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Enzymatic Synthesis of Optically Active Tertiary Alcohols: Expanding the Biocatalysis Toolbox

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Enantiopure tertiary alcohols are very valuable building blocks for the synthesis of many different natural products and pharmaceuticals. As a consequence, several chemical and enzymatic strategies to afford such chiral structures have been described. Promising enzymatic approaches with agents such as epoxide hydrolases, dehalogenases and hydroxynitrile lyases have been reported, as well as dihydroxylation by microorganisms. Apart from those valuable options, the hydrolase-based kinetic resolution of tertiary alcohols has been known for the last three decades, as several wild-type enzymes have been reported to be able to accept these sterically hindered molecules. More recently, the existence of an amino acid motif within an enzyme's active site has been identified as highly relevant for the acceptance of such bulky structures. This discovery clearly facilitates the identification of novel biocatalysts for this application. Although several tertiary alcohols have been successfully resolved with wild-type biocatalysts, enantioselectivities have often been too low for synthetic purposes. These limitations have recently been overcome by accessing enzymes from the metagenome through directed evolution or by rational protein design. This minireview describes the state of the art in this area, highlighting aspects of basic academic research into the practical application of biocatalysts for the synthesis of optically active tertiary alcohols.

Introduction

The production of building blocks containing quaternary carbon centres is highly relevant for various applications in fields ranging from natural products to pharmaceuticals. Within this sphere, access to enantiopure tertiary alcohols represents an important, but also challenging, task.[1] Consequently, tremendous effort has been spent on the development of synthetic routes to produce such quaternary structures, as comprehensively discussed in several reviews.[2] However, the availability of well established and standardized methods for the production of large numbers of enantiopure tertiary alcohols with high yields and purities is still an important synthetic challenge. Consequently, there is a need for new synthetic strategies aiming towards furnishing chiral tertiary alcohols.

Driven by the demand for greener, environment-friendly processes, biocatalysis is currently attracting increasing attention from chemical industries, as in many cases enzymes can meet sustainability targets in terms of selectivity, energy savings and higher atom efficiency. In this respect, a considerable number of biocatalytic processes have already been implemented on a commercial scale.[3] The key success factor is the impressive development of molecular biology techniques, which are changing the approaches by which enzymes and cells are searched for, found, characterized and used. Even more so, the possibility of producing tailor-made enzymes on large scales is a crucial asset for practical purposes. Likewise, modern biological methods lead to much more reliable biocatalysts, an aspect that has traditionally been an important issue for the systematic use of enzymes, since nonreproducible results had been achieved in many cases, even when dealing with the same biocatalyst from the same commercial supplier.

This article provides an overview of biocatalytic routes to producing chiral tertiary alcohols. It should be noted that, in contrast to the enzymatic production of optically active primary and secondary alcohols, the frequently used ketoreductases (or alcohol dehydrogenases) represent no alternative here, as no corresponding ketone for the generation of a tertiary alcohol by reduction can exist. Consequently, researchers have to rely mostly on hydrolases—namely lipases and esterases—as the most important enzymes for the kinetic resolution of tertiary alcohols.[3,4,9] Actually, though, their reactivity without the need for external cofactors, their robustness, their commercial availability at competitive prices and their wide enzymatic promiscuity make these biocatalysts highly versatile for many useful synthetic reactions.[3,9,10] This article therefore emphasizes the use of lipases or esterases and their optimization by directed evolution and rational protein design, although some other interesting enzymatic approaches are also covered.

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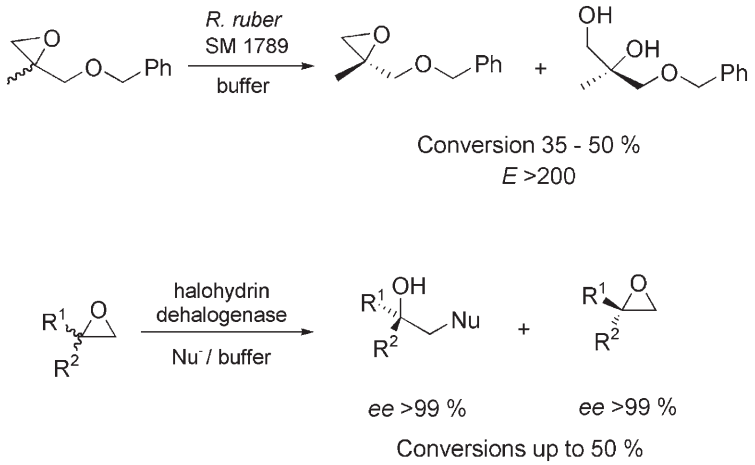
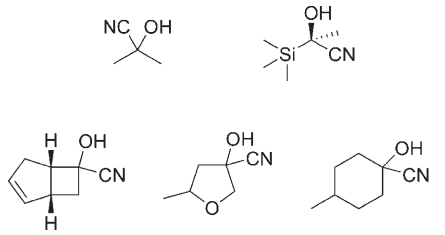
Epoxide Hydrolases, Dehalogenases, Hydroxynitrile Lyases, Dihydroxylations

Several enzymatic approaches have been proposed as effective tools for the synthesis of optically active tertiary alcohols.[4] For instance, successful kinetic resolutions of racemic oxiranes catalysed by epoxide hydrolases from *Rhodococcus ruber*[5] and from *Bacillus subtilis*[1c] have been reported. In both cases the ring-opening of racemic epoxides in aqueous media in the kinetic resolution gave the corresponding tertiary alcohols with high enantioselectivities (*E* from 70 to >200) and in good yields (Scheme 1). Very recently, a new approach to the production of tertiary alcohols, again involving the use of quaternary racemic epoxides, was disclosed. In this case, however, the oxirane rings were stereoselectively opened by a halohydrin dehalogenase with high enantioselectivities by use of different nucleophiles (i.e., CN^-, N_3^-, Cl^-, $HCOO^-$; Scheme 1).[6] Here, quaternary racemic epoxides were the best substrates in terms of enantioselectivity, as was confirmed by molecular modelling studies on 2-ethyl-2-methyloxirane. Remarkably, this is an interesting case of highly efficient chiral discrimination by an enzyme towards a substrate that bears very similar substituents, both in electronic and in steric terms.[6]



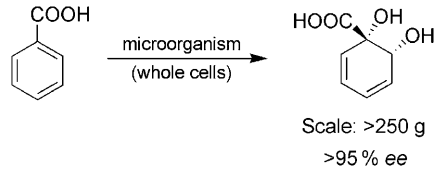
Scheme 1. Epoxide hydrolases or a halohydrin dehalogenase can be used for the stereoselective ring-opening of epoxides to yield tertiary alcohols.[1c,5,6]

Alternatively, hydroxynitrile lyases can be used to form quaternary cyanohydrins directly from ketones and HCN. These enzymes have been subjected to intensive research during the last decades (Scheme 2). Recently, processes based on hydroxynitrile lyase have led to high enantioselectivities by means of smart tools of substrate engineering.[8] Examples at industrial scales, employing tailor-made enzymes and affording productivities of up to 1 kg L^{-1} per day and optical purities of up to >98% *ee*, have also been reported and have recently been reviewed.[7]



Scheme 2. Some examples of chiral tertiary cyanohydrins produced with the aid of hydroxynitrile lyases.[7]

Another very promising approach for the biotechnological production of chiral tertiary alcohols is based on biooxidation processes.[11] A reaction that might be of interest for synthetic purposes is that of enantioselective biodihydroxylation of an aromatic carboxylic acid to afford a diol containing a tertiary alcohol moiety (Scheme 3).[12]



Scheme 3. Example of preparative biotransformation with whole cells of mutants of *Alcaligenes eutrophus* B9 or *Pseudomonas putida* U103.[12]

These bio-dihydroxylations represent a powerful synthetic tool, as two stereogenic centres can be produced concomitantly starting from benzoic acid and related compounds.[12] Interestingly, this reaction was reported as early as in 1971, when Reiner and Hegeman disclosed the performance of a mutant strain of *Alcaligenes eutrophus* that, when growing in the presence of benzoic acid, was able to accumulate the *cis*-1,2-dihydroxylation product. Presumably, this was due to a defect in the metabolic pathway that usually produces catechol from benzoate.[13] Some mutants of *Pseudomonas putida* have also displayed such performance.[12d,e] Remarkably, this preliminary finding recently led to an optimized process at a scale of >250 g product with >95% *ee* with an ~18 h fermentation time at 30 °C,[12c] which obviously shows the importance that such type of bioprocesses might have in future.

Lipases and Esterases

Application of wild-type enzymes

A range of lipases and esterases able to accept tertiary alcohols—coming from plants and mammals, but mainly from microorganisms—have in the past been identified from the natural pool. In general, their biological roles with respect to the conversion of tertiary alcohols are unknown, though in some cases these enzymes have been associated with certain metabolic degradation pathways such as, for instance, esterases involved in the hydrolysis of tertiary esters derived from the metabolic degradation of methyl *tert*-butyl ether (MTBE).[14] In other cases the presence of selective linalyl esterases from

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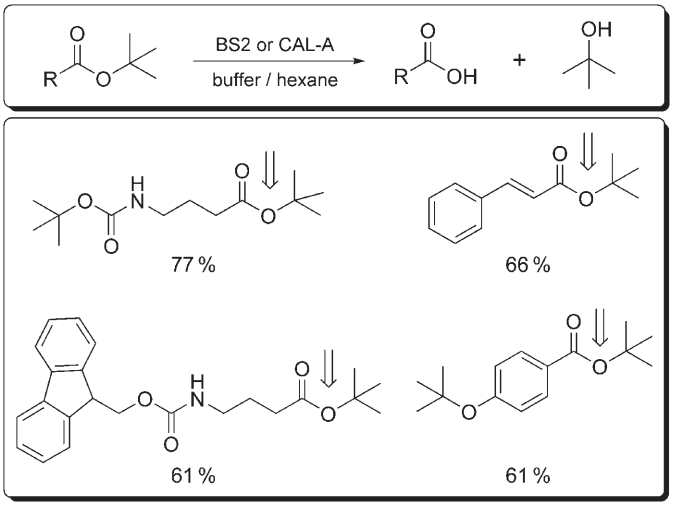
plant extracts and microorganisms has been reported.[4,15] Enzymes capable of hydrolysing aliphatic *tert*-butyl esters have also been described,[16] many of them derived from thermophilic sources.[17] Modern metagenomic approaches have been useful as well.[18] The majority of lipases and esterases found to be active towards the sterically demanding tertiary alcohols (or their esters) bear GGG(A)X motifs in the oxyanion holes next to their active sites.[19] Since this structural motif seems to play a relevant role in the acceptance of tertiary alcohols by hydrolases it is discussed in more detail below. Furthermore, this motif has guided the identification of a broad set of further biocatalysts obtained from metagenomic resources.[18]

In addition to the use of wild-type hydrolases in the kinetic resolution of tertiary alcohols, it was found that they could also be efficiently applied in the selective removal of carboxyl protecting groups. Biocatalysis has been successfully used in several strategies for protection/deprotection in organic synthesis, as summarized in several extensive reviews.[20]

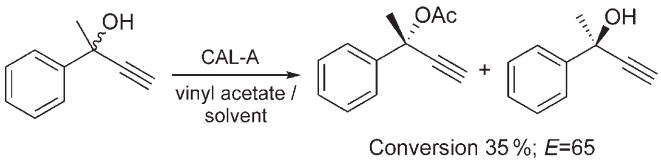
The high (chemo)selectivity that enzymes usually display is clearly an asset for these applications. Enzymes capable of hydrolysing tertiary structures are very useful, as they display high selectivity towards this functionality without affecting other protected functional groups.[20,21] For instance, an esterase from *Bacillus subtilis* (BS2) and lipase A from *Candida antarctica* (CAL-A) were able to hydrolyse tertiary esters selectively when other amino acid protecting groups were present in the molecules (Scheme 4).



Scheme 4. Hydrolase-based removal strategy for *tert*-butyl groups. The arrow indicates the cleavage site. More examples can be found in the literature.[20,21]

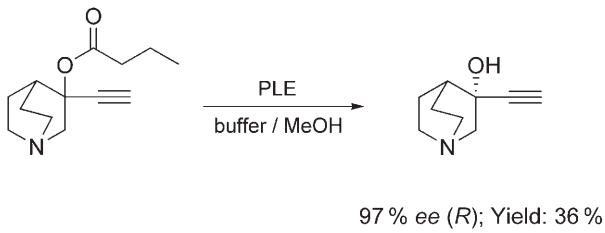
One remarkable example of an enzyme obtained from the natural pool is CAL-A. Originally isolated from soil samples taken from Lake Vanda in Antarctica, which is perennially covered by ice, the yeast *C. antarctica* produces two interesting lipases that have found many practical applications. Lipase B (CAL-B) is probably the most extensively used hydrolase in biocatalysis, as it displays an enormous versatility, combined with high thermostability.[3,9,10] In contrast, CAL-A has attracted much less attention, although it displays unique features among hydrolases, which confer on it great potential as a practical biocatalyst for many different applications.[22] For instance, CAL-A shows high thermostability and robustness, is able to accept *trans*-fatty acids and is a very versatile biocatalyst for conducting dynamic kinetic resolutions (DKRs) of cyanohydrins. By virtue of these promising performances, cloning and overexpression of CAL-A in *E. coli* have been reported by several groups,[23] together with its crystal structure.[23a]

As well as for deprotection, CAL-A has also been used for the synthesis of *tert*-butyl esters through esterifications under solvent-free conditions with fatty acids as acyl donors, and also through transesterifications between tributyrin and *tert*-butanol.[24] Furthermore, CAL-A was found to be an efficient biocatalyst in the resolution of (±)-2-phenylbut-3-yn-2-ol by transesterification with vinyl acetate as acyl donor (Scheme 5). Other wild-type hydrolases, such as *Candida rugosa* lipase, displayed lower enantioselectivities towards this specific substrate.[25]



Scheme 5. CAL-A-catalysed kinetic resolution of (±)-2-phenylbut-3-yn-2-ol with vinyl acetate as acyl donor.[25]

Likewise, porcine hydrolases have also been found to accept tertiary alcohols. For instance, the synthesis of precursors of several fungicides was effectively conducted with porcine pancreatic lipase (PPL) by enantioselective hydrolysis of the racemates under aqueous conditions.[26] PPL has also been successfully used in other kinetic resolutions to afford tertiary alcohols.[1i,27] The well known pig liver esterase (PLE) can also be used to resolve tertiary alcohols. Coope and Main reported PLE-catalysed enantioselective kinetic resolutions of quinuclidine esters to produce chiral tertiary esters in acceptable yields (for a kinetic resolution) and enantioselectivities (Scheme 6).[28]



Scheme 6. PLE-catalysed enantioselective hydrolysis of quinuclidine esters.[28]

PLE has been shown to be an active biocatalyst towards other tertiary alcohols,[19] as well as towards a plethora of (more or less) hindered structures.[9b,29] Coming from an animal source, its application in certain fields, such as for the produc-

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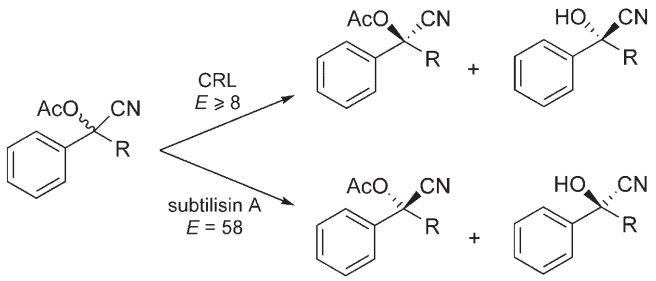
tion of pharmaceuticals, is forbidden. Very recently, a successful PLE overexpression in *E. coli* has been reported,[30] and several PLE mutants have already been described and reviewed.[9b,29–31] Much more promising research is expected from this field in the coming years as well, as this successful cloning should provide pure *rec*-PLE for large-scale processing. Problems relating to the enzyme's animal origin and to the presence of mixtures of isoenzymes are expected to be solved in this way.

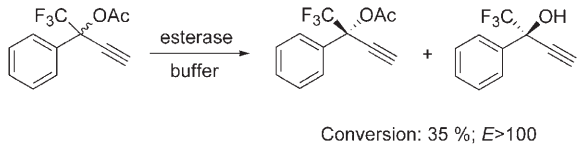
Another relevant synthetic application in the field of tertiary alcohols and hydrolases lies in the resolution of racemic cyanohydrins. These chemicals are very useful building blocks, and, as mentioned above, several studies relating to hydroxynitrile lyases have been published.[7,8] In addition to this approach, the hydrolytic resolution of racemic cyanohydrin esters is also a promising synthetic alternative. *Pichia miso* and *Bacillus coagulans* strains were reported to be active in such hydrolyses, presumably due to the presence of hydrolases.[32] To complement this approach, some isolated hydrolases have very recently been shown to be efficient catalysts in the stereoselective hydrolysis of α,α-disubstituted cyanohydrin acetates. The kinetic resolution of these tertiary esters with the protease subtilisin A or lipase from *C. rugosa* (CRL; Scheme 7) have been reported.[33] Remarkably, the two hydrolases displayed opposite enantioselectivities, which allows the production of both possible isomers with high purities and yields.



Scheme 7. Hydrolase-catalysed resolution of cyanohydrin acetates.[33]

Interestingly, the GGG(A)X amino acid motif has also been found in CRL, and other kinetic resolutions of tertiary alcohols with this biocatalyst have been described, but enantioselectivities were usually unsatisfactory.[34]

Probably the best studied hydrolase is an esterase from *Bacillus subtilis* (BS2).[36a] BS2 esterase has recently been cloned and overexpressed in *E. coli* and has been a major target for protein engineering to resolve tertiary alcohols efficiently,[37] and so it is discussed in detail below. Furthermore, it shows a promiscuous amidase activity.[36b] Finally, a recent screening of enzymes obtained from the metagenome provided a set of hydrolases active towards tertiary alcohols.[18] Although most of those new wild-type enzymes displayed low enantioselectivities, one of them was able to hydrolyse 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate at $E > 100$ (Scheme 8).[18] Obviously, the metagenomic approach should become increasingly important in many biocatalytic disciplines, as it can provide access to a diverse range of enzymes from "nonculturable" biodiversity.

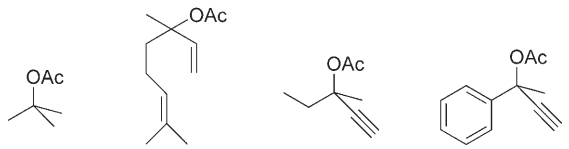


Scheme 8. Hydrolase-based kinetic resolution of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate catalysed by a new esterase isolated from the metagenome.[18]

In this section it has been shown that hydrolases isolated from the natural pool are able to accept tertiary alcohols and can readily be used for several tertiary alcohols of interest. However, if the biochemical properties, and especially the enantioselectivities, are unsatisfactory, protein engineering is the method of choice for further improving the biocatalysts, especially when they are already available recombinantly and thus easily amenable to mutagenesis. The following sections therefore focus on these aspects from an academic and a practical viewpoint.

Insights into proteins: the importance of the GGG(A)X motif

The ability of lipases and esterases containing α/β-hydrolase folds to accept and to enantiomerically resolve tertiary alcohols has successfully been associated with structural properties of their active sites. To understand this capability, a screening of 25 commercial enzymes was conducted, with the hydrolyses of four different tertiary alcohol acetates being evaluated (Scheme 9). In this study, only lipase from *C. rugosa* (CRL) and



Scheme 9. Substrates used for the identification of new enzymes capable of converting tertiary alcohol esters.[18,19]

lipase A from *C. antarctica* (CAL-A) were found to be active.[19b]

A further study based on the 3D structure of CRL revealed that its active site was wider than those of enzymes unable to convert tertiary alcohols. Subsequently, the ability of CRL to hydrolyse tertiary alcohol esters was then related to an amino acid pattern in the oxyanion hole, the binding site that stabilizes the oxyanions of the tetrahedral intermediates in the hydrolysis of esters. This so-called GGG(A)X motif was first identified in an extensive study of sequence–structure–function relationships of 92 microbial serine hydrolases,[38] in which two main types of oxyanion hole structures were found. In the first—more common—type the C-terminal neighbour of a conserved glycine is involved in the stabilization of the anionic carbonyl oxygen atom of the tetrahedral intermediate (GX

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type). In the so-called GGG(A)X type, however, the oxyanion hole residue is shifted by one position towards the C terminus relative to the GX type and, in most cases, three glycines are present in the oxyanion hole.[38] (In a few other hydrolases, the third glycine is replaced by alanine.[19a]) This GGG(A)X pattern has been found mainly in carboxylesterases and short-chain fatty acid-specific lipases, often of eukaryotic origin. Table 1 lists examples of enzymes and substrates used.

Comparison of several structures of GGG(A)X and GX hydrolases revealed that the alcohol binding pockets in GGG(A)X hydrolases are 1.5–2 Å wider than those of the GX hydrolases.[19b] In GX hydrolases the backbone carbonyl C atom of the X residue is oriented towards the binding pocket, which was assumed to induce a repulsive interaction with the substituents of the quaternary Cα atom of the tertiary alcohols. In GGG(A)X hydrolases, however, the GGG(A)X loop is likely to provide a more flexible structure. This concept was verified by the identification of three GGG(A)X esterases that showed activity towards esters of tertiary alcohols.[19b] One of these esterases, the acetylcholine esterase (AChE) from *Bungarius fasciotus*, displayed a moderate enantioselectivity ($E=10$) towards 2-phenylbut-3-yn-2-yl acetate.[19a] Furthermore, attempts to create activity towards tertiary alcohols in the GX esterases from *Pseudomonas fluorescens* and *Bacillus stearothermophilus* by directed evolution failed.[19b] In view of the difficulty in inducing activity towards tertiary alcohol esters by random mutagenesis, this negative result seems to confirm the importance of the GGG(A)X pattern in the active site.

Furthermore, the GGG(A)X concept proved to be successfully applicable to the identification of active esterases by a screening for activity in the hydrolysis of *tert*-butyl acetate by a series of metagenome-derived esterases bearing the GGG(A)X motif. Thus, 75% of the screened enzymes were active and for the "inactive" hydrolases it was assumed either that the amount of enzyme was too low or that they might have different substrate preferences.[18] However, the enantioselectivities of almost all enzymes towards the three chiral tertiary alcohol acetates (Scheme 9) were very low (up to $E=5$). Interestingly, one of the studied enzymes, the so-called esterase 34, showed an excellent enantioselectivity ($E>100$, Scheme 8) towards (*S*)-1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate, while all GGG(A)X esterases studied so far have a preference for the *R* enantiomer.[18] As the gene encoding esterase 34 was isolated at high temperature (106 °C, pH 6) it is likely that the origin of the enzyme was a hyperthermophilic organism. The thermostable esterase from the hyperthermophilic archeon *Pyrobaculum calidifontis* VA1 also features a GGG(A)X motif and was active in the hydrolysis of *tert*-butyl acetate.[17] The fact that the GGG(A)X motif seems to occur frequently in hyperthermophile organisms[17] makes them attractive candidates for the identification of new enzymes for the hydrolysis of tertiary alcohols. A metagenome approach including sample isolation in hot sites and further selection for activity towards tertiary alcohols would be therefore a tempting thought.

Nevertheless, it appears that activity towards tertiary alcohols is not restricted to GGG(A)X-type α/β-hydrolases (Table 1), although only very few examples have been described. For instance, G(X)-type lipases from *Burkholderia cepacia* and *Rhizomucor miehei* were reported to be active towards cyanohydrin acetates[33] and cyclic tertiary alcohol esters,[35] respectively. Interestingly, these lipases were also studied with the compounds shown in Scheme 9 and were not active in this instance. Activity towards tertiary alcohols has also been found in enzymes that do not strictly belong to the class of α/β-hy-

Table 1. Selected examples of microbial-, plant- and mammalian-derived hydrolases active in the bioconversion of tertiary alcohols.

Enzyme	Type	Comment	References
Bungarius fasciotus AchE[a]	GGG(A)X	$E>10$ towards 2-phenylbut-3-yn-2-yl acetate	[19]
Electrophorus electricus, AChE	GGG(A)X	–	[19]
human AChE	GGG(A)X	–	[19]
Bacillus subtilis BS2 esterase	GGG(A)X	enantioselective towards tertiary alcohols; removal of protecting groups	[19, 21, 36, 37]
Burkholderia cepacia lipase	GX	active towards several cyanohydrin acetates	[33]
Burkholderia gladioli esterase EstB	homology to β-lactamases	enantioselective towards linalyl acetate	[15d, g, h]
Burkholderia sp. YY62 hydrolase	unknown	*tert*-butyl octanoate synthesis	[16]
C. antarctica lipase A	similarity to peptidases	*tert*-butyl ester synthesis; removal of protecting groups; enantioselective towards tertiary alcohols	[20–22, 23a, 24, 25]
C. rugosa lipase	GGG(A)X	enantioselective towards tertiary alcohols and cyanohydrin esters	[19, 33, 34]
esterase 34	GGG(A)X	metagenome-derived esterase; $E>100$ towards a certain substrate	[18]
esterases from *Mycobacterium*	unknown	presumably involved in the hydrolysis of tertiary esters produced from the metabolic degradation of MTBE	[14]
esterases from *Lippia alba*, *Mentha citrata*	unknown	plant esterase; active in the hydrolysis of linalyl acetate	[15a]
linalyl acetate hydrolases from *Rhodococcus ruber*	unknown	enantioselective in the hydrolysis of linalyl acetate	[4, 15]
Rhizomucor miehei lipase	GX	enantioselective towards tertiary structures	[35]
pig liver esterase	GGG(A)X	enantioselective towards quinuclidine esters	[19 28]
porcine pancreas lipase	GGG(A)X	enantioselective towards tertiary alcohols	[1b, 26, 27]
Pyrobaculum calidifontis VA1 esterase	GGG(A)X	active in the hydrolysis of *tert*-butyl acetate	[17 25]
subtilisin A	protease	enantioselective towards several cyanohydrin acetates	[33]

[a] AChE: acetyl choline esterase.

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drolases: EstB from *Burkholderia gladioli* hydrolyses linalyl acetate.[15d] This esterase bears the common Gly-X-Ser-X-Gly consensus, which is, however, apparently not involved in catalysis. Moreover, the active site of Estb resembles that of β-lactamases.[15g,h]

Lipase A from *C. antarctica* was first proposed to be a GGG(A)X hydrolase.[19] The recently solved crystal structure,[23a] however, revealed that the initially proposed catalytically active serine is not actually involved in catalysis. Nevertheless, the low similarity of both sequence and structure[23a] of CAL-A to those of other known hydrolases and the fact that most related enzymes belong to the peptidases[23a] underline the fact that CAL-A is an enzyme with unique properties.[22] Other proteases such as, for instance, subtilisin A were also active in the hydrolysis of tertiary structures such as cyanohydrin acetates with low to good enantioselectivities (Scheme 7).[33]

Apart from these examples, it has in some other cases not yet been clarified whether or not enzymes capable of converting tertiary alcohols can be classified as GGG(A)X hydrolases, as structural information is not available.

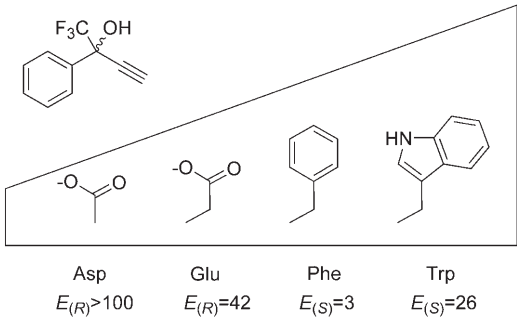
Regardless of these considerations, the observation that it is mostly GGG(A)X hydrolases that are active towards tertiary alcohols in the case of classical α/β-hydrolase fold lipases and esterases has markedly improved understanding of the molecular basis of substrate acceptance, thus facilitating the identification of new hydrolases capable of resolving tertiary alcohol acetates.

Protein engineering of esterases

Modern methods of protein design, either through rational approaches or by directed evolution, have substantially expanded the toolbox available for understanding and improving biocatalysts at a molecular level. Indeed, protein design has proved to be a versatile approach for improving the enantioselectivity of esterase BS2 from *Bacillus subtilis* towards tertiary alcohols, as the enantioselectivity could be shifted in both directions, resulting in highly selective mutants for both enantiomers.

In a first attempt, the efficacy of the wild-type BS2, with a low enantioselectivity in the hydrolysis of 2-phenylbut-3-yn-2-yl acetate, was increased sixfold (up to $E=19$) by exchanging a glycine in position 105 for alanine.[19a] It must be noted, however, that in this particular case a non-enzymatic hydrolysis of this compound—therefore nonselective, by a S_N1 mechanism[34e]—considerably decreased the overall enantioselectivity of the reaction. This was easily overcome by the addition of the water-miscible co-solvent DMSO, resulting in a better E value ($E=54$ in 20% *v/v* DMSO instead of $E=19$) for the overall reaction.[37b] Moreover, the chemically stable trifluoromethyl analogue was converted with excellent enantioselectivity ($E>100$), whereas wild-type BS2 had an E value of 42 towards this compound.[37b] Gly105 lies in the active site of BS2 and is part of the GGG(A)X motif. The observation that mutant G105A with the Ala-Gly-Ala sequence instead of Gly-Gly-Gly in the oxyanion hole still retained—though decreased tenfold—activity towards tertiary alcohols demonstrates that small changes in the GGG(A)X motif do not inevitably suppress this activity. The effect of the substitution by alanine on enantioselectivity was attributed to an unfavourable interaction between the phenyl ring of the more slowly reacting enantiomer and the sterically more demanding alanine side chain. Thus, a series of α-acetylenic tertiary alcohol acetates could be resolved with excellent enantioselectivities by kinetic resolution catalysed by BS2 G105A. Small changes in substrate structure, however, strongly affected enantioselectivity and activity, resulting in a rather limited substrate scope.[37a]

As well as position Gly105, the N-terminal neighbour Glu188 of the catalytically active Ser189 turned out to be a second key residue for control of enantioselectivity. By rational protein design[37b] and directed evolution[37c] a relation between the size of this residue and the enantioselectivity of BS2 esterase was found: whereas smaller residues favour the conversion of the *R* enantiomer—as was the case with glutamate (WT) or aspartate (mutant) in position 188—other variants with larger residues, such as phenylalanine or tryptophan, displayed an inversed enantiopreference (Scheme 10).



Scheme 10. Influence of the size of residue 188 in esterase BS2 from *B. subtilis* on the enantioselectivity in the conversion of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate. The wild-type enzyme has a glutamate in this position. Adapted from Bornscheuer et al.[37c]

The most striking example was the recent identification of a double mutant (E188W/M193C) capable of preferentially converting the *S* enantiomer with excellent enantioselectivity ($E_{(S)}=64$).[37c] Here, the E188W displayed low *S* selectivity ($E_{(S)}=26$) and the M193C had *R* preference ($E_{(R)}=16$). Thus, only the combination of both mutations gave high enantioselectivity towards the *S* enantiomer. It was suggested that the two enantiomers have opposite orientations in the active site. While the phenyl ring of the *S* enantiomer is oriented towards the GGA motif, the phenyl ring of the *R* enantiomer points towards the amino acid residue 188, and thus experiences unfavourable interactions with larger substituents at this position (Figure 1).

Conclusions

In the past few years, considerable progress has been made in the identification of enzymes capable of catalysing the enantioselective synthesis of tertiary alcohols. Epoxide hydrolases, dehalogenases and hydroxynitrile lyases have thus been described, together with some biodihydroxylation processes.

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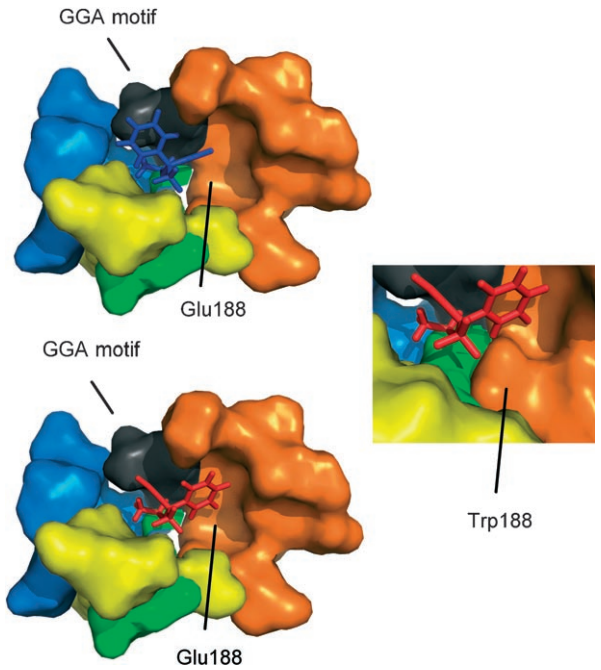

Figure 1. Orientation of the two enantiomers of 1,1,1-trifluoro-2-phenylbut-3-yn-2-yl acetate in the active site of BS2 esterase. Above: The phenyl ring of the *S* enantiomer (blue) points out of the active site. Below: The phenyl ring of the *R* enantiomer (red) is accommodated in a hydrophobic pocket. Right: Substitution of Glu188 by the bulky tryptophan results in unfavourable interactions between the phenyl ring of the *R* enantiomer. Adapted from ref. [37c].

Apart from these strategies, the use of lipases and esterases is of pivotal importance. Especially, the discovery of a GGG(A)X motif has facilitated the discovery of new hydrolases capable of accepting these sterically demanding substrates. Furthermore, protein engineering has substantially helped in the understanding and improvement of enzymes to make them synthetically useful. The substrate ranges and specific activities of most biocatalysts acting on tertiary alcohols are still too limited to allow their broad use in organic synthesis, however. Further efforts will therefore have to be made in order to make the enzymatic conversion of tertiary alcohols as straightforward as the resolution of primary and secondary alcohols.

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