ChemPubSoc Europe

DOI: 10.1002/cbic.201500074

CHEMBIOCHEM Communications

Two Subtle Amino Acid Changes in a Transaminase Substantially Enhance or Invert Enantiopreference in Cascade Syntheses

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Amine transaminases (ATAs) are powerful enzymes for the stereospecific production of chiral amines. However, the synthesis of amines incorporating more than one stereocenter is still a challenge. We developed a cascade synthesis to access optically active 3-alkyl-substituted chiral amines by combining two asymmetric synthesis steps catalyzed by an enoate reductase and ATAs. The ATA wild type from *Vibrio fluvialis* showed only modest enantioselectivity (14% *de*) in the amination of (*S*)-3-methylcyclohexanone, the product of the enoate-reductase-catalyzed reaction step. However, by protein engineering we created two variants with substantially improved diastereoselectivities: variant Leu56Val exhibited a higher *R* selectivity (66% *de*) whereas the Leu56Ile substitution caused a switch in enantiopreference to furnish the *S*-configured diastereomer (70% *de*). Addition of 30% DMSO further improved the selectivity and facilitated the synthesis of (1*R*,3*S*)-1-amino-3-methylcyclohexane with 89% *de* at 87% conversion.

The enantioselective synthesis of chiral primary amines has advanced remarkably during the last decade,[1] and some enzymatic processes can now outperform well-established chemical processes. One benchmark example is the manufacture of Sitagliptin, the active pharmaceutical ingredient (API) of Januvia, which demonstrates the potential of amine transaminase technology.[2]

Many APIs contain multiple stereocenters, and their preparation is therefore challenging. An elegant solution with regard to atom economy is the combination of different (bio)catalytic asymmetric synthesis reactions facilitating the stepwise creation of several stereogenic centers. If reaction conditions are compatible, the synthesis can be carried out as a one-pot cascade reaction. This biomimetic approach represents an exciting recent development in "white biotechnology".[3] In a cascade, intermediate downstream and purification steps are unnecessary, and so cascades also represent a very promising approach from a green chemistry point of view. For example, Sehl et al. used the combination of a thiamine diphosphate (ThDP)-dependent acetohydroxy acid synthase and amine transaminases to produce the 1,2-amino alcohols pseudo- and norpseudoephedrine.[4] Several other cascades with up to 13 different enzymes have been reported recently, thus showing the general applicability of this strategy.[5] A great advantage is the modularity of this approach, because different combinations of enzymes with distinct enantiopreferences result in a spectrum of products with different diastereomeric configurations.

We were interested in the synthesis of ring-substituted exocyclic amines because they are employed as building blocks for the production of pharmaceuticals and fine chemicals.[6] Typically, in reactions employing ATAs, acyclic ketones containing one large and one small substituent apiece have been used as substrates,[7] and the molecular mechanisms governing substrate recognition and enantioselectivity are fairly well understood: the active sites of an *R*- and an *S*-selective ATA differ in the localization of the small and the large binding pocket, thus forcing the ketone into one specific binding mode, leading to the formation of a distinct amine enantiomer.

In contrast, only a few reports on the amination of (substituted) cyclic ketones have been published.[6b,8] Structurally, cyclic ketones are challenging with respect to activity and stereoselectivity[9] because they do not possess the well-defined large and small substituents that are usually important for achieving highly selective transamination. A few cyclic substrates with large substituents (e.g., 2-(1*H*-benzoimidazol-2-yl)-1-methylpiperidin-4-one,[6b] 2-[2-(3,4-dimethoxyphenyl)ethoxy]cyclohexanone,[6a] or methyl (3-oxocyclohexyl)acetate[8]) were investigated recently, but cyclic compounds only bearing single small alkyl substituents have not been explored. Therefore, we envisioned the synthesis of 1-amino-3-methylcyclohexanes (**3**) as model compounds by combining two asymmetric synthesis steps based on 1) an enoate reductase (ERED), and 2) an amine transaminase and starting from 3-methylcyclohex-2-enone (**1**, Scheme 1).

An efficient cascade requires: 1) a highly enantioselective ERED, 2) that the precursor **1** should not be an ATA substrate (or otherwise, **1** must be fully consumed in the first step), to avoid the formation of byproducts, and 3) that the ATA should introduce the amino group with the desired enantiopreference

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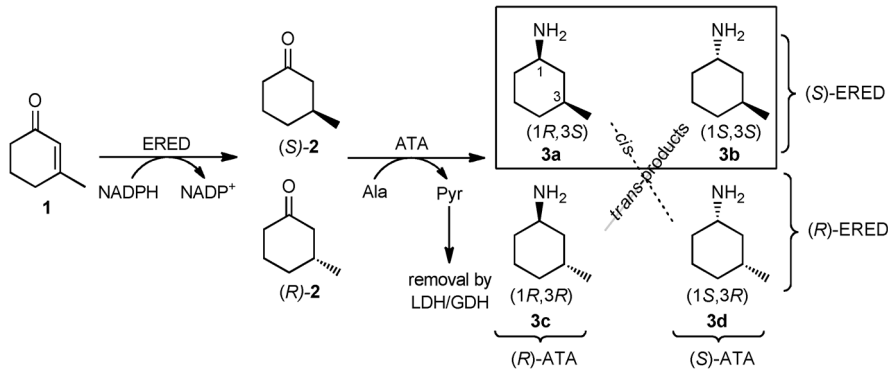
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201500074.



Scheme 1. The combination of an ERED with an ATA in a cascade reaction enables access to optically pure 1-amino-3-methylcyclohexanes. Glucose dehydrogenase (GDH) can be used for cofactor recycling in the ERED-catalyzed reaction, whereas lactate dehydrogenase (LDH) and GDH allow a shift of the equilibrium in the ATA-catalyzed reaction. Note that different combinations of EREDs and ATAs with complementary enantiopreferences are required to obtain all four diastereomers. The boxed diastereomers were obtained in this study.

and with high enantioselectivity, preferentially regardless of the configuration of the 3-methyl group. Enoate reductases, which are flavin-mononucleotide-containing (FMN-containing) NAD(P)H-dependent oxidoreductases, are well known to convert α/β-unsaturated ketones or aldehydes into the corresponding enantiopure compounds.[10] ATAs introduce amine groups, often with excellent enantioselectivities,[7b,11] into a wide range of ketones, but enantioselectivity drops drastically if the size difference between the two substituents on either side of the carbonyl group is too small or if the ketones are small cyclic molecules such as our target ketone **2**. If (dia)stereoselectivity is not sufficient for a ketone bearing a chiral center in the α- or β-position, this can in principle be improved by protein engineering. One example was shown by Limanto et al., who used ATA to convert 2-[2-(3,4-dimethoxyphenyl)-ethoxy]cyclohexanone;[6a] after three rounds of evolution selective enzymes for both chiral centers were found.

The aim of this work was to demonstrate the applicability of the envisioned cascade and, in this context, to identify enantiocomplementary ATAs, preferably with high stereoselectivity towards **2**. Identification of suitable ATAs can be achieved by methods of protein discovery[7b,12] and protein engineering,[2a,13] which can be guided by bioinformatics tools. One of these tools is the 3DM software package, which connects a high-quality, structure-guided sequence alignment of a protein superfamily[14] with bioinformatics analysis tools. 3DM integrates data from various sources, including sequences, structural information, protein ligand contacts, and mutational data from the literature, using a unified amino acid residue numbering system for all proteins. We have previously used this platform to guide the degree of randomization for the creation of "small, but smart" libraries in multiple-site saturation mutagenesis experiments.[15] In this work, we have used information from 3DM to guide the identification of variants of the ATA from *Vibrio fluvialis* (ATA-Vfl) with suitable stereoselectivity and enantiopreference for the synthesis of different diastereomers of **3**, because an initial screening had identified this ATA as having sufficient activity. Unfortunately, ATA-Vfl exhibited only moderate selectivity, with *rac*-**2** having been fully converted after 18 h. The observed composition of 30:26:40:4 for the four amine diastereomers **3a**–**3d** clearly shows a low enantioselectivity in the amination reaction. Remarkably, the ratios of the (1*R*)- to the (1*S*)-amines of 30:26 and 40:4 indicate that ATA-Vfl introduces the amino group with *R* enantiopreference (see below; Figure S4 A and B in the Supporting Information). This is in contrast with the well-documented[16] *S* enantiopreference of ATA-Vfl and the previously reported high *S* selectivity in the conversion of the related six-membered cyclic ketone methyl (3-oxocyclohexyl)acetate.[8] Obviously, the type and size of substituents at the β-position in a cyclic ketone such as **2** strongly influence enantioselectivity and -preference in the amination reaction.

To identify amino acid positions governing enantioselectivity, we modeled the internal aldimine intermediate of **2** into the structure of ATA-Vfl by using *YASARA*[17] (Figure 1 A). From visual inspection, we chose Leu56 as target for mutagenesis because its side chain would be in contact with the methyl substituent in the PLP intermediates of **3b** and **3c**, so mutations of Leu56 might induce a change in selectivity. Analysis of the amino acid distribution at position 56 by use of the 3DM database[14] identified six amino acids (Ile, Val, Ala, Trp, Tyr, Phe) besides leucine as most frequently occurring in PLP fold type I proteins (Figure 1 B). In a subset containing only subfamilies harboring *S*-selective ATA sequences, leucine is found in 90% of the sequences. These six mutants were generated by Mega-Whop PCR, overexpressed in *E. coli* Bl21 (DE3), and purified by His-tag affinity chromatography (Figures S1–S3). Only the Leu56Ile, Leu56Val, and Leu56Ala mutants showed sufficient activity. Because of its lower activity, in combination with enantioselectivities that were similar to those of the Leu56Val mutant, the Leu56Ala mutant was also excluded.

GC analysis revealed that variant Leu56Ile showed an improved 1*R* enantioselectivity in the amination of the *R* enantiomer of *rac*-**2** (Table 1, entry 2). Interestingly, the enzyme aminated (*S*)-**2** with inverted enantiopreference. The Leu56Val mutation improved the *R* enantioselectivity in the amination of (*S*)-**2**, but had no effect on the *R* enantiomer of **2** (Table 1, entry 3, Figure S4 B). In the case of **2**, the carbonyl group is part of a flexible aliphatic ring: **2** is an almost symmetric compound because only a small methyl group is present in the β-position. Thus, the typical steric constraints originating from the interaction of a substrate bearing well-defined small and large substituents with the enzyme's active site[7a,16b] do not apply for **2**. This difference could lead to the switch of the commonly observed stereoselectivity of the ATA from *V. fluvialis* resulting in the *R* configuration here. We were thus able to show that subtle changes in the active site geometry produced

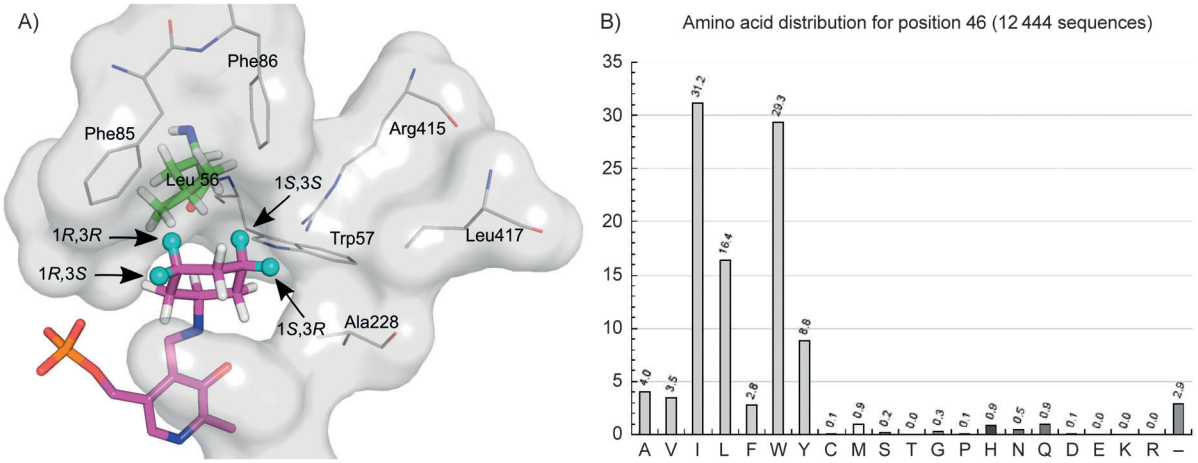


Figure 1. A) View of the active site of ATA-Vfl together with the modeled external aldimine of **2**. The PLP intermediate and Leu56 are shown as magenta and green sticks, respectively. The cyan spheres indicate which hydrogen atom is replaced by a methyl group if one of the amines **3a**–**3d** is bound to PLP. Note that the side-chain Arg415 can also adopt a conformation flipping outside the active site.[20] B) Amino acid distribution of PLP fold type I proteins at position 56 of ATA-Vfl. In the 3DM database this position corresponds to number 46.

Table 1. Diastereomeric purities of the products of the transamination of substrate **2** and of the simultaneous cascade reactions starting from **1** with employment of OYE as the ERED and ATA-Vfl or its mutants in the absence or presence of the organic solvents DMF or DMSO.

	Enzyme	Substrate	DMSO [%]	DMF [%]	*t* [h]	Diastereomeric composition **3a** (1*R*,3*S*)	**3b** (1*S*,3*S*)	**3c** (1*R*,3*R*)	**3d** (1*S*,3*R*)	*de* [%][a]	Conversion [%]
1	ATA-Vfl	*rac*-**2**	0	1	18	30	26	40	4	n.a.	99
2	ATA Leu56Ile	*rac*-**2**	0	1	18	7	47	46	n.d.	n.a.	99
3	ATA Leu56Val	*rac*-**2**	0	1	18	40	16	39	5	n.a.	99
4	OYE + ATA-Vfl	**1**	1	0	48	56	44	n.d.	n.d.	12	99
5	OYE + ATA Leu56Ile	**1**	1	0	48	14	86	n.d.	n.d.	71	97
6	OYE + ATA Leu56Val	**1**	1	0	48	83	17	n.d.	n.d.	66	99
7	OYE + ATA-Vfl	**1**	0	1	48	57	43	n.d.	n.d.	14	97
8	OYE + ATA Leu56Ile	**1**	0	1	48	15	85	n.d.	n.d.	70	95
9	OYE + ATA Leu56Val	**1**	0	1	48	82	18	n.d.	n.d.	65	98
10	OYE + ATA-Vfl	**1**	30	0	72	88	12	n.d.	n.d.	76	88
11	OYE + ATA Leu56Ile	**1**	30	0	72	59	41	n.d.	n.d.	18	81
12	OYE + ATA Leu56Val	**1**	30	0	72	94	6	n.d.	n.d.	89	87

n.d.: not detected. [a] The enantioselectivity of the ERED was >99% *ee* for the *S* enantiomer.

by small side chain variations at position 56 (located at the boundary of the two binding pockets) significantly affected diastereoselectivity.

To provide access to highly optically pure diastereomers, we then investigated the one-pot reaction starting with the reduction of the α/β-unsaturated ketone **1** by using the enoate reductases from *Saccharomyces carlsbergensis* (OYE) or from *Pseudomonas putida* (XenB). This afforded optically pure (*S*)-**2** with >99% *ee*[18] (Figure S8). Because OYE showed quantitative conversion, this enzyme was used for further investigations. To shift the equilibrium of the amination reaction, the well-established lactate dehydrogenase/glucose dehydrogenase (LDH/GDH) system was used;[16a,19] it also served to recycle the NADPH required for the ERED. Interestingly, **1** was not converted by ATAs, so the process could be performed as a cascade reaction in a simultaneous mode (both enzymes being present from the beginning). This setup was used in all subsequent experiments. In accordance with the results described above, application of the Leu56Val variant increased the diastereomeric purity of (1*R*,3*S*)-**3a** from 14% *de* (WT) to 65% *de* (mutant). In contrast, the mutant Leu56Ile gave the (1*S*,3*S*)-amine **3b** with 70% *de* (Table 1, entries 8 and 9, Figures S5–S7).

Because the ERED reaction exclusively produced the *S* enantiomer of **2**, it was possible to quantify the selectivity of the amine transaminase by determining the ratio of *cis* and *trans* products **3a** and **3b**. All ^{1}H NMR resonances of both diastereomers in the mixture were assigned through DQF-COSY experiments. The downfield-shifted H1 protons of the *cis* and the *trans* diastereomer significantly differ in their chemical shifts, by nearly 0.5 ppm, and were subsequently used for a stereochemical assignment with a one-dimensional NOESY version employing selective refocusing (Figure 2). Thus, on excitation of the H1 resonance at 2.62 ppm in the selective 1D NOESY experiment, NOEs develop for H3 and H5a as well as for the two directly neighboring H2a and H6a protons (Figure 2B). This places H1 and H3 on the same side of the cyclohexane ring system in close proximity, thus confirming the *cis* form with both substituents being in an equatorial position. On the other hand, upon excitation of the other H1 resonance at 3.08 ppm a noticeable NOE develops for the methyl group at position 7 in addition to the two H2a and H6a neighboring protons (Figure 2C). These NOEs, indicative of short interproton distances, reveal a *trans* configuration with the methyl group oriented on the same side as H1. Based on the unambiguous assignment

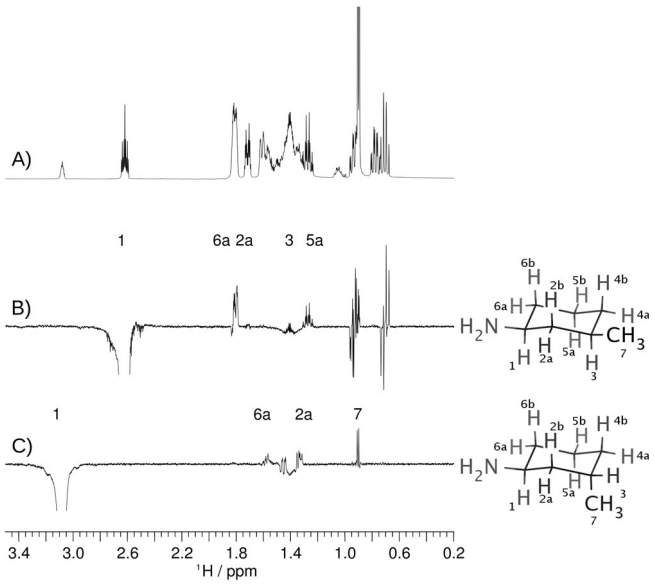


Figure 2. NMR spectra of the commercial 3-methylcyclohexylamine standard. A) Normal 1D spectrum. B), C) 1D NOESY spectra of B) the *cis*, and C) the *trans* diastereomer with selective excitation of the corresponding H1 protons at 2.62 and 3.08 ppm, respectively. The two antiphase multiplets at high field in (B) are artifacts arising from polarization transfer between H1 and the two scalar coupled H2b and H6b protons.

of the H1 protons of the two diastereomers, a preparative-scale cascade reaction (77% isolated yield) with the ERED and the ATA-Leu56Ile was found to result in a preference for the *trans* product **3b** over the *cis* product **3a** (Figure S9).

The dependence of enantioselectivity on the type of solvent is well known in traditional chemical synthesis,[21] but has also been described for enzyme reactions, including for ATAs.[22] Therefore we sought to identify suitable co-solvents that might further improve the enantioselectivity of the ATA reaction. Indeed, addition of DMSO or DMF during the transamination of *rac*-**2** (with ATA-Vfl and its mutants) results in increased formation of the *cis* products (Table 1, Figure S10).

The cascade reactions (Scheme 1) were then also investigated with the co-solvents DMSO and DMF. Fortunately, all of the enzymes (ATA, OYE, LDH, and GDH) are active in the presence of DMSO. Although OYE tolerated 30% DMSO, as reported earlier,[23] the enzyme was inactive in 30% DMF. High levels of DMSO increased the preference for the *cis* product **3a** in all three cases. We were pleased to find that, by adding 30% DMSO, the diastereomeric excess of the *cis* product (1*R*,3*S*)-**3a** could be substantially improved to 76 and 89% *de* in the presence of ATA-Vfl or the mutant Leu56Val, respectively. Because mutant Leu56Ile forms the *trans* product **3b**, DMSO addition had no useful effect for preparative purposes, but we observed a switch in diastereoselectivity towards a slight preference for the *cis* product **3a** (Table 1, entry 11). As described in other studies, such as the hydrolysis of α,α-disubstituted malonate esters,[24] the mechanisms of these solvent effects are currently not understood.

Our results demonstrate the advantage of a cascade based on the use of EREDs and ATAs for the successive introduction of the two stereogenic centers to yield particular diastereomers of the cyclic, alkyl-substituted amine **3**. Thus, we consider this cascade to represent a valuable synthetic strategy for the production of compounds of this class. Mutations leading to very small variations in the active site can strongly influence the diastereoselectivity of the amine transaminase from *V. fluvialis*, and this is further improved by use of co-solvents.

Acknowledgements

We thank the European Union (KBBE-2011-5, grant no. 289350) and the DFG (grant no. Bo1862/6-1) for financial support. We are grateful to Prof. Jon D. Stewart (University of Florida, USA) for the gene encoding OYE1 and Prof. Byung-Gee Kim (Seoul National University, Korea) for the gene encoding the ATA from V. fluvialis.

Keywords: amine transaminases · cascade synthesis · enantiopreference · enzyme catalysis · protein engineering

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Received: February 10, 2015
Published online on ■■ ■■, 0000

COMMUNICATIONS

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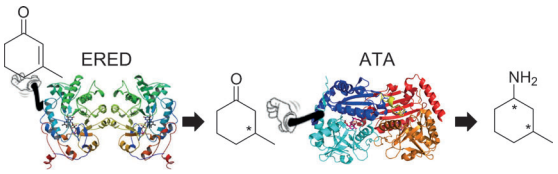
Two Subtle Amino Acid Changes in a Transaminase Substantially Enhance or Invert Enantiopreference in Cascade Syntheses



A single mutation, a big difference: An amine transaminase (ATA) and an enoate reductase (ERED) combined in a cascade reaction allowed the production of amines containing two chiral centers. Single-point mutations of the ATA substantially improved (L56V) or even inverted (L56I) its selectivity and gave products with high diastereomeric excess.