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Engineering the Active Site of the Amine Transaminase from *Vibrio fluvialis* for the Asymmetric Synthesis of Aryl–Alkyl Amines and Amino Alcohols

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Although the amine transaminase from *Vibrio fluvialis* has often been applied as a catalyst for the biocatalytic preparation of various chiral primary amines, it is not suitable for the transamination of α-hydroxy ketones and aryl-alkyl ketones bearing an alkyl substituent larger than a methyl group. We addressed this problem through a systematic mutagenesis study of active site residues to expand its substrate scope towards two bulky ketones. We identified two mutants (F85L/V153A and Y150F/V153A) showing 30-fold increased activity in the conversion of (*S*)-phenylbutylamine and (*R*)-phenylglycinol, respectively. Notably, they facilitated asymmetric synthesis of these amines with excellent enantiomeric purities of 98% *ee*.

Enantiomerically pure amines and amino alcohols play a fundamental role in the pharmaceutical industry. One in four of the 200 top-sold drugs contains a chiral amine moiety and these drugs had a total market value of more than 88 billion USD in 2013 according to Weber and Sedelmeier.[1] When it comes to the choice of the synthetic strategy for the preparation of the amine building blocks, amine transaminases (ATAs) are increasingly recognized as an attractive option as they facilitate a one-step asymmetric synthesis starting from the corresponding prochiral ketone.[2] A very impressive example is the application of an engineered (*R*)-selective ATA from *Arthrobacter sp.* (ATA117-mut), which is currently being used for the production of sitagliptin, the active ingredient of the drugs Januvia and Janumet.[3] This example demonstrates the importance of protein engineering of wild-type amine transaminases to expand their limited substrate scope. Known wild-type ATAs are not able to convert bulky compounds demanded by the pharmaceutical industry. Compared to the success story of engineered (*R*)-selective transaminases with relaxed substrate specificity, (*S*)-selective ATAs that convert a range of bulky ketones with similar efficiency as the engineered ATA117-mut are still not available, despite the progress of first engineering studies.[4] The crystal structures of several (*S*)-selective ATAs were solved recently, enabling a detailed understanding of the mechanism of substrate binding.[5]

Both (*R*)- and (*S*)-selective ATAs that were found in nature possess a large and a small pocket in their active sites (Figure 1a).[5a,6] Although the large pocket can accommodate substituents with a rather broad size distribution, such as small alkyl to naphthyl groups, the small pocket creates a strict steric constraint: if the size of the small substituent exceeds that of a methyl group, activity drops significantly.[6] For instance, ketones with a hydroxymethyl group as small substituent are hardly accepted.[7] This active site architecture limits the substrate scope, but at the same time contributes to the usually high enantioselectivity of these ATAs.

Midelfort et al.[4a] and Park et al.[4b] recently reported the first attempts of rational engineering: they identified key residues via bioinformatic methods or structural inspection and investigated up to two substitutions per position by site-directed mutagenesis to achieve the transamination of their bulky target ketones. By combining eight mutations in *Vibrio fluvialis* ATA, a β-keto ester bearing a long (6 carbon) alkyl chain could be converted employing 1-phenylethylamine **1b** as amino donor, affording the amine imagabalin at 28% yield via asymmetric synthesis. A single mutant in *Paracoccus denitrificans* ATA[4b] showed increased activity in the deamination of 1-alkyl substituted benzyl amines and the amination of 2-oxo-octanoate. Interestingly, this study showed that larger *n*-alkyl substituents are accepted in the small binding pocket if the substrate bears an α-carboxylate functional group instead of a large hydrophobic substituent such as a phenyl group.

Despite these first successes, further efforts are needed to create an (*S*)-selective ATA that is useful for asymmetric synthesis of bulky amines. In the present study, we systematically address this problem by a (partial) saturation mutagenesis of all amino acids that form the small binding pocket of the ATA of *Vibrio fluvialis*.

We employed 1-phenylbutane-1-one **2a** and the hydroxy ketone 2-hydroxyacetophenone **3a** as model substrates (Table 1). The amine product (*R*)-phenylglycinol **3b** is a building block for many important pharmaceuticals, such as an inhibitor of the 3-phosphoinositide-dependent protein kinase-1 (PDK1), which was identified as a target enzyme for cancer therapy.[8] Additionally, **3b** is applied as a chiral auxiliary in the synthesis of some of the top selling drugs, saxagliptin[9] (treatment of type 2 diabetes), femoxetine and paroxetine[10] (antidepres-

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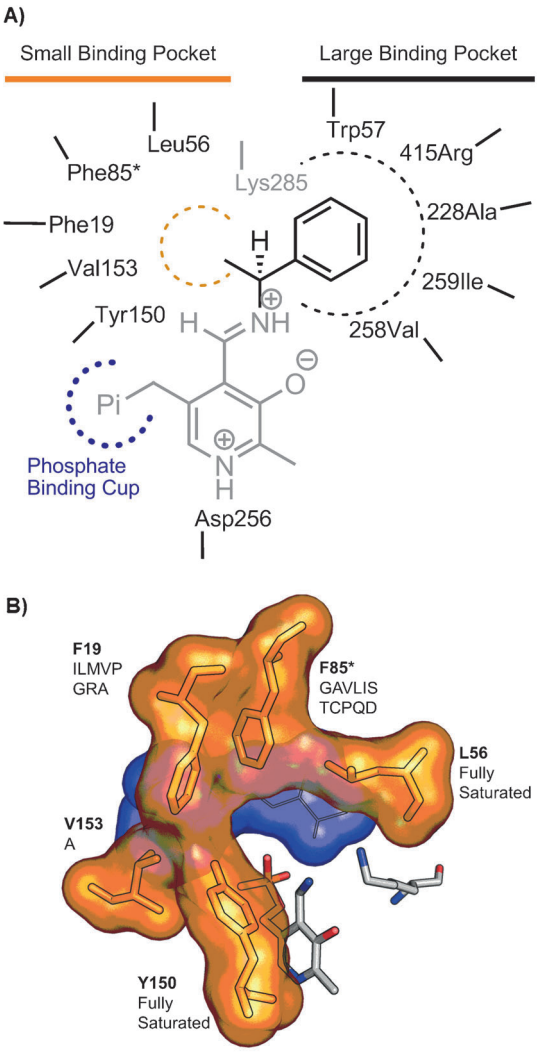


Figure 1. Active site architecture of *Vibrio fluvialis* ATA (PDB-code: 4E3Q). A) Schematic drawing of residues that form the large and small binding pocket around the external aldimine intermediate (PLP-Schiff' base with (*S*)-1-phenylethylamine). B) View of the small binding pocket. The side-chains of five residues form a hydrophobic shell by π-π stacking interactions. Y165 and F86* are omitted for clarity. The catalytic lysine and PMP are shown as gray sticks; the targeted residues for mutagenesis and their surface are shown in orange and are labeled by one-letter abbreviation and number. The libraries were constructed by choosing those residues that were potentially able to decrease the steric hindrance in the pocket, that is, smaller and aliphatic residues. Residues with different chemical properties derive from the selection of the randomized codons (see the Supporting Information). The phosphate-binding cup is shown in blue.

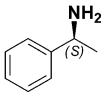
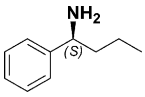
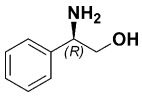
sants). These substrates exert an increased steric demand on the small binding pocket compared to the well-known acetophenone **1a**, which is often employed as a benchmark substrate. Furthermore, (*S*)-phenylbutylamine **2b** and (*R*)-phenylglycinol **3b** facilitate an easy activity screening owing to the increase in UV-absorption upon deamination to their corresponding ketones **2a** and **3a**. Hence, the acetophenone assay[11] could be used with slight modifications to screen for active variants with high throughput and sensitivity (see Figure S1 in the Supporting Information). We assumed that mutations which would facilitate asymmetric synthesis of **2b** and **3b** would also lead to higher activity in the deamination reaction, and thus be detected during screening.

When pyruvate is used as the amino acceptor, the reaction is virtually irreversible, reducing the screening time and reaction complexity compared to asymmetric synthesis. As this assay works with both enantiomers, we were also able to determine if a mutation affected the enzyme's enantioselectivity. Based on the crystal structure of the transaminase of *Vibrio fluvialis* (PDB-code: 4E3Q), the small binding pocket, located at the interface of the homodimer, is composed of eight residues that completely surround the cofactor, from Tyr150 at the active site entrance (*re*-face of PLP[12]) to the catalytic Lys285 on the other (*si*-) side (Figure 1B, the residues that belong to the second monomer are indicated with an asterisk).[4a] Residues Tyr165, Tyr150, Phe19, Phe85*, and Phe86* create a continuous π–π stacked shell and thus form a relatively hydrophobic environment. Three of the eight residues, Gly320*, Phe321*, and Thr322*, were excluded in our mutagenesis strategy, as they are known as the "phosphate-binding cup"[13] and are responsible for the coordination of the PLP phosphate group. All the other positions (Phe19, Leu56, Phe85*, Tyr150 and Val153) were sifted through for improved properties in the transamination between the enantiopure amines and pyruvate. In a first step, we generated libraries with a (partial) saturation at each position. The allowed residues in the partially saturated libraries included those amino acids that aim to create more space in the pocket, while still maintaining structural solidity (Figure 1B). The quality of each library was checked to ensure 99% library coverage during the screening,[14] then each library was screened using the modified acetophenone assay.[11] Variants with improved activities were purified and further investigated to confirm their improved properties. Finally, the best mutants were combined to elucidate positive additive effects. When positions 19 and 56 were randomized, no improved variants were found for the substrates tested (data not shown).

Single mutations at positions 85, 150, and 153 led to up to 40-fold improvements. For the conversion of **3b**, the results suggest that the positioning of its hydroxyl group in the small binding pocket is hindered by the presence of the Tyr150 in the wild-type scaffold (Figure 1), whose hydroxyl group occupies the required space. The simple substitution Y150F improves the template's performance 23-fold, whereas the best mutant discovered in this project is the double mutant Y150M/V153A for a final improvement of the reaction velocity of 53-fold. By combining the mutations F85L and V153A it was possible to achieve a 26-fold improvement towards **2b**. We then tested the ability of the best mutants to catalyze the asymmetric synthesis of our target amines, using the LDH/GDH system to shift the equilibrium.[15]

Regrettably, the mutants carrying the mutation Y150M had a decreased activity towards **2a**. The Y150M-containing mutants were not among the best mutants for the transamination of **3a**, either. Consequently, we investigated with increased enzyme concentrations all the different improved variants identified by the screening in the asymmetric synthesis reaction and we found that variant Y150F/V153A formed **3b** with

Table 1. Specific activities of the purified wild-type and variants identified during the screening.

	 **1b**	 **2b**		 **3b**	
Variant	SA [U mg^{-1}][a]	SA [U mg^{-1}][a]	Conv [%][b]	SA [U mg^{-1}][a]	Conv [%][b]
VF-wt	1.33 ± 0.02	0.19 ± 0.00	19	0.01 ± 0.00	14
F85L	0.58 ± 0.01	0.32 ± 0.00	51	0.00 ± 0.00	0
Y150F	0.97 ± 0.02	0.22 ± 0.01	20	0.23 ± 0.00	93
Y150M	0.68 ± 0.03	0.39 ± 0.01	8	0.41 ± 0.02	47
V153A	5.59 ± 0.07	3.36 ± 0.03	71	0.11 ± 0.00	49
F85L/Y150F	0.55 ± 0.02	0.35 ± 0.00	52	0.07 ± 0.00	30
F85L/Y150M	0.69 ± 0.04	0.74 ± 0.01	14	0.35 ± 0.00	0
F85L/V153A	2.38 ± 0.14	4.99 ± 0.07	93	0.03 ± 0.01	0
Y150F/V153A	1.60 ± 0.02	0.82 ± 0.02	44	0.34 ± 0.01	92
Y150M/V153A	1.36 ± 0.03	0.65 ± 0.02	8	0.53 ± 0.03	23
F85L/Y150F/V153A	0.98 ± 0.01	1.55 ± 0.03	79	0.14 ± 0.01	32
F85L/Y150M/V153A	0.47 ± 0.01	0.02 ± 0.00	1	0.00 ± 0.00	0

[a] SA = Specific activity: The reaction was followed at 245, 242, and 252 nm for the detection of **1a**, **2a**, and **3a**, respectively, using 2.5 mM of the amino donor and 2.5 mM of pyruvate in 50 mM HEPES buffer pH 7.5 containing 1.66% DMSO at 30 °C. The activities were calculated as U mg^{-1} (purified enzyme). One Unit is defined as the conversion of 1 µmol of product per minute. Values and standard deviations given are based on three measurements. [b] Conversion reached in asymmetric synthesis: Reaction conditions for the synthesis of **2b**: 0.5 mg mL^{-1} purified enzyme, 1 mL reaction volume, 10 mM **2a**, 150 mM L-alanine, 30% DMSO, 14 days, 30 °C, 50 mM HEPES buffer pH 7.5, 0.1 mM PLP. Reaction conditions for the synthesis of **3b**: 0.5 mg mL^{-1} purified enzyme, 1 mL reaction volume, 10 mM **3a**, 250 mM L-alanine, 10% DMSO, 4 days, 30 °C, 50 mM HEPES buffer pH 7.5, 0.1 mM PLP. Both reactions were followed via GC analysis using a Hydrodex-β-TBDAC chiral column and in both cases the LDH/GDH system was used to shift the equilibrium.[15]

88% conversion after 24 h. In agreement with the previous observations, the asymmetric synthesis of **3b** is possible when the active site is freed from the Tyr150's hydroxyl group and Y150F is now the key mutation to accomplish this transamination. Variant F85L/V153A afforded **2b** with quantitative conversion after 5 days (Table S1).

The asymmetric synthesis of **2b** and **3b** was then confirmed on a semi-preparative scale (Table 2) employing 0.2 mmol ketone and 0.2 mol% catalyst. We also observed a beneficial effect of the above-identified mutations on the activity of the ATA towards branched-chain α-keto acids, where the activity was increased by up to 6-fold in the synthesis of L-Leu, L-Ile, and L-Val (Table S2).

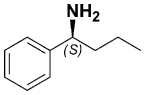
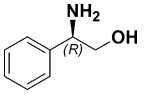
Our results demonstrate the difficulty of broadening the substrate specificity of (*S*)-selective ATAs. All amino acids that contribute to the small binding pocket are located at the dimer interface of the enzymes (except Y150) and they are placed on different loops. Although the placement of smaller residues in positions 19, 56, and 85 would theoretically excavate the small binding pocket and provide more space for bulky substrates, mutations at these sites seem to affect other properties of the enzyme (such as flexibility) important for folding, catalysis or stability. Other than the effect of F85L on **2b**, all mutations in these positions were detrimental. This supports previous findings by Park et al. and Humble et al. where mutations at the structurally equivalent positions led to a drastic drop in activity in the ATAs from *Paracoccus denitrificans* and *Chromobacterium violaceum*.[4b,16]

Most of the variants screened from those libraries showed little-to-no residual activity in our initial screening assays compared to the wild-type. In position 150, a Phe or Tyr is found in virtually all ATA-sequences, as this position is important for the positioning and π-stacking with PLP. The presence of the Tyr hydroxyl group, however, determines the acceptance of the hydroxylated substrates **3a** and **3b** in the forward or backward reaction, respectively. This result is in agreement with those presented in an independent study performed on the ATA from *Chromobacterium violaceum*, and published by Deszcz et al. contemporaneously to ours.[17]

The different activities of Y150F- and Y150M-containing variants in the kinetic resolution or asymmetric synthesis underline the importance of screening variants under conditions as close to the desired synthetic application as possible. V153A showed the largest contribution towards a relaxed active site able to accept bulkier substrates, which was also observed by Park and coworkers.[4b] This second shell residue is not in contact with the bound substrate, but its mutation to alanine might increase the active site's flexibility.

Finally, we conclude that 1) there is no easy solution to generally expand the substrate scope of *Vibrio fluvialis* ATA by modifying single residues of the small binding pocket, and 2) creating space is not sufficient to yield an efficient amine transaminase

Table 2. Asymmetric synthesis results with the best (purified) variants.

Mutant	Product		Conv. [%]	*ee* [%]	Yield [%]
F85L/V153A		**2b**	>98[a]	98	53
Y150F/V153A		**3b**	>98[b]	98	60

The reaction progress was followed via TLC analysis (0.2 mM detection limit of the ketone). To shift the equilibrium, the LDH/GDH system was applied.[15] [a] Reaction conditions: 1 mg mL^{-1} purified enzyme, 20 mL reaction volume, 10 mM **2a**, 150 mM L-alanine, 30% DMSO, 14 days, 30 °C, 50 mM HEPES buffer pH 7.5, 0.1 mM PLP. [b] Reaction conditions: 1 mg mL^{-1} purified enzyme 20 mL reaction volume, 10 mM **3a**, 250 mM L-alanine, 10% DMSO, 3 days, 30 °C, 50 mM HEPES buffer pH 7.5, 0.1 mM PLP.

activity. Instead, second shell residues should be included in further mutagenesis studies. The prediction of further useful mutations is not possible at the moment, because the underlying factors governing catalytic efficiency are not yet understood and hence, depending on the chemical structure of the substrate, different mutations are needed to enhance activity.

Experimental Section

All experimental details are presented in the Supporting Information.

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Keywords: amine transaminase · biocatalysis · protein engineering · substrate scope

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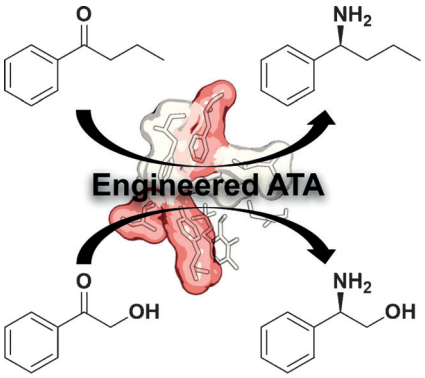
Excavating the active site: The constrained active site of the amine-transaminase from *Vibrio fluvialis* was engineered in order to achieve the conversion of bulky ketones. This led to the discovery of mutants with a 30-fold increase in activity which enabled the asymmetric synthesis of (*S*)-phenylbutylamine and (*R*)-phenylglycinol



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