Highly enantioselective kinetic resolution of two tertiary alcohols using mutants of an esterase from Bacillus subtilis

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Enzyme-catalyzed kinetic resolutions of secondary alcohols are a standard procedure today and several lipases and esterases have been described to show high activity and enantioselectivity. In contrast, tertiary alcohols and their esters are accepted only by a few biocatalysts. Only lipases and esterases with a conserved GGG(A)X-motif are active, but show low activity combined with low enantioselectivity in the hydrolysis of tertiary alcohol esters. We show in this work that the problematic autohydrolysis of certain compounds can be overcome by medium and substrate engineering. Thus, 3-phenylbut-1-yn-3-yl acetate was hydrolyzed by the esterase from Bacillus subtilis (BS2, mutant Gly105Ala) with an enantioselectivity of $E = 56$ in the presence of 20% (v/v) DMSO compared to $E = 28$ without a cosolvent. Molecular modeling was used to study the interactions between BS2 and tertiary alcohol esters in their transition state in the active site of the enzyme. Guided by molecular modeling, enzyme variants with highly increased enantioselectivity were created. For example, a Glu188Asp mutant converted the trifluoromethyl analog of 3-phenylbut-1-yn-3-yl acetate with an excellent enantioselectivity ($E > 100$) yielding the (S)-alcohol with >99% ee. In summary, protein engineering combined with medium and substrate engineering afforded tertiary alcohols of very high enantiomeric purity.

Keywords: tertiary alcohol/enantioselectivity/enzyme catalysis/esterase/rational protein design

Introduction

The synthesis of optically pure tertiary alcohol is still a challenge, not only in biocatalysis but also in classical stereoselective synthesis (Corey and Guzman-Perez, 1998; Christoffers and Mann, 2001; Christoffers and Baro, 2005). A promising route to an optically pure compound is via hydrolase-catalyzed kinetic resolution of chiral tertiary alcohols. While carboxylester hydrolases are widely used for the synthesis of optically pure secondary alcohols and to a smaller extent also for the resolution of primary alcohols and carboxylic acids (Bornscheuer and Kazlauskas, 2005), there are only few examples of their utilization in the hydrolysis of esters of tertiary alcohols. These are generally not accepted as substrates by almost any carboxylester hydrolases of commercial interest, probably due to the sterically demanding structure of those compounds (O’Hagan and Zaidi, 1992, 1994; Schlacher et al., 1998). We recently discovered that the only hydrolases which are active towards this class of substrates are characterized by a highly conserved GGG(A)X-motif (Henke et al., 2002), which is located in the active site and contributes to the formation of the so-called oxyanion hole (Pleiss et al., 2000). This binding pocket stabilizes the oxyanion in the tetrahedral intermediate formed during the catalytic cycle of ester hydrolysis (Bryan et al., 1986; Whiting and Peticolas, 1994; Ordentlich et al., 1998). The two groups, GGG(A)X- and GX-hydrolases, differ significantly in the structure of the catalytic site (Pleiss et al., 2000).

However, a detailed study using three model compounds showed that the enantioselectivity of the GGG(A)X-hydrolases is very low and did not exceed an $E$-value of 3 in the kinetic resolution of 3-phenylbut-1-yn-3-yl acetate (1a, Figure 1) using an esterase from Bacillus subtilis (BS2). On the basis of a computer modeling, two mutants were predicted (Gly105Ala, Ala400Leu), of which the Gly105Ala variant led to an increase in enantioselectivity to $E = 19$ (Henke et al., 2003). Although this represents a 6-fold increase in enantioselectivity, this value is still not synthetically useful to obtain both enantiomers in optically pure form. Alternatively, transesterification of a racemic tertiary alcohol using vinyl acetate as acyl donor in organic solvents was performed using lipase A from Candida antarctica. Autohydrolysis of 3-phenylbut-1-yn-3-yl acetate could be avoided and an $E = 65$ could be reached, but the reaction suffered from very long reaction times and a maximum conversion of 35% (Krishna et al., 2002).

Materials and methods

General

All chemicals were purchased from Fluka (Buchs, Switzerland), Sigma (Steinheim, Germany) and Merck (Darmstadt, Germany), unless stated otherwise. Restriction enzymes and polymerases were obtained from New England Biolabs GmbH (Beverly, MA, USA) and Roboklon GmbH (Berlin, Germany). MWG-Biotech (Easthleigh, UK), MWG-Biotech (Ebersberg, Germany) provided primers and performed sequence analysis. NMR spectroscopy experiments were performed on an ARX300 device (Bruker, Karlsruhe, Germany).

Synthesis of tertiary alcohols

(R, S)-3-Phenylbut-1-yn-2-ol (1b) was purchased from Ubichem plc (Eastleigh, UK). (R, S)-4,4,4-Trifluoro-3-phenylbut-1-yn-2-ol (2b) was synthesized as described...
(O’Hagan and Zaidi, 1992). A solution of 2,2,2-trifluoroacetophenone (5.05 g, 29 mmol) in tetrahydrofuran (20 ml) (THF) was added to a refluxing solution of ethynyl magnesium bromide (1.2 eq.) in THF (70 ml). The mixture was heated under reflux until all of the ketone was consumed (control via thin-layer chromatography). Water (20 ml) was added to the cooled reaction mixture and the product was extracted into diethyl ether (3 × 100 ml), washed with saturated NaHCO$_3$ solution twice with ethyl ether (100 ml) and evaporated under reduced pressure. The residue was distilled to give the alcohol as a colorless liquid (510 mg, 2.7 mmol, 46%). The NMR data matched the literature data (O’Hagan and Zaidi, 1992).

**Synthesis of tertiary alcohol acetates**

Acetyl chloride (1.2 ml, 15 mmol, 3 eq.) was added dropwise to a solution of the appropriate alcohol (5 mmol) in pyridine (6 ml) while the flask was cooled on ice and the mixture was stirred for another 15 min. Then the ice bath was removed and the mixture was stirred at ambient temperature for 16 h. The solution was extracted twice with diethyl ether (100 ml). The organic layer was washed twice with saturated NaHCO$_3$ solution and three times with 5% aqueous solution of NaHSO$_4$. The organic layer was dried over anhydrous Na$_2$SO$_4$ before the solvent was removed under reduced pressure. $(R, S)-3$-Phenylbut-1-yn-2-yl acetate (1a): chromatography on silica gel (hexane : dichloromethane 1 : 1) yielded the product as a colorless liquid (510 mg, 2.7 mmol, 54%). The NMR data matched the literature data (O’Hagan and Zaidi, 1992). $(R, S)-4,4,4$-Trifluoro-3-phenylbut-1-yn-2-yl acetate (2a): chromatography on silica gel (hexane : dichloromethane 1 : 1) yielded the product as a colorless liquid (550 mg, 2.3 mmol, 46%). The NMR data matched the literature data (O’Hagan and Zaidi, 1992).

**Expression and protein analysis of BS2**

*Escherichia coli* DH5$\alpha$ and JM109 were used as hosts for the transformation of plasmid DNA. The strains were grown in LB liquid media and on LB agar plates supplemented with 100 $\mu$g ml$^{-1}$ ampicillin at 37°C (Sambrook et al., 1989). The vector pGaston with a rhamnose-inducible promoter was used for the expression of BS2 (Henke et al., 2003). Cells were grown to an optical density of 0.5 at 600 nm. Then esterase production was induced upon addition of rhamnose (final concentration 0.2% v/v) and cultivation continued for 5 h. Cells were collected by centrifugation (15 min, 4°C, 4000 g) and washed twice with sodium phosphate buffer (100 mm, pH 7.5, 4°C). Cells were disrupted by sonication on ice for 10 min at 50% pulse and centrifuged to separate soluble from insoluble fractions. To produce a larger amount of enzyme for use in preparative scale, cells were disrupted using a French press (French Pressure cell press, Thermo Spectronic, USA). The supernatant was aliquoted and stored at −20°C. His-tag purification was performed following the standard protocol by using BD Talon$^\text{TM}$ IMAC resin from Clontech, Laboratories Inc., Mountain View, USA. Elution was done by pH-shift to pH 5. Protein content was determined using Bradford reagent with bovine serum albumin as standard.

Esterase activity was determined spectrophotometrically by hydrolysis of p-nitrophenol acetate (pNPA, 1 mm) in sodium phosphate buffer (10 mm, pH 7.5, 10% (v/v) DMSO). p-Nitrophenol released was quantified at 410 nm ($e = 15 \times 10^3$ M$^{-1}$ cm$^{-1}$). One unit (U) of activity was defined as the amount of enzyme releasing 1 $\mu$mol p-nitrophenol per min under assay conditions (Krebsfinger et al., 1998). Proteins were also analyzed by a 12% separating and 4% stacking sodium dodecyl sulphate polyacrylamide gel (Sambrook et al., 1989). After electrophoresis, the gel was first activity-stained (Krebsfinger et al., 1998) with $\alpha$-naphthyl acetate and Fast Red$^\text{TM}$ followed by Coomassie brilliant blue staining.

**Site-directed mutagenesis**

For site-directed mutagenesis, complementary primers bearing the nucleotides to be changed and the vector pGaston encoding for the wild-type gene of BS2 were used for PCR with *Pfu* DNA-polymerase and the following reaction conditions: (i) 95°C, 30 s and (ii) 18 cycles: 95°C, 50 s; 55°C, 60 s; 68°C, 10 min. The PCR mixture was treated afterwards with *DpnII* to digest methylated template DNA. The mutated plasmids were transformed into competent *E. coli* cells prepared by the transformation and storage solution method, and transformed according to a standard protocol (Sambrook et al., 1989). The mutants were confirmed by sequencing. The following primers were used:

- Ala107Trp: 5' - GTGGATTCCAGGAGGCTGTTTATCTAGACGCGG-3'
- Ala400Trp: 5' - GCCGGCTCAAAATAAGCGTTTCAGTGTTAGAGC-3'
- Glu188Asp: 5' - GTAACAGTTTGGAGATTCCGCCGGCGGATGAG-3'
- Glu188Phe: 5' - GTAACAGTTTGGAGATTCCGCCGGCGGAGG-3'
- Glu188Gln: 5' - GTAACAGTTTGGAGATTCCGCCGGCGGAGG-3'
- Ala107Trp: 5' - CTGCGCGGCGGCGGCGGCGGCGGCGG-3'
- Ala400Trp: 5' - CCGCGCGGCGGCGGCGGCGGCGGCGG-3'
- Glu188Asp: 5' - GGTGGGCGGCGGCGGCGGCGGCGGCGG-3'
- Glu188Phe: 5' - GGTGGGCGGCGGCGGCGGCGGCGGCGG-3'
- Glu188Gln: 5' - GGTGGGCGGCGGCGGCGGCGGCGGCGG-3'

**General method for esterase-catalyzed small-scale resolutions**

To a stirred solution of substrate (25 mm) in phosphate buffer (100 mm, pH 7.5) and the appropriate amount of cosolvent, the esterase solution was added. The reaction mixture was stirred in a thermostaker (Eppendorf, Hamburg, Germany) at 37°C for 20 min. The reaction mixture was then extracted twice with 400 $\mu$l dichloromethane. The combined organic
layers were dried over anhydrous sodium sulphate and the organic solvent was removed under nitrogen. The samples were analyzed by gas chromatography. 1b was analyzed using a chiral Hydrodex®-β-3B (Heptakis-(2,6-di-O-methyl-3-O-pentyl)-β-cyclodextrin; 25 m, 0.25 mm) column, and 1a was analyzed using a chiral heptakis-(2,3-di-O-acetyl-6-O-t-butylmethylisilyl)-cyclodextrin column (provided by Prof. König, University of Hamburg, Germany), both installed in a GC-14A gas chromatograph (Shimadzu, Tokyo, Japan) with hydrogen as the carrier gas. 2a and 2b were analyzed using the Hydrodex®-β-3B column in a GC-MS (model QP-2010, Shimadzu, Tokyo, Japan). Enantioselectivity and conversion were calculated according to Chen et al. (1982).

Esterase-catalyzed kinetic resolution in preparative scale
Esterase solution (12 ml, 108 U) was added to a stirred solution of 2a (90 mg, 0.37 mmol) in DMSO (4 ml) and phosphate buffer (4 ml, 100 mM, pH 7.5). The reaction mixture was stirred for 15 min at 30°C. The reaction mixture was then extracted twice with 10 ml dichloromethane. The organic layers were combined and washed with brine (2 x 10 ml), water (2 x 10 ml) and dried over anhydrous NaHSO4. The solvent was removed under reduced pressure. Chromatography on silica gel (hexane:dichloromethane 2:1) yielded 2b (28 mg, 0.14 mmol, 38%, ee = 94%) and 2a (20 mg,) as colorless liquids (20 mg, 0.08 mmol, 22%, ee > 99%).

Molecular modeling
Molecular modeling was performed using the SYBYL molecular modeling package version 7.0 (Tripos Inc.) on an SGI Octane UNIX workstation. The enzyme-substrate system was described by using the Kollman All Atom force field (Weiner et al., 1984, 1986) for the purpose of energy minimization calculations and MD simulations. The Kollman All Atom types for the tetrahedral intermediate were assigned as follows: aromatic carbon, CA; tetrahedral carbon, CT; hydrogen, HC; oxyanion oxygen, OH; and non-oxyanion oxygen, OS. A new Kollman All Atom type for the carbon atoms of the ethinyl group was defined as CX. Non-standard partial charges were calculated by using the Pullman method (Berthod and Pullman, 1965; Berthod et al., 1967) with a formal charge of −1 for the substrate oxyanion.

Non-standard Kollman All Atom force field parameters were assigned in analogy to the existing parameters for vdw radius, bond length, angle and torsion angle bending as follows: (i) CX, vDW radius 1.7 Å, mass 12.01 g mole −1; (ii) OH−CT−OS; (iii) CA−CT−OS; (iv) CX−CT−OS; (v) CX−CX−HC; (vi) OS−CT−OS, for (ii)−(vi): 109.5°, force constant 50 kJ mol−1 degree−2; (vii) CA−CT−CX 109.5°; (vIII) CT−CT−CX, for (vII)−(vIII): 109.5°, force constant 63 kJ mol−1 degree−2; (ix) CT−CX−CX 180°, force constant 125 kJ mol−1 degree−2; (x) CX−CX 1.2 Å, force constant 650 kJ mol−1 Å−2; (xi) CT−CX 1.5 Å, force constant 300 kJ mol−1 Å−2; (xii) 1.08 Å, force constant 34 kJ mol−1 Å−2; (xiii) CA−CT−CX−CX; (xiv) OS−CT−CX−CX, (xv) CT−CT−CX−CX, (xvi) CT−CX−CX−HC, for (xIII)−(xVI): 0°, periodicity 0, force constant 0 kJ mol−1 degree−2.

A non-bonded cut off distance of 8 Å and a distance-dependent dielectric function with a scaling factor of 1 were used in all calculations. An NTV ensemble (i.e., constant number of atoms, temperature and volume), a temperature of 300 K after a warm-up phase and a time step of 1 fs were used in all MD simulations. Energy minimizations were performed by using the Powell method (Powell, 1977). All crystal structure coordinates were obtained from the Protein Data Bank (Berman et al., 2000).

Preparation of the free enzyme
The preparation of the free enzyme was done similar to the procedure described for C. antarctica Lipase B (Raza et al., 2001). The crystal structure of the highly homologous B. subtilis p-Nitrobenzyl Esterase (Schmidt et al., 2007) (PDB code 1QE3, Spiller et al., 1999) was used as the starting point for the modeled tetrahedral intermediate. Hydrogen atoms were added to the enzyme. The positions of the water hydrogen atoms and then the enzyme hydrogen atoms were optimized by using a consecutive series of short (1 ps) MD runs and energy minimizations. This series of optimization steps was repeated until the energy of the system was stable. Thereafter, an iterative series of energy minimizations were performed on the water hydrogens, enzyme hydrogens and full water molecules. Finally, the whole system was energy minimized.

Preparation of the tetrahedral intermediate
The catalytic histidine, His399, was defined as protonated. The general position of the tetrahedral intermediate of 1a and the orientation of its acyl moiety were modeled by superimposing the catalytic triad of BS2 with the catalytic triad of C. rugosa lipase in complex with a tetrahedral inhibitor (PDB code 1LPM, Cylger et al., 1994). The alcohol moiety was modeled in both (R) and (S) configurations. For each enantiomeric configuration of the alcohol moiety, three binding modes fitted sterically into the active site. To avoid local minima, for each binding mode, the Ser 189-bound substrate was submitted to an MD simulation of 1 ps. Finally, energy minimization of the whole system was performed to produce the starting structure for the subsequent MD simulations.

Molecular dynamics simulations
The energy-minimized free enzyme and each energy-minimized enzyme substrate system were run through a MD warm-up phase to a temperature of 300 K in a series of 30 short (1 ps) steps of 10 K intervals. Thereafter, an MD simulation of 200 ps was performed at 300 K for each system. A sample structure was extracted at every 0.2 ps from each simulation.

Results and discussion
In this study, we applied two complementary strategies to achieve a highly enantioselective kinetic resolution: Medium-engineering via addition of cosolvents was used to suppress the autohydrolysis of acetate 1a; rational protein design of BS2 was further investigated, to suggest alternative mutants with stronger influence on the enantioselectivity. In addition, the more stable trifluoromethyl analog 2a was synthesized and included in these studies (Fig. 1). These strategies resulted in a highly enantioselective kinetic resolution of both acetates with E-values exceeding 100.
Medium engineering

The addition of water-miscible organic cosolvents such as dimethyl sulfoxide (DMSO) is a promising and quite frequently used method to improve the enantioselectivity of hydrolytic enzymes (Faber, 2004). The influence of DMSO on the enzymatic hydrolysis of 1a was investigated for wild-type BS2, and the mutants Gly105Ala and Glu188Asp (Table I). Best results were achieved by the addition of 20% (v/v) DMSO resulting in substantially higher ee-values for the Gly105Ala mutant (corresponding to observed enantioselectivity equal to $E = 56$ vs. $E' = 28$ ($E'$ stands for observed enantioselectivity, which is a result of enzyme hydrolysis and autohydrolysis)) and the Glu188Asp mutant ($E' = 41$ vs. $E' = 8$). Of course, this effect was much less pronounced for the wild-type esterase ($E' = 5$ vs. $E' = 3$) as the enzyme-catalyzed hydrolysis had a low true enantioselectivity. Higher concentrations of DMSO led to enzyme inactivation (data not shown). We mostly attribute the increase in enantioselectivity to the suppression of the autohydrolysis. We used the program KRESH developed by Faber et al. (Faber, K., Mischitz, M., and Klewein, A., Institute of Organic Chemistry, Graz University of Technology, Stremayergasse 16, A-8010 Graz, Austria) to calculate true $E$-values. In all cases, we could see that the mutants had very high enantioselectivity with $E$-values exceeding 100. With these high $E$-values, we could not detect any effect of DMSO on the enantioselectivity. Therefore, we ascribe the effect of DMSO to repression of autohydrolysis, leaving the increase of enantioselectivity to the mutations only.

To avoid competing autohydrolysis in the resolution of 1a, the trifluoromethyl analog 2a (Fig. 1) was used. Due to the electron-withdrawing nature of the CF$_3$-group, 2a is known to be resistant against autohydrolysis (O’Hagan and Zaidi, 1992). Thus, the wild-type of BS2 preferably converted (R)-1a and (S)-2a. No autohydrolysis was observed when using 2a. Hence, the enantioselectivity of wild-type BS2 and the mutant Gly105Ala increased considerably (Table I).

Rational protein design

Molecular dynamics was used to study the interaction between the enantiomeric substrates and the enzyme in transition state. The tetrahedral intermediate was used as a model of the transition state as often done when studying serine hydrolases (Haeffner et al., 1998; Henke et al., 2003) (Fig. 2A). The position of the acetyl moiety of 1a and the oxyanion hole was defined by superimposing the active site of BS2 on the highly similar structure of C. rugosa lipase with a bound transition state inhibitor (Cygler et al., 1994). The positions of the acetyl and the oxyanion hole were also in agreement with the same interactions in C. antarctica lipase B (Haeffner et al., 1998). The large active site of BS2 allows the alcohol part of the substrate to adopt several possible orientations. Thus, by manual docking three binding modes for each enantiomer were found. By comparing the binding modes and studying the interactions between the enzyme and the enantiomeric alcohols moieties, we looked for mutations that either would obstruct the reaction for the slow enantiomer or facilitate it for the fast one.

![Table 1. Influence of substrate and medium engineering on the kinetic resolution of 1a and 2a](https://academic.oup.com/peds/article-abstract/20/3/125/2274240)
Fig. 3. Snap shots from MD simulations showing the tetrahedral intermediate of \(1a\) and the catalytically important amino acids His399 and Glu310 in the active site of BS2. The upper panel shows how His399 bends down and disrupts the hydrogen bonds to the tetrahedral intermediate to form one to Glu188 instead. The lower panel shows how this is avoided by the mutation Glu188Asp, which shifts the carboxyl away from His399 rendering it impossible to form the unproductive hydrogen bond. Substrate \(1a\) is seen in green except for the tetrahedral carbon and its oxygens, which are shown in white and red, respectively. The functional groups of His399, Glu310 and Glu188 are color coded by type of atom (blue for nitrogen and light blue for hydrogen). The oxyanion hole is formed from the backbone amide hydrogens of Ala107, Gly 106 and Gly105.
p-nitrophenyl acetate (pNPA). Further, no improvement of the enantioselectivity was seen. Thus, the strategy of introducing steric repulsion into the active site of BS2 based on manual docking did not prove to be a promising approach.

The binding modes were studied by molecular dynamics simulations in order to identify stable productive ones. The hydrogen bonding network of a productive binding mode should have the typical H-bond pattern (Fig. 2A) of the tetrahedral intermediate of serine hydrolases (Uppenberg et al., 1994). The hydrogen bonding pattern was monitored during several molecular dynamics runs of 100 ps in order to investigate which of the six identified binding modes could lead to a reaction. The H-bonds of the oxyanion hole and between His399 and Glu310 were intact during all MD simulations. However, the two hydrogen bonds between His399 and the tetrahedral intermediate were only kept in one binding mode of the (S)-1a configuration, suggesting a stable hydrogen bonding network, while no catalytically competent binding mode could be found for (R)-1a. According to the modeling, the imidazol group of His399 moved downwards and formed a hydrogen bond with Glu188, the neighboring amino acid of the catalytically active Ser189 (Figs 2B and 3). Fig. 4 shows a section of 14 ps of the MD simulation of (S)-2a in BS2. The maximal distance that allows the formation of hydrogen bonds (2.8 Å) is indicated in gray shade.

To study the role of Glu188 on the bending of His399, a model of the mutant Glu188Asp was investigated. The residue 188 in Glu188Asp would still have the negative charge while the distance from the carboxylate to His399 would increase. In two investigated binding modes of 1a in Glu188Asp (one (R) and one (S) configuration), the entire hydrogen bonding network stayed intact over 80% of the simulation period, while the hydrogen bond between His399 and the carboxylate at 188 was not formed. Based on these observations, three mutations were introduced at position Glu188 by the site-directed mutagenesis and analyzed experimentally. Glu188Asp was chosen, as the side-chain is still negatively charged, but has a smaller size. Substituting Glu188Gln, the charge was removed without altering the size of the functional group. Glu188Phe was created in order to introduce steric hindrance very close to the catalytically active serine. It turned out that Glu188Gln had a reduced activity in the hydrolysis of pNPA and a lower enantioselectivity towards 1a and 2a than the wild-type enzyme (Table II).

The substitution of Glu188 with phenylalanine resulted in a reduced activity towards pNPA (40%) and, surprisingly, in an inversion of the enantiopreference in the conversions of 1a ($E_S = 2$) and 2a ($E_R = 4$). Inversion of enantioselectivity of lipases and esterases towards tertiary alcohols acetates is still a challenge. A successful example of an enantiopreference switch towards a secondary alcohol by rational protein design has been reported recently: A single amino acid exchange in the active site of C. antarctica lipase B of a tryptophane by an alanine resulted in an altered enantiopreference towards 1-phenylethanol from (R) to (S) (Magnusson et al., 2005). Additionally, the inversion of enantioselectivity towards esters of sec-alcohol was also achieved by directed evolution (Koga et al., 2003; Zha et al., 2001). An inversion of the enantioselectivity of BS2 towards the tertiary alcohol linalool has been reported for the mutant Gly105Ala (Henke et al., 2003).

We were very pleased to find that the Glu188Asp mutant converted 2a with an excellent enantioselectivity ($E_S > 100$), which was also confirmed by a preparative scale experiment yielding (R)-2a at 94%ee and (S)-2b at >99%ee with
fluoromethyl analog of hydrolysis for reactions performed in the presence of the could be substantially increased by the suppression of auto-
topic of two tertiary alcohols.

Enantioselectivity. The effective combination of both strat-
tegies facilitated the highly enantioselective kinetic resolution


to elucidate, but an effect on the bending of His399 appears
to be most likely.


Conclusions

We could demonstrate that the enantioselectivity of the ester-
ase BS2 towards esters of the tertiary alcohols \( \text{1a} \) and \( \text{2a} \)
could be substantially increased by the suppression of auto-
hydrolysis for reactions performed in the presence of the water-miscible cosolvent DMSO, use of the more stable tri-
fluoromethyl analog of \( \text{1a} \) and by rational protein design.
The last strategy led to a variant with inverted enantioselectivity,
but especially to one mutant with synthetically useful enantioselectivity. The effective combination of both strat-
egies facilitated the highly enantioselective kinetic resolution of two tertiary alcohols.

Acknowledgements

The authors gratefully acknowledge Matthias Höhne and Anett Kirschner for helpful discussions and Aurelio Hidalgo for providing the altered pg-BS2 construct. This work was supported by the Deutscher Akademischer Austauschdienst (DAAD, Germany) and The Swedish Foundation for International Cooperation in Research and Higher education (STINT, Sweden).

References


