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Isopropylamine as Amine Donor in Transaminase-Catalyzed Reactions: Better Acceptance through Reaction and Enzyme Engineering

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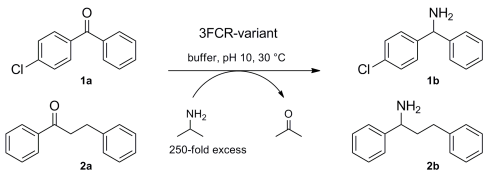
Abstract: Amine transaminases (ATA) have now become frequently used biocatalysts in chemo-enzymatic syntheses including industrial applications. They catalyze the transfer of an amine group from a donor to an acceptor leading to an amine product with high enantiopurity. Hence, they represent an environmentally benign alternative for waste intensive chemical amine synthesis. Isopropylamine (IPA) is probably one of the most favored amine donors since it is cheap and achiral, but nevertheless there is no consistency in literature concerning reaction conditions when IPA is best to be used. At the same time there is still a poor understanding which structural properties in ATA are responsible for IPA acceptance. Herein, we demonstrate, on the basis of the 3FCR enzyme scaffold, a substantial improvement in catalytic activity towards IPA as the amine donor. The asymmetric synthesis of industrial relevant amines was used as model reaction. A systematic investigation of the pH-value as well as concentration effects using common benchmark substrates and several ATA indicates the necessity of a substrate- and ATA-dependent reaction engineering.

Transaminases are versatile and widely used biocatalysts for the production of chiral amines. They catalyze the transfer of an amine group from an amine donor to a ketone or aldehyde acceptor, following a Ping-Pong Bi-Bi-reaction mechanism utilizing pyridoxal-5'-phosphate (PLP) as cofactor, revealing the amine product in a high enantiopurity. Transaminases belong to the fold types I and IV of PLP-dependent enzymes and are additionally divided into six subclasses, depending on the natural substrate and especially the position of the transferred amine group and/or carboxyl moiety.[1,2] Amine transaminases (ATA), a subgroup of ω-TA (class III transaminase family), are of special interest for the chemo-enzymatic application. In contrast to ω-TA, which accept carbonylic substrates with a distal carboxylate group, ATA tolerate substrates without a carboxyl moiety and therefore a wide range of ketones and aldehydes. Hence, in the last decade ATA became very attractive targets for enzyme engineering[3–13] representing an environmentally benign alternative for the chemical transition metal-catalyzed amine synthesis in pharmaceutical and agrochemical industries.[14] For instance, the production of imagabaline,[10] (*S*)-rivastigmine[15,16] or (*S*)-ivabradine[17] has been realized on larger scale using ATA. Certainly, one of the most notable examples is the manufacturing of (*R*)-sitagliptin in >200 g/L scale after several rounds of protein engineering of the selected ATA.[3] In this specific example, enzyme engineering resulted not only in a better substrate acceptance or higher temperature and solvent stability, but also in an enhanced isopropylamine (IPA) acceptance. The best variant contained 27 mutations. To have the enzyme accepting the bulky prositagliptin ketone, the most mutations were around the active site region. However, to ensure acceptance/tolerance of IPA many mutations were globally distributed over the entire protein since the majority of wild-type ATA do not accept it well as the amine donor. More recently, we could also design (*S*)-selective ATAs for the asymmetric synthesis of chiral amines from sterically demanding bulky ketones.[6,13,18]

The asymmetric synthesis is the most convenient and economically favored route to a target chiral amine. In contrast to the kinetic resolution mode, the asymmetric synthesis starts from the prochiral ketone and results in the desired chiral product with a theoretical yield of 100%.[19–21] The downside of this strategy is a very often unfavorable reaction equilibrium, which makes strategies for equilibrium shifting necessary. Therefore several equilibrium displacement techniques were established, for instance involving enzymatic cascades in order to remove co-products,[19,22–26] utilization of 'smart-donors' which are converted into sacrificial co-substrates after transamination[27–32] or application of the amine donor in large excess.[33] In fact, IPA is the industrially favored amine donor for asymmetric syntheses since it is cheap, achiral – so the enantioselectivity of the ATA has not to be considered – and the by-product acetone is supposed to have a drastic lower reactivity in the back reaction.[33] Additionally, in terms of shifting the equilibrium, acetone can be easily removed from the reaction solution.[3] In general, it was reported in many cases that an excess of IPA needs to be applied to drive the transamination of various substrates to the desired product side[8,13,18,23,34–44] when enzyme engineering did not lead to a better IPA acceptance, as mentioned above. In particular, different reaction conditions were reported such as varying donor-acceptor-ratio from 1.5-fold[39] over 40-fold[38] to 200-fold[8] and a pH range from 7.3[41] to 9.5.[45]



Scheme 1. Model reactions and preparative scale reactions with IPA as amine donor using substrates as reported in literature.[6]

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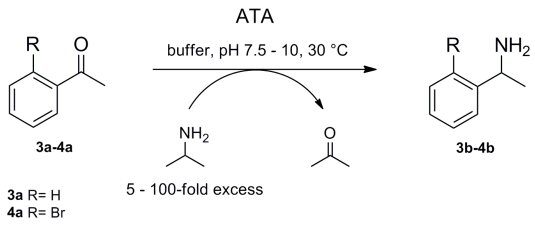
Table 1. Comparison of the presented variants of 3FCR regarding initial activity measurements with racemic **1b** or **2b** and asymmetric synthesis of **1b** or **2b** using IPA as the amine donor. * n.d.: not detectable

ATA variant	Specific activity [mU mg^{-1}][a]		Substrate conversion [%][b]			
	***rac*-1b**	***rac*-2b**	1a	ee_P [%][c]	2a	ee_P [%][c]
3FCR-QM	218.6 ± 16.8	104.9 ± 1.6	12.8 ± 1.2	> 99 (*R*)	n.d.*	-
3FCR-QM-W59L	88.1 ± 4.2	70.3 ± 1.5	6.8 ± 1.5	86 (*R*)	8.9 ± 1.8	> 99 (*S*)
3FCR-QM-W59L-R420A	141.2 ± 1.6	63.5 ± 0.6	50.6 ± 3.6	91 (*R*)	23.8 ± 2.5	> 99 (*S*)
3FCR-QM-W59L-R420W	70.9 ± 0.7	90.9 ± 4.3	83.5 ± 4.0	80 (*R*)	85.8 ± 3.5	> 99 (*S*)

[a] Assay conditions for the kinetic resolution: 0.5 mM *rac*-**1b** or 0.25 mM *rac*-**2b**, pentanal in equimolar ratio, 10% (v/v) DMSO, CHES pH 10 (50 mM), 0.045 – 0.07 mg mL^{-1} purified enzyme, 30 °C.
[b] Assay conditions for the asymmetric synthesis: 2 mM ketone (**1a** or **2a**), 0.5 M IPA, 30% (v/v) DMSO, CHES buffer pH 10 (50 mM), 0.1 mM PLP, 0.89 - 1.2 mg mL^{-1} purified enzyme (substrate to enzyme ratio, s/e ~ 0.35 – 0.47 w/w), 30 °C. Conversion was determined after 20 h via gas chromatography (GC) with 2-iodoacetophenone as internal standard for the quantification of amine product formation. Samples were taken in triplicate from three parallel reactions.
[c] The enantiomeric excess was determined via chiral GC analysis using a Hydrodex-ß-TBDAc column (Macherey & Nagel).

So far, there has been no study, in which the influence of IPA on transaminase activity has been explored to cover both aspects, the acceptance of IPA as amine donor (requiring identification of optimal pH values, concentration dependency as well as mutations in the active site region) and the influence of IPA to the overall protein stability as this amine donor also has solvent effects on the enzyme. In this work, crucial amino acid residues around the active site were identified for IPA acceptance and also a systematic investigation was performed for a range of ATA, different ketone substrates and different reaction conditions with the aim of providing a more generic solution for ATA-catalyzed asymmetric synthesis using IPA.

Recently, we reported the engineering of the ATA from *Ruegeria* sp. TM1040 (PDB-ID: 3FCR) for the acceptance of bulky ketones where the corresponding amines have pharmaceutical relevance.[6,13,18] The best variants from this approach (e.g. one quadruple mutant 3FCR-Y59W-Y87F-Y152F-T231A, 3FCR-QM) did not accept IPA well as the amine donor.[13,18] We already identified position 59 in 3FCR (according to Protein Data Bank numbering) as very crucial for the activity towards aromatic and bulky substrates.[6,46] Interestingly, the homolog position in the ω-transaminase from *Ochrobactrum anthropoi* (OATA, W58) was reported to play a comparable role in which the mutation OATA-W58L led to a better acceptance of aromatic ketones and amines.[12] However, at the same time this mutation was obviously responsible for a higher affinity towards IPA, which was proven by lower K_M values and explained by steric interference of W58 with one of the methyl groups of IPA. Because of the notable sequence identity between OATA (PDB-ID 5GHG) and 3FCR of approx. 43% we decided to focus again on position 59 in the 3FCR scaffold. Starting from the variant 3FCR-QM as template we saturated position 59 and screened the NNK library against IPA using the glycine oxidase (GO) assay, as described previously.[47] It turned out that the only variant with significant higher activity towards IPA as amine donor contained the mutation 3FCR-QM-W59L which led to a 4.6-fold higher activity compared to the template (see Table S1, Supporting Information). Moreover, transaminases are remarkable for their dual substrate recognition facilitated by a flexible arginine residue, which is forming a salt bridge to the carboxylate function of the respective substrate. It is highly conserved in e.g. amino acid transaminases, ornithine transaminases and amine transaminases.[1,46] Notably, the ω-amino acid transaminase from *Bacillus anthracis* (Ban-TA) represents an exception as the 'flipping arginine' is naturally replaced by a glycine, which means that no movement of an amino acid residue is required for substrate recognition. Ban-TA showed provable activity towards propylamines (e.g. IPA) as amine donors.[48] However, when the choice of substrates make the coordination of acidic moieties obsolete, the role of the 'flipping arginine' (position 420 in 3FCR) is questionable and in addition a strong positive charge at the entrance of the active site tunnel was considered to be detrimental for the accessibility of IPA on the PLP molecule. We used 3FCR-QM as template and introduced at position 420 several amino acids with different characteristics (hydrophobic, basic and acidic). The variants were interrogated in our chosen asymmetric synthesis model reactions (Scheme 1), because the GO-assay demands coordination of glyoxylate and therefore was not suitable for the purpose of a screening. The best-performing variant was the mutation R420W (Table S2, SI), which we later introduced into the 3FCR-QM-W59L variant. Additionally, we produced the variant 3FCR-QM-W59L-R420A in order to provide an amino acid residue at this position, which causes more space and low interference at the entrance of the active site tunnel. Both variants were compared in the mentioned model reactions with IPA revealing a drastic improvement in conversion of **1a** and **2a** compared to 3FCR-QM and 3FCR-QM-W59L (Table 1), especially in case of 3FCR-QM-W59L-R420W. Remarkably, the mutation at position 59 in 3FCR-QM – which showed a significant improvement in the GO-assay with IPA – did not lead to a better conversion of **1a** to **1b** but at the same time a detectable product formation of **2b**. These results (especially for 3FCR-QM-W59L-R420W) are more meaningful when the activities in kinetic resolution mode using *rac*-**1b** and *rac*-**2b** are considered.



Scheme 2. Reactions studied for the investigation of conditions using IPA as amine donor. Various donor-acceptor ratio and pH values were compared.

All three variants of 3FCR-QM showed lower activities for both racemic amines (Table 1). For comparison, pentanal was used as amine acceptor for all four enzymes because pyruvate was not a suitable amine acceptor anymore mainly due to the lack of the 'flipping arginine' (details in Fig. S1, SI).[49] These results are demonstrating that the introduced mutations in 3FCR-QM were not responsible for a better substrate acceptance of **1a** and **2a** – since mutations at position 59 have a great impact on the reactivity towards bulky substrates in 3FCR – but rather they did lead to a higher catalytic activity in asymmetric synthesis mode with IPA as the amine donor. The best two variants 3FCR-QM-W59L-R420A/W were subjected to the preparative scale production of amines **1b** and **2b** in a 80 mg scale applying 0.5 M IPA (see experimental section). The identity of the products was confirmed via GC-MS, ^{1}H- and ^{13}C-NMR spectroscopy (see SI).

Next, we investigated the influence of reaction conditions on the transaminase reactivity with IPA as amine donor. For that we included our presented variants of 3FCR-QM and additionally several commonly known wild-type ATA, which in pre-tests showed significant conversion with IPA. Those wild-type ATA were utilized in engineering and asymmetric synthesis approaches before.[4–6,21,26,46,49–53] For instance, Afu-TA (ATA from *Aspergillus fumigatus*) and Spo-TA (ATA from *Silicibacter pomeroyi*) were recently used for the production of halogenated chiral amines[54] and Arth-TA (ATA from *Arthrobacter* sp.) for the synthesis of e.g. (*R*)-3,4-dimethoxy-amphetamine.[20,21] 3FCR wild-type and 3FCR-QM were excluded from these experiments due to their low reactivity in pre-tests. We selected commonly used aromatic benchmark substrates for the evaluation of amine donors[26,31,32,38] (**3a** and **4a**, Scheme 2) and tested different pH values as well as donor-acceptor ratio (Fig. 1). The amination of acetophenone **3a** to 1-phenylethylamine **3b** is challenging due to the unfavored equilibrium,[14,55] so significant effects were expected after reaction engineering. Additionally, we investigated one halogenated derivative of acetophenone (**4a**), since Cassimjee *et al.*[26] show-cased significant differences in conversion with substituted acetophenones and IPA using the ATA from *Chromobacterium violaceum* (Cvi-TA). The donor-acceptor ratio varied in a range of 5-fold – 100-fold excess of IPA at the respective pH optimum of the ATA (Fig. S2, SI).

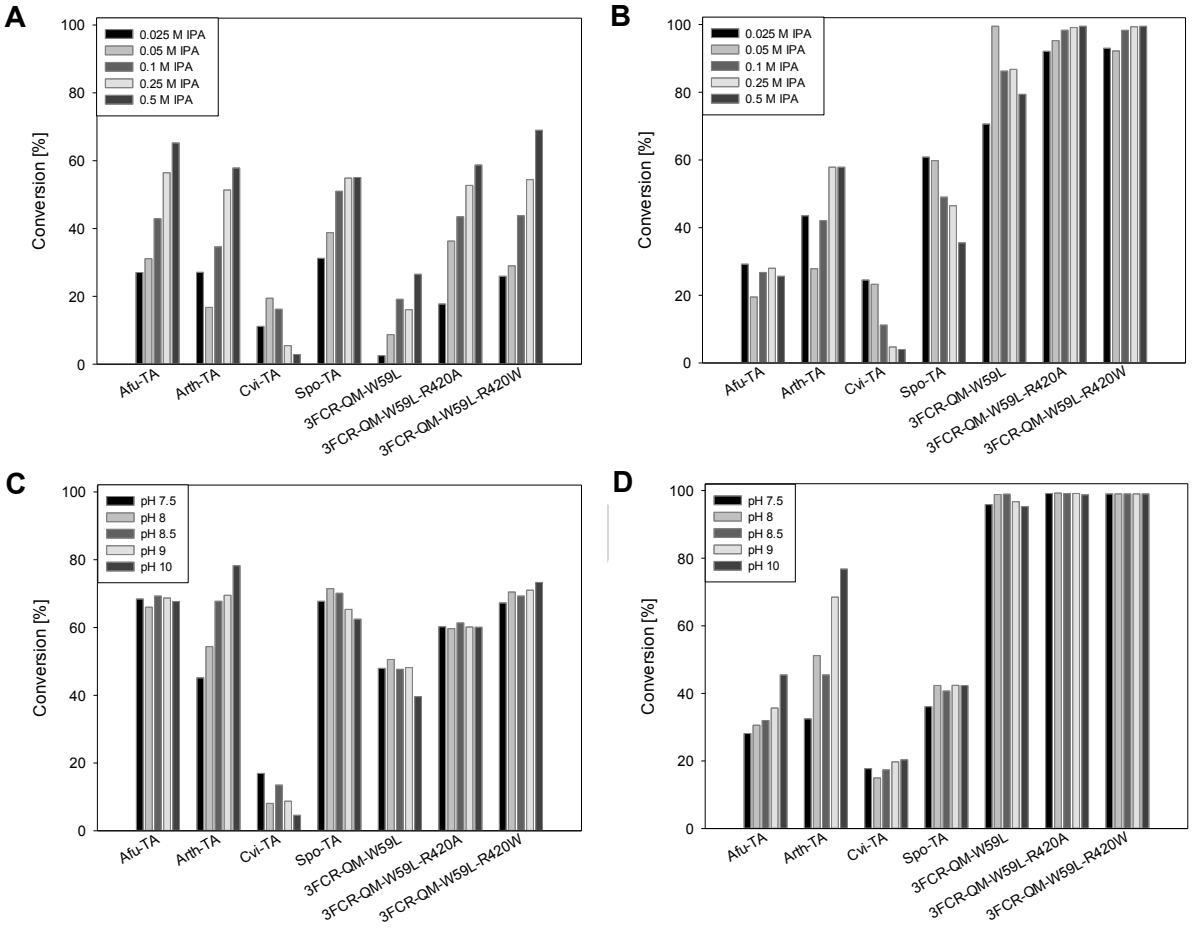


Fig. 1. Asymmetric synthesis of **3b** (A, C) and **4b** (B, D) using IPA as amine donor. The influence of different donor-acceptor-ratio (A, B) and pH values (C, D) was investigated. Wild type ATA from *Aspergillus fumigatus* (Afu-TA), *Arthrobacter* sp. (Arth-TA), *Chromobacterium violaceum* (Cvi-TA), *Silicibacter pomeroyi* (Spo-TA) and the presented variants of 3FCR (ATA from *Ruegeria* sp. TM1040) were used. **A**, **B** IPA was applied in the following excess related to the amine acceptor: from left to right 5-fold, 10-fold, 25-fold, 50-fold and 100-fold (in a range of 0.025 – 0.5 M). The concentration of the ketone substrates was fixed at 5 mM. **C, D** Different pH values were set using Davies buffer[56]: from left to right pH 7.5, pH 8, pH 8.5, pH 9 and pH 10. The shown conversion levels are mean values out of duplicates. For single values see Supporting Information. General reaction conditions: 1 mg mL^{-1} purified enzyme, 5 mM **3a** (substrate to enzyme ratio, s/e ~ 0.6 w/w) or **4a** (s/e ~0.9 w/w), 5% (v/v) DMSO, 0.1 mM PLP, 50 mM HEPES/CHES buffer pH 7.5 or 9 (according to each pH optimum, see Supporting Information), 30 °C. Samples were taken after 20 h and analyzed via gas chromatography with 2-iodoacetophenone as internal standard.

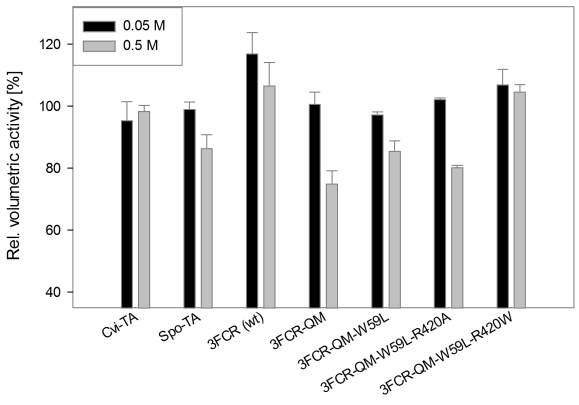


Fig. 2. Volumetric activities (U/mL) determined via the initial activity assay after incubation with IPA over 8 h (two concentrations were used as indicated). Relative activities were normalized to an IPA free control experiment. Reaction conditions: 0.05 – 0.1 mg mL^{-1} purified protein, 1.25 mM (*S*)-PEA (1-phenylethylamine), pyruvate or pentanal in equimolar ratio, 0.5 – 5% (v/v) DMSO in CHES pH 9 (50 mM). The formation of acetophenone was quantified at 245 nm at 30 °C. All measurements were performed in triplicate. The compatibility of this assay under these conditions was ensured by previous solvent exchange (Fig. S5 and S6, Supporting Information).

In case of acetophenone **3a** the gradual increase of the IPA concentration resulted in an increased conversion of **3a**. The only exception out of all investigated ATA was Cvi-TA showing a decreased conversion level of **3a** at high IPA concentrations. For 2'-bromoacetophenone **4a** no general statement in terms of IPA excess effect could be made. While the results for Arth-TA showed an increased activity at higher IPA concentrations, Spo-TA and Cvi-TA were apparently less active the more IPA was applied. But for the rest of the investigated ATA there was no significant difference in conversion over the whole range of applied IPA concentrations indicating a more favored equilibrium situation. This was in line with previous results from literature mentioned above.[38] All three 3FCR-QM variants showed indeed the highest conversion of **4a**, in case of 3FCR-QM-R420A and 3FCR-QM-R420W even with quantitative conversion. The effect of the pH on the conversion level of **3a** and **4a** was investigated over a range of pH 7.5 – 10, which was considered as the common pH range for the most ATA reactions in literature. For these experiments the IPA concentration was fixed to 0.5 M to ensure no limitations here. Beside two significant exceptions (Afu-TA and Arth-TA) the pH value did not influence the catalytic activity of the ATA in both of the showed model reactions with IPA. The two mentioned exceptions showed the trend that a more basic pH is more beneficial for transamination reactions with IPA since the concentration of unprotonated IPA is certainly higher (Fig. 1C, 1D). Especially Arth-TA is interesting in this manner since the best conversion of both **3a** and **4a** was reached at pH 10 and in contrast the pH optimum of this enzyme is at pH 8 (see SI). But the majority of the ATA showed a similar activity in both reactions regardless of pH optima (see SI for all ATA) and protonation state of IPA. To further support the results from Fig. 1 we looked at enzyme stability in presence of IPA. Beside the three presented variants of 3FCR-QM we chose exemplary Cvi-TA and Spo-TA to cover all variations of catalytic activity from Fig. 1. 3FCR wild-type and 3FCR-QM were included for comparison. After incubation with IPA (final concentration of 0.05 and 0.5 M) over 8 h at 30 °C the residual activity of the ATA were quantified via the initial activity assay (acetophenone assay,[57] Fig. 2). Interestingly, the majority of the 3FCR variants were apparently affected by IPA incubation and not any wild-type enzyme. But a considerable detrimental effect due to IPA incubation was not obvious for any investigated ATA. Additionally, protein melting points were determined in the presence of IPA (see Table S5, SI) giving a similar result. This experiment on ATA stability demonstrated that a higher tolerance towards IPA was not the reason for the better conversion in the investigated reactions by 3FCR-QM variants.

Conclusively, the results from the pH and donor-acceptor-ratio experiments revealed that both aspects should be considered as enzyme as well as substrate dependent (or a combination of both). Therefore no general guideline for an optimal reaction setup can be derived. It appears that every ATA reaction using IPA needs to be optimized relating to the mentioned aspects since they likely have overlapping effects, e.g. pH optima of the enzyme, pH/solvent stability and protonation state of IPA. Through this reaction engineering even wild-type enzymes could reach moderate to good activities with IPA as shown here and also in previous works.[54] Thus, we demonstrated a remarkable increase in catalytic activity towards IPA as amine donor mutating amino acid residues around the active site in the 3FCR-QM scaffold, namely positions 59 and 420. It has to be highlighted that the presented 3FCR-QM variants exhibit a good to excellent performance in all shown reactions, e.g. over a broad range of pH values and donor-acceptor ratio. The mutation 59L is an important key mutation for the catalytic activity towards IPA since the conversion in reactions with **2a** – **4a** was already substantially increased. It should be noted however that in terms of better IPA acceptance the role of both positions (59 and 420) is definitely substrate dependent. With the variant 3FCR-QM-W59L-R420W a general approach for an improved IPA acceptance for the investigated reactions was identified.

Experimental Section

All chemicals and kits were purchased either from Sigma Aldrich (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Acros/Thermo Fisher Scientific (Waltham, USA) in analytical grade. The ketones **1a** and **2a** as well as the corresponding racemic amines (Scheme 1) were kindly provided by F. Hoffmann-La Roche.

Enzyme expression, cell lysis and protein purification

Information about the plasmids containing genes of ATAs from *Chromobacterium violaceum*, *Silicibacter pomeroyi*, *Arthobacter* sp., *Aspergillus fumigatus* and *Ruegeria* sp. TM1040 are given in Table S3 (Supporting Information). The protein expression was done in Terrific Broth (TB) media with 100 µg mL^{-1} ampicillin or 50 µg mL^{-1} kanamycin at 160 rpm and 20°C. After the optical density at 600 nm (OD_{600}) reached 0.5 – 0.7, expression was induced by adding 0.2 mM isopropyl β-D-1-thiogalactopyranoside (final concentration). After 18 h the cultures were centrifuged (4,000 x *g*, 15 min, 4 °C) and washed with lysis buffer (HEPES (50 mM pH 7.5), 0.1 mM PLP, 300 mM NaCl). Cell disruption was performed via sonication using the Bandelin Sonoplus HD 2070 (8 min, 50% pulsed cycle, 50% power) on ice followed by centrifugation in order to remove cell debris (12,000 x *g*, 45 min, 4 °C, Sorvall centrifuge). The supernatant containing the crude ATA was stored at 4 °C until use. Metal affinity chromatography was used to purify all enzymes with an Äkta purifier and a 5-mL HiTrap Fast Flow column (GE Healthcare, Freiburg, Germany). Elution was mediated by the lysis buffer additionally containing 300 mM imidazole. The desalting step was

performed using three HiTrap desalting columns in line (each 5 mL; GE Healthcare, Freiburg, Germany) and lysis buffer without NaCl. The protein concentration was determined via the Pierce BCA Protein Assay Kit according to the manual.

Point mutations via *QuikChange* mutagenesis

Variants of 3FCR-QM were produced using a modified version of the *QuikChange* PCR method. Primers were designed with the desired mismatches to provide the desired mutations. For each PCR, Pfu buffer, 0.2 mM dNTPs, 0.2 ng μL^{-1} parental plasmid, 0.2 µM of each primer and 0.2 µL of Pfu Plus! DNA polymerase were applied. A DMSO concentration of 3% (v/v) was set. The amplification was performed as follows: (a) 94 °C, 2 min; (b) 25 cycles: 94 °C, 30 s; 55 °C or 60 °C, 30 s; 72 °C, 7:05 min (c) 72 °C, 14 min. The PCR product was digested with DpnI (20 µL mL^{-1}) for 2 h at 37 °C and the restriction enzyme was inactivated by incubation at 80 °C for 20 min. Chemo-competent Top10 *E. coli* cells were transformed with the PCR product. After confirmation of the correct sequence the plasmids were isolated from Top10 and chemo-competent *E. coli* BL21 (DE3) cells were transformed for protein expression as described above.

Determination of transaminase activity

The characterization of the ATA was done via the initial activity assay (acetophenone assay) according to Schätzle *et al.*[57] with slight modifications. In the reaction solution the concentrations of the amine donor ((*R*)-/(*S*)-1-PEA **3b**, *rac*-**1b** or *rac*-**2b**) and the acceptor pyruvate or pentanal was set to 1.25 mM or as indicated in 0.5% - 10% (v/v) DMSO. Briefly, 10 µL of a pre-diluted ATA solution was mixed with the respective buffer (according to the pH optimum of each ATA, HEPES or CHES buffer) and the reaction was initiated by the addition of the 4-fold concentrated stock of reaction solution. The formation of the corresponding ketone was quantified at 245 nm (**2a** and **3a**) or 265 nm (**1a**), respectively using the Tecan Infinite M200 Pro (Crailsheim, Germany) at 30 °C. One unit (U) of ATA activity was defined as the formation of 1 µmol of **1a** – **3a** per minute (**1a** ε= 16.56 $M^{-1}cm^{-1}$, **2a** ε= 9.65 $M^{-1}cm^{-1}$, **3a** ε= 12 $M^{-1}cm^{-1}$). All measurements were performed in triplicate. The pH optimum of each ATA was determined by using Davies[56] buffer with a pH value as indicated.

Asymmetric synthesis of the chiral amines 1b **–** 4b

Biotransformations were performed in 0.25 mL scale using 1.5 mL glass vials at 30 °C and 950 rpm shaking. The reaction mixtures contained 1 mg mL^{-1} purified ATA, 5 mM ketone, 30% DMSO as co-solvent, the respective concentration of IPA (from a 4 M IPA-HCL stock solution, pH 7) in HEPES, CHES (50 mM) or Davies buffer as indicated. The final pH was checked. Additionally, control experiments with desalting buffer instead of enzyme were performed. After 20 h incubation, the reaction was quenched by adding 3 M NaOH (resulting in pH ≥12). Samples for gas chromatography (GC) analysis were taken immediately after quenching.

GC analysis

Samples of 100 µL were withdrawn for chiral GC analysis and extracted with 300 µL of ethyl acetate containing 1 mM 2'-iodoacetophenone as internal standard for quantification. The organic layers were dried over anhydrous $MgSO_4$ and derivatized (when necessary) with *N*-Methyl-bis-trifluoroacetamide (MBTFA) by adding 7.5 µL of the commercial stock solution to 100 µL of the organic layer and incubation at 60 °C for 30 min. Afterwards, the samples were analyzed immediately using the Hydrodex-ß-TBDAc column (Macherey & Nagel). For the analysis of substances **1** and **2** the following temperature gradient program was established: initial temperature 140 °C, kept for 15 min, linear gradient to 180 °C with a slope of 15 °C min^{-1}, kept for 35 min, linear gradient to 220 °C with a slope of 15 °C min^{-1}, kept for 10 min. For **3**: initial temperature 120 °C, kept for 5 min, linear gradient to 220 °C with a slope of 10 °C min^{-1}, kept for 5 min. For **4**: initial temperature 100 °C, kept for 7.5 min, linear gradient to 220 °C with a slope of 5 °C min^{-1}, kept for 5 min. For retention times see Supporting Information. The conversion of **1a** and **2a** was determined by quantification of amine product formation via calculation of the response factor. In case of **3a** and **4a** the same principle was followed regarding substrate consumption. Each sample included the mentioned internal standard and was set in relation to ATA-free control experiments.

Preparative scale synthesis of 1b **and** 2b

The conversion of **1a** and **2a** to the corresponding amine was performed in preparative scale. 87 mg **1a** (using 3FCR_QM_W59L_R420A) and 84 mg **2a** (using 3FCR_QM_W59L_R420W) were applied respectively in an Erlenmeyer flask and dissolved in DMSO (5% final concentration). CHES buffer (50 mM final, pH 9) and isopropylamine (0.5 M final concentration) were added under stirring. The pH was adjusted with aqueous HCl. In the end the reaction was started by addition of 1 mg mL^{-1} enzyme which led to a final working volume of 0.2 L and a final substrate concentration of 2 mM (**1a** s/e ~ 0.43, **2a** s/e ~ 0.42). The reaction mixture was incubated for 48 h at 30 °C under agitation. For the quantification of the conversion samples were taken, extracted as described above and analyzed via GCMS. The reaction was stopped when no further conversion was observed during reaction monitoring (for **1a** 53%, for **2a** 27%). The following reaction workup was done: After reaction quenching with 10 mL 3 M NaOH to a pH of >12, an extraction with 1x 0.2 L and 1x 0.1 L hexane was performed in a separation funnel. The combined organic layers were dried over anhydrous $MgSO_4$ and evaporated under vacuum to a volume of 0.5 mL. The crude reaction product was applied to a silica column with ethyl acetate as mobile phase. The fractionation monitoring was done via TLC. Fractions containing the respective amine product were pooled and evaporated under vacuum until dryness. The consistency of the amine products was a yellow oil. Each product was confirmed via GCMS and 10-12 mg each were subjected to ^{1}H- and ^{13}C-NMR spectroscopy (see Supporting Information). **1b**, 53% conv., 35% isolated yield (not optimized), 55.5%*ee*. **2b**, 27% conv., 33% isolated yield (not optimized), 98%*ee*.

ATA stability in the presence of IPA

Enzyme stability was investigated by incubation samples of ATA with IPA (0, 0.05 and 0.5 M final concentration) for 8 h at 30 °C at 950 rpm shaking. Afterwards a solvent change was performed via PD columns® (GE Healthcare, Freiburg, Germany) according to the manual. Fractions of 0.5 mL were taken and the most active one was subjected to further investigations. The residual enzyme activity was measured via the initial activity assay. For reaction conditions see figure capture. Additionally, melting points T_M of each subjected ATA in presence of 0, 0.05 and 0.5 M IPA (final concentration) were determined using the Prometheus NT.48 device from nanotemper® (see Supporting Information). The protein concentration was set to 1 mg mL^{-1} in HEPES buffer pH 7.5 (50 mM) including 50 µM of PLP. The heating rate was set to 0.5 °C min^{-1} from 20 – 95 °C. Inflection points (or melting points, respectively) were determined by the first derivative of the measured fluorescence at a 330/350 nm ratio.

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[1] F. Steffen-Munsberg, C. Vickers, H. Kohls, H. Land, H. Mallin, A. Nobili, L. Skalden, T. van den Bergh, H.-J. Joosten, P. Berglund, *Biotechnol. Adv.* **2015**, *33*, 566–604.
[2] N. V. Grishin, M. A. Phillips, E. J. Goldsmith, *Protein Sci.* **1995**, *4*, 1291–1304.
[3] C. K. Savile, J. M. Janey, E. C. Mundorff, J. C. Moore, S. Tam, W. R. Jarvis, J. C. Colbeck, A. Krebber, F. J. Fleitz, J. Brands, *Science* **2010**, *329*, 305–309.
[4] A. Nobili, F. Steffen-Munsberg, H. Kohls, I. Trentin, C. Schulzke, M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2015**, *7*, 757–760.
[5] M. Genz, O. Melse, S. Schmidt, C. Vickers, M. Dörr, T. van den Bergh, H.-J. Joosten, U. T. Bornscheuer, *ChemCatChem* **2016**, *8*, 3199–3202.
[6] I. V. Pavlidis, M. S. Weiß, M. Genz, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *Nat. Chem.* **2016**, *8*, 1076–1082.
[7] B.-K. Cho, H.-Y. Park, J.-H. Seo, J. Kim, T.-J. Kang, B.-S. Lee, B.-G. Kim, *Biotechnol. Bioeng.* **2008**, *99*, 275–84.
[8] D. F. Dourado, S. Pohle, A. T. Carvalho, D. S. Dheeman, J. M. Caswell, T. Skvortsov, I. Miskelly, R. T. Brown, D. J. Quinn, C. C. Allen, *ACS Catal.* **2016**, *6*, 7749–7759.
[9] K. Deepankumar, S. P. Nadarajan, S. Mathew, S.-G. Lee, T. H. Yoo, E. Y. Hong, B.-G. Kim, H. Yun, *ChemCatChem* **2015**, *7*, 417–421.
[10] K. S. Midelfort, R. Kumar, S. Han, M. J. Karmilowicz, K. McConnell, D. K. Gehlhaar, A. Mistry, J. S. Chang, M. Anderson, A. Villalobos, *Protein Eng. Des. Sel.* **2013**, *26*, 25–33.
[11] S.-W. Han, E.-S. Park, J.-Y. Dong, J.-S. Shin, *Adv. Synth. Catal.* **2015**, *357*, 2712–2720.
[12] S.-W. Han, E.-S. Park, J.-Y. Dong, J.-S. Shin, *Adv. Synth. Catal.* **2015**, *357*, 1732–1740.
[13] M. S. Weiß, I. V. Pavlidis, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *ChemBioChem* **2017**, *18*, 1022–1026.
[14] M. Höhne, U. T. Bornscheuer, *ChemCatChem* **2009**, *1*, 42–51.
[15] M. Fuchs, D. Koszelewski, K. Tauber, W. Kroutil, K. Faber, *Chem. Commun.* **2010**, *46*, 5500–5502.
[16] M. Fuchs, D. Koszelewski, K. Tauber, J. Sattler, W. Banko, A. K. Holzer, M. Pickl, W. Kroutil, K. Faber, *Tetrahedron* **2012**, *68*, 7691–7694.
[17] S. Pedragosa-Moreau, A. Le Flohic, V. Thienpondt, F. Lefoulon, A.-M. Petit, N. Ríos-Lombardía, F. Morís, J. González-Sabín, *Adv. Synth. Catal.* **2017**, *359*, 485–493.
[18] M. S. Weiß, I. V. Pavlidis, P. Spurr, S. P. Hanlon, B. Wirz, H. Iding, U. T. Bornscheuer, *Org. Biomol. Chem.* **2016**, *14*, 10249–10254.
[19] M. Höhne, S. Kühl, K. Robins, U. T. Bornscheuer, *ChemBioChem* **2008**, *9*, 363–365.
[20] A. Iwasaki, Y. Yamada, Y. Ikenaka, J. Hasegawa, *Biotechnol Lett* **2003**, *25*, 1843–1846.
[21] A. Iwasaki, Y. Yamada, N. Kizaki, Y. Ikenaka, J. Hasegawa, *Appl Microbiol Biotechnol* **2006**, *69*, 499–505.
[22] D. Koszelewski, I. Lavandera, D. Clay, G. M. Guebitz, D. Rozzell, W. Kroutil, *Angew. Chem.* **2008**, *120*, 9477–9480.
[23] M. D. Truppo, J. D. Rozzell, J. C. Moore, N. J. Turner, *Org. Biomol. Chem.* **2009**, *7*, 395–8.
[24] D. Koszelewski, I. Lavandera, D. Clay, D. Rozzell, W. Kroutil, *Adv. Synth. Catal.* **2008**, *350*, 2761–2766.
[25] P. Tufvesson, J. Lima-Ramos, J. S. Jensen, N. Al-Haque, W. Neto, J. M. Woodley, *Biotechnol. Bioeng.* **2011**, *108*, 1479–1493.
[26] K. E. Cassimjee, C. Branneby, V. Abedi, A. Wells, P. Berglund, *Chem. Commun.* **2010**, *46*, 5569–5571.
[27] A. Gomm, W. Lewis, A. P. Green, E. O'Reilly, *Chem.- Eur. J.* **2016**, *22*, 12692–12695.
[28] A. P. Green, N. J. Turner, E. O'Reilly, *Angew. Chem. Int. Ed.* **2014**, *53*, 10714–10717.
[29] L. Martınez-Montero, V. Gotor, V. Gotor-Fernández, I. Lavandera, *Adv. Synth. Catal.* **2016**, *358*, 1618–1624.
[30] S. E. Payer, J. H. Schrittwieser, W. Kroutil, *Eur. J. Org. Chem.* **2017**, *2017*, 2553–2559.
[31] I. Slabu, J. L. Galman, C. Iglesias, N. J. Weise, R. C. Lloyd, N. J. Turner, *Catal. Today* **2017**, doi.org/10.1016/j.cattod.2017.01.025.
[32] J. L. Galman, I. Slabu, N. J. Weise, C. Iglesias, F. Parmeggiani, R. C. Lloyd, N. J. Turner, *Green Chem.* **2017**, *19*, 361–366.
[33] I. Slabu, J. L. Galman, R. C. Lloyd, N. J. Turner, *ACS Catal.* **2017**, *7*, 8263–8284.
[34] F. G. Mutti, C. S. Fuchs, D. Pressnitz, J. H. Sattler, W. Kroutil, *Adv. Synth. Catal.* **2011**, *353*, 3227–3233.
[35] W. Neto, M. Schürmann, L. Panella, A. Vogel, J. M. Woodley, *J. Mol. Catal. B Enzym.* **2015**, *117*, 54–61.
[36] A. Cuetos, M. Garcıa-Ramos, E.-M. Fischereder, A. Dıaz-Rodrıguez, G. Grogan, V. Gotor, W. Kroutil, I. Lavandera, *Angew. Chem.* **2016**, *128*, 3196–3199.
[37] U. Kaulmann, K. Smithies, M. E. Smith, H. C. Hailes, J. M. Ward, *Enzyme Microb. Technol.* **2007**, *41*, 628–637.
[38] C. E. Paul, M. Rodrıguez-Mata, E. Busto, I. Lavandera, V. Gotor-Fernández, V. Gotor, S. Garcıa-Cerrada, J. Mendiola, Ó. de Frutos, I. Collado, *Org. Process Res. Dev.* **2014**, *18*, 788–792.
[39] E.-S. Park, J.-Y. Dong, J.-S. Shin, *Org. Biomol. Chem.* **2013**, *11*, 6929–6933.
[40] M. Truppo, J. M. Janey, B. Grau, K. Morley, S. Pollack, G. Hughes, I. Davies, *Catal Sci Technol* **2012**, *2*, 1556–1559.
[41] M. Svedendahl, C. Branneby, L. Lindberg, P. Berglund, *ChemCatChem* **2010**, *2*, 976–980.
[42] D. Koszelewski, K. Tauber, K. Faber, W. Kroutil, *Trends Biotechnol.* **2010**, *28*, 324–32.
[43] L. H. Andrade, W. Kroutil, T. F. Jamison, *Org. Lett.* **2014**, *16*, 6092–6095.
[44] T. Börner, S. Rämisch, E. R. Reddem, S. Bartsch, A. Vogel, A.-M. W. H. Thunnissen, P. Adlercreutz, C. Grey, *ACS Catal.* **2017**, *7*, 1259–1269.
[45] H. Mallin, M. Höhne, U. T. Bornscheuer, *J. Biotechnol.* **2014**, *191*, 32–7.
[46] F. Steffen-Munsberg, C. Vickers, A. Thontowi, S. Schätzle, T. Meinhardt, M. Svedendahl Humble, H. Land, P. Berglund, U. T. Bornscheuer, M. Höhne, *ChemCatChem* **2013**, *5*, 154–157.
[47] M. S. Weiß, I. V. Pavlidis, C. Vickers, M. Höhne, U. T. Bornscheuer, *Anal. Chem.* **2014**, *86*, 11847–53.
[48] F. Steffen-Munsberg, P. Matzel, M. A. Sowa, P. Berglund, U. T. Bornscheuer, M. Höhne, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 4511–4521.
[49] M. Genz, C. Vickers, T. van den Bergh, H.-J. Joosten, M. Dörr, M. Höhne, U. T. Bornscheuer, *Int. J. Mol. Sci.* **2015**, *16*, 26953–26963.
[50] A. Iwasaki, K. Matsumoto, J. Hasegawa, Y. Yasohara, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 1563–1573.
[51] M. Höhne, S. Schätzle, H. Jochens, K. Robins, U. T. Bornscheuer, *Nat. Chem. Biol.* **2010**, *6*, 807–813.
[52] K. E. Cassimjee, M. S. Humble, H. Land, V. Abedi, P. Berglund, *Org. Biomol. Chem.* **2012**, *10*, 5466–5470.
[53] F. Steffen-Munsberg, C. Vickers, A. Thontowi, S. Schätzle, T. Tumlirsch, M. Svedendahl Humble, H. Land, P. Berglund, U. T. Bornscheuer, M. Höhne, *ChemCatChem* **2013**, *5*, 150–153.
[54] A. W. H. Dawood, R. O. M. A. de Souza, U. T. Bornscheuer, *ChemCatChem* **2018**, *10*, 951–955.
[55] J. S. Shin, B. G. Kim, *Biotechnol. Bioeng.* **1999**, *65*, 206–11.
[56] M. Davies, *Analyst* **1959**, *84*, 248–251.
[57] S. Schätzle, M. Höhne, E. Redestad, K. Robins, U. T. Bornscheuer, *Anal. Chem.* **2009**, *81*, 8244–8248.

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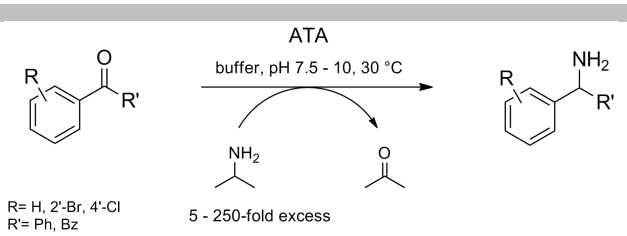
Improved variants for higher catalytic activity towards isopropylamine (IPA) as amine donor were created by protein engineering and applied in the asymmetric synthesis of industrial relevant amines. A systematic investigation of pH and an excess of IPA indicates the necessity of substrate- and ATA-dependent optimizations.



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