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MINI-REVIEW

Biocatalytic synthesis of optically active tertiary alcohols

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Abstract The enzymatic preparation of optically pure tertiary alcohols under sustainable conditions has received much attention. The conventional chemical synthesis of these valuable building blocks is still hampered by the use of harmful reagents such as heavy metal catalysts. Successful examples in biocatalysis used esterases, lipases, epoxide hydrolases, halohydrin dehalogenases, thiamine diphosphate-dependent enzymes, terpene cyclases, -acetylases, and -dehydratases. This mini-review provides an overview on recent developments in the discovery of new enzymes, their functional improvement by protein engineering, the design of chemoenzymatic routes leading to tertiary alcohols, and the discovery of entirely new biotransformations.

Keywords Tertiary alcohols · Esterase · Enantioselectivity · Protein engineering · Enzyme catalysis

Introduction

The synthesis of chiral tertiary alcohols (TAs) is still a challenging task for the organic chemist. Despite considerable progress, a standard method for the synthesis of the large number of bioactive compounds bearing a quaternary center has not been found yet. Impressive research effort has been dedicated to the development of catalytic methods, using chiral auxiliaries, ligands, and catalysts (Hasegawa et al. 2010; Sonawane et al. 2011). A very interesting recent development is the conversion of optically pure secondary alcohols to their tertiary counterparts by addition of chiral carbenoids to boron reagents. Conditions and reagents determine whether the reaction proceeds by inversion or retention of the stereocenter (Stymiest et al. 2008). For a comprehensive overview over the chemical synthesis by catalytic formation of quaternary stereocenters, including tertiary alcohols, the reader is referred to recent excellent articles and a book (Christoffers and Baro 2005; Cozzi et al. 2007; Seebach 2011).

Despite the impressive progress, chemical approaches often suffer from the application of highly toxic reagents, the use of elevated or very low temperatures. While these conditions are suitable for the lab bench, large-scale applications require reactions that should have no harmful impact on the environment. Sustainable strategies for the preparation of optically pure TAs would thus be highly desirable, as this functional group is present in a large number of natural products and can serve as building block for valuable pharmaceutical compounds (Cozzi et al. 2007). For instance, linalool is a widely used fragrance product for perfumes, soaps, and detergents, and is applied in the hopping of beer (Crowell et al. 2002). The large number of pharmaceutical products includes efavirenz, used as treatment of HIV, and intermediates for the semisynthetic production of taxol, one of the most efficient cytostatic anticancer drugs of today (Hou and Adachi 2005).

Enzymes thus might contribute sustainable methods for the production of optically pure TAs. Recently,

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considerable progress has been made in the biocatalytic or biotransformation-based preparation of these sterically demanding substrates. This includes the expansion of the catalytic scope of known biocatalysts by protein engineering or the discovery of novel enzymes and biotransformation routes. This review first gives a short outline of recent trends and developments using rather mature enzymatic methods, mostly with α/β-hydrolase fold enzymes. Second, examples with novel catalytic principles recently discovered in nature will be presented with a particular focus on successful examples for the optimization of these biocatalysts by protein engineering. In particular, enzymatic addition of water to C–C double bonds in terpenes and C–C bond-forming reactions such as carboligation or cyclization have recently been discovered as very promising methods for the production of optically pure tertiary alcohols.

Synthesis of chiral tertiary alcohols using isolated enzymes

The most frequently used enzymatic route for the preparation of tertiary alcohols is the kinetic resolution of racemates catalyzed by hydrolases such as esterases (Kourist et al. 2008a), lipases (Bosley et al. 1994; O'Hagan and Zaidi 1992), proteases (Holt et al. 2007), halohydrin dehalogenases (Majeric-Elenkov et al. 2007) or epoxide hydrolases (Steinreiber and Faber 2001). In the case of primary or secondary alcohols, the ketoreductase-catalyzed desymmetrizations of prostereogenic substrates are often preferred over kinetic resolution because it offers 100% conversion, whereas a separation of enantiomers from a racemic mixture has a maximal yield of 50%. However, a ketone needed as precursor of a tertiary alcohol simply cannot exist (the central carbon atom can have only four bonds!), which makes the kinetic resolution still a competitive reaction. The use of hydrolases for the preparation of tertiary alcohols has been reviewed recently (Kourist et al. 2008a).

Esterases and lipases

The application of esterases or lipases for the kinetic resolution of tertiary alcohols was initially hampered by the fact that most of the commercially available enzymes did not accept TA as substrates, which made the resolution of remote centers or activated substrates the preferred method for the preparation of tertiary alcohols (O'Hagan and Zaidi 1992; Pogorevc and Faber 2000). For instance, in a study using 25 commercially available esterases and lipases, only lipase from *Candida rugosa* and lipase A from *Candida antarctica* showed significant activity (Henke et al. 2002). An analysis of structure–function relationships of the active site region of an esterase revealed the molecular basis for this limitation. The activity towards tertiary alcohols could be associated with the composition of the oxyanion hole, the binding site that stabilizes the tetrahedral intermediate in the hydrolysis of esters. In most lipases and esterases, a highly conserved glycine, followed by a bulky hydrophobic residue (denoted as X), is present. The carbonyl group of the peptide backbone of this so-called GX motif points towards the alcohol moiety of the tetrahedral intermediate and prevents binding of substrates bearing three substituents on the alcohol atom (Fig. 1).

Several subclasses of the α/β-hydrolase fold superfamily, however, have a wider active site with a conserved motif containing three glycines or two glycines and an alanine followed by a bulky residue X (Pleiss et al. 2000). Enzymes belonging to this so-called GGG(A)X family can be found among the subfamilies with similarity to acetylcholine esterase and hormone-sensitive lipase-like enzymes (Kourist et al. 2010a, b). Indeed, it could be then experimentally verified that lipases and esterases of the GGG(A)X class are able to hydrolyse esters of tertiary alcohols with moderate to good activity. Successful examples include enzymes from animals such as pig liver esterase or an acetylcholine esterase from the snake bander krait (Henke et al. 2002), fungi (Henke et al. 2002), gram-positive or gram-negative bacteria (Herter et al. 2011; Kourist et al. 2007a), archaea (Hotta et al. 2002), and from noncultivatable (metagenome-derived) microorganisms (Kourist et al. 2007b, 2008a; Rashamuse et al. 2009). The observation that the GGG(A)X motif determines activity of esterases or lipases towards tertiary alcohols hence substantially facilitated the discovery of further enzymes.

It should be noted, however, that hydrolytic activity towards tertiary alcohols is not exclusively restricted to GGG(A)X enzymes. The GX-type lipase from *Burkholderia cepacia* does not convert arylaliphatic tertiary alcohols, but is active and highly enantioselective towards tertiary cyanohydrins (Holt et al. 2007), which however can be related to the rather unstable nature of these compounds. A remarkable example for an active enzyme from the natural pool is lipase A from *C. antarctica* (CAL-A). CAL-A displays unique catalytic properties and has been used in many different applications (Dominguez de Maria et al. 2005). The protein sequence and the three-dimensional structure of CAL-A differ considerably from that of other known α/β-hydrolase fold enzymes (Ericsson et al. 2008). It was suggested that an aspartic acid residue contributes to the stabilization of the intermediate oxyanion hole and that the enzyme cannot be classified to belong to the GX or the GGG(A)X class, but rather represents an entirely different configuration (Fig. 1). Two enzymes from *Ustilago maydis* and *Kluyveromyces lactis* have been recently discovered to

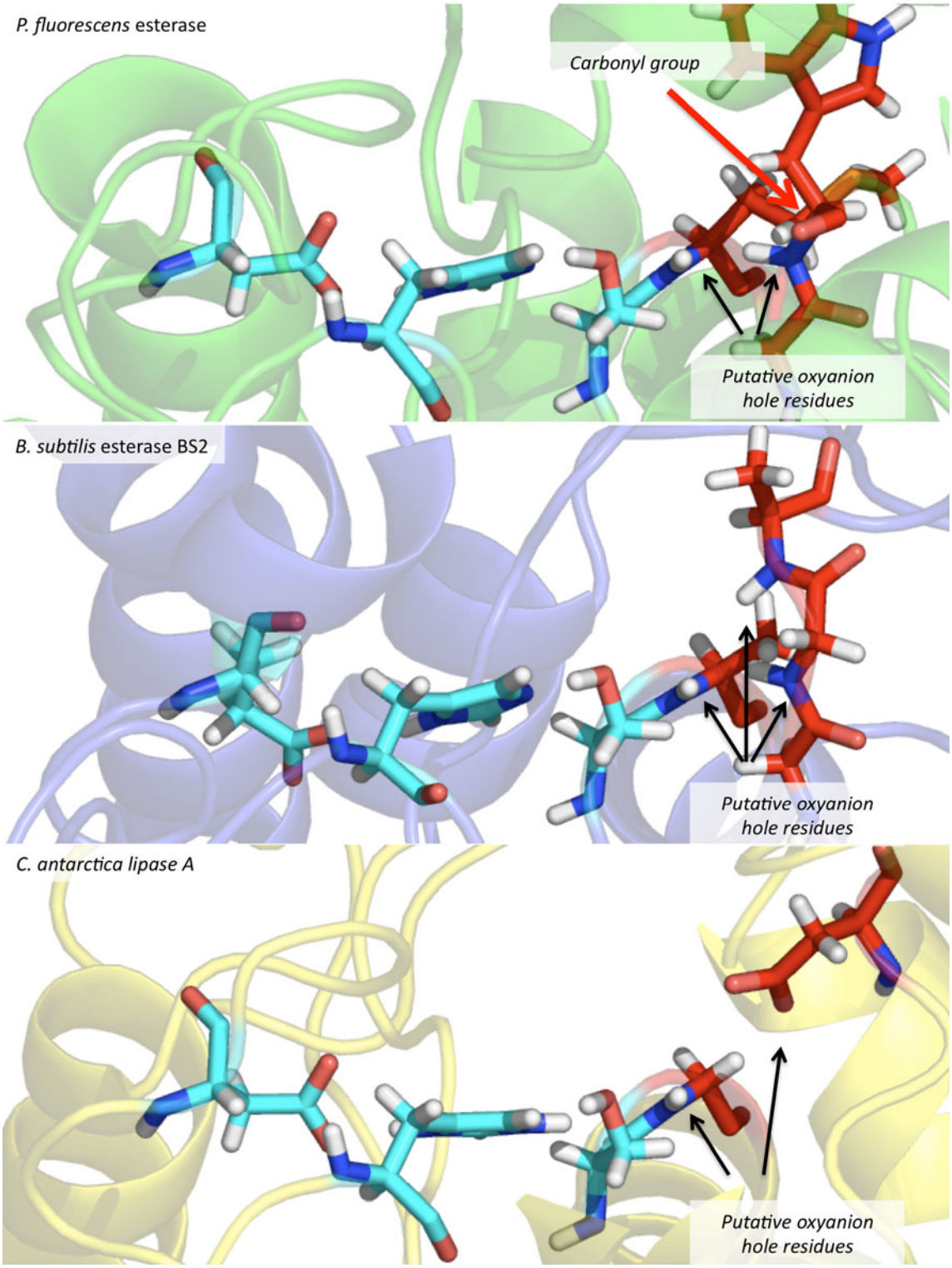


Fig. 1 In the conversion of tertiary alcohols by carboxyl hydrolases, the carboxyl group O atom of the ester is fixed by hydrogen bonds in to the oxyanion hole residues (highlighted as *red sticks*). Consequently, this structural element plays a decisive role both in the activity and the enantiorecognition towards TAs. Enzymes bearing a so-called GX motif in the oxyanion hole (*P. fluorescens* esterase, pdb-entry 3IA2, *top*) do not convert TAs, which has been explained by a backbone carbonyl group (highlighted with a *red arrow*) that prevents binding of the third substituent of the TA. Enzymes from the GGG(A)X type such as *B. subtilis* esterase (pdb-entry 1QE3, *center*) and CAL-A (pdb-entry 2VEO, *bottom*) bearing an aspartate in the oxyanion hole can convert TAs

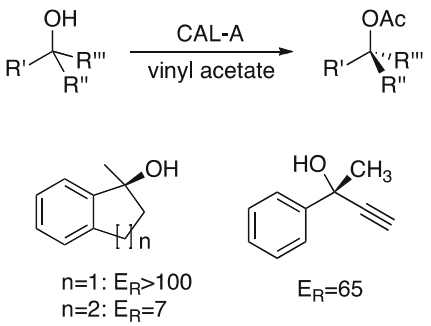
be similar to CAL-A, but their activity towards tertiary alcohols still needs to be verified (Ericsson et al. 2008).

Activity and enantioselectivity of CAL-A towards tertiary alcohols are excellent. CAL-A has been used in the synthesis of *tert*-butyl oleate (Bosley et al. 1994) and chiral tertiary alcohol esters through transesterification in organic media (Scheme 1). Krishna et al. (2002) applied immobilized CAL-A and the thermostable esterase from *Pyrobaculus calidifontis* for the resolution of a tertiary alcohol with good enantioselectivity. The choice of the solvent and the water activity played an important role for the optimization of the conditions. In 2008, Özdemirhan et al. used CAL-A as cross-linked enzyme aggregates to prepare aromatic ring fused cyclic tertiary alcohols by transesterification, giving rise to the products in good optically purity (up to 91%ee) at 25% conversion (Özdemirhan et al. 2008). In contrast, the stability of most esterases in organic solvent is usually poor, making hydrolysis the reaction of choice.

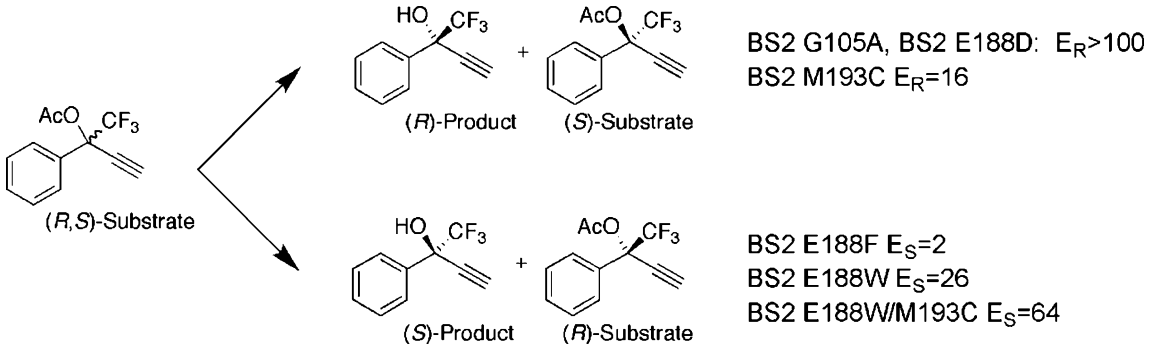
Microorganisms are a promising source for enzymes active and enantioselective towards tertiary alcohols. Herter et al. recently isolated bacterial strains by an enrichment and screening strategy, which led to the isolation of 14 strains with the ability to utilize *tert*-butyl acetate as sole carbon source. Five of these strains belong to gram-negative cell-wall type microorganisms. This is notable as most other bacterial esterases with activity towards tertiary alcohols have been isolated from gram-positive species. Several of the strains had excellent enantioselectivity in kinetic resolution experiments (with *E* values up to 70), some showed even inverse enantiopreference (Herter et al. 2011). Paravidino et al. (2010) demonstrated the catalytic potential of fungal mycelia and cell-bound hydrolases when they showed that several enzymes had good activity and reasonable selectivity (with *E* values up to 40) in the kinetic resolution of tertiary cyanohydrins.

Protein engineering has emerged as a powerful tool for the generation of improved biocatalysts (Lutz and Bornscheuer 2008) and also has helped to substantially expand the catalytic toolbox for the synthesis of optically pure tertiary alcohols. α/β-Hydrolase fold enzymes often combine a broad substrate spectrum with high enantioselectivity. In addition, they are characterized by a high "plasticity," e.g., the substrate recognition and product formation can be influenced to a large extent by the exchange of amino acids in the active site region (Kourist et al. 2010a, b). An early attempt using directed evolution of esterases from *Bacillus stearothermophilus* or *Pseudomonas fluorescens* by simple error-prone PCR to create activity for the conversion of tertiary alcohols failed (Henke et al. 2002). It was proposed that the configuration of the oxyanion hole in these GX hydrolases by backbone residues prevents activity towards tertiary alcohols and cannot be introduced by just a few randomly inserted amino acid exchanges. A sequence-guided approach using GGG(A)X enzymes proved to be more successful as Bassegoda et al. could strongly increase the activity of an esterase from *Paenibacillus barcinonensis* (Bassegoda et al. 2010). Despite its structural similarity to GGG(A)X enzymes, the activity towards tertiary alcohols of this esterase was poor. An analysis of the active site revealed that the enzyme belonged to the GGG(A)X type, but bears a serine in the third position. Back mutation to the common GGG-X motif increased activity. A systematic variation of the GGG(A)X motif in this esterase from *P. barcinonensis* (Bassegoda et al. 2010) and pig liver esterase (Gall et al. 2010) showed that some exchanges to alanine in the first and third position (leading to AGGX, AGAX, and GGAX motifs) are tolerated, while the second glycine appears to be crucial to maintain activity towards tertiary alcohols.

Also by rational protein design, the first glycine has been shown to be a key residue strongly affecting enantioselectivity in esterase BS2 from *Bacillus subtilis* (Heinze et al. 2007; Henke et al. 2003) as replacement with alanine increased the *E* value (WT, E=42; G105A, E >100). Despite only moderate identity of 49% to the esterase from *P. barcinonensis*, the same effect was observed for the latter (Bassegoda et al. 2010), underlining the significance of the first glycine for the stereodiscrimination. A glutamate in the N-terminal position of BS2 was identified as another key residue for enantioselectivity and its replacement by an Asp residue also substantially improved the *E* value (WT, E=42; E188D, E>100; Heinze et al. 2007). The most striking achievement was made by simultaneous saturation mutagenesis of position E188 and two neighboring amino acids, which lead to a complete inversion of the enantioselectivity of BS2 for one double mutant identified after high-throughput screening of approx. 5,000 variants (Bartsch et al. 2008, Scheme 2). Whereas the wild-type favored the (*R*) enantiomer with an E>100, the best double mutant favored the (*S*) enantiomer with E=64. Interestingly, the corresponding single mutants either showed low (*R*) or low (*S*) enantiopreference and



Scheme 1 Examples for the lipase-catalyzed transesterification of tertiary alcohols (Krishna et al. 2002; Özdemirhan et al. 2008)

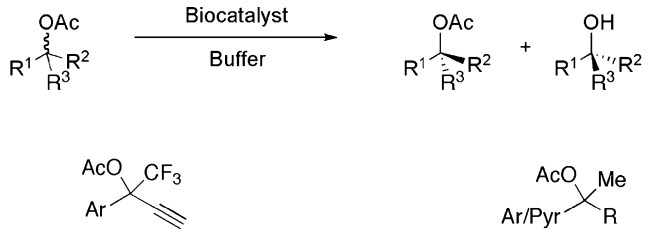


Scheme 2 Complete inversion of the enantioselectivity of esterase BS2 from *B. subtilis* by simultaneous saturation mutagenesis (Bartsch et al. 2008)

hence only the simultaneous saturation mutagenesis applied in the protein engineering approach could lead to the discovery of this double mutant with desired enantiopreference and high enantioselectivity. Thus, molecular modeling methods in combination with high-throughput screening proved to be useful for the identification of key residues and drastic changes in selectivity.

Until recently, most of the studies for the esterase/lipase-catalyzed synthesis of tertiary alcohols had been restricted to a few model substrates. We have then studied the substrate scope of these enzymes in broad detail and successful examples include a range of aryl- and pyridylaliphatic tertiary alcohols (Scheme 3), which were converted with excellent enantioselectivity (Kourist et al. 2007a;

Scheme 3 Examples for the kinetic resolution of tertiary alcohol acetates using recombinant esterases, commercially available proteases/lipases, and fungal/bacterial wild-type whole-cell biocatalysts



Ar = Ph; *p*-Me-C_6H_4; *p*-Cl-C_6H_4; *p*-F-C_6H_4; *m*-F-C_6H_4; *m,p*-bi-F-C_6H_4; 2-thienyl.

E from 12 to >100

Esterase BS2 variants; Est34 (from metagenome)

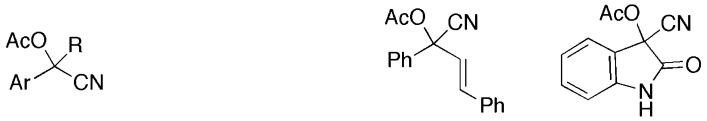
Kourist et al. 2007a,b

Ar/Pyr = Ph, 2-pyr; 3-pyr; 4-pyr.
R = Ethinyl, ethenyl, CN

E from 51 to 100

Pig liver esterase, esterase BS2, Est8 (from metagenome)
whole-cell biocatalysts

Nguyen et al. 2010, Herter et al. 2011



Ar = Ph; *p*-MeO-C_6H_4; *p,m*-bi-MeO-C_6H_4; 2-thienyl.
R = Me; Et.

E from 7 to 58

Subtilisine A, *Candida rugosa* lipase, *Burkholderia cepacia* lipase

Holt et al. 2007

E = 2.4 E=42

Mycelia from *Aspergillus oryzae*; *Kluyveromyces marxianus*.

Paravidino et al. 2010

Nguyen et al. 2010). Despite the high structural similarity of tertiary cyanohydrins to arylaliphatic tertiary alcohols, the enantioselectivity of GGG(A)X hydrolases in their resolution was mostly poor (Nguyen et al. 2010; Wiggers et al. 2009). This striking observation has been attributed to the ability of cyanohydrins to act as hydrogen-bond donor, which can have a strong effect on the substrate recognition in the active site. Nevertheless, the resolution of tertiary cyanohydrins was successfully accomplished by applying serine proteases (Holt et al. 2007) and fungal mycelia containing esterases biocatalysts (Paravidino et al. 2010) with excellent enantioselectivity. Enzymatic synthesis of cyanohydrins is often hampered by the low stability of the products in aqueous solution. Hence, only the remaining starting compounds (e.g., the cyanohydrin esters) can be isolated, which reduces the overall efficiency of the process beside the risk that highly toxic HCN can be formed. Performance of the reaction in organic solvents might be a solution for this problem.

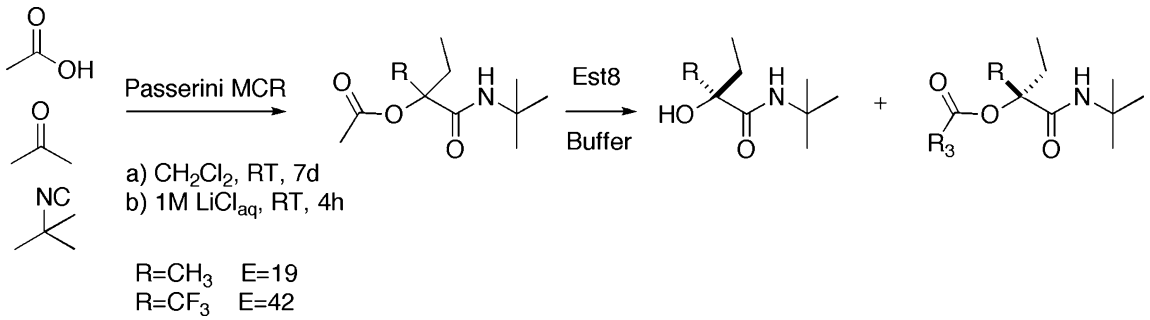
The hydrolase-catalyzed kinetic resolution could successfully be combined with a convenient substrate preparation by the Passerini multicomponent reaction (Scheme 4). This straightforward two-step method provided an easy access to protected enantioenriched 2-hydroxy-2-methylbutyric acid, a building block used for the synthesis of a COX inhibitor. The tertiary hydroxycarboxylamides proved to be difficult substrates. Out of 40 tested enzymes, only 3 showed activity. The best enzyme, GGG(A)X esterase Est8 from BRAIN (Zwingenberg, Germany) had a good enantioselectivity of E=42 (Kourist et al. 2008b).

Enzymatic ring opening of epoxides

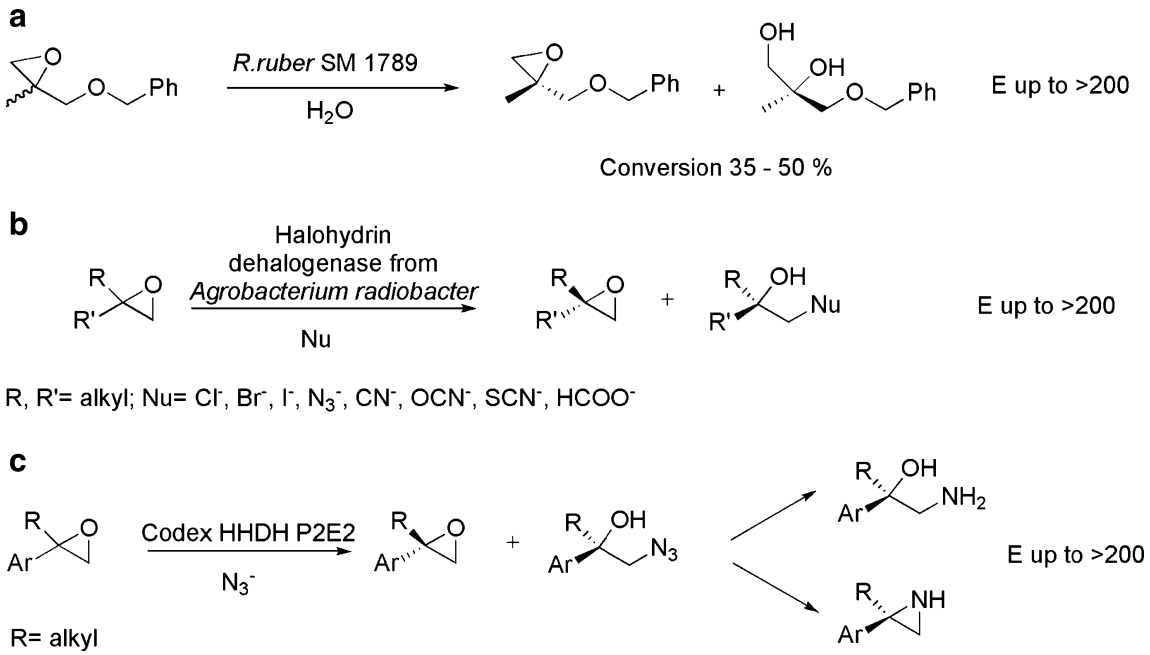
The ring opening of racemic 2,2-disubstituted epoxides catalyzed by epoxide hydrolases (Hellström et al. 2001) is an efficient route to furnish enantiopure tertiary alcohols (Scheme 5). For a general overview on the use of epoxide hydrolases for this aim, the reader is referred to several reviews (Steinreiber and Faber 2001; Kourist et al. 2008a; Choi 2009). Interestingly, halohydrin dehalogenases such as HheC from *Agrobacterium radiobacter* AD1 have a promiscuous activity in the ring opening of epoxides using different nucleophiles (Scheme 6). This kinetic resolution provides access to β-substituted tertiary alcohols and epoxides with excellent yields and enantiopurity (up to >99%ee, Majeric-Elenkov et al. 2007). Also, the enzymatic ring opening of terminal epoxides with cyanide as nucleophile furnished 5-substituted oxazolidinones with high enantioselectivity (69–98%ee, Majeric-Elenkov et al. 2008). While these examples focused on aliphatic epoxides, researchers from Merck substantially expanded the scope of the method towards arylaliphatic substrates. Molinaro et al. (2010) prepared enantiopure 1-azido-2-arylpropan-2-ols in excellent optical purity. The products were used as intermediates for the preparation of enantiomerically enriched amino alcohols and aziridines containing a quaternary chiral center.

Hydroxynitrile lyases

Hydroxynitrile lyases from plants (HNLs) can form tertiary cyanohydrins by the addition of the nucleophile CN^- to ketones (Andexer et al. 2009; Holt and Hanefeld 2009; Purkarthofer et al. 2007). This reaction was used by Avi et al. for the preparation of a series of heterocyclic tertiary cyanohydrins in high optical purity (Avi et al. 2004). Interestingly, the opposite enantiopreferences of HNLs from *Hevea brasiliensis* (HbHNL) and *Prunus amygdalus* (PaHNL) led to the formation of *cis*- and *trans*-isomers, respectively. Later, the same authors used a docking/protection group strategy for the synthesis of 2-hydroxy-2-methylbutyric acid (Fechter et al. 2007). In the direct preparation from 2-butanone, the enantioselectivities of HbHNL and PaHNL were poor (54%ee and 76%ee). This was explained by the high similarity of both substituents at the carbonyl group. By masking one substituent with spatial demanding thiol ethers, the enantioselectivity of HbHNL could be increased to 95%ee (Scheme 6). After hydrolysis of the



Scheme 4 Chemoenzymatic preparation of hydroxycarboxylamides by combined Passerini multicomponent reaction and esterase-catalyzed kinetic resolution (Kourist et al. 2008b)

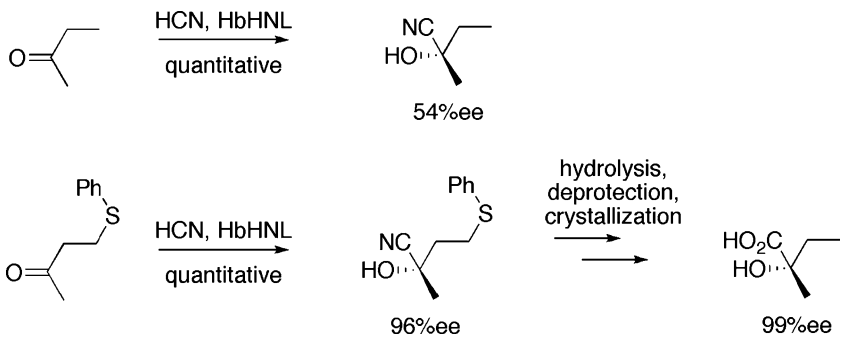


Scheme 5 Enantioselective ring opening of epoxides to yield β-substituted tertiary alcohols. While epoxide hydrolases employ water as nucleophile (**a**), halohydrin dehalogenases can introduce several nucleophiles. The method has been used successfully for the conversion of aliphatic (**b**) and arylaliphatic (**c**) epoxides

nitrile and crystallization, the thiol ether group was deprotected to furnish the final product in excellent enantiopurity. Interestingly, both enantiodivergent HNLs showed the same enantiopreference towards the thiol-protected substrates.

Carboligation

Thiamine diphosphate (ThdP)-dependent enzymes have been used for a long time in biocatalysis. Among other reactions, they catalyze the carboligation of two aldehydes to furnish 2-hydroxyketones. While this reaction can also be done using organocatalysis (Enders et al. 2007), the analog aldehyde–ketone cross-coupling has not been possible yet, due to an increased steric hindrance and lower electrophilicity of the ketone group. Recently, Lehwald et al. have isolated a ThdP-dependent flavoenzyme that catalyzes the formation of 2-hydroxyketones bearing a tertiary alcohol group (Lehwald et al. 2010). Their finding was based on the observation that YerE from *Yersinia pseudotuberculosis* catalyzes the transfer of activated acetaldehyde to the carbonyl group of a carbohydrate-derived ketone, namely cytidine-5′-diphosphate-3,6-di(deoxy)-4-keto-D-glucose, in a biosynthetic pathway leading to yersiniose A. YerE has some structural homology to bacterial acetohydroxyacid synthases (AHAS). AHAS are ThdP-dependent enzymes that catalyze the formation of (*S*)-acetolactate and (*S*)-acetohydroxybutyrate by condensation of pyruvate or 2-oxobutyrate with activated acetaldehyde, which itself stems from the decarboxylation of pyruvate. AHAS have been studied mostly for the fermentative production of branched amino acids. As they are highly enantioselective enzymes and also accept



Scheme 6 The high enantioselectivity of HbHNL towards masked 2-butanone equivalents was used for the preparation of 2-hydroxybutyric acid in excellent optical purity (Fechter et al. 2007)

unnatural substrates (Chipman et al. 1998), they would be useful biocatalysts for the preparation of optically pure tertiary alcohols. Indeed, YerE is able to activate nonsugar ketones in cross-coupling reactions (Scheme 7), giving rise to optically pure α-acetyl tertiary alcohols with yields up to 40% and good enantiopurity in several cases (up to 91%ee). The substrate scope of the method is broad and includes several small cyclic and open-chain aliphatic ketones. The reaction is only hampered by the formation of the side product acetolactate, which originates from the competing cross-coupling of two pyruvate molecules. From the investigation of analog enzymes and the determination of their three-dimensional structures with bound substrates, more insights into this fascinating reaction can be expected.

Dehydratases

The bacterial degradation and synthesis of terpenes is a promising source for uncovering new reactions in the synthesis of enantiopure terpene alcohols. Recently, Hiseni et al. reported the cloning and overexpression of two carotenoid 1,2-hydratases (CrtC) from biosynthetic pathways of carotenoid molecules in the photosynthetic bacteria *Rubrivivax gelatinosus* and *Thiocapsa roseopersicina*. CrtC hydrates the terminal carbon–carbon double bonds of lycopene. As enzymes of the carotenoid biosynthesis are associated to the membrane, the functional expression of the dehydratases in *Escherichia coli* was hampered by inclusion body formation and required a careful optimization of the cultivation conditions. Using purified enzyme, 30% conversion of lycopene to the tertiary alcohols 1-hydroxy-lycopene and 1,1′-dihydroxy-lycopene was achieved (Scheme 8), with a ratio of the mono- and dihydroxylated product of 2:1. The enzyme seems to be specific for C40 terpenes, as C5, C6, and C15 terpenes were not converted. Using geranylgeraniol (C20) as substrate, however, some activity was found (5% conversion). Interestingly, CrtC does not need any cofactor.

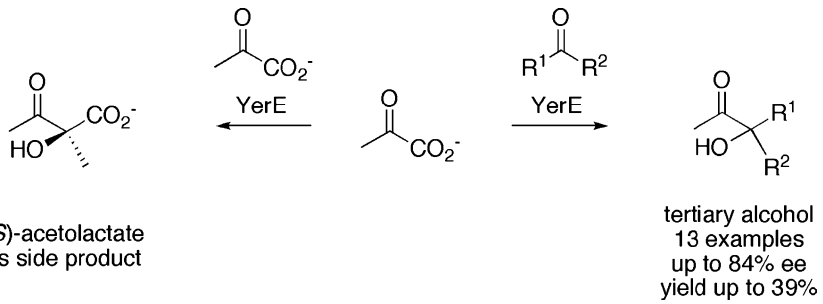
Brodkorb et al. (2010) identified a linalool dehydratase-isomerase when they studied the mineralization of monoterpenes by anaerobic bacteria. By cultivation approaches, the anaerobic strain *Castellaniella defragrans* was found to degrade monoterpenes. The authors could elucidate a simple pathway that involves the hydration of myrcene to linalool, isomerisation of geraniol, and further oxidation to geranic acid, which is then metabolized by β-oxidation (Scheme 8). The initial step, the hydratation of myrcene to linalool, is catalyzed by an apparently cofactor-free enzyme of 43 kDa. The dehydratase catalyzed the reaction in both directions. Interestingly, it displayed high enantioselectivity ($E>100$) when acting on racemic linalool—exclusively one enantiomer was converted to myrcene (Brodkorb and Harder 2009). The fact that the amino acid sequence was not similar to any described enzyme underlines that Nature's rich catalytic diversity still contains a high number of yet undisclosed reactions.

Synthesis of chiral tertiary alcohols using biotransformation with microorganisms

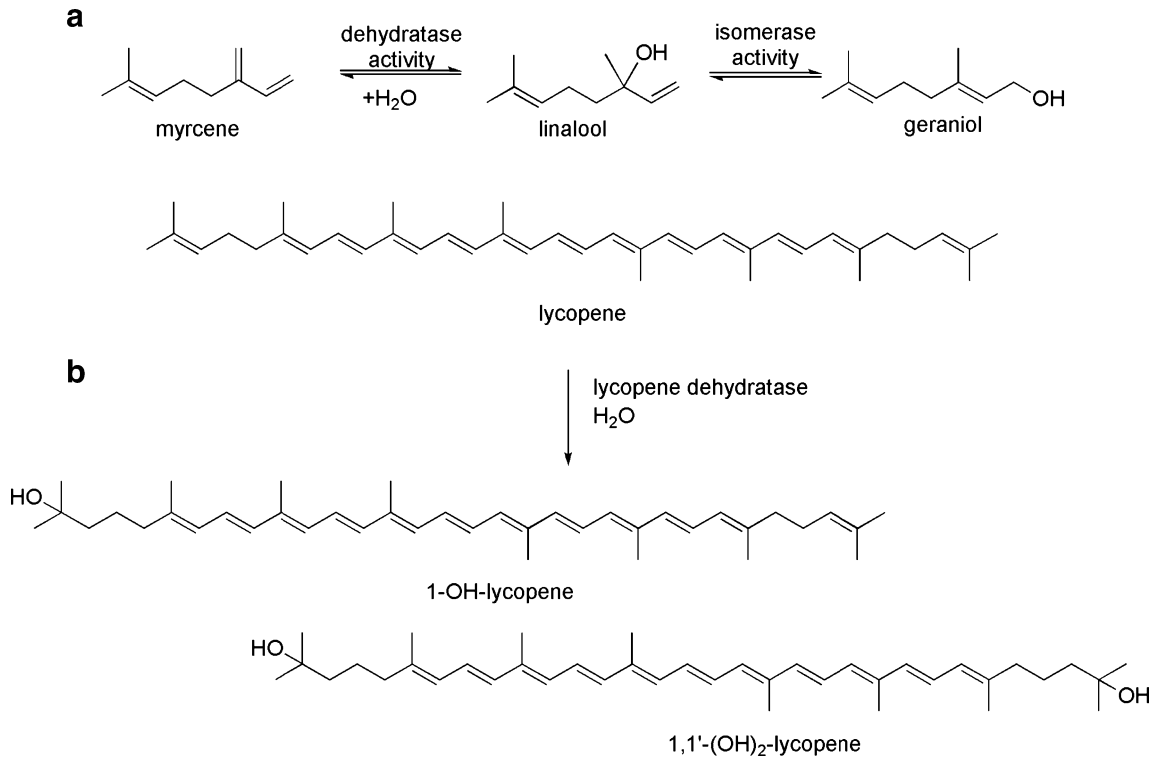
The examples given in the chapters above were solely based on the use of isolated enzymes. However, if for instance the desired reaction is cofactor dependent, the isolated enzyme is rather unstable or membrane bound or several enzyme-catalyzed reaction steps have to take place, then the use of biotransformations with whole cell systems is a useful alternative. It must be kept however in mind that side reactions can occur due to the presence of many other enzymes in whole cells, the addition of organic solvents to facilitate substrate solubility is usually restricted to keep the cells in a viable state, and that substrate access into the cell and product release must be ensured. The use of dioxygenases for the dihydroxylation of aryl carboxylates (Mihovilovic et al. 2004), a very successful approach done in multigram scale, has been reviewed recently (Kourist et al. 2008a).

Terpene cyclases

With approximately 30,000 compounds, terpenes constitute an extremely diverse group of isoprenoid natural compounds. Among them, a large number are chiral tertiary



Scheme 7 Synthesis of optically pure tertiary alcohols catalyzed by the ThdP-dependent carboligase YerE from *Y. pseudotuberculosis* (Lehwald et al. 2010)



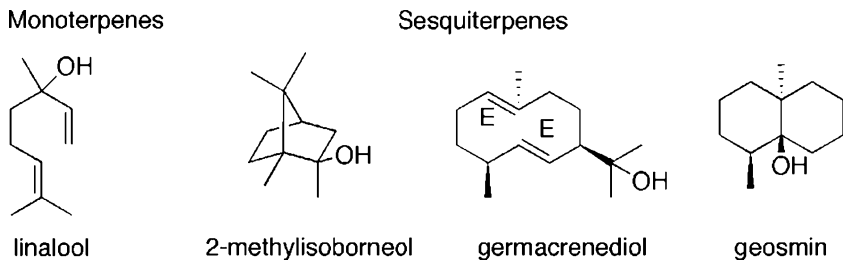
Scheme 8 Addition of water to terpenes catalyzed by bacterial dehydratases. **a** Anaerobic linalool dehydratase-isomerase from *C. defragrans* (Brodkorb et al. 2010); **b** lycopene dehydratase (Hiseni et al. 2011) from photosynthetic bacteria

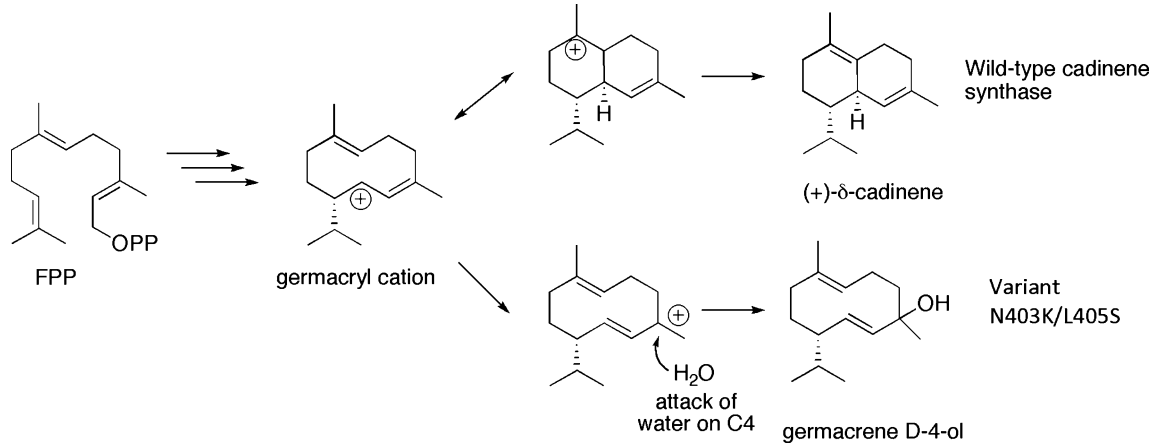
alcohols such as linalool, *cis*- and *trans*-sabinene hydrate, α-terpineol, or terpinen-4-ol (Scheme 9).

Terpene synthases catalyze the formation of the vast diversity of terpenes from a few simple precursors such as acetyl-CoA via the mevalonic acid, or in the plastids of plants and several bacteria by the so-called non-mevalonate pathway (Bohlmann et al. 1998). The high diversity of monoterpene skeletons is accounted for by the large number of terpene synthases and the fact that several of them can catalyze the formation of more than one product. The isolation of several terpene synthases from plants and the heterologous expression of their genes have facilitated our understanding of the molecular mechanism of this diversity, which is based on the formation of a carbocation by metal ion-dependent ionization (Bohlmann et al. 1998; Degenhardt et al. 2009). This substantially facilitates the production of terpenes by fermentation. Despite the fact that the differing toxicity of terpenes requires careful case-to-case optimizations, the fermentative production of terpenes by overexpression of terpene cyclases and of pathways for the synthesis of terpene precursors becomes possible. One impressive example is the production of a diterpenoid taxol precursor in *E. coli* with a titer as high as 1 g L^{-1} (Ajikumar et al. 2010). Regarding tertiary alcohols, several linalool synthases have been cloned and characterized, among them from *Mentha citrata* (Crowell et al. 2002) or lavender (Landmann et al. 2007). They both contain a bivalent metal ion as cofactor and act on geranyl diphosphate as substrate and produce (*R*)-linalool as single product with an enantiopurity of 96%ee or 98.5%ee, respectively.

In 2006, Yoshikuni et al. accomplished a switch of the selectivity of a cotton sesquiterpene synthase from the formation of δ-cadinene to the tertiary alcohol germacrene

Scheme 9 Tertiary alcohols from isoprenoid metabolism in plants





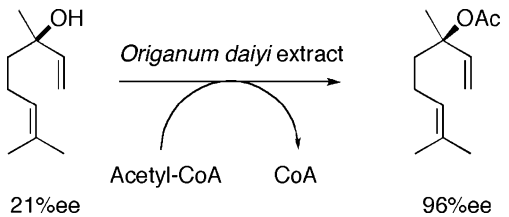
Scheme 10 The formation of (+)-∂-cadinene from farnesyl diphosphate (*FPP*) catalyzed by sesquiterpene synthase from cotton occurs via two cyclization reactions. In variant N403K/L405S, an intermediate cation was exposed to water, leading to the promiscuous formation of the tertiary alcohol germacrene D-4-ol. The mutation is positioned in the so-called G helix that shields the intermediary cations in the active site from water exposure (Yoshikuni et al. 2006)

D-4-ol by directed evolution (Yoshikuni et al. 2006). As no practicable method for the screening or selection of sesquiterpene synthesis has been developed yet, the authors combined low-throughput GC/MS analysis (limited to 100 clones per day) with a reporter protein. By fusing the synthase to chloramphenicol acetyltransferase, variants with lower solubility (87% of the epPCR library) could be eliminated and thus the screening effort was greatly reduced. After screening 100 correctly folded variants, mutant L405S was shown to produce the tertiary alcohol germacrene D-4-ol. A further round of screening led to the isolation of a double mutant N403K/L405S with a selectivity towards germacrene D-4-ol of 53%, the 47% being the natural product δ-cadinene. The improved variants were analyzed on the basis of a homology model of the enzyme. In the conversion of farnesyl pyrophosphate to δ-cadinene, two cyclization steps take place. The formation of the tertiary alcohol after the first cyclization was explained by an increased accessibility of the intermediate cation to a water molecule, which led to a quenching at the C4 atom (Scheme 10). Position 405 is situated on the so-called G helix. This structural element is believed to shield the substrate carbocation from solvent exposure. The homology model showed that the C4 atom indeed was situated closely to the G helix, which is a plausible explanation for the effect of the L405S mutation on the product formation by the enzyme.

Microbial terpene synthases have been less frequently reported. Terpene synthases identified in *Actinomycetes* were overexpressed in *E. coli* and shown to synthesize the volatile monoterpenoid alcohol 2-methylisoborneol from methylated geranyldiphosphate (Komatsu et al. 2008). The latter was furnished by a methyltransferase acting on the C2 position of geranyl diphosphate in the presence of S-adenosine methionine. An interesting example is the synthesis of the tertiary sesquiterpene alcohol geosmin via germacrenedienol in *Streptomyces avermitilis* or *Streptomyces coelicolor* (Cane and Watt 2003). Recently, Agger et al. have identified a germacrenediol synthase from the nitrogen-fixing freshwater cyanobacterium *Nostoc punctiforme*. The enzyme was cloned and heterologously expressed in *E. coli* JM109. Using cell-free extracts, evidence for the formation of germacrenediol was found in analytical scale biotransformations (Agger et al. 2008).

Terpene transferase activity

Terpene alcohol acetyl transferases constitute an important class of enzymes for the biosynthesis of enantiopure tertiary alcohol esters. In 2008, Larkov et al. have characterized the enantioselectivity of several acetyl transferases from plants (Larkov et al. 2008). They deduced the enantioselectivity of the acetylation from the observation that in some species, the esters of tertiary terpene alcohols like linalool, *cis*-and *trans*-sabinene hydrate and terpineol are isolated in much higher enantiopurity than alcohol, while in others the optical purity of alcohol and ester is similar. For instance, (*R*)-linalool is found in *Origanum daiyi* with 21%ee, while



Scheme 11 Extracts from *O. daiyi* show enantioselectivity in the enzymatic acetylation of linalool (Larkov et al. 2008)

the corresponding acetate can be isolated with 96%ee (Scheme 11). In *Origanum majorana*, however, both compounds are isolated with low enantiopurity. Using cell-free protein extracts, they could verify the enantioselectivity of the acetyl transferases in vitro. Purification and characterization of the isolated enzymes would for sure shed more light on this interesting reaction. The acetyl transferases of the plant extracts presumably apply acyl-coenzyme A thioesters as substrates. Consequently, the technical use of these enzymes will presumably be restricted to whole-cell catalysts, where this expensive cofactor can easily be regenerated.

Conclusions

From the examples covered in this review, several lessons may be drawn. First, the enzymatic preparation of tertiary alcohols offers a number of solutions for their optically pure synthesis under environmentally friendly conditions. Reactions using isolated enzymes such as the hydrolase-catalyzed kinetic resolution of esters, cyanohydrins, and epoxides have emerged as versatile, robust catalytic methods with often excellent enantioselectivity and yields in the preparation of enantiopure tertiary alcohols. However, the very limited substrate spectra of most of the enzymes still represent a bottleneck for a wider application.

Second, combinations of chemical synthesis such as a multicomponent reaction with a biocatalysis resolution step offer the additional advantage of higher total yields and reduction of solvents and waste.

Third, the recent advances in molecular biology techniques have greatly expanded the discovery and recombinant expression of novel biocatalysts for synthetic purposes as exemplified for metagenome-derived enzymes. Furthermore, protein engineering has substantially increased the toolbox for understanding and improving these biocatalysts on a molecular level and has contributed considerably in the creation of highly selective enzyme variants for the preparation of optically pure tertiary alcohols. Generally, it might be concluded that a key to success often lies in the combination of a good rational such as known structure–function relationships from 3D-structure analysis with a robust and efficient high-throughput screening method.

Finally, the examples of terpene synthesis and degradation and the C–C bond-forming carboligation demonstrate that Nature's diversity bears still a tremendous potential for the discovery of new biocatalytic principles. Notably, these enzymes were isolated from microorganisms that can easily be grown in the laboratory. With the rise of sophisticated tools to explore noncultivatable microbial consortia by second-generation sequencing, still more fascinating biocatalysts are to be expected.

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