the application of enzymes into important types of reactions should give synthetic chemists in academia and industry an understanding of synthetically useful catalytic activities so that they can use the correct catalyst/biocatalyst for a particular transformation. Hence, biocatalysis offers new opportunities in the retrosynthetic analysis of key reaction steps for the synthesis of complex organic molecules and intermediary products.

2. Specific Hydrogenations of Activated C=C Bonds

Asymmetric hydrogenation processes are of prime importance in organic synthesis and are often a critical step in an overall synthesis. The asymmetric reduction of C=C bonds is a powerful tool for the creation of up to two stereocenters (Figure 1).

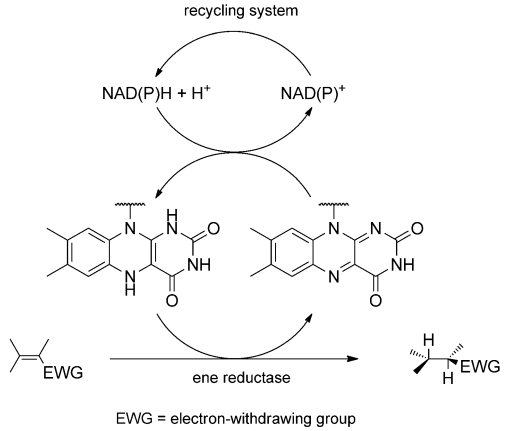


Figure 1. Asymmetric reduction of an α,β-unsaturated alkene catalyzed by ene reductases from the Old Yellow Enzyme family.

Currently, such reactions are carried out with transition-metal-based homogeneous catalysts[16] or metal-free organocatalysts for transfer hydrogenation.[17] A new approach for the asymmetric reduction of C=C double bonds uses catalysts of the Old Yellow Enzyme (OYE) family.[18] The members of this family, also referred to as ene reductases (EC 1.3.1.31), are flavin mononucleotide (FMN) containing, NAD(P)H-dependent redox catalysts. The reaction proceeds in two steps: the reductive half-reaction involves the reduction of the FMN cofactor by NAD(P)H, while the oxidative half-reaction proceeds by the reduction of the activated alkene through hydride transfer from the reduced FMN (Figure 2).

Overall, the reaction resembles an asymmetric Michael-type addition of a hydride and proton to activated alkenes with exclusive relative *trans* stereospecificity. The first biocatalytic attempts focused on whole cell bioreductions to avoid the requirements for external NAD(P)H cofactor recycling and protein purification. The vast majority of such experiments have been performed with baker's yeast. Although excellent stereoselectivities were achieved, the

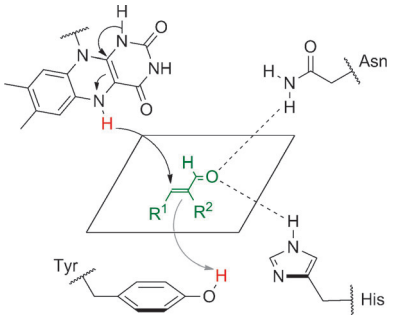


Figure 2. Oxidative half-reaction catalyzed by OYE. The reaction proceeds through hydride transfer from reduced FMN onto the Cβ atom of the substrate and protonation of the Cα atom. The reduction of FMN takes place in the reductive half-reaction at the expense of NAD(P)H.

chemoselectivities and overall yields with whole cells are often poor. This is due to the presence of competing alcohol dehydrogenases, which catalyze carbonyl reduction and other side reactions, such as ester hydrolysis and acyloin reactions. These disadvantages can now be avoided by using recombinant ene reductases together with suitable redox enzymes for the efficient recycling of the expensive NAD(P)H cofactor.

The asymmetric reduction of conjugated C=C bonds by ene reductase enzymes has received great interest in preparative organic chemistry. The expanding spectrum of stereospecific reductions catalyzed by ene reductases represents a viable alternative to transition-metal-catalyzed reductions. A major challenge in using this system involves the dependency of these reductases on the reduced nicotinamide cofactor NAD(P)H, which provides the reducing equivalents needed for reduction of the alkene. The expensive cofactors, the necessity of a cofactor regeneration system, as well as the partly unsatisfactory chemoselectivity in regard to unsaturated aldehyde and ketone reductions has led to a series of synthetic NADH mimics being studied recently.

Crude preparations of the thermophilic ene reductase YqjM from *Bacillus subtilis* were used in this reduction. High conversions and enantioselectivities along with high chemoselectivities were obtained under non-optimized conditions when employing these cofactor mimics.[19] Enzymes of the OYE family are widespread in nature and can be found in several microorganisms and plants. More than 25 members have so far been characterized biochemically, and their catalytic abilities have been investigated. Overall, these enzymes exhibit a remarkably broad substrate scope and catalyze the reduction of acyclic and cyclic α,β-unsaturated ketones, aldehydes, nitroalkenes, carboxylic acids, and esters, as well as nitrate esters, nitroglycerin, nitroaromatic explosives, and cyclic triazines.[20] Some ene reductases can also catalyze the disproportionation reaction of cyclohex-2-enone, thereby forming equimolar amounts of cyclohexanone and phenol. This disproportionation has been considered to be a minor side reaction and demonstrates the catalytic promiscuity of Old Yellow Enzymes.[21]

Furthermore, Faber and co-workers provided evidence that ene reductases are versatile catalysts for the non-redox isomerization of α,β-unsaturated γ-butyrolactone **1**.[22] The

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

reductive dehalogenation of β-haloacrylic ester derivatives **4** was found to proceed through an enzymatic C=C bond reduction followed by a spontaneous β-elimination of the unstable intermediate.[23] The bioreduction of β,β-disubstituted nitroalkenes **6** is somewhat surprising in light of the reaction proceeding through the Nef pathway (to form **8**)[24] or through the formation of 1,2-oxazete intermediates[25] in racemic forms. The reaction pathway depends on the substitution pattern and on the biocatalyst applied (Figure 3).

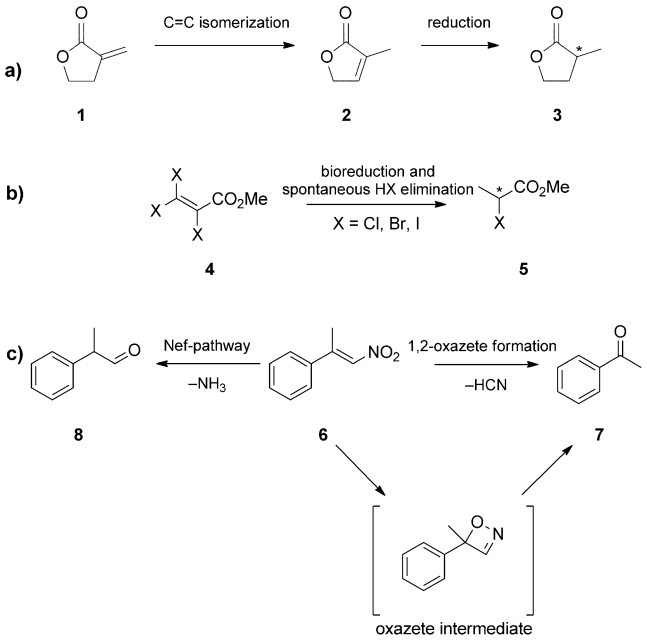
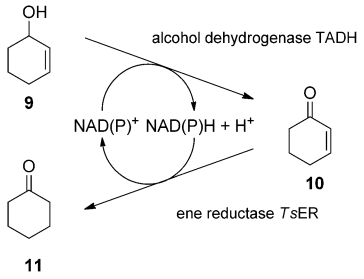


Figure 3. Promiscuous activities of ene reductases. a) Biocatalytic C=C bond reduction and isomerization of α,β-unsaturated γ-butyrolactone **1**. b) Enzymatic bioreduction of β-halo-α,β-unsaturated carboxylic esters by sequential C=C bond reduction and spontaneous β-elimination of the unstable, saturated intermediate. c) Reductive transformation of β,β-disubstituted nitroalkenes, which proceeds through a Nef reaction pathway or the reduction of the nitro moiety to form 1,2-oxazete derivatives.

A recent study demonstrated the isomerization of allylic alcohols to saturated carbonyl products occurred by a bioenzymatic cascade (Scheme 1). In this cascade, a $NAD(P)^+$-dependent alcohol dehydrogenase (ADH) catalyzed oxidation of the allylic alcohol cyclohexenol **9** was coupled to an NAD(P)H-dependent ene reductase catalyzed reduction of the α,β-unsaturated ketone **10** to form the saturated carbonyl analogue cyclohexanone **11**.[26] The ADH from *Thermus* sp. ATN1 (TADH) as well as the ene reductase from *Thermus scotoductus* SA-01 (*Ts*ER) were chosen as model enzymes. A total yield of 66% has been obtained after 24 h reaction time under optimized transformation conditions.

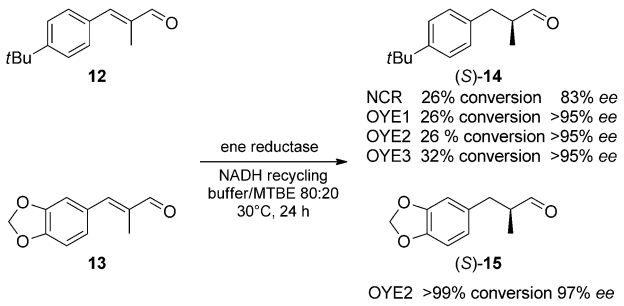
The catalytic potential of OYEs has been studied extensively over the last five years, which has resulted in highly stereoselective C=C reductions of industrially interesting compounds on a preparative scale that are difficult to perform by conventional techniques. The following examples demon-



Scheme 1. Enzymatic cascade for the redox isomerization of allylic alcohols.

strate the broad applicability of these enzymes towards different highly functionalized Michael systems as substrates. Furthermore, the first proof of principles showed that OYEs can even be used to catalyze the reverse reaction, the regioselective synthesis of α,β-unsaturated ketones from their saturated analogues.[27]

The asymmetric (bio)catalytic reduction of C=C bonds goes in hand with the creation of (up to) two chiral centers and, thus, is one of the most widely employed strategies for the production of chiral materials. For the chemoenzymatic asymmetric synthesis of the fragrance aldehydes Lilial (**14**) and Helional (**15**), Stückler et al. have studied the enzymatic reduction of the corresponding cinnamaldehyde precursors **12** and **13** using cloned and overexpressed ene reductases (Scheme 2).[28] These bioreductions with ene reductase



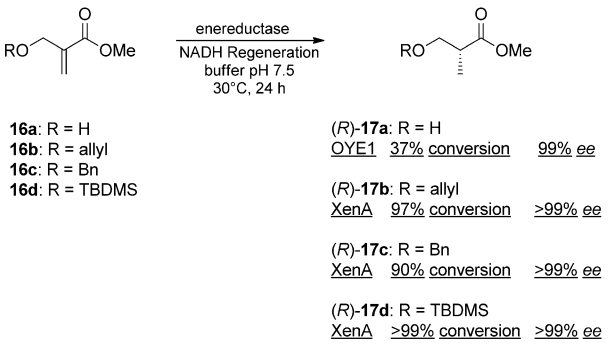
Scheme 2. Reductase-catalyzed synthesis of chiral α-methyl dihydrocinnamaldehyde derivatives. MTBE = *tert*-butyl methyl ether.

enzymes proved to be very slow under standard conditions in neat aqueous buffer at pH 7.5. To provide sufficient solubility to the lipophilic α-methyl dihydrocinnamaldehyde derivatives **12** and **13** (in the $g\,L^{-1}$ concentration range), 20% *tert*-butyl methyl ether was added as cosolvent, which led to an increased reaction rate. *R* Enantiomers were obtained in moderate enantiomeric excess (maximum 53% *ee* for α-methyl dihydrocinnamaldehydes) by using ene reductase enzymes YqjM from *Bacillus subtilis* and OPR1 from *Lycopersicon esculentum*. The corresponding *S* enantiomers were produced with excellent stereoselectivities (up to 97% *ee*) by using OYE1–OYE3 from *Saccharomyces* sp. and NCR from *Zymomonas mobilis*. The bioreduction of **12**

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

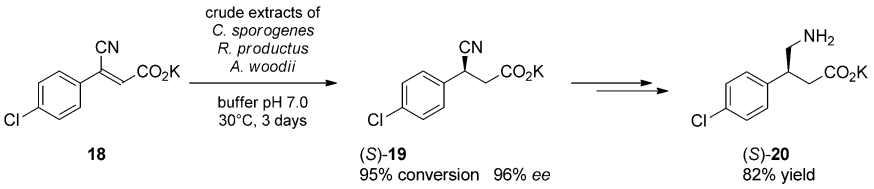
furnished Lilial (**14**) with only moderate conversions of up to 32%, while quantitative conversion rates could be observed using **13** and OYE2, which led to Helional (**15**).

Based on the above, a direct synthesis of "Roche ester" compounds was developed. The "Roche ester" is an important chiral building block for the synthesis of vitamins, antibiotics, and several fragrance and natural products. Stückler et al. have used 11 OYEs to synthesize the "Roche ester" by the biocatalytic reduction of methyl 2-hydroxymethylacrylates **16** with various protective groups (Scheme 3).[21] Flavoproteins OYE1–OYE3 from *Saccharomyces* sp. and YqjM from *Bacillus subtilis* were found to reduce unprotected methyl 2-hydroxymethylacrylate **16a** to the corresponding (*R*)-propionate compound **17a** with almost perfect stereoselectivity (99% *ee*) in whole cell transformations. OYE1 resulted in the highest conversion of 37% when NADH was used as a reducing cofactor. Protection of the hydroxy group with allyl (**16b**) and benzyl ether groups (**16c**) led to higher conversion rates. The *O*-allyl (**16b**) and *O*-benzyl ether substrates (**16c**) were converted by all 11 reductase enzymes tested, with OYE3 and XenA from *Pseudomonas putida* showing almost quantitative conversion (up to 97%) and *R* stereopreference. Only XenA was able to reduce the bulky *tert*-butyldimethylsilyloxy derivative **16d** with high enantiomeric excess (>99% *ee*) and conversion (99%).



Scheme 3. Asymmetric bioreduction of methyl 2-hydroxymethylacrylate derivatives for the production of "Roche esters". Bn = benzyl, TBDMS = *tert*-butyldimethylsilyl.

Chiral γ-amino acids and derivatives thereof have been examined for their importance in the treatment of a range of central nervous system disorders. Fryszkowska et al. have developed a new asymmetric method for the preparation of γ-amino butyric acid (GABA) derivatives by using reductases as an efficient alternative to classical catalysis. β-Aryl-β-cyano propanoic acid derivative **18** was synthesized from the respective aryl acetonitrile and glyoxalic acid, and employed in bioreductions with Old Yellow Enzymes. Crude extracts of *Clostridium sporogenes* DSM795, *Ruminococcus productus* DSM3507, and *Acetobacterium woodii* DSM1030 catalyzed the formation of the respective (*S*)-β-aryl-β-cyanopropanoic acids in quantitative yields (>99%) and excellent enantioselectivities (up to >99% *ee*). All reactions were performed at pH 7.0 to minimize the racemization of the products under aqueous reaction conditions. This strategy has been applied in the chemoenzymatic highly asymmetric synthesis of the β-aryl-substituted γ-aminobutyric acid derivative (*S*)-baclofen [(*S*)-**20**] from the (*Z*)-3-(4-chlorophenyl)-3-cyanopropenoic acid potassium salt **18** via intermediate (*S*)-**19** (Scheme 4).[29]



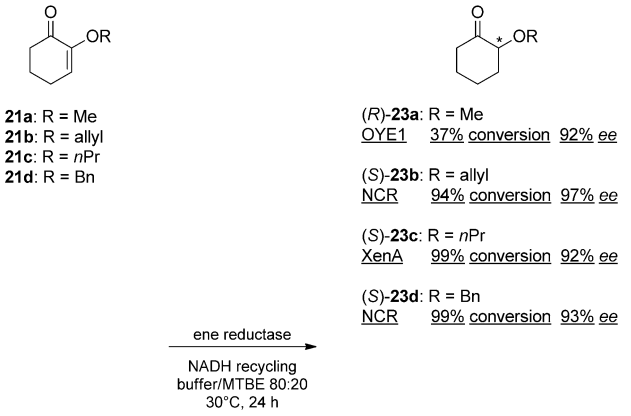
Scheme 4. Enantioselective biocatalytic reduction of the 3-cyanopropenoic acid salt in the synthesis of (*S*)-baclofen.

Furthermore, ene reductases were also used for the reduction of α-alkoxycycloenones to acyloins. Various *O*-protecting groups—methyl (**21a** and **22b**), propyl (**21c**), allyl (**21b**), benzyl (**21d** and **22a**)—were investigated.[30] Chiral acyloins are used as intermediates in several processes. The configuration of the *O*-protected acyloins could be controlled efficiently by the size of the protecting group. Cyclohexenone derivatives with a methyl group furnished *R*-configured acyloins [(*R*)-**23a**] whereas long-chain analogues (propyl, allyl, and benzyl) led to the mirror image *S* products [(*S*)-**23b–d**] with very high enantiomeric excesses (92–97% *ee*). The reactions were performed in the presence of 20% (v/v) *tert*-butyl methyl ether as cosolvent. Interestingly, no switch in the enantioselectivity was observed with cyclopentenone derivatives: invariably the (*S*)-acyloins (*S*)-**24a**,**b** (up to >99% *ee*, Scheme 5) were generated.

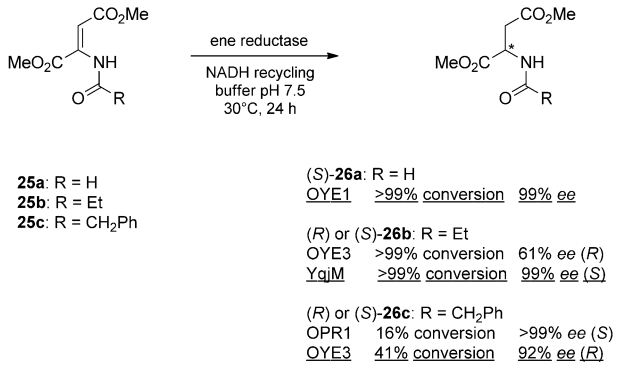
Chiral amino acids are important building blocks and intermediates in the chemical and pharmaceutical industry. Recently, α,β-dehydroamino acid derivatives have been used as substrates in the asymmetric synthesis of amino acids by reductases (Scheme 6).[31] The small *N*-acylaminofumaric diester **25a** was converted quantitatively and with high stereoselectivity under standard conditions into (*S*)-**26a** (99% *ee*) when using purified OYE1 and OYE3 from *Saccharomyces* sp. A further increase in the length of the amide chain to propionyl (**25b**) resulted in a switch of the stereopreference with a moderate enantiomeric excess [61% *ee* for (*R*)-**26b**] on using the reductase enzyme OYE3. The most active enzyme YqjM from *Bacillus subtilis* produced the *S* enantiomer of **26b** with high conversion (>99%) and high stereoselectivity (99% *ee*). An analogous switch was observed by a further variation of the *N*-protective group to *N*-phenylacetyl: while OYE3 afforded the *R* enantiomer [(*R*)-**26c** 92% *ee*], OPR1 furnished the *S* enantiomer in high enantiomeric excess (>99%).

The asymmetric reductions of citral and ketoisophorone with a wide range of ene reductases have already been

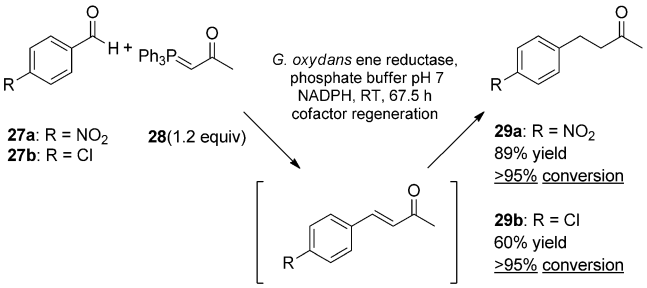
 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 5. Asymmetric synthesis of O-protected acyloins by using ene reductases.



Scheme 6. Asymmetric bioreduction of α,β-dehydroamino acid derivatives.



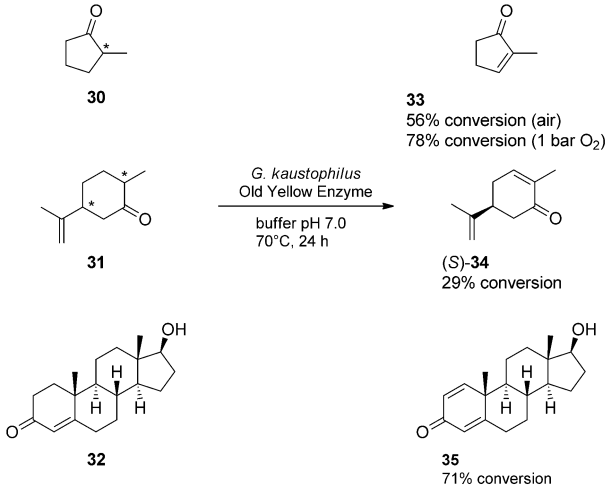
Scheme 7. Combination of the Wittig reaction with enzymatic C=C bond reduction.

investigated in great detail. All of the reductases resulted in either poor selectivities or low conversions. A good alternative to overcome these limitations is the reductase from *Gluconobacter oxydans*,[32] which is applicable to the enantioselective hydrogenation of citral (mixture of geranial and neral) and ketoisophorone.[33] Studies on the cofactor dependency of the reductase revealed that NADPH is preferred over NADH in the reduction, with an eightfold higher specific activity at pH 5.5 and 45 °C reaction temperature. In a recent example, this recombinant reductase from *Gluconobacter oxydans* was combined with a Wittig reaction in a chemoenzymatic one-pot multistep process in water (Scheme 7).[34]

In this cascade reaction, *p*-nitro- and *p*-chlorobenzaldehydes (**27a** and **27b**, respectively) were converted completely into the unsaturated ketone intermediates by using equimolar ylide **28**. Subsequent enzymatic reduction using the ene reductase from *Gluconobacter oxydans* resulted in the formation of saturated ketone products **29a** and **29b**, respectively, with excellent conversion (>95%) and high yields (60–89%).

A further example of the catalytic potential of ene reductases is the enzymatic desaturation of a C–C bond adjacent to a carbonyl group for synthesis of a number of α,β-unsaturated compounds. Schittmayer et al. recently identified such an enzymatic desaturation during a study of a thermostable Old Yellow Enzyme from *Geobacillus kaustophilus* DSM7263 (*Gk*OYE).[27] *Gk*OYE is able to reduce typical substrates, such as cyclopentenone and cyclohexenone, in the presence of NADPH at 70 °C. In addition to the expected "enone" reduction, *Gk*OYE also catalyzes the reverse reaction, the desaturation of C–C bonds (Scheme 8). This reaction proceeds without the need for any nicotinamide oxidizing cofactor, albeit at the expense of molecular oxygen. Conversions of up to 78% were achieved for different substrates (including testosterone). Various substituted cyclic ketones were tested as substrates to determine the regio- and enantioselectivity of these dehydrogenation reactions.

The dehydrogenation of 2-methylcyclopentanone (**30**) furnished 2-methylcyclopentenone (**33**; 56% with air and 78% with 1 bar O_2); however, the stereopreference was low as both enantiomers were transformed at comparable rates.



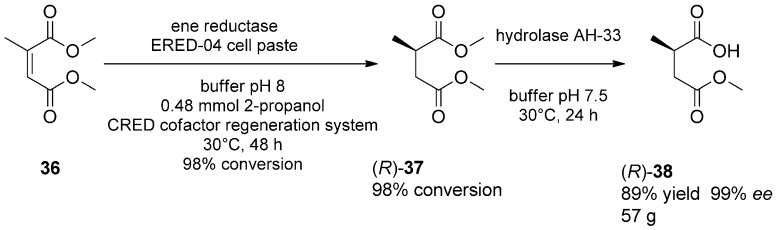
Scheme 8. Selective ketone dehydrogenation catalyzed by an Old Yellow Enzyme.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

Of the substrates tested, only dihydrocarvone (**31**) could be converted selectively (99% *ee*, *S* enantiomer) within 24 h. Although, OYE-catalyzed desaturation is still in its infancy, this opens up new synthetic routes to a number of α,β-unsaturated ketones. Thus, new ketosteroides **35** as well as small α,β-unsaturated ketones (which are often valuable flavor and aroma-defining compounds) could be synthesized.

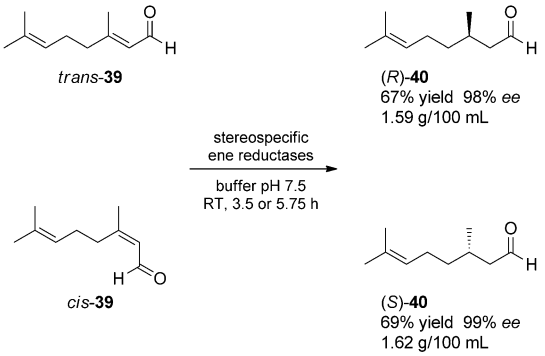
Representatives of the Old Yellow Enzyme family and homologues thereof have been further applied in the enzymatic synthesis of a variety of valuable products. Enantiomerically pure (*R*)-flurbiprofen and derivatives were synthesized using the thermophilic reductase YqjM from *Bacillus subtilis*.[35] Further biocatalytic approaches for the production of methyl (*S*)-2-bromobutanoate[36] by using Old Yellow Enzymes has been reported. Bromobutanoates are useful key intermediates in the synthesis of therapeutic agents for the treatment of non-insulin-dependent type 2 diabetes mellitus. The stepwise formation of the fragrant intermediate dihydrocarveol by combining an ene reductase enzyme with a carbonyl reductase has also recently been employed.[37]

Retrosynthetic analysis for the generation of valuable chiral building blocks was used by Almac for the synthesis of (*R*)-monoacids using ene reductase and hydrolase enzymes on a large scale. Scientists at Almac developed a process for the production of monoacid building blocks in a three-enzyme one-pot process (Scheme 9).[38] A 98% conversion of 70 g of substrate **36** was obtained after 48 h by using 70 g of ene reductase ERED-04 cell paste (from the SelectAZyme EESK-1300 screening kit). The product (*R*)-**37** was then hydrolyzed with 25 g of hydrolase AH-33 under concomitant control of the pH value to afford 57 g of the desired monoacid (*R*)-**38** (99% *ee*) after 22 h. The NADH cofactor was regenerated by using a coupled-enzyme approach in which 2-propanol is oxidized to acetone by a solvent-stable carbonyl reductase (A131 from the SelectAZyme CRED enzyme kit). This highly efficient NADH recycling system was developed by Glueck and co-workers.[39]



Scheme 9. Reaction cascade for the synthesis of monoacids on a large scale.

The synthesis on a gram scale of enantiopure citronellal, a valuable building block in asymmetric synthesis, was investigated by the research group of Stewart. They constructed and screened a library of 17 biocatalysts that were overexpressed in *E. coli* as fusion proteins with glutathione *S*-transferase (GST) to allow a one-step purification. From this set of ene reductases, OYE2.6 from *Pichia stipites* and NemA from *E. coli* were used as free enzymes in a single-phase system to reduce citral with very good *R* and *S* selectivity, respectively (Scheme 10).[40] Geranial (**39**), the *trans* isomer of citral, was used as the substrate for the biocatalytic alkene reduction. By using a crude extract of GST-OYE2.6 treated with ammonium sulfate, geranial (*trans*-**39**) was converted into 1.59 g of (*R*)-citronellal [(*R*)-**40**] in 67% yield (95% conversion) and 98% *ee* after 5.75 h. No reduction of the carbonyl group was observed. A similar strategy has been used for the bioreduction of the *cis* isomer neral (*cis*-**39**). Neral was added in three equal portions and afforded 1.62 g of (*S*)-citronellal [(*S*)-**40**] in 69% yield (>98% conversion) and 99% *ee* after 3.5 h.



Scheme 10. Biocatalytic production of gram-scale quantities of (*R*)- and (*S*)-citronellal.

The substrate selectivity, promiscuity, as well as stereoselectivity of Old Yellow Enzyme family members and analogues thereof have been explored in various studies. Despite many successes in creating chiral building blocks with these enzymes, however, there is no ene reductase described that is capable of reducing 3-alkyl-substituted cyclic alkenes. Directed evolution with iterative saturation mutagenesis (ISM) has been applied to broaden the substrate spectrum of the ene reductase YqjM from *Bacillus subtilis*. Twenty amino acid positions were selected as potential saturation mutagenesis sites by studying the X-ray structure of YqjM containing the inhibitor *p*-hydroxybenzaldehyde. In the first round of saturation mutagenesis, nine mutants were identified that showed increased activity towards *m*-methylcyclohexenone (model substrate). Wild-type YqjM shows only low conversion (3%) and moderate enantioselectivity (77% *ee*, *R* enantiomer) towards the model substrate. Furthermore, *R*- and *S*-selective variants were identified that gave *ee* values of up to 91% (*R*) and 84% (*S*), respectively. A double mutation led to a 130-fold rate increase (99% conversion) in the reduction of the cyclic model substrate while maintaining the high enantioselectivity (*R* enantiomer up to 94% *ee*). In the case of the *S*-selective system, two double mutants showed relatively high enantioselectivity (up to 99% *ee*), but low conversion (<20%). These selective YqjM variants evolved for the model reaction were tested in the asymmetric reduction of a set of 3-substituted cyclo-

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

hexenone and cyclopentenone analogues. Substrates bearing various alkyl residues as well as a second functionality (an ester group) were successfully reduced by the variants to furnish the corresponding products with excellent enantiomeric excess and conversion (up to 99% *ee* and 99%, respectively).[41]

3. Enzymes for the Preparation of Chiral Amines and Amino Acids

Chiral amino acids and amines are core components of a large number of pharmaceuticals, agrochemicals, and other specialty chemicals.[42] The formation of such molecules continues to be of great interest in industrial biocatalysis. Classical chemical methods for the production of enantiomerically pure amines and amino acids achieve the resolution through crystallization with chiral carboxylic acids,[43,44] reduction of C=N bonds,[45] the so-called "Dutch resolution",[46] Bucherer–Bergs variants of the Strecker synthesis,[47] and amidocarbonylation catalyzed by transition metals.[48] Enzymatic methods used to prepare enantiomerically pure chiral amines and amino acids are mostly based on enzyme-catalyzed kinetic resolutions of racemic substrates by lipases or acylases.[44,49] The enantioselective desymmetrization of 3,4-substituted *meso*-pyrrolidines by the variant D5 of the monoamine oxidase MAO-N from *Aspergillus niger* offers an alternative for the synthesis of amines and non-natural amino acids.[50] The ability to catalyze stereospecific reactions has also stimulated research on amino acid dehydrogenases. Members of this amino acid dehydrogenase superfamily are appealing because of their unique catalytic properties and stereospecificities: they catalyze the reversible $NAD(P)^+$-linked oxidative deamination of amino acids.[51] By altering the specificity of the leucine dehydrogenase enzyme by active-site-targeted protein engineering, an amine dehydrogenase has recently been created that is able to catalyze the synthesis of chiral amines from prochiral ketones and ammonia.[52] Furthermore, asymmetric approaches, such as deracemization through the combined use of an enantioselective amine oxidase and a nonselective reducing agent,[53] or the conversion of ketones to amines using transaminases (aminotransferases), have proven successful. The use of ammonia lyases and aminomutases for the asymmetric synthesis of amino acids and chiral amines has provided an alternative and highly attractive option. The benefit and application as well as challenges that are associated with the usage of these enzymes will be discussed in greater detail in this section. A more detailed survey of transaminases[54–56] and ammonia lyases/aminomutases[57–59] and their potential application in biocatalysis can be found in a number of recent reviews.

3.1. Transaminases as Biocatalysts

Many advances have been made in the past few years in the development of transaminases as effective biocatalysts for the preparation of enantioenriched as well as enantiopure amines.[55,60–62] Transaminases (EC 2.6.1.x) are pyridoxal-5′-phosphate (PLP) dependent enzymes that catalyze the direct amination of ketones to amines in high yield and enantiomeric excess by making use of the NH_2 group of a donor molecule (Figure 4).[56,60]

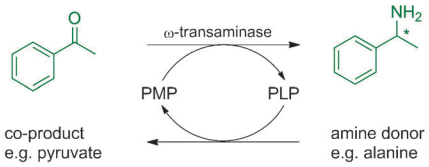


Figure 4. Synthesis of amines with ω-transaminases. PMP = pyridoxamine 5-phosphate.

More than 20 ω-transaminases have been characterized from different microbial origins, and all show similar substrate specificities. Transaminases can be applied in preparative approaches for the production of chiral amines and amino acids in three different ways: 1) kinetic resolution of racemic amines into an enantiomer and ketone with a theoretical yield of $\leq 50\%$, 2) asymmetric synthesis from prochiral ketones to produce enantiomers with a yield of $\leq 100\%$ in the presence of a favorable equilibrium, and 3) deracemization to convert a racemate into a single enantiomer with 100% theoretical yield (Figure 5).[63]

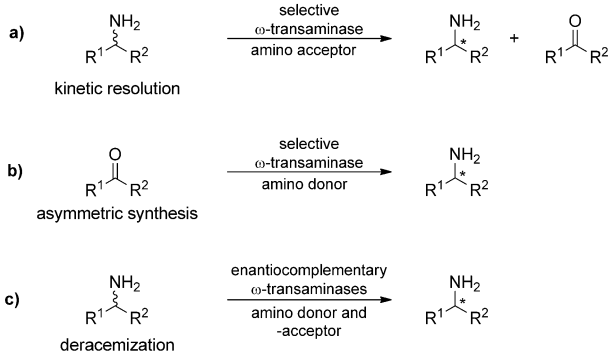


Figure 5. ω-Transaminase-catalyzed reactions.

The asymmetric synthesis approach is more favored as it can generate 100% theoretical yield. Although deracemization also leads to an equally high yield, it is the most complex reaction. The synthesis of enantiopure amines by deracemization has been achieved using either a dynamic kinetic resolution (DKR) or two enantiocomplementary ω-transaminases in a two-step one-pot process.[64,65] In the proposed mechanism, the amine donor substrate forms a Schiff base with the PLP cofactor and forms a quinonoid moiety after proton abstraction. Subsequent proton rearrangements and a hydrolysis reaction yields pyridoxamine-5′-phosphate (PMP) and the corresponding ketone. The enantiospecificity presumably results from an unfavored coordination of one enantiomer in a catalytically productive conformation

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

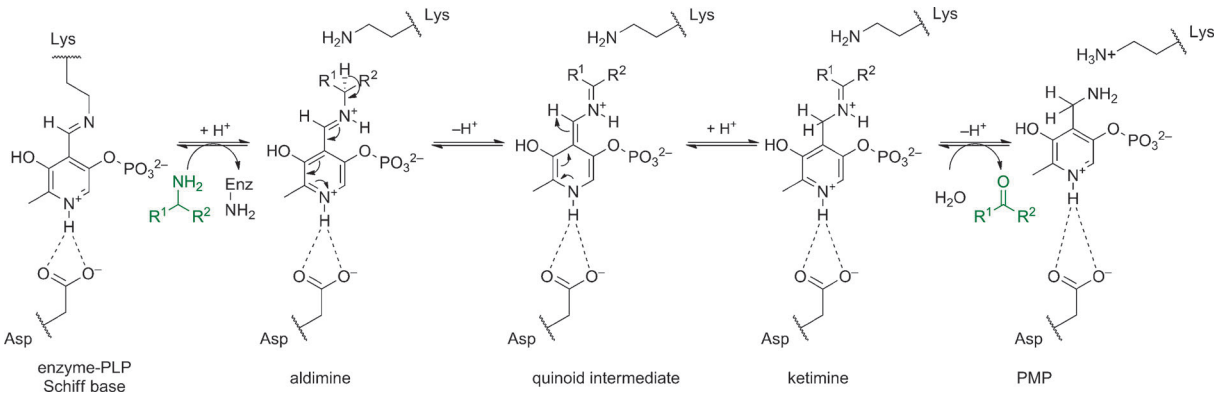


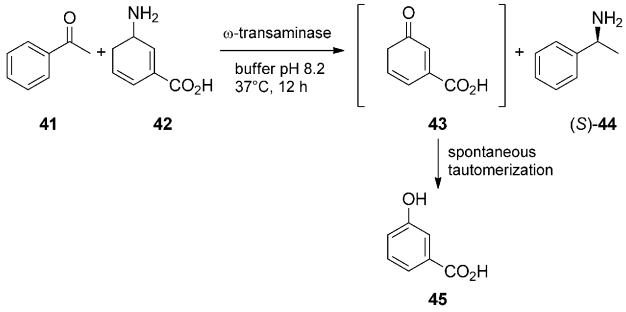
Figure 6. The transaminase reaction is divided into two steps: 1) an oxidative deamination of an amine donor and 2) a reductive amination of an amine acceptor. In the first step, PLP is used as a carrier to transport an amine group and electrons between the amine donor and the amine acceptor. A proton rearrangement followed by hydrolysis reversibly converts PLP into PMP. The amine group from PMP is transferred to the amine acceptor to regenerate PLP.

(Figure 6). It was also demonstrated that ω-transaminases can be employed as lyophilized cell-free extracts in organic solvents by using isopropylamine as the amine donor. The highest activity (99% conversion) in the preparation of enantiopure amines has been found with *tert*-butyl methyl ether as the organic solvent. In addition, it has been shown that isopropylamine functioned better as the amine donor under solvent conditions compared to purely aqueous ones in the bioamination of a set of ketones.[66]

However, the unfavorable reaction equilibrium is the major hurdle in the asymmetric synthesis of chiral amines from achiral ketones. The removal of the coproduct, which is the product generated in the deamination of the amine donor, is crucial to overcome this unfavorable reaction equilibrium and to drive the reaction towards the product side. Several enzymatic strategies exist for the removal of the coproduct pyruvate so as to shift the thermodynamically unfavorable equilibrium.

The decarboxylation of pyruvate to yield acetaldehyde and CO_2 by pyruvate decarboxylases (PDC) constitutes a simple process for pyruvate removal. Acetolactate synthase is also used to convert two molecules of pyruvate into acetolactate, which decomposes to acetoin and CO_2. A significant excess of alanine as the amine donor is required for these two methods, but no cofactor recycling system is needed, and as a result of carbon dioxide formation the reaction equilibrium is irreversibly shifted towards the product side.[67] The amine donor can also be recycled using an amino acid dehydrogenase (often alanine dehydrogenase), which catalyzes the reductive amination of the by-product α-keto acid. In this case, the amino acid dehydrogenase recycles pyruvate to alanine by employing a standard NAD(P)H regeneration system consisting of formate and formate dehydrogenase (FDH) or glucose and glucose dehydrogenase (GDH).[68]

The kinetic resolution of amines using transaminases remains an option in many cases, but needs stoichiometric quantities of pyruvate as the amine acceptor. The process for recycling pyruvate requires an amino acid oxidase, which catalyzes the in situ oxidation of the alanine amine donor in the presence of molecular oxygen.[69] The reduction of pyruvate using lactate dehydrogenase is also a generally applied method. Lactate dehydrogenase requires the cofactor NADH, thus either formate dehydrogenase (FDH) or glucose dehydrogenase (GDH), an excess of alanine, and formate or glucose as reducing agents are needed to recycle the NADH cofactor. Furthermore, Wang et al. have presented the asymmetric synthesis of chiral amine (*S*)-**44** using commercially available 3-aminocyclohexa-1,5-dienecarboxylic acid (**42**) as the amine donor and various ω-transaminases (Scheme 11). The produced ketone **43** was effectively removed by a subsequent tautomerization to 3-hydroxybenzoic acid (**45**). The application of this approach resulted in quantitative formation of (*S*)-**44** (99%) with an excellent enantiomeric excess (>99%).[70]



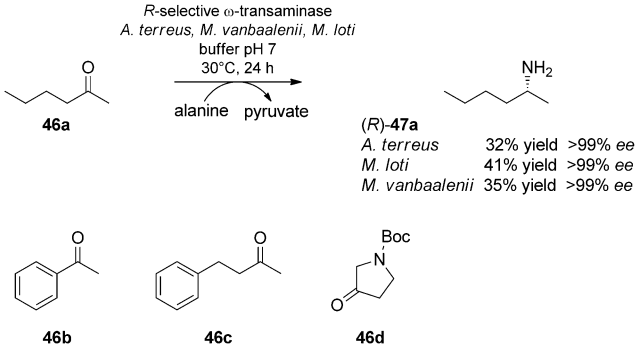
Scheme 11. Asymmetric synthesis of a chiral amine with an artificial amine donor.

3.1. Production of Chiral Amines

So far, more than 20 *S*-selective ω-transaminases have been described and characterized.[60] As a result of only one transaminase enzyme with *R* selectivity (from *Arthrobacter* sp.)[71] having been been reported, Höhne et al. have developed an in silico approach to discover enzymes with desired complementary enantiopreference by circumventing

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

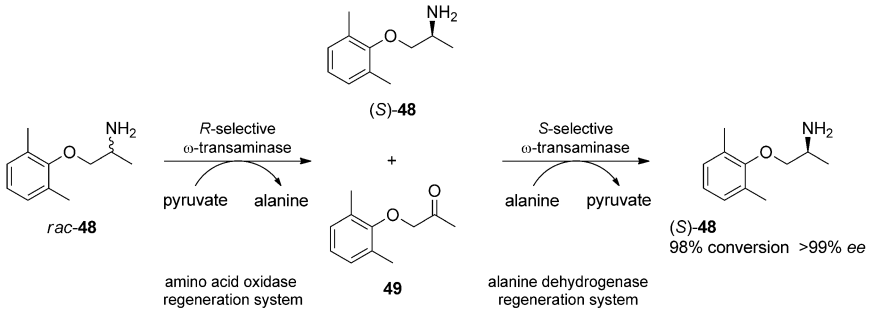
directed evolution concepts (Scheme 12).[72] This included the prediction of key features of the desired enzyme by analyzing the structural information available, followed by the design and application of a sequence-based algorithm. 21 sequences matched the criteria for the selection of a putative *R*-selective enzyme, from which 17 genes were identified as possessing activity and *R* selectivity. From a panel of four different ketones **46a–d**, the asymmetric synthesis of the best substrate 2-hexanone (**46a**) with three



Scheme 12. Asymmetric synthesis of (*R*)-amines using newly identified *R*-selective transaminases. Boc = *tert*-butyloxycarbonyl.

transaminases from *Aspergillus terreus*, *Mycobacterium vanbaalenii*, and *Mesorhizobium loti* resulted in the formation of (*R*)-2-aminohexane [(*R*)-**47a**] in moderate yields (32–41 %) and excellent enantiopurities (> 99 % *ee*).

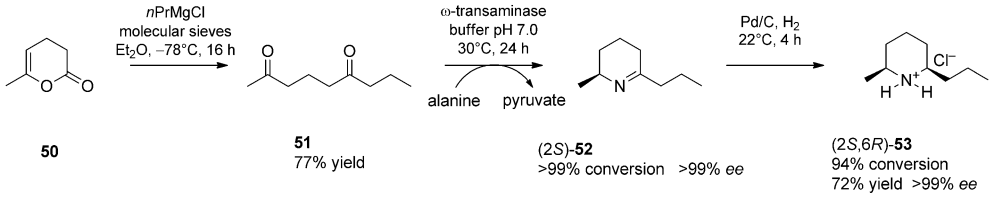
Recently, the deracemization of α-chiral primary amines catalyzed by ω-transaminases has been described.[65,73] Depending on the order of the enantiocomplementary enzymes employed, the *S* and the *R* products were accessible. As a consequence, the deracemization of 1-(2,6-dimethylphenoxy)-2-propanamine (mexiletine, *rac*-**48**), a chiral orally effective antiarrhytmic agent, has been analyzed in great detail (Scheme 13). The approach used for this deracemization is based on a two-step one-pot process comprising a kinetic resolution and stereoselective amination step catalyzed by stereocomplementary transaminases. The synthesis of enantiopure (*R*)-mexiletine [(*R*)-**48**; > 99 % *ee*] was achieved with a high conversion of > 99 % via **49**, by employing the transaminases from *Chromobacterium violaceum* CV-ωTA and ATA-117. The combination of the two commercial transaminases ATA-117 and ATA-113 resulted in the formation of the *S* product (*S*)-**48** (> 99 % *ee*) with excellent conversion (98 %) after 24 h.[74]



Scheme 13. Deracemization process of mexiletine by ω-transaminases to form *S* isomers.

Moreover, ω-transaminases enable the regio- and stereoselective asymmetric monoamination of aliphatic 2,6-diketones without the need for protecting groups (Scheme 14). Simon et al. have reported the synthesis of the natural alkaloid (+)-dihydropinidine [(2*S*,6*R*)-**53**], a potential antifeedant against the pine weevil *Hylobius abietis*. Starting from dihydropyrane-2-one (**50**), enantiopure (2*S*,6*R*)-**53** was obtained in three steps in a yield of 72 % and an enantiomeric excess of > 99 %. The synthesis of the dehydropiperidine intermediate **52** was achieved with an excellent conversion of > 99 % and very high enantioselectivity (> 99 % *ee*) via the corresponding diketone **51** by employing the ω-transaminase from *Chromobacterium violaceum*.[75]

Amines are a versatile class of compounds used frequently in organic synthesis. Therefore, transformation of easily accessible alcohols to amines is an important reaction for



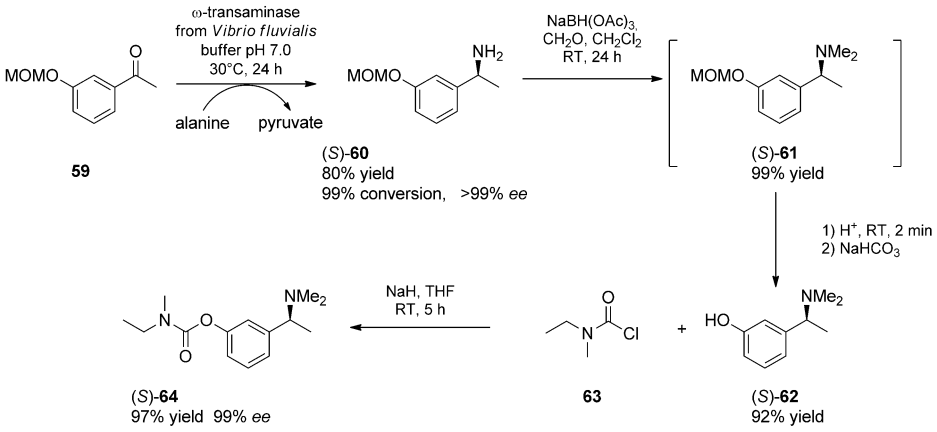
Scheme 14. Synthesis of (2*S*,6*R*)-**53** by employing an ω-transaminase.

the synthesis of a variety of organic compounds. The most common approaches for the synthesis of amines from alcohols comprise their conversion into the corresponding halides or sulfonates, nucleophilic substitution by azide anions, and finally the reduction of the azide to an amine or reductive amination. Alternatively, they can be prepared by a two-step artificial multienzyme cascade.

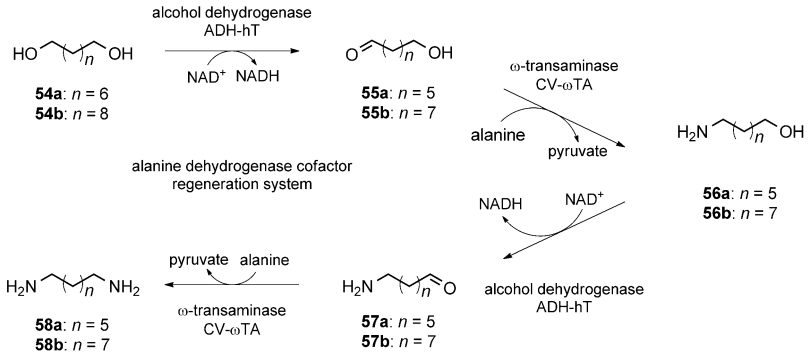
Such an artificial multienzyme network has been designed for the asymmetric amination of secondary alcohols to the corresponding α-chiral primary amines through the use of alcohol dehydrogenase and transaminase enzymes. Conversions of the alcohol to the amine of up to 91 % were obtained under optimized reaction conditions.[76] A similar system has been proposed for the synthesis of diamines, which are common building blocks for polyamides.[77] The thermostable alcohol dehydrogenase from *Bacillus stearothermophilus* ADH-hT and ω-transaminase from *Chromobacterium viola-*

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

ceum (Scheme 15) afforded amines as well as diamines with high conversions (99%). These products have been obtained by oxidation of the diols **54a**,**b** to the corresponding aldehyde-functionalized intermediates **55a**,**b** which in turn were transformed to the amine **56a**,**b**. A subsequent oxidation to intermediates **57a**,**b** followed by amination with transaminase enzyme afforded diamines **58a**,**b**. Alanine dehydrogenase was chosen for regeneration of the cofactor. In the case of 1,8-octanediol (**54a**), 98% of the



Scheme 16. Chemoenzymatic asymmetric synthesis of (*S*)-Rivastigmine. MOM = methoxy methyl ether.

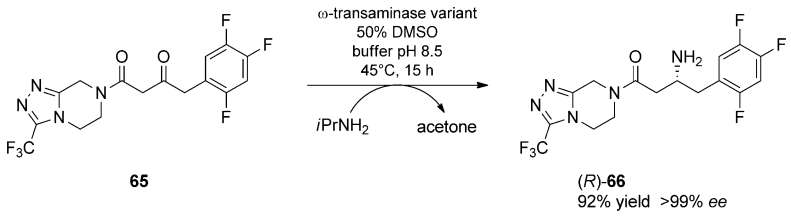


Scheme 15. Amination of diols by using an artificial multienzyme cascade.

starting material has been converted (46% diamine **58a** and 52% amino alcohol **56a**). In contrast, the transformation of 1,10-decanediol (**54b**) resulted in the synthesis of pure diamine (99% diamine **58b**, < 1% **56b**). The water-miscible solvent 1,2-dimethoxyethane (10% v/v) was used to increase the solubility of the poorly soluble diol compounds **54a**,**b**. The diamination of 1,10-decanediol (**54b**) was also performed on a preparative scale (174 mg of substrate), thereby leading to a 94% conversion and a 74% yield of the diamine product **58b** in 20 h.

A biocatalytic process based on ω-transaminases was shown to be an efficient tool for the preparation of (*S*)-Rivastigmine, a drug for the treatment of Alzheimer's disease (Scheme 16). The key building block for the Rivastigmine synthesis, the methoxymethyl ether protected α-methylbenzylamine **60**, was obtained from the corresponding acetophenone substrate **59** through an enzyme-catalyzed asymmetric transamination using stereocomplementary ω-transaminases. DMSO or CH_2Cl_2 were used as cosolvents (10% v/v) to increase the solubility of the substrate and to improve the reaction rates. Enantiopure (*S*)-**60** was obtained in 80% yield (99% conversion) and excellent enantiopurity (> 99% *ee*) using ω-transaminase from *Vibrio fluvialis*, while the corresponding *R* enantiomer (*R*)-**60** was accessed in a moderate 56% yield (76% conversion) and a high enantiomeric excess of 98% by using the stereocomplementary enzyme ATA-117. Moreover, MOM-protected amine precursor **59** was used in a four-step chemoenzymatic synthetic procedure to give (*S*)-Rivastigmine [(*S*)-**64**] in 71% overall yield and 99% *ee*.[78]

Recently, a combined approach of molecular modeling with directed evolution has emerged to improve the substrate specificity and stability of a transaminase so as to replace an established rhodium-based chiral catalyst in an industrial process. The tailoring of the *R*-selective commercially available transaminase ATA-117 provided an enzyme with increased activity for the synthesis of sitagliptin [(*R*)-**66**], a compound for treating diabetes (Scheme 17). Docking studies using prositagliptin ketone substrate **65** suggested that the enzyme would be unable to bind the sterically demanding ketone in the active site, which consists of a large and small binding pocket. In subsequent rounds of evolution (11 rounds), a final enzyme catalyst was obtained that overall contains 27 mutations and shows four orders of magnitude improved activity over the initial transaminase. In parallel, chemical process development was performed with respect to the stability of the enzyme at high concentrations of substrate, DSMO (cosolvent), and

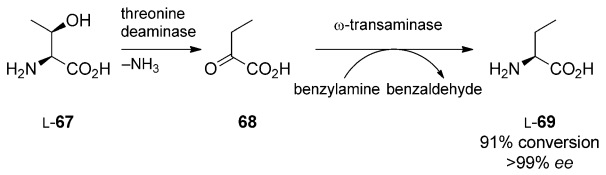


Scheme 17. Evolved transaminase biocatalysts for the manufacture of sitagliptin. *i*PrNH$_2$ = isopropylamine.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

amine donor isopropylamine, as well as the stability of the reaction medium at elevated temperatures. In the optimized final process, 6 g L^{-1} of the evolved transaminase variant were capable of converting 200 g L^{-1} of prositagliptin ketone (**65**) into enantiopure sitagliptine product [(*R*)-**66**] with > 99 % *ee* and 92 % yield at 45 °C in 50 % DMSO over 15 h.[79]

Among the different biocatalytic methods available to produce non-natural amino acids, ω-transaminases are the most prominent biocatalysts for the synthesis of non-natural α- and β-amino acids.[62,80] Enantiopure, non-natural amino acids represent highly valuable compounds for the pharmaceutical, cosmetic, food, chemical, and agricultural industries. Therefore, amine group transfers have been coupled with other useful reactions to produce compounds other than chiral amines. Park et al. have developed a one-pot conversion of L-threonine (L-**67**) into L-homoalanine (L-**69**) by using threonine deaminase and ω-transaminase (ω-TA) in a coupled enzyme reaction approach (Scheme 18). An *S*-specific threonine deaminase from *E. coli* (ilvA) was used in the conversion of L-threonine (L-**67**) to 2-oxobutyrate (**68**). In the second step, **68** was asymmetrically converted into L-homoalanine (**69**) using a novel ω-transaminase from *Paracoccus denitrificans* PD1222 and benzylamine as the amine donor. After 5 h, this coupled enzyme reaction afforded L-**69** with 91 % conversion and an enantiomeric excess of > 99 %.[81]



Scheme 18. One-pot conversion of L-threonine into L-homoalanine. Reaction conditions: 10 mM substrate, 20 mM benzylamine, 1 μM PLP, buffer pH 7.0, 37 °C, 300 min.

A related system was described for the coupling of a tyrosine aminotransferase with an ω-transaminase for the simultaneous production of aromatic L-amino acids and *R*-configured amines.[82] Among several enzymatic methods that have been employed for the synthesis of amino acids and amines, transaminases are promising because of their broad substrate specificity. Furthermore, the stereospecific transfer of an amine group by transaminases can be coupled to C–C bond-forming reactions to produce industrial synthons such as amino alcohols. In this light, the two-step biocatalytic preparative synthesis of chiral amino alcohols from achiral starting materials with transketolase and transaminase enzymes was described. This one-pot synthesis combining an enzyme-catalyzed formation of an asymmetric C–C bond with a stereospecific transfer of an amine group onto an L-erythrulose or (3*S*)-dihydroxypentanone intermediate was investigated for the production of 2-amino-1,3,4-butanetriol or 2-aminopentane-1,3-diol.[83] Amino alcohols are an important class of compounds, being employed as chiral auxiliaries and ligands in organic and medicinal chemistry. In addition, as a result of the pharmacological interest in fluorinated α-amino acids, Bea et al. have investigated the asymmetric synthesis of (*R*)-fluoroalanine. In this approach, an extractive biphasic reaction system to avoid product inhibition was used to synthesize (*R*)-fluoroalanine from 3-fluoropyruvate and (*S*)-α-methylbenzylamine as the amine donor with recombinant ω-transaminase from *Vibrio fluvialis* JS17. (*R*)-Fluoroalanine was obtained with 70 % conversion and > 99 % enantiomeric excess by using this biphasic system.[84]

3.2. Amino Acid Ammonia Lyases

In contrast to transaminases, amino acid ammonia lyases are a class of enzymes that carry out electrophilic catalysis without a metal or common cofactor. Ammonia lyases are capable of cleaving carbon–nitrogen bonds without hydrolysis or oxidation mechanisms. There are a number of ammonia lyases that catalyze the reversible formation of α,β-unsaturated bonds through the elimination of ammonia from their amino acid substrates. Aromatic amino acid ammonia lyases catalyze the reversible deamination of aromatic amino acids, such as phenylalanine, tyrosine, and histidine, into their corresponding achiral α,β-unsaturated carboxylic acids.[57,59,85] Two further candidates were identified, namely aspartate ammonia lyase and 3-methylaspartate ammonia lyase.[86] Ammonia lyase catalyzed reactions can be applied in the synthesis of natural and non-natural amino acids through an amination of α,β-unsaturated carboxylic acids or a kinetic resolution of amino acid racemates (Figure 7).

Aromatic amino acid lyases contain a 4-methylideneimidazol-5-one (MIO) group for substrate activation.[87,88] MIO is formed by spontaneous cyclization within a conserved alanine-serine-glycine sequence, with the concomitant elimination of two molecules of water.[87,89] The exact mechanism of ammonia elimination by ammonia lyases has been disputed for almost 50 years. The first reported mechanism proposed by Hanson and Havir was an E_1cb mechanism, which was

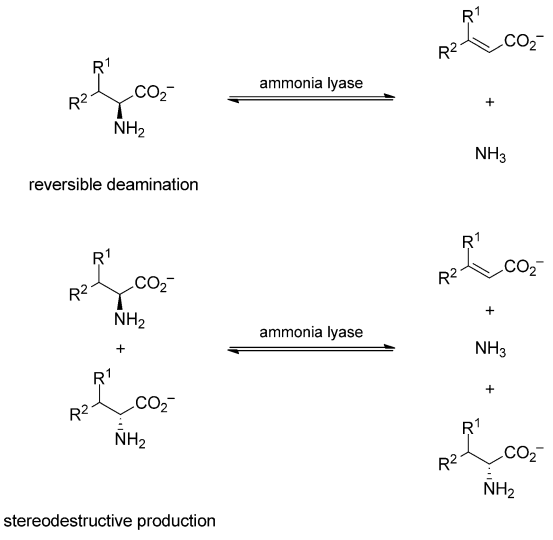


Figure 7. Ammonia lyase catalyzed reactions used for the stereoselective production of amino acids by two alternative strategies.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

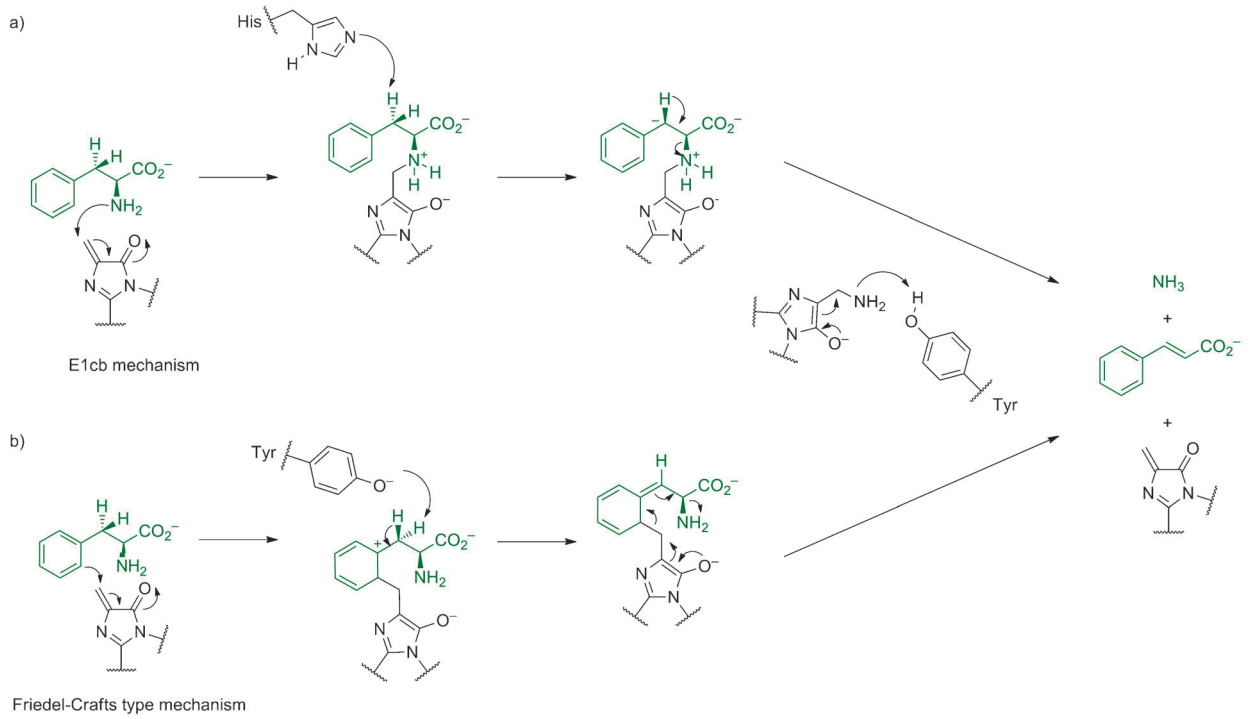


Figure 8. The two mechanisms proposed for MIO-dependent ammonia lyases: a) An E_1cb mechanism with an electrophilic attack of the MIO group on the amine of the substrate; b) a Friedel–Craft-type mechanism is initiated by electrophilic attack of MIO on the aromatic ring. These mechanisms lead to activation and, therefore, abstraction of the β-proton followed by elimination of ammonia and regeneration of the MIO group. Histidine (E1cb mechanism) or tyrosine (Friedel—Crafts-type mechanism) act as the catalytic bases to abstract the proton from C3.

superseded a year later by a mechanism involving a Friedel–Crafts-type attack of the MIO group on the phenyl moiety of L-phenylalanine (Figure 8).[90] The carboxylate oxygen atoms are involved in hydrogen-bonding networks with glutamine, arginine, and tyrosine.

A recent study by Bartsch and Bornscheuer based on docking and MD simulations on the phenylalanine ammonia lyase from *Petroselinum crispum* suggests that the deamination of aromatic amino acids can occur by both mechanisms—the Friedel–Crafts and the E_1cb mechanism.[91]

Phenylalanine ammonia lyase (PAL, EC 4.3.1.5) is a very important plant enzyme that catalyzes the conversion of L-phenylalanine into *trans*-cinnamic acid, itself the precursor of a great variety of phenylpropanoids such as lignins, flavonoids, and coumarins. Numerous PAL enzymes have been identified in microorganisms, especially in various yeasts, with many examples found within the red yeast basidiomycetes *Rhodotorula*.[92] PAL is specific for L-phenylalanine and to a lesser extent L-tyrosine.[59] PAL has been used to synthesize a wide range of L-arylalanines containing differently substituted phenyl, polycyclic aromatic, as well as heterocyclic rings and L-phenylalanine methyl ester by the addition of ammonia to their corresponding unsaturated precursors.[93]

Aspartate ammonia lyase (EC 4.3.1.1), also referred to as aspartase, plays an important role in bacterial metabolism of nitrogen by catalyzing the reversible conversion of L-aspartate into fumarate and ammonia. This ammonia lyase has been identified in several anaerobic microorganisms which are part of the *Enterobacteriaceae* family. Aspartate ammonia lyase is one of the most specific enzymes known, as no other substrate can replace L-aspartate in the deamination reaction. The high selectivity of aspartate ammonia lyase for its natural substrate limits the practical application of this enzyme. The reverse reaction catalyzed by aspartase from *E. coli* (AspA)—the amination of fumarate—is used commercially in the industrial production of the artificial sweetener aspartame (*N*-L-α-aspartyl-L-phenylalanine 1-methyl ester).[94] An excess of ammonia is used to drive the equilibrium from fumarate towards L-aspartate. The broader substrate specificity of 3-methylaspartate ammonia lyase (MAL, EC 4.3.1.2) makes this biocatalyst more versatile than aspartate ammonia lyase. In nature, MAL catalyzes the reversible α,β-elimination of ammonia from L-*threo*-3-methylaspartate and L-*erythro*-3-methylaspartate to yield mesaconate. A number of substituted amines with fumarates are accepted and, thus, generate substituted aspartic acid products.[58] A general acid–base reaction mechanism via an enolate intermediate was proposed for both ammonia lyases (Figure 9). The substrate specificity for these enzymes partly arises from specific hydrogen bonds between the enzyme and the carboxylate groups of the substrate. In the case of 3-methylaspartate ammonia lyase, glutamine and histidine support the Mg^{2+} ion in carboxylate coordination.[86]

The use of ammonia lyases as biocatalysts for the preparation of enantiomerically pure amino acids is of high interest. Weiner et al. studied the deamination of amino acids other than the natural substrate by using the thermostable aspartate ammonia lyase from Bacillus sp. YM55-1. Despite

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

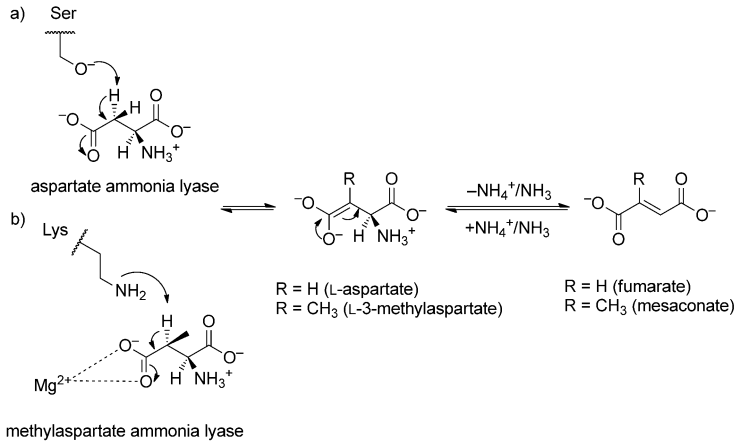
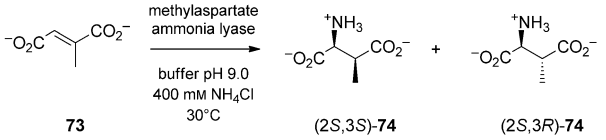


Figure 9. Proposed reaction mechanism for a) aspartate and b) methylaspartate ammonia lyases. A base in the active site abstracts the proton from the C3-position of the substrate. An enolate intermediate is formed which is stabilized by amino acid residues in the active site or in the case of MAL by interactions with a Mg^{2+} ion. Collapse of the intermediate is followed by Cα–N bond cleavage, which results in the elimination of ammonia.

the fact that no alternative amino acid could be identified to replace L-aspartate as the substrate, four nucleophiles **71a–d** showed promising activities in the amination reaction (Scheme 19). The stereoselective amination of fumarate **70** with the small amine nucleophiles hydroxylamine (**71a**), hydrazine (**71c**), methoxylamine (**71b**), as well as methylamine (**71d**) in phosphate buffer/D_2O was monitored by ^{1}H NMR spectroscopy. The conversions and steady-state kinetics of the amination of fumarate (**70**) demonstrated that both **71a** and **71c** are excellent nucleophilic substrates. 200 mM fumarate (**70**) were completely converted after incubation times of 30 min and 24 h, respectively. In contrast, **71b** and **71d** afforded the corresponding N-substituted aspartic acids **72b** and **72d** in yields of 11% and 95%, respectively, after incubation for 7 days.[95]

Methylaspartate ammonia lyase (MAL) from *Clostridium tetanomorphum* is able to catalyze the stereo- and regioselective addition of ammonia to several mesaconate derivatives to form various 3-alkyl- and 3-halo-substituted aspartic acids.[96] MAL shows some reasonable activity towards small, substituted amines and fumarates.[97] A structure-based engineering of the lyase to accept a variety of substituted amines and fumarates for the asymmetric synthesis of aspartic acid derivatives has recently been described.[98]

Guided by the structure of *Clostridium amalonaticus* methylaspartate ammonia lyase in complex with the natural substrate (2*S*,3*S*)-3-methylaspartic acid, three active-site residues in *Clostridium tetanomorphum* were selected for mutagenesis. These residues are reported to be important for the activity and stereoselectivity of MAL-catalyzed reactions. Therefore, Raj et al. have altered the diastereoselectivity of the 3-methylaspartate ammonia lyase by applying structure-based mutagenesis (Scheme 20). 3-MAL catalyzes the reversible rapid *anti* and much slower *syn* addition of ammonia to mesaconate **73** to

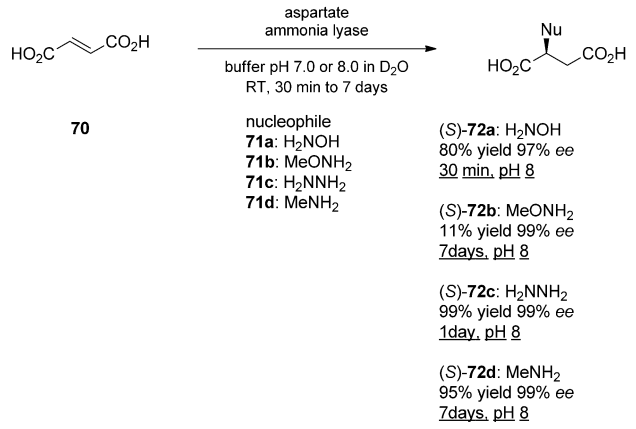


Scheme 20. Methylaspartate ammonia lyase catalyzed amination of mesaconate.

afford (2*S*,3*S*)-3-methylaspartic acid [(2*S*,3*S*)-**74**] and (2*S*,3*R*)-3-methylaspartic acid [(2*S*,3*R*)-**74**]. As a result, the amination of mesaconate using mutant enzymes led only to (2*S*,3*R*)-3-methylaspartic acid (2*S*,3*R*)-**74**, with no detectable formation of (2*S*,3*S*)-**74**.[99]

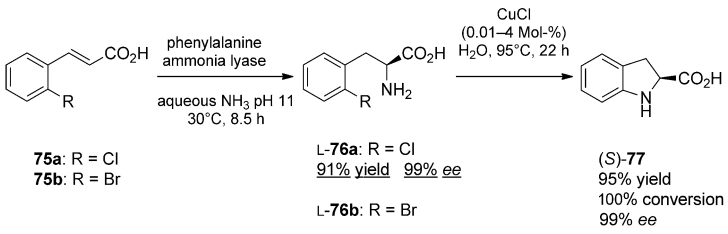
Recently, de Lange et al. have demonstrated a synthetic route that combines biocatalysis and homogeneous catalysis for the formation of enantiopure (*S*)-2-indolinecarboxylic acid [(*S*)-**77**], a key intermediate for ACE (angiotensin-converting enzyme) inhibitors (Scheme 21). The synthesis of this compound is traditionally achieved by preparation of the racemic precursor by a Fischer indole synthesis followed by a classical or enzymatic resolution. The enantioselective amine addition of ammonia to *ortho*-substituted cinnamic acids **75a,b** was catalyzed by the PAL from *Rhodotorula glutinis* DSMPAL01 and was followed by a copper-catalyzed ring-closing reaction of the corresponding enantiopure L-*o*-halophenylalanines L-**76a,b**. An excess of aqueous NH_3 (13% v/v) was used.

The cyclization products were obtained in excellent yields (95%) and enantioselectivities (99%) by using a CuCl catalyst. Having thus optimized the methods for the ring closure of both halogenated phenylalanines L-**76a,b**, (*S*)-2-indolinecarboxylic acid [(*S*)-**77**] was obtained in 60% total yield and an enantiomeric excess of 99% after an additional crystallization step.[100]



Scheme 19. Biocatalytic synthesis of N-substituted aspartic acid derivatives by aspartate ammonia lyase.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 21. Chemoenzymatic asymmetric synthesis of (*S*)-2-indolinecarboxylic acid.

α product). The absolute configuration of the product was L for the α-phenylalanine and *R* for the β-phenylalanines.[101]

As already described above, the asymmetric addition of ammonia results in a product mixture containing both α- and β-amino acids, which is a disadvantage of this system. Isomer-selective liquid–liquid extraction (ILLE) with a $[PdCl_2(PPh_3)_2]$ complex host has been used for the separation of mixtures of α- and β-phenylalanine isomers. This extraction step is a refinement of enantioselective liquid–liquid extraction (ELLE). In the latter case, the enantiopure host is used as an extractant to react enantiospecifically and reversibly with a racemic substrate.[105] Thus, an optimized extraction system for the purification of β-phenylalanine from a reaction mixture was obtained in a PAM-catalyzed enantioselective addition of ammonia to (*E*)-cinnamic acid.[106] Wu et al. have successfully altered the phenylalanine aminomutase from *Taxus chinensis* into a selective β-lyase by applying a structure- and mechanism-based protein-engineering approach. This led to the synthesis of almost pure (*R*)-β-phenylalanine and derivatives through the asymmetric amination of cinnamic acid.[107]

To generate enantiopure β-phenylalanines, Wu et al. established an efficient tandem biocatalytic process from aromatic β-amino acid racemates (Scheme 22). In their approach, enantiopure (*S*)-β-phenylalanines (*S*)-**78a,b** were prepared by coupling the PAM-catalyzed stereoselective isomerization with the PAL-catalyzed deamination of L-**79a,b**. This resulted in the formation of cinnamic acids **80a,b** using PAM from *Taxus chinensis* and PAL from *Rhodosporidium toruloides*. Hence, the use of this biocatalytic tandem reaction led to full substrate conversions and excellent enantioselectivities (> 99 % *ee*) of (*S*)-β-phenylalanine (*S*)-**78a,b** after 48 h.[108]

PAM was also applied to the dynamic kinetic resolution of various substituted α-arylalanines **81a–e** to produce (*R*)-β-arylalanines (*R*)-**82a–e**. For this, the aminomutase-catalyzed

3.3. Aminomutases

Aminomutases are a subfamily of MIO-containing enzymes that are responsible for the biosynthesis of β-amino acids in structurally diverse natural products. One member, phenylalanine aminomutase, is able to produce aromatic β-amino acids by a stereospecific isomerization of phenylalanines to the corresponding β-isomers, which can be found in a range of natural and pharmaceutical products.[101]

The high sequence homology of aminomutases to ammonia lyases indicates that the mechanism of aminomutases is similar to that of ammonia lyases, and that the former may have evolved from the latter.[102] Support for this mechanism came from the finding that the aminomutase activity proceeds through a readdition of the amine by 1,4-conjugate addition to a cinnamate intermediate. The reaction is reversible.[103]

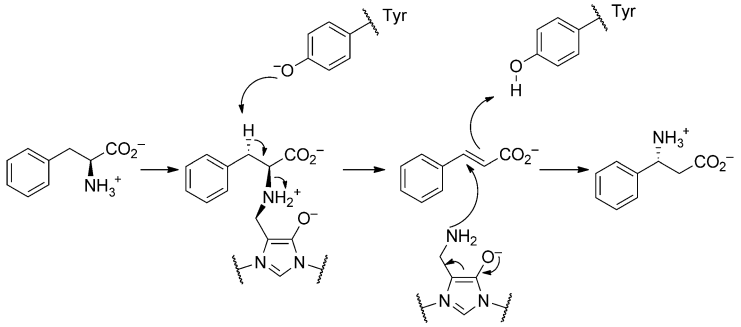
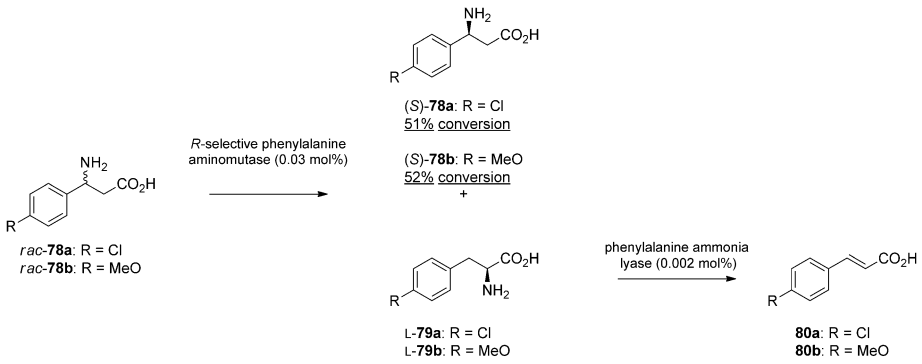


Figure 10. Reaction mechanism of aminomutases with α-phenylalanine entering the active site of aminomutase where the amine group and the proton are oriented in a geometry that is suitable for the elimination step. A nucleophilic attack of the amine group of the substrate on the MIO group procedes through a tyrosine base, followed by elimination of MIO-NH_2 to form cinnamic acid. After rotation of the cinnamate intermediate, the readdition of the amino group to the β-position and of α-proton to the α-position occurs.

Recently a mechanism for the aminomutase reaction has been proposed. The migratory hydrogen atom and amino group are eliminated heterolytically from the substrate in a stepwise manner and held by the enzyme (Figure 10). The rotation of the cinnamate intermediate in the active site allows the aminomutase to reattach the amino group to the opposite site which results in the formation of β-phenylalanine in the *R*-configuration. The carboxylate oxygen atoms form hydrogen bonds with the asparagine, arginine and glutamine residues in the active site of phenylalanine aminomutase.[104]

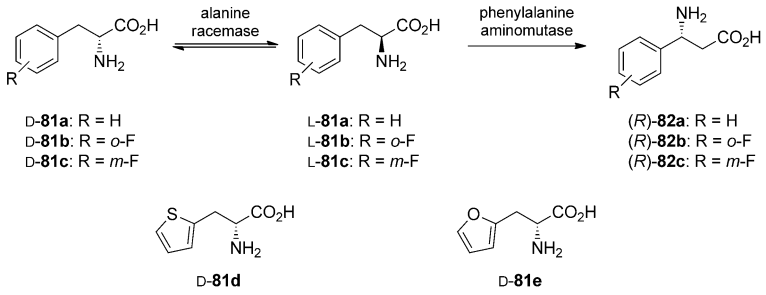
Enantiopure β-amino acids, which are not as abundant in nature as α-amino acids, are highly valuable building blocks for peptidomimetics and the synthesis of bioactive compounds. Wu et al. have demonstrated the enantioselective synthesis of non-natural α- and β-amino acids by using phenylalanine aminomutase (PAM). The enzyme has a broad substrate scope and gives substituted α- and β-phenylalanines with excellent enantiomeric excess (> 99 %). The addition of ammonia to the cinnamic acid intermediate normally results in a 1:1 mixture of α- and β-phenylalanine, with an excellent enantioselectivity for both isomers (> 99 % *ee*). The best results for the synthesis of β-amino acids were obtained using cinnamic acid derivatives having a methyl or a methoxy group substituent at the *para* position (only 4–14 % of the α product was detected). In contrast, the cinnamic acid substrate with nitro substitution on the aromatic ring promoted the addition of ammonia to the α position (98 % formation of the

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 22. Biocatalytic process for the preparation of enantiomerically pure β-phenylalanine derivatives. Conditions for the biocatalytic tandem reaction: buffer pH 8.8, 3% glycerol, 37°C, 48 h.

isomerization was coupled to an alanine racemase catalyzed epimerization step (Scheme 23). A PAM from *Taxus* plants catalyzed the stereospecific isomerization of L-α-arylalanines L-**81a–e** to (*R*)-β-arylalanines (*R*)-**82a–e**. It could be demonstrated in experimental studies that the formation of the (*R*)-



Scheme 23. Dynamic kinetic resolution of α-arylalanines to enantiopure β-arylalanines. Reaction conditions: buffer pH 8.0, 5% glycerol, 31°C, 20 h.

β product is slightly more preferred than the L-arylalanine substrate. As a consequence of the nonproductive accumulation of the D-α-arylalanine isomers D-**81a–e**, the catalysis of PAM was coupled with racemization of the α-arylalanine enantiomers by using a pyridoxal-5′-phosphate-dependent alanine racemase from *Pseudomonas putida* KT2440. This led to an increase in the formation of enantiopure (*R*)-β-arylalanines with high enantiomeric excesses (> 99%) from racemic α-arylalanine mixtures D,L-**81a–e**. The natural product β-phenylalanine **82a** showed the largest mol% increase (19%) from phenylalanine, followed by **82c** (13%), **82d** (7%), **82b** (6%), and **82e** (4%) compared to the conversion of the α-arylalanines under kinetic resolution conditions with PAM alone. A series of non-natural *o*- and *m*-substituted α-arylalanines L-**81a–c** and β-heterocyclic α-alanines L-**81d,e** have been employed in the coupled racemase-aminomutase resolution reactions.[109]

4. Biocatalysis as an Alternative for the Production of Chiral Epoxides and Epoxide Ring Opening Products

The production of chiral epoxides through chemical synthesis needs expensive catalysts and often results in products with a low enantiomeric excess. In the last decade, biocatalytic approaches for the kinetic resolution of racemic epoxides as well as stereoselective epoxide ring opening have evolved into a remarkable synthetic tool.[110,111] Processes utilizing these enzymes have been patented by several companies such as BASF, DSM, and Codexis.[112] Epoxide hydrolases (EHs) catalyze the enantioselective hydrolysis of epoxides and are applied in the synthesis of chiral epoxides as well as 1,2-diols. Halohydrin dehalogenases (HHDHs) catalyze the conversions of vicinal halohydrins into the corresponding epoxides. They are also able to catalyze the stereoselective ring opening of epoxides with various nucleophiles, which provides another biocatalytic strategy for the preparation of chiral compounds. The principle mechanism differs between EHs and HDHHs. Most epoxide hydrolases are part of the α/β-hydrolase enzyme family and possess a catalytic triad. Part of this triad is a nucleophilic aspartate that carries out an attack on a carbon atom of the epoxide ring, thereby forming a transient covalent intermediate, whose ester bond is then hydrolyzed (Figure 11A). A minor class of epoxide hydrolases, with the limonene 1,2-epoxide hydroxylase (LEH) from *Rhodococcus erythropolis* as a prominent example, act through a distinct mechanism.[113] LEH catalyzes the direct hydrolysis of epoxides by activating a water molecule in the active site, thereby enabling the nucleophilic attack while the oxygen atom of the oxirane leaving group is protonated by an aspartate (Figure 11B). The proposed mechanism for HHDH involves the activation of the epoxide by hydrogen bonding, which allows the attack of a nucleophile that is expected to bind in a halide-binding pocket (Figure 11C).

4.1. Epoxide Hydrolases

Epoxide hydrolases are ubiquitous in nature and can be found in various living organisms.[114] Although mammalian enzymes were studied initially, much effort was made in the 1990s to identify and characterize microbial epoxide hydrolases. Although epoxide hydrolases are limited to water as the nucleophile for opening the epoxide ring, they have attracted much attention in organic synthesis. Chiral epoxides can be easily obtained from racemic substrates by kinetic resolution,

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

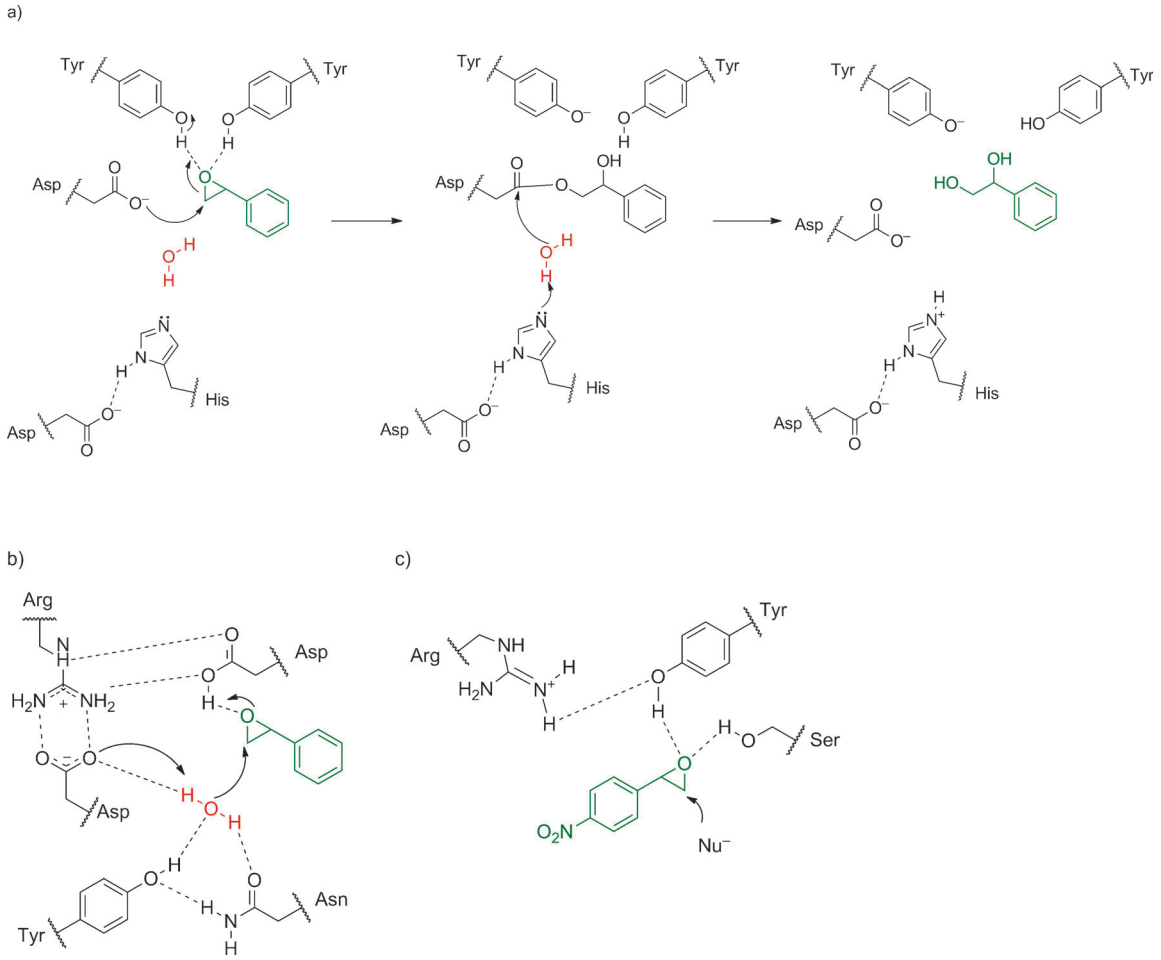
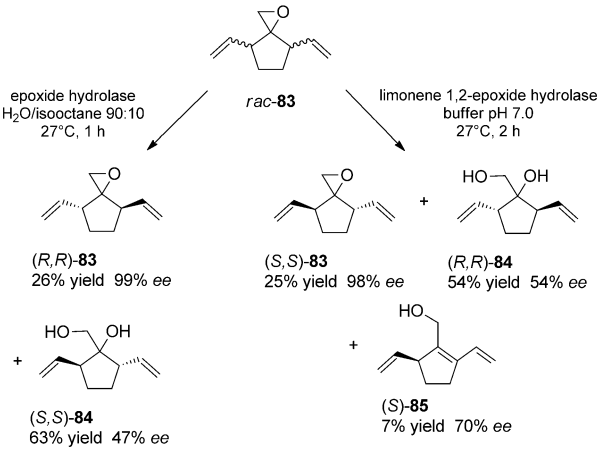


Figure 11. a) The proposed reaction mechanism for epoxide hydrolases suggests that the substrate epoxide is polarized by two tyrosine residues (hydrogen bond with the epoxide oxygen atom), the nucleophilic aspartate attacks the epoxide, usually at the least sterically hindered and most reactive carbon atom, and the ester intermediate is hydrolyzed. b) In contrast, in the mechanism of limonene 1,2-epoxide hydrolases, the epoxide hydrolysis seems to occur without the formation of a covalent enzyme–substrate intermediate. In this mechanism, tyrosine and asparagine position a water molecule in a favorable position for epoxide attack. An aspartate-arginine-aspartate triad is proposed to be involved in proton donation and abstraction during the reaction, with one aspartate donating a proton to the oxirane ring of the substrate, while the other aspartate abstracts a proton from the water molecule (facilitation of a nucleophilic attack on the epoxide carbon atom). The arginine residue positions the carboxylate groups of the two aspartates and also assists in charge stabilization. c) In the catalysis by halohydrin dehalogenases, the catalytically active residues serine, tyrosine, and arginine are involved. These amino acids activate the epoxide for a nucleophilic attack by a halogen. The conserved serine ensures proper positioning of the substrate, whereas arginine plays a critical role by lowering the pK_a value of the catalytically active tyrosine.

whereas chiral 1,2-diols can be synthesized from racemic epoxides in enantioconvergent processes. In the early days, biocatalytic studies often focused on monosubstituted epoxides such as derivatives of styrene oxide as well as aryl glycidyl ether, which are important key intermediates. During the last decade, research on disubstituted epoxides remarkably broadened the synthetic applications of these enzymes. Besides high enantiopurities, short reaction times and often very high substrate concentrations characterize processes with these cofactor-independent enzymes.

The kinetic resolution of a variety of 2,2-disubstituted epoxides has been studied in detail by Faber and co-workers, using epoxide hydrolases from different microbial strains.[115] Many of these 2,2-disubstituted substrates are available with a methyl group as substituent. However, several studies showed that even sterically more challenging compounds can be kinetically resolved to generate sterically demanding 2,2-disubstituted epoxides. Together with the well-established monosubstituted compounds, these enable the enantioselective synthesis of various terminal epoxides, arguably the most useful subset of these compounds. The *trans*-spiroepoxide *rac*-**83** is an important building block for the synthesis of 11-heterosteroids, which are of current pharmacological interest. The kinetic resolution of the epoxide substrates was achieved using two enantiocomplementary enzymes, namely *Aspergillus niger* epoxide hydrolase (*An*EH) and limonene 1,2-epoxide hydrolase (LEH).[116] *An*EH resolves *rac*-**83** to provide the (*R*,*R*)-**83** epoxide with high enantioselectivity (99% *ee*), while LEH resolves *rac*-**83** to give (*S*,*S*)-**83** with 98% *ee*. The preparative conversions (1 g scale) at high

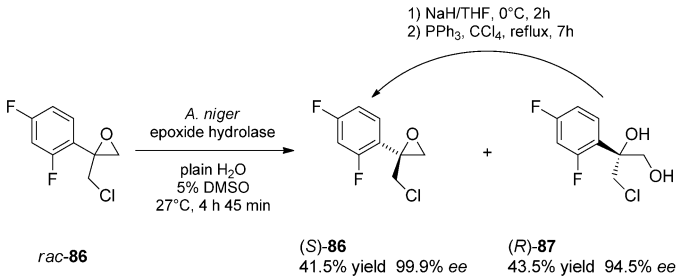
 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 24. Kinetic resolution of the *trans*-spiroepoxide using epoxide hydrolase *An*EH and limonene 1,2-epoxide hydrolase.

substrate concentrations ($100\,g\,L^{-1}$ *An*EH, $8\,g\,L^{-1}$ LEH) afforded the epoxides in good yields (Scheme 24).

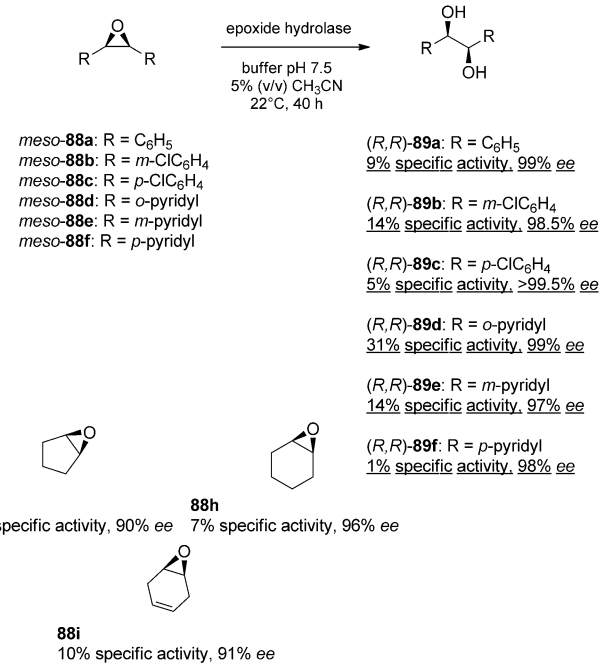
Furthermore, Monfort et al. described a highly productive process for the preparation of an enantiopure azole antifungal key synthon D0870 (*S*)-**86**.[117] The kinetic resolution of the 2,2-disubstituted epoxide *rac*-**86** was performed in a two-phase plain water/DMSO system with a substrate concentration of $500\,g\,L^{-1}$ in the presence of commercially available *An*EH (Scheme 25). The preparative conversion under these



Scheme 25. Kinetic resolution of 1-chloro-2-(2,4-difluorophenyl)-2,3-epoxypropane using an epoxide hydrolase.

conditions afforded the unreacted epoxide (*S*)-**86** (41.5% yield, 99.9% *ee*) as well as the diol (*R*)-**87** (43.5% yield, 94.5% *ee*) in high yield and high enantiomeric excess after almost 5 h. As a complement to this process, the chemical cyclization of diol (*R*)-**87** to epoxide (*S*)-**86** was obtained in an overall enantioconvergent process by treatment with NaH and triphenylphosphine.

Asymmetric biocatalytic hydrolysis of 1,2-disubstituted epoxides is often applied in processes to generate the corresponding 1,2-diols (Scheme 26). Although *meso*-epoxides constitute a relative narrow subset of *cis*-disubstituted epoxides, their desymmetrization is an attractive route that can furnish a 100% theoretical yield of the corresponding *trans*-diols. A landmark with respect to enzymatic hydrolysis of *meso*-epoxides *meso*-**88a–i** was achieved in 2004 by



Scheme 26. Epoxide hydrolase catalyzed desymmetrization of *meso*-epoxides.

scientists at Diversa.[118] More than 50 novel microbial epoxide hydrolases were identified by sequence- and activity-based screening of DNA libraries generated from various environmental samples. Three epoxide hydrolases (epoxide hydrolase BD10721, BD9883, BD8877) were found to catalyze the formation of the (*R*,*R*)-diol products (*R*,*R*)-**89a–i** from a diverse set of heterocyclic, alicyclic, and bulky epoxides **88a–i**. The corresponding (*R*,*R*)-diols (*R*,*R*)-**89a–i** were formed with high enantiopurities and turnover frequencies. The synthetic utility and simplicity was confirmed by treating 1.0 g of *cis*-stilbene *meso*-**88a** with 109 mg cell lysate, which led to (*R*,*R*)-1,2-diphenyl-1,2-ethanediol [(*R*,*R*)-**89a**] in a yield of 83% and 99% *ee*. In addition, these studies further identified the first examples of (*S*,*S*)-diol-producing epoxide hydrolases for the desymmetrization of *meso*-epoxides, although their specific activities were lower than those of (*R*,*R*)-diol-producing epoxide hydrolases.

Recently, Zheng and Reetz used iterative saturation mutagenesis (ISM) to optimize the stereoselectivity of limonene 1,2-epoxide hydrolase.[119] The enantioselectivity of this enzyme with non-natural substrates is generally poor and, therefore, limits its utilization in synthetic organic chemistry. By grouping residues which align the active site and performing saturation mutagenesis iteratively using a reduced amino acid alphabet, less than 5000 mutants had to be screened using the desymmetrization of cyclopentene oxide (**90a**) as the model substrate.

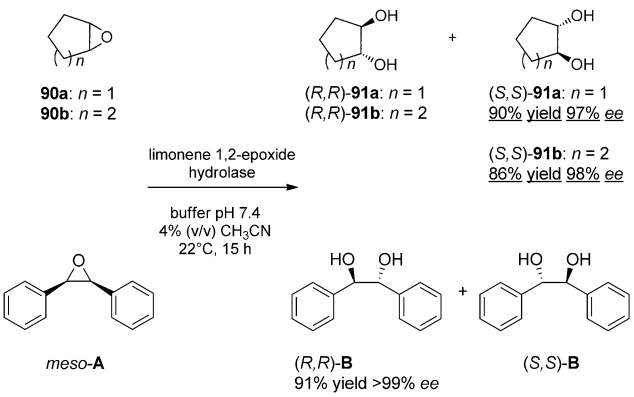
Although wild-type limonene 1,2-epoxide hydrolase resulted in an *ee* value of 14% (*R*,*R*), mutants were identified with improved *R*,*R* selectivity and inverted *S*,*S* selectivity. Mutant H178 converted the test substrate **90a** into the (*S*,*S*)-diol (*S*,*S*)-**91a** (93% *ee*) with inverted stereoselectivity, while mutant H173 generated the (*R*,*R*)-diol (*R*,*R*)-**90a** with

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

improved enantioselectivity (80% *ee*). The substrate scope of the lead mutants was further studied and these enzymes proved to be excellent catalysts for the desymmetrization of other *meso*-epoxides such as *meso*-**90b** and *meso*-**A**.

Three *meso*-epoxides were selected for an up-scaling with substrate concentrations of up to 30 g L^{-1}. High enantioselectivities (97–99% *ee*) and yields (86–91%) were achieved, with *meso*-**A** showing the highest yield (Scheme 27). This result is indicative of ISM being a fast method in protein engineering to generate enantioselective catalysts. LEH variants for the desymmtrization of *meso*-epoxides were engineered, with the (*S*,*S*)-diol-producing mutants being of special interest since there natural occurrence is scarce.

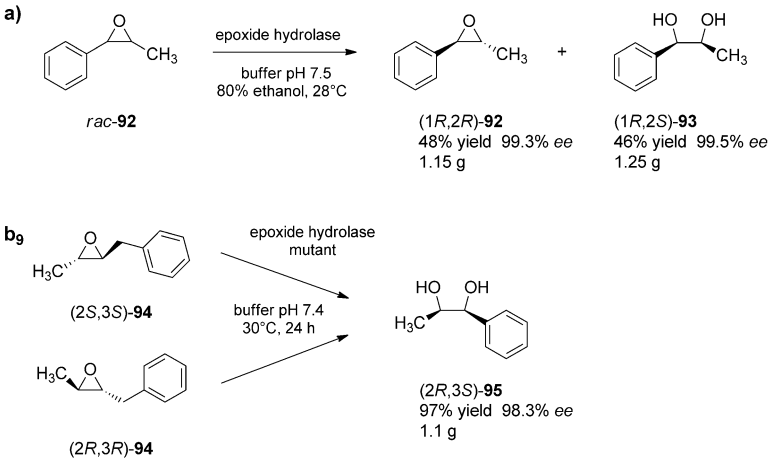
Furthermore, 1,2-*trans*-disubstituted epoxides have been used as substrates. Recently, Kotik et al. identified an interesting epoxide



Scheme 27. Scale-up of the desymmetrization of selected *meso*-epoxides using engineered epoxide hydrolases.

hydrolase (Kau2) from a metagenomic DNA library of biofilter-derived biomass.[111] Besides stereoselectivity with different monosubstituted epoxides, Kau2 showed high enantioselectivity and activity in the kinetic resolution of *trans*-1-phenyl-1,2-epoxypropane (*rac*-**92**). The corresponding (1*R*,2*R*)-epoxide (1*R*,2*R*)-**92** (48% yield, 99.3% *ee*) and the (1*R*,2*S*)-diol (1*R*,2*S*)-**93** (46% yield, 99.5% *ee*) could be obtained, respectively, in an up-scaling experiment with a substrate concentration of 80 g L^{-1} (Scheme 28a).

Reetz and co-workers applied laboratory evolution with epoxide hydrolase from *Aspergillus niger* and *trans*-2-benzyl-3-methyloxirane (**94**) as the substrate, the conversion of which is extremely slow and sluggish by wild-type *An*EH.[120] Two variants exhibited high enantioselectivitiy ($E > 200$) in the kinetic resolution of the test substrate. Later, using the same substrate, enantioconvergence was demonstrated with one of these mutants, thereby generating the single enantiopure (2*R*,3*S*)-diol (2*R*,3*S*)-**95** from the racemic substrate **94**. This is based on the regioselective hydrolysis of the (2*S*,3*S*)-epoxide (2*S*,3*S*)-**94** at the 2-position as well as the mediation of the



Scheme 28. Conversion of *trans*-disubstituted epoxides with epoxide hydrolase.

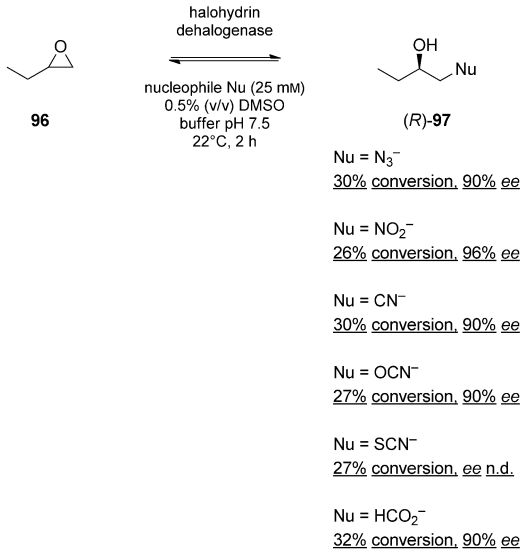
reaction of the enantiomeric (2*R*,3*R*)-epoxide (2*R*,3*R*)-**94** regioselectively at the 3-position (Scheme 28b). Further iterative saturation mutagenesis with this mutant generated variants with higher reaction rates, while maintaining the enantioconvergence (92% conversion, >99% *ee*).[121] It is worth mentioning that the Jacobsen catalyst is unable to convert such racemic *trans*-disubstituted epoxides **94**.[122]

4.2. Halohydrin Dehalogenases

Halohydrin dehalogenases (HDHHs) are bacterial enzymes involved in the biodegradation of halogenated organic compounds. Several microorganisms possess halohydrin dehalogenases, with the enzyme from the epichlorhydrin-degrading bacterium *Agrobacterium radiobacter* AD1 (HheC) being the best studied one. HheC catalyzes the dehalogenation of 1,3-dichloropropanol and 1-chloropropane-2,3-diol to produce an epoxide and HCl, as well as the reverse reaction. The X-ray structure of HheC combined with various ligands revealed a spacious halide-binding pocket and contributed to a better understanding of the catalytic mechanism and the enantioselectivity of the enzyme.[123] Halohydrin dehalogenases exhibit high regio- and enantioselectivity towards aliphatic and aromatic substrates, and thus are useful tools for the production of enantiopure epoxides as well as their ring-opening products.[124] The diversity of non-halide nucleophiles which can be used for the opening of epoxide rings further expands the synthetic application, especially considering that β-functionalized alcohols are often required as pure enantiomers in the synthesis of pharmaceutical and biologically active compounds.

Janssen and co-workers demonstrated that HheC is able to catalyze the epoxide ring opening reaction with various small anionic nucleophiles with high enantiomeric excesses and high yields (Scheme 29).[125] Sufficient activity (k_{cat} values of 2–100 s^{-1}) for preparative application was demonstrated with nucleophiles such as azides, cyanides, nitrites, cyanates, and thiocyanates by using 1,2-epoxybutane (**96**) as test substrate. All these reactions showed a predominant attack

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 29. Versatility of ring-opening reactions catalyzed by halohydrin dehalogenase. n.d. = not determined.

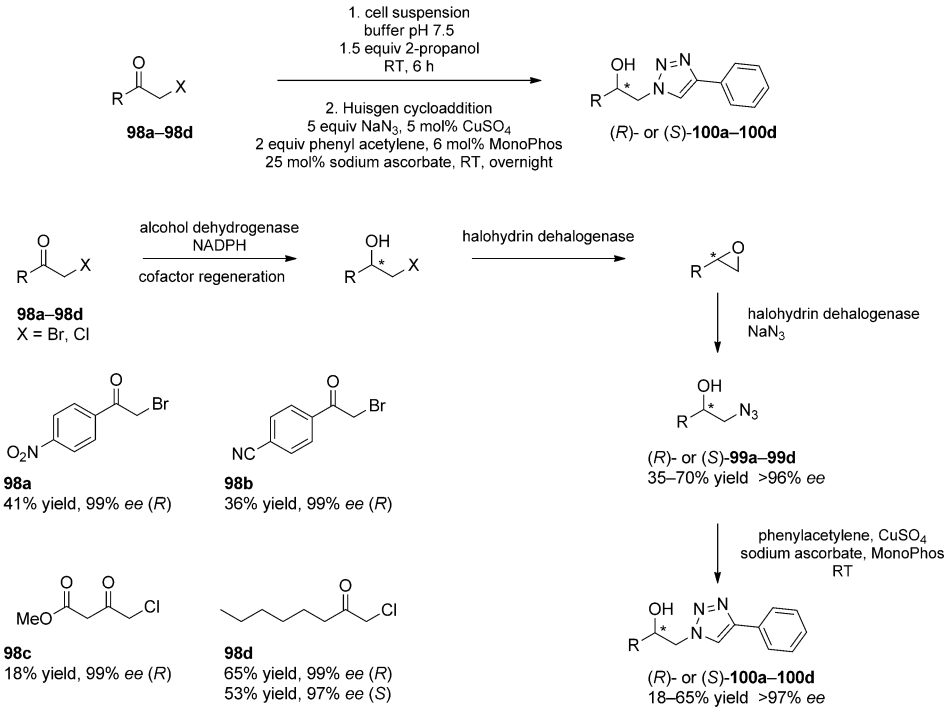
at the less-substituted carbon atom, with a strong enantiopreference for the (*R*)-epoxide **97**. In addition to **96** as the substrate, more sterically demanding aromatic substrates as well as disubstituted epoxides were successfully opened with different nucleophiles.

The combination of a multienzymatic cell cascade with halohydrin dehalogenases offers a valuable method for the one-pot transformation of simple, prochiral substrates into enantiopure, functionalized products. In this respect, designer cells which overexpress one or more biocatalysts have been applied in a one-pot combination of multienzymatic cells with the Huisgen cycloaddition. *R* and *S* enantiomers of different β-hydroxytriazoles **100a–d** were synthesized with high enantiomeric excesses from α-halo ketones **98a–d** via the corresponding azide intermediates **99a–d** (Scheme 30).[126] β-Hydroxytriazoles have been reported to be β-adrenergic receptor blockers, and thus important pharmacophores.

The use of recombinant microbial whole cell catalysts was combined with the Huisgen reaction that uses a copper catalyst. Two constructs were made that combined alcohol dehydrogenases and halohydrin dehalogenases with opposite stereoselectivity. The first constructed cells, also named CT cells, consisted of an overexpressed *R*-selective alcohol dehydrogenase AdhT from *Thermoanaerobacter* sp. and halohydrin dehalogenase HheC from *Agrobacterium radiobacter* AD1. The constructed *S*-selective cells (BL cells), comprised an alcohol dehydrogenase AdhL from *Lactobacillus brevis* and HheBGP1, the halohydrin dehalogenase from *Mycobacterium* sp. GP1. Combining CT designer cells and click chemistry led to good results with α-bromo ketones as substrates possessing electron-withdrawing groups in the *para* position of the phenyl ring [*p*-NO_2 (**98a**) and *p*-CN (**98b**)]. *R*-Configured products (*R*)-**100a**,**b** could be isolated in yields of 36–41% and high enantiomeric excesses (> 99%).

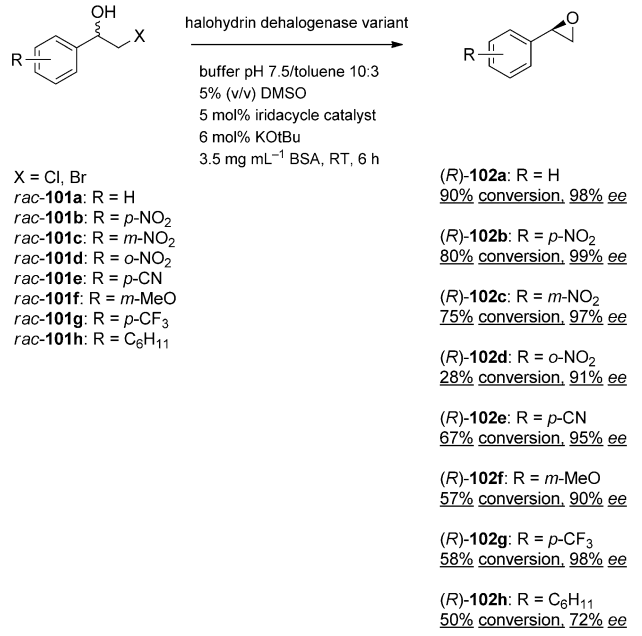
In a similar way, two additional aliphatic substrates methyl-4-chloroacetoacetate (**98c**) and 1-chloro-2-octanone (**98d**) were converted in yields of 18 and 65%, respectively, and 99% *ee*. BL cells with opposite enantiopreference were applied to obtain the other enantiomer of β-hydroxytriazole. As the halohydrin dehalogenase HheB prefers aliphatic compounds, 1-chloro-2-octanone (**98d**) was converted into the corresponding *S* product (*S*)-**100d** in 53% yield and high enantiomeric excess (97%).

Halohydrin dehalogenases were also used in kinetic resolution experiments to generate epoxides in enantioselective conversions. Haak et al. reported the first direct chemoenzymatic dynamic kinetic resolution of racemic β-haloalcohols **101a–h** to provide the corresponding epoxides (*R*)-**102a–h**.[127] An activated iridacycle catalyst was found to be the most active catalyst for the racemization of β-haloalcohols **101a–h**. The dynamic kinetic resolutions were performed in a water/toluene biphasic system containing 5% DMSO as cosolvent. Bovine serum albumin (BSA) was required for stabilization of the enzyme. A variant of HheC was used to



Scheme 30. Multistep catalytic cascade reaction using designer cells for the enantioselective production of β-hydroxytriazoles.

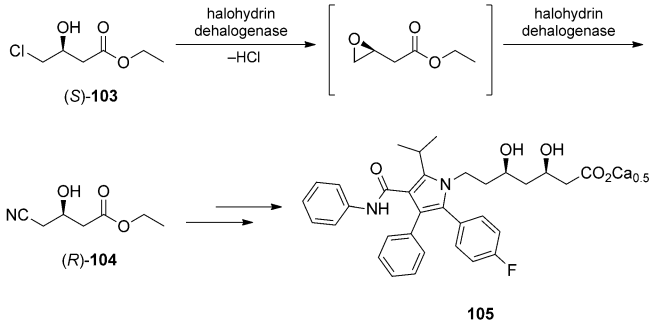
© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim



Scheme 31. Dynamic kinetic resolution of β-haloalcohols. BSA = bovine serum albumin.

synthesize different aromatic (*R*)-epoxides (*R*)-**102a–h** in good yields and excellent enantioselectivities (Scheme 31).

An application of halohydrin dehalogenases for epoxide ring opening with a non-natural nucleophile is realized in the commercial manufacture of the cholesterol-lowering agent atorvastin (**105**), the active ingredient of Lipitor (annual sales exceed $10 billion). In this case, an enzymatic synthesis of the key chiral building block ethyl (*R*)-4-cyano-3-hydroxybutyrate [(*R*)-**104**] in a biocatalytic two-step process was developed (Scheme 32).[128]



Scheme 32. Biocatalytic synthesis of a key intermediate in the manufacture of atorvastatin. Reaction conditions for enzymatic cyanation: 70 g substrate, buffer pH 7.3, 25% NaCN solution, 40°C, 18 h.

The first step (not shown) involves the ketoreductase-catalyzed biocatalytic reduction of ethyl-4-chloroacetoacetate to the corresponding (*S*)-**103**. The enantioselective reduction is coupled to a cofactor-regeneration step using glucose as reductant and a $NADP^+$-dependent glucose dehydrogenase GDH. DNA shuffling was applied to improve the activity and the stability of the reducing enzyme while maintaining the high enantioselectivity. DNA shuffling resulted in a 13-fold and 7-fold improved activity for GDH and KRED, respectively. The enantiopure product (*S*)-**103** is obtained in 96% yield and 99% *ee*.

In a second step, recombinant halohydrin dehalogenase is employed to catalyze the irreversible formation of the hydroxynitrile (*R*)-**104** via the epoxide intermediate by using cyanide as a nucleophile at neutral pH. Iterative rounds of DNA shuffling were applied to overcome the low activity and poor stability of the wild-type HHDH for the cyanation reaction. Compared to the wild-type enzyme, the presence of high concentrations of product HHDH led to the activity increasing 2500-fold. This led to a high product yield (92%) and an enantiomeric excess of >99%.

5. Conclusion

The trend in the last 10 years has been a growing interest in enzyme-based catalysts and a change in our perspective on the use of biocatalysis for organic syntheses. Research and development within the biocatalysis sector gained further momentum and, thus, showed that in addition to the existing and technically used hydrolases and ketoreductases, novel biocatalysts are gradually filling the gap of desired chemical reactions of interest. In addition to the challenges to provide new selective enzymes and variants suitable for individual biocatalytic processes, another facet is the optimization of existing enzymes with respect to their stability under given process conditions and their concentrations as well as production rates. The development of enzyme toolkits that consist of a diverse collection of enzymes and variants thereof will help to sample the chemical space and to enable an accelerated technology transfer to industry. While there are promising efforts directed at the employment of enzymes and variants for a number of particular synthesis steps, there is room for improvement in regard to retrosynthetic analyses. A further increase in the range of available enzyme catalysts for process chemists in the future will enable our vision and ultimate goal of having biocatalysis alongside chemocatalysis to be realized. The employment of chemical reactions that can be used in one-pot processes through synthetic or multi-enzymatic cascades is highly desired in particular, as cascade reactions that form a number of bonds by one operation contribute towards a reduction in the number of reactions as well as unnecessary downstream processing steps.

This Review describes how these demands are being addressed, particularly by the use of various C=C bond-reducing as well as C–C, C–O, and C–N bond-forming biocatalysts. It can be expected that many more enzymes will be applied in key chemical reaction steps with the next generation of promising enzymes such as cyclases, berberine-bridge enzymes, pictet-spenglerases, or imine-reductases, to name a few, including also the desired iminase from N. J. Turner which is already in the starting blocks. Developments in enzyme engineering, de novo protein design, as well as the search and discovery of new enzymes will further expand the repertoire of biocatalysis in the coming years. Moreover,

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

enzymes with their specialized catalytic mechanisms are believed to be more widely applicable than it might have seemed a few years ago. Numerous studies show that the existing catalytic centers can, analogous to classical catalysis, be exploited for mechanistically related reactions. In this regard, existing enzymes can be adapted for the catalysis of synthetically important reactions not previously observed in nature. The ideas and advances in this area (so called chemistry-based enzyme engineering) are particularly suitable for the provision of new catalysts.

We thank Dr. Rainer Stürmer from Stürmer Scientific and Dr. Michael Breuer from BASF SE for their thoughtful comments on the Review.

Received: March 15, 2013
Published online: February 12, 2014

[1] G. Grogan, *Annu. Rep. Prog. Chem. Sect. B* **2009**, *105*, 206 – 231; A. Schmid, J. S. Dordick, B. Hauer, A. Kiener, M. Wubbolts, B. Witholt, *Nature* **2001**, *409*, 258 – 268; K. M. Köller, C.-H. Wong, *Nature* **2001**, *409*, 232 – 240; S. Panke, M. Held, M. Wubbolts, *Curr. Opin. Biotechnol.* **2004**, *15*, 272 – 279; C. Wandrey, A. Liese, D. Kihumbu, *Org. Process Res. Dev.* **2000**, *4*, 286 – 290; K. Faber, W. Kroutil, *Curr. Opin. Chem. Biol.* **2005**, *9*, 181 – 187; U. T. Bornscheuer, G. W. Huisman, R. J. Kazlauskas, S. Lutz, J. C. Moore, K. Robins, *Nature* **2012**, *485*, 185 – 194.

[2] M. Breuer, K. Ditrich, T. Habicher, B. Hauer, M. Kesseler, R. Stürmer, T. Zelinski, *Angew. Chem.* **2004**, *116*, 806 – 843; *Angew. Chem. Int. Ed.* **2004**, *43*, 788 – 824.

[3] E. Busto, V. Gotor-Fernández, V. Gotor, *Chem. Soc. Rev.* **2010**, *39*, 4504 – 4523; R. N. Patel in *Biocatalysis in the Pharmaceutical and Biotechnological Industry*, CRC, Boca Raton, **2007**, pp. 103 – 158; R. Schoevaart, M. van Vliet, *Spec. Chem. Mag.* **2007**, *27*, 38 – 39; V. Gotor-Fernández, R. Brieva, V. Gotor, *J. Mol. Catal. B* **2006**, *40*, 111 – 120; E. Busto, V. Gotor-Fernandez, V. Gotor, *Chem. Rev.* **2011**, *111*, 3998 – 4035.

[4] M. A. J. Veld, A. R. A. Palmans, *Adv. Polym. Sci.* **2010**, *237*, 55 – 78.

[5] D. Liu, P. Trodler, S. Eiben, K. Koschorreck, M. Müller, J. Pleiss, S. C. Maurer, C. Branneby, R. D. Schmid, B. Hauer, *ChemBioChem* **2010**, *11*, 789 – 795.

[6] P.-O. Syrén, E. Lindgren, H. W. Hoeffken, C. Branneby, S. Maurer, B. Hauer, K. Hult, *J. Mol. Catal. B* **2010**, *65*, 3 – 10; S. Warwel, G. Steinke, M. R. Klass, *Biotechnol. Tech.* **1996**, *10*, 283 – 286; L. N. Monsalve, E. M. Rustoy, A. Baldessari, *Biocatal. Biotransform.* **2011**, *29*, 87 – 95.

[7] B. Hauer, B. C. Kvarnström, R. Schmid, S. C. Maurer, P. Trodler, D. Liu, M. Müller, (BASF AG), WO 2009080676 A120090702, **2009**; B. Hauer, D. Häring, C. Branneby, M. Rusnak, D. Liu, R. Schmid, (BASF AG), WO 2007113336 A2 20070607, **2007**; D. Häring, T. L. Herrera, B. Hauer, T. Friedrich, A. Göthlich, (BASF AG), WO 2007063037 A2 20070607, **2007**; D. Haering, U. Meisenburg, B. Hauer, F. Dietsche, (BASF AG), WO 2007014935 A1 20070208, **2007**; B. Hauer, H. W. Höffken, B. C. Kvarnström, H. Schwab, S. Feichtenhofer, M. Buchebner, B. Pohn, (BASF AG), WO 2010007155 A1 20100121, **2010**; B. Hauer, B. C. Kvarnström, K. Hult, A. Magnusson, A. Hamberg, (BASF AG), WO 2011033039 A1 20110324, **2011**.

[8] J. P. Adams, A. J. Collins, R. K. Henderson, P. W. Sutton, *Practical Methods for Biocatalysis and Biotransformations* (Eds.: J. Whittall, P. Sutton), Wiley, Chichester, **2010**, pp. 1 – 82.

[9] W. Kroutil, H. Mang, K. Edegger, K. Faber, *Curr. Opin. Chem. Biol.* **2004**, *8*, 120 – 126; W. Hummel, *Trends Biotechnol.* **1999**, *17*, 487 – 492; A. Weckbecker, H. Gröger, W. Hummel, *Adv. Biochem. Eng./Biotechnol.* **2010**, *120*, 195 – 242; M. L. Hall, A. S. Bommarius, *Chem. Rev.* **2011**, *111*, 4088 – 4110; D. Monti, G. Ottolina, G. Carrea, S. Riva, *Chem. Rev.* **2011**, *111*, 4111 – 4140.

[10] J. C. Moore, D. J. Pollard, B. Kosjek, P. N. Devine, *Acc. Chem. Res.* **2007**, *40*, 1412 – 1419.

[11] H. Leisch, K. Morley, P. C. K. Lau, *Chem. Rev.* **2011**, *111*, 4165 – 4222.

[12] N. J. Turner, *Chem. Rev.* **2011**, *111*, 4073 – 4087; J. H. Schrittwieser, J. Sattler, V. Resch, F. G. Mutti, W. Kroutil, *Curr. Opin. Chem. Biol.* **2011**, *15*, 249 – 256; V. Resch, J. H. Schrittwieser, E. Siirola, W. Kroutil, *Curr. Opin. Biotechnol.* **2011**, *22*, 793 – 799.

[13] M. Brovetto, D. Gamenara, P. S. Mendez, G. A. Seoane, *Chem. Rev.* **2011**, *111*, 4346 – 4403.

[14] B. M. Nestl, B. A. Nebel, B. Hauer, *Curr. Opin. Chem. Biol.* **2011**, *15*, 187 – 193; G. A. Strohmeier, H. Pichler, O. May, M. Gruber-Khadjawi, *Chem. Rev.* **2011**, *111*, 4141 – 4164.

[15] P. Lorenz, J. Eck, *Eng. Life Sci.* **2004**, *4*, 501 – 504; P. A. Romero, F. H. Arnold, *Nat. Rev. Mol. Cell Biol.* **2009**, *10*, 866 – 876; M. J. Dougherty, F. H. Arnold, *Curr. Opin. Biotechnol.* **2009**, *20*, 486 – 491; J. D. Bloom, F. H. Arnold, *Proc. Natl. Acad. Sci. USA* **2009**, *106*, 9995 – 10000; D. Röthlisberger, O. Khersonsky, A. M. Wollacott, L. Jiang, J. DeChancie, J. Betker, J. L. Gallaher, E. A. Althoff, A. Zanghellini, O. Dym, S. Albeck, K. N. Houk, D. S. Tawfik, D. Baker, *Nature* **2008**, *453*, 190 – 195; L. Jiang, E. A. Althoff, F. R. Clemente, L. Doyle, D. Rothlisberger, A. Zanghellini, J. L. Gallaher, J. L. Betker, F. Tanaka, C. F. Barbas III, D. Hilvert, K. N. Houk, B. L. Stoddard, D. Baker, *Science* **2008**, *319*, 1387 – 1391; J. B. Siegel, A. Zanghellini, H. M. Lovick, G. Kiss, A. R. Lambert, J. L. St. Clair, J. L. Gallaher, D. Hilvert, M. H. Gelb, B. L. Stoddard, K. N. Houk, F. E. Michael, D. Baker, *Science* **2010**, *329*, 309 – 313.

[16] W. S. Knowles, *Angew. Chem.* **2002**, *114*, 2096 – 2107; *Angew. Chem. Int. Ed.* **2002**, *41*, 1998 – 2007; R. Noyori, *Angew. Chem.* **2002**, *114*, 2108 – 2123; *Angew. Chem. Int. Ed.* **2002**, *41*, 2008 – 2022.

[17] J. W. Yang, M. T. H. Fonseca, N. Vignola, B. List, *Angew. Chem.* **2005**, *117*, 110 – 112; *Angew. Chem. Int. Ed.* **2005**, *44*, 108 – 110.

[18] R. Stürmer, B. Hauer, M. Hall, K. Faber, *Curr. Opin. Chem. Biol.* **2007**, *11*, 203 – 213; C. K. Winkler, G. Tasnadi, D. Clay, M. Hall, K. Faber, *J. Biotechnol.* **2012**, *162*, 381 – 389.

[19] C. E. Paul, S. Gargiulo, D. J. Opperman, I. Lavandera, V. Gotor-Fernandez, V. Gotor, A. Taglieber, I. W. C. E. Arends, F. Hollmann, *Org. Lett.* **2013**, *15*, 180 – 183.

[20] H. S. Toogood, J. M. Gardiner, N. S. Scrutton, *ChemCatChem* **2010**, *2*, 892 – 914.

[21] C. Stückler, T. C. Reiter, N. Baudendistal, K. Faber, *Tetrahedron.* **2010**, *66*, 663 – 667.

[22] K. Durchschein, S. Wallner, P. Macheroux, K. Zangger, W. M. F. Fabian, K. Faber, *ChemBioChem* **2012**, *13*, 2346 – 2351.

[23] G. Tasnádi, C. K. Winkler, D. Clay, M. Hall, K. Faber, *Catal. Sci. Technol.* **2012**, *2*, 1548 – 1552.

[24] K. Durchschein, B. Ferreira-da Silva, S. Wallner, P. Macheroux, W. Kroutil, S. M. Glück, K. Faber, *Green Chem.* **2010**, *12*, 616 – 619.

[25] K. Durchschein, W. M. F. Fabian, P. Macheroux, K. Zangger, G. Trimmel, K. Faber, *Org. Biomol. Chem.* **2011**, *9*, 3364 – 3369.

[26] S. Gargiulo, D. J. Opperman, U. Hanefeld, I. W. C. E. Arends, F. Hollmann, *Chem. Commun.* **2012**, *48*, 6630 – 6632.

[27] M. Schittmayer, A. Glieder, M. K. Uhl, A. Winkler, S. Zach, J. H. Schrittwieser, W. Kroutil, P. Macheroux, K. Gruber, S. Kambourakis, J. D. Rozzell, M. Winkler, *Adv. Synth. Catal.* **2011**, *353*, 268 – 274.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[28] C. Stückler, N. J. Müller, C. K. Winkler, S. M. Glück, K. Gruber, G. Steinkellner, K. Faber, *Dalton Trans.* **2010**, *39*, 8472 – 8476.
[29] A. Fryszkowska, K. Fisher, J. M. Gardiner, G. M. Stephens, *Org. Biomol. Chem.* **2010**, *8*, 533 – 535.
[30] C. K. Winkler, C. Stückler, N. J. Müller, D. Pressnitz, K. Faber, *Eur. J. Org. Chem.* **2010**, 6354 – 6358.
[31] C. Stückler, C. K. Winkler, M. Hall, B. Hauer, M. Bonnekessel, K. Zangger, K. Faber, *Adv. Synth. Catal.* **2011**, *353*, 1169 – 1173; R. Stürmer, B. Hauer, T. Friedrich, K. Faber, M. Hall, P. Macheroux, C. Stückler, (BASF AG), WO 2009074524 A2 20090618, **2009**.
[32] P. Schweiger, H. Gross, S. Wesener, U. Deppenmeier, *Appl. Microbiol. Biotechnol.* **2008**, *80*, 995 – 1006; B. Yin, X. Yang, G. Wei, Y. Ma, D. Wei, *Mol. Biotechnol.* **2008**, *38*, 241 – 245; E. Shinagawa, Y. Ano, O. Adachi, K. Matsushita, *Biosci. Biotechnol. Biochem.* **2008**, *72*, 260 – 264.
[33] N. Richter, H. Gröger, W. Hummel, *Appl. Microbiol. Biotechnol.* **2011**, *89*, 79 – 89.
[34] M. Kraußer, T. Winkler, N. Richter, S. Dommer, A. Fingerhut, W. Hummel, H. Gröger, *ChemCatChem* **2011**, *3*, 293 – 296.
[35] J. Pietruszka, M. Schölzel, *Adv. Synth. Catal.* **2012**, *354*, 751 – 756.
[36] E. Brenna, F. G. Gatti, A. Manfredi, D. Monti, F. Parmeggiani, *Org. Process Res. Dev.* **2012**, *16*, 262 – 268.
[37] X. Chen, X. Gao, Q. Wu, D. Zhu, *Tetrahedron: Asymmetry* **2012**, *23*, 734 – 738.
[38] D. Mangan, I. Miskelly, T. S. Moody, *Adv. Synth. Catal.* **2012**, *354*, 2185 – 2190.
[39] K. Tauber, M. Hall, W. Kroutil, W. M. F. Fabian, K. Faber, S. M. Glück, *Biotechnol. Bioeng.* **2011**, *108*, 1462 – 1467.
[40] D. J. Bougioukou, A. Z. Walton, J. D. Stewart, *Chem. Commun.* **2010**, *46*, 8558 – 8560.
[41] D. J. Bougioukou, S. Kille, A. Taglieber, M. T. Reetz, *Adv. Synth. Catal.* **2009**, *351*, 3287 – 3305.
[42] N. J. Turner, R. Carr, *Biocatalysis in the Pharmaceutical and Biotechnology Industries* (Ed.: R. N. Patel), CRC, Boca Raton, **2007**, pp. 743 – 755.
[43] C.-D. Graf, J. Rieke-Zapp, (Sanofie), WO 2012019967 A1 20120216, **2012**.
[44] N. Turner, *Curr. Opin. Chem. Biol.* **2004**, *8*, 114 – 119
[45] S. Guizzetti, M. Benaglia, *Eur. J. Org. Chem.* **2010**, 5529 – 5541; F. Spindler, H.-U. Blaser, *Transition Metals for Organic Synthesis*, *Vol. 2*, (Eds.: M. Beller, C. Bolm), Wiley-VCH, Weinheim, **2004**, pp. 113 – 123.
[46] R. M. Kellogg, B. Kaptein, T. R. Vries, *Top. Curr. Chem.* **2007**, *269*, 159 – 197.
[47] T. Mita, J. Chen, M. Sugawara, Y. Sato, *Angew. Chem.* **2011**, *123*, 1429 – 1432; *Angew. Chem. Int. Ed.* **2011**, *50*, 1393 – 1396; J. Martens, *ChemCatChem* **2010**, *2*, 379 – 381; S. J. Zünd, M. P. Coughlin, M. P. Lalonde, E. N. Jacobsen, *Nature* **2009**, *461*, 968 – 970; Y. Ohfune, K. Sakaguchi, T. Shinada, *ACS Symp. Ser.* **2009**, *1009*, 57 – 71.
[48] B. A. Arndtsen, *Chem. Eur. J.* **2009**, *15*, 302 – 313; A. J. von Wangelin, H. Neumann, M. Beller, *Top. Organomet. Chem.* **2006**, *18*, 207 – 221; M. Beller, M. Eckert, *Angew. Chem.* **2000**, *112*, 1026 – 1044; *Angew. Chem. Int. Ed.* **2000**, *39*, 1010 – 1027.
[49] A. Luna, I. Alfonso, V. Gotor, *Org. Lett.* **2002**, *4*, 3627 – 3629; F. Messina, M. Botta, F. Corelli, M. P. Schneider, F. Fazio, *J. Org. Chem.* **1999**, *64*, 3767 – 3769; M. I. Youshko, R. F. van, R. A. Sheldon, *Tetrahedron: Asymmetry* **2001**, *12*, 3267 – 3271; K. Ditrich, F. Balkenhohl, W. Ladner, (BASF AG), DE 19534208 A1 19970320, **1997**; F. Balkenhohl, B. Hauer, W. Landner, U. Pressler, (BASF AG), DE 4332738 A1 19950330, **1995**
[50] V. Köhler, K. R. Bailey, A. Znabet, J. Raftery, M. Helliwell, N. J. Turner, *Angew. Chem.* **2010**, *122*, 2228 – 2230; *Angew. Chem. Int. Ed.* **2010**, *49*, 2182 – 2184.
[51] Y. Li, H. Ogola, Y. Sawa, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 503 – 516.
[52] M. J. Abrahamson, E. Vázquez-Figueroa, N. B. Woodall, J. C. Moore, A. S. Bommarius, *Angew. Chem.* **2012**, *124*, 4036 – 4040; *Angew. Chem. Int. Ed.* **2012**, *51*, 3969 – 3972.
[53] C. J. Dunsmore, R. Carr, T. Fleming, N. J. Turner, *J. Am. Chem. Soc.* **2006**, *128*, 2224 – 2225.
[54] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* **2010**, *28*, 324 – 332.
[55] M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2009**, *1*, 42 – 51.
[56] D. Zhu, L. Hua, *Biotechnol. J.* **2009**, *4*, 1420 – 1431.
[57] L. Poppe, J. Rétey, *Angew. Chem.* **2005**, *117*, 3734 – 3754; *Angew. Chem. Int. Ed.* **2005**, *44*, 3668 – 3688; N. J. Turner, *Curr. Opin. Chem. Biol.* **2011**, *15*, 234 – 240.
[58] Y. Asano, Y. Kato, C. Levy, P. Baker, D. Rice, *Biocatal. Biotransform.* **2004**, *22*, 133 – 140.
[59] M. J. MacDonald, G. B. D'Cunha, *Biochem. Cell Biol.* **2007**, *85*, 273 – 282.
[60] L. H. Andrade, A. V. Silva, P. Milani, D. Koszelewski, W. Kroutil, *Org. Biomol. Chem.* **2010**, *8*, 2043 – 2051.
[61] N. J. Turner, *Curr. Opin. Chem. Biol.* **2010**, *14*, 115 – 121; M. S. Malik, E.-S. Park, J.-S. Shin, *Appl. Microbiol. Biotechnol.* **2012**, *94*, 1163 – 1171.
[62] S. Mathew, H. Yun, *ACS Catal.* **2012**, *2*, 993 – 1001.
[63] N. J. Turner, M. D. Truppo, *Chiral Amine Synthese* (Ed.: T. C. Nugent), Wiley-VCH, Weinheim, **2010**, pp 431 – 459.
[64] D. Koszelewski, D. Clay, K. Faber, W. Kroutil, *J. Mol. Catal. B* **2009**, *60*, 191 – 194.
[65] D. Koszelewski, D. Clay, D. Rozzell, W. Kroutil, *Eur. J. Org. Chem.* **2009**, 2289 – 2292.
[66] F. G. Mutti, W. Kroutil, *Adv. Synth. Catal.* **2012**, *354*, 3409 – 3413.
[67] M. Höhne, S. Kühl, K. Robins, U. T. Bornscheuer, *ChemBioChem* **2008**, *9*, 363 – 365; H. Yun, B.-G. Kim, *Biosci. Biotechnol. Biochem.* **2008**, *72*, 3030 – 3033.
[68] D. Koszelewski, M. Goeritzer, D. Clay, B. Seisser, W. Kroutil, *ChemCatChem* **2010**, *2*, 73 – 77; D. Koszelewski, I. Lavandera, D. Clay, G. M. Gübitz, D. Rozzell, W. Kroutil, *Angew. Chem.* **2008**, *120*, 9477 – 9480; *Angew. Chem. Int. Ed.* **2008**, *47*, 9337 – 9340.
[69] M. D. Truppo, N. J. Turner, J. D. Rozzell, *Chem. Commun.* **2009**, 2127 – 2129.
[70] B. Wang, H. Land, P. Berglund, *Chem. Commun.* **2013**, *49*, 161 – 163.
[71] A. Iwasaki, Y. Yamada, N. Kizaki, Y. Ikenaka, J. Hasegawa, *Appl. Microbiol. Biotechnol.* **2006**, *69*, 499 – 505.
[72] M. Höhne, S. Schätzle, H. Jochens, K. Robins, U. T. Bornscheuer, *Nat. Chem. Biol.* **2010**, *6*, 807 – 813; M. Höhne, U. Bornscheuer, K. Robins, S. Schätzle, (Lonza AG), WO 2011026556 A1 20110310, **2011**.
[73] I. Lavandera, D. Koszelewski, W. Kroutil (Angewandte Biokatalyse Kompetenzzentrum GmbH), AT 507050 A1 20100115, **2010**.
[74] D. Koszelewski, D. Pressnitz, D. Clay, W. Kroutil, *Org. Lett.* **2009**, *11*, 4810 – 4812.
[75] R. C. Simon, B. Grischek, F. Zepeck, A. Steinreiber, F. Belaj, W. Kroutil, *Angew. Chem.* **2012**, *124*, 6817 – 6820; *Angew. Chem. Int. Ed.* **2012**, *51*, 6713 – 6716.
[76] K. Tauber, M. Fuchs, J. H. Sattler, J. Pitzer, D. Pressnitz, D. Koszelewski, K. Faber, J. Pfeffer, T. Haas, W. Kroutil, *Chemistry* **2013**, *19*, 4030 – 4035.
[77] J. H. Sattler, M. Fuchs, K. Tauber, F. G. Mutti, K. Faber, J. Pfeffer, T. Haas, W. Kroutil, *Angew. Chem.* **2012**, *124*, 9290 – 9293; *Angew. Chem. Int. Ed.* **2012**, *51*, 9156 – 9159.
[78] M. Fuchs, D. Koszelewski, K. Tauber, W. Kroutil, K. Faber, *Chem. Commun.* **2010**, *46*, 5500 – 5502.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[79] S. Lutz, *Science* **2010**, *329*, 285 – 287; C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, P. N. Devine, G. W. Huisman, G. J. Hughes, *Science* **2010**, *329*, 305 – 309; F. G. Mutti, J. Sattler, K. Tauber, W. Kroutil, *ChemCatChem* **2011**, *3*, 109 – 111; A. A. Desai, *Angew. Chem.* **2011**, *123*, 2018 – 2020; *Angew. Chem. Int. Ed.* **2011**, *50*, 1974 – 1976; C. Savile, E. Mundorff, J. C. Moore, P. N. Devine, J. M. Janey, (Codexis Inc.), WO 2010099501 A2 20100902, **2010**; G. Hughes, P. N. Devine, F. J. Fleitz, B. T. Grau, J. Limanto, C. Savile, (Codexis Inc.), WO 2011005477 A1 20100113, **2011**.

[80] J. Rudat, B. Brucher, C. Syldatk, *AMB Express* **2012**, *2*, 11 – 21.

[81] E. Park, M. Kim, J.-S. Shin, *Adv. Synth. Catal.* **2010**, *352*, 3391 – 3398.

[82] B.-K. Cho, H. J. Cho, S.-H. Park, H. Yun, B.-G. Kim, *Biotechnol. Bioeng.* **2003**, *81*, 783 – 789; B.-K. Cho, H. J. Cho, H. Yun, B.-G. Kim, *J. Mol. Catal. B* **2003**, *26*, 273 – 285.

[83] C. U. Ingram, M. Bommer, M. E. B. Smith, P. A. Dalby, J. M. Ward, H. C. Hailes, G. J. Lye, *Biotechnol. Bioeng.* **2007**, *96*, 559 – 569; M. E. B. Smith, B. H. Chen, E. G. Hibbert, U. Kaulmann, K. Smithies, J. L. Galman, F. Baganz, P. A. Dalby, H. C. Hailes, G. J. Lye, J. M. Ward, J. M. Woodley, M. Micheletti, *Org. Process Res. Dev.* **2010**, *14*, 99 – 107.

[84] H.-S. Bea, S.-H. Lee, H. Yun, *Biotechnol. Bioprocess Eng.* **2011**, *16*, 291 – 296.

[85] Y. Asano, Y. Kato, C. Levy, P. Baker, D. Rice, *Biocatal. Biotransform.* **2004**, *22*, 133 – 140; L. Poppe, C. Paizs, K. Kovács, F.-D. Irimie, B. Vértessy, L. Pollegioni, S. Servi *in Methods in Molecular Biology*, *Vol. 794*, Humana, New York, **2012**, pp. 3 – 19.

[86] M. de Villiers, V. Puthan Veetil, H. Raj, J. de Villiers, G. J. Poelarends, *ACS Chem. Biol.* **2012**, *7*, 1618 – 1628.

[87] J. C. Calabrese, D. B. Jordan, A. Boodhoo, S. Sariaslani, T. Vannelli, *Biochemistry* **2004**, *43*, 11403 – 11416.

[88] H. Ritter, G. E. Schulz, *Plant Cell* **2004**, *16*, 3426 – 3436; T. F. Schwede, J. Rétey, G. E. Schulz, *Biochemistry* **1999**, *38*, 5355 – 5361.

[89] M. Baedeker, G. E. Schulz, *Eur. J. Biochem.* **2002**, *269*, 1790 – 1797.

[90] K. R. Hanson, E. A. Havir, *Arch. Biochem. Biophys.* **1970**, *141*, 1 – 17; B. Schuster, J. Rétey, *Proc. Natl. Acad. Sci. USA* **1995**, *92*, 8433 – 8437; M. Langer, A. Pauling, J. Rétey, *Angew. Chem.* **1995**, *107*, 1585 – 1587; *Angew. Chem. Int. Ed. Engl.* **1995**, *34*, 1464 – 1465.

[91] S. Bartsch, U. T. Bornscheuer, *Angew. Chem.* **2009**, *121*, 3412 – 3415; *Angew. Chem. Int. Ed.* **2009**, *48*, 3362 – 3365.

[92] R. R. Fritz, D. S. Hodgins, C. W. Abell, *J. Biol. Chem.* **1976**, *251*, 4646 – 4650.

[93] G. B. D'Cunha, V. Satyanarayan, P. M. Nair, *Enzyme Microb. Technol.* **1994**, *16*, 318 – 322; A. Gloge, B. Langer, L. Poppe, J. Rétey, *Arch. Biochem. Biophys.* **1998**, *359*, 1 – 7; A. Gloge, J. Zon, A. Kovari, L. Poppe, J. Rétey, *Chem. Eur. J.* **2000**, *6*, 3386 – 3390; C. Paizs, A. Katona, J. Rétey, *Eur. J. Org. Chem.* **2006**, 1113 – 1116.

[94] M. Wubbolts in *Enzyme Catalysis in Organic Synthesis*: *A Comprehensive Handbook*, 2nd ed. (Eds.: K. Daruz, H. Waldmann), Wiley-VCH, Weinheim, **2008**, pp. 686 – 697; A. Liese, K. Seelbach, A. Buchholz, J. Haberland, *Industrial Biotransformations* (Eds.: A. Liese, K. Seelbach, C. Wandrey), Wiley-VCH, Weinheim, **2006**, pp. 95 – 392.

[95] B. Weiner, G. J. Poelarends, D. B. Janssen, B. L. Feringa, *Chem. Eur. J.* **2008**, *14*, 10094 – 10100.

[96] N. P. Botting, M. Akhtar, M. A. Cohen, D. Gani, *Biochemistry* **1988**, *27*, 2953 – 2955.

[97] M. Akhtar, N. P. Botting, M. A. Cohen, D. Gani, *Tetrahedron* **1987**, *43*, 5899 – 5908; M. S. Gulzar, M. Akhtar, D. Gani, *J. Chem. Soc. Perkin Trans. 1* **1997**, 649 – 656.

[98] H. Raj, W. Szymanski, J. de Villiers, H. J. Rozeboom, V. P. Veetil, C. R. Reis, M. de Villiers, F. J. Dekker, S. de Wildeman, W. J. Quax, A.-M. W. H. Thunnissen, B. L. Feringa, D. B. Janssen, G. J. Poelarends, *Nat. Chem.* **2012**, *4*, 478 – 484.

[99] H. Raj, B. Weiner, V. P. Veetil, C. R. Reis, W. J. Quax, D. B. Janssen, B. L. Feringa, G. J. Poelarends, *ChemBioChem* **2009**, *10*, 2236 – 2245.

[100] B. de Lange, D. J. Hyett, P. J. D. Maas, D. Mink, F. B. J. van Assema, N. Sereinig, A. H. M. de Vries, J. G. de Vries, *ChemCatChem* **2011**, *3*, 289 – 292.

[101] B. Wu, W. Szymanski, P. Wietzes, S. de Wildeman, G. J. Poelarends, B. L. Feringa, D. B. Janssen, *ChemBioChem* **2009**, *10*, 338 – 344.

[102] C. L. Steele, Y. Chen, B. A. Dougherty, W. Li, S. Hofstead, K. S. Lam, Z. Xing, S.-J. Chiang, *Arch. Biochem. Biophys.* **2005**, *438*, 1 – 10; W. Mutatu, K. L. Klettke, C. Foster, K. D. Walker, *Biochemistry* **2007**, *46*, 9785 – 9794.

[103] S. D. Christenson, W. Liu, M. D. Toney, B. Shen, *J. Am. Chem. Soc.* **2003**, *125*, 6062 – 6063; S. D. Christenson, W. Wu, M. A. Spies, B. Shen, M. D. Toney, *Biochemistry* **2003**, *42*, 12708 – 12718.

[104] N. D. Ratnayake, U. Wanninayake, J. H. Geiger, K. D. Walker, *J. Am. Chem. Soc.* **2011**, *133*, 8531 – 8533; L. Feng, U. Wanninayake, S. Strom, J. Geiger, K. D. Walker, *Biochemistry* **2011**, *50*, 2919 – 2930.

[105] B. J. V. Verkuijl, A. J. Minnaard, J. G. de Vries, B. L. Feringa, *J. Org. Chem.* **2009**, *74*, 6526 – 6533.

[106] B. J. V. Verkuijl, W. Szymanski, B. Wu, A. J. Minnaard, D. B. Janssen, J. G. de Vries, B. L. Feringa, *Chem. Commun.* **2010**, *46*, 901 – 903.

[107] B. Wu, W. Szymanski, G. G. Wybenga, M. M. Heberling, S. Bartsch, S. de Wildeman, G. J. Poelarends, B. L. Feringa, B. W. Dijkstra, D. B. Janssen, *Angew. Chem.* **2012**, *124*, 497 – 501; *Angew. Chem. Int. Ed.* **2012**, *51*, 482 – 486.

[108] B. Wu, W. Szymanski, S. de Wildeman, G. J. Poelarends, B. L. Feringa, D. B. Janssen, *Adv. Synth. Catal.* **2010**, *352*, 1409 – 1412.

[109] B. M. Cox, J. B. Bilsborrow, K. D. Walker, *J. Org. Chem.* **2009**, *74*, 6953 – 6959.

[110] N. Bala, S. S. Chimni, *Tetrahedron: Asymmetry* **2010**, *21*, 2879 – 2898; E. J. de Vries, D. B. Janssen, *Curr. Opin. Biotechnol.* **2003**, *14*, 414 – 420; E. Y. Lee, M. L. Shuler, *Biotechnol. Bioeng.* **2007**, *98*, 318 – 327; M. Widersten, A. Gurell, D. Lindberg, *Biochim. Biophys. Acta Gen. Subj.* **2010**, *1800*, 316 – 326; M. Kotik, A. Archelas, R. Wohlgemuth, *Curr. Org. Chem.* **2012**, *16*, 451 – 482.

[111] M. Kotik, V. Stepanek, M. Grulich, P. Kyslik, A. Archelas, *J. Mol. Catal. B* **2010**, *65*, 41 – 48.

[112] B. Hauer, (BASF AG), WO 2007099107 A1 20070907, **2007**; J. H. Lutje Spelberg, D. B. Janssen, (Rijsksuniversiteie te Groningen), EP 1158054 A1 20011128, **2001**; D. Mink, J. H. Lutje Spelberg, E. J. de Vries, (DSM IP Assets B.V.), WO 2007137816 A1 20071206, **2007**; D. Mink, S. J. H. Lutje, E. J. de Vries (DSM IP Assets B.V.), WO 2007128469 A1 20071115, **2007**.

[113] Q. Q. Hou, X. Sheng, J. H. Wang, Y. J. Liu, C. B. Liu, *Biochim. Biophys. Acta Proteins Proteomics* **2012**, *1824*, 263 – 268.

[114] S. Barth, M. Fischer, R. D. Schmid, J. Pleiss, *Bioinformatics* **2004**, *20*, 2845 – 2847; A. J. Fretland, C. J. Omiecinski, *Chem.-Biol. Interact.* **2000**, *129*, 41 – 59; B. van Loo, J. Kingma, M. Arand, M. G. Wubbolts, D. B. Janssen, *Appl. Environ. Microbiol.* **2006**, *72*, 2905 – 2917.

[115] W. Krenn, I. Osprian, W. Kroutil, G. Braunegg, K. Faber, *Biotechnol. Lett.* **1999**, *21*, 687 – 690; M. Mischitz, W. Kroutil, U. Wandel, K. Faber, *Tetrahedron: Asymmetry* **1995**, *6*, 1261 – 1272; I. Osprian, W. Kroutil, M. Mischitz, K. Faber, *Tetrahedron: Asymmetry* **1997**, *8*, 65 – 71.

[116] A.-L. Bottalla, M. Ibrahim-Ouali, M. Santelli, R. Furstoss, A. Archelas, *Adv. Synth. Catal.* **2007**, *349*, 1102 – 1110.

 © 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim

[117] N. Monfort, A. Archelas, R. Furstoss, *Tetrahedron* **2004**, *60*, 601 – 605.
[118] L. Zhao, B. Han, Z. Huang, M. Miller, H. Huang, D. S. Malashock, Z. Zhu, A. Milan, D. E. Robertson, D. P. Weiner, M. J. Burk, *J. Am. Chem. Soc.* **2004**, *126*, 11156 – 11157.
[119] H. Zheng, M. T. Reetz, *J. Am. Chem. Soc.* **2010**, *132*, 15744 – 15751.
[120] M. T. Reetz, D. Kahakeaw, R. Lohmer, *ChemBioChem* **2008**, *9*, 1797 – 1804.
[121] H. Zheng, D. Kahakeaw, J. P. Acevedo, M. T. Reetz, *ChemCatChem* **2010**, *2*, 958 – 961.
[122] E. N. Jacobsen, *Acc. Chem. Res.* **2000**, *33*, 421 – 431.
[123] R. M. de Jong, J. J. W. Tiesinga, H. J. Rozeboom, K. H. Kalk, L. Tang, D. B. Janssen, B. W. Dijkstra, *EMBO J.* **2003**, *22*, 4933 – 4944; R. M. de Jong, J. J. W. Tiesinga, A. Villa, L. Tang, D. B. Janssen, B. W. Dijkstra, *J. Am. Chem. Soc.* **2005**, *127*, 13338 – 13343.
[124] J. H. L. Spelberg, L. Tang, R. M. Kellogg, D. B. Janssen, *Tetrahedron: Asymmetry* **2004**, *15*, 1095 – 1102; J. H. L. Spelberg, L. Tang, M. van Gelder, R. M. Kellogg, D. B. Janssen, *Tetrahedron: Asymmetry* **2002**, *13*, 1083 – 1089.
[125] G. Hasnaoui-Dijoux, M. M. Elenkov, J. H. L. Spelberg, B. Hauer, D. B. Janssen, *ChemBioChem* **2008**, *9*, 1048 – 1051.
[126] W. Szymanski, C. P. Postema, C. Tarabiono, F. Berthiol, L. Campbell-Verduyn, S. de Wildeman, J. G. de Vries, B. L. Feringa, D. B. Janssen, *Adv. Synth. Catal.* **2010**, *352*, 2111 – 2115; L. S. Campbell-Verduyn, W. Szymanski, C. P. Postema, R. A. Dierckx, P. H. Elsinga, D. B. Janssen, B. L. Feringa, *Chem. Commun.* **2010**, *46*, 898 – 900.
[127] R. M. Haak, F. Berthiol, T. Jerphagnon, A. J. A. Gayet, C. Tarabiono, C. P. Postema, V. Ritleng, M. Pfeffer, D. B. Janssen, A. J. Minnaard, B. L. Feringa, J. G. de Vries, *J. Am. Chem. Soc.* **2008**, *130*, 13508 – 13509.
[128] S. K. Ma, J. Gruber, C. Davis, L. Newman, D. Gray, A. Wang, J. Grate, G. W. Huisman, R. A. Sheldon, *Green Chem.* **2010**, *12*, 81 – 86.

© 2014 Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim