Book of Abstracts

Transam 2.0 - Chiral Amines Through (Bio)Catalysis

04 – 06 March 2015
University of Greifswald, Institute of Biochemistry
Felix-Hausdorff-Str. 4, Greifswald, Germany

Organized by the division Biotechnology / Enzyme Technology of Euro Fed Lipid

Organizers
Uwe Bornscheuer, University of Greifswald, Greifswald, Germany
Matthias Höhne, University of Greifswald, Greifswald, Germany
The organizers gratefully acknowledge the following organizations:
Program

Wednesday, March 4th

18.00-19.00 Registration, mounting of posters

Session I

19.00-19.05 Opening remarks (U. Bornscheuer, Per Berglund)

19.05-19.50 Keynote Lecture I
M. Beller (LIKAT, Rostock, Germany): "Homogeneous and Heterogeneous Catalysis: Tools for Efficient Synthesis of Amines"

19.50-20.30 Speed talk poster presentations (2 min each) – even numbered posters

20.30-21.30 Welcome reception and poster session

Thursday, March 5th

8.00-8.30 Registration

Session IIa: Transaminases Chair person: Nick Turner

08.30-09.15 Keynote Lecture II
J. Woodley (DTU, Copenhagen, Denmark): "Comparison of Biocatalytic Routes and Processes to Optically Active Amines"

09.15-09.30 E. O'Reilly (MMU, Manchester, UK): "New Strategies for Chiral Amine Synthesis using ω-Transaminases"

09.30-09.45 S. Heintz (DTU, Copenhagen, Denmark): "Microfluidics for Development and Testing of ISPR Options for the Synthesis of Chiral Amines using ω-Transaminases"

09.45-10.00 E. Heuson (Université Blaise Pascal, ICCF, Aubière, France): "A Novel General Screening Assay for Transaminases Discovery"

10.00-10.30 Coffee break

Session IIb: Transaminases Chair person: Matthias Höhne

10.30-11.00 J. Littlechild (Exeter Univ., Exeter, UK): "Structural Studies on Transaminase Enzymes and Applications in Biocatalysis"

11.00-11.15 C. Grey (Lund University, Lund, Sweden): "Improving ω-Transaminase Catalysed Reactions through Process Engineering: In situ Product Removal and Immobilization"


11.30-12.30 Lunch

12.30-13.30 Poster session
### Session III: Enzyme Cascades  Chair person: Wolfgang Kroutil

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<td>&quot;Synthetic Enzyme Cascades to Pharmaceutically relevant Amino Alcohols - Setup and Optimisation Strategies&quot;</td>
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<td>14.00-14.20</td>
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<td>E. Fischereder (Univ. Graz, Graz, Austria)</td>
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### Session IV: COST Action SysBiocat Working Group Meeting  Chair person: Stefano Servi

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Friday, March 6th

Session V: COST Action SysBiocat Working Group Meeting  Chair person: Gideon Grogan

08.30-09.15  Keynote lecture III
A. Bommarius (GeorgiaTech, Atlanta, USA):
"(R)- and (S)-Amines through Amine Dehydrogenase Catalysis"

09.15-09.45  G. J. Poelarends (Univ. Groningen, Groningen, The Netherlands):
"Enzymatic Synthesis of Substituted Aspartic Acids"

09.45-10.15  T. R. Ward (Univ. Basel, Basel, Switzerland):
"Artificial Metalloenzymes for the Synthesis of Chiral Amines and Derivatives"

10.15-10.45  Coffee break

10.45-11.05  A. Hummel (Bielefeld University, Bielefeld, Germany):
"Ene-reductase-catalyzed Access towards Chiral Amines"

11.05-11.35  K. Baldenius (BASF, Ludwigshafen, Germany):
"150 Years BASF - We Create Chemistry ...with a little Help of Enzymes!"

11.35-11.55  S. Borchert (Enzymicals, Greifswald, Germany):
"Development of Biocatalytic Processes for the Production of Chiral Amines from Screening to Batch Process"

12.00-13.00  Lunch

13.00-14.00  Poster session

Session VI: Imine Reductases and Other Enzymes  Chair person: Gerrit Poelarends

14.00-14.30  W. Kroutil (Univ. Graz, Graz, Austria):
"Extending the Biocatalytic Toolbox to Access Chiral Amines"

"Lipases in Dynamic Kinetic Resolution of Alcohols and Amines. Model Studies and Directed Evolution"

15.00-15.20  D. Mink (DSM Chemical Technology R&D BV, Geleen, Netherlands):
"Application of Transaminases for the Synthesis of Pharma Intermediates and Materials Science Products"

15.20-15.50  Coffee break

15.50-16.05  M. Gand (Greifswald University, Greifswald, Germany):
"Biocatalytic Preparation of Chiral Secondary Amines: Identification of Novel Imine Reductases"

16.05-16.25  M. Mayo (Codexis, Redwood City, USA):
"Imine Reductases: Engineering of a Novel Enzyme for the Production of Chiral Secondary and Tertiary Amines"

16.25-16.55  G. Grogan (Univ. York, York, UK):
"Structure, Mechanism and Application of NADPH-Dependent Imine Reductases"

17.00  Closing remarks (U. Bornscheuer, M. Höhne)
Maps for orientation

- Theatercafe
- Institute of Biochemistry
- Europa hotel

(city center)
Lectures
Homogeneous and Heterogeneous Catalysis: Tools for Efficient Synthesis of Amines
Matthias Beller
Leibniz-Institut für Katalyse an der Universität Rostock,
Albert-Einstein-Str. 29a, 18059 Rostock, Germany

Despite numerous important methodological advancements in all areas of chemistry, still most organic synthesis as well as the industrial production of chemicals can be improved. Currently, more than 80% of all products of the chemical industry are made via catalysis. In this regard, the development of new and more efficient catalysts constitutes a key factor for achieving a sustainable production of amines, too. Here, several examples will be presented in the talk; e.g. the use of mixtures for the synthesis of bulk amines. Furthermore, it will be shown that recently developed molecular-defined as well as nano-structured cobalt and iron catalysts enable us to perform catalytic hydrogenation processes to give aromatic amines with high yields and unprecedented selectivity. Specific examples which demonstrate the potential of selective catalytic processes also include the reduction of peptides.

Comparison of Biocatalytic Routes and Processes to Optically Active Amines

John M Woodley\textsuperscript{1}, Maria T Gundersen\textsuperscript{1}, Gustav Rehn\textsuperscript{1}, Nicholas J Turner\textsuperscript{2},
\textsuperscript{1}Technical University of Denmark, Lyngby, Denmark, 
\textsuperscript{2}University of Manchester, Manchester, UK

The synthesis of optically active chiral amines is of major industrial importance [1]. Given its importance it is therefore not surprising that there are multiple methods and routes to synthesize such molecules. Interestingly, there are at least six routes using biocatalysis alone, including the well-known $\omega$-transaminases and monoamine oxidases, as well as newer enzymes such as amine dehydrogenases and imine reductases [2]. Some of the enzymes are used for asymmetric synthesis, some resolution and some, both operating modes. Likewise, each of these routes uses different reagents, has a different atom economy and operates optimally under different conditions, with associated advantages and disadvantages. While the majority of reported research on biocatalytic methods to produce chiral amines describe the details of each methodology (and associated enzyme), in this presentation the routes will be compared based on some common criteria and metrics. Furthermore, potential processes using each of the routes will be compared. At an early stage, during biocatalytic retro-synthesis it is necessary to compare routes [3], and the template for making such comparisons should include both chemistry, as well as process considerations, alongside economic and environmental considerations [4]. We believe the template presented here to be of general applicability and use the example of optically active amine production as a test case.

New Strategies for Chiral Amine Synthesis using ω-Transaminases

Elaine O'Reilly, Anthony P. Green and Nicholas J. Turner
Manchester Metropolitan University
Manchester, UK

The widespread application of ω-transaminases as biocatalysts for chiral amine synthesis has been hampered by fundamental challenges including unfavorable positions of equilibria and product inhibition. In this study we report a highly efficient process which allows reactions to proceed in high conversion using only 1.0 equivalent of a diamine donor (o-xylylenediamine) and without the need for by-product removal. This operationally simple methodology is compatible with the most widely used (R)- and (S)-selective ω-TAs and is particularly suitable for the conversion of substrates with unfavorable positions of equilibria. Significantly, spontaneous polymerization of the isoindole by-product generates colored derivatives, providing a high-throughput screening platform to identify desired ω-TA activity.
Microfluidics for Development and Testing of ISPR Options for the 
Synthesis of Chiral Amines using ω-transaminases.

Søren Heintz¹, Rolf H. Ringborg¹, Gustav Rehn¹,², Tim Börner², Carl Grey², Patrick 
Adlercreutz², Ulrich Krühne¹, Krist V. Gernaey¹ & John M. Woodley¹

¹Department of Chemical and Biochemical Engineering, The Technical University of 
Denmark, DK-2800 Kgs. Lyngby, Denmark
²Department of Biotechnology, Lund University, 22100 Lund, Sweden

The synthesis of valuable chiral amines, building blocks in many pharmaceuticals and 
precursors, using ω-transaminases is very attractive. This is caused by the fact that 
transaminases potentially can ensure high yields of optically pure chiral amines, through 
simple process routes that operate at mild reaction conditions [1]. However, the 
development of new transaminase based processes is quite time and cost intensive, i.e. 
many modified variants of the catalysts and multiple process options have to be 
evaluated. Additionally many process challenges, e.g. unfavorable thermodynamics and 
inhibitory effects, must be overcome in order to reach feasible process targets [2]. The 
development of such processes may therefore greatly benefit from the application of 
specialized and advanced technologies, such as microfluidics. The focus of this 
contribution is the use of basic microfluidic modules for the development of 
transaminase based processes with integrated separation methods, e.g. in-situ product 
removal (ISPR) by liquid-liquid extraction. More precisely this work applies a 
combination of microfluidic experimental platforms, with well-defined fluid dynamic 
behavior, in combination with modelling tools to greatly increase the process 
knowledge. In this contribution a demonstration system will be presented, applying the 
conversion of benzylacetone to 1-methyl-3-phenylpropylamine using various amine 
donors, where liquid-liquid extraction is integrated with a reactor module to achieve high 
product titer with a minimal impact on the biocatalyst. The concept of the platform is 
based on the work performed by Rehn et al. [3]

References:
Bioengineering 108 (2011) 1479-1493
A Novel General Screening Assay for Transaminases Discovery

Heuson, E.¹; Gefflaut, T.¹; Charmantray, F.¹; de Berardinis, V.²

¹Université Blaise Pascal, ICCF, Aubière, France; ²Génoscope, Evry, France

Transaminases (TA) offer an efficient access to chiral amines which are found in numerous pharmaceuticals, and therefore have gained considerable attention in the past few years.¹ With the aim of mining biodiversity to isolate new useful biocatalysts within the TA family, we have developed a general sensitive and colorimetric high throughput screening (HTS) assay for the detection of both α-transaminases and amine-transaminases. This method is based on the use of a β-amino sulfinic acid (BAS) as amino donor substrate. The β-keto sulfinic acid formed from the BAS through transamination instantaneously decomposes, in aqueous buffer, into sulfite and a carbonyl derivative. Sulfite ions can then be easily detected by spectrophotometry using Ellman’s reagent, thus allowing direct kinetic measurements.²

We first developed a direct assay aiming to reveal BASTAs: ie enzymes active with any chosen acceptor and a BAS as donor. These BASTA catalyse an irreversible transamination, provided that they don’t accept as substrate, the carbonyl derivative resulting from SO₂ elimination. In a second assay (coupled assay), a generic substrate of TA (Glu or Ala) used as primary amino donor is regenerated using an auxiliary BASTA. This coupled reaction allows again the activity detection as well as an easy equilibrium shifting. The combination of these two complementary assays thus constitutes a highly efficient way to identify a virtually unlimited range of transaminase activities. Primary results obtained by screening a collection of enzymes from biodiversity will be discussed.

Structural Studies on Transaminase Enzymes and Applications in Biocatalysis

C. Sayer\textsuperscript{1}, M. Isupov\textsuperscript{1}, J. Ward\textsuperscript{2}, J. Littlechild\textsuperscript{1}

\textsuperscript{1}Henry Wellcome Building for Biocatalysis, Biosciences, College of Life and Environmental Sciences, University of Exeter, Stocker Road, Exeter, EX4 4QD, UK
\textsuperscript{2}Research Department of Structural and Molecular Biology, University College London, Darwin Building, Gower Street, London WC1E 6BT, UK

The transaminases (TAs) catalyse the transfer of an amino group from an amino acid to a keto acid using the cofactor pyridoxal 5'-phosphate (PLP) and are important for the production of optically pure amines and amino alcohols used in the synthesis of many important drugs.

We have solved the structures of TAs of the Pfam classes, III, IV and V in order to further understand their mechanism and substrate specificities.

The class III \(\omega\)-amino acid TAs catalyse transamination of \(\omega\)-amino acids such as \(\beta\)-alanine or \(\gamma\)-aminobutyric acid where the transferred amino group is not adjacent to the carboxyl group. The crystal structures and inhibitor complexes of two (S)-selective \(\omega\)-TAs from \textit{Pseudomonas aeruginosa} and \textit{Chromobacterium violaceum} have been determined to understand differences in their substrate specificity.

The class V TAs include the serine:pyruvate transaminases (SPATs) which have broad substrate specificity including activity with \(\beta\)-hydroxyl substrate. The structure of the thermophilic archaeal \textit{Sulfolobus solfataricus} SPAT has been determined to 1.8 Å resolution and in complex with the inhibitor, gabaculine. This has shown the conformational changes at the active site during the course of the catalytic reaction.

The structure of the class IV \textit{Nectria haematococca} transaminase enzyme has been determined in the holo and inhibitor bound form which offers a detailed insight into the structural basis for substrate specificity and enantioselectivity of (R)-selective amine:pyruvate transaminases.

Studies on other novel thermophilic class IV and class V1 sugar transaminases are being studied as part of an ERA-IB grant Thermogene. Biochemical and structural studies of some representatives of these new transaminase enzymes will also be reported.

Improving $\omega$-Transaminase Catalysed Reactions through Process Engineering: *In situ* Product Removal and Immobilization

Carl Grey, Department of Biotechnology, Lund University, Lund, Sweden

The synthesis of chiral amines using $\omega$-transaminase catalysis is currently of great interest. Many of these reactions, however, suffer from poor yield and low final product concentration, due to product inhibition and unfavourable thermodynamic equilibrium. To address these problems we have explored the concept of using supported liquid membrane (SLM) for selective *in situ* product removal, in which the amine product is removed and up concentrated (leaving the amine donor substrate behind). The SLM consists of a hollow fibre membrane contactor in which a hydrophobic solvent resides within the pores of the polymeric membrane. The product amine is transferred from the reaction solution at high pH, through the solvent and finally trapped in the strip solution kept at low pH. By recirculating the reactor effluent through the membrane contactor, *in situ* product removal is achieved. If the pH and the volume in the strip solution is sufficiently low, high product concentrations (>100 g/l) can be achieved.

To improve biocatalyst handling, reuse and flexibility regarding reactor configuration we have investigated different options of whole cell immobilization. A strategy involving chitosan flocculation was found to have many interesting features, such as simplicity, high immobilization yield, possibility of reaching high loadings without any serious mass transfer issues. Chitosan immobilised cell preparation has been tested in several different types of reactors, such as stirred tank reactor (STR), packed bed reactor (PBR) and continuous STR (CSTR).
EziG™-immobilized ω-Transaminase
Karim Engelmark Cassimjee, EnginZyme AB
Stockholm, Sweden

EziG™ is a robust and inert porous material developed for immobilization of His-tagged enzymes directly from cell lysate. It has a core consisting of controlled porosity glass with a surface modified by polymer coatings to control the hydrophobicity and to enable the attachment of a protein affinity tag. This yields a porous support material with a large surface area, high binding capacity, excellent fluid properties, and a tailored surface, which is suitable for biocatalysis in aqueous or organic media. Finally, the protein affinity tags provide a non-destructive, fast and selective binding, whereby a pure immobilized preparation can be achieved in one step directly from cell lysate.

By applying this immobilization technique to His$_6$-tagged ω-transaminase, a versatile and reusable enantioselective catalyst was reached. The immobilization method and applications with ω-transaminase are presented.

Synthetic Enzyme Cascades to Pharmaceutically Relevant Amino Alcohols - Setup and Optimisation Strategies

Torsten Sehl\textsuperscript{a}, Vanessa Erdmann\textsuperscript{a}, Robert Simon\textsuperscript{b}, Benjamin Lichman\textsuperscript{c}, Ulf Menyes\textsuperscript{d}, Wolfgang Kroutil\textsuperscript{b}, John M Ward\textsuperscript{c}, Helen C Hailes\textsuperscript{c}, Dörte Rother\textsuperscript{a*}

\textsuperscript{a} IBG-1: Biotechnology, Forschungszentrum Jülich GmbH, Germany
\textsuperscript{b} Department of Chemistry, University of Graz, Austria
\textsuperscript{c} Departments of Biochemical Engineering & Chemistry, University College London, UK
\textsuperscript{d} Enzymicals AG, Greifswald, Germany; * do.rother@fz-juelich.de

A substantive subgroup within the broad field of pharmaceutically relevant amines is based on the functional motif of vicinal amino alcohols. One example within this compound class are nor(pseudo)ephedrines (N(P)E) having sympathomimetic function.\textsuperscript{[1]} Besides their pharmacological interests, N(P)Es are used as synthons, ligands, and chiral auxiliaries in organic syntheses.\textsuperscript{[2]} However, asymmetric syntheses of N(P)Es are challenging and require either multi-step preparative routes, expensive reagents or lack high selectivities.

Here we present modular biocatalytic 2-step 1-pot approaches for the synthesis of N(P)Es from inexpensive starting materials. By flexible combination of ThDP-dependent enzymes with $\omega$-transaminases from enzyme toolboxes, all possible enanti- and diastereomers of N(P)E are accessible.\textsuperscript{[3]} Due to multi-parameter reaction optimisation including design-of-experiment and evaluation of various process strategies, cascades with high space-time-yields (26 g L\textsuperscript{-1} d\textsuperscript{-1}) and high final stereoselectivities ($ee/de >99 \%$) could be gained. Utilisation of whole cell catalysts and addition of organic solvents are able to reduce costs and enhance space-time-yields further. The access of ($1S,2S$)-NE, also called cathine, provided a special challenge, since in nature no ($S$)-selective enzyme for the synthesis of the ($S$)-intermediate phenylacetylcarbinol was found so far. This gap could be now filled by rational enzyme design.\textsuperscript{[4]} With new ($S$)-selective pyruvate decarboxylase variants in hands, not only cathine, but also substituents thereof are accessible, broadening the product platform of optically pure vicinal amino alcohols. In addition, we present an eco-efficient recycling cascade mode, where the co-product of the second step is recycled in the first step.\textsuperscript{[5]}

Synthesis of Chiral β-amino Acids applying an Enzymatic Reaction Cascade

C. Slomka¹*, U. Engel¹, S. Zhong², C. Syldatk¹, S. Bräse² and J. Rudat¹

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² Karlsruhe Institute of Technology (KIT), Institute of Organic Chemistry Karlsruhe, Germany
*christin.slomka@kit.edu

The use of β-amino acids is gaining importance for the synthesis of enantiopure fine chemicals and pharmaceuticals. Since the Hydantoinase Process for production of optically pure α-amino acids is well-established in industry, the application of a modified Hydantoinase Process (fig. 1) has been proposed in order to obtain β-amino acids. [1]

By screening of bacterial strains with known hydantoinase activity and further investigations, the enantioselective conversion of several unnatural dihydropyrimidines to N-carbamoyl-β-amino acids was shown using whole-cell biocatalysis. [2, 3] Based on this work, novel substrates for the synthesis of β-amino acids were successfully tested. Since transport limitations of applied substrates and resulting products as well as low substrate solubilities are restricting factors, enzymatic reaction cascades in cell-free systems will be investigated aiming at applications in microfluidic reaction systems.

References

Figure 1: Proposed modified Hydantoinase Process, modified after Slomka 2014 [1]
Monoterpenic indole alkaloids are structurally complex natural products with well established biological activity.\textsuperscript{1,2} One biosynthetic pathway to this highly valuable compounds is initiated by the enzyme called strictosidine synthase (STR) which condenses tryptamine and secologanin via a "Pictet-Spengler" condensation to form exclusively C1-(S)-strictosidine.\textsuperscript{1} To tap the substrate scope of STR's, α-methyltryptamines (AMT's; 2a-f) were applied in the biotransformation with the natural aldehyde secologanin, thereby introducing a second a-chiral centre next to the non-aromatic nitrogen, to give diastereomerically pure strictosidine derivatives (3a-d).

Scheme 1: Cascade combining ω-transaminases (ω-TA) and strictosidine synthases (STR) to synthesize diastereomerically pure strictosidine derivatives 3a-d.

For this purpose, a cascade combining ω-transaminases (ω-TA) and strictosidine synthases was designed. Thereby the ω-TA reductively aminates the corresponding prochiral ketones (1a-e) and gives access to the desired α-methyltryptamines (2a-e) in optically pure form; the latter were directly condensed with secologanin by the STR's. By the choice of the appropriate ω-TA both enantiomers of the α-methyltryptamines (2a-e) were accessible, whereby the STRs showed a clear stereopreference for one of the amine enantiomers.


High-throughput Solid-phase Screening, Biocatalyst Evolution and Development of a Multi Enzymatic Cascade Process for the Formation of D-arylalanines by Phenylalanine Ammonia Lyases

Fabio Parmeggiani, Sarah L. Lovelock, Nicholas J. Weise, Syed Ahmed, Nicholas J. Turner,
Manchester Institute of Biotechnology and School of Chemistry, University of Manchester, Manchester, United Kingdom

The catalytic asymmetric hydroamination of 2-arylpropenoic acids mediated by phenylalanine ammonia lyases (PALs) is a very attractive and atom-efficient approach to the synthesis of enantiomerically enriched amino acids. [1-2] A number of PAL enzymes have been identified in various organisms and employed for the production of L-arylalanines in very high ee. [3-4]

It has been recently shown that a few cinnamic acid derivatives bearing electron-withdrawing groups on the ring do not afford the corresponding phenylalanine with high ee, unlike most of the accepted substrates, and that the ee decreases over time. [5]

In an attempt to find PAL variants able to form mixtures enriched in the D-enantiomers, we have developed a solid-phase screening method to identify the best candidates in large site-saturation mutagenesis libraries at multiple positions. This method allows thousands of variants to be screened, in the form of colonies plated on nylon membranes, leading to the identification of the most promising starting points for directed evolution.

The best variants were exploited in a new one-pot one-step approach, coupling the PAL amination with a chemoenzymatic deracemisation (based on stereoselective oxidation and non-selective reduction). Starting from cheap and easily synthesized cinnamic acids, substituted D-phenylalanines were obtained in high yield and excellent optical purity. The system was also extended to the preparation of those L-phenylalanines that are obtained in low ee with PAL amination.

References
Cascade Reactions via Amine Biocatalysis

Nicholas J. Turner, School of Chemistry & MIB, University of Manchester
131 Princess Street, Manchester, M1 7DN, UK.

This lecture will describe recent work from our laboratory aimed at developing new biocatalysts for enantioselective organic synthesis, with a particular emphasis on enzymes involved in amine biocatalysis. By applying the principles of 'biocatalytic retrosynthesis' it is possible to design new synthetic routes to target molecules in which biocatalysts are used in the key bond forming steps.\(^1\)

The integration of several biocatalytic transformations into multi-enzyme cascade systems has also been a focus of recent work in our laboratories. In this context MAO-N has been used in combination with other biocatalysts and chemocatalysts in order to complete a cascade of enzymatic reactions.\(^2-4\) Other engineered biocatalysts that can be used in the context of cascade reactions include \(\omega\)-transaminases,\(^5\) phenylalanine ammonia lyases,\(^6\) amine dehydrogenases, imine reductases and artificial enzymes.\(^7\)

We are also developing combined oxidase/metal-catalyst systems for the selective oxidation of amines and alcohols to amides/lactams/aldehydes and carboxylic acids.

References

Novel Fluorogenic Assay for Transaminase Engineering

Wolf-Dieter Fessner, Thomas Scheidt
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Direct asymmetric synthesis by using ω-Transaminases (TA) offers a green route from prochiral ketones to enantiopure amines, which are valuable as chiral building blocks or active pharmaceutical ingredients. Although TA show excellent enantiospecificity, until recently most known TA were (S)-selective. Natural TA are also limited in their substrate scope. Thus, development of smart TA assay methods is of vital importance for enantioselectivity typing and for screening of novel promiscuous enzymes from natural sources or for directed protein evolution to broaden their substrate tolerance.

Traditionally, TA screening is based on in vivo selection for microbial ability to grow on chiral amines as the sole nitrogen source, or on colorimetric in vitro assays that determine formation of ketone or secondary amino acid byproducts. Here we report on the development of the first highly sensitive fluorogenic assay in high-throughput format for the enantiospecific determination of TA activity. The assay principle relies on the conversion of a set of non-fluorescent amine precursors, which can be synthesized easily in optically pure form to incorporate broad structural variations as required for custom projects in protein engineering.


Looking on Transaminase Libraries in a New Way: the splitGFP-system to Normalize Enzyme Activities

M. Doerr, J. S. Aberturas, U. Bornscheuer, Institute of Biochemistry,
University Greifswald. Greifswald, Germany
mark.doerr@uni-greifswald.de

High-throughput screenings of large transaminase mutant libraries contain an inherent number of hidden active mutants, easily classified as false negatives that are very difficult to detect with conventional screening methods. To overcome this limit, we successfully applied the splitGFP system, developed by Geoffrey Waldo and coworkers [1], for the first time to enzyme screening to the best of our knowledge. We screened an error prone PCR library of an aminotransferase from *Vibrio fluvialis* in our robotic platform [2] and used the fluorescence signal of the splitGFP to normalize the enzyme activities without the need of laborious and time consuming purification in 96 well microtiter plate format (s. Scheme). With this approach we were able find new mutants that would have been invisible by activity-only based screenings. The presented splitGFP technique is widely applicable for many protein engineering tasks.

Scheme 1: Cartoon of the splitGFP-*Vibrio fluvialis* ATA assembly. It can be excited at 488 nm and shows fluorescence at 530 nm [1]; source of the crystal structures: [3].

References
(2) LARA | Laboratory Automation Robotic Assistant Biochemistry Greifswald; http://lara.uni-greifswald.de
Transaminase Catalysis: Enzyme Engineering and Amine Synthesis

Per Berglund, Mattias Anderson, Shan Chen, Henrik Land, Fabian Steffen-Munsberg, Bo Wang, Uwe Bornscheuer, Matthias Höhne, Maria Svedendahl Humble
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Amine transaminases catalyze industrially important transamination reactions for amine production (1). These enzymes are PLP-dependent and belong to two different fold families (2). Several crystal structures from each family are known, which increases the possibility of exploring them further through rational design.(3)

We have studied several issues in transamination during the past few years, involving either chiral or achiral amines. Equilibrium displacement (4), stereoselectivity improvement (5), cascade reactions (6) and catalytic promiscuity are some examples that will be discussed.

(R)- and (S)-Amines through Amine Dehydrogenase Catalysis

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Three amine dehydrogenases (AmDHs) have been developed from LeuDH (L-AmDH)\(^1\), PheDH (F-AmDH)\(^2\), and a chimera of both (cFL1-AmDH)\(^3\). Through protein engineering of the amino acid dehydrogenase scaffold, the amine dehydrogenase now catalyzes the reduction of prochiral ketones to chiral amines, while the original activity is either strongly diminished or completely eliminated. Expansion of the binding pocket has led to a catalytic constant \(k_{cat}\) of p-fluoro phenyl acetone (pFPA) of \(11 \text{ s}^{-1}\) at 50\(^\circ\)C.

Previous work involving the AmDH included the development of a biphasic organic solvent system to allow for conversion of hydrophobic substrates.\(^4\) All current AmDHs catalyze formation of the (R)-amine during reductive amination of standard substrates. To further extend our repertoire of products, we conducted oxidative amination of racemic amines in a biphasic organic solvent system resulting in successful conversion to the (S)-amine. For proof of concept, we have produced (S)-methylbenzylamine ((S)-MBA) from the racemate, catalyzed by the chimeric cFL1-AmDH. We will discuss enantiomeric purity of the product, related degree of conversion, and reaction parameters such as residence time and enzyme loads, as well as additional products.

References
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Enzymatic Synthesis of Substituted Aspartic Acids

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This lecture will describe recent work from our laboratory aimed at the structure-based engineering of methylaspartate ammonia lyase to enhance its diastereoselectivity and expand its substrate scope. The designed enzymes open new opportunities for the asymmetric synthesis and kinetic resolution of aspartic acid derivatives, which are highly valuable as tools for neurobiological research and as chiral building blocks for pharma- and nutraceuticals. Newly developed chemical synthesis routes to racemic mixtures of various substituted aspartic acids will also be discussed. Finally, the characterization of a new C-N lyase, which is involved in the metabolism of a biodegradable metal chelator, will be described.
Artificial metalloenzymes result from incorporation of a catalytically competent organometallic moiety within a host protein. We and others have been exploiting the potential of either streptavidin or carbonic anhydrase for the creation of artificial metalloenzymes, Figure. In the former case, the abiotic cofactor can be anchored thanks to biotin whereas in the latter, an arylsulfonamide can be used. Such artificial metalloenzymes are optimized either by chemical (variation of the anchor-spacer-ligand moiety) or genetic means. These chemogenetic schemes were applied to optimize the performance for eight different catalyzed transformations as well reaction cascades in the presence of natural enzymes.[1] In this context, the preparation of enantiopure amines has provided a propitious playground to test novel concepts including: engineering enzyme cascades,[2] dative anchoring of the cofactor,[3] in silico modelling, screening crude cellular extracts[4] etc.

Following a general introduction on the topic of artificial metalloenzymes, the current challenges and opportunities will be detailed.

Reactions implemented thus far:
- Hydrogenation (up to 96 % ee)
- Transfer Hydrogenation (up to 98 % ee)
- Allylic Alkylation (up to 95% ee)
- C-H Activation (up to 86 % ee)
- Suzuki Cross coupling (up to 90 % ee)
- Olefin Metathesis (up to 100 TONs)
- Alcohol Oxidation (up to 250 TONs)
- Sulfoxidation (up to 93 % ee)
- Dihydroxylation (up to 98 % ee)

Figure. Artificial metalloenzymes are obtained upon supramolecular incorporation of a abiotic cofactor within a host protein.

Ene-reductase-catalyzed Access towards Chiral Amines

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We developed a highly enantioselective chemoenzymatic route towards pharmaceutically valuable amines via the chemical Henry reaction coupled with an ene-reductase-catalyzed reduction (Scheme 1). The key step, the biocatalytic reduction of α-methylated nitroalkenes, could be realized for different substrates with conversion rates up to 99 % and enantiomeric excesses of typically 90-95 % ee with a recombinantly expressed, purified ene-reductase from *Gluconobacter oxydans* (1). The resulting nitroalkanes can be reduced to the corresponding chiral amines, which can be applied as intermediates in the synthesis of pharmaceuticals like Tamsulosin or Selegilin.

Scheme 1. Synthetic concept for the construction of chiral amines by a chemoenzymatic route coupling the chemical Henry reaction with the biocatalytic reduction by an ene-reductase followed by the reduction of the nitro group.

150 years BASF – We Create Chemistry….with a little Help of Enzymes!

Kai Baldenius, BASF SE; Ludwigshafen, Germany

In the last decades the application of enzymes for enantioselective syntheses has made its way to industrial use. BASF has led this development with its Chipros® product line of chiral intermediates with a particular focus on chiral amines. However, the horizon for enzymatic catalysis is now going beyond pharma intermediates. Examples for non-chiral volume chemical application will be given and the current status of transaminase in this field discussed.
Optically active amines play an important role in the pharmaceutical, agrochemical, and chemical industries. They are frequently used as synthons for the preparation of various pharmaceutically active substances. Consequently, there is a need for efficient methods to obtain the desired enantiomer of a given target structure in optically pure form. Beside a range of chemical methods using for example, asymmetric synthesis with transition metal catalysts, enzymes represent a useful alternative to access this important class of compounds. For a selection of biocatalysts regarding the synthesis of chiral amines see scheme 1.

Scheme 1. Biocatalytic routes for the synthesis of chiral amines

This talk will give an overview of challenges and solutions within projects covering the varying stages of process of development: from catalyst screening to reaction design.
Extending the Biocatalytic Toolbox to Access Chiral Amines

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Lipases and mono amine oxidases were probably the first enzymes employed to prepare amines in optically pure for synthetic purposes. In the last years various other types were identified as suitable catalysts. In the presentation $\omega$-transaminases, the berberine bridge enzyme (BBE) and Pictet-Spenglerases, especially the strictosidine synthase will be discussed. Recent examples for each enzyme will be given. For instance, $\omega$-transaminases were used for the synthesis of ramatroban\(^1\) or the dynamic kinetic resolution of 2-phenylpropanal derivatives to yield $\omega$-chiral primary amines.\(^2\) The berberine bridge enzyme was successfully used for the deracemisation of racemic 1-benzyl-1,2,3,4-tetrahydroisoquinolines to optically pure berbines in theoretically 100% yield.\(^3\) More recently, the potential of strictosidine synthases were tested for the transformation of tryptamines and aldehydes. Although according to literature the substrate spectrum seem s to be rather limited, a broader scope of aldehydes can indeed be transformed.\(^4\)

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4 Unpublished results.
Our laboratory is deeply involved in the discovery and development of new reaction methodology based on catalysis that can be used in organic synthesis. In particular we have used metal catalysis and biocatalysis in our research. The combination of different types of catalysis in tandem processes has been utilized, and our group has contributed to the development of combined metal and enzyme catalysis for efficient preparation of enantiomerically pure compounds. The development of coupled catalysis, where different catalysts work together has led to dynamic kinetic asymmetry transformations (e.g. dynamic kinetic resolution).

In the present lecture it is demonstrated how the combination of a catalytic racemization procedure and an enzymatic resolution can lead to a dynamic kinetic resolution (DKR) if the enzyme and racemization catalysts are compatible with one another. The advantage with this approach is that all of the racemic substrate can be transformed into enantiomerically pure product in up to 100% yield.\(^1\) Efficient procedures for DKR of alcohols and amines by combining an enzyme with a metal racemization catalyst have been used in enantioselective organic synthesis of biologically active compounds.\(^2\)

The mechanism of the racemization of alcohols catalyzed by \((\eta^5\text{-pentaphenylcyclopentadienyl})\text{RuCl(CO)}_2\) was recently studied.\(^3\) The racemization involves an alkoxide complex that undergoes a \(\beta\)-elimination followed by re-addition of the hydride.

In this lecture we also discuss a well-characterized hybrid catalyst in which \textit{Candida antarctica} lipase B and a nanopalladium species are co-immobilized into the compartments of mesoporous silica.\(^4\) This hybrid catalyst mimics a metalloenzyme and was used for dynamic kinetic resolution of a primary amine in high yield and excellent enantioselectivity.

To improve the enantioselectivity and substrate scope of the lipases used, directed evolution and engineering of these enzymes have been carried out. This has led to more enantioselective variants. Recently we were able to screen large libraries in organic solvents, which provided improved enzyme variants that can be used in the DKR reactions developed in our laboratory.\(^5\) These DKR reactions are run as transacylation reactions in a dry organic solvent.

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Application of Transaminases for the Synthesis of Pharma Intermediates and Materials Science Products

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Royal DSM N.V. is a global life science and material science company actively developing sustainable processes for production of chemical building blocks and intermediates from renewable resources. As an industry leader in sustainability DSM takes a multidisciplinary approach including chemo- and biocatalysis, organic synthesis and metabolic engineering. In this approach sustainability of process concepts and designs are evaluated early on to focus development on the most cost-efficient and sustainable option.

In this presentation we will highlight aspects of biocatalyst identification, strain development and reaction engineering in the field of transaminase technology. This will be illustrated with examples of typical chiral pharmaceutical chemical intermediates and functional amines for material science applications. Aspects of identification of novel transaminases, screening, computational tools for prediction and concepts for shifting the reaction equilibrium as well as application of transaminases for the synthesis of chiral and functional amines exemplified in this presentation.
Biocatalytic Preparation of Chiral Secondary Amines: Identification of Novel Imine Reductases

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For the enantioselective synthesis of secondary cyclic amines, imine reductases (IRED) [1] are a promising alternative compared to other biocatalytic strategies such as monoamine oxidases [2]. We characterized two novel IREDs from *Paenibacillus elgii* B69 and *Streptomyces ipomoeae* 91-03, which were identified by amino acid similarity search. Another IRED from *Pseudomonas putida* KT2440 was found in the protein databank (PDB-code 3L6D) by analyzing structures of proteins belonging to the superfamily of the known IREDs, but with unknown function. All three enzymes were recombinantly expressed in *E. coli*, followed by a detailed investigation of their substrate scope [3].

Despite the acceptance of cyclic amines, also acyclic amines could be identified as substrates for the newly discovered as well as the formerly known IREDs. The enzyme from *P. putida* showed the highest apparent $E$-value of approximately $E_{\text{app}} = 52$ for (R)-methylpyrrolidine. A phylogenetic analysis revealed that this enzyme forms a new clade of possible IREDs. It also differs in the placement of the active site residues: H180 could be confirmed to be relevant for catalysis by mutagenesis, as the mutant H180V shows a 10-fold reduced activity. Unexpectedly, this residue is located one turn aside compared to the position of the catalytic D187 of *Streptomyces kanamyceticus* IRED.

Imine Reductases: Engineering of a Novel Enzyme for the Production of Chiral Secondary and Tertiary Amines

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Chiral secondary and tertiary amines are important building blocks in pharmaceutical industry; however the existing chemical methods for their synthesis are lengthy, expensive, and often fail to produce enantiomerically pure products. There are currently no efficient biocatalytic routes known to produce this class of chiral amine compounds. Here we report using Codexis’ CodeEvolver® directed evolution technology to engineer an imine reductase (IRED)¹ into a catalyst for the efficient production of chiral secondary and tertiary amines². In 10 rounds of evolution, the newly developed enzyme was shown to fully convert 14 g/L ketone substrate to the desired tertiary amine in 24 hours. This enzyme shows wide substrate scope, including accepting primary and secondary amines, along with electron deficient amines such as anilines and is very amenable to focused evolution strategies. To date, this is the first known biocatalytic route to selectively generate tertiary amines using reductive amination at a commercially viable scale.

Reductases catalysing the NADPH-dependent asymmetric reduction of prochiral ketones (KREDs) are well established, but enzymes that catalyse the asymmetric reduction of prochiral imines, ‘IREDs’ have received more limited attention. In this presentation we will describe structural and mechanistic investigations into NADPH-dependent IREDs that begin to shed light on the molecular determinants of substrate specificity and asymmetric selectivity in these enzymes. We also show that IREDs have potential for the asymmetric reduction of a wide range of prochiral imine substrates and therefore constitute a valuable potential source of biocatalysts for the production of chiral amine intermediates.

Posters
Enzymatic Cascade Reactions for an Efficient Production of Chiral Hydroxy Amino Acids.

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Hydroxy amino acids in nature have various bioactivities (such as anti-diabetes, anti-inflammatory, anti-oxidative effect) and are constituents for bioactive peptides. This makes them very attractive compounds as fine chemicals and building blocks for drugs. Recently, a bacterial isoleucine dioxygenase (IDO) was reported to catalyze the asymmetric oxidation of natural and artificial aliphatic L-amino acids [1]. While natural amino acids are easily available as pure L-enantiomers, non-natural amino acids are produced mainly in racemic form. Due to the high enantioselectivity of IDO, the conversion of racemic amino acids using IDO results in 50% maximum yield as only the L-enantiomer is converted. We aimed to construct efficient cascade enzymatic reactions producing optically pure hydroxy amino acids from pro-chiral or racemic substrates. In this study, we compare three alternative reaction concepts combining other amino acid converting enzymes in order to overcome the yield limitation. The poster discusses limitations and chances of the three approaches.

Recent Achievements for Biocatalytic Asymmetric Chiral Amine Synthesis from Substituted Ketones

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Chiral amine synthesis is highly encouraged by chemoenzymatic reaction concepts and unusual enzyme actions. Protein engineering extended the scope of accessible enzymes and enhancing their new actions which is specific development in chiral amine synthesis. For example, mutagenesis of an L-amino acid dehydrogenase produced an enzyme amine dehydrogenase due to which ketones directly go for a symmetric amination with ammonia. Synthesis of secondary amines by developing different approaches is another trend in chiral amine chemistry. However, secondary amine can be synthesized by good selection of substrate for transaminases of amine. Moreover, two novel biocatalyst Pictet-Spenglerases and imine reductases have been used for the synthesis of secondary amines. These examples show that how effectively the biocatalytic amine synthesis is used for industrial scale preparation of some pharmaceutical related substances. By comparing process parameters like space-time yield and turnover number, we can reveal that these biocatalytic planes are growing and can already contend with conventional chemical methods.
The Crystal Structure of an (R)-selective ω-transaminase from *Aspergillus terreus* as Prerequisite for Protein Engineering

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Omega-transaminases are of great importance as biocatalysts for the direct synthesis of chiral amines, which are important building blocks for the synthesis of pharmaceutical products, fine chemicals and agrochemicals. In Nature, (S)-selective amine transaminases are more abundant than the (R)-selective enzymes, and therefore more information concerning their structures is already available.

The crystal structure of an (R)-ω-transaminase from *Aspergillus terreus* was determined by X-ray crystallography at a resolution of 1.6 Å. The structure of the protein is a homodimer that displays the typical type IV fold of PLP-dependent aminotransferases. The PLP-cofactor observed in the structure is present in two states (i) covalently bound to the active site lysine (the internal aldimine form) and (ii) substrate/product adduct (the external aldimine form) and free lysine. Docking studies indicate that (R)-transaminases follow a dual binding mode, in which the large binding pocket can harbour the bulky substituent of the amine or ketone substrate and the α-carboxylate of pyruvate or amino acids, and the small binding pocket accommodates the smaller substituent. A flexible arginine residue in the large binding pocket might be involved in the dual binding mode, as the R128A variant showed significantly reduced activity towards pyruvate. The insights in the active site were subsequently used to engineer the protein for improved activity and substrate scope.
Metabolically Active Baker’s Yeast as Platform Host for Recombinant \( \omega \)-transaminase-oxidoreductase Coupled Reactions

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Whole-cell biocatalysis refers to the conversion of a substrate to a given product catalyzed by one or several specific enzymes inside a microorganism that can be either metabolically active or inactive. Main advantages of using a metabolically active microorganism as catalyst include the possibility to express multiple enzymes simultaneously and the possibility to exploit cell metabolism for driving the biocatalytic reaction(s). However, a bioprocess based on living microbial cells may be inefficient due to inhibition or inactivation of metabolism by substrates, intermediates or products. When exposing a microbial cell to the harsh conditions that exist within a bioreactor during reaction progress, it is therefore imperative to understand physiological and metabolic responses of the microbial cell to design an efficient process.

In this study, we explored different genetic and metabolic aspects of using engineered baker’s yeast as platform host for \( \omega \)-transaminase-oxidoreductase-catalyzed conversion of racemic 1-phenylethylamine to \((R)\)-1-phenylethylamine and \((R)\)-1-phenylethanol (Figure 1). Particularly, the capacity of the cell’s central carbon metabolism to sustain the recombinant enzymes with amine acceptor pyruvate and regeneration of co-factor NADPH was investigated. We will report on results showing the capacity of recombinant yeast as catalyst for efficient coupling of PLP- and NADPH-dependent reactions.

Figure 1. Yeast catalyzed model reaction under current investigation.
Structural Snapshots Illustrate the Catalytic Mechanism of EDDS lyase

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Widespread use of metal chelators often leads to their buildup in the environment. Hence, biodegradable alternatives are highly desirable. We have structurally and functionally characterized a novel lyase from the aspartase/fumarase superfamily\textsuperscript{1} involved in the degradation of a metal chelator, ethylenediamine-\textit{N,N’}-disuccinic acid (EDDS). EDDS is reversibly broken down by EDDS lyase to form fumaric acid and ethylenediamine.\textsuperscript{2,3} We have solved a series of crystal structures of EDDS lyase in complex with EDDS, fumarate, and the reaction intermediate, \textit{N}-(2-aminoethyl)aspartic acid, which gives us a comprehensive view of the catalytic mechanism of this enzyme.

References

Screening of Transaminase Activity for the Production of Unnatural Amino Acids

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Enantiomerically pure amines represent highly valuable building blocks found in 70% of all pharmaceuticals, with the global market estimated at US$ 9.5 billion per annum. Synthetic methods to introduce the amine group can be non-trivial, time consuming, require undesirable solvents, energetic reagents or scarce metals as catalysts. A “green alternative” to the conventional metal catalysed reductive aminations would be the use of microbial transaminase (TA) as biocatalysts. Microbial transaminases (TA) are well established biocatalysts for the synthesis of the chiral amines. Given the prevalence of chiral amines in pharmaceutical preparations and the development of biocatalytic processes in that industry there is a drive to develop either new TAs as biocatalysts or to broaden the range of the activity of established TAs mainly through protein engineering.

The aim of this work was to screen for TAs from different bacterial strains that could be used for synthesis of various unnatural amino acids. Different primer sets were designed based on known TA sequences and were used to amplify TA genes from strains belonging to Arthrobacter, Mesorhizobium, Corynebacterium, Nocardioides, Pseudomonas, Chromobacterium, and Vibrio species. Amplified TA genes were verified by sequencing and overexpressed in E. coli BL21 (DE3). Recombinant TAs were assessed as catalysts for asymmetric synthesis of amines.

In parallel, a number of enzymatic assays were assessed. Wild type and recombinant strains were used as sources of TA activity. The assay based on using (S)-α-methylbenzylamine as an amino group donor in the reaction of asymmetric synthesis of amines proved to be the most reliable and compatible with different amino group acceptors.
ω-amino Acid Production by a Transaminase/Lipase Enzyme Cascade

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Transaminases are a powerful tool for the synthesis of chiral amines and amino acids, due to their wide substrate spectrum and their high enantio- and stereoselectivity. Taking into consideration the growing importance of optically pure β-amino acids as building blocks for peptidomimetics or other bioactive compounds new synthesis strategies have to be found.

In earlier studies a transaminase was found capable to convert β-amino acids [1]. For the asymmetric synthesis of β-amino acids the major challenge is the spontaneous decarboxylation of the β-keto acid precursor [2]. Consequently β-keto acid esters were used as starting substrates, hydrolyzed by a lipase to the corresponding β-keto acid. This freshly prepared β-keto acid is subsequently converted to the β-amino acid by the transaminase (Fig. 1). To find the perfect reaction conditions and to adjust the specific activities of the enzymes, several parameters (like buffer systems or enzyme immobilization) have to be investigated.

Figure 1: Synthesis of β-amino acids by an enzymatic reaction cascade.

Tailor-made Transaminations

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Enzymicals AG is a German biotech-company specialized in the development, production and utilization of biocatalysts. We produce chemicals with special emphasis on chiral building blocks and provide customer-based solutions for enzyme discovery, protein engineering and process development.

Biocatalysts can simplify, or in some instances even enable, the production process of complex chemicals and drug intermediates. Various enzymatic routes can be employed for the synthesis of optically active amines originating from the enzyme classes of hydrolases, oxidoreductases and transferases. Especially the application of transaminases is currently undergoing a constant acceptance and increasing application because of the possibility to obtain a 100% yield starting from easily accessible prochiral ketones (see scheme 1).

Scheme 2 General reaction concept
Application of Membrane Contactor as a Process Intensification Approach for Chiral Amine Synthesis

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The use of asymmetric synthesis (AS) for chiral amines production using prochiral precursors has been favoured over kinetic resolution (KR) using racemic mixtures due to the high theoretical yields (and the less expensive nature of the achiral precursors compared to the racemic mixtures). Compared to the KR route, \( \omega \)-transaminase catalyzed AS reactions are however limited due to unfavourable thermodynamic equilibrium and product (and substrate) inhibition issues\(^1\). Using isopropyl amine (IPA) as a cheap amine donor, investigations on the asymmetric amination of benzyl acetone (BA) showed that the 1-methyl-3-phenylpropylamine (MPPA) product exhibited severe product inhibition.

\[
\begin{align*}
\text{BA} + \text{IPA} & \xrightarrow{\text{TA}} \text{MPPA} + \text{Acetone} \\
\end{align*}
\]

Approaches to remove the MPPA product which would prove beneficial to reduce its inhibitory effects, while providing an equilibrium shift aiding more product formation, were investigated. Using the BA reaction system as a case study, the extent of amine conversion in different organic solvents was studied. This is with the aim of developing an intensified process where the MPPA is subsequently extracted in an aqueous phase using a microporous membrane contactor. Of the three solvents (n-heptane, toluene and MTBE) tested, conducting the transamination reaction in n-heptane was seen to exhibit the best conversions (33% conversion, on basis of product formed-10 mg enzyme (ATA 50)/mL, 10 mM BA, 100 mM IPA). With respectively \( \approx 93\% \) and 81% of the MPPA and acetone concentration extracted from the solvent phase, sodium acetate buffer (pH 3) was selected as a suitable extractant. Further process optimization and a demonstration of a coupled system is currently under investigation. The study results will be detailed in this paper with the implications for the use of the proposed conversion and separation approach for chiral amine synthesis discussed.

Enzyme Characterization in Microreactors by UV-Vis

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In protein engineering mutants are often purely selected on the basis of activity [1], this simplifies the analysis and enables high throughput screening. At the later stage of development, few mutants show comparable performance and this basis for selection becomes indistinct. The basis can at this point be improved by characterization of the enzyme performance where also inhibition and toxicity effects are taken into account. Enzyme characterization is here defined as the effect on initial rate of pH, Enzyme, Substrate, co-Substrate, Product and co-Product concentration [2]. From this investigation, it will be possible to determine whether the enzyme meets the criteria for process requirements or not. The development of the process will determine the requirements and this can also reach a state of maturity that resolves obstacles, lowers criteria and paves the way for implementation. As an example ω-transaminase is here investigated, which facilitates the exchange of an amine- and keto-group stereoselectively. The characterization will be carried out in a microreactor [3], this size is currently the only concept that can facilitate this thorough analysis, as the enzyme resource is scarce at this point of development. In the case where the reaction operates with UV active components, UV can be used to detect compounds with high sensitivity supplemented by multivariate data analysis. The spectra are here decorrelated and regressed to yield concentrations of individual compounds. HPLC systems are built for handling small quantities of liquids and the UV detectors for these proves to be fitting excellent. Enzyme characterization will therefore be carried out by combination of a microreactor with a diode array detector from an HPLC system.

References:
Biosynthesis of Cationic Cysteine Derived *gemini* AAS Surfactants towards Gene and Drug Delivery Complex

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Amino acid-based surfactants (AAS) are very attractive compounds regarding their multifunctionality, biocompatibility and renewable sources of raw materials. Among them gemini AAS have shown to interact with biological macromolecules, to form supramolecular complexes with bioactive molecules, like DNA. Cationic gemini AAS are based on cysteine scaffold, making use of a disulfide bond, that links the cysteine residues of the two monomeric units of the gemini structure, to enhance transfection efficiency exploiting the high intracellular levels of glutathione (GSH), an endogenous reducing agent. These redox-triggered biosurfactants are expected to act as bioresponsive systems that deliver genes to the target cells where efficient nucleic acid release will result from cleavage of the disulphide linker by intracellular GSH. Therefore, the goal of this work was the development of new biocompatible and biodegradable gemini AAS with improved performances for technological and biomedical applications. The new AAS was prepared from cystine (disulfide-bonded cysteine) by amide bond formation between the carboxyl groups and long-chain alkylamines (fatty amines) (e.g. dodecylamine). A green chemistry-based approach to the biosynthesis of the gemini AAS were developed using the lipases: Lipozyme® TL IM, Lipozyme® RM and PPL. This enzyme was immobilized, in-house, in sol-gel lens. In order to improve reaction rates and yields, due to the low and different solubility of substrates, the media design included biphasic (e.g. Tris buffer with n-hexane or 2-propanol), co-solvent (e.g. MeOH, DMSO) or “solid” (55% Dowtherm®A) systems. Samples from the media modification assays were analysed by HPLC-MS/MS, leading to the identification and quantification of the *gemini* AAS in most media optimized. For the first time a biosystem, in liquid and solid media, using immobilised lipases was used with success in the production of cationic gemini AAS from cystine. The combination of dimeric AAS activity and the ability to form complex aggregates with DNA is very promising to gene therapy and as a new vehicle for (bio)drug delivery and transfection.
Fast Selection Method for Simple Scale-up of $\omega$-transaminase Reactions

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For some biocatalytic processes, such as small singular batches, the speed of implementation is key to success, in some cases even more than an optimal process. To use of biocatalysis in such applications we have developed a fast and easy step-wise selection procedure which evaluates if a given $\omega$-transaminase ($\omega$-TA) reaction is suitable for a “simple scale-up”, defined as a system without the need for extensive process development or specialized equipment set up. The three step evaluation procedure consists of (1) thermodynamic assessment, (2) biocatalyst activity screening and (3) determination of product inhibition. Each step of the method has a threshold value for easy implementation. In a case study the method has been applied to the reaction of two ketones previously found to be reactive with $\omega$-TA, 4-bromoacetophenone$^1$ and 1-Boc-3-piperidone$^{2,3}$, with the donors, isopropylamine and $\omega$-methylbenzylamine. One reaction pair (1-Boc-3-piperidone and isopropylamine) was selected for intensification and subsequently ran at 25 mL scale. The best product yields were obtained with the 50 g/L reaction giving a yield of 70% (S)-1-Boc-3-amino-piperidine, the reaction was further optimized up to intensified up to 75 g/L substrate, however lower yields where obtained.

Aldolase-Transaminase loop for \(\alpha\)-amino-\(\gamma\)-hydroxy acids synthesis


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Aldolases (AL) and transaminases (TA) constitute efficient tools for the development of new biocatalytic processes\(^1,2\) and the combination of AL and TA in a bienzymatic cascade constitute an interesting approach to prepare \(\alpha\)-amino-alcohols with high stereoselectivity. We have designed a new cascade type, in which the donor substrate of AL is generated from the amino donor substrate of TA. This “AL-TA loop” process brings the benefits of high atom economy and overall equilibrium shift.

As a proof of concept, we have combined several pyruvate-aldoleses (PyrAL) with aspartate aminotransferase (AspAT) to prepare 4-hydroxy-glutamic acids (scheme).

Aspartic acid (Asp) was used as amino donor and converted to oxaloacetate (OA) through transamination. Decarboxylation of OA was catalysed by the PyrAL itself, thus giving pyruvate (Pyr) as the donor substrate for aldolisation with glyoxylic acid. Therefore, a catalytic amount of Pyr proved sufficient for reaction completion whereas decarboxylation and thermodynamically favored aldolisation ensured the overall equilibrium shift. AspAT offered exclusive access to Glu analogues of the \(L\)-series. Moreover, use of 2 different PyrAL with complementary stereoselectivities allowed to prepare separately syn and anti-isomers both isolated with high yields (> 95 %) and high purity (> 95% de). We will now try to implement this AL-TA loop principle for the preparation of a variety of target compounds through the combination of various AL and TA isolated by screening enzyme collections built by a genome mining approach.

Carboligation combined with Reductive Amination – In two Enzymatic Steps to Amino Alcohols

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Amino alcohols are valuable compounds for the pharmaceutical industry and find various applications in organic synthesis. An example of valuable compounds with diverse applications are Nor(pseudo)ephedrines, N(P)E. Asymmetric synthesis of these chiral compounds require either multi-step preparative routes, expensive reagents and/or lack high selectivities\cite{1,2}. We have developed 1-pot strategies for the synthesis of nor(pseudo)ephedrine isomers in only 2-steps and high optical purities\cite{1,3}. In in first step the ThDP-dependent carboligases produce PAC followed by subsequent reductive amination catalyzed by ω-transaminases in the second step. This gave access to N(P)Es in space-time yields up to ~26 g l\textsuperscript{-1} d\textsuperscript{-1}. Optical purities of ee >99 \%, de >98 \% were feasible for (1\text{R},2\text{R})-NPE and (1\text{R},2\text{S})-NE\cite{4-5}.

The production of (1\text{S},2\text{S})-NPE and (1\text{S},2\text{R})-NE in high optical purities is more challenging, since for the first reaction step only (\text{R})-selective ThDP-dependent carboligases exist in nature, but an (\text{S})-selective catalyst is required. By enzyme engineering of a ThDP-dependent pyruvate decarboxylase via several rounds of side directed mutagenesis, a catalyst for the (\text{S})-selective synthesis of the intermediate phenylacetylcarbinol is now accessible yielding >97 \% ee using whole cells or isolated enzyme\cite{6}.

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\bibitem{6} D. Rother, M. Pohl, T. Sehl, L. Marx, R. Westphal, patent app. no. 10 2014 013 644.2 and 10 2014 013 644.2
\end{thebibliography}
High-throughput Screening of epPCR-based Transaminase Libraries by Application of a Glycine Oxidase Solid-phase Assay

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Transaminases represent one of the most important enzyme types within the biocatalytic toolbox for the synthesis of chiral amines as they allow asymmetric synthesis with quantitative yields and high enantioselectivity. However, for application as efficient biocatalysts, numerous criteria should be met, such as broad substrate scope (also towards bulky substrates), high thermal and organic solvent stability, high enantioselectivity, minimized substrate and product inhibition and a shift towards product formation in the asymmetric synthesis mode. Besides rational protein engineering approaches, directed evolution still represents the key to engineer proteins in order to fit the manufacturing process. However, this inevitably results in huge libraries, predominantly containing inactive or less active variants. In order to enable high-throughput prescreening of large epPCR-libraries for directed evolution of transaminases a coupled solid-phase assay with glycine oxidase and horseradish peroxidase was developed. Transaminase activity is detected upon transfer of an amine group from an amino donor substrate to glyoxylate, generating glycine, which is subsequently oxidized by glycine oxidase, releasing hydrogen peroxide in turn. Horseradish peroxidase uses the hydrogen peroxide to produce benzoquinone, which forms a red quinone imine dye by a subsequent condensation reaction. As glycine does not carry a chiral center, both (R)- and (S)-selective transaminases accepting glyoxylate as amino acceptor are amenable to screening. The solid-phase assay features prescreening of up to 1000 variants per 8 cm diameter plate with a sensitivity as low as 10 mU/mg of specific activity of a well over-expressed transaminase.

In this poster we provide a detailed insight in the application of our recently reported [1] assay to ongoing research, using it to improve the performance of transaminases.

Sequential Immobilization of Catalysts for Application in Cascade Reactions

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Immobilization of catalyst on solid supports can confer a number of advantages including ease of recovery and re-use and the capability of using biocatalysis in solutions such as in nonaqueous solvents, where the enzyme is insoluble [1, 2]. The use of mesoporous materials (MPS) as supports offers some valuable advantages and, more interestingly, can provide a more stable environment in comparison to planar surfaces. The large regular repeating porous structures of MPS enables the adsorption of high loadings of catalytically active enzymes within the pores. Individually optimized heterogeneous catalysts such as metal based catalysts and enzymes can be prepared and combined for use in cascade reactions.

We have developed a detailed protocol for the successful immobilisation of enzymes on MPS [3] including lipase CALB [4], glucose oxidase and alanine racemase [5]. Here we report the preparation of styrene oxide using a chemo-enzymatic cascade system. The catalytic system involves the enantioselective epoxidation of styrene by manganese complexes (1,4-dimethyl-1,4,7-triazacyclononane) using H$_2$O$_2$ generated by immobilized glucose oxidase. The intermediate styrene oxide can then be further converted to amino-phenylethanol by haloalcohol dehalogenase.

In the Search of Novel $\omega$-transaminases from hot Terrestrial Environments

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Transaminases (TAs) represent an attractive option for the synthesis of chiral amines, valuable building blocks for the preparation of many pharmaceutical agents.

In particular, $\omega$-TAs are of industrial interest because they are capable of performing reductive amination using a broad range of amine donors and acceptors. To date, an increasing array of $\omega$-TAs are available to investigate their industrial application, but little is known about thermostable $\omega$-TAs which could be advantageous for industrial processes requiring harsh conditions such as high temperature and/or the presence of organic solvents.

Aim of the presented work is to obtain novel thermostable $\omega$-TAs from thermostable microorganisms by metagenomic mining.

Hot spring samples were collected in Iceland, China and Italy, DNA was extracted from these samples and sequenced. A bioinformatics search of the metagenomic assemblies was carried out using as queries representative $\omega$-TA sequences, e.g. from Chromobacterium violaceum and Vibrio fluvialis. Four complete sequences showing good similarity with $\omega$-TA from C. violaceum were identified, cloned from the metagenomic DNA and expression trials for the recombinant production of the proteins in E. coli were performed. Functional characterization of the successfully overexpressed enzymes is currently under investigation.

This work has been carried out in the framework of the HOTRAM project, a FP7 Marie Curie IEF post-doctoral program (grant agreement PIEF-GA-2013-622732).
Expanding the Substrate Scope of an Amine Transaminase via Protein Engineering of its Active Site towards Bulky Substrates

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The preparation of complex bulky amines, which are desired building blocks for pharmaceuticals and agrochemicals, is challenging. Nature already has a rich reservoir of enzymes, from which a suitable candidate can be chosen as a starting point for the development of biocatalysts capable of producing such amines. In contrast to classical approaches such as screening of strain collections, modern developments in the area of metagenomics offer enormous potential. In a recent study we identified four crystal structures with unknown function from the protein database ((PDB) codes: 3HMU, 3I5T, 3FCR, and 3GJU), where we could show that all four are transaminases.¹,² Based on extensive analysis using the 3DM-database provided within NewProt, homolog libraries were constructed with three templates: 3HMU, 4E3Q and 2YKY. A position located in the small binding pocket (SBP) was proposed to be responsible for the steric limitation of substrate recognition. This position, with Phe in 3HMU and 4E3Q or Arg in 2YKY, was randomized with all the possible small and aliphatic residues.³ This substitution revealed mutation F85L in the ATA from *Vibrio fluvialis* (4E3Q) that produced a variant 1.5x more active towards bulky substrates. After the initial results, the analysis on the ATA from *Vibrio fluvialis* was continued, aiming to further improve its performance in the catalysis of more challenging bulky substrates. Therefore a larger library comprising 16,384 variants targeting seven sites simultaneously has been prepared and is currently screened on our robotic platform⁴ using a modified acetophenone assay⁵ for six reactions comprising three amine donors and two amine acceptors.

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Plug & Play Biocatalysis for Synthesis of Chiral Amines with Transaminases

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The application of ω-transaminases (or amine transaminases, ATAs) for the preparative synthesis processes of chiral amines is often hampered by limited acceptance of appropriate amine donors at high substrate concentration. The majority of wild-type enzymes neither accepts isopropylamine (IPA) very well nor are very active at process relevant substrate concentrations. Enzyme engineering is the key technology to create an ATA platform which can be applied for preparative chiral amine syntheses. We identified a set of unique (R)- and (S)-selective wild-type ATAs from biodiversity screenings and selected scaffolds of either type to create a mutant enzyme platform. The c-LEcta enzyme engineering platform consists of a fully established method portfolio to make each step in directed evolution highly efficient. The platform allows fast access to optimized enzymes and utilizes process relevant screening conditions. A set of mutant enzymes was specifically developed with this platform for chiral amine synthesis process applications at high substrate loads, showing high thermostability and acceptance of IPA. In order to make these enzymes available at any scale, scalable enzyme production processes were implemented for reference scaffold enzymes of each platform. The availability of a set of high quality ATA enzymes, which were obtained by enzyme engineering and are pre-qualified for a wide range of important structural patterns, applicable for preparative synthesis processes and rapid production at any scale make plug & play biocatalysis possible. The enzymes are available from c-LEcta starting in amounts required for screening up to kg and ton-scale in timeframes that are consistent with the development routine in the pharmaceutical industry.
A Concept for Simultaneous Product and Coproduct Removal with an Integrated Downstream Step: Enzymatic Cascade and SLM Extraction

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A continuous process concept for chiral amine synthesis is presented, which simultaneously overcomes the thermodynamic and kinetic hurdles in amine transaminase-catalyzed reactions: enzyme inhibition of product and coproduct as well as increasing the synthetic yield by displacing the reaction equilibrium. This was achieved by combining the supported liquid membrane (SLM) methodology with an enzymatic auxiliary reaction and utilizing alanine as amine donor substrate. The use of alanine (instead of isopropyl amine) in combination with SLM omits amine donor extraction and affords high product purity. The SLM facilitates the equilibrium shift by selective amine product extraction and the enzymatic auxiliary reaction minimizes coproduct inhibition. Advantageously, because this system allows amine product enrichment, poorly water-soluble ketones can be employed as substrate. Moreover, this process concept features an integrated downstream step facilitating amine recovery. For a continuous (industrial) process, however, the enzyme(s) stability needs to be maintained.
Methoxamine Synthesis in a Biocatalytic One-pot Two-step Approach

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Many chiral vicinal amino alcohols are of direct pharmaceutical use or constitute building blocks for medicinal substances. Since the synthesis of amino alcohols in chemical reactions often reveals low stereoselectivities or affords multi-steps, biocatalytic cascade reactions using ThDP-dependent enzymes and \(\omega\)-transaminases are a valuable alternative.

In previous studies,\textsuperscript{[1,2]} all four stereoisomers of the sympathomimetic amino alcohol nor(pseudo)ephedrine could be achieved in a one-pot two-step reaction with high conversions and \textit{de}le\textit{ee} values. Now this concept is transferred to substituted amino alcohols to broaden the product platform towards pharmaceutically potent vicinal amino alcohols by establishing two-step cascade reactions producing 2-amino-1-(2,5-dimethoxyphenyl)-propan-1-ol, also called methoxamine, with high space-time-yields and stereoselectivities.

\begin{center}
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=0.7\textwidth]{figure1.png}};
\end{tikzpicture}
\end{center}

\textbf{Figure 1: One-pot two-step reaction to methoxamine.}

First results reveal that all single steps of the cascade reactions are achievable. An outlook on current reaction optimisation and process engineering strategies to find an optimal operation mode for the whole cascade will be given.

One-pot Triangular Chemoenzymatic Cascades for the Syntheses of Chiral Alkaloids from Dopamine

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Multi-enzyme cascade reactions can provide access to complex chiral compounds from low cost starting materials in one pot. Here we describe novel biocatalytic routes to (S)-benzylisoquinoline alkaloids (BIAs) using the enzymes transaminase (TAm) and norcoclaurine synthase (NCS) in a one-pot, one-substrate ‘triangular’ cascade. The addition of a biomimetic chemical step to this process enables the one-pot, three-step formation of a (S)-tetrahydroprotoberberine alkaloid (THPB) from dopamine. These reactions proceed quickly (< 4 h), operate with high atom economy, and generate products with excellent enantioselectivity [1]. Employment of up to two C-C bond forming steps allows for the rapid generation of molecular complexity under mild conditions. This work demonstrates the potential of in vitro biocatalytic approaches to the synthesis of natural products and related compounds from simple starting materials.

Single-point Mutation Increases the Serine: Pyruvate α-transaminase Activity in CV2025 ω-transaminase

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Transaminases (TAm) have been widely applied for the production of various α-amino acids and chiral amines [1,2,3]. In contrast to α-TAs that exclusively transfer the amino group from a carbon atom to an α-carbon of amino acids, ω-TAs accept donors with amines distal to the carboxyl group [3]. Protein engineering efforts aiming at the improvement of enantioselectivity or modulation of substrate specificity of TAs have previously been reported [4,5]. However, engineering α-TAm type activity within an ω-TAm enzyme by directed evolution has not yet been explored. The introduction of single-point mutations into Chromobacterium violaceum ω-TAm (CV2025) was found to significantly change the substrate specificity towards L-serine and thus towards α-TAm-type serine-pyruvate aminotransferase activity. The results indicated that Y153, W60 and F88 in CV2025 are important positions for defining ω-TAm substrate specificity. In particular, Y153 appears to be a key residue which controls access to the cofactor and which can be altered to allow L-serine access. Thus, redesigning the enzyme by saturation mutagenesis of single amino acid residues is sufficient to significantly modulate the substrate specificity, without requiring remodelling of the backbone and this work will be described.

Furthermore, several (S)- and (R)-selective ω-transaminases were screened towards their ability to convert various aromatic compounds. Amongst these transaminases, several were identified showing excellent conversion of the aromatic ketones towards their corresponding amines using methylbenzylamine as amino donor and these substrate selectivities will be presented.

Metagenomics: A New Strategy to find Transaminases for Biocatalysis


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The metagenome contains the DNA from all organisms in an environment, including all uncultivable bacteria and thus enables access to many genes for enzymes inaccessible by classical means. Our aim is to use a metagenomics approach to identify new biocatalysts, in particular transaminases (TAs) for the synthesis of chiral building blocks or bioactive molecules not easily accessible using classical organic chemistry. In our approach, the DNA from an environment is extracted, analysed and sequenced. Subsequently a contiguous (contig) read library is generated in silico and formatted into a BLAST database. Once the library is created, it can be searched by enzyme type. Here, 11 putative TAs from a tongue metagenomic sample have been identified, cloned and overexpresssed. The 11 TAs were screened as crude cell lysates using several assays against a set of substrates covering a wide structural diversity (aromatic, cyclic, aliphatic, functionalized ketones). Several TAs have been identified with activities towards different substrates and the results will be presented. Available TAs from the current UCL TAs toolbox have also been screened against several interesting aromatic ketones. These results will also be presented.

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

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Amine transaminases (ATA) are powerful enzymes for the stereospecific production of chiral amines. However, the synthesis of amines bearing 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 catalysed by enoate reductase and ATA. The ATA wild-type from \textit{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 diastereoselectivity: variant Leu56Val exhibited a higher (R)-selectivity (66\%de) whereas the Leu56Ile substitution caused a switched enantiopreference to furnish the (S)-configured diastereomer (70\%de). Addition of 30\% DMSO further improved the selectivity and facilitated the synthesis of (1R,3S)-1-amino-3-methylcyclohexane with 89\%de at 87\% conversion.
Selective ISPR in Asymmetric Synthesis of Chiral Amines using a Supported Liquid Membrane

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Asymmetric synthesis using ω-transaminase (ω-TA) is an attractive option to produce chiral amines of industrial importance. However, the reaction can be hampered by product inhibition and/or an unfavourable equilibrium position which then makes high yields difficult to achieve, often necessitating efficient in situ (co)product removal, (ISPR).

The presented work describes the selective ISPR of a chiral amine produced through asymmetric synthesis by employing a supported liquid membrane (SLM) prepared in a hollow fibre membrane contactor. The extraction selectivity is based on the difference in the pKa value and hydrophobicity of the amine product compared to the other components. The SLM strategy was successfully applied to the asymmetric synthesis of (S)-α-methylbenzylamine, increasing the conversion from 50% to 98% in a model system using isopropylamine as the amine donor and immobilized E. coli cells containing ω-TA from A. citreus [1]. In further studies, the system has shown promising potential also for continuous operation, allowing a very high product concentration to be accumulated in the stripping phase and simple regeneration of the SLM unit at suitable time intervals. Thus, the SLM approach should be a valuable addition to the existing ISPR techniques for improving ω-TA catalyzed asymmetric synthesis.

References
Engineering of $\omega$-transaminase from *Halomonas elongata* Adapting the Catalytic Pocket to the Synthesis of Unsaturated $\beta$-amino Acids

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Classical chemical synthesis of small molecules and pharmaceuticals generally exploits reactions that are often plagued by suboptimal yields and a considerable amount of toxic waste, especially when it comes to asymmetric synthesis and metal catalysts. Biocatalysis and the use of enzymes offer a valid alternative often able to reduce the number of synthetic steps while yielding a cleaner and greener process. Enzymes are traditionally strongly regio-chemo- and enantioselective, minimizing the need for selective protection of similarly reactive positions, requiring fewer separation and purification steps, and virtually eliminating the possibility of contamination of the reaction as the enzymes can easily be removed from the environment through precipitation or immobilization. Among the various classes of enzymes, transaminases are particularly interesting, since they can catalyze the amino transfer between two molecules, a challenging reaction to perform with traditional synthetic approaches. Specifically, $\omega$-transaminases can be applied to the production of chiral amines or $\beta$-amino acids, useful building blocks for pharmaceutical compounds.

Enzymes have clearly a number of drawbacks such as poor stability in harsh conditions typical of industrial set-ups, and poor versatility. A solution is to investigate enzymes from extremophile organisms, which are naturally adapted to “extreme” environments of high temperatures and low water concentrations. The $\omega$-transaminase from *Halomonas elongata* (HEwT), a halotolerant organism, has been shown to be resistant to organic solvents and to accept a variety of amino donors and acceptors making it an excellent candidate for evolution and incorporation in the synthesis of dehydro-$\beta$-amino acids. Generating a series of dehydro-$\beta$-amino acids is the target of this proposal, they are valuable intermediates in the synthesis of dehydropeptides (short, non-natural peptide sequences with an inherited rigidity), which have shown improved biological activity and selectivity when tested to prevent blood-vessel formation (angiogenesis), a key feature in cancer treatment.
A Continuous-flow Methodology for the Amidation of rac-N-Boc Amino acid Thioesters in DKR Conditions.

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Amide bond formation is a labour intensive process often involved in the synthesis of fine chemicals; esters and thioesters aminolysis is a practical method of amide synthesis avoiding inconvenient carboxylate activation. In recent evaluations of chemical processes trying to comply to the rule of GMF and Green Chemistry, amide bond formation was identified as one of the most utilized and problematic synthetic step in the pharmaceutical industry [1].

We have recently developed an efficient methodology for the hydrolysis/amidation of N-protected amino acid thioesters under Dynamic Kinetic Resolution (DKR) conditions, where the relatively high acidity of the α-hydrogen was exploited in a base-catalysed substrate racemization occurring in the same pot with a selective, protease-mediated, hydrolysis [2] or aminolysis/amoniolysis reaction [3].

Here we present a new continuous-flow DKR procedure for L-AA-amides formation which exploits an immobilized protease (optimised alcalase™ sol-gel entrapment system). The racemization of the unreacted enantiomer was carried out by the synergic action of DBU and heating [4].


Immoblization of L-aspartate oxidase from *Sulfolobus tokodaii*

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L-Amino acid oxidases are FAD-containing enzymes catalyzing the stereospecific oxidative deamination of L-amino acids to α-keto acids, ammonia and hydrogen peroxide. Our group recently and efficiently produced an L-aspartate oxidase (active on L-aspartate and L-asparagine only) from the thermophilic archea *Sulfolobus tokodaii*, (StLASPO), as a recombinant protein in *E. coli* and fully characterized it. The recombinant flavoenzyme shows distinctive features that makes it attractive for biotechnological applications (it is highly thermostable, it is stable in a broad pH range, it tightly binds the FAD cofactor and it shows a low Km for dioxygen).\(^{[1]}\) In this work immobilization studies on this novel biocatalyst have been carried out. Immobilized enzyme was used for kinetic resolution of racemic mixture of D,L-aspartate achieving e.e. > 99% in less than 1 hour, experiments on enzyme reusability and preliminary scaling up studies were also carried out. The best results in terms of immobilization yield have been obtained when StLASPO was immobilized on the amino support Relizyme™ HA403/S R and on the epoxy support SEPABEADS® EC-EP/S, as well as cross-linked enzyme aggregates (CLEA). Indeed, the Relizyme-StLASPO immobilized enzyme was efficiently reused in 5 cycles keeping full oxidation of L-aspartate in less than 2 hours.\(^{[2]}\)

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Facile Access to Optically Active 1,3-aminoalcohols by Combining Ketoreductase and Amine Transaminase Catalysed Reactions

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The biocatalytic synthesis of chiral amines has become a valuable addition to the chemists’ toolbox. However, the efficient asymmetric synthesis of functionalized amines bearing more than one stereo center remains still a challenge. By employing a ketoreductase (KRED) and two enantiocomplementary amine transaminases (ATA) we developed a biocatalytic route towards all four diastereomers of the 1,3-amino alcohol 4-amino-1-phenylpentane-2-ol. Starting from a racemic hydroxyketone a kinetic resolution using a KRED provided optically active hydroxyketone and the corresponding diketone. Further transamination of the hydroxyketone was performed by either an (R)- or (S)-selective ATA, yielding two distinctive 1,3-amino alcohol diastereomers. The remaining two diastereomers were accessible in two subsequent asymmetric steps. Regio- and enantioselective reduction of the diketone by the same KRED yielded the hydroxyketone with the opposite stereoconfiguration. Eventually, the subsequent transamination of the crude product with either an (R)- or (S)-selective ATA yielded the two remaining diastereomers.
Exploring the Stability of 
*Chromobacterium violaceum* ω-transaminase

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The ω-transaminase from *Chromobacterium violaceum* should be considered to be a thermostable enzyme due to its high melting point (74°C) in buffer solution (Humble *et al.* 2012). Despite the high melting point of the enzyme, it can not be successfully applied at elevated temperatures or in organic solvents in free form, even though thermostability often is correlated to other forms of enzyme stability. To explore this phenomenon further, the effects of different substrates or additives were explored by melting point measurements.

A well known method to stabilize proteins is the use of additives (Stepankova *et al.* 2013). In our previous study, addition of glycerol and NaCl to the enzyme solution improved the thermostability (Humble *et al.* 2012). Currently, the reason for enzyme destabilization and stabilization using different reaction conditions (for example, additives, organic solvents and temperature) is under investigation.


New ω-transaminases from Databases and Metagenome Sequences for the Stereoselective Synthesis of Chiral Amines

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Chiral amines are important building blocks for the production of fine chemicals and pharmaceuticals. Due to their capability to generate such amines in a stereoselective manner with high efficiency ω-transaminases received considerable attention during the last years.

To expand the range of these very useful enzymes evocatal screened its proprietary metagenome sequences as well as public databases. Besides the sequences which are publicly available we could identify about 550 sequences for (R)- as well as (S)-selective transaminases from our proprietary metagenome sequences. Sequences from different families were made available and have been studied in detail. Characterization of the proteins concerning their pH and temperature optima as well as their catalytic properties towards a broad range of carbonyl compounds was performed. In addition, production processes making these enzymes available on technical scale were developed.

As a result, 13 new (R)- as well as (S)-selective ω-transaminases have been made available that were proven to have a broad substrate range and that are appropriate to be used in technical applications. These enzymes will be commercially available in evocatal’s ω-transaminase kit soon. Since evocatal has a large number of transaminase sequences from the metagenome much more promising enzymes can be made available easily.
Engineering and Immobilization of Transaminases for Continuous-flow process applications

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Chiral amines are desired building blocks for the industrial production of pharmaceuticals and agrochemicals. Amine Transaminases (ATA) are highly versatile for the synthesis of optically active amines from a prochiral ketone by asymmetric synthesis.¹ Continuous-flow processes emerged to a promising method for asymmetric synthesis in the organic chemistry recently. Flow reactors can perform at higher temperatures and pressures. Additionally, they are distinguished for high mass or temperature transfer, easy downstream processing, avoiding intermediate isolation and reducing waste production, which agrees with the concept of green chemistry.²

Industrial application of biocatalysts requires their immobilization. In previous works, ATAs from Gibberella zeae ((R)-selective), Aspergillus fumigatus ((R)-selective) Ruegeria pomeroyi ((S)-selective) and Rhodobacter sphaeroides 2.4.1 ((S)-selective) were immobilized successfully on Chitosan beads using glutaraldehyde as crosslinking agent.³⁴ A higher thermostability and acceptable yields in conversion of α-methylbenzylamine could be obtained. Thus, we aim for the immobilization of ATAs in a continuous-flow system to improve the asymmetric synthesis of ATAs and hence the production of chiral amines. The combination of directed evolution and rational design of biocatalysts⁵ will be used to improve the catalytic activity and immobilization efficiency on several immobilization matrices to reveal an attractive industrial ATA with highest possible enantioselectivity, regioselectivity and specific activity towards a desired compound.

The pyridoxal-5'-phosphate (PLP)-dependent enzyme superfamily is grouped into five fold classes with separate evolutionary origins, resulting in similar reactions catalyzed by proteins with significantly different tertiary structures [1]. The reaction specificity is largely determined by the active site structure [2], and PLP-dependent enzymes often have promiscuous activity with related enzymes. It has been shown that with just one to three mutations close to the PLP binding site the enzyme can be changed from a racemase to a transaminase [3], a racemase to an aldolase [4], and a transaminase to a decarboxylase [5].

The fungal alanine racemase from *Tolypocladium inflatum*, which to our knowledge is the only canonical amino acid racemase within PLP Fold Type I [6], will be investigated for this approach. Here we present the promiscuous activity and substrate scope of the wild type, as well as variants with rational mutations in the active site.

THERMOGENE- Novel thermostable enzymes for industrial biotechnology

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There is an increasing demand for new thermostable enzymes with enhanced performance and/or novel functionalities that provide savings in time, money and energy for industrial processes in the areas of high value chemical production and other "white" biotechnology applications. The THERMOGENE project aims to identify transferase enzymes from microorganisms inhabiting natural hot environments. The project involves sampling from natural thermal environments, isolation of phylogenetically diverse thermophilic species with the desired enzymatic activities, sequencing of metagenomes or genomes of selected microorganisms, both new isolates and microorganisms from previously obtained culture collections of thermophiles, genome assembly, gene mining and functional classification of predicted proteins, high-throughput cloning, expression and activity screening, detailed biochemical and structural characterisation of selected novel enzymes.

THERMOGENE focuses on the discovery of selected transferase enzymes with known and potential commercial applications. These include enzymes which transfer 2-carbon units, transketolases; transfer amine groups, transaminases; transfer isoprenyl or prenyl groups, prenyltransferases and which transfer methyl and hydroxymethyl groups, methyl and hydroxymethyl transferases.

The Exeter group have solved the structures of the several transaminase enzymes of Pfam classes, III, IV and V in order to further understand their mechanism and substrate specificities.

The new THERMOGENE studies are concentrating on other less well studied thermophilic class IV (branched chain) and class V1 (sugar transaminases). Several new transaminases from novel thermophilic genomes and metagenomes have been identified and cloned and over-expressed in Escherichia coli. Biochemical and structural studies of some representatives of these new transaminase enzymes will be reported.

Integrated Reaction Approach For The
Production of Optically Pure Amines Involving
\(\omega\)-Transaminases

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Optically pure amines are important building blocks in pharmaceuticals and biological relevant fine chemicals. There is a high demand for the desired enantiomer in optically pure form. One promising, but also very challenging, route is the asymmetric synthesis catalyzed by \(\omega\)-transaminases.

Though asymmetric synthesis is advantageous over kinetic resolution it suffers from unfavorable equilibrium in the direction of the desired aromatic amine. Detailed studies to overcome this phenomenon were evaluated: Equilibrium constants of various amine donor and acceptor pairs were determined, high molar excess of the amine donor was applied and product removal strategies such as stripping of the ketone by-product and \textit{in-situ} product removal of the product amine were implemented. Theoretical conversions were calculated and compared to the practically obtained data.

Different (S)- and (R)-selective transaminases were tested in view of substrate specificity, activity and stability. All obtained data were implemented in calculating the overall performance of transaminase-catalyzed asymmetric synthesis of enantiomeric pure amines.

\[\text{Reaction Scheme: General transaminase reaction involving various aromatic ketones and aliphatic amines as starting material.}\]