## Expanding the catalytic activity of amine dehydrogenases

*Rational enzyme engineering via computational analysis and application in organic synthesis*

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## Summary

a-Chiral amines are of great relevance in pharmaceutical manufacturing as these amines are involved in a variety of biological activities. Over the past years, their importance as building blocks in many Active Pharmaceutical Ingredients (APIs) was highlighted in a large number of scientific reports. It is estimated that a-chiral amines constitute approximately 40% of the optically active drugs that are currently commercialized mainly as single enantiomers. Their share is expected to increase due to stringent regulations for market approval concerning safety and efficiency, which are both directly related to the (enantio)purity of the compounds. Asymmetric synthesis of a-chiral amines is hard to achieve using traditional synthetic chemistry that involves multiple synthetic steps and often requires the use of toxic transition-metal-based catalysts. Consequently, various enzymatic methods have been developed that provide sustainable synthetic alternatives. Among them, amine dehydrogenases (AmDHs) constitute a recently developed enzyme family that possesses tremendous potential for the synthesis of a-chiral amines due to the elevated atom economy of the enzymatic reductive amination and exquisite enantioselectivity of AmDHs. The diversity of substrate scope, reactivity and enantioselectivity among the known engineered AmDHs is, however, poor. Overcoming these limitations are the main objectives of this thesis. Using a computational based rational approach, we were able to create novel AmDHs with increased and complementary substrate scope than the ones developed so far. The new AmDHs developed showed impressive enantioselectivity, increased thermal stability, and were applied for the asymmetric synthesis of chiral amines (APIs intermediate) at large scale (≥500 mg). Moreover, in contrast to the common belief that AmDHs accept only ammonia as amino donor, we have shown that AmDHs enable the synthesis of secondary and tertiary amines in enantioenriched form using small aliphatic or cyclic-aliphatic amines as amino donors. Through the combination of practical lab experiments and computational simulations, we gained new insights into the catalytic mechanism

of these AmDHs, thus revealing new aspects related to their applicability and current limitations in organic synthesis. The applicability of AmDHs in the kinetic resolution of racemic mixtures of amines was also demonstrated using an optimized AmDH-NOx system to give access to pharmaceutically relevant optically active amines possessing (*S*)-configuration. Starting from a promiscuous amino acid dehydrogenase scaffold (*L*-lysine-(ε-deaminating)-dehydrogenase from *G. stearothermophilus*), we were able to create enzymes that show both ADH activity as well as AmDH activity towards benzylic substrates. By optimizing and controlling the conditions of the biocatalytic transformation, these enzymes were used either for the preparation of a-chiral amines or primary alcohols with high efficiency. Amination of benzylalcohol to benzylamine was achieved by a single enzyme providing the first example of biocatalyst showing alcohol aminase activity. Finally, the scarce availability of (*S*)-selective AmDHs motivated us to develop a high-throughput screening methodology to detect (*S*)-configured amines in aqueous solution. This strategy possesses several advantages over the previously reported screening methodologies as it is highly enantioselective, can be used for the screening in the more desirable reductive amination reaction direction, and allows *in situ* purification of the biocatalyst. The latter feature enables to avoid any possible interference of other enzymes produced by the host organism as one of the steps of the assay. These features significantly reduce the possibility to obtain false positive results. This assay is expected to be used for screening potential (*S*)-selective AmDHs in the future.

## Samenvatting

Chirale a-amines zijn betrokken bij een verscheidenheid aan biologische activiteiten. Om die reden zijn ze van groot belang bij de productie van geneesmiddelen. Gedurende de afgelopen jaren is de rol van chirale a-amines als bouwstenen van actieve farmaceutische grondstoffen (ook wel Active Pharmaceutical Ingredients - API´s) benadrukt in een groot aantal wetenschappelijke artikelen. Naar schatting vormen chirale a-amines ongeveer 40% van de optisch actieve geneesmiddelen die momenteel hoofdzakelijk als enkele enantiomeren worden verkocht. Er wordt verwacht dat hun aandeel zal toenemen als gevolg van strenge voorschriften voor marktgoedkeuring omtrent veiligheid en efficiëntie, die beide rechtstreeks verband houden met de (enantiomere) zuiverheid van de verbindingen. Asymmetrische synthese van chirale a-amines is moeilijk te bereiken met behulp van traditionele synthetische chemie. Vaak zijn er meerdere synthesestappen nodig en is het gebruik van giftige overgangsmetaal-katalysatoren vereist. Als gevolg hiervan zijn er verschillende enzymatische methodes ontwikkeld die duurzame synthetische alternatieven bieden. Amine dehydrogenases (AmDHs) vormen een recent ontwikkelde enzymfamilie met een enorm potentieel voor de synthese van chirale a-amines vanwege de hoge atoomeconomie van de enzymatische reductieve aminering en de uitstekende enantioselectiviteit. Echter, de substraatomvang, reactiviteit en enantioselectiviteit van alle bekende AmDHs vertonen maar weinig diversiteit. Het overwinnen van deze beperkingen is het hoofddoel van dit proefschrift. Met behulp van een computationeel gebaseerde rationele benadering waren we in staat om nieuwe AmDHs te creëren met een grotere en complementaire substraatomvang in vergelijking met de AmDHs die tot nu toe zijn ontwikkeld. De nieuwe AmDHs vertoonden indrukwekkende enantioselectiviteit, verhoogde thermische stabiliteit en werden toegepast voor de asymmetrische synthese van chirale amines (APIs intermediair) op grote schaal (≥500 mg). Bovendien hebben we, ondanks de algemene overtuiging dat AmDHs alleen ammoniak als aminodonor

accepteren, aangetoond dat onze AmDHs de synthese van secundaire en tertiaire amines in enantio-verrijkte vorm mogelijk maken door kleine alifatische of cyclisch-alifatische amines te gebruiken als aminodonoren. Door een combinatie van praktische laboratoriumexperimenten en computationele simulaties hebben we nieuwe inzichten verkregen in het katalytische mechanisme van deze AmDHs. Hierdoor zijn nieuwe aspecten onthuld met betrekking tot de toepasbaarheid en de huidige beperkingen van deze enzymen in organische synthese. De toepasbaarheid van AmDHs in de kinetische resolutie van racemische mengsels van amines werd aangetoond met behulp van een geoptimaliseerd AmDH-NOx systeem. Dit enzymatische systeem gaf toegang tot farmaceutisch relevante optisch actieve amines met (*S*)-configuratie. Uitgaande van een promiscue aminozuur dehydrogenase scaffold (*L*-lysine-(ε-deaminerende)-dehydrogenase van *G. stearothermophilus*) hebben we enzymen gecreëerd die zowel ADH activiteit als AmDH activiteit vertonen richting benzylische substraten. Door de omstandigheden van de biokatalytische transformatie te optimaliseren en te beheersen, konden deze enzymen worden gebruikt voor de efficiënte productie van chirale α-amines of primaire alcoholen. Dezelfde enzymen werden benut voor de directe aminering van benzyl alcohol tot benzylamine door een enkel enzym. Hiermee leverden we het eerste voorbeeld van een biokatalysator die 'alcohol aminase' activiteit vertoont. Ten slotte motiveerde de schaarse beschikbaarheid van (*S*)-selectieve AmDHs ons om een high-throughput screeningmethode te ontwikkelen voor de detectie van (*S*)-geconfigureerde amines in waterige oplossing. Onze strategie heeft verschillende voordelen ten opzichte van de eerder gepubliceerde screeningsmethodes, aangezien deze zeer enantioselectief is, kan worden gebruikt voor de screening in de meer gewenste reductieve aminering reactie richting en *in situ* zuivering van de biokatalysator mogelijk maakt. Het laatste kenmerk stelt ons in staat om elke mogelijke tussenkomst van andere enzymen te voorkomen, waardoor de kans op vals-positieve uitslagen aanzienlijk kleiner is. Naar verwachting zal deze assay in de toekomst worden gebruikt voor het screenen van potentiële (*S*)-selectieve AmDHs.

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## List of publications

**Published:**

1. **Kinetic resolution of racemic primary amines using *Geobacillus stearothermophilus* amine dehydrogenase variant**

**Vasilis Tseliou**, Tanja Knaus Jan Vilím, Marcelo F. Masman and Francesco G. Mutti
ChemCatChem 2019, 12, 1-6
DOI: 10.1002/cctc.201902085

2. **Generation of amine dehydrogenases with increased catalytic performance and substrate scope from ε-deaminating L-lysine dehydrogenase**

**Vasilis Tseliou**, Tanja Knaus, Marcelo F. Masman, Maria L. Corrado and Francesco G. Mutti
Nature Communications 2019, 10, 3717
DOI: 10.1038/s41467-019-11509-x

- **Patent application filed '*L-Lysine epsilon-dehydrogenase variants and uses thereof*'**
  application number EP19157237.9

3. **Mechanistic insight into the catalytic promiscuity of amine dehydrogenases: asymmetric synthesis of secondary and primary amines**

**Vasilis Tseliou**. Marcelo F. Masman, Wesley Böhmer, Tanja Knaus and Francesco G. Mutti
ChemBioChem 2019, 20, 1–14
DOI: 10.1002/cbic.201800626

4. **A biocatalytic method for the chemoselective aerobic oxidation of aldehydes to carboxylic acids**

Tanja Knaus, **Vasilis Tseliou**, Luke D. Humphreys, Nigel S. Scrutton and Francesco G. Mutti
Green Chemistry 2018, 20, 3931-3943
DOI: 10.1039/C8GC01381K

5. **Molecular characterization of pyrethroid resistance in the olive fruit fly *Bactrocera oleae***

Nena Pavlidi Anastasia Kampouraki, **Vasilis Tseliou**, Nicky Wybouw, Wannes Dermauw, Emmanouil Roditakis, Ralf Nauen, Thomas van Leeuwen and John Vontas
Pesticide Biochemistry and Physiology 2018, 148, 1–7
DOI: 10.1016/j.pestbp.2018.03.011

6. **Functional characterization of glutathione (*S*)-transferases associated with insecticide resistance in *Tetranychus urticae*.**

Nena Pavlidi, **Vasilis Tseliou**, Maria Riga, Ralf Nauen, Thomas van Leeuwen, Nicolaos Labrou and John Vontas
Pesticide Biochemistry and Physiology 2015, 121, 53–60
DOI: 10.1016/j.pestbp.2015.01.009

**In preparation**

1. **Exploiting the catalytic promiscuity of L-lysine-ε-dehydrogenase for the synthesis of primary alcohols and (a)chiral amines**

Tseliou *et al*.
In preparation for Chemistry: A European Journal

2. **Substrate scope, applications and structural insights of amine dehydrogenases in asymmetric synthesis of a-chiral Amines**

Tseliou *et al*.
In preparation as a review for ACS catalysis