

Transaminase mediated cascade reactions

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Abbreviations

AAO	Amino acid oxidase
4-AAP	4-Aminoantipyrine
ACP	Acetophenone
ACT	Acetone
ADCL	4-amino-4-deoxychorismate lyase
ALA	Alanine
AlaDH	Alanine dehydrogenase
AlaR	Alanine racemase
ALDH	Aldehyde dehydrogenase
ALS	Acetolactate synthase
ATA	Aminotransferase
BCAT	Branched-chain amino acid transaminase
Bm_ygjG	<i>Bacillus megaterium</i> ygjG putrescine transaminase
BMy_ygjG	<i>Bacillus mycoides</i> ygjG putrescine transaminase
CALB	<i>Candida antartica</i> Lipase B
CAR	Carboxylic acid reductase
Cv-TA	<i>Chromobacterium violaceum</i> transaminase
DAB	3,3'-Diaminobenzidine
DAO	Diamino acid oxidase
DAT	D-amino acid transaminase
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
Ec_ygjG	<i>Escherichia coli</i> ygjG putrescine transaminase
<i>Ee</i>	Enantiomeric excess
ERED	Ene-reductase
Et	Ethyl
FDH	Formate dehydrogenase
GDH	Glucose dehydrogenase
GC	Gas chromatography
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HRP	Horseradish peroxidase

IPA	Isopropyl amine
IRED	Imine reductase
INT	Iodonitrotetrazolium chloride
KRED	Ketoreductase
LAAO	L-amino acid oxidase
LDH	Lactate dehydrogenase
MAO	Monoamine oxidase
MAO-N	Monoamine oxidase-N
MBA	α -methylbenzylamine
Me	Methyl
MeOH	Methanol
MPMS	1-Methoxy-5-methylphenazinium methyl sulfate
MS	Mass spectrometry
NAD(H)	Nicotinamide adenine dinucleotide
NADP(H)	Nicotinamide adenine dinucleotide phosphate
NCS	Norcochlorine synthase
NMR	Nuclear magnetic resonance
Oa_TA	<i>Ochrobactrum anthropi</i> transaminase
ORF	Open reading frame
pATA	Putrescine transaminase
PAL	Phenylalanine ammonia lyase
PDC	Pyruvate decarboxylase
<i>P. putida</i>	<i>Pseudomonas putida</i>
Pp_spuC	<i>Pseudomonas putida</i> spuC transaminase
Pc_spuC	<i>Pseudomonas chlororaphis</i> subsp. <i>Aureofaciens</i> spuc transaminase
Pf_spuC	<i>Pseudomonas fluorescens</i> spuc transaminase
PCR	Polymerase chain reaction
Ph	Phenyl
P _i	Phosphate
PLP	Pyridoxal 5'-phosphate
PYR	Pyruvate
<i>spuC</i>	Gene encoding for the SpuC transaminase (spermidine and putrescine utilisation)
TA	Transaminase
ThDP	Thiamine diphosphate

TTC	Triphenyl tetrazolium chloride
Vf_TA	<i>Vibrio fluvialis</i> transaminase
<i>ygjG</i>	Gene encoding for the YgjG putrescine transaminase
ω -TA	ω -transaminase

Abstract: Transaminase mediated cascade reactions

A thesis submitted to The University of Manchester for the degree of Doctor of Philosophy in the Faculty of Science and Engineering (School of Chemistry), 2017

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In all its forms, catalysis accounts for 80% of all chemical processes worldwide, with biocatalysis playing a major part, as a “white biotechnological” tool in sustainable industrial production. Biocatalysis complements the traditional chemical synthetic methods and offers a distinct approach to the synthesis of high-value chiral molecules.

Chiral amine units are highly common in both the pharmaceutical and agrochemical industries. Biocatalytic strategies for the synthesis of chiral amines rely heavily on the use of ω -transaminases, enzymes impeded by their thermodynamic limitations in the synthetic direction, amongst others. This thesis describes an attractive alternative strategy to both the equilibrium-shifting problem and the low-cost availability amine donor, by employing biogenic polyamines, which are ubiquitous in all types of living cells. Moreover, they can be used in biomimetic enzymatic cascades for the synthesis of simple heterocyclic scaffolds of more complex alkaloids.

The present thesis shows the research carried out as part of this PhD and it is formatted as a series of manuscripts, part of which have either been published or recently accepted for publication, together with a manuscript which is planned to be submitted to a peer-review journal in the near future.

The thesis is structured as follows:

Chapter 1: a review of the current development of transaminases as biocatalysts, from their discovery and characterisation, to their development and use in multi-enzymatic cascades

Chapter 2: a research article on the discovery and characterisation of three novel putrescine transaminases (YgjG pATA) with high specificity for small aliphatic terminal diamine

Chapter 3: a research article on the discovery, characterization and use as biocatalysts of a class of diamine transaminases (Spuc_TA) with the ability to accept both mono- and diamine substrates

Chapter 4: a research article on the application of the diamine transaminase Spuc_TA in a multi-enzyme cascade reaction, using a novel amine donor (n-butylamine)

Chapter 5: a research article on the synthesis of hygrine and pelleterine type alkaloids *via* a putrescine transaminase/ lipase cascade.

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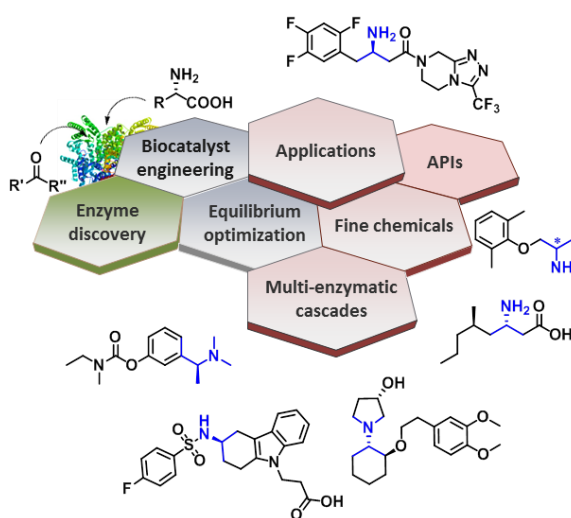
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Pentru Mami si Moni

Chapter 1. Transaminases (TAs)

Iustina Slabu¹, James L. Galman¹, Richard C. Lloyd^{2,3}, Nicholas J. Turner¹



1.1 Foreword

This chapter consists of a review article published in *ACS Catalysis* on 18th of October 2017. Due to its comprehensive nature, this review is used as an introduction and overview of the transaminase family enzymes. The review was conceived as a holistic approach of the aspects involved in the discovery and development as biocatalysts of transaminases, addressing to the most important challenges encountered in these processes. At the moment of publication, the manuscript did not include the work described in Chapter 5.

1.2 Acknowledgments

The present review was a collaborative effort between the doctoral candidate and James L. Galman¹, under the supervision of Richard C. Lloyd^{2,3} and Nicholas J. Turner¹. The doctoral candidate wrote the following sections: 1.3 Abstract; 1.4 Introduction; 1.5.2 Enzyme assays; 1.6.2 Equilibrium displacement techniques; 1.7 Synthetic applications, and co-edited the manuscript as a whole.

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1.3 Abstract

Transaminases have attracted considerable interest in their use as biocatalysts for the synthesis of compounds containing chiral amine units, which are wide spread within the pharmaceutical, agrochemical and fine chemical industry. Recent developments in enzyme- and process-engineering have expedited their use in asymmetric synthesis; however industrial applications are still hindered by a number of factors, including equilibrium thermodynamics, product inhibition and poor substrate tolerance. Detailed and comprehensive approaches to each of these challenges have been reported during the last two decades; the most representative enzyme discovery and screening strategies, protein and equilibrium engineering and immobilization techniques are reviewed herein. Furthermore, we present a detailed look into the applications of transaminases for the synthesis of a variety of amine-containing compounds and the integration of transaminases into multi-enzymatic systems that allow access to a variety of highly complex products for the end user.

1.4 Introduction

1.4.1 Pyridoxal-5'-phosphate (PLP) -dependent enzymes

PLP-dependent enzymes are widespread in cellular processes and intimately associated with biosynthetic pathways involving amino compounds¹. From actively participating in the biosynthesis of amino acids^{2,3,4,5} to being involved in the biosynthesis or degradation of aminosugars⁶, polyamines^{7,8,9} and alkaloids¹⁰, the PLP-dependent enzymes are characterised by the plethora of chemical reactions they catalyze.

Several PLP-dependent enzymes have been identified as drug-targets for a number of therapeutic strategies, *e.g.* serine hydroxymethyltransferase has been targeted in cancer therapy (SHMT2)^{11,12} due to its involvement in folate metabolism; γ -aminobutyric transaminase (GABA-TA) has been targeted for the treatment of epilepsy¹¹ and ornithine decarboxylase¹³ in the treatment of African sleeping sickness.

Based on the fold type, the PLP-dependent enzymes are classified into 7 major groups (Figure 1), with transaminases belonging to fold types I and IV^{2,14}. The first attempts to

establish a classification of PLP-dependent enzymes were reported by Grishin *et al.*¹⁵, refined later by Perracchi *et al.*²

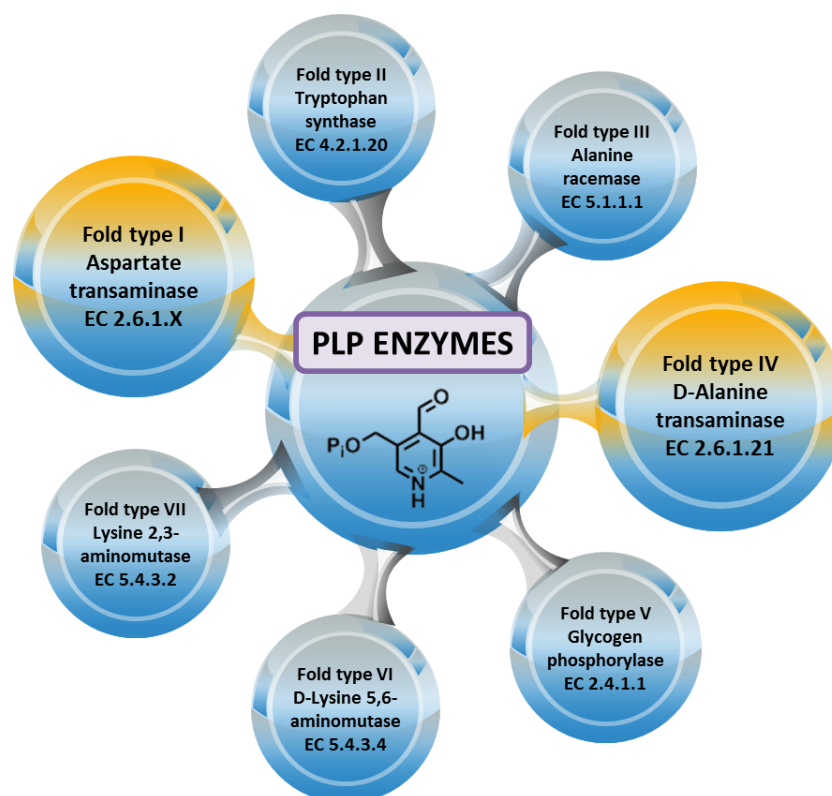


Figure 1. Classification of PLP enzymes based on fold types^{2,14}.

Currently, approximately 238 distinct catalytic functions are attributed to this group of enzymes¹⁶, which include racemization, transamination, decarboxylation, elimination, retro-aldol cleavage and Claisen condensation amongst others.

1.4.2 Transaminases TA

Transaminases are a class of pyridoxal-5'-phosphate PLP-dependent enzymes that catalyze the transfer of an amino group between an amino donor and an amino acceptor – *i.e* a compound containing a carbonyl functional group.

Based on the position of the transferred amino group, transaminases have been broadly grouped into α -TAs (catalyze the transfer of the amino group at the α -carbon) and ω -TAs (the amino group transferred in the reaction is located further away from the carboxylic moiety). Mehta *et al.*¹⁷ classified transaminases depending on sequence and structure similarity and grouped them in six subgroups or classes, with classes I and II including L-

aspartate transaminase and L-alanine transaminase, class III ω -transaminases, class IV D-amino acid transaminases and branched chain transaminases (BCAT), class V L-serine transaminase and class VI sugar transaminase (Figure 1).

The ω -TAs group includes some of the most interesting transaminases, both in terms of functionality and synthetic applications. Of particular interest are the amino transaminases (ATAs), enzymes that belong to both fold type I and fold type IV and which do not require the presence of a carboxylic group in the substrate molecule, being able to accept a large variety of carbonyl compounds, both aldehydes and ketones, as substrates. The amine transaminases constitute the main subject of this review, given their immense interest and use in chemo-enzymatic routes for the production of high-value chiral amines in pharmaceutical and agrochemical industries.

Transaminases follow a ping-pong bi-bi reaction mechanism, in which the enzyme bounces back from its intermediate form to its standard state, regenerating the PLP cofactor in the process. The overall process of transamination is composed of two half-reactions, the second one being the exact reversed sequence of events of the forward reaction.

1.4.3 Mechanism of biocatalysis of PLP-dependent enzymes

The common step (Figure 2, Step II) of the reactions catalysed by PLP enzymes is the formation of the external aldimine between the PLP and the substrate (Step I). PLP stabilizes the negative charge development at C_{α} in the external aldimine in the following step¹ and the fate of this adduct decides the type of reaction catalysed by each of the VII classes of PLP-enzymes. A notable exception from this is represented by a glycogen phosphorylase (EC 2.4.1.1)¹⁸ fold V which uses the phosphate group of internal aldimine in its biocatalytic mechanism.

Two chemical reactions follow: decarboxylation and deprotonation resulting in formation of a carbanionic quinonoid intermediate (Step III). A series of distinct retro-reactions (retro-Claisen cleavage and retro-aldol cleavage) also occur *via* C_{α} - C_{β} bond breaking (glycine C-acetyltransferase; serine hydroxymethyltransferase)¹⁴ or *via* β -deprotonation (radical mechanism, lysine-2,3-aminomutases). Decarboxylation reactions are typical for

ornithine decarboxylases. Deprotonation at the α -position by the ϵ -amino group of the catalytic lysine residue generates a quinonoid intermediate, stabilized by resonance. Reprotonation of $C\alpha$ position on the opposite face is performed by amino acid racemases and epimerases, whilst the protonation of the $C4'$ of the PLP generates a ketimine, central to the transaminases biocatalytic mechanism.

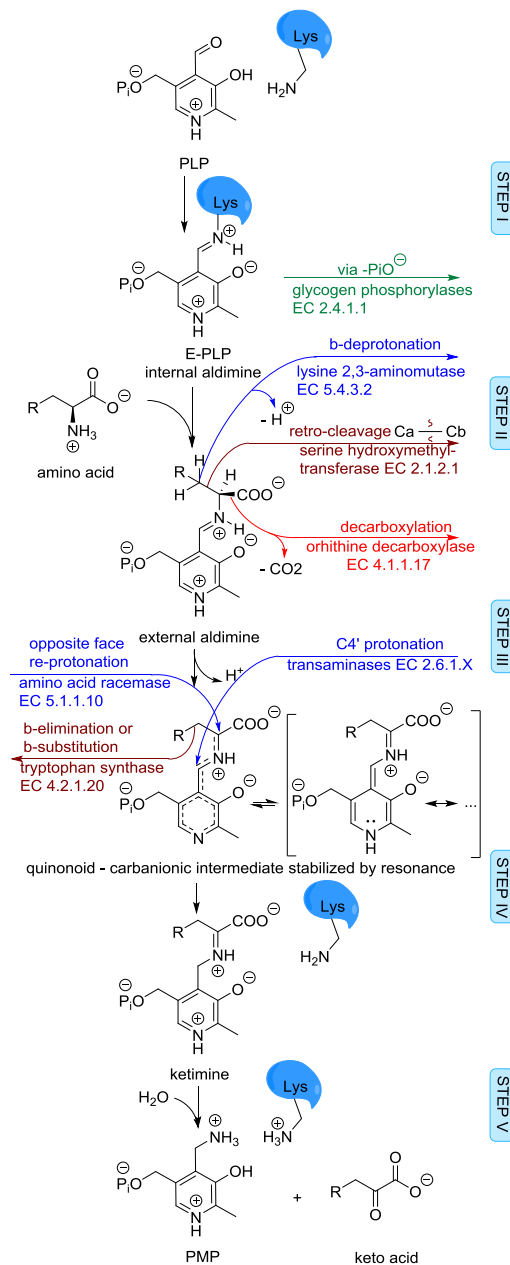


Figure 2. General mechanism of biocatalysis of the PLP-dependent enzymes, with short descriptions for the main classes of reaction catalysed. The five-step sequence describes in detail the first half of the mechanism of biocatalysis of transaminases (E.C. 2.6.2.X)¹⁴

The ketimine formed by re-protonation at C4' of PLP undergoes hydrolysis to form PMP and the free product (amino acid), thus completing first half of reaction in the transaminase mechanism. Kinetic studies have shown that the enzyme-PMP complex rapidly forms the apoenzyme as it is released from the enzyme active site. Crystal structure analysis reveals how the topology of the enzyme-cofactor binding site is contributory to the significant increase in the formation of insoluble higher aggregates from the apoenzyme.^{19,20} To impede this process it has been shown that an increase in PLP supply between 0.1-10 mM is essential for ATA operational stability, however higher PLP concentrations inhibit the enzyme-PMP complex resulting in aggregation.

The PMP will form an external aldimine with the second substrate participant to the reaction and this adduct will follow a mirrored sequence of events, completing the second half reaction, characteristic for the ping-pong bi-bi reaction mechanism of transaminases.

1.4.4 Scope of the review

Transaminases are industrially relevant enzymes and are useful in generating a large number of diverse compounds that contain chiral amine functionality. Transaminases have shown a rapid development over the past few decades, both in terms of enzyme discovery and industrial applications. Several previous reviews²¹⁻²⁵ focused on each individual aspect of their use as biocatalysts, *i.e.* gene discovery, enzyme engineering, biocatalyst formulation and synthetic applications. In this review, we give an updated holistic approach from the characterization of transaminases as biocatalysts, to their development and investigating their capability to participate in multi-enzymatic systems and in the synthesis of chiral building blocks used in the production of active pharmaceutical ingredients (APIs), agrochemicals and fine chemicals.

1.5 Discovery of transaminases

1.5.1 Gene mining

1.5.1.1 Enrichment cultivation: A functional driven approach

In the last decade, enrichment cultures of bacterial communities has led to the discovery of class III and class IV transaminase enzymes with novel activity beyond L- or D-alanine as an amino donor. This functional-driven approach was based on screening bacterial isolates expressing genes with a desirable trait that can actively metabolise amines of interest as the sole nitrogen source with minimal sugar based media. After several rounds of enrichment cultivation, the increase in biomass led to the identification of strains from 16S rRNA gene analyses.

Several research groups have identified microorganisms from soil samples at diverse environmental sites or from screening in-house culture collections for the chiral resolution of racemic amines using pyruvate as the keto acceptor. *Bacillus thuringiensis* JS64, *Klebsiella pneumonia* JS2F, and *Vibrio fluvialis* JS17 were amongst the earliest identified microorganisms to perform (*S*)-selective whole cell biotransformation on α -methylbenzylamine (MBA) and *sec*-butylamine and affording >95% of the (*R*)-enantiomer and attaining the theoretical maximum yield of 50%.²⁶

In hindsight, whole cell biocatalysis using cultured environmental samples has several complications such as enrichment bias due to competitive growth rates, inhibitory effects and bacteriophages: a major component in microbial soil flora, their prolific presence are likely to infect and lyse targeted bacteria in many enrichment cultures and effectively distort the distribution of bacterial isolates²⁷. Access to culture collections can alleviate these problems, however; significant drawbacks can arise despite isolating strains individually from other external environmental factors. A laborious screening of more than 100 lyophilized whole preparations from diverse range of microorganism was used to identify bacterial strains which contained ω -transaminase activity²⁸. Surprisingly a few from the genus *Pseudomonas* were capable of performing kinetic resolutions and *Janibacter terrae* DSM 13953 gave high conversion (51%) on a preparative scale within 48 hours with high *ees* >99%, (*R*)-isomer (α -MBA). Unfortunately none of these

microorganisms was able to perform sufficiently well in the amination direction regardless of the presence of coupling the reaction with a pyruvate decarboxylase to shift the equilibrium to product formation. The authors acknowledged that the major limitations were a result of poor transaminase gene expression within the host organism, generation of side products from other endogenous enzymes and/or poor mass transfer rate through the cell membrane.

A powerful yet challenging approach relies on identifying promiscuous transaminase phenotypes in the native organism involving a combination of genomic and proteomic studies. One such study involved *Bacillus megaterium* SC6394 (Bm-TA)²⁸ that showed initial whole cell activity with α -MBA. The cells were cultivated, harvested and lysed affording cell free extract. Soluble proteins were purified and fractionally collected according to their molecular weight and were assayed for their biochemical properties. Isolated protein hits were further digested into smaller peptide fragments and analysed via mass spectrometry (MALDI, ESI etc.) resulting in the identification of partial amino acid sequences.

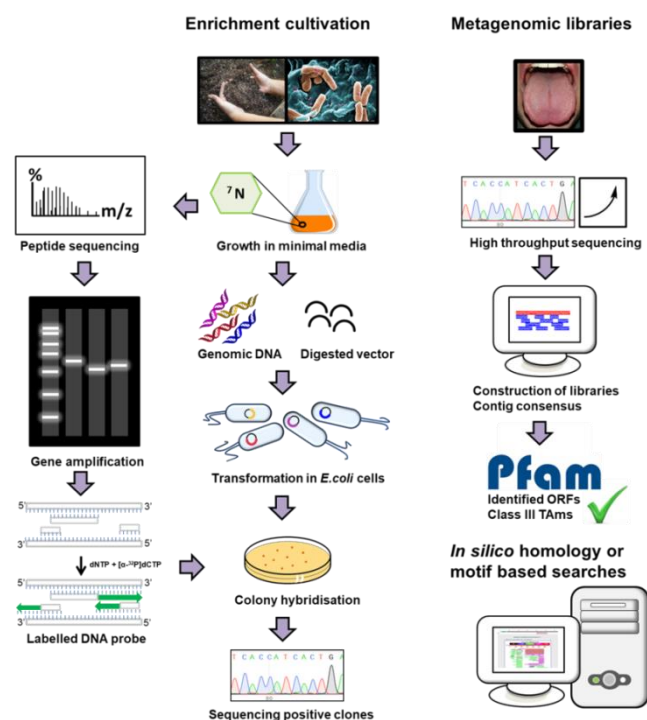


Figure 3. A schematic representation of the current strategy at identifying and isolating transaminase genes from function-driven and structure-driven approaches.

Degenerate primers based on the inferred amino acid sequences were used to amplify the gene from the genomic DNA extracted from the bacterial isolates that had been digested with a variety of restriction endonucleases. These genomic fragments were subcloned in a propagating vector and transformed in a suitable host bacterium. Clones were then probed for the labelled PCR gene fragment by colony hybridisation and the resulting positive hits were sequenced (Figure 3).

The identity of the transaminase DNA sequence from *Bacillus megaterium* SC6394 provided molecular biologists with the necessary information to overexpress the transaminase gene in heterologous hosts (such as *E.coli*) in addition to sequence similarity searches of comparable homologues in public databases. Other microorganisms such as *Vibrio fluvialis* JS17 (Vf-TA) which contains a widely studied (S)-selective transaminase, *Pseudomonas fluorescens* KNK08-18²⁹ (Pf-TA), *Alcaligenes denitrificans* Y2k-2³⁰ (Ad-TA), *Mesorhizobium* sp. LUK³¹ (Ms-TA), *Pseudomonas* sp. AAC³² and most notably the first (R)-selective transaminase from *Arthrobacter* sp. KNK168³³ (Ar-TA) with relaxed substrate specificity were identified in this manner.

1.5.1.2 Metagenome mining: a sequence driven approach

Metagenomics is a cultured-independent analysis of different microbial communities to assess the genetic diversity by non-selective means. Metagenomics is a growing field, yet still in its infancy and provides an alternative method to enrichment cultivation through the analysis of unbiased environmental samples. Moreover, metagenomics offers the opportunity to search both exotic and everyday environments ranging from deep sea vents, ocean surfaces and volcanic hot springs to commensal mammalian hosts.³⁴ These uncultured latent microorganisms can be elucidated via genomic assemblage to determine their metabolic functions and discover if they present novel taxonomic ranks of prokaryotic life³⁵.

There are generally two approaches to metagenomics analyses: 'function-driven' and 'sequence-driven' analysis have been used to obtain biological data useful for industrial application. Although there have been other industrial enzymes that has been discovered via a functional driven approach^{36,37} the work of Ward *et al.* on *in silico* sequence driven methods for discovering novel genes (transaminases) in commensal microflora from the

essential for functional-genomic analysis. Bioinformatics has provided researchers with similarity searching programs such as BLAST (Basic Local Alignment Search Tool) that uses an algorithm providing statistical estimates of amino-acid sequences or the DNA sequences to reliably infer homology from public database sequences. Homology based searches from previously discovered gene sequences have been the best choice for an efficient and cost effective approach towards gene mining of ω -transaminase genes. Genes can be cloned from cultured strains or as purchased synthetic codon optimised genes for effective heterologous gene expression in *E.coli* cells.

Kim et al. identified and characterised β -ATA genes (a subgroup of ω -ATA) from two independent bacterial isolates. *Mesorhizobium sp.* LUK³¹ was shown to synthesize enantiomerically pure L- β -aminobutyric acid (L-BABA) from the lipase mediated decarboxylation of the β -ketoester; however, low conversions were obtained (<20%). An alternative strategy was sought using a complementary β -ATA gene from *Alcaligenes denitrificans* Y2k-2, was shown to perform a kinetic resolution of 50 mM DL- β -aminobutyric acid (BABA) producing optically active D- β -BABA with 99% enantiomeric excess. Interestingly, the genes have a low protein sequence identity (~20%) but a high statistical estimate ($E > 50$) which is generally considered a more reliable measure of sequence homology. Further homologues were identified, *Caulobacter crescentus* TA⁴⁰ (65% protein sequence identity to *A. denitrificans* TA) that has high activity for short-chained β -amino acids, *Polaromonas sp.* JS666⁴¹ (54% sequence identity towards *Mesorhizobium sp.* LUK) which can resolve aromatic β -amino acids and *Burkholderia graminis* C4D1M⁴² TA (56% sequence identity towards *Mesorhizobium sp.* LUK) was shown to successfully synthesize chiral γ -amino acids.

The isolation and heterologous expression of *Vibrio fluvialis* JS17 TA gene was found to perform the best kinetic resolutions and asymmetric syntheses at producing pure chiral amines compared to other microorganism screened. This discovery heralded an influx of homology searches using Vf-TA as a reference protein ensuring functional critical (conserved) residues are highlighted and are part of the alignment. These ω -TAs, now referred to as amine transaminases are useful for industrial biocatalysis as they can effectively achieve chiral asymmetric synthesis from the corresponding prochiral ketone

independent of an α -carboxylic acid moiety. Some important examples are *Chromobacterium violaceum*⁴³ (38%) transaminase Cv-TA and *Parracoccus denitrificans*⁴⁴ (94%) Pd-TA, although they have comparable preferences for aromatic amines as the amino donor their main focus was the amination of 2-keto-1,3-diols and the production of L-homoalanine an unnatural amino acid, respectively.

Recent independent studies have identified a putrescine transaminase gene *spuC*, which is part of the polyamine and utilization pathway of *Pseudomonas aeruginosa* PAO1 and shown to have high sequence identity with Cv-TA (55-60%). The natural function of these ω -TAs is relatively unknown, although recent studies seem to indicate the involvement in the arginine decarboxylation pathway since an analogous function is found in *E.coli yjgG* encoding for a putrescine transaminase with strict preference for diamines and pyruvate/ α -ketoglutarate as ketone acceptor⁴⁵. The *spuC* genes from *Pseudomonas* species, in particular *P. putida* (Pp-*spuC*), *P. chlororaphis subsp. aureofaciens* (Pc-*spuC*) and *P. fluorescens* (Pf-*spuC*) have activity towards both mono- and biogenic diamines and hence are promising candidates for industrial applications.⁴⁶ The SpuC protein from *Halomonas elongata* DSM 2581 a moderate halophile, was shown to have cosolvent tolerance up to 20% with probable diamine activity⁴⁷ (61% identity to Pp-*spuC*).

1.5.1.4 In silico motif based assignments

The annotations of new sequences deposited daily are mostly predictions inferred from protein domains, families and functional residues and this can be completed efficiently into databases. However, proteins can have several motifs with different evolutionary origins, and hence a general homologous multiple sequence alignment would be considered although sometimes it is inadequate to predict the biological function of annotated enzymes against experimentally derived ones. By 2006, only one (*R*)-amine transaminase from *Arthrobacter sp. KNK168* had showed diverse activity with a variety of ketone acceptors. In 2010, Höhne *et al.* inferred from protein topologies, crystal structures and biochemical information of the transaminase family that (*R*)-selective amine transaminases belong within fold class IV. Most notably their research hypothesized patterns of evolutionarily conserved amino acid residues (or motifs) from D-amino acid transferases (DATs), 4-amino-4-deoxychorismate lyase (ADCL) and the L-

branched chain amino acid aminotransferase (BCAT) considered to be a plausible ancestor of the (*R*)-amine specific transaminase. An *in silico* annotation algorithm was developed that carefully analysed the motifs of multiple sequence alignments and jettisoned incorrectly labelled BCAT, DAT and ADCL from searched public databases. This led to the identification of 21 putative (*R*)-transaminases from approximately 6000 candidates, and of these candidates, 17 enzymes were experimentally confirmed. Following their lead, several groups have validated this motif based *in silico* strategy and found interesting genes from various prokaryotes and eukaryotes with a variety of amination and kinetic resolution activities.^{48,49}

1.5.2 Enzyme assays

The rapid development in processes employing transaminases, together with the increasing number of commercially available enzymes, has highlighted the need for high-throughput and efficient activity assays towards a wide range of substrates. From an industrial perspective,⁵⁰ an assay must allow screening for the desired conversion and lead to the identification of the most reliable hits. Very often, enzyme assays rely on artificial substrates, designed to elicit a response in a colorimetric or fluorometric assay. These substrates may not always be accepted by the target enzymes due to either steric or catalytic incompatibility.

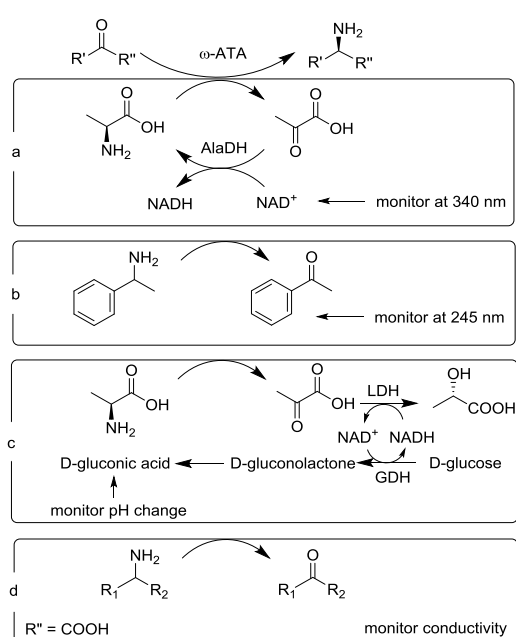
Robustness, reliability and sensitivity are very important especially in high-throughput screening of colonies, where the assay must discriminate between true positive clones and false positives. Delivering a very clear change of response is essential, although the degree of accuracy might vary, depending of the type of library screened. Screening of metagenomics libraries requires a less accurate assay than screening directed-evolution libraries, for which a quantitative assay is essential.

The most industrially relevant feature is the integral screening time, which quantifies the time needed from the start of the screening process to the generation of the first screening results. Although the ultra-high-throughput methods can generate a large number of results very quickly, their development can be time-consuming. Sometimes, a

generic assay is preferred, as it can generate results much faster and it allows a rapid start of the screening.

1.5.2.1 Liquid phase assays

The development of high throughput screening methods has been accelerated during recent decades due to the rapid increase in discovery of enzymes and their application in synthesis of high value compounds. The number of transaminases catalysing the reductive amination of a diversity of substrates has increased greatly and the number of distinct screening assays mirrors this trend, both in number and substrate diversity.



Scheme 2. High-throughput screening methods employing UV, pH and conductometric analysis

Keto acids/amino acids pairs are the natural substrates for some transaminases, therefore the early approaches to enzyme screening were based on direct detection of the resulting amino acids by capillary electrophoresis,⁵¹ ion-exchange liquid chromatography^{5,52} or direct spectrophotometric detection (*e.g.* formation of phenyl pyruvate monitored at 318 nm).⁴ Furthermore, coupling the main transamination process with a dehydrogenase allowed spectrophotometric monitoring of the NAD(P)H cofactor at 340 nm (Scheme 2a), enabling rapid screening; *e.g.* glutamate dehydrogenase³², malate dehydrogenase⁵³, alanine dehydrogenase⁵⁴ and succinic semialdehyde dehydrogenase.⁵⁵ An early

colorimetric assay was based on formation of a coloured complex between o-amino benzaldehyde and 1-pyrroline or 1-piperidine (440 nm) (Scheme 3f), allowing characterization of polyamine transaminases⁵⁶⁻⁵⁹ with limited applications in terms of amine substrate spectrum.

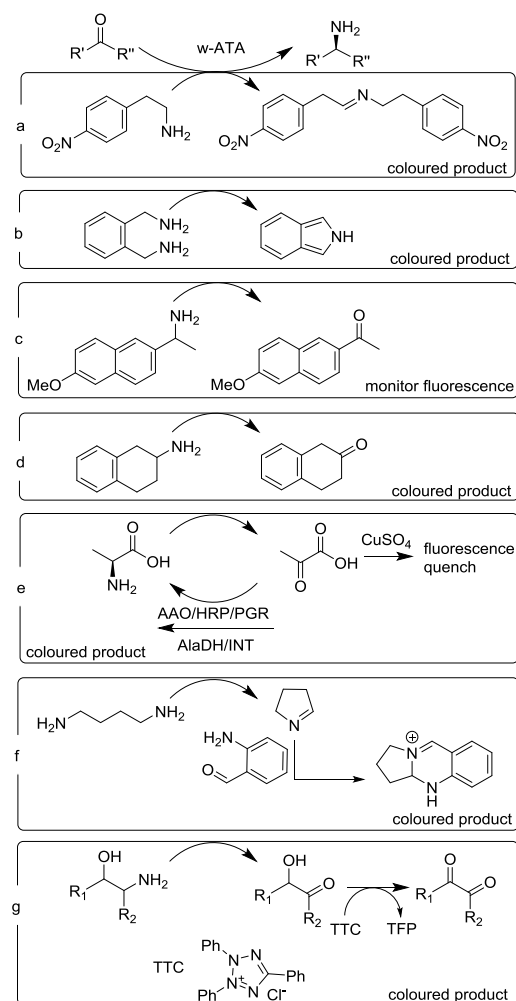
Monitoring changes in physical properties of the reaction system presented an alternative way of screening enzymes. For instance, Truppo *et al.*⁶⁰ coupled the transamination reaction with a lactate dehydrogenase/ glucose dehydrogenase system and monitored the pH change with the aid of a pH indicator dye (Scheme 2c). The system required tuning of the buffer strength for accurate pH variation detection. Likewise, Schätzle *et al.*⁶¹ analysed the substrate specificity of ω -transaminases by monitoring the changes in the conductivity of the reaction medium, using amines and keto acids as substrates (Scheme 2d).

One of the first high throughput transaminases screening methods relied upon monitoring the formation of coloured complexes between Cu(II) ions and α -amino acids (595 nm)⁶²; however the method suffered from interference from other amino acids in the cell extract and enzyme inactivation due to the chromogenic agent. An updated version of this assay was later applied in the characterization of a glutamic-pyruvic transaminase⁶³ which catalyses the conversion of alanine to pyruvate (Scheme 3e). Dissociation of the Cu(II)-L-alanine complex results in free Cu(II) ions which combine with BSA leading to fluorescence quench (monitor at 340 nm).

Screening large libraries of enzymes variants requires assays that are facile to perform and result in rapid and easily observable response. Using (*S*)- or (*R*)-MBA and pyruvate as substrates pair, Schätzle *et al.* developed a rapid kinetic assay that allowed qualitative and quantitative screening of transaminases by measuring acetophenone produced at 300 nm (Scheme 2b). The assay allowed for some flexibility in amine substrate spectrum, with a variety of substituted α -MBA and 1-aminotetraline derivatives used in screening process.^{26,64}

A set of epPCR mutant libraries (Cambrex enzymes) was assessed using (*S*)-2-aminotetraline derivatives, which were converted to the corresponding 2-tetralone

derivatives, that become coloured under air exposure⁶⁵ (Scheme 3d). Large panels of amines can be screened using pyruvate as amine acceptor and measuring the residual pyruvate in a complex with 2,4-dinitrophenylhydrazine (coloured complex, 450 nm)⁶⁶, whilst the formation of a fluorescent ketone from 1-(6-methoxynaphth-2-yl)ethylamine and pyruvate allows enzyme screening⁶⁷ (Scheme 3c).



Scheme 3. Colorimetric and fluorimetric screening techniques for assaying transaminases activity

Specialised and artificial substrates that elicit coloured responses are increasingly applied in colorimetric screening techniques. 2-(4-nitrophenyl)ethan-1-amine and several of its derivatives were applied in liquid-phase and solid-phase screening of several transaminases, based upon the ability of the resulting ketones to form coloured enamines (Scheme 3a). Some background interference was reported, however the method presents high sensitivity with the additional advantage of allowing screening of various ketones

and transaminases with a single amine donor.⁶⁸ In a similar manner, *o*-xylylene diamine⁶⁹ generates a coloured precipitate resulted from polymerisation of the reactive isoindole intermediate (Scheme 3b). Although non-linear and presenting a strong background colour, the assay is useful in solid-phase screening of enzymes and ketones. Alternatively, L/D-cysteine sulfinic acid can be used as amine donor in an ω -transaminase/aspartate transaminase coupled system, in which the released sulphur dioxide is detected by colorimetric sulphite titration with Ellman's reagent. The assay is particularly useful in screening keto acids and enzyme libraries.⁷⁰

Various dyes have been employed in designing enzyme assays, resulting in development of effective and sensitive enzyme assays. Our group has also reported recently a coupled enzyme assay that allows screening of enzymes and amine substrates using pyruvate as amine acceptor. The main transamination reaction is coupled with an L- or D-amino acid oxidase and horseradish peroxidase, the resulting H₂O₂ being detected in combination with pyrogallol red.⁷¹ (Scheme 3e) A modified version of this assay was later reported for solid-phase colonies screening,⁷² in which the transaminase and the amino acid oxidase were co-expressed in the cytoplasm of *Escherichia coli* cells on a single agar plate. To circumvent the toxicity of the L-amino acid oxidase to the cellular metabolism, a filtered sandwich technique was designed, in which an alanine racemase was expressed on a secondary membrane.

A glycine oxidase-based assay⁷³ that uses glyoxylate as amine acceptor was described by Bornscheuer *et al.*, based on a similar transaminase/amino acid oxidase principle; the technique was applied successfully to substrate profiling of (*R*)- and (*S*)- transaminases, being devoid of toxicity issues encountered with L-amino acid oxidase AAO.

Tetrazolium salts have been used in another coupled assay, involving transaminases and amino acid dehydrogenases.^{74,46} The alanine produced is converted back to pyruvate using the appropriate alanine dehydrogenase, whilst the phenazine-tetrazolium system (MPMS+INT) is used for colour development. Using this assay, the polyamines substrate panel of a novel class of bifunctional mono- and diamine transaminases was characterized. Alternatively, α -amino alcohols⁷⁵ and α -hydroxy ketones^{76,77} were

screened, using pyruvate as amine acceptor and α -MBA or alanine as amine donors, respectively (Scheme 3g)

1.5.2.2 Solid phase assays

Solid phase assays are very attractive because they are relatively easy to perform, are high-throughput by nature and the colorimetric changes are easy to detect by naked eye, without requiring specialized equipment.

Three different types of solid-phase assays have been described, allowing for the screening of transaminases and various amine substrates,^{72,73} transaminases and various ketone substrates^{68,69} and transaminases in presence of a fixed set of substrates.^{65,78} These assays have been described above and a summary is presented in Figure 4.

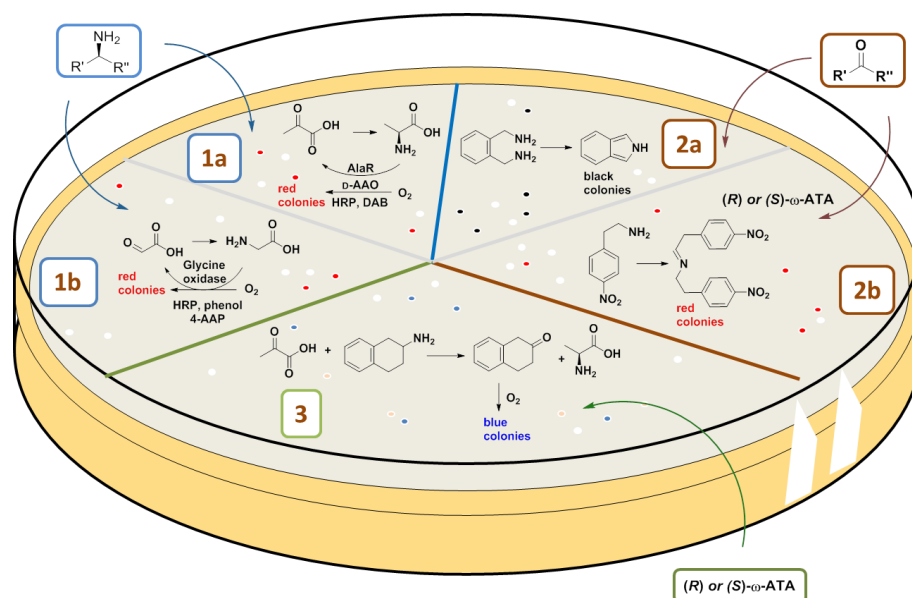


Figure 4. Solid-phase high-throughput screening strategies for transaminase activity applied to assess amine donor spectrum (**1a-b**), ketone spectrum (**2a-b**) and transaminases with a set pair of substrates (**3**).

1.6 Engineering

1.6.1 Enzyme engineering

Many tools are now readily available that assist in the engineering of the transaminase enzymes via two complementary approaches: rational design or random mutagenesis.

The appropriate technique can be chosen based on prior knowledge of the enzyme structure-function relationship, biochemical activity and evolution history or the availability and efficiency of the selection/screening strategy. This section will cover a selection of both random mutagenesis and site directed mutagenesis strategies that have been implemented to increase/alter substrate scope and increase the stability of transaminase enzymes, a full comprehensive review can be found elsewhere.²²

Wild type transaminases isolated from enrichment cultures (Vf-TA) or from in-house culture collections such as *Arthrobacter citreus* transaminase (Ars-TA) were further improved by error prone PCR utilising high throughput screening strategies (reviewed above). A complementary directed evolution strategy used enrichment culture to screen and isolate mutants from a random library which produced a mutant (P233L/V297A) resistant to product inhibition by 2-butanone and 2-heptanone. Successful resolution of 150 mM 2-aminoheptane gave (*R*)-2-aminoheptane (>99% *ee*) with 53% conversion. The mesophilic transaminase from Ars-TA required five rounds of evolution acquiring 17 amino acid changes and gave a variant with improved activity from 5.9 IU/g to 1582.8 IU/g for the production of (*S*)-aminotetralin. The newly improved mutant reduced reaction time, enzyme loading and increased optimum temperature activity to from 30 °C to 55 °C.⁶⁵

Before the first ω -transaminase was crystallised and characterised, researchers relied on previously deposited homologous protein crystal structures with similar active sites and highly conserved sequences (Figure 5), using these as templates to build and predict unknown protein structures. Online homology modelling web servers such as SWISS-PROT⁷⁹ have provided useful information for protein engineering, however the precise amino acid interaction can be difficult to envisage.

Necessary refinements for model validation are then calculated by energy minimisation and side chain optimisation to relieve steric and torsional constraints. As an example, Vf-TA was compared to known primary sequences and assigned to the class III family of aminotransferases with 2,2-dialkylglycine decarboxylase (PDB: 1DGE) used as a template to construct the homology model.⁸⁰ The active site of Vf-TA of fold class I was shown to

have two binding pockets: the large (P-pocket) and the small binding pocket (O-Pocket). Steric hindrance in the large pocket from W147 and W57 was relieved by introducing glycine at these two positions, resulting in an increase in activity for aliphatic amine substrates without the loss of activity towards aromatic amines.

The semi conserved tryptophan residue (W57) is present in other transaminases from *O.anthropi*⁸¹ Oa-TA and *C.violaceum*⁸², point mutations at this residue to W58L and W60C respectively were shown to have improved enantioselectivities and activities towards aliphatic and aromatic ketones. Interestingly the highly conserved neighbouring residue L57 in Oa-TA was mutated to alanine and exhibited high activity towards 2-oxopentanoic acid and the corresponding amine L-norvaline; the synthetic utility of this variant was demonstrated by affording product with >99.1% conversion and an enantiomeric excess >99%.⁸³

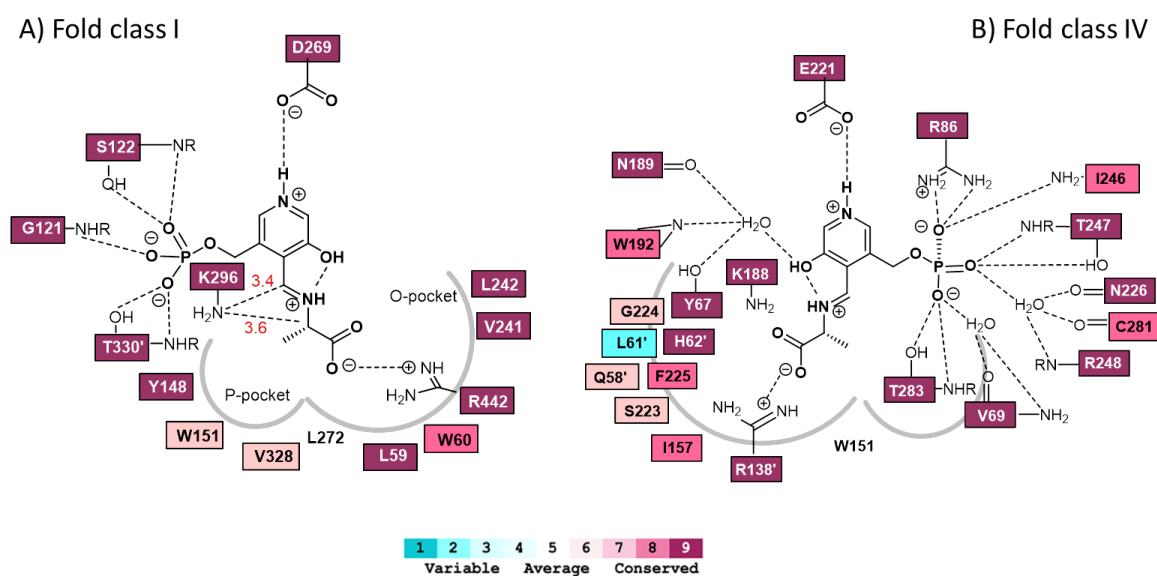


Figure 5. Schematic diagram showing the hydrogen bonds and electrostatic interactions stabilizing bound PLP-Ala (external aldimine state) in the active site BM-ATA⁸⁴ (PDB:5G2Q) of type fold I (A) and an ATA-117 of type fold IV (B, unpublished data). The P-pocket (small) and O-pocket (large) are shown by the thick grey lines. Distances (in Å) between the Lys269 amino group and the C4' and Cα atoms of PLP-L-Ala are indicated by red labels, and with dashed lines. The degree of conservation is indicated by different colours, and is based on a multiple-sequence alignment analysis of published transaminases.

The semi conserved tryptophan residue (W57) is present in other transaminases from *O.anthropi*⁸¹ Oa-TA and *C.violaceum*⁸², point mutations at this residue to W58L and W60C respectively were shown to have improved enantioselectivities and activities towards aliphatic and aromatic ketones. Interestingly the highly conserved neighbouring residue L57 in Oa-TA was mutated to alanine and exhibited high activity towards 2-oxopentanoic acid and the corresponding amine L-norvaline; the synthetic utility of this variant was demonstrated by affording product with >99.1 conversion and an enantiomeric excess >99%.⁸⁵

Since the depositing of a 1.9 Å resolution crystal structure of Vf-TA (PDB: 4E3Q) attempts have been made to increase the substrate scope beyond the preferred methyl phenyl ketones by mutating active site residues in the small binding pocket.

A combination of two mutants were identified (F85L/V153A) and (Y150F/V153A) which showed 30-fold increased activity towards (*S*)-phenylbutylamine and (*R*)-phenylglycinol from their corresponding ketones. Recently a structural motif for fold class I transaminase was identified for the asymmetric synthesis of bulky chiral amines from previous mutational studies on *Ruegeria sp.* TM1040 (Rsp-TA) which exhibited low activity. An *in silico* molecular docking of energy minimised quinoid intermediates in the active site complex was performed and identified four crucial amino acid motifs (Y59W, Y87F and Y152F and T213A). These motifs were engineered into other protein scaffolds that share >70% protein sequence identity with Rsp-TA and afforded >95% conversion with high ees (63-99%).⁸⁶

Since the discovery of the (*R*)-selective transaminase from *Arthrobacter sp.* KNK168 (Figure 5), its homolog ATA-117 from Codexis has been engineered extensively for the industrial production of sitagliptin, in which a variant was used for amination of the pro-sitagliptin ketone in the synthesis of the API, achieving 10-13% increase in overall yield.⁸⁷ A combination of computational modelling, structure aided design and iterative site saturation mutagenesis strategies were used to enlarge both the small and large binding pockets. A total of 27 mutations which were not exclusive to the enzyme active site but also located in the dimer interface presumably strengthened protein stabilisation as observed from the increased solvent tolerance to 50% DMSO.

Most recently the company c-LEcta found from their metagenomics library a tetrameric transaminase with high sequence identity to a class III of fold I type from *Pseudomonas fluorescens*,⁸⁸ which had relatively high activity for 4-phenylbutan-2-one with IPA as the amine donor. Using their multi-dimensional-mutagenesis (MDM) and automated generation of mutant (AGM) technologies they targeted 32 amino acid residues around the enzyme active site. Only two point mutations Q161I and Y164L in close proximity to the bound PLP cofactor were necessary for stabilising the cofactor-binding intermediate this led to improved enzyme activity, thermostability and biphasic (aqueous/non polar) reaction conditions.⁸⁹

1.6.2 Equilibrium displacement techniques

Transaminases are used in industry for the synthesis of chiral amines using two complementary approaches: kinetic resolution of racemic amines and asymmetric amination of pro-chiral ketones. Both methods present several challenges, which are related to the unfavourable thermodynamic equilibrium, the narrow substrate scope and substrate/product inhibition, meaning that they do not match to industrial processes requirements. These challenges and solutions have been the subject of several reviews.^{22,90,21,24}

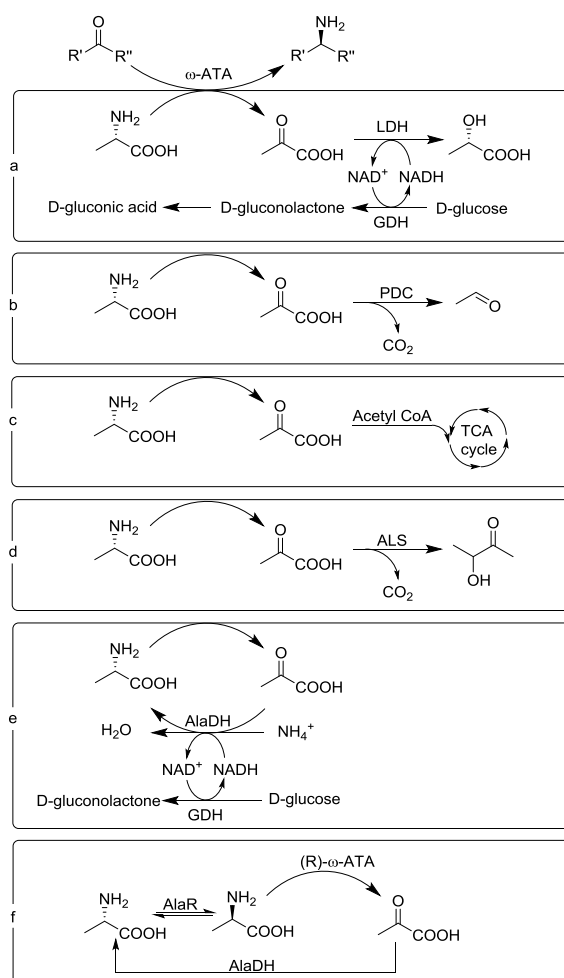
Kinetic resolution is mainly affected by the reversible nature of the transamination reaction, which means that the process is prone to both substrate and product inhibition. The unfavourable thermodynamic equilibrium and the low reactivity of the ketones are critical factors in the asymmetric synthesis. Equilibrium constants of $K_{eq} = 10^{-3}$ M have been reported for the synthesis of α -MBA from acetophenone and L-alanine⁶⁴, with indications of even lower values from other studies.^{91,92}

Several strategies have been designed to improve the efficiency of transamination reactions: removal and/or recycling of the inhibitory product *via* a physical process or a chemo-enzymatic cascade; auto-degradation of products generated from smart co-substrates and use of amine donor in large excess for pushing the equilibrium towards products.

The physical removal methods include *in situ* product removal systems (ISPR) that are able to extract the inhibitory product from the reaction mixture via supported liquid membrane reactors^{93,94,95,96} use of a co-solvent in an biphasic system,⁹⁷⁻⁹⁹ evaporation of volatile co-products¹⁰⁰ or precipitation of insoluble products.¹⁰¹

1.6.2.1 Co-substrate and/or co-product removal and/or recycling

As the natural substrate for most transaminases, alanine is the amine donor of choice for many transamination processes; however the resulting pyruvate is inhibitory to the enzyme and it must be removed from the system or recycled back to alanine. Designing multi-enzyme systems for efficient pyruvate removal or recycling must harmonize the optimal conditions (pH, temperature and solvent) for each enzyme component, without affecting their activities and selectivity.



Scheme 4. Equilibrium-displacement techniques based on pyruvate removal from the system (3a-f)

One of the most frequently used methods of pyruvate removal from the reaction mixture is by converting it to lactate, using a lactate dehydrogenase LDH, coupled with a glucose dehydrogenase GDH, for the NADH cofactor recycling, at the expense of glucose (Scheme 4a). The method was firstly reported by Shin and Kim¹⁰² who used coupled LDH with a whole-cell mediated transamination reaction, for the synthesis of (S)-MBA using Vf-TA as biocatalyst.

Truppo *et al.*⁹¹ designed a high-throughput screening method that integrated the LDH/GDH cascade and allowed both screening for transaminase activity using a pH sensitive dye and monitoring the course of the large-scale reaction by automated base addition. The three-enzyme system was successfully used for the conversion of various ketones to the corresponding chiral amines^{103–106} and in the chemo-enzymatic synthesis of (S)-Rivastigmine.¹⁰⁷

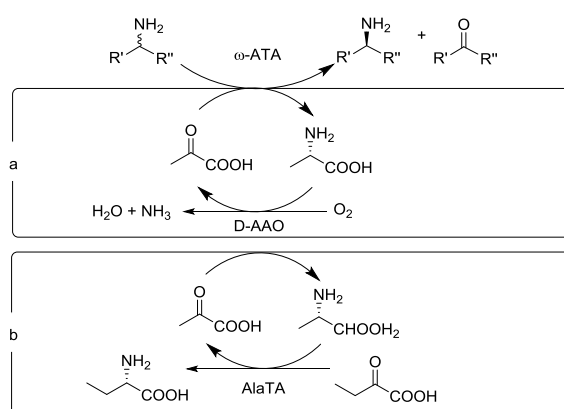
Similar approaches employed a pyruvate decarboxylase¹⁰⁸ (Scheme 4b) and a metabolically driven equilibrium shift that uses the native metabolism of the cell was designed by Han and Shin.¹⁰⁹ The carbon-starved *E.coli* cells harbouring the ω -TA gene from *Ochrobactrum anthropi*, were used for the synthesis of (S)-*sec*-butylamine on a 50mM scale, with only 2 eq. of L-alanine, with a 75% conversion (Scheme 4c), surpassing the thermodynamic limit reported by the group previously (Keq= 6.8×10^{-3}).¹¹⁰

A strategy aimed at reducing the costs associated with the use of expensive D-alanine was firstly reported by Soda *et al.* in a four enzyme-system²⁵ that couples a thermostable D-amino acid transaminase with a L-alanine dehydrogenase L-AlaDH and an alanine racemase AlaR, while the NAD(H) cofactor is recycled using a formate dehydrogenase FDH. The system was developed further by Richter *et al.*¹⁰⁵ and applied to transamination of ketones, using an ω -transaminase (Scheme 4f). Yun and Kim¹¹¹ co-expressed the transaminase from *Vibrio fluvialis* with an acetolactate synthase (ALS) from *Bacillus subtilis* for removing pyruvate as unstable acetolactate (Scheme 4d).

Alanine dehydrogenase AlaDH has been coupled either with glucose dehydrogenase⁹¹ or formate dehydrogenase FDH¹¹² for cofactor recycling. Amino acid oxidase AAO¹¹³ was

employed in kinetic resolution of racemic amines, reducing the pyruvate loading and driving the equilibrium towards accumulation of desired amine enantiomer (Scheme 5a).

A multi-enzyme system designed by Cho *et al.*¹¹⁴ uses tyrosine transaminase and aspartate transaminase for synthesis of (S)- α -amino acids from corresponding α -ketoacids and S-aspartic acid. The branched chain transaminase¹¹⁵ from *E.coli* and a ω -TA from *Ochrobactrum anthropi* were coupled for the simultaneous synthesis of chiral amines and unnatural branched chain amino acids, using L-homoalanine as a “shuttling substrate” between the two enzymes (Scheme 5b).



Scheme 5 Equilibrium-displacement techniques based on recycling alanine

Non-enzymatic methods include the use of biphasic systems such as buffer/organic solvent, *e.g.* cyclohexanone,²⁶ dioctylphthalate¹¹⁴, isooctane⁹⁸ and toluene.⁹⁷ Alternative methods are based on exploiting physical properties of co-products, such as low boiling point (resolution of sec-butylamine¹⁰⁰ or poor solubility (synthesis of L-homophenylalanine).¹⁰¹

The development of enzyme immobilization techniques advanced the development of *in situ* product removal strategies (ISPR), such as flat-membrane reactors with countercurrent circulation in organic solvent flows¹⁰², supported liquid membranes coupled with uphill amine extraction strategies^{93,94,95} or a combination of enzymatic cascades/physical removing methods.⁹⁵ ISPR methods offer a more physical approach to the equilibrium problem by capturing or isolating the amine product and they are mainly developed for industrial applications. Methods involving ionic exchange resins or silica-gel catch-and-release¹¹² suffer from non-scalability for industrial use.

1.6.2.2 Use of IPA in excess

Isopropyl amine is commonly employed in a large excess (1 M) as the amino donor in transamination reactions, as it is economically affordable and it is converted to the volatile acetone, following the transamination process. The use of a high excess of IPA was initially studied for comparison purposes in amination of a group of ketones⁹¹ using a set of Codexis enzymes. With an achiral substrate, all the amine can be incorporated to the ketone acceptor, making it economically attractive. In addition, the co-product acetone can be easily removed from the system, due to its low boiling point. In specific cases such as the synthesis of non-proteinogenic amino acids in an α -TA/ ω -TA cascade, IPA is usually the amino donor of choice, as the resulting acetone is a poor substrate for these enzymes. Nevertheless, extensive enzyme engineering is required,⁸⁷ for both improving acceptance of substrate and stability of enzyme for industrial use.

Acetone can also be removed enzymatically, by coupling the transamination process with a narrow substrate spectrum yeast alcohol dehydrogenase YADH from *S. cerevisiae*¹¹⁶ and a formate dehydrogenase for cofactor recycling. The resulting isopropyl alcohol can also serve as solvent for the poorly soluble ketone substrates. A recent chemical engineering approach couples the transamination reaction with an Oppenauer oxidation reaction, in which the secondary alcohol entering the chemical oxidation reaction must be a precursor of the ketone used in the biocatalyzed transamination reaction.¹¹⁷ Although both reactions have unfavourable equilibria, by coupling them together, the equilibrium is shifted towards amination of the ketones.

For industrial scale use, the removal of acetone can present additional challenges, the removal methods including sweeping or sparging of acetone using a carrier gas. Tufvesson *et al.*¹¹⁸ have investigated a model for acetone removal based on its dependence to temperature and sparging gas flowrate and found that the K_{eq} and the volatility of the ketone substrate are critical parameters to consider in designing such process strategies.

1.6.2.3 Use of sacrificial co-substrates

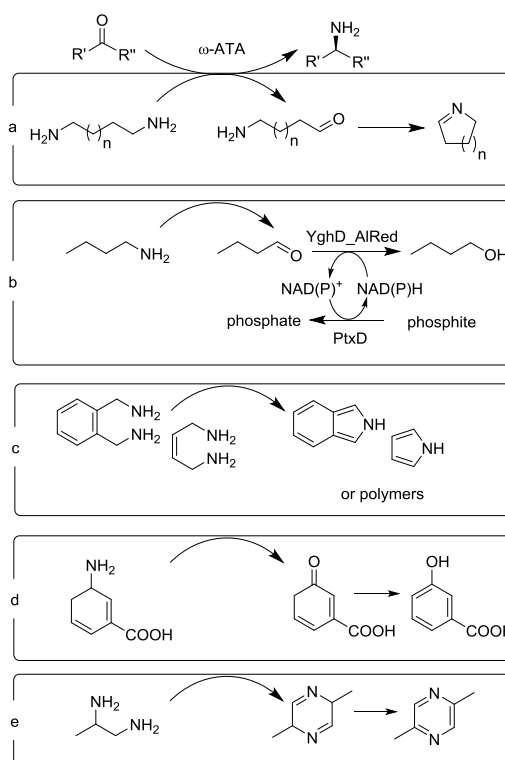
The use of sacrificial co-substrates that will generate a spontaneous reaction following the main transamination process is one of the more modern approaches to the equilibrium problem. Such substrates result in very reactive co-products which will auto-degrade or polymerise, irreversibly driving the equilibrium towards amination of ketones.

Early investigations of such substrates included the use of ornithine in a tyrosine TA/ornithine TA system¹¹⁹ or lysine based on the rapid cyclization of the resulting semialdehyde to the corresponding cyclic amino acid. Deracemization of β -amino acids in presence of pyruvate was driven to completion by the spontaneous decarboxylation of the resulting β -keto acids.⁴¹

Our group has reported a cost-effective and environmentally benign three-enzyme system which allows reductive amination of prochiral ketones using near-stoichiometric concentration of *n*-butylamine.¹²⁰ The system employs the newly described SpuC transaminases from *P. putida* in conjunction with the aldehyde reductase YqhD from *E.coli* and a phosphite dehydrogenase PtxD from *P. stutzeri* for the removal of the reactive co-product butanal as inert *n*-butanol, thus driving the equilibrium towards amination of ketones (Scheme 6b)

3-aminocyclohexa-1,5-dienecarboxylic acid was used as amino donor in the transaminations of prochiral ketones due to the spontaneous tautomerization / aromatization of the resulting ketone, which led to formation of 3-hydroxybenzoic acid.¹²¹ (Scheme 6d). *ortho*-Xylylene diamine was tested as substrate for transamination based on the ability of the resulting *iso*-indole product to rapidly polymerize to a series of dark-coloured compounds.⁶⁹ Similarly, *cis*-but-2-ene-1,4-diamine was used as substrate for a series of commercial enzymes, resulting in formations of reactive semialdehydes that cyclize and polymerize rapidly, allowing quantitative amination of ketones.¹²² (Scheme 6c)

Biogenic polyamines are a suitable alternative to the designer substrates, being affordable, readily available and easy to integrate into a microbial cell catalyst for production of chiral amines.



Scheme 6. Transaminase equilibrium displacement using sacrificial co-substrates

We have recently described and characterized two distinct groups of diamine transaminases: the YgjG transaminases (*E.coli* K12), that have a high specificity for terminal aliphatic diamines⁴⁵ and the SpuC transaminases (*P. putida*) that accept both mono- and poly- biogenic amine, and are able to transaminate a wide range of ketones (Scheme 6a). Following transamination, these diamines are converted to aminoaldehydes, which cyclize and irreversibly polymerize, thus driving the equilibrium. Similar results were obtained using a commercial transaminase¹²³ and , more recently, using vicinal 1,2-diamines¹²⁴ (Scheme 6e)

1.7 Synthetic applications of transaminases

1.7.1 Synthesis of amines and amino alcohols

Chiral amines benefit from a constantly increasing chiral toolbox, a plethora of diverse enzymes being available for their synthesis. Used in single-enzyme processes or in elaborate bio-/hybrid synthetic pathways, transaminases have emerged as powerful catalysts for the synthesis of an abundance of chiral amine compounds of great interest in the pharmaceutical, chemical and agrochemical industries.

Two main strategies are used for chiral amine synthesis: kinetic resolution of racemates and asymmetric synthesis from prochiral carbonyl precursors.

1.7.1.1 (Dynamic) Kinetic resolution

Kinetic resolution methods usually have low atom efficiency and require a transaminase and an amine acceptor in stoichiometric amounts, allowing for theoretical yields of 50%, while producing a low value ketone co-product, which can potentially have an inhibitory effect on the enzyme. Nevertheless, the use of pyruvate favours the transamination equilibrium due to its high acceptance by the enzyme.

Various strategies have been designed for overcoming the inhibition caused by the ketone co-product, which include the use of a biphasic system,^{64,125,97} an enzyme-membrane reactor for ketone removal^{96,126} or reduce pressure system¹⁰⁰ for ketone removal via extraction or evaporation. Whole-cell approaches have also been investigated, by exploiting endogenous oxidoreductases for reducing resulting acetophenone to benign 1-phenyl-ethanol.¹²⁷ A whole-cell yeast biocatalyst (*S. cerevisiae*), expressing the ω -TA from *Capsicum chinense*¹²⁸ exploited the cellular metabolism for *in vivo* production of pyruvate from glucose, was used for the kinetic resolution of α -MBA,¹²⁹ however the amine substrate concentration had to be kept reasonably low (15 mM).

Interestingly, some transaminases display very little susceptibility to product inhibition; for example the ω -TA from *O. antropi*¹³⁰ allowed for efficient kinetic resolution of α -MBA on a 500mM scale, the (*R*)-MBA enantiomer reaching a 95.3% *ee* after only 3h. Similarly, the (*R*)- ω -TA from *Mycobacterium vanbaalenii*¹³¹ was applied to the kinetic resolution of a series of aliphatic and aromatic amines on 100 mM substrate loading, the (*S*)-amines being obtained with perfect *ees*, using the more affordable acetone as amine acceptor.

Improving the conversions in terms of economic and yield efficiency requires a distinct approach which allows theoretical yields of 100% to be obtained. This can be achieved by a dynamic kinetic resolution or deracemization approach which relies on enzyme cascades or easy-to-racemize carbonyl precursors.

Two enantiocomplementary transaminases can be coupled in a one-pot two-step deracemization process; the first step affords the enantiopure desired amine in 50% yield, whilst the ketone co-product is transaminated in the second step to the same enantiomer using a ω -TA with opposite enantioselectivity. By carefully choosing the amine acceptor, Shin *et al.*¹³² performed simultaneous deamination/transamination steps for the deracemization of a series of aromatic amine. α -Ketoglutarate was used as amine acceptor for (*S*)-TA from *Polaromonas* sp. JS666, whilst the (*R*)-TA from *Mycobacterium vanbaalenii* used L-alanine as amine donor, as opposed to poorly accepted L-glutamate.

O'Reilly and co-workers¹³³ have described an one-pot cascade using ω -transaminase/monoamine oxidase for the synthesis of 2,5-disubstituted pyrrolidines starting from the corresponding 1,4-diketone precursors. The two stereogenic centres are set sequentially, initially by the transaminase, followed by a stereo-inversion performed by the monoamine oxidase/ NH_3BH_3 system (Scheme 7a).

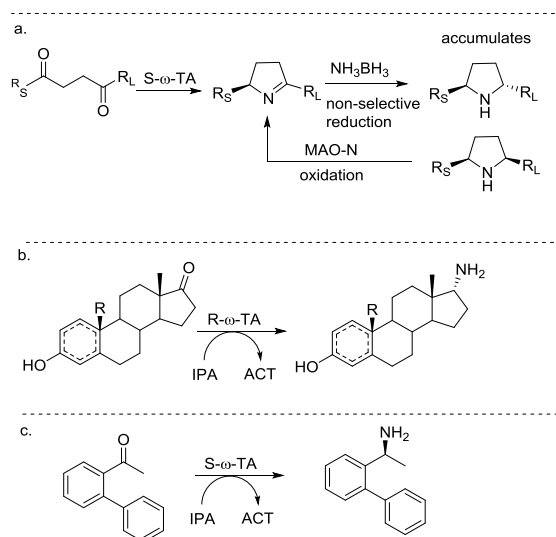
Koszelewski *et al.*¹³⁴ reported the deracemization of a group of pharmacologically relevant amines, employing the (*R*)-selective ATA117 and (*S*)-selective ATA113 Codexis enzymes. A heat-treatment step resulting in deactivation of the first enzyme was necessary to avoid enzyme cross-talking. The system was further developed by including an amino acid oxidase in the first biocatalytic step¹¹³ for pyruvate recycling, thus improving cost-efficiency and it was tested successfully in the deracemization of mexiletine, an antiarrhythmic agent.¹³⁵

A single-transaminase dynamic kinetic resolution process was used in amination of enantio-enriched 4-phenylpyrrolidin-2-one, to give a key intermediate in the synthesis of 3-phenyl-GABA¹³⁴ from cinnamyl alcohol, based on rapid racemisation of the intermediate aldehyde. A similar strategy was adopted for the synthesis of a series of β -chiral primary amines from precursor 2-phenylpropanal derivatives.¹³⁶

1.7.1.2 Asymmetric synthesis

A large number of chiral primary amines based on a variety of molecular scaffolds have been synthesized in an asymmetric synthesis fashion, using transaminases.

We have recently investigated and reported the synthesis of a large range of aliphatic and aromatic chiral amines from the corresponding ketones, using biogenic polyamines as amine donors, in small excess¹²⁰ The reactions were catalysed by a newly described class of diamine transaminases, SpuC transaminases that combine efficiently equilibrium displacement potential of the diamine donor with broad ketones substrate spectrum, enabling a greener strategy for the production of valuable chiral amine units.



Scheme 7. Selected examples of using ω -transaminases for the synthesis of (a) enantio-enriched 1,5-disubstituted pyrrolidines; (b) enantiopure 17- α -amino steroids and (c) chiral amines with bulky substituents

Aryl- and alkyl-ketones with various boron functionalities¹³⁷ and organohalogenated acetophenone derivatives^{138,139} have been successfully converted to the corresponding amines, under mild conditions, using stereo-complementary transaminases.

Substrates such as cyclic ketones are usually more challenging as they do not display distinct small/large substituent areas, which are characteristic for the transaminase biocatalytic mechanism. However, the transamination of several 2-substituted cyclohexylketones has been reported¹⁴⁰ using the transaminase from *C. violaceum*, with good diastereoselectivity, allowing access to compounds with two chiral centres in one step, starting from achiral precursors. Similarly, 17- α -amino steroids were obtained from corresponding ketones using a variant of the TA from *Arthrobacter* sp. in very good isolated yields (Scheme 7b), 83-89%, this method representing the shortest synthetic route described to date.¹⁴⁰

An evolved mutant of the transaminase from *Ruegeria sp.*, 3FCR¹⁴¹ was used in the asymmetric reductive amination of the bridged bicyclic amine (*exo*-3-amino-8-azabicyclo[3.2.1]oct-8-yl-phenyl-methanone), with 99.5% selectivity. A similar combination of *in silico* design techniques and *in vitro* screening resulted in a variant of the (*S*)-TA from *Vibrio fluvialis*, Vf-TA with 7 mutated residues (W57F/R88H/V153S/K163F/I259M/R415A/V422A) that shows a 1716-fold improvement in reductive amination of 2-acetylbiphenyl substrate, allowing the synthesis of (*S*)-1-(1,1'-biphenyl-2-yl)ethanamine in >99% ee, with a retention of 90% of activity after 18 h at 50° C¹⁴² (Scheme 7c).

Furfuryl amines, which are important building blocks in pharmaceutical products, such as antiseptics and diuretic agents, have been synthesized from furfural derivatives using the (*S*)- ω -transaminase from *Chromobacterium violaceum* DSM30191 and the (*R*)- ω -transaminases from *Arthrobacter sp.* variant ArRMut11 and *Mycobacterium vanbaalenii*.¹⁴³ The method shows a valuable alternative to the chemical synthesis of these compounds, which presents difficulties due to the sensitivity of the furan ring, as well as using a sustainable feedstock for the synthesis of valuable fine chemicals.

1.7.2 Synthesis of amino acids

Over the past two decades, a variety of (chemo)enzymatic strategies for the synthesis of optically pure amino acids have been developed and they have been the subject of a series of reviews.^{144–146} Both natural and unnatural amino acids are essential chiral building blocks in drug development in modern medicinal chemistry and in manufacturing of industrial products.

Non-proteinogenic chiral α -amino acids are key intermediates in synthesis of various drugs, e.g. D-alanine as precursor for Abarelix¹⁴⁷ and L-homophenylalanine as component of antiepileptic drugs¹⁴⁸, as well as intermediates in synthesis of biocompatible plastics industry.¹⁴⁹

Chiral β -amino acids are very often building blocks in bioactive molecules such as antitumour (Paclitaxel)¹⁵⁰, antiviral agents (CCR-5 receptor antagonist Maraviroc)¹⁵¹ or pharmaceutically relevant hybrid peptides, because they provide higher stability against

peptidases, while retaining the biological activity and reducing rejection by the human body.¹⁵²

The synthesis of γ -amino acid derivatives has gained enormous interest as the γ -aminobutyric acid (GABA) is a molecule of interest in several major neurological disorders, such as Huntington's and Parkinson's disease and epilepsy,¹⁵³ peptidomimetics¹⁵¹ and antineoplastic agents.¹⁵⁴

The industrial methods of choice for the synthesis of chiral amino acids rely mostly on microbial fermentation strategies (cell factories)¹⁵⁵; however they suffer from limited flexibility towards structurally diverse unnatural amino acids and unreliable behaviour of the cellular production systems.¹⁵⁶ *In vitro* production systems, such as isolated enzyme systems can offer more adaptability by avoiding complexity of the cellular metabolic pathways and they enable synthesis of a broad variety of natural and unnatural amino acids analogues.

By combining an ornithine δ -transaminase OTA [EC 2.6.1.13] from *Bacillus subtilis rocD* with a tyrosine transaminase from *E. coli* K12 *tyrB*, Li *et al.*¹¹⁹ synthesised L-2-aminobutyrate from 2-ketobutyric acid and L-*tert*-leucine from trimethylpyruvic acid. By exploiting the spontaneous cyclization of the resulted reactive δ -aminoaldehyde to pyrroline-5-carboxylic acid, the conversions of the two amino acids were greatly improved, the novel α -/ ω -transaminase coupling emerging as a powerful synthetic tool for amino acid preparation. Based on a similar equilibrium-shifting technique, L-phenylalanine was synthesized in 97% yield and >99% *ee* from 2-oxo-4-phenylbutanoic acid and L-lysine using an engineered variant R292E/L18H of the aspartate transaminase from *E. coli*.¹⁵⁷

Using primary amines as amino donor, as opposed to the traditional use of more expensive D-aspartic acid, with the (*S*)-TA from *O. anthropi* and the (*R*)-TA from *Arthrobacter sp. KNK168*, Park *et al.*^{158,159} synthesised a series of α -amino acids in high conversions and excellent *ees* from the corresponding α -ketoacids.¹⁵⁸ Furthermore, L- and D-homoalanine were accessed from the readily available L-threonine via a threonine

deaminase/ ω -transaminase cascade, on a 100mM scale, conversions of 90% and >99.9% ee being achieved after 90 min (Scheme 8a).

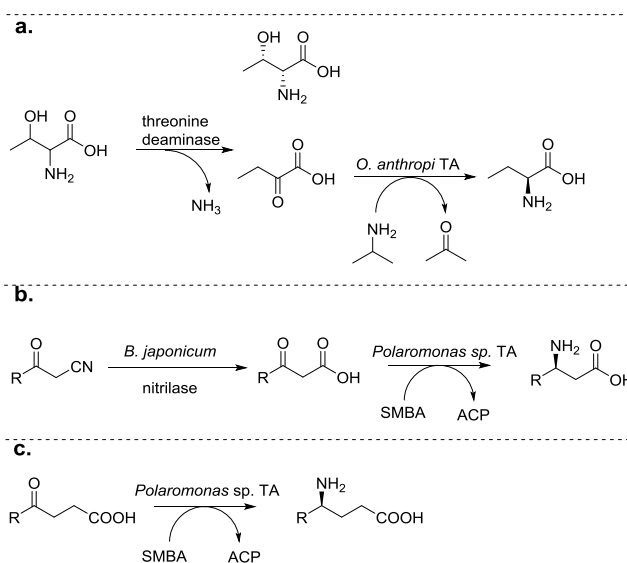
The (S)- selective ω -TA from *O. antropi* was also coupled with a branched-chain aminotransferase BCTA from *E.coli* for the synthesis of L-homoalanine, using 2-oxobutyric acid as shuttle substrate between the two enzymes.¹¹⁵ An improved version of this cascade was used in synthesis of L-valine, L-Leucine and L-isoleucine *via* kinetic resolution of corresponding racemates, by recycling L-homoalanine co-substrate and using a biphasic system to alleviate acetophenone inhibition.¹⁶⁰ Further evolution of the same (S)- selective ω -TA from *O. antropi* resulted in a mutant (L57A) which was used successfully in the asymmetric synthesis of L-norvaline and L-norleucine.⁸³

An industrially-scalable biosynthesis of L-norleucine, an unnatural amino acid used in residue-specific protein labelling, was achieved by overexpression of the *leuABCD* gene, responsible for synthesis of L-norleucine in *E.coli*.¹⁶¹ The metabolically engineered methionine auxotrophic *E.coli* strain was used in synthesis of L-norleucine from glucose on a 5 g/L scale.

L-homoserine, an unnatural amino acid used as precursor in biocompatible plastic industry was prepared via an aldolase/transaminase one pot cascade, using affordable formaldehyde and alanine.¹⁶² The protein insolubility problem of the pyruvate aldolase YfaU from *E.coli* K-12 was circumvented by expressing it as a fusion protein with maltose binding protein from *E.coli* at N-terminus and the system was optimized by continuous feeding of formaldehyde, increasing the yield of L-homoserine to 86%.

The biocatalytic methods of β -amino acids synthesis are more challenging due to the instability of the β -keto acids precursors. (3S)-amino-3-phenylpropionic acid was synthesized in modest conversion (20%) from the ethylbenzoylacetate precursor and (S)-MBA, via a lipase (*C. rugosa*) / ω -transaminase (*Mesorhizobium* sp. strain LUK) cascade reaction.³¹ An optimized version of this cascade was later reported using the ω -TA from *Polaromonas* sp. JS666 in combination with the same lipase from *C. rugosa*, the lipase concentration and the solvent were found to be limiting factors in the reaction system, which was successfully used in synthesis of ten aromatic β -amino acids.¹⁶³

The poor stability of the β -keto acids was exploited successfully in the kinetic resolution of racemates using the ω -transaminase from *Burkholderia graminis* C4D1M and benzaldehyde as amine acceptor,⁴² a more economically efficient alternative to expensive pyruvate. Excellent conversions to (*R*)- β -amino acids have also been achieved in kinetic resolution of racemic β -amino acids using the lipase/transaminase cascade mentioned above.¹⁶⁴



Scheme 8. Selected examples of synthesis of chiral (a) α -amino acids; (b) β -amino acids and (c) γ -amino acids from their corresponding α -, β - or γ -keto acids, using an ω -transaminase.

Overcoming the instability of the β -keto acids was investigated via a nitrilase/transaminase cascade system.¹⁶⁵ β -keto nitriles were hydrolysed to corresponding β -keto acids by a nitrilases from *Bradyrhizobium japonicum*, followed by transamination using the ω -TA from *Polaromonas* sp. JS666. As the β -keto nitriles are poor substrates for the nitrilase, several nitrilase variants have been constructed and tested. The cascade offered modest conversions, but excellent *ees* for a series of β -keto nitriles (Scheme 8b).

A recently characterized (*S*)-selective transaminase from *Burkholderia vietnamiensis* showed promising activity on a series of aliphatic β -keto esters, the first one of this kind.¹⁶⁶ Ethyl propionylacetate and ethyl acetoacetate were successfully transaminated to their corresponding β -amino esters in 17% and 89% conversion, respectively, with >99%

ee. This reaction could potentially be coupled with a further lipase-catalysed hydrolysis process, affording the chiral β -amino acids of interest.

Compared to α - and β -amino acids, the bioenzymatic routes to γ -amino acids are not as well developed. Shon *et al.*¹⁶⁷ have used the ω -TA from *Polaromonas* sp.⁴¹ and *Burkholderia graminis* C4D1M for producing enantiopure γ -amino acids via asymmetric synthesis and kinetic resolution. Both enzymes successfully resolved the racemates, using pyruvate as amine acceptor. The asymmetric synthesis route used (*S*)-MBA for amination of the corresponding γ -keto acids, in a biphasic system with isooctane, for acetophenone removal (Scheme 8c).

A broad-substrate specific thermostable ω -transaminase from *S. thermophiles* was expressed in *E.coli* BL21 host and used in synthesis of β - and γ -amino acids via kinetic resolution and asymmetric synthesis.¹⁶⁴ The enzyme showed increased selectivity towards aromatic substrates and the optically pure (*S*)- γ -amino acids were obtained in good conversions (>73%) and high ee (>99%). The (*R*)- γ -amino acids were synthesized by kinetic resolution of racemates, using pyruvate as amine acceptor.

A recombinant whole-cell *E.coli* biocatalyst, expressing both the alcohol dehydrogenase AlkJ ADH from *P. putida* GPo1 and the ω -transaminase from *Silicibacter pomeroyi* was used by Park *et al.* for the synthesis of long-chain ω -amino acids from their correspondent long-chain fatty acids. This *in vivo* cascade system is of particular interest for polymers and polyamides production, allowing access to valuable building blocks such as 11-aminoundecanoic acid, from renewable fatty acids sources.¹⁶⁸

1.7.3 Cascades

Multi-enzyme cascade reactions are now being reported as well as multiple single-step processes and represent a major opportunity for developing cleaner, more efficient and environmentally benign chemical technologies. Enzymes are ideal for use in these cascades, as they are intrinsically green, allow shortening the reaction times, avoid the use of protecting groups and minimize the use of organic solvents and chemical reagents, thus reducing the waste.

Over the years, various transaminase-mediated cascades have been described, representing different cascade designs, *i.e.* linear cascades, consisting of one-pot sequential reactions, orthogonal cascades that use concomitant enzymatic reactions for co-product removal or recycling, parallel cascades that couple two distinct enzymatic processes involving the same co-factor or co-substrate and cyclic cascades, that are mainly designed for dynamic kinetic resolution processes¹⁶⁹

1.7.3.1 Functional group interconversion mediated by transaminases/oxidoreductases cascades

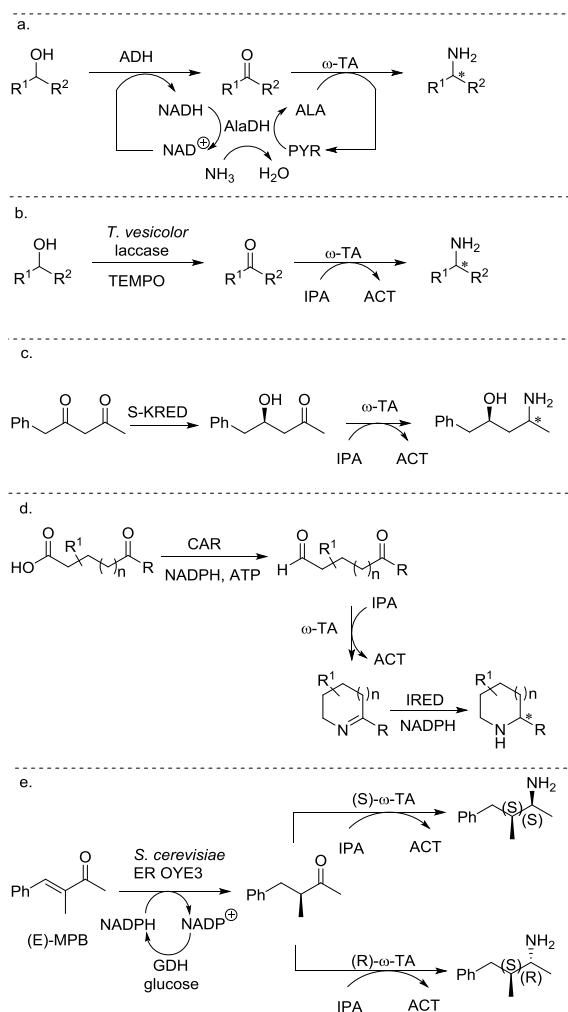
The combination of transaminases and oxidoreductases usually follows a linear design in which the starting materials are converted to the desired chiral amines via sequential reactions.

Monti *et al.*¹⁷⁰ have used the ene-reductase *S. cerevisiae* ER OYE3 coupled with several commercial transaminases for the synthesis of diastereomerically enriched (2*S*,3*S*)-3-methyl-4-phenylbutan-2-amine and (2*R*,3*S*)-3-methyl-4-phenylbutan-2-amine from the corresponding unsaturated ketone (Scheme 9e). A set of (*S*)- and (*R*)-selective Codexis transaminases was screened and the best candidates showed almost quantitative conversions, with perfect *ee* values. In addition, the intermediate un-saturated amine was not observed, the transaminases showing a clear preference towards amination of the saturated ketone. Similar substrates, such as 3-methylcyclohex-2-enone have also been successfully converted to the corresponding (1*S*,3*S*)- and (1*S*,3*R*)-1-amino-3-methylcyclohexane using the same cascade. The missing diastereomer, (1*R*,3*R*)-1-amino-3-methylcyclohexane was later synthesised via a similar cascade, using the YqjM Cys26Asp/Ile69Thr variant of the xenobiotic reductase A (XenA) from *Pseudomonas putida* ATCC 17453.¹⁷¹

Amination of the hydroxyl group of primary and secondary alcohols has been reported and their conversion to valuable chiral amine units has been achieved by coupling various oxidases with the required enantioselective transaminase.²⁴

Primary alcohols have been converted to the corresponding terminal amines via a two-step oxidation-transamination process; for example, long-chain 1, ω -alkanediols have

been converted to 1, ω -diamines, building blocks for polymers, using a redox-neutral alcohol dehydrogenase/transaminase cascade, at the expense of ammonia. In a similar manner, the antifungal reagent naftifine has been synthesized in 51% overall yield from the cinnamic alcohol precursor in a cascade employing the galactose oxidase from *Fusarium* NRRL 2903 and the (*S*)-selective transaminase from *V. fluvialis*¹⁷² (Scheme 9a).



Scheme 9. Selected examples of ω -transaminases-oxidoreductases cascades. (a) and (b): synthesis of primal chiral amines from their corresponding secondary alcohols; (c) synthesis of chiral 1,3-amino-alcohols from 1,3-diketones, *via* consecutive biocatalyzed reduction/transamination; (d) linear cascade for the synthesis of chiral di-substituted piperidines from 1,5-keto acids; (e) coupling of ene-reductases with ω -transaminases for the reduction/transamination of unsaturated ketones.

Preparation of chiral *sec*-amines from *sec*-alcohols has been approached using artificial redox-neutral cascades that employ alcohol dehydrogenases of appropriate enantioselectivity to obtain the ketones, which are afterwards transaminated to the chiral amines of interest.^{173,174} Furthermore, a single whole-cell system harbouring a P450 monooxygenase [Y96F], (*R*)- and (*S*)-selective alcohol dehydrogenases [LbRADH and ReSADH], and a ω -transaminase was described by Both *et al.*¹⁷⁵ for the synthesis of enantiopure (*R*)-1-phenylethanamine derivatives from the corresponding ethylbenzenes.

An elegantly designed cascade has been recently described by Martinez-Montero *et al.*¹⁷⁶ by using a non-stereoselective laccase/TEMPO system for alcohols oxidation, followed by ketone transamination reaction. A series of organofluorinated amines have been synthesised from corresponding alcohols in this manner, in good conversions (67-99%) an excellent *ees* (90-99%) (Scheme 9b)

Chiral 1,2- or 1,3-aminoalcohols are common structural motifs in natural products and drugs, as well as being important building blocks in chiral ligands and chiral auxiliaries. Their synthesis is typically challenging as it requires establishing two chiral centres, however the synthetic difficulties can be overcome by using multi-enzymatic cascades. Following the screen of a panel of commercially available keto-reductases, Kohls *et al.*¹⁷⁷ identified an (*S*)-selective KRED which afforded enantiopure 1,3-hydroxy ketones via dynamic kinetic resolution of the racemic ketone. Subsequent coupling with two enantiocomplementary transaminases allowed access to all 4 diastereomers of 4-amino-1-phenylpentane-2-ol (Scheme 9c).

For the synthesis of (1*S*,2*S*)-norpseudoephedrine and (1*S*, 2*R*)-norephedrine, Sehl *et al.*¹⁷⁸ have designed a transaminase/alcohol dehydrogenase cascade, starting from 1-phenylpropan-1,2-dione. Optimization studies showed that higher yields were obtained when the alcohol dehydrogenase was added only after the inactivation of the transaminase.

Chiral mono- and disubstituted piperidines and pyrrolidines have been synthesized starting from dicarbonyl precursors. A three-enzyme system approach to the synthesis of chiral pyrrolidines and piperidines was described by France *et al.*¹⁷⁹, where keto acid

derivatives were initially reduced to ketoaldehydes via carboxylic acid reductase-mediated reaction followed by the transamination and reduction of the imine intermediate using an imine reductase IRED (Scheme 9d). The hybrid *in vivo/in vitro* enzymatic cascade was later shown to mirror a natural pathway for the synthesis of polyketide-derived indolizidines from polyketide chains in *Streptomyces sp.* NCIB 11649.^{180,181}

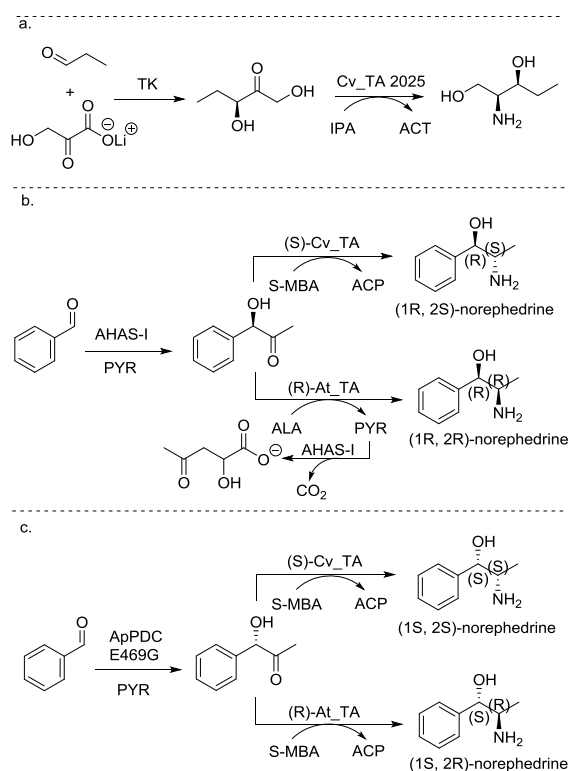
1.7.3.2 Building up C-C complexity *via* transaminase/ligase cascade

A different approach to multi-enzymatic routes to chiral amino alcohols includes the use of transaminases coupled with transferases or ligases enzymes. Rother *et al.*¹⁸² have designed a sequential one-pot two-steps cascade for the synthesis of (1*R*,2*R*)-norpseudoephedrine and (1*R*,2*R*)-norephedrine by combining the thiamine diphosphate (ThDP)-dependent acetohydroxyacid synthase I (AHAS-I) from *E. coli* with an *S*- or *R*-transaminase, respectively. The cascade was designed in a manner that allowed recycling the pyruvate substrate between the two enzymatic steps: an initial pyruvate decarboxylation and ligation with benzaldehyde to yield the (*R*)-phenylacetylcarbinol intermediate, followed by reductive amination with and *S*- or *R*-selective transaminase, using alanine as amine donor (Scheme 10b). Sequential addition of the enzymes improved the cascade efficiency; however the metabolic integration of the system showed substantial difficulties.

In the absence of a natural *S*-selective ThDP-dependent carbonylase, the remaining (1*S*,2*S*)-norpseudoephedrine and (1*S*,2*R*)-norephedrine isomers were synthesized using a structure-based designed variant of the pyruvate decarboxylase from *Acetobacter pasteurianus* (Scheme 10c). The variant displayed reduced stereoselectivity, the *S*-phenylacetylcarbinol being obtained in only 70% *ee* and the subsequent transamination step lowered the overall diastereomeric excess even more.¹⁷⁸

A similar pyruvate-recycling concept has been applied to synthesis of γ -hydroxy- α -amino acids through aldolase/ α -transaminase cascades.¹⁸³ A set of 19 pyruvate aldolases were tested and coupled with an aspartate transaminase from *E. coli*, which uses L-aspartic acid as amine donor. The stereocentre at the γ -position was determined by the pyruvate

aldolase, while the transaminase controlled the configuration of the amine and the cascade was successfully used in the synthesis of 4,5-dihydroxynorvaline, which was obtained in 83% conversion as a 7/93 *syn/anti* mixture.

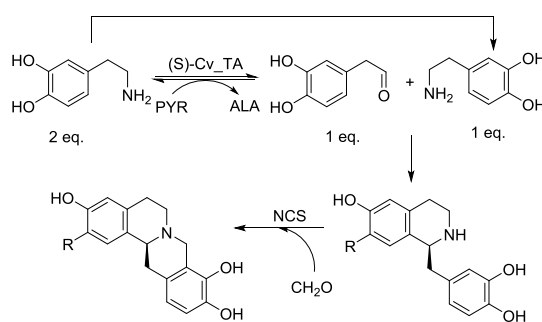


Scheme 10. Selected examples of coupling of ω -transaminase with transketolases (a); (ThDP)-dependent acetoxyacid synthase I (AHAS-I) (b) and pyruvate decarboxylase ApPDC (c) for the synthesis of diastereomerically-enriched 1,2-amino alcohols.

Chiral 2-amino-1,3-diols have been synthesised in a two-step biocatalytic system starting from simple and achiral materials such as propanal and hydroxypyruvate, using an engineered transketolase TK D469T from *E.coli* and the *S*-selective transaminase from *C. violaceum*.¹⁸⁴ The process was successfully tested on a preparative scale synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol which was obtained in 61% conversion and 98% *ee*. More recently, a transaminase from thermostable *Thermosinus carboxydvorans* has been coupled with the thermostable transketolase from *Geobacillus stearothermophilus* for the synthesis of naturally rare L-erythro (3*S*,4*S*) ketoses. One of the major advantages of this process is represented by the *in situ* generation of the labile β -hydroxypyruvate, offering an environmentally-friendly procedure for the synthesis of this class of ketoses.

1.7.3.3 Integration of transaminases in biomimetic synthetic pathways

A recent development in transaminase-mediated cascades is the use of enzymes derived from biosynthetic pathways. A triangular cascade using the transaminase from *C. violaceum* Cv_2025 and norcoclaurine synthase NCS was described by Hailes *et al.*¹⁸⁵ as a route to (*S*)-benzylisoquinolines and (*S*)-tetrahydroprotoberberines alkaloids (Scheme 11). Optimization of the pyruvate loading, use of purified enzymes and control of the enzyme loading allowed the synthesis of (*S*)-benzylisoquinolines in good conversions (87%) and 99% *ee*. A chemical extension of the cascade was performed by addition of formaldehyde, which resulted in the synthesis of the (*S*)-10,11-dihydroxy-tetrahydroprotoberberine analogues, as the major isomer.



Scheme 11. Transaminase/ norcoclaurine synthase cascade

More recently, a three-step (chemo)enzymatic cascade that includes a carboligase (ThDP-dependent acetoxy acid synthase I from *E.coli*, ECAHAS-I), a transaminase (*C. violaceum* 2025, Cv-TA) and the norcoclaurine synthase variant from *Thalictrum flavum* (Δ TfNCS-A79I) was designed for the synthesis of stereochemically complementary 1,3,4-trisubstituted-tetrahydroisoquinolines from cheap and achiral starting materials. The modularity of the carboligase/transaminase cascade allows for all 4 isomers of the amino alcohols to be synthesized and the availability of several aldehydes as substrates for the NCS enzyme helps enlarging the panel of trisubstituted tetrahydroisoquinolines that can be obtained via this cascade.¹⁸⁶

In a similar manner, strictosidine synthase from *O. pumila* has been coupled with the (*S*)-transaminase from *S. pomeroyi* or the (*R*)-transaminase from *Arthrobacter sp.* for the synthesis of 3-methylated strictosidine derivatives.¹⁸⁷ The initial transaminase-catalysed

step established the stereogenic centre at the beta-carboline core, while the strictosidine synthase catalysed a Pictet-Spengler coupling of the secologanin with the newly formed α -methyltryptamines.

These systems emphasise the advantages of enzymatic cascades, by displaying high atom economy and rapid build-up of molecular complexity *via* C-C bond forming steps.

1.7.3.4 Hybrid chemo-enzymatic cascades

Hybrid chemo-enzymatic cascades usually exploit the potential further reactivity of the transamination product and offer the double advantage of generating novel compounds while displacing the equilibrium towards products side.

We have recently described a chemo-enzymatic cascade for the one-pot synthesis of the alkaloid ficuseptine⁴⁵ using the newly-characterized YgiG putrescine transaminases from *E. coli* K12. Using inexpensive putrescine and pyruvate as starting materials, a reactive 1-pyrroline intermediate was synthesized which subsequently participate in a Chichibabin-type pyridine synthesis upon addition of two equivalents of phenylacetaldehyde.

2,6-disubstituted piperidines have been prepared via a chemo-enzymatic cascade reaction from corresponding 1,5-diketone precursors. The ketones were converted to cyclic imines following a transaminase reaction and the final piperidine scaffolds were obtained via a diastereoselective chemical reduction process.¹⁸⁸ A similar approach was reported for the synthesis of the (-)-pinidinone alkaloid¹⁸⁹; however in this instance a transaminase/intramolecular aza-Michael reaction was designed, which used unsaturated diketones and isopropyl amine as starting materials. Careful optimization of the system, avoiding possible enone carbonyl reductive amination or double bond reduction, allowed synthesis of the target alkaloid in 90% conversion and >99% de.

An artificially metabolic pathway that couples heterogeneous metal, organocatalytic and enzymatic routes has been designed by Cordova *et al.*¹⁹⁰ for the synthesis of capsaicin analogues. Vanillyl alcohol was oxidised to vanillin using heterogeneous Pd(0) nanoparticles in an initial step, followed by transamination of vanillin to vanillylamine, that was subsequently converted to capsaicin analogues via a lipase-catalysed reaction.

The simultaneous use of organo- and biological catalysts has been investigated for the synthesis of sterically hindered β -substituted cycloalkanols.¹⁹¹ The hydroxyl groups were oxidised to ketone functionalities using 2-azaadamantane *N*-oxyl, AZADO, and then further converted to chiral amino groups via enzymatic transamination. Diastereomerically enriched products were obtained under optimized conditions that enabled epimerization and thereby dynamic kinetic resolution of the diastereomers; the use of high pH and a benzyloxy- protecting group increased the acidity of the proton in the α - position.

1.7.4 Transaminase-mediated routes to small molecule pharmaceuticals

Now established as green and scalable technology for the synthesis of a large range of natural products and pharmaceutically relevant amine-containing molecules (Scheme 10), biocatalysis benefits from a continually expanding number of new enzymes and processes catalysed by them. Furthermore, significant advances in directed evolution techniques and in the design of novel enzymatic cascade broaden the potential applications of engineered biocatalysts.

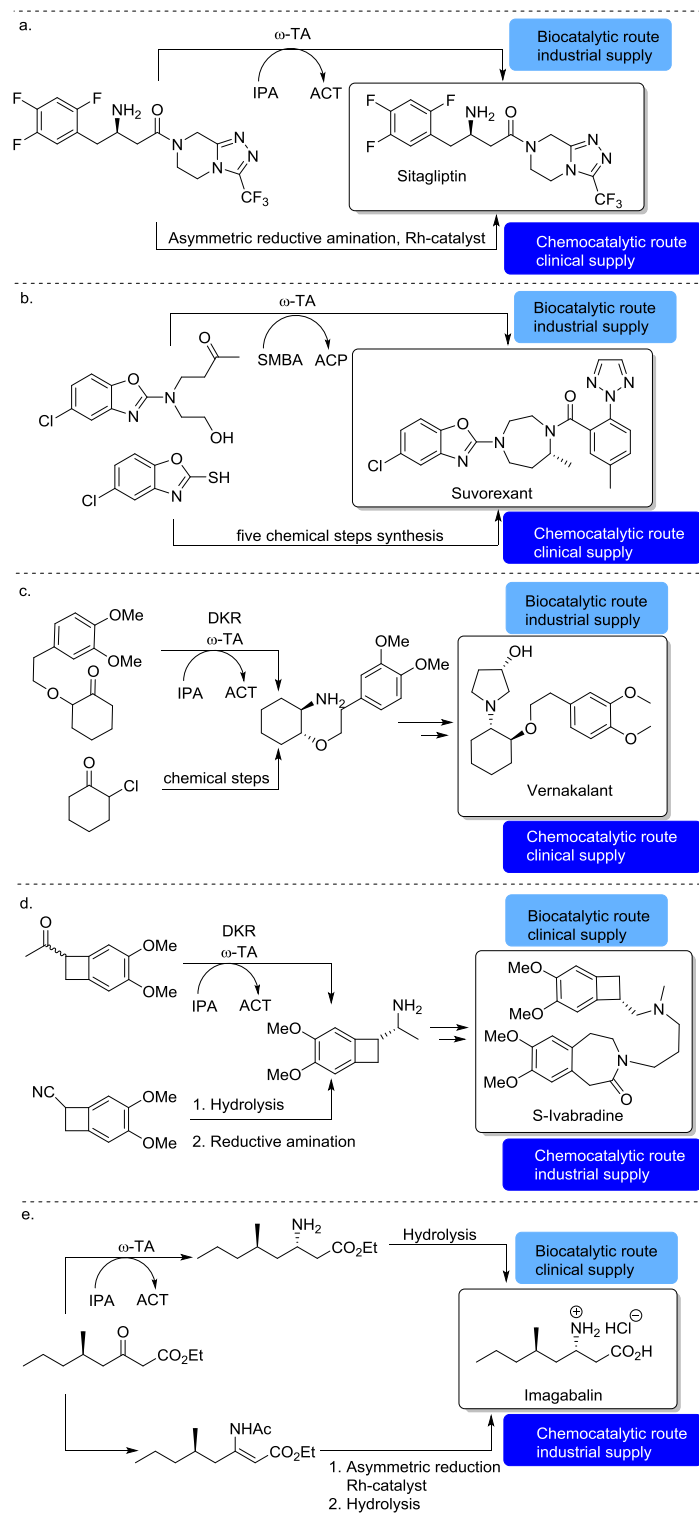
A series of parameters needs to be optimized for assembling successful biocatalytic processes, which relate not only to the activity and selectivity of an enzyme, but also to its compatibility with other components of the catalytic system in order to enable the synthesis of the product of interest in the most economical fashion.

One of the first uses of transaminase in the synthesis of API intermediates was reported by Shin and Kim¹⁹² who synthesized L-2-aminobutyric acid from 2-oxobutyric acid using the ω -TA from *Vibrio fluvialis* JS17 (Vf-TA) and benzylamine as amine donor. A biphasic system (buffer: hexane), for controlling inhibition caused by benzaldehyde, generated the target compound in 96% conversion with 50mM substrate concentration. Subsequently, a short and straightforward synthesis employed the same Vf-TA for the synthesis of (*S*)-rivastigmine, a potent drug for treatment of Alzheimer's disease.¹⁹³ A four-step chemo-enzymatic cascade starting from the acetophenone precursor afforded the optically pure target compound in 71% overall yield.

The antiarrhythmic agent (*R*)-mexiletine¹³⁵ was prepared *via* a deracemization cascade from the racemic mexiletine, by employing two enantiocomplementary TAs, at the expense of pyruvate. The optically pure (*R*)-mexiletine was obtained in >99% conversion *via* a two-step procedure, the first step involving the (*S*)-selective Cv-TA and L-AAO from *Crotalus atrox* for the kinetic resolution of the racemate, followed by the asymmetric transamination of the resulting ketone with the enantiocomplementary (*R*)-TA ATA 117 coupled the LDH/GDH pyruvate removing system.

The first scaled industrial application of a transaminase was reported in the sitagliptin manufacturing process. Following multiple rounds of protein engineering that included semi-rational design for altering substrate specificity and directed evolution *via* random mutagenesis for improving thermo- and solvent stability and for removing substrate and product inhibition, a stable and highly useful ω -TA was created. Exceptional stability of the engineered enzyme allowed synthesis of the key precursor amine at substrate concentrations >250 g/L in 50% DMSO at 50 °C (Scheme 12a).

The newly developed biocatalyst was further used in the synthesis of MK-6096 (filorexant), a potent dual Orexin receptor antagonist. The chirality on the α -methylpiperidine core was introduced *via* transamination with the Sitagliptin TA starting from a prochiral keto-ester¹⁹⁴ and D-alanine as amine donor, coupled with the LDH/GDH pyruvate removing cascade, allowing synthesis of the chiral lactam on a kilogram scale. An improvement on the synthetic efficiency of this system was later reported, by using a different keto acid precursor, allowing for a shorter synthetic pathway.¹⁹⁵ An alternative enzyme/amine donor system (CDX-017/isopropyl amine) promoted the spontaneous lactamization of the amino ester intermediate, affording more simplicity to the biocatalytic process. The third generation synthesis of MK-6096 improved on both conversion and economic efficiency by employing a crystallization-induced dynamic resolution of the chiral lactam intermediate,¹⁹⁶ allowing a 40% yield over 4 steps.



Scheme 12. Examples of biocatalytic synthetic routes to active pharmaceutical small molecules performed on industrial (a-c) or multi-kg (d,e) scale.

Similarly challenging drug molecules bearing the chiral amine moiety employed the same Sitagliptin transaminase, shortening the synthetic pathways. Suvorexant¹⁹⁷, a dual Orexin inhibitor¹⁹⁸ was synthesized in 4 linear steps *via* a transamination/ring annulation in 43%

overall yield (Scheme 12b). The chiral indole core of the selective CRTH2 antagonist MK-7246 was accessed using the Sitagliptin TA from the precursor prochiral ketone,¹⁹⁹ resulting in a scalable and manufacturing route.

Further engineering of the Vf-TA resulted in evolved variants tailored for specific ketone substrates such as 2-acetyl-5-fluoropyrimidine, intermediate in synthesis of JAK2 kinase inhibitor AZD1480^{125,97} and the complex ketone precursor of Saridegib²⁰⁰ used in cancer therapy. The Vf-TA r414 variant displayed a 60-fold increase in activity towards (*R*)-ethyl 5-methyl-3-oxooctanoate substrate,²⁰¹ allowing a short quantitative synthesis of Imagabalin²⁰² (Scheme 12e), which was obtained in 95% *de*.

The transaminase from *C. violaceum* was used to prepare of (*R*)-2,3,4,9-tetrahydro-1*H*-carbazol-3-amine via asymmetric synthesis from the corresponding ketone in the synthesis of Ramatroban;²⁰³ the optically pure product was isolated in excellent yield 96% through an alternative freeze-drying work-up, on a 500mg scale.

The increasing availability of commercial preparations of transaminases enabled suitable biocatalysts to be identified that were able to accept bulky substrates and establish two stereogenic centres in a single process. The commercial transaminase ATA-036 from Codexis was used in the transamination of the 4-piperidone derivative in synthesis of a smoothed receptor inhibitor²⁰⁴ enabling a 10:1 diastereomeric ratio of the target amine, whilst an evolved variant ATA-013 (evolution 3) allowed efficient synthesis of Vernakalant²⁰⁵ from an inexpensive α -chloroketone via ZnCl₂-mediated α -etherification and subsequent dynamic asymmetric transamination (Scheme 12c).

Cascade reactions involving transaminase and spontaneous cyclization have been used in the development of dynamic kinetic resolution routes to cyclic piperidines, contained in targets such as the poly(ADP-ribose)polymerase inhibitor Niraparib²⁰⁶ and 3-phenyl-GABA.¹³⁴ A similar dynamic kinetic resolution method was applied in the synthesis of the *N*-methylated (*S*)-amine intermediate in the 4-step route to the heart-rate reducing agent Ivabradine,²⁰⁷ which was obtained in 50% overall yield and excellent enantiopurity (Scheme 12d).

1.8 Future perspectives

There is currently considerable interest in TAs as biocatalysts in the production of chiral amines in which high diastereo-, regio- and enantioselectivity is required during the transformation. The expanding toolbox of enzymes available for the synthesis of chiral amines opens new synthetic routes and disconnections and enables asymmetric synthesis methods catalysed by ω -transaminases alongside reductive aminases²⁰⁸ or ammonia lyases²⁰⁹, while dynamic kinetic resolution processes use monoamine oxidases¹³³ and hydrolases.²⁵

Such products are essential building blocks of intermediary metabolites and pharmaceutical drugs, such as the widely acclaimed manufacture of sitagliptin on an industrial scale highlighting the need for cost and green effective approaches for their chemical syntheses.

Recent attention has focused on the need to overcome problems in the use of TAs in the synthesis of chiral amines; the conversion of the ketone to the amine is hindered by the reaction equilibrium, non-enzymatic competitive substrate and/or product inhibition and a poor substrate tolerance profile. Some solutions have been demonstrated here that utilises *in situ* product removal techniques (ISPR), combining enzyme cascades or displacing the equilibrium with sacrificial amine donors, the latter in particular have garnered considerable interest from academia. Studies have shown biogenic diamines to be effective donors in the asymmetric synthesis of chiral amines from their corresponding prochiral ketones.

Recent advances in metabolic engineering of host strains for the production of secondary metabolites could potentially reduce waste and omit extraneous amine reagents to create a self-sufficient microbial cell factory for chiral amine synthesis. Furthermore, several non-natural amino acids such as taurine and β -alanine that are produced naturally in the cell have untapped potential to be effective amino donors. In Nature these substrates are used in the same family of class III transaminase enzymes as well as belonging to the same class I fold of the promiscuous ω -TAs making it an ideal candidate for asymmetric synthesis.

Predicting the suitability of substrate-donor-enzyme combinations for intensification and scale-up in industrial applications has been probed recently through a stepwise evaluation process.²¹⁰ Thermodynamic (reaction engineering) and kinetic aspects (biocatalyst engineering) have been previously investigated in isolation and often catalyst engineering studies overlooked process engineering attempts. This selection procedure integrates the two approaches and offers a set of criteria for choosing optimal substrate-donor-biocatalyst combination.

Although a broad range of TA enzymes are now available to the end user, there are still substrates that are difficult to aminate. The future of discovering novel transaminase genes perhaps lies in the construction of metagenomics libraries. This approach has provided us with an alternative and powerful method for retrieving genes from uncultured microorganisms with high genetic diversity not known in public databases. Sequence driven analysis of these metagenomics libraries relies on conserved family residues providing us with predicted biological information; however, some transaminase genes maybe far too divergent to make feasible assumptions at the identification of genes with the desired biochemical properties. Combination of sequence and functional driven approaches may be best suited using the principles of enrichment cultivation to be able to enrich sequences which contain our interested transaminase clone.

1.9 References

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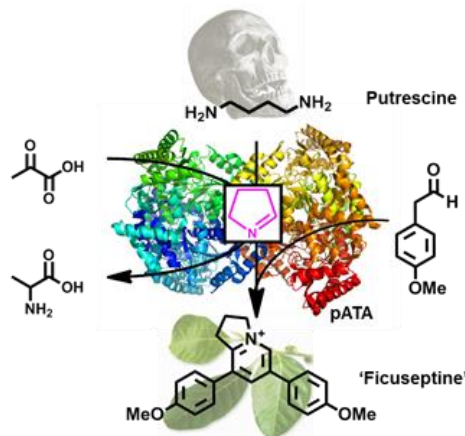
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Chapter 2. Putrescine Transaminases for the Synthesis of Saturated *N*-Heterocycles from Polyamines

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2.1 Foreword

This chapter consists of a research paper published in *ChemCatChem* on 18th of February 2016 and it presents the discovery, characterization and use as biocatalysts of the YjGj putrescine transaminases (pATA), enzymes that display specificity for short-chain aliphatic terminal diamines. This paper was featured on the inside back cover of this issue.

2.2 Acknowledgments

This research was a collaborative effort between the doctoral candidate and the following researchers: James L. Galman¹ and Nicholas J. Weise¹, under the supervision of Richard C. Lloyd^{2,3} and Nicholas J. Turner¹. The doctoral candidate co-wrote the manuscript of the main paper and wrote the supplementary information and performed the following experimental work: protein expression, purification and specific activity studies, substrate spectrum investigation, analytical scale and preparative scale biotransformations for synthesis of compounds **12-18** and **20**, including their isolation, purification and spectral data collection and interpretation.

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2.3 Abstract

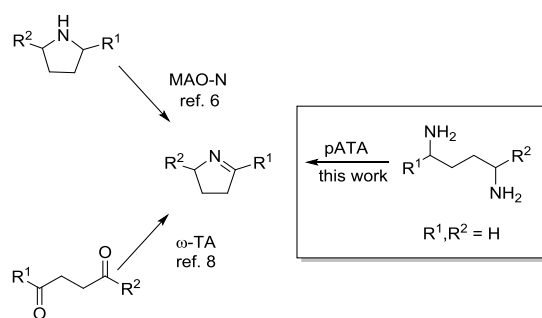
Putrescine transaminase (pATA; EC 2.6.1.82) catalyses the transfer of an amino group from terminal diamine donor molecules to keto acid acceptors using pyridoxal-5'-phosphate (PLP) as a cofactor. The *ygjG* genes from *Escherichia coli* K12, *Bacillus megaterium* and *B. mycooides* were successfully cloned and expressed in *E. coli* BL21(DE3) cells.

The three putrescine transaminases were all shown to prefer diaminoalkanes as substrates thereby generating cyclic imines from the ω -aminoaldehyde intermediates. Addition of a mild chemical reducing agent rapidly reduced the imine intermediate *in situ*, to furnish a range of N-heterocycle products. We have applied pATA in a biomimetic synthesis of 2,3-dihydro-1H-indolizinium containing targets, notably the bioactive alkaloid ficuseptine.

2.4 Introduction

Saturated nitrogen containing heterocycles are prevalent in organic chemistry and are frequently found in natural products¹ as well as being used as intermediates for target molecule synthesis. In particular, substituted pyrrolidines, piperidines and azabicyclic motifs are found in bioactive natural products^{2,3}, as well as currently marketed agrochemicals, fine chemicals and pharmaceuticals. A number of synthetic strategies have been developed for preparation of N-heterocyclic compounds including hydroamination, C-H amination and cyclization reactions⁴.

During the last decade, microbial or enzymatic catalysis has gained considerable interest for the production of enantiopure amine containing building blocks⁵. Biocatalysts are typically highly regio- and stereoselective and can be used under mild conditions. Successful biocatalytic strategies have been reported for the synthesis of chiral cyclic amines from different enzyme classes, including monoamine oxidases⁶, imine reductases⁷ and ω -transaminases⁸.



Scheme 1. Selected biocatalytic strategies for the synthesis of cyclic imines.

Polyamines such as putrescine and cadaverine are biogenic diamines present in almost all living cells, where they modulate cellular proliferation and protein synthesis⁹. These polyamines are also used industrially for the production of commercial products such as plastics, fabric softeners and petroleum additives¹⁰. Recently researchers have elegantly shown the potential to engineer overproducing strains of bacteria such as *Corynebacterium glutamicum* and *E. coli* to provide an alternative to polyamine production¹¹ which at present is largely achieved by chemical synthesis.

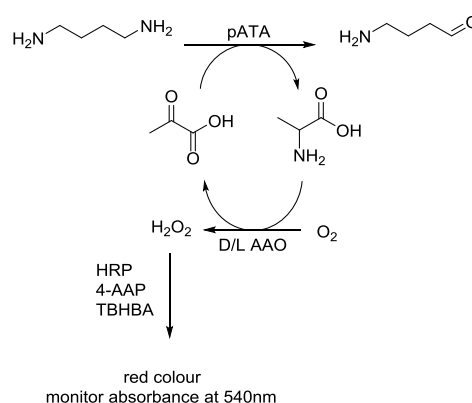
Three catabolic routes for degradation of putrescine have been proposed, namely oxidative deamination by amine oxidases, transamination by transaminases or *via* glutamylation of putrescine with subsequent oxidations steps to γ -aminobutyric acid (GABA)¹². Amongst these, the transaminase pathway for catabolism of putrescine has not previously been investigated for potential application in biocatalysis.

Transaminases are PLP-dependent enzymes that have shown remarkable versatility in the asymmetric synthesis of chiral amino building blocks⁸. Terminal diamines present themselves as attractive alternative substrates for transaminases. In addition to their relatively low cost and high availability, upon transamination diamines are converted to reactive amino aldehydes¹³ (Scheme 1), which spontaneously cyclise to cyclic imines, thus driving the reaction towards product formation. We envisaged using pATAs to generate imines *via* this process for further application in the synthesis of *N*-heterocycles as described herein.

2.5 Results and discussion

In order to identify suitable candidate transaminases with activity towards diamines, we initially screened ω -transaminases from *Chromobacterium violaceum*,¹⁴ *Alcaligenes denitrificans*,¹⁵ *Arthrobacter citreus*¹⁶, commercially available ATA-113 and *Arthrobacter sp.* (ATA-117)¹⁷ against a panel of polyamines using our well-developed L/D-amino acid oxidase colorimetric assay.¹⁸ However, none of these ω -transaminases showed any activity towards either putrescine **1c** or cadaverine **1d** as the amino donor with pyruvate as keto acceptor. We therefore targeted the *ygjG* gene coding for putrescine transaminase from *E. coli* K12 (*Ec-ygjG*)^{19,20} for which little data has been reported regarding substrate scope.

A BLAST analysis of the *ygjG* gene revealed two other putative transaminase genes from *Bacillus megaterium* (*BM-ygjG*) and *B. mycoides* (*BMy-ygjG*) with protein sequence identities of 57% and 58%, respectively. The *ygjG* genes were subcloned into a pET-28b vector followed by overexpression in *E. coli* BL21(DE3) cells. Purified recombinant His₆-tag protein was obtained and employed in specific activity experiments. In parallel reactions, either L-AAO from *Crotalus adamanteus* or D-AAO from porcine kidney was added to reactions containing the diamine and *Ec-ygjG* with pyruvate as amine acceptor (Scheme 2).



Scheme 2. Putrescine transaminase/amino acid oxidase coupled liquid phase colorimetric assay, using amino acid oxidase (L-AAO); horseradish peroxidase (HRP); 4-aminoantipyrine (4-AAP); 2,4,6-tribromo-3-hydroxy-benzoic acid (TBHBA). Assay conditions: diamine substrate 10 mM, pyruvate 10mM, TBHBA 4.5mM, 4-AAP 1.5 mM, PLP 0.1 mgmL⁻¹, HRP 0.1 mgmL⁻¹, L-AAO 4.5 U mL⁻¹, pATA (purified enzyme) 0.2 mgmL⁻¹.

In the L-AAO experiment, generation of L-alanine was confirmed as the product of transamination whereas the use of D-AAO did not lead to the production of D-alanine. We subsequently used this method to screen all three putrescine transaminases *Ec-yggG*, *BMy-yggG* and *BM-yggG* against a broad range of diamines and polyamines in order to assess substrate scope (Table 1 and Figure 1).

In the diaminoalkane series **1a-i** the length of the alkyl chain was found to be an important parameter in determining the relative activities of the substrates (Table 1). No activity was observed with diamines possessing alkyl chains with 2 or 3 C- atoms **1a**, **1b**, possibly due to *in situ* generation of a bridged bicyclic complex with PLP, thereby effectively inhibiting the catalytic role of the cofactor.²¹ The highest activity was observed with putrescine **1c** and thereafter subsequently decreased in a step-wise manner with increasing chain length from **1d-f**. Diamines containing greater than 8 C atoms **1g-i** had correspondingly lower measureable activity. In addition, we examined a series of diamines containing heteroatoms **2a-c** and **3a-b**, and branched diamine **4**. All of these diamines were shown to be active although the lower activities of the bulky substrates can possibly be rationalised by the presence of a small substrate-binding site entrance with a highly hydrophobic channel to the active site cavity compared to other class III transaminases.²²

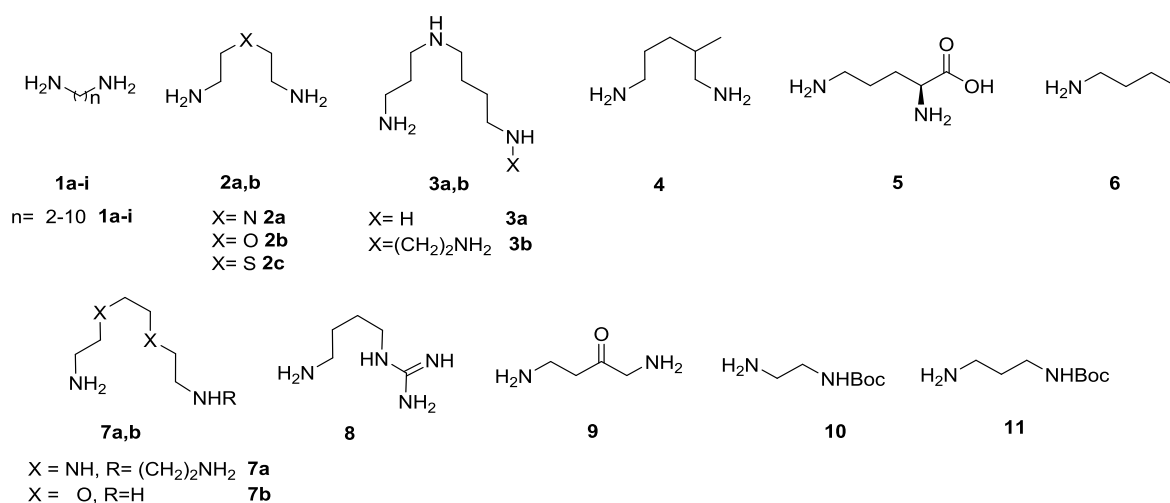


Figure 1. Diamines tested as substrates for the novel ω -transaminases

Interestingly putrescine transaminase pATA was found to exhibit high selectivity for diamines compared to monoamines of comparable size, *e.g.* putrescine **1c** showed high activity with *Ec-ygjG* pATA whereas the corresponding monoamine butyl amine **6**, showed no detectable activity. Ornithine **5**, mono *N*-Boc protected derivatives of **1a** and **1b** (amines **10** and **11** respectively) and other diamines **7a-b**, **8** and **9** were also found to be inactive substrates, suggesting that the *ygjG* enzyme is strictly involved in the degradation of linear diamines.

Table 1. Specific activities of *Ec-ygjG* pATA, *BM-ygjG* pATA and *BMy-ygjG* pATA on polyamines **1-6**. The rates were determined by measuring the absorbance at 510 nm using a horse radish peroxidase (HRP)/L-AAO colorimetric assay with purified enzyme.

Substrate	Specific activity (mU mg ⁻¹)		
	<i>Ec_ygjG</i>	<i>BM_ygjG</i>	<i>Bmy_ygjG</i>
1a	0.62	0.41	0.22
1b	0.83	0.39	0.21
1c	270.04	41.54	103.86
1d	251.13	62.31	83.08
1e	164.42	20.77	51.93
1f	57.70	5.81	14.54
1g	27.04	4.15	10.38
1h	18.90	2.07	5.19
1i	8.10	0.83	3.11
2a	62.10	12.46	27.00
2b	124.21	20.77	41.54
2c	13.05	4.15	10.38
3a	121.51	16.61	41.54
3b	16.20	8.30	5.19
4	40.50	10.38	24.92
5	0.04	0.04	0.04
6	0.51	0.50	0.45

Assay conditions: 10 mM polyamine substrate, pATA (purified enzyme) 0.2 mgmL⁻¹, KiPO₄ buffer pH 8.0 at 30°C.

A recent report suggested that the *N*-2 nitrogen atom of **1c** is stabilised *via* a hydrogen bond to a conserved Q119 residue²² which is also found in a range of γ -aminobutyrate (GABA): α -ketoglutarate aminotransferases with low putrescine amino donor specificity.²³ The catalytic role of Q119 was therefore examined by mutating this residue to alanine

which resulted in a 60% loss of activity with **1c** as substrate (Figure 1). This reduced activity is consistent with the role of Q119 in stabilizing the orientation of putrescine bound as the PLP-substrate imine although it is clearly not crucial for catalysis. Furthermore, for substrates with increasing chain length from C5 to C8, the effect of the mutation on activity was less pronounced.

Further insights into the factors affecting substrate recognition were gained by mutation of other active site residues of the *Ec-ygjG* transaminase. According to the published crystal structure of this enzyme (PDB: 4UOX), the substrate-binding residues are not conserved between *Ec-ygjG* and its structural homologues²², unlike the PLP-binding residues.

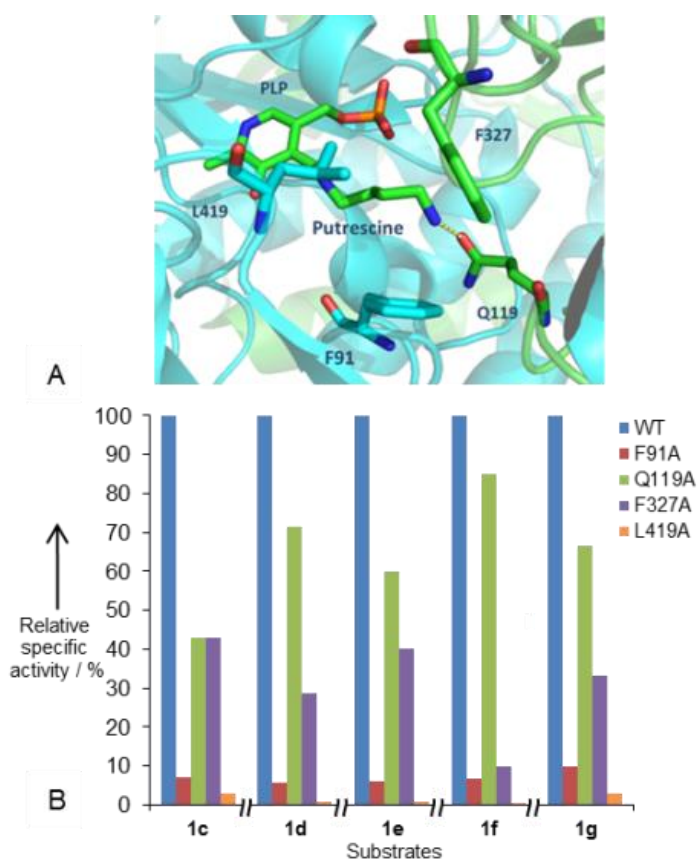
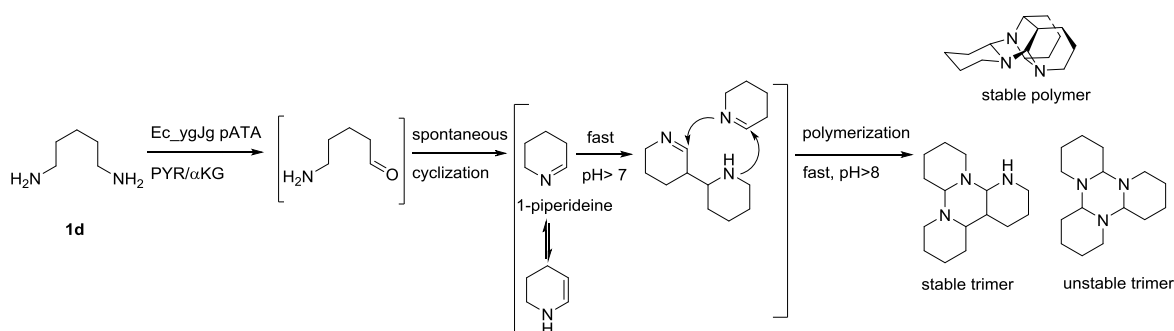


Figure 2 (A) PyMol representation of the pATA enzyme (PDB: 4UOX), highlighting the important residues in the enzyme substrate cleft. (B) The effect of mutation to alanine of substrate-recognition residues on the specific activities of the *Ec-ygjG* transaminase in the 1c-1g series. Substrates were normalised with respect to activity of wild type enzyme.

A combination of bulky residues that protrude into active site cleft (F327, F91 and L419) ensures that the entrance to the active site is hydrophobic and narrow, allowing the

enzyme to discriminate between diamines **1c** and **5** that only differ by the presence of a carboxylate group. These residues are unique to this transaminase and it has been suggested that they are responsible for substrate binding²⁴ (Figure 2A). A series of point mutations to alanine were performed in order to examine the effect of each of these residues (F91A; F327A; L419A) on transamination of diaminoalkanes **1c-g**. Interestingly, mutation of F to A at position 91 resulted in a complete loss of activity and similarly, the same effect was observed for L419A. Substitution of F with A at position 327 resulted in a *ca.* 50% loss of activity for the shorter chain diamines **1c-e**, the decrease being more pronounced for longer chain diamines **1f** and **1g**. These results indicate that F91 and L419 residues are important in catalysis; however further mutagenesis studies are required in order to elucidate completely the factors responsible for substrate binding and catalysis (Figure 2B).

In order to develop preparative scale reactions using putrescine transaminase, we initially focussed on using cadaverine **1d** as a model substrate. Transamination of **1d** yielded 1-amino-5-pentanal which underwent cyclisation to 1-piperidine followed by rapid pH dependent polymerisation resulting in a mixture of oligomers that is unable to revert back to the imine monomer.^{25,26} (Scheme 3).



Scheme 3. Spontaneous polymerisation of the reactive intermediate imine 1-piperidine and formation of a complex mixture of stable oligomers^{25,26}

The imine is only stable at low pH values which are not compatible with the transamination reaction. An alternative approach was therefore pursued, involving *in situ* reduction of the imine intermediate to prevent polymerisation. Several chemical reducing agents were tested, including NH_3BH_3 , NaCNBH_3 , and $\text{Na}(\text{AcO})_3\text{BH}$ with pyruvate and α-ketoglutarate as the keto acceptor (Table 2).

In all cases, GC analysis revealed clean monomeric product peaks. Both Na(AcO)₃BH and NaCNBH₃ reduced the imines with high conversions with no loss of transaminase activity. Using Na(AcO)₃BH (10 eq) the highest conversions were observed for **1c-e**, while the presence of a heteroatom in similar substrates (**2a** and **2b**) resulted in lower conversion values (Table 2).

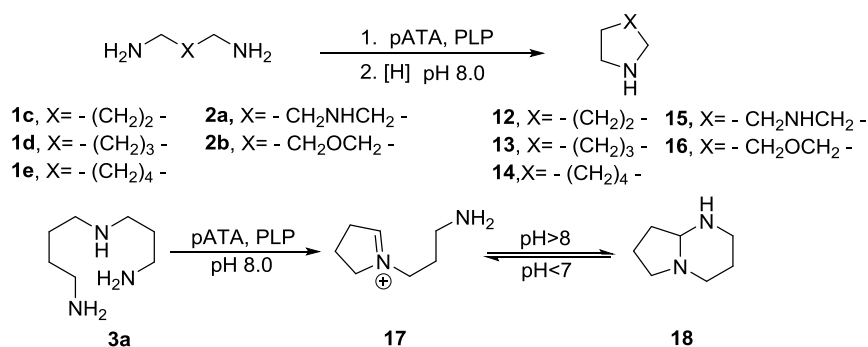
Table 2. Reducing reagent optimisation

Reducing reagent	Amino acceptor	Conversions	
		1c	1d
NaCNBH ₃ ^[a]	pyruvate	97%	88%
NH ₃ BH ₃ ^[a]	pyruvate	55%	40%
Na(CH ₃ CO ₂) ₃ BH ^[a]	pyruvate	97%	98%
<i>R</i> -IRED	pyruvate	20%	79%
<i>R</i> -IRED	α-ketoglutarate	79%	80%
<i>S</i> -IRED	pyruvate	50%	79%
<i>S</i> -IRED	α-ketoglutarate	79%	99%

Reaction conditions: 5 mM diamine; 10 mM keto acid, 50 mM Tris-HCl, 1 mM PLP; cell-free lysate; [a] 10 eq. reducing reagent ; [b] wet cell pellet 1mg/mL

Interestingly, substrate **3a** gave the bicyclic product **18**. Preparative scale studies resulted in isolation of an open chained *N*-substituted pyrroline **17** from acidic workup confirming that transamination occurs at the amine of the 4C terminus as expected. Conjugate addition of the 3C terminal amine of **17** is pH dependent and generated the fused bicyclic structure **18** under basic conditions in 60% isolated yield.

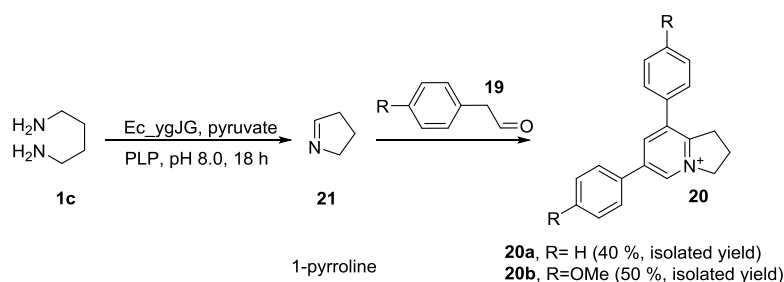
As an alternative to chemical reduction of the imine we examined the use of imine reductases. One-pot enzyme cascades with **1c** and **1d** using *Ec-ygjG* with (*R*)- or (*S*)-imine reductase (IRED) from *Streptomyces* sp. GF3587²⁷ and *Streptomyces* sp. GF3546²⁸ were examined respectively. Interestingly, both (*S*)- and (*R*)-IREDs showed good to excellent conversions (up to 99%) highlighting that the potential for combining these two enzymes *in vivo* for the conversion of polyamines to *N*-heterocycles (Table 2).

Table 3. Transamination of polyamines using pyruvate/ α -ketoglutarate as keto acceptors.

Substrate	Product	Conversions (%)					
		Ec-ygjG		BM-ygjG		BMy-ygjG	
1c		98 ^[a]	98 ^[b]	97 ^[a]	95 ^[b]	97 ^[a]	95 ^[b]
1d		99 ^[a]	99 ^[b]	97 ^[a]	95 ^[b]	97 ^[a]	95 ^[b]
1e		97 ^[a]	93 ^[b]	97 ^[a]	95 ^[b]	97 ^[a]	95 ^[b]
2a		15 ^[a]	15 ^[b]	10 ^[a]	14 ^[b]	15 ^[a]	12 ^[b]
2b		60 ^[a]	53 ^[b]	72 ^[a]	72 ^[b]	65 ^[a]	64 ^[b]
3a^[c]		51 ^[a]	72 ^[b]	56 ^[a]	58 ^[b]	39 ^[a]	25 ^[b]

[a] Keto acceptor: pyruvate [b] Keto acceptor: α -ketoglutarate. Reaction conditions: 400 μ L reaction, 5 mM diamine; 10mM keto acceptor, buffer: 50mM TRIS, 300 mM NaCl, 1mM PLP; 10eq Na(AcO)₃BH; cell-free lysate. [c] No addition of reducing reagent. Conversions were measured by GC-FID by comparison with commercially available standards, using an empty vector control as baseline.

Finally, in order to demonstrate the utility of putrescine transaminase in synthesis, we have developed a biomimetic one-pot route to 2,3-dihydro-1*H*-indolizinium motifs, notably the antibacterial and antifungal alkaloid ficuseptine.²⁹ Starting from **1c** we prepared **20a** (40%, isolated yield) and analogue **20b** (50%), both of which have previously been reported requiring a 5-step chemical synthesis²⁹ or a similar biomimetic route under harsh acidic conditions.³⁰ (Scheme 4)



Scheme 4. Bioimimetic formation of 2,3-dihydro-1*H*-indolizinium motifs using 2 molecules of aldehyde **19** and the *in situ* product 1-pyrroline **21** to generate **20a-b**.

2.6 Conclusions

In summary, three putrescine transaminases, with broad specificity for terminal aliphatic diamines, have been characterised and their potential for biocatalysis subsequently investigated. These transaminases use pyruvate and α -keto-glutarate as keto acceptors and have been combined with a chemical reducing reagent to generate a range of different *N*-heterocycles in good yield. Further engineering of these enzymes should lead to new biocatalysts for the synthesis of precursors for pyrrolidine and piperidine-derived alkaloids.

2.7 Experimental section

2.7.1 Materials and methods

General: All biotransformations were carried out in TRIS buffer (50mM, pH 8.0, 1 mM PLP) at 37°C in 4 mL vials. GC-MS spectra were recorded on a Hewlett Packard HP 6890 equipped with a HP-1MS column, a HP 5973 Mass Selective Detector and an ATLAS GL FOCUS sampling robot. GC-FID analysis was performed on Agilent 6850 equipped with a Gerstel Multipurpose sampler MPS2L and a Varian CP CHIRASIL-DEX CB 25 m x 0.25 mm DF=0.25 column.

Materials: Commercially available reagents were used throughout without further purification. Putrescine and all other reagents were purchased from Sigma Aldrich (St Louis, MO, USA) or Acros including anhydrous solvents. Restriction enzymes, T4 ligase, *taq* polymerase, dNTPs and broad protein marker (2-212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* DH5 α and BL21 (DE3) cells were

purchased from New England Biolabs (Ipswich, MA, USA). Expression vector pET-28b was purchased from Novagen (Darmstadt, Germany) and was used for gene expression. Commercially available transaminases, ATA113 and ATA117 were purchased from Codexis in the form of lyophilised cell extract. *Chromobacterium violaceum* (Cv-TA) was purchased from LG standards (ATCC 12472, NCIMB 9178). Putrescine transaminase gene *ygjG* from *Bacillus megaterium* (NCIMB 9376) and *Bacillus mycooides* (8096) was isolated from NCIMB culture collection.

2.7.2 Molecular biology protocols

2.7.2.1 Cloning of *ygjG* gene (UNIPROT accession no. G2RVC5) from *Bacillus megaterium* and *Bacillus mycooides* (UNIPROT accession no. C3AJ99)

The coding region of the *ygjG* gene was amplified by colony PCR from *Bacillus megaterium* using forward primer (5'-CAGCCCATATGGAAACGAATGTGAAAAGTAAATCTAATGAA-3') and reverse primer (5'-GTGCTCGAGTTATTGTACTTTTACTTGTGCCAACACTGAT-3') with *NdeI* and *XhoI* restriction sites underlined respectively. The following PCR protocol was used: 5 min denaturation at 95 °C and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s elongation at 68 °C with a 5 min final extension time at 68 °C. The PCR product was cloned into ZERO Blunt TOPO PCR cloning vector (K2830-20) following the manufacturer's protocol. The *ygjG* gene from *Bacillus mycooides* was amplified using forward (5'-CAGCCCATATGGAAACGAATGTGAAAAGTAA-ATCTAATGAA-3') and reverse (5'-GTGCTCGAGTTATTGTACTTTTACTTGTGCCAACACTGAT-3') primers (*XhoI/NdeI* restriction sites underlined) and was cloned into TOPO PCR cloning vector as above. Both *ygjG* genes were subcloned into pET-28b expression vector containing an *N*-terminal His₆ tag with a thrombin linker.

2.7.2.2 Subcloning of *ygjG* gene into pET28b-Ec-pATA

A codon optimised putrescine transaminase *ygjG* gene (UNIPROT accession no. P42588) from *E. coli* was synthesized by GeneArt® life technologies containing the restriction sites *NdeI* and *XhoI* downstream from the ribosome binding site. The gene was subcloned into a pET-28b vector containing an *N*-terminal His₆ tag with a thrombin linker. The inserted

gene was confirmed by DNA sequencing (Eurofins). The pET-28b expression vector was transformed into *E.coli* BL21 (DE3) cells and grown on LB agar plates containing kanamycin.

2.7.2.3 Expression and purification of recombinant protein

The protein expression of *ygjG* genes in pET28b expression vector was transformed into the strain *E. coli* BL21 (DE3) for yielding *E. coli* BL21 (pET28b-pATA). The freshly-prepared strain *E. coli* BL21 (pET28b-pATA) was cultivated in 500 mL of the LB medium supplemented with 50 µg/mL kanamycin in 1-L Erlenmeyer flasks at a rotary shaking rate of 220 rpm at 37°C. The recombinant protein expression was induced by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) (0.2 mM, final) when A₆₀₀ reached 0.6 - 0.8. The cell cultures were incubated at 18 °C for 16 h. The cells were harvested by centrifugation (4°C, 3,250xg, 20 min) and resuspended (1g of wet cell paste/10 mL) in 50 mM Tris-HCl, pH 8.0, 300 mM NaCl and 1 mM PLP. The cell pellets were lysed in an iced bath by ultrasonication by Soniprep 150 (20 cycles of 20s on/20s off.). After centrifugation (4°C, 16,000xg, 20 min) the supernatant was used for protein purification by a Ni-NTA agarose column. The enzyme was washed with 50 mM Tris-HCl, 30 mM imidazole and 1 mM PLP at pH 8.0 and eluted with 50mM Tris-HCl, 300 mM imidazole, 100 mM NaCl and 1mM PLP at pH 8.0. The collected fractions was washed twice with 50 mM Tris-HCl buffer containing 1 mM PLP and concentrated in a micropore filter. The enzyme was flash frozen and stored at -80°C. The purity was analysed by SDS/PAGE and the protein was more than 95% pure.

Table 4. Purification of recombinant Ec_ygjG; BM_ygjG and BMy_ygjG pATA expressed in *E.coli*.

Recombinant protein	Culture medium volume (L)	Cell pellet (g)	Yield pure protein (g)	Purity (%)
Ec_ygjG	0.5	8	0.250	94
BM_ygjG	0.5	7.8	0.250	95
BMy_ygjG	0.5	7.5	0.280	98

2.7.2.4 Protein determination and SDS-PAGE analysis

Concentrations of soluble protein was analysed by SDS-PAGE with 15% resolving gel and 5% stacking gel in a Tris-glycine buffer system. The gel was stained with Bio-Rad Coomassie Blue 250 kit (Bio-Rad lab, Munich, Germany), with bovine serum albumin (BSA) as a standard protein.

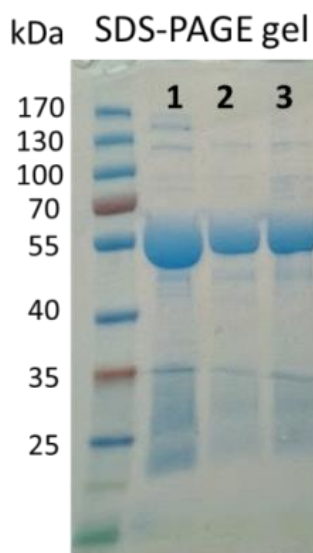


Figure 3. SDS-PAGE analysis of the novel ω -transaminases; lane 1: Ec-ygjG; lane 2: BM-ygjG; lane 3: BMy-ygjG.

2.7.2.5 Protocol for site-directed mutagenesis for generating Ec-ygjG transaminase mutants

Site directed mutants were introduced into the *ygjG* gene cloned in the pET14b expression vector. Synthetic oligonucleotide primers were purchased from Eurofins (Table S1) and were designed to contain the desired mutation and to anneal to the complementary DNA strand. The mutagenesis procedure followed the Thermo Scientific Phusion[®] Site-Directed Mutagenesis Kit, using Phusion high-fidelity DNA polymerase from New England Biolabs (Ipswich, MA, USA). The PCR thermocycler was Eppendorf temperature gradient Mastercycler (Hauppauge, NY). The mutated plasmids were overexpressed in *E. coli* BL21 (DE3). All microorganisms were cultivated in Luria-Bertani (LB) medium at 37°C with kanamycin 50 $\mu\text{g}/\text{mL}$. *E. coli* BL21 (DE3) were prepared as described elsewhere. High transformation efficiency of *E. coli* BL21 (DE3) cells were also

purchased from Invitrogen (Carlsbad, CA). All isolated plasmids were sequenced (Eurofins) to confirm desired mutations.

Table 5. Primers for site directed mutagenesis

Primer	Sequence
For G119A	CAT AGC GCA GAA CTG CTG GAT CC
Rev G119A	CAG CGG CTG TTT TGC CAG CTG
For F91A	CTG GGT GGT GCT GGC ATT TTT AAC
Rev F91A	ACA ATC AAT AAA TTC TTG
For L419A	GGT ACA GCG AAT AAT GCA AAA AC
Rev L419A	TGC AAC CAG AAC ACG CTG ACG
For F327A	GAT AAC CCG GGT CTG CAT ACC ACC
Rev F327A	AAA CAG AAC GCT AAA AAC TTC

2.7.3 Protein activity assays

L-amino acid oxidase (L-AAO) activity assay screen^[S1]

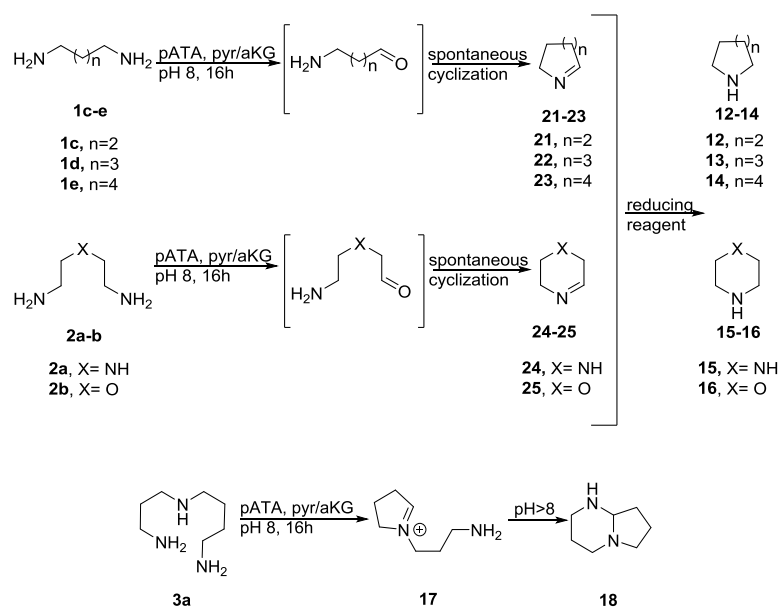
A stock solution of substrate and reagents was made up of the following; 4.95 mM 2,4,6-Tribromo-3-hydroxybenzoic acid (TBHBA), 1.65 mM 4-aminoantipyrine, 11 mM sodium pyruvate, 6 mg mL⁻¹ HRP, 10 mg mL⁻¹ PLP and 11 mM of the amine donor in MeOH was added to 50 mM Tris-HCl buffer and adjusted to pH 8.0. The stock solution (90 µL) was dispensed to individual wells in a 96 well plate. 5 µL of L-AAO (4.5 U mL⁻¹) was added to each well and the assays were initiated upon addition of putrescine transaminase (purified enzyme, 0.2 mg mL⁻¹). Experiments were run on a spectrophotometer at 30 °C with the measured activity at a wavelength of 510 nm and the absorbance taken every 20 seconds. One Unit (U) is defined by one µmole of produced per minute. One unit of enzyme activity (U) was defined as the amount of enzyme required to produce one µmole of H₂O₂ produced per min.

2.7.4 Biotransformations

2.7.4.1 pATA-mediated reductive amination of polyamines

Analytical scale: Biotransformations were carried out in 4mL vials, in a volume of 400 μ L. To a mixture of polyamine substrate (5 mM from a 100 mM stock in Tris-HCl buffer, prepared as described above) and keto donor (10 mM from a 100 mM stock in Tris-HCl buffer), was added the reducing reagent* (10 eq) and the pH was adjusted to 8. The enzyme was added as cell-free lysate (370 μ L from a 0.1 g/mL wet cell pellet stock). The reactions were incubated at 37°C and 250 rpm for 12 hours, followed by addition of derivatizing reagent (30 μ L (AcO)₂O and 10 μ L Et₃N). The reaction mixtures were allowed to derivatize for 15 min at rt and then were extracted with 400 μ L DCM. Samples were analysed by GC-MS and GC-FID as detailed below.

* The reducing reagents were not used in the analysis of biotransformations involving spermine **3a**.



Scheme 5. Products of the transamination of substrates **1c-e**, **2a-b** and **3a**. Conversions of the substrates **1c-e** and **2a-b** were measured on GC-FID by comparison with commercially available standards. Conversion of **3a** was measured on GC-FID using 1,5-Diazabicyclo(4.3.0)non-5-ene (DBN) as internal standard.

2.7.4.2 GC-assay conditions

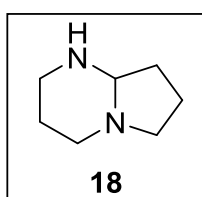
GC-MS method: Hewlett Packard HP 6890 equipped with a HP-1MS column, a HP 5973 Mass Selective Detector and an ATLAS GL FOCUS sampling robot; method: 90°C for 0 min, then 10°C min⁻¹ to 200°C, hold for 10 min

GC FID method: Agilent 6850 equipped with a Gerstel Multipurposesampler MPS2L and an Agilent CHIRASIL-DEX CB 25 m x 0.25 mm DF=0.25 column; method: 100°C for 10 min, then 5°C min⁻¹ to 200°C, hold for 5 min. The amines (both starting materials and the products) were derivatized and analysed as their corresponding acetate derivatives.

Table 6. Retention times for the biotransformation products GC-FID method

Product	R _t (min)
12	15.6
13	17
14	20.4
15	29.4
16	18.3
18	10.4

2.7.4.3 Preparative scale reactions

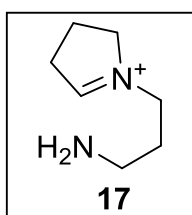


1,5-diazabicyclo[4.4.0]decane 18

Spermidine (0.39 mL, 2.5 mmol, 1 eq, 50 mM final concentration) and sodium pyruvate (0.440 g, 4 mmol, 1.6 eq, 80 mM final concentration) were dissolved in 20 mL Tris-HCl buffer (50 mM TRIS-HCl, 300 mM NaCl, 1 mM PLP) and the pH was adjusted to 8. Clarified cell lysate (30 mL from 3 g of fresh cell pellet) was added to the mixture and the reaction was incubated at 37 °C, 250 rpm for 48 hours and monitored by GC-FID as detailed above. The reaction mixture was centrifuged at 3,250 x g for 15 minutes and the pH of the supernatant was adjusted at pH 12 with NaOH 3M. The reaction was extracted with DCM (3 x 50 mL) and the combined organics

were dried on MgSO₄ and concentrated in vacuo to afford the product as yellow oil (0.1593 g, 51% yield).

¹H NMR (400 MHz, CHCl₃-*d*) δ ppm 1.32-1.84 (m, 5H), 1.89-1.99 (m, 1H), 2.07-2.16 (q, *J*=8.84 Hz, 1H), 2.19-2.28 (td, *J*=11.62, 3.16 Hz, 1H), 2.59-2.69 (td, *J*=13.20, 3.41 Hz, 1H), 2.78-2.85 (dd, *J*=9.28, 6.25 Hz, 1H), 2.93-3.03 (td, *J*=8.91, 2.15 Hz, 1H) 3.05-3.20 (m, 2H); ¹³CNMR (400 MHz, CHCl₃-*d*) δ ppm 19.0, 26.0, 29.9, 45.5, 51.4, 51.7, 78.7.



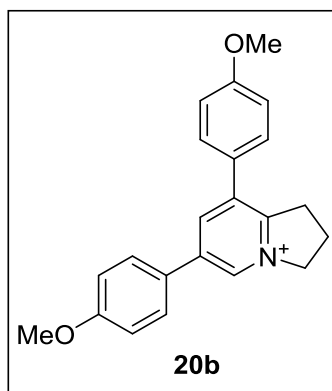
1-(3'-aminopropyl)-3,4-dihydro-2H-pyrrolium 17

Compound **17** was prepared identically as **18** above and isolated following an acidic work up of the reaction.

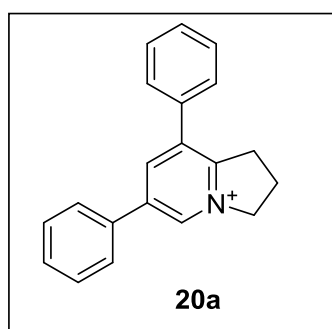
¹HNMR (400 MHz, MeOH-*d*₄) δ ppm 2.25-2.35 (m, 2H), 2.35-2.45 (m, 2H), 3.07-3.17 (t, *J*= 8.0 Hz, 2H), 3.27-3.37 (t, *J*= 8.0 Hz, 2H), 4.10-4.20 (t, *J*= 8.0 Hz, 2H), 4.25-4.35 (t, *J*= 8.0 Hz, 2H), 8.98 (s, 1H). ¹³CNMR (400 MHz, MeOH-*d*₄) δ ppm 20.5, 25.8, 37.16, 37.62, 52.1, 60.2, 183.7

General method for the synthesis of 2,3-dihydro-6,8-diphenyl-1H-indolizinium hydroxide^[S3] **20a** and 2,3-dihydro-6,8-bis(4-methoxyphenyl)-1H-indolizinium hydroxide (ficuseptine)^[S2] **20b**

Putrescine dihydrochloride (0.5 g, 3.1 mmol, 1eq, 25 mM final concentration) and sodium pyruvate (0.545 g, 4.9 mmol, 1.6 eq, 40 mM final concentration) were dissolved in 50 mL Tris-HCl buffer (50 mM Tris-HCl, 300 mM NaCl, 1 mM PLP) and the pH was adjusted to 8. To this mixture, a solution of phenylacetaldehyde (**20a**) or 4-methoxyphenylacetaldehyde (**20b**) (6.2 mmol, 2 eq, 50 mM final concentration) in 5 mL MeOH was added and the volume of the solution was adjusted to 95 mL by addition of Tris-HCl buffer pH 8. Clarified cell lysate (30 mL from 3 g of fresh cell pellet) was added to the mixture and the reaction was incubated at 37° C, 250 rpm for 48 hour. The mixture was centrifuged at 3,250xg for 15 minutes and the pH of the supernatant was adjusted at pH 10 with NaOH 1M. The reaction was extracted with DCM (3 x 50 mL) and the combined organics were dried on MgSO₄ and concentrated in vacuo. The product was purified by flash chromatography (4:1 CHCl₃:MeOH).



Yellow powder (0.255g, 50% yield). ^1H NMR (400 MHz, CHCl_3-d) δ ppm 2.46-2.56 (t, $J=7.20$ Hz, 2H) 3.44-3.53 (t, $J=7.20$ Hz, 2H), 5.40-5.50 (t, $J=7.20$ Hz, 2H), 7.40-7.55 (m, 8H), 7.78-7.87 (d, $J=7.20$ Hz, 2H), 8.27 (s, 1H) 10.02 (s, 1H); ^{13}C NMR (400 MHz, CHCl_3-d) δ ppm 22.25, 32.3, 60.48, 127.6, 128.3, 129.5, 129.8, 130.12, 130.3, 131.3, 132.7, 138.9, 140.9, 141.6, 151.52.



Light brown solid (0.39 g, 40% yield). ^1H NMR (400 MHz, CHCl_3-d) δ ppm 2.38-2.64 (m, 2H), 3.41-3.5 (t, $J=8.46$ Hz, 2H), 3.7 (s, 3H), 3.8 (s, 3H), 5.2-5.3 (t, $J=7.20$ Hz, 2H), 6.84-6.94 (d, $J=8.46$ Hz, 2H), 6.93-7.03 (d, $J=8.46$ Hz, 2H), 7.35-7.45 (d, $J=8.72$ Hz, 2H), 7.67-7.77 (d, $J=8.72$ Hz, 2H), 8.09 (s, 1H) 9.81 (s, 1H); ^{13}C NMR: (400 MHz, CHCl_3-d) δ ppm 22.3, 32.3, 53.8,

55.4, 60.2, 114.9, 115.3, 125, 126.5, 128.9, 129.7, 138.14, 138.5, 139, 140.33, 152.2, 160.9, 161.2

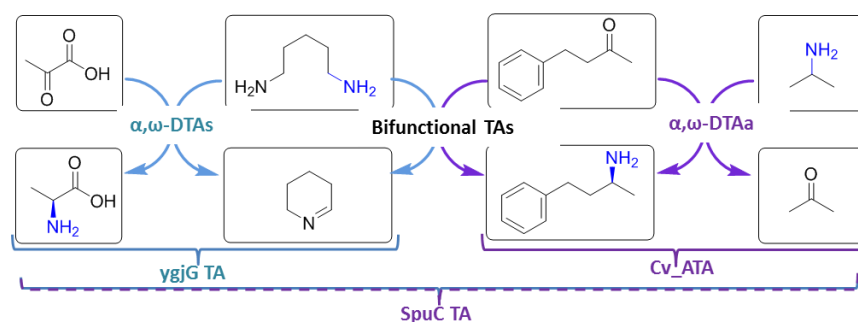
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Chapter 3. Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases

James L. Galman¹, Iustina Slabu¹, Nicholas J. Weise¹, Cesar Iglesias⁴, Fabio Parmeggiani¹, Richard C. Lloyd^{2,3}, Nicholas J. Turner¹



3.1 Foreword

Chapter 2 described the ygjG putrescine transaminases (ygjG pATA), a group of enzymes that specialise in transamination of short-chain aliphatic terminal diamines. They feature a narrow amine substrate spectrum and only accept pyruvate and α -ketoglutarate as amine acceptors, which make them unsuitable for use in transamination equilibrium-shifting approaches. For this purpose, a novel group of transaminases (SpuC TAs) has been studied and the results are presented herein. These enzymes have the ability to accept both mono- and diamines as amine donors, in addition to featuring a relaxed ketone donor spectrum. They allow reductive amination of a large variety of prochiral aliphatic and aromatic ketones, as described below.

This chapter consists of a research paper published in *Green Chemistry* on 12th of August 2016 and it describes the discovery, characterisation and use as biocatalysts of a group of SpuC transaminases. The paper was chosen to feature on the front cover of this issue.

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3.2 Acknowledgements

The research paper was a collaborative effort between the doctoral candidate and the following researchers: James L. Galman¹, Nicholas J. Weise¹, Cesar Iglesias⁴ and Fabio Parmeggiani¹, under the supervision of Richard C. Lloyd^{2,3} and Nicholas J. Turner¹. The doctoral candidate wrote the supplementary information and helped editing the final manuscript and performed the following experimental work: protein expression, purification and characterisation, protein activity and kinetics assays, substrate spectrum characterization, analytical and preparative scale biotransformations; analysis, collection and interpretation of the spectral data of the products obtained.

3.3 Abstract

The discovery and characterisation of enzymes with both monoamine and diamine transaminase activity is reported, allowing conversion of a wide range of target ketone substrates with just a small excess of amine donor. The diamine co-substrates (putrescine, cadaverine or spermidine) are bio-derived and the enzyme system results in very little waste, making it a greener strategy for the production of valuable amine fine chemicals and pharmaceuticals.

3.4 Introduction

Biocatalytic strategies for the synthesis of high added-value optically pure chiral intermediates provide extraordinary benefits to the pharmaceutical and agrochemical industries, due to the excellent activity, stereo-/regioselectivity and stability of many biocatalysts, often unmatched by their corresponding chemical equivalents.¹ There are obvious sustainability benefits to such approaches, including renewable resourcing of recombinant biocatalysts, solvent- and metal-free chemical transformations and ambient temperature/pressure of operation. In spite of this highly cited “greenness”, the concept of any given biocatalytic transformation being effectively environmentally benign has been questioned in several instances.² Many widely used biotransformation protocols require, for example, expensive cofactor supplementation and/or large excesses of co-substrate to combat poor atom efficiency and unfavourable equilibria.

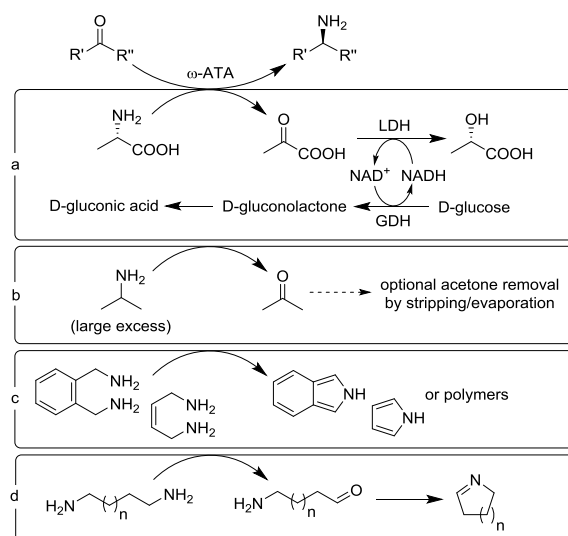
The asymmetric amination of ketones to enantiomerically enriched amines mediated by ω -amine transaminases (ω -ATAs, E.C. 2.6.1.18) is undoubtedly one of the most studied and exploited biotransformations which has become integrated into modern industrial practice. The broad substrate tolerance of this class of enzymes is one of their most appealing features in synthetic applications. A prominent example of this is the commercial manufacture of sitagliptin – probably one of the most cited success stories of industrial biocatalysis.³

For these reactions, the thermodynamic equilibria are often unfavourable and hence different methods for shifting the equilibrium have been sought to address this long-standing challenge (Scheme 1).⁴ One of the earliest approaches, still heavily relied on, was the coupling of the enzymatic reaction with a secondary concomitant irreversible reaction (*e.g.*, conversion of pyruvate by-product to L-lactate with lactate dehydrogenase, paired with an oxidoreductase to recycle NAD(P)H, Scheme 1a).^{5,6} Whilst producing naturally-degradable biochemicals as by-products, this method has the obvious disadvantage of requiring two additional enzymes and an expensive cofactor to achieve high conversion of the starting ketone.

On the other hand, for industrial scale application, the approach of choice is the use of a sacrificial amine donor in large excess to drive the equilibrium towards the desired product. Isopropylamine is widely used for this purpose, because of its effectiveness and availability.⁷ This approach has proven very cost-efficient in many cases, however, a 100-fold excess (or higher) is required to drive equilibrium. Also, in order to improve the yields, the removal of the volatile acetone waste product by evaporation or stripping needs to be implemented using specialised temperature platforms and heat-stable enzymes.⁸

More recently, the use of synthetic diamines such as *o*-xylylenediamine⁹ and but-2-ene-1,4-diamine¹⁰ has been the focal point to displace the equilibria of ω -ATA reactions by spontaneous cyclisation and subsequent ring aromatisation of the aminocarbonyl by-

product (Scheme 1c). However, these donors are usually expensive, highly toxic and often form difficult to remove polymers following aromatisation, adding to downstream processing costs.



Scheme 1. Different approaches to shifting the thermodynamic equilibrium of ω -ATA transaminations.

In contrast, biogenic terminal diamines present themselves as renewably-sourced alternative amino donors. In addition to their simple bio-based production and relatively low cost, upon transamination they are converted into reactive amino aldehydes which spontaneously convert to cyclic imines, thus driving the equilibrium towards amination of ketones. Enzymes active on such compounds, known as α,ω -diamine transaminases (α,ω -DTAs), have exploited these advantages for the synthesis of *N*-heterocycles precursors.¹¹ However, these enzymes were found to have strict preference for pyruvate over other ketones, making them unsuitable for the synthesis of a broad range of amines. Nevertheless, if the substrate specificity could be addressed, biogenic diamines would offer a distinct approach to equilibrium issues (Scheme 1d), intrinsically “greener” than previous ones, since near-stoichiometric loadings of sacrificial diamines could be applied, in principle.

Herein, we report the discovery and characterisation of a panel of bifunctional α,ω -DTA/ ω -ATA enzymes that readily accept cheap and easily-accessible mono-/diamine donors as well as possessing broad ketone acceptor scope, allowing an equilibrium shift

to reach theoretical yield of 100% aminated product with almost stoichiometric donor loadings.

3.5 Results and discussion

The lack of activity of commonly used ω -ATAs towards simple aliphatic diamines such as putrescine (1,4-diaminobutane, 1a), cadaverine (1,5-diaminopentane, 1b) and spermidine (1,8-diamino-4-azaoctane, 1c) has been previously reported.^{10,11} Indeed, the only relevant example is a single commercial transaminase (ATA256) recently found through extensive screening of a large biocatalyst panel from Codexis, highlighting the rarity of such activity.¹² This transaminase was shown to have activity with a narrow range of ketone acceptors, and its commercial nature precludes evolutionary, sequence and structural insight into its substrate scope or optimisation through engineering. To address this gap in the transaminase toolbox, we undertook a search for new candidate enzymes able to accept diamine donors.

As a promising starting point for the development of an enzyme with broader substrate range we identified the putrescine transaminase gene *spuC*, part of the polyamine uptake and utilization pathway in *Pseudomonas aeruginosa* PAO1.¹³ The *spu* (spermidine utilisation) operon, consisting of 9 genes, was previously characterised as responsible for spermidine transport and the catabolic route of putrescine in the arginine decarboxylase pathway. Genes with analogous functions are found in *E. coli* such as the complementary *yjgG* gene encoding for putrescine transaminase that suggests a common pathway between the two microorganisms.¹⁴

To probe the evolutionary relationships between known putrescine transaminases and well-characterised ω -TAs, a multiple sequence alignment was performed, from which a cladogram could be constructed to infer the order of divergence events (Figure 1). Interestingly, it was found that sequences with reported diamine transaminase activity clustered within the ω -ATA sequences as polyphyletic groups. *SpuC* from *P. aeruginosa* (PA-*SpuC*) was revealed to have high sequence identity with the well-studied Cv-ATA from *Chromobacterium violaceum* (55%)^{15,16} and only 24% identity with the more functionally similar *E. coli* *YjgG* protein.¹⁴ This indicated two separate acquisitions of such activity in

this family, with one convergence (Pa-SpuC) occurring relatively recently in evolutionary history compared to the other.

As previous reports with the more ancient putrescine transaminases (Ec-, Bme- and Bmy-YgjG) revealed minimal acceptance of monoamine substrates,¹¹ it is probable that any ancestral ω -TA function of these enzyme has been lost. Due to the higher sequence similarity between the Pa-SpuC and characterised ω -ATA biocatalysts, this enzyme was chosen for investigation of potential latent ω -ATA side-activity. Previous experiments carried out with Pa-SpuC revealed that, although it was likely to be involved in the catabolism of diamines,¹³ minimal activity was observed with putrescine and derived amides.¹⁷ In light of this, a selection of predicted orthologues were mined from publically-available sequence data, cloned and expressed to access the natural diversity of SpuC enzymes with respect to activity and potential monoamine/diamine acceptance.

A Basic Local Alignment Search Tool (BLAST) analysis of the characterised spuC gene from the pathogenic *P. aeruginosa* PAO1 strain revealed other homologous genes from Pseudomonas species, in particular *P. putida* (Pp-spuC), *P. chlororaphis subsp. aureofaciens* (Pc-spuC) and *P. fluorescens* (Pf-spuC) all with an aligned protein sequence identity of 69% (UNIPROT). The three putative spuC genes were cloned from our in-house NCIMB culture collection and subcloned into apET-28b vector followed by overexpression in *E. coli* BL21(DE3) cells. All three enzymes were successfully overproduced and purified as recombinant N-terminal His₆-tagged protein for screening against a representative panel of diamine and monoamine donors.

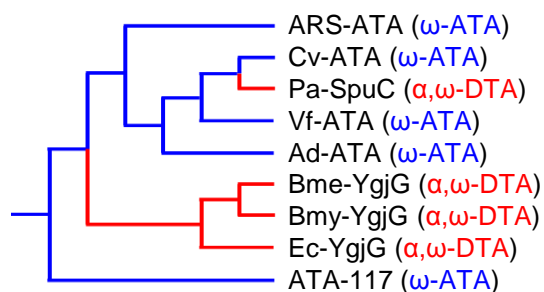


Figure 1. Cladogram showing evolutionary relationships between various wild-type transaminase enzymes as inferred from amino acid sequence identity.

In order to determine the activity of the SpuC enzymes, an adapted version of the previously described L-amino acid dehydrogenase colorimetric assay¹⁸ was employed, using pyruvate as the amino acceptor, L-alanine dehydrogenase from *Bacillus megaterium* to regenerate pyruvate with concomitant reduction of NAD⁺, and the phenazine-tetrazolium system (MPMS+INT) for colour development (Scheme 2). The results for amines **1a-f** are summarised in Table 1 (additional mono- and diamines were tested, and kinetic constants were determined, see Section 3.7.3).

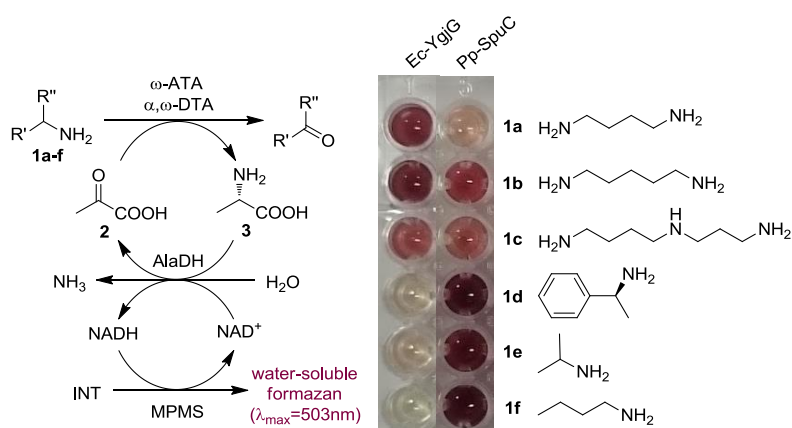


Table 1. Specific activities of the novel SpuC enzymes against amine donors **1a-f**, compared with Ec-YgjG and Cv-ATA.

Subs.	Specific activity (U/mg) ^a				
	Pp-SpuC	Pf-SpuC	Pc-SpuC	Ec-YgjG	Cv-ATA
1a	1.3	0.4	1.4	2.7	n.d.
1b	27.3	16.5	15.1	2.7	7.7
1c	15.7	16.5	21.5	2.1	n.d.
1d	23.2	25.7	30.4	n.d.	28.9
1e	20.4	18.5	18.7	0.05	5.6
1f	16.5	19.3	13.5	n.d.	14.1

Experimental conditions: 5 mM **1a-1f**, 1 mM **2**, 1 mM PLP, 0.2 mg mL⁻¹ purified ATA, 100 mM sodium phosphate buffer, pH 8.0, 30°C. a: one unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of alanine per min at 30°C. n.d.: not determined (too low activity).

Our initial findings showed that all the recombinant SpuC proteins displayed high activity with the biogenic diamine donors **1a-1c** of interest with comparable specific activities within the different *Pseudomonas* species.

Despite a 2-fold increase in activity of Ec-YgjG with putrescine **1a**, Pp-SpuC exhibited a 10-fold greater activity with cadaverine **1b**, Pc-SpuC with spermidine **1c**, with little or no activity displayed with Cv-ATA. Surprisingly, the SpuC enzymes also gave promiscuous activity with industrially relevant monoamines (*S*)-methylbenzylamine **1d**, isopropylamine **1e** and butylamine **1f**, which have been extensively studied with ω -ATAs, but none has been reported to have high activity with biogenic amines. Further studies on the diaminoalkane series was in close agreement with Cv-ATA as the carbon backbone increased in size ($>C_6$), in contrast to the diminished activity presented in Ec-YgjG pATA. This pattern is in part due to the narrow hydrophobic channel approaching the enzyme active site elucidated from recent crystal structures.¹⁹

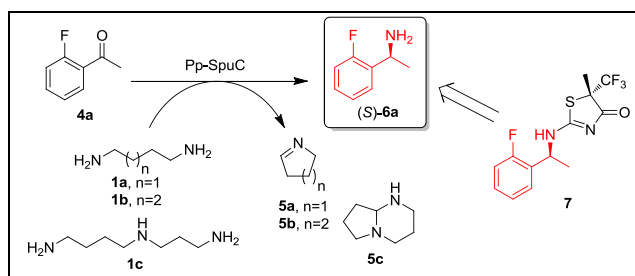
Encouraged by these preliminary studies, we turned to the investigation of non-keto acid prochiral acceptors for preparative applications. As a model keto acceptor for reaction optimisation we chose *o*-fluoroacetophenone **4a**, since the chiral amine (*S*)-**6a** produced after transamination can be coupled to rhodanine scaffolds resulting in heterocycle analogues (*e.g.*, **7**) with a range of pharmacological activities such as the clinical treatment of type II diabetes mellitus.^{20,21}

Biotransformations were conducted using our SpuC enzymes using different amounts of the diamine donors **1a-c**. The best conversions (Table 2) were obtained with Pp-SpuC (that also showed the highest specific activity for **1a-b** before), although the values for Pf-SpuC and Pc-SpuC were very similar.

Remarkably, high conversions were observed at near-stoichiometric amounts of diamine donors **1a-1c**, indicating an efficient displacement of the equilibrium *via in situ* cyclisation, providing a greener substitute than 50 equivalents of volatile isopropylamine.

The transamination of **1a-b** gave ω -aminoaldehydes which underwent spontaneous cyclisation to 1-pyrroline **5a** and 1-piperidine **5b**, respectively (as confirmed by GC-MS). Biotransformations were conducted at pH 9.0 and it was previously shown *via* ¹H NMR that the imine monomers are stable at conditions between pH 7-13.²² Amino donor **1c**, instead, afforded the corresponding amino-imine that spontaneously cyclises a second time to yield the fused bicyclic structure **5c** under biotransformation conditions.

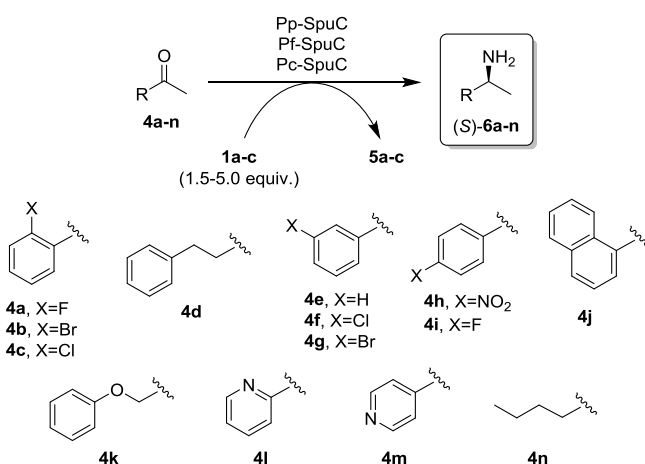
Table 2. Testing of different diamine loadings in the transamination of **4a** mediated by Pp-SpuC.



Diamine equiv.	1a		1b		1c	
	C (%) ^a	ee (%) ^a	C (%) ^a	ee (%) ^a	C (%) ^a	ee (%) ^a
1.5	84	>99	95	>99	83	>99
5	91	>99	96	>99	78	>99
10	87	>99	95	>99	73	>99
25	77	>99	83	>99	63	86

Experimental conditions: 5 mM **4a**, 1 mM PLP, 1% v/v DMSO, 2 mg mL⁻¹ purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h. a: measured by HPLC on a chiral stationary phase. C = conversion. ee = enantiomeric excess.

Interestingly, increasing the concentration of **1c** not only did not improve the conversion to **6a**, it was even detrimental, possibly due to inhibition by the *N*-heterocycle side-product. The optical purity of the product was almost invariably >99% with all three enzymes tested.



Scheme 3. Panel of aromatic and aliphatic ketones tested with SpuC enzymes.

In order to test the feasibility of larger scale processes we also performed a preparative biotransformation with **4a** and 2 equivalents of **1b**, purifying the product by column

chromatography after extraction. The procedure afforded (*S*)-**6a** with an isolated yield of 79% and >99% ee.

Finally, to prove the wide applicability of this procedure, we also tested a panel of prochiral ketones **4a-n** that afford synthetically useful chiral amines of interest for the production of fine chemicals and pharmaceuticals (Scheme 3). Even though the monomeric imines **5a** and **5b** are known to oligomerise in aqueous environment,¹² the corresponding products (as well as the bicyclic product **5c**) are water soluble and do not interfere with the standard work-up. Therefore, the chiral amine products of the transamination **6a-n** can be easily recovered by extraction (together with residual ketone) for HPLC analysis.

Table 3. Amination of ketones **4a-n** with diamine donors **1a-c** (only the best conversion obtained among the three SpuC enzymes is shown).

Substrate	Diamine 1a			Diamine 1b			Diamine 1c		
	SpuC	C (%) ^a	ee (%) ^a	SpuC	C (%) ^a	ee (%) ^a	SpuC	C (%) ^a	ee (%) ^a
4a	Pf	92	>99	Pc	97	>99	Pc	97	>99
4b	Pc	89 ^b	>99	Pc	93	>99	Pc	93	>99
4c	Pf	75	>99	Pc	91	>99	Pc	96	>99
4d	Pf	34 ^b	75	Pc	72 ^b	74	Pp	47	>99
4e	Pf	12 ^b	>99	Pp	42 ^b	>99	Pc	30	>99
4f	Pc	35 ^b	>99	Pc	62 ^b	>99	Pc	74	>99
4g	Pp	63 ^b	>99	Pp	80 ^b	>99	Pc	86	>99
4h	Pc	99 ^b	>99	Pc	93	>99	Pc	99	>99
4i	Pp	12	>99	Pc	35	>99	Pf	14	>99
4j	Pc	37 ^b	>99	Pc	80 ^b	>99	Pc	38	>99
4k	Pp	92	>99	Pc	87	>99	Pf	81	>99
4l	Pp	79	>99	Pp	96	>99	Pf	91	>99
4m	Pp	62 ^b	>99	Pp	95	>99	Pp	55	>99
4n	Pp	99 ^b	92	Pf	99 ^b	>99	Pp	91	95

Experimental conditions: 5 mM **4a-n**, 1.5 equiv. **1a-c**, 1mM PLP, 1% v/v DMSO, 2 mg mL⁻¹ purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h. a: determined by HPLC on a chiral stationary phase. b: in these cases 5. equiv. of amine donor were used to increase the conversion values. C = conversion. ee = enantiomeric excess.

The highest conversions obtained for each substrate among the three SpuC enzymes are listed in Table 3. The (*S*)-enantiomer of the product was formed with all substrate/enzymes pairs, most often with perfect enantioselectivity. Only substrates **4d**, **i**, **k** and **n** afforded in some cases slightly lower ee values (56-96%), however, at least one of the three SpuC enzymes tested provided >99% ee. This emphasises the importance of screening several different members of the family for each application.

The sterically hindered *ortho*-substituted ketone substrates **4a-c** afforded high conversions with high enantioselectivities (>99%). This high conversion is presumably due to a stabilising interaction between the amine formed and the halogen atom at the *ortho*-position, that drives the transamination reaction more readily,²³. Likewise, a similar stabilising effect of the intramolecular *H*-bond with the newly formed amine and vicinal oxygen atom of **4k** has been suggested,²⁴ that would account for the good conversion (41-59%) of this substrate.

For non-activated ketone substrates (**4d-g**, **j** and **n**) the conversions appeared considerably lower, therefore we tested also a slightly higher loading of amine donors **1a-b** (5 equiv.), leading to modest to excellent conversions. Interestingly, however, with spermidine **1c** comparable conversions were reliably obtained at near-stoichiometric amounts (1.5 equiv.) compared to the better amino donor **1b** (5 equiv.). This effect can be rationalised by the higher stability of the bicyclic amine product **5c**.

A few examples of the pharmaceutical relevance of the chiral amine products thus obtained are several *N*-Methyl-D-aspartate (NMDA) glycine-site antagonists used in the treatment neuropathic pain (*i.e.*, Parkinson's disease), synthesised from (*S*)-**6l-m**,²⁵ or the analogues of the antiarrhythmic mexiletine obtained from (*S*)-**6k**.²⁶ All these compounds were obtained in almost quantitative conversion and excellent enantioselectivity (Table 3).

3.6 Conclusions

In summary, we report the identification, heterologous production and characterisation of SpuC orthologues, a class of transaminases that uniquely allow the conversion of numerous ketone substrates at the expense of natural diamines. The enzymes were

found to afford high conversion of a broad range of substrates with a modest excess of amine donor, giving good to excellent enantiomeric excess values for pharmaceutically relevant chiral amines.

The application of these bifunctional SpuC enzymes effectively combines the appealing substrate breadth of traditional ω -ATAs with the equilibrium shifting potential of diamine donor acceptance by α,ω -DTAs. This shift, requiring just 1.5-5.0 equivalents of bio-derived putrescine or cadaverine, represents a clean, 'green' and effective synthetic strategy, as opposed to the widespread use of a 50-fold excess of conventional, often poorly-accepted monoamines. The method also mitigates the need for expensive cofactor supplementation and complex regeneration systems often employed in conjunction.

The approaches developed in this work are particularly relevant to recent advances in metabolic engineering of host strains for enhanced fermentation of putrescine,²⁷ potentially enabling the creation of an integrated microbial cell catalyst for chiral amine synthesis.

3.7 Experimental section

3.7.1 Materials and methods: Commercially available reagents were used throughout without further purification. Putrescine and all other reagents were purchased from Sigma Aldrich (St Louis, MO, USA) or Acros including anhydrous solvents. Restriction enzymes, T4 ligase, *taq* polymerase, dNTPs and broad protein marker (2-212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* DH5 α and BL21 (DE3) cells were purchased from New England Biolabs (Ipswich, MA, USA).

Expression vector pET-28b was purchased from Novagen (Darmstadt, Germany) and was used for gene expression. *Chromobacterium violaceum* (Cv-TA) was purchased from LG standards (ATCC 12472, NCIMB 9178). *Pseudomonas fluorescens* (Asc no. 13500), *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84, (Asc. no 9392), *Pseudomonas putida* NBRC 14161 (Asc no. 9494), and *Bacillus megaterium* (Asc. No 9376) were purchased from the NCIMB culture collection (Aberdeen, UK).

3.7.2 Molecular biology protocols

3.7.2.1 Cloning of *spuC* genes from *Pseudomonas* strains

The coding region of the *spuC* gene was amplified by colony PCR from *Pseudomonas putida* NBRC 14161, *Pseudomonas chlororaphis subsp. aureofaciens* 30-84, and *Pseudomonas fluorescens* using the primers detailed in Table 4.

The *NdeI* and *XhoI* restriction sites are underlined. The following PCR protocol was used: 5 min denaturation at 95 °C and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s elongation at 68 °C with a 5 min final extension time at 68 °C. The PCR product was cloned into ZERO Blunt TOPO PCR cloning vector (K2830-20) following the manufacture's protocol.

Table 4. Primers for cloning the *spuC* gene from *Pseudomonas putida* NBRC 14161, *Pseudomonas chlororaphis subsp. aureofaciens* 30-84, and *Pseudomonas fluorescens*.

Pf- <i>spuC</i>	(5'-CAGCC <u>CATATG</u> ATGACCCGCAATAACCCGCAAACCCGTGAA-3') (5'-CACCTCGAGTTAGCCTTGAACGCACTGAGCGTCAGGTCC-3')
Pp- <i>spuC</i>	(5'- AGTCGAC <u>CATATG</u> AGCACCAACAACCCGCAAACCCG-3') (5'- ATTCTGGCTCGAGCTACCGAATCGCCTCAAGGGTC-3')
Pc- <i>spuC</i>	FW: (5'- GCAAG <u>CATATG</u> AYCAGCAACAAYCCGCAAACCCGTG-3') RV: (5'- GCAAGCTCGAGTTAGCCCTGYAAYGCACTCARS GTC -3')

The alanine dehydrogenase (*Aldh*) gene from *Bacillus megaterium* was amplified using forward (5'-GCAGCCCATATGATTATTGGCGTACCAAAGAAATC-3') and reverse (5'-GCAAGCTCGAGTTAGATAGAAGCTAATTCTTTTTCAAGAG-3') primers (*XhoI/NdeI* restriction sites underlined) and was amplified and cloned into TOPO PCR cloning vector as above.

Pf-*spuC* and *Aldh* genes were subcloned into pET-28b expression vector containing an N-terminal His₆ tag with a thrombin linker. Pc-*spuC* and Pp-*spuC*, was subcloned into pET-22b expression vector containing a C-terminal His tag with a thrombin linker. The inserted genes were in-frame downstream from the ribosome binding site as confirmed *via* DNA sequencing (Eurofins).

Table 5. Sequence-identity matrix heat map of the transaminases used to draw the cladogram in Figure 1.

[%]	ATA-117	ARS-ATA	Ec-YgjG	Bme-YgjG	Bmy-YgjG	AdATA	VfATA	CvATA	PaSpuC
ATA-117	100.0	16.7	17.0	18.1	17.7	16.4	15.0	16.1	15.5
ARS-ATA	16.7	100.0	25.6	27.8	29.2	25.8	22.9	25.7	28.3
Ec-YgjG	17.0	25.6	100.0	61.5	60.0	28.8	25.0	26.7	28.3
Bme-YgjG	18.1	27.8	61.5	100.0	85.8	29.8	26.0	27.9	28.4
Bmy-YgjG	17.7	29.2	60.0	85.8	100.0	30.3	24.6	25.9	28.7
AdATA	16.4	25.8	28.8	29.8	30.3	100.0	28.9	32.8	35.9
VfATA	15.0	22.9	25.0	26.0	24.6	28.9	100.0	37.3	38.3
CvATA	16.1	25.7	26.7	27.9	25.9	32.8	37.3	100.0	59.6
PaSpuC	15.5	28.3	28.3	28.4	28.7	35.9	38.3	59.6	100.0

3.7.2.2 Expression and purification of HisTag recombinant proteins

Protein expression protocols

The *spuC* genes from *Pseudomonas putida* NBRC 1416 and *Pseudomonas chlororaphis* subsp. *aureofaciens* 30-84, were transformed into *E. coli* BL21 (DE3) for yielding *E. coli* BL21 (pET-22b(+)-4-Pp_spuC) and *E. coli* BL21 (pET-22b(+)-4-Pc_spuC). Similarly, the *spuC* gene from *Pseudomonas fluorescens* F113 in pET-28b(+) expression vector and the *ald* gene from *Bacillus megaterium* DSM 319 in pET-22b(+)-4 expression vector were used to yield *E. coli* BL21 (pET-28b(+)-Pf_spuC) and *E. coli* BL21 (pET-22b(+)-4-Bm_AlaDh), respectively.

The freshly-prepared strains were cultivated in 600 mL of the LB medium supplemented with 50 µg/mL antibiotic (ampicillin or kanamycin) in 1-L Erlenmeyer flasks at a rotary shaking rate of 220 rpm at 37°C. The recombinant protein expression was induced by adding isopropyl b-D-1-thiogalactopyranoside (IPTG) (0.2 mM, final) when A₆₀₀ reached 0.6 - 0.8. The cell cultures were incubated at 18 °C for 16 h. The cells were harvested by centrifugation (4°C, 3,250xg, 20 min).

Protein purification

For purification purposes, cell pellets were resuspended (1g of wet cell paste/10 mL) in HEPES buffer (100mM, 1mM PLP, 5mM imidazole, pH 8). The cell pellets were lysed in an

iced bath by ultra-sonication by Soniprep 150 (20 cycles of 20s on/20s off). After centrifugation (4°C, 16,000xg, 20 min) the supernatant was used for protein purification manually or using an AKTA Pure system. Crude extract was then loaded onto a 5 mL HisTrap column. Purification was achieved manually or automated using an AKTA Pure system, using de-gassed HEPES buffer 1 (100 mM, 1mM PLP, 30mM imidazole, pH 8.0) and an elution buffer of HEPES buffer 2 (100 mM, 1mM PLP, 300mM imidazole, pH 8.0).

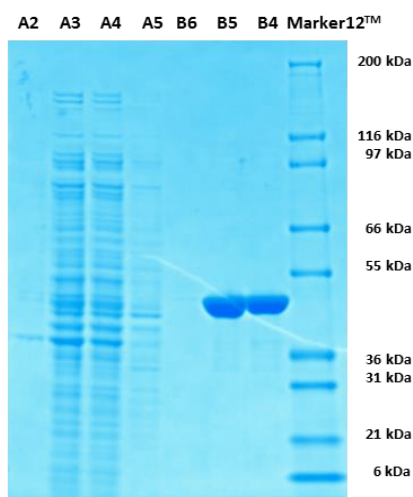


Figure 2. Representative SDS-PAGE analysis for the purification of the recombinant Pp-SpuC protein on AKTA (A2-A5 and B6: flow-through of crude extract during column loading; B5 and B4: fractions of Pp-SpuC protein post HisTag purification; Mark12™ Unstained Standard protein ladder).

The column was initially washed with 5 column volumes HEPES buffer 1. The protein was then eluted with 10 column volumes of 100% elution buffer (50 mM HEPES, 1mM PLP, pH 9.0). 5 mL fractions were collected during the elution phase and a protein gel was run to identify fractions containing the transaminase protein, before these were pooled together. The protein solutions were concentrated in an Amicon® Ultra-15 10K centrifugal filter device and the purified enzyme was flash frozen and stored at -80°C. The purity was analysed by SDS/PAGE and the protein was more than 95% pure.

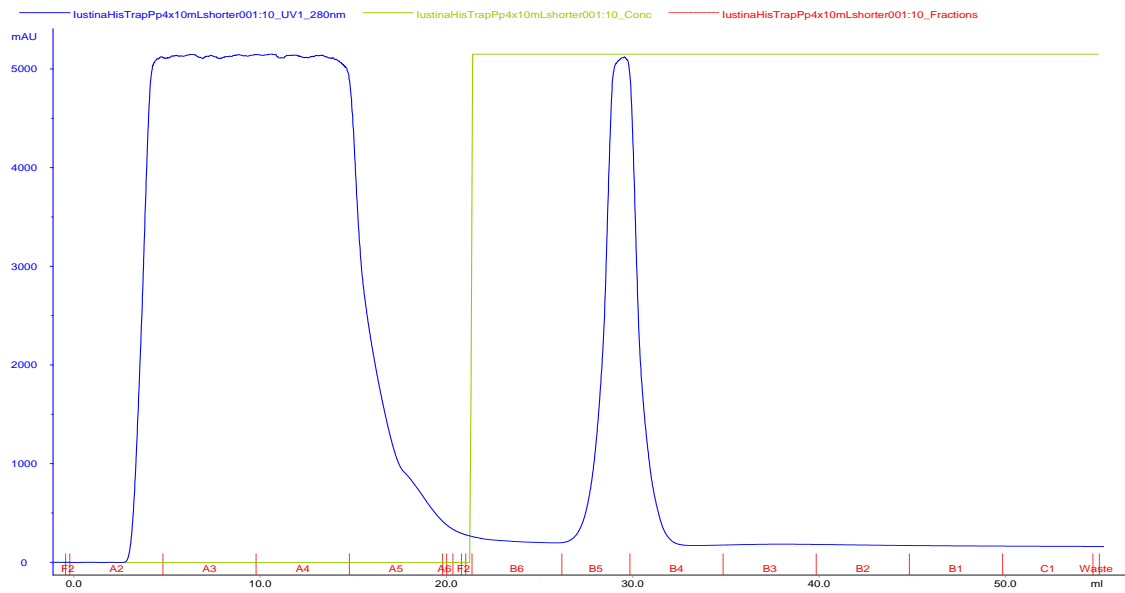


Figure 3. Representative chromatogram for purification of the His₆-tagged Pp-SpuC recombinant protein by metal ion affinity chromatography. Absorbance at 280 nm is shown in blue and the imidazole concentration of the buffer used in green (maximal value: 300 mM). The fractions collected (5mL each) are shown in red.

Protein determination and SDS-PAGE analysis

Concentrations of soluble protein was analysed by SDS-PAGE with 15% resolving gel and 5% stacking gel in a Tris-glycine buffer system. The gel was stained with Bio-Rad Coomassie Blue 250 kit (Bio-Rad lab, Munich, Germany), with bovine serum albumin (BSA) as a standard protein.

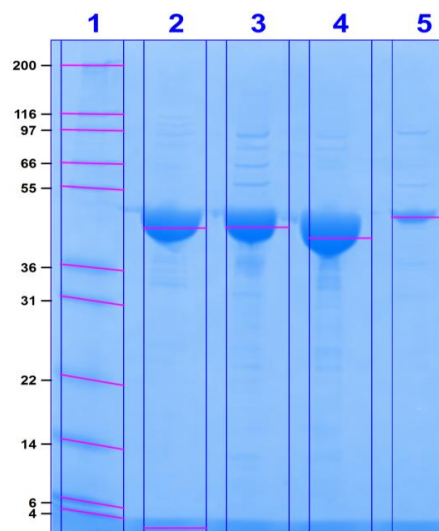


Figure 4. SDS-PAGE analysis of the three *spuC* recombinant proteins and Cv_TA (lane 1: Marker 12 ; lane 2: Pp_spuC; lane 3: Pf_spuC; lane 4: Pc_spuC; lane 5: Cv_TA)

Table 6. Purification of recombinant *Pp_spuC*, *Pf_spuC* and *Pc_spuC* expressed in *E.coli*.

Recombinant protein	Culture medium volume (L)	Cell pellet (g)	Yield pure protein (g)	Purity (%)
Pp_spuC	0.6	5.8	0.200	98
Pf_spuC	0.6	6	0.250	94
Pc_spuC	0.6	5	0.390	95

3.7.3 Spectrophotometric activity analysis and determination of kinetic constants

L-Alanine dehydrogenase (L-AlaDh) activity assay screen

The transaminase activity was determined on a Tecan Sunrise™ plate reader, using a modified alanine dehydrogenase/ transaminase assay method. The spectrophotometric assay monitors the reduction of the tetrazolium salt INT by NADH, using 1-methoxy PMS as catalyst ($\lambda = 503\text{nm}$, $\epsilon = 5286.2\text{ M}^{-1}\text{cm}^{-1}$).

Solution 1 (double-fold concentrated stock) was made up of the following: 2 mM nicotinamide adenine dinucleotide (NAD^+ , 100 mM in water), 0.6 mM 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2H-tetrazolium (INT, 60 mM in DMSO), 0.01 mM 1-methoxy-5-methylphenazinium methyl sulfate (1-methoxy PMS, 1 mM in water), 0.05 mg/mL alanine dehydrogenase from *B. megaterium* (Bm_AlaDh) in 100 mM sodium phosphate buffer pH 8.

The 96-well substrate microplates contained 5 mM amine substrate (double-fold concentrated, in 100 mM sodium phosphate buffer, pH 8) and 0.2 mg/mL transaminase (double-fold concentrated purified enzyme in 100 mM HEPES buffer with 1 mM PLP, pH 8). 100 μL of solution 1 were added to the substrate microplate and the assay was initiated by addition of 2 mM sodium pyruvate (10 mM in water, double-fold concentrated).

Experiments were run on a spectrophotometer at 37 °C with the measured activity at a wavelength of 503 nm and the absorbance taken every 13 seconds for 60 minutes. One Unit (U) is defined by one μmole of product per minute.

Specific activities against substrates **1a-k** are shown in Table 7.

Table 7. Specific activities for substrates **1a-k** determined using the L-AlaDh-TA assay.

substrate	Specific activity U/mg				
	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	1.3	0.4	1.4	2.7	nd
1,5-diaminopentane	27.3	16.5	15.1	2.7	7.7
spermidine	15.7	16.5	21.5	2.1	nd
(S)-methylbenzylamine	23.2	25.7	30.4	nd	28.9
isopropylamine	20.4	18.5	18.7	0.05	5.6
butylamine	16.5	19.3	13.5	nd	14.1
1,6-diaminohexane	14.9	18.1	13.5	0.6	10.9
1,7-diaminoheptane	15.2	24.7	14.7	0.45	18.4
1,8-diaminooctane	7.5	2.7	2.3	0.33	4.4
1,9-diaminononane	13	4.4	3.1	nd	4.6
1,10-diaminodecane	2.1	3.8	1.7	nd	2.3

Determination of kinetic parameters for substrates 1a-k

Kinetic parameters (Tables 8-10) were deduced by non-linear least-square regression analysis based on Michaelis-Menten kinetics.

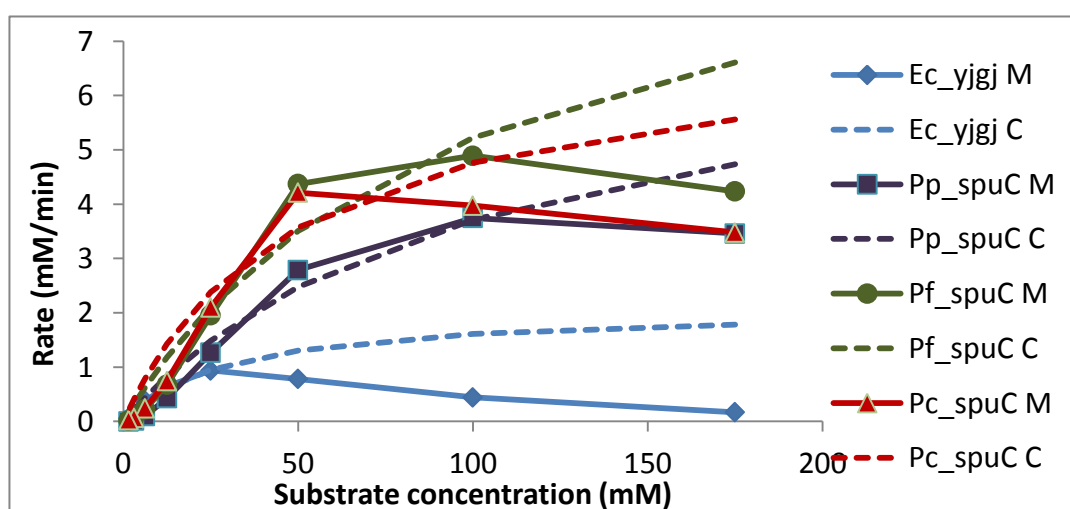


Figure 5. Determination of kinetic constants for Ec_yjgj, Pp_spuC, Pf_spuC and Pc_spuC for spermidine substrate using the L-Aladh/TA liquid phase assay. The measured (M, dotted line) and the calculated (C, solid line) rates of reactions for each protein are plotted against the substrate concentration. The kinetics parameters are determined using non-linear least-squares regression method.

Table 8. Values of Km for substrates **1a-k** determined using the L-AlaDh-TA assay.

substrate	Km (mM)				
	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	62.34	52.99	59.87	0.47	Nd
1,5-diaminopentane	25	32.38	22.99	0.34	48.04
1,6-diaminohexane	1.41	2.31	4.05	2.54	3.92
1,7-diaminoheptane	1.06	1.02	0.64	0.44	1.09
1,8-diaminooctane	0.59	0.39	0.52	0.53	0.73
1,9-diaminononane	3.48	2.13	1.64	nd	2.11
1,10-diaminodecane	0.91	2.14	0.17	nd	0.34
spermidine	20.82	16.95	25.00	29.9	nd
(S)-methylbenzylamine	1.25	4.64	3.81	nd	1.06
butylamine	5.86	6.05	5.86	nd	10.19

Table 9. Values of Vmax for substrates **1a-k** determined using the L-AlaDh-TA assay.

substrate	Vmax (mM/min)				
	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	0.13	0.04	0.14	0.27	nd
1,5-diaminopentane	2.73	1.65	1.51	0.27	0.77
spermidine	1.57	1.65	2.15	2.1	nd
(S)-methylbenzylamine	2.32	2.57	3.04	nd	2.89
butylamine	1.65	1.93	1.35	nd	1.41
1,6-diaminohexane	1.49	1.81	1.35	0.06	1.09
1,7-diaminoheptane	1.52	2.47	1.47	0.05	1.84
1,8-diaminooctane	0.75	0.27	0.23	0.03	0.44
1,9-diaminononane	1.3	0.44	0.31	nd	0.46
1,10-diaminodecane	0.21	0.38	0.17	nd	0.23

Table 10. Values of Kcat for substrates **1a-k** determined using the L-AlaDh-TA assay.

substrate	Kcat (s ⁻¹)				
	Pp_spuC	Pf_spuC	Pc_spuC	Ec_yjgj	Cv_TA
1,4-diaminobutane	1.11	0.34	1.19	2.33	nd
1,5-diaminopentane	23.15	14.38	12.86	2.33	6.68
spermidine	13.32	14.38	18.31	18.17	nd
(S)-methylbenzylamine	19.68	22.41	25.89	nd	25.09
butylamine	13.99	16.82	1.50	nd	12.24
1,6-diaminohexane	12.64	15.77	11.49	0.51	9.46
1,7-diaminoheptane	12.89	21.53	12.51	0.38	15.97
1,8-diaminooctane	6.36	2.35	1.95	0.28	3.82
1,9-diaminononane	11.03	3.83	2.64	nd	3.99
1,10-diaminodecane	1.78	3.31	1.44	nd	1.99

3.7.4 Biotransformations

3.7.4.1 Analytical scale

All biotransformations were carried out in 2 mL Eppendorf tubes, in a volume of 0.5 mL. To a mixture of amine substrate (25 mM from a 500 mM stock in 50 mM HEPES buffer, 1mM PLP, pH 9) and ketone (5 mM from a 500 mM stock solution in DMSO or MeOH), was added the enzyme (2mg/mL, purified or as cell-free lysate, prepared as described). The reactions were placed in a shaking incubator at 30°C and 250 rpm for 12 hours. The reactions were quenched by addition of 10 M NaOH (100µL), followed by extraction with methyl tert-butyl ether (300 µL). The organic phase was dried on MgSO₄ and analysed on normal phase chiral HPLC or GC-FID. For the GC-FID analysis, the samples were derivatized to the corresponding acetamides by addition of 30 µL Et₃N and 20 µL Ac₂O.

3.7.4.2 Preparative scale

In a 250 mL conical flask, the amine (20 mM, 2 eq) was dissolved in 50 mM HEPES buffer, containing 1mM PLP, pH 9 and the transaminase, Pp_spuC (60 mg from a stock of 20 mg/mL in buffer prepared as above) was added. The pH of the mixture was adjusted to 9 and the ketone (10 mM, 1eq) in DMSO (2.5% v/v) was added. The reaction was placed in a shaking incubator, 200 rpm, 30°C for 24h and the progress was followed by HPLC, with samples prepared as described in the analytical scale procedure. Samples were taken every hour for an initial period of 5 hours, followed by sampling at 19h, 21h and 29 h, respectively. The time course of the reaction is shown in Figure 6.

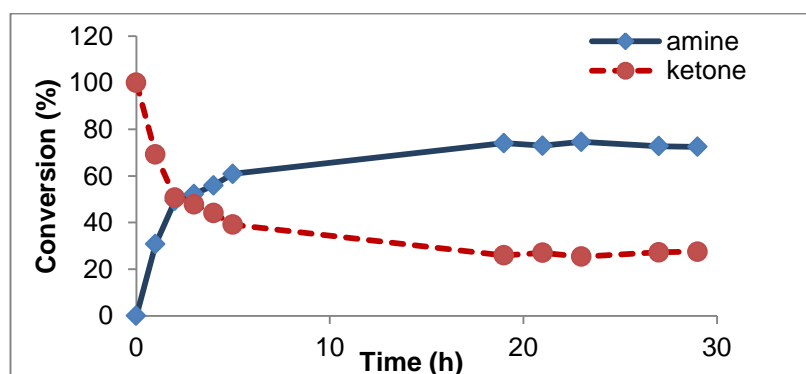
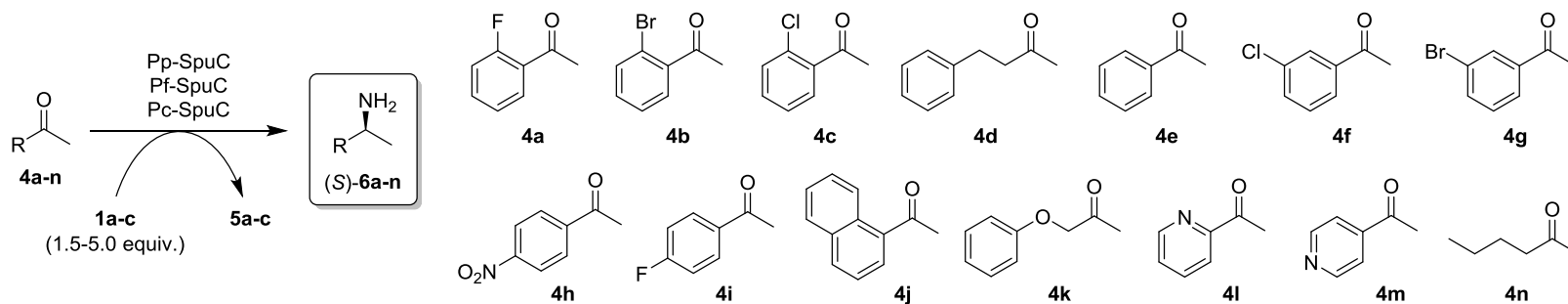


Figure 6. Kinetics of the transamination of 2-fluoroacetophenone (10 mM) by Pp_spuC, using cadaverine **1b** as amino donor (20 mM, 2 eq).

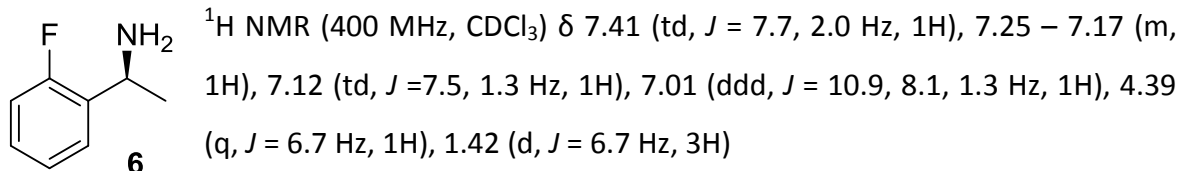
Table 11. Amination of ketones **4a-m** with diamine donors **1a-c**.



Substrate	Diamine 1a						Diamine 1b						Diamine 1c					
	Pp-SpuC		Pf-SpuC		Pc-SpuC		Pp-SpuC		Pf-SpuC		Pc-SpuC		Pp-SpuC		Pf-SpuC		Pc-SpuC	
	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a	c (%) ^a	ee (%) ^a
4a	84	>99	92	>99	92 ^b	>99	95	>99	95	>99	97	>99	83	>99	92	>99	97	>99
4b	80	>99	68	>99	89 ^b	>99	68	>99	65	>99	93	>99	67	>99	65	>99	93	>99
4c	67	>99	75	>99	88 ^b	96	88	>99	83	>99	91	>99	79	>99	81	>99	96	>99
4d	37 ^b	69	34 ^b	75	51 ^b	9	64 ^b	>99	59 ^b	90	72	74	47	>99	41	50	90	36
4e	12 ^b	>99	12 ^b	>99	13 ^b	82	42 ^b	>99	25 ^b	>99	34	>99	13	>99	13	>99	30	>99
4f	5 ^b	>99	33 ^b	>99	35 ^b	>99	16 ^b	>99	44 ^b	>99	62	>99	8	>99	27	>99	74	>99
4g	63 ^b	>99	5 ^b	>99	85 ^b	85	80 ^b	>99	46 ^b	>99	56	>99	62	>99	13	>99	86	>99
4h	41	>99	57	>99	99 ^b	>99	59	>99	63	>99	93	>99	55	>99	63	>99	99	>99
4i	12	>99	11	>99	13 ^b	94	28	80	13	>99	35	>99	13	>99	14	>99	37	93
4j	32 ^b	>99	27 ^b	>99	37 ^b	>99	59 ^b	>99	10 ^b	>99	80	>99	28	>99	12	>99	38	>99
4k	92	>99	91	>99	35 ^b	>99	99	96	98	>99	87	>99	88	56	81	>99	60	>99
4l	79	>99	77	>99	29 ^b	83	96	>99	58	>99	5	>99	65	>99	91	>99	71	>99
4m	58	>99	49	>99	62 ^b	>99	95	>99	73	>99	57	>99	55	>99	11	>99	44	>99

Expt. Cond.: 5 mM **4a-n**, 1.5 equiv. **1a-c**, 1mM PLP, 1% v/v DMSO, 2 mg mL⁻¹ purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h. a: determined by HPLC on a chiral stationary phase. b: in these cases 5. equiv. of amine donor were used to increase the conversion values.

After 30 h, the reaction was stopped by addition of 10 M NaOH until the pH reached 12. The reaction was filtered through a short pad of Celite and extracted with MTBE (3 x 10 mL); the combined organics were washed with brine (1 x 20 mL) and dried on Na₂SO₄ and concentrated in vacuo to afford the amine as a pale yellow oil (55 mg).



3.7.5 Analytical methods

3.7.5.1 GC-assay conditions

Hewlett Packard HP 6890 equipped with a HP-1MS column, a HP 5973 Mass Selective Detector and an ATLAS GL FOCUS sampling robot; method: 90°C for 0 min, then 10°C min⁻¹ to 200°C, hold for 10 min. GC FID method: Agilent 6850 equipped with a Gerstel Multipurposesampler MPS2L and an Agilent CHIRASIL-DEX CB 25 m x 0.25 mm DF=0.25 column; method: 120°C isothermal, 15 min. The amines were analyzed by GC-FID as their corresponding acetamides, following derivatization as described previously.

3.7.5.2 HPLC assay conditions

Chiral normal phase HPLC was performed on an Agilent system (Santa Clara, CA, USA) equipped with a G1379A degasser, G1312A binary pump, a G1367A well plate autosampler unit, a G1316A temperature controlled column compartment and a G1315C diode array detector. CHIRALCEL[®] OD-H Analytical (Daicel, Osaka, Japan), 250 mm length, 4.6 mm diameter, 5 μm particle size) column was used. The typical injection volume was 15 μl and chromatograms were monitored at 265 nm. Solvent mixtures are given in *n*-hexane/isopropanol ratios (+0.1% diethylamine v/v).

Table 12. Retention times for substrates and products

Ketone	Product	Amine	<i>n</i>-Hex/<i>i</i>-PrOH ratio
4a (4.59 min)	6a	(<i>R</i>)- 7.3 min, (<i>S</i>)- 7.81 min	95:5
4b (6.93 min)	6b	(<i>S</i>)- 9.83 min	95:5
4c (5.82 min)	6c	(<i>R</i>)- 7.3 min, (<i>S</i>)-9.07 min	95:5
4d (8.44 min)	6d	(<i>R</i>)- 13.01 min, (<i>S</i>)-18.28 min	95:5
4e (5.93 min)	6e	(<i>S</i>)- 11.09 min	95:5
4f (5.57 min)	6f	(<i>S</i>)- 9.41 mn	95:5
4g (5.81 min)	6g	(<i>S</i>)- 10.07 min	95:5
4h (11.37 min)	6h	(<i>S</i>)- 14.89 min	90:10
4i (5.53 min)	6i	(<i>S</i>)- 8.92 min	95:5
4j (9.05 min)	6j	(<i>S</i>)- 15.57 min	95:5
4k (10.5 min)	6k	(<i>R</i>)- 11.41 min, (<i>S</i>)- 17.87 min	90:10
4l (4.92 min)	6l	(<i>S</i>)- 8.3 min	90:10
4m (4.96 min)	6m	(<i>S</i>)- 11.45 min	90:10

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Chapter 4. *n*-Butylamine as an alternative amine donor for the stereoselective biocatalytic transamination of ketones

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4.1 Foreword

The SpuC transaminases described in Chapter 3 display a broad amine substrate spectrum and can accept both mono- and polyamines as amine donors, offering distinct advantages over the *ygjG* putrescine transaminases described in Chapter 2. This chapter exploits the ability of SpuC to transaminate mono-amines for the conversion of prochiral ketones to their corresponding chiral amines. More specifically, *n*-butylamine, a low-cost and readily available amine donor, is used as substrate for SpuC transaminases, in a tandem co-product (*n*-butanal) removal/cofactor (NAD(P)H) regeneration cascade system.

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4.2 Acknowledgements

The research paper presented here was a collaborative effort between the doctoral candidate and the following researchers: James L. Galman¹, Cesar Iglesias⁴ and Nicholas J. Weise¹ under the supervision of Richard C. Lloyd^{2,3} and Nicholas J. Turner¹. The doctoral candidate co-wrote the manuscript and performed the following experimental work: proteins expression and purification, optimisation of the product removal/cofactor recycling reaction system, analytical scale and preparative scale biotransformations.

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4.3 Abstract

Formal reductive amination has been a main focus of biocatalysis research in recent times. Among the enzymes able to perform this transformation, pyridoxal-5'-phosphate-dependent transaminases have shown the greatest promise in terms of extensive substrate scope and industrial application

Despite concerted research efforts in this area, there exist relatively few options regarding efficient amino donor co-substrates capable of allowing high conversion and atom efficiency with stable enzyme systems. Herein we describe the implementation of the recently described *spuC* gene, coding for a putrescine transaminase, exploiting its unusual amine donor tolerance to allow use of inexpensive and readily-available *n*-butylamine as an alternative to traditional methods. *Via* the integration of *SpuC* homologues with tandem co-product removal and cofactor regeneration enzymes, high conversion could be achieved with just 1.5 equivalents of the amine with products displaying excellent enantiopurity.

4.4 Introduction

Chiral amine motifs are rapidly becoming common in both pharmaceutical and agrochemical industries and almost half of the active pharmaceutical ingredients (APIs) contain at least one chiral centre with a chiral amine unit. Approximately 95% of all drugs are predicted to be chiral by 2020 and share a market of almost \$5bn together with agrochemical, flavour and fragrance industries¹. Special emphasis is now placed on the development of safe and efficient chiral amine synthetic methods.

Complementing the traditional chemical synthetic methods, biocatalysis offers a distinct approach to the synthesis of high-value enantiopure amines, often using small, low-cost achiral starting materials. Biocatalytic routes to chiral amines have evolved significantly during the past decade, from kinetic resolution of amines using lipases, to the successful use of transaminases, imine reductases and ammonia lyases, amongst others².

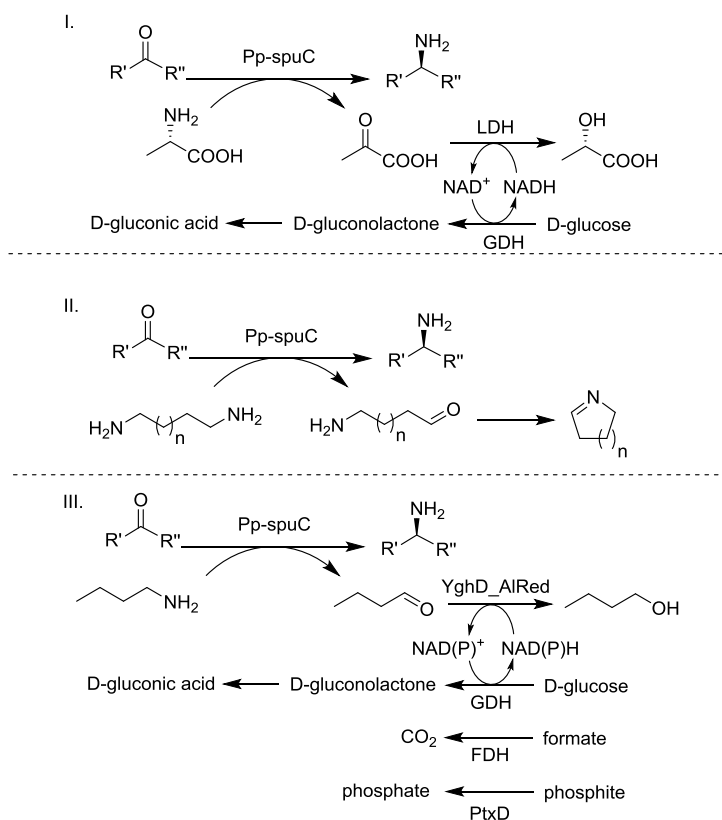
Transaminases (**EC 2.6.1.18**) perform the transfer of an amino group from an amino donor to a prochiral ketone or aldehyde acceptor, employing pyridoxal-5'-phosphate PLP as a cofactor in this process. Asymmetric chiral amine synthesis catalysed by transaminases is a robust and simple method, but it has specific challenges in becoming a green and cost effective approach.

Particular attention has been drawn to overcome problems in the synthesis reaction; in which, transamination of prochiral ketones using alanine as the amino donor is hindered by a poor equilibrium constant and the occurrence of an inhibitory co-product³. In the last decade, several *in situ* pyruvate product removal strategies have been devised to displace the equilibrium, such as coupling the enzymatic reaction with a secondary concomitant irreversible reaction with oxidoreductases that recycles cofactor NAD(P)H.

The most well-known method involves the use of lactate dehydrogenase (LDH) or formate dehydrogenase (FDH) in the presence of excess alanine and a sacrificial reducing agent (formate or glucose) to generate faster reaction rates (Scheme 1, I)^{3,4}. Recently the use of diamine donors has been the focal point to displace the equilibria *via* ring aromatisation/cyclisation (Scheme 1, II)⁵. Although still in its infancy these diamine donors have proven to be successful in the transamination reaction, even though their full potential has yet to be achieved^{5,6}. Other amino donors such as isopropylamine are the preferred industrial choice since the amine is readily available and economically efficient; however, a 50-100 fold amine donor excess is required with further downstream processing required utilising specialised temperature platforms to remove highly soluble volatile coproducts *via* evaporation⁷. The selection of the amine donor is crucial and ideally must satisfy requirements of both atom (near-stoichiometric amounts to be used) and economic efficiency (low cost). In addition, the co-product has to be easy to remove or recycle and should not complicate the main product purification step.

Previous cost effective and environmentally benign strategies were attempted to replace isopropylamine or alanine with *n*-butylamine **1** as amino donor with commercially available transaminases but only negligible product formation was detected. Presumably,

the reactive aldehyde product (butanal **2**) can bind irreversibly to the enzyme affecting the overall catalytic activity⁸. Herein we circumvented the limitations and implemented a novel enzyme cascade that harnesses the high activity of recently published Pp-spuC transaminase in tandem with a well-studied aldehyde reductase YqhD from *E.coli*^{9,10}.



Scheme 1. Summary of transamination equilibrium-driving strategies involving the novel SpuC bifunctional transaminases. Each class of amino donor presents a specific method of co-product recycling/removal. **I. Amino acids**- alanine is used together with the LDH/GDH system for the removal of pyruvate; **II. Short-chain aliphatic diamines**- the spontaneous cyclization of the reactive amino-aldehyde intermediate provides the driving force of the reaction; **III. *n*-Butylamine**- the inhibitory butanal is removed *via* enzymatic reduction coupled with various NAD(P)⁺ recycling systems.

The *yqhD* gene product is described as a scavenger of reactive aldehydes in non-metabolic pathways and has NADPH-dependent reductase activity towards simple aldehydes with a high turnover number for *n*-butanal **2** and was also shown to be effective in the industrial production of isobutanol¹¹. Aldehyde reductase (YqhD) requires

NADPH as cofactor to reduce the aldehyde efficiently; however, an alternative cofactor recycling system was sought after previous experience with glucose dehydrogenase led to some reduction of the ketone substrates.

Our attention was focused on the phosphite dehydrogenase (PtxD) enzyme¹² as part of an NAD(P)⁺ recycling cascade that has rarely been exploited and presents itself with several advantages over the ubiquitous formate dehydrogenase and/or glucose dehydrogenase systems. Such difficulties include low specific activities and high production costs of FDHs^{13,14}, and the generation of acidic byproducts (gluconic or carbonic acid) that require a controlled addition of base not suitable for pH sensitive reactions. PtxD presents an attractive alternative that catalyzes a near irreversible reaction with a thermodynamically favorable equilibrium constant in order to reduce nicotinamide cofactors NAD(P)⁺, with the generation of innocuous phosphate as the byproduct and with negligible effects on pH.

The present work describes the implementation of a novel enzyme cascade transaminase/aldehyde reductase/phosphite dehydrogenase, which explores the use of *n*-butylamine as an alternative and inexpensive amino donor. The toxic co-product butanal is converted to the environmentally benign *n*-butanol by the YqhD aldehyde reductase and phosphite dehydrogenase PtxD is used as part of NAD(P)H recycling system. The novel enzyme system is tested on a diverse panel of ketones substrates and the substitution of NADP⁺ with more the more economically affordable NAD⁺ is also investigated.

4.5 Results and discussion

4.5.1 An enzyme cascade to displace the equilibrium towards chiral amine products

We have recently described and characterized a group of SpuC enzymes from *Pseudomonas* species: *P. putida* (Pp-spuC), *P. chlororaphis* subsp. *Aureofaciens* (Pc-spuC), and *P. fluorescens* (Pf-spuC), which accept a wide range of amine donors and facilitate the reductive amination of ketones using near stoichiometric amounts of diamine donors⁵.

The three SpuC enzymes were purified as recombinant His₆-tagged proteins and screened against a panel of potential mono- and di-amino donors using our modified L-amino acid dehydrogenase colorimetric screen using pyruvate as the amino acceptor¹⁵. Due to the high activity of the newly characterised SpuC enzymes against *n*-butylamine **1**, we directed our investigations to a non-ketoacid prochiral acceptor with potential bioactive motifs.

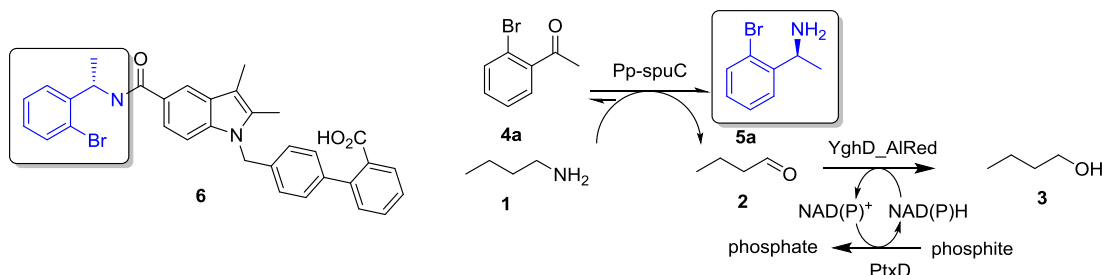
Biotransformations were conducted using Pp-spuC with varying concentration of *n*-butylamine **1**. Not surprisingly low conversions of 10-14% were achieved with increased amine loading suggesting an inhibitory effect on product formation. This problem was addressed by coupling the reaction with an aldehyde reductase YqhD from *E.coli* requiring NADP⁺ for the biotransformation to proceed. In the light of the high costs of the reduced cofactor our attention was drawn to an *in situ* recycling system using NADP⁺ and a phosphite dehydrogenase PtxD from *P. stutzeri*.

The keto acceptor of choice was *o*-bromoacetophenone **4a**, as the chiral amine product **5a** can be coupled to biphenylmethyldole scaffolds (**Table 1**) reported to exhibit antidiabetic properties with the potential to clinically treat type II diabetes **6**¹⁶.

The wildtype PtxD poorly accepts NADP⁺, instead we utilised an engineered variant with relaxed nicotinamide specificity containing point mutations at E175A and A176R that has been reported to have a 3.6-fold and 1000-fold increase in catalytic efficiency for NAD⁺ and NADP⁺ respectively¹⁷.

Remarkably near-stoichiometric amounts of *n*-butylamine **1** with catalytic amounts of NADP⁺ were able to efficiently drive the reaction whilst procuring the chiral amine product (92% conversion, Table 1, entry 5) and producing *n*-butanol **3**, a byproduct which has considerable interests as an alternative fuel to gasoline, due to its high hydrophobicity.

Table 1. Exploring selected conditions for the amination of the ketone **4a**. **Entries 1-4:** increasing concentrations of *n*-butylamine **1**, no butanal removing system; **Entries 5-6:** comparison between NADP⁺ and NAD⁺, when the AIRed/PtxD system is employed; **Entries 7-8:** use of the butanal **2** removing system with commercially available transaminases ATA-117 and ATA -113.



Entry	ATA	AIRed/PtxD NAD(P) ⁺ recycling	Equiv. of 1	Conv. (%)	ee (%)
1	Pp_spuC	No	1.5	23	>99
2	Pp_spuC	No	3	14	>99
3	Pp_spuC	No	5	10	>99
4	Pp_spuC	No	20	10	>99
5	Pp_spuC	Yes – NADP ⁺	1.5	92	>99
6	Pp_spuC	Yes –NAD ⁺	1.5	84	>99
7	ATA-117	Yes –NAD ⁺	1.5	27	>99
8	ATA-113	Yes –NAD ⁺	1.5	55	>99

Experimental conditions: 5mM **4a**, 100mM Na₃PO₃, 1 mM NAD⁺, AIRed 1mg/mL, PtxD 1 mg/mL, TA 2mg/mL, 50 mM HEPES buffer, 1 mM PLP, pH 9, 37°C, 16h

A further cost efficient strategy using NAD⁺ was sought with substrate **4a** afforded a slightly lower conversion to the amine product **5a**, on the grounds of the strong affinity for NADP⁺/NADPH. Nevertheless, the aldehyde reductase *yhqD* can utilize NAD(P)H towards a synthetically viable route for generating chiral amines with high conversions and excellent *ees*. Commercially available aminotransferases ATA-113 and (*R*)-selective ATA-117 from Codexis were tested with the AIRed/PtxD cascade using catalytic amounts of NAD⁺ and gave 55% and 27% conversion respectively. The modest conversions can be

attributed to the poor acceptance of *n*-butylamine as the amino donor, in comparison to Pp-spuC which was shown to have a relaxed donor amine profile.

4.5.2 Biocatalytic conversions of non-ketoacid prochiral acceptor substrates

A second set of experiments employed a panel of prochiral methyl ketones that can generate synthetically useful motifs. Initially, these ketone substrates (Figure 1) were tested against the novel NAD(P)H recycling cascade using Pp-spuC as biocatalyst and a very small excess of *n*-butylamine **1** (1.5eq.) as the amine donor.

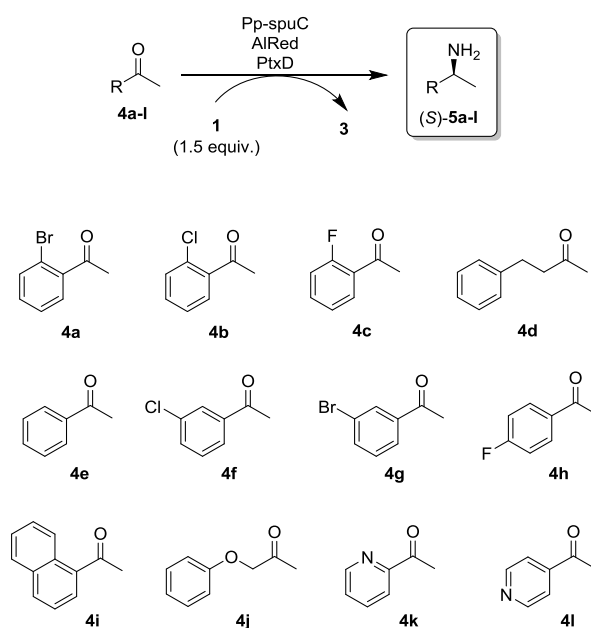


Figure 1. Panel of prochiral ketone substrates employed in this study.

For many of the substrates (**4a-c**, **4f-g**, **4k-l**) negligible variations in the conversions were noticed when using catalytic amounts of NAD^+ as a more economically affordable substitute for NADP^+ , both cascades performing equally well in removing the butanal **2** co-product (Figure 2). The remaining substrates show a noticeably higher conversion with NADP^+ . The system allowed for conversions of over 80% for *ortho*-substituted acetophenones **4a-c**, whilst the *meta*-substituted acetophenones **4f-g** have performed rather modestly, with conversions in the range of 60-70%. However, a decrease of approximately 30% in the conversion values, between the two cascade systems, has been noted when using ketones containing mono-substituted aromatic rings; relatively low

conversions of 20-30% were recorded in this case. Without exceptions, the enantiomeric excess for the amines **5a-l** were consistent between complementary approaches.

Encouraged by these results, we investigated other SpuC proteins from *Pseudomonas* species, Pf-spuC and Pc-spuC. Our previous studies have shown that these enzymes have comparable activity on these classes of substrates; only small differences were observed between the three transaminases. The reactions were performed using exclusively NAD⁺ as cofactor in the ARed/PtxD butanal removing system, as it was found to be an excellent replacement for the costly NADP⁺.

Remarkably, using only a small excess of amino donor *n*-butylamine **1** (1.5 eq.) it was possible to convert activated *ortho*-substituted acetophenones **4a-c** to the corresponding amines **5a-c** in very good conversions (>84%) and excellent *ees* (>99%) (Table 2).

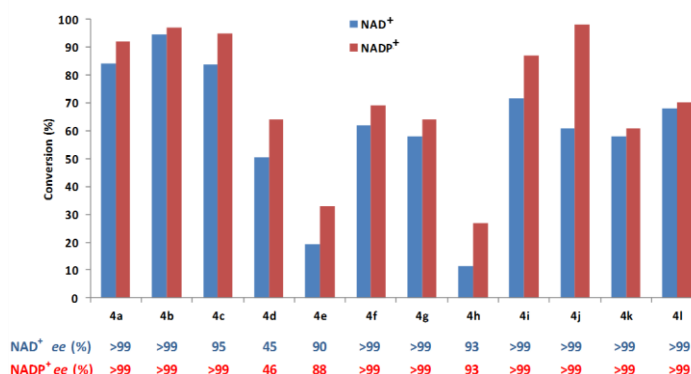


Figure. 2. Comparison of the amination of substrates **4a-j** using *n*-butylamine **1** as amino donor and the Pp-spuC/ARed/PtxD cascade, employing the NADP⁺ cofactor and NAD⁺, respectively.

Furthermore, *n*-butylamine **1** proved to be a better amino donor than previously reported diamine, 1,4-diaminobutane^{5b}, resulting in higher conversion to the majority of the chiral amines **5a-l**. The effect of the arene substitution on the conversion and enantioselectivity of the enzymes is consistent to what was previously described with this class of transaminase biocatalysts^{5b}.

Table 2. Amination of ketones **4a-l** using *n*-butylamine **1** as amino donor

Substrate	Pp-spuC		Pf-spuC		Pc-spuC	
	Conv. (%)	ee (%)	Conv. (%)	ee (%)	Conv. (%)	ee (%)
4a	84	>99	62	>99	76	>99
4b	95	>99	86	96	85	97
4c	84	>99	55	>99	85	>99
4d ^(a)	51	46	30	51	42	23
4e	19	88	11	>99	7	>99
4f	62	>99	24	>99	20	>99
4g	58	>99	19	>99	23	>99
4h	11	93	8	>99	16	>99
4i	72	>99	32	>99	29	>99
4j	61	>99	62	12	87	35
4k	71	>99	31	>99	39	>99
4l	87	>99	57	>99	68	>99

Experimental conditions: 5mM **4a-l**, 7.5mM *n*-butylamine, 100mM Na₃PO₃, 1mM NAD⁺, AlRed 1mg/mL, PtxD 1mg/mL, ATA 2mg/mL, 50mM HEPES buffer, 1mM PLP, pH 9, 37°C, 16h. (a) low *ee* value obtained for this substrate; possibly due to an increased binding flexibility of this ketone, resulting in relaxed stereospecificity.

4.5.3 Time course of the transamination of 2-bromoacetophenone **4a**

The transamination of 2-bromoacetophenone **4a** to the corresponding amine **5a** was performed at time courses over 20-25 h using 1mg/mL of purified Pp-spuC enzyme and using 1.5 equivalents *n*-butylamine **1** donor and catalytic amount of NAD⁺ cofactor (Figure 3). The temperature was maintained at 30 °C and optimum pH 9 during our investigations. The substrate concentration was kept low due to the insolubility of the substrates. In addition, the substrate and product are known to be inhibitions for several omega-transaminases, that share comparable sequence identity. To overcome the solubility issues, other alternatives should be tested, including screening a variety of bio-compatible solvents (methanol, isopropanol, isoamylacetate, toluene, MTBE, etc) and performing the biocatalysed reactions at higher temperatures.

Conversions to the amine product were obtained after 30 minutes initiation of the 3 enzyme cascade. After 5 h the reaction had reached 50% conversion to the amine product

with >99% *ees*. After 25 hours the reaction reached 84% product conversion and did not progress any further, presumably due to the inactivation of the three-enzyme system.

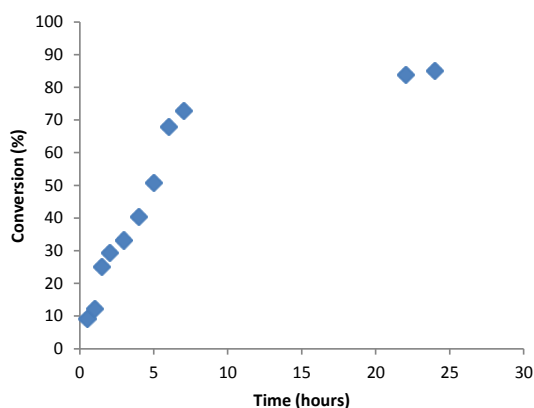


Fig. 3. Kinetics of the transamination of 2-bromoacetophenone **4a** (5 mM) by Pp-spuC, using *n*-butylamine **1** as amino donor (7.5 mM, 1.5 eq) and the AlRed/PtxD cofactor recycling system.

4.6 Conclusions

We have reported the development and demonstration of a three-enzyme cascade enabling the amination of prochiral ketones with near-stoichiometric concentrations of co-substrate. The use of enzymes of the SpuC class of transaminase biocatalysts was shown to enable use of the alternative and otherwise poorly accepted amine donor *n*-butylamine. Issues surrounding the unfavourable equilibrium and reactive coproduct of this transformation were addressed through combination with an efficient aldehyde reductase, driving the reaction to near-completion and giving butanol as a benign side-product. To eliminate enzymatic cross-talk between the reductase and widely-used cofactor recycling systems, such as glucose dehydrogenase, an engineered dual-specificity phosphite-metabolising enzyme was employed with for either NADPH or NADH regeneration. The utility of this strategy was demonstrated for a range of pharmaceutically-relevant chemical transformations, including the production of enantiopure (*S*)-*ortho*-bromophenylethanamine **5a**.

4.7 Experimental

4.7.1 Materials and methods

The ketones (**4a-l**), amines (**5a-l**) and buffers were purchased from Sigma Aldrich (St Louis, MO, USA) or Alfa Aesar (Haverhill, MA, USA) and were used throughout without further purification. The HPLC solvents were purchased from Sigma Aldrich. Restriction enzymes, T4 ligase, *Taq* polymerase, dNTPs and broad protein marker (2–212 kDa) were purchased from New England Biolabs (Ipswich, MA, USA). *Escherichia coli* DH5 α and BL21 (DE3) cells were purchased from New England Biolabs (Ipswich, MA, USA). Phusion polymerase was purchased from Thermo Fisher (Waltham, MA, USA). Expression vector pET-28b was purchased from Novagen (Darmstadt, Germany) and was used for gene expression.

Pseudomonas fluorescens (KX954133), *Pseudomonas chlororaphis* subsp. *aureofaciens*30–84(KX954134), *Pseudomonas putida* NBRC 14161 (T2HES1) were obtained from NCIMB culture collection. The phosphite dehydrogenase (PtxD) gene from *P. stutzeri* was previously subcloned into a pBAD expression vector and was gratefully received from Professor Nigel Scrutton of University of Manchester. Commercially available transaminases ATA-113 and ATA-117 were purchased from Codexis in the form of lyophilised cell extract.

4.7.2 Cloning of the YqhD gene from E. coli K-12

The coding region of the *yqhD* gene (UNIPROT acc. no. Q46856) was amplified using Phusion polymerase *via* colony PCR from *E. coli* K-12 using the oligonucleotide primers as follows: (i) Forward 5'-ATATGGCTAGCATGAACAACCT-TTAATCTGCACACCC-3' and (ii) Reverse complementary 5'- TGGTGCTCGAGTTAGCG-GGCGGCTTCGTATATACGG -3' with *Nhe*I and *Xho*I restriction sites underlined respectively.

The following PCR protocol was used: 5 min denaturation at 95 °C and then 30 cycles of 30 s denaturation at 95 °C, 30 s annealing at 58 °C and 90 s elongation at 68 °C with a 5 min final extension time at 68 °C. The PCR product was cloned into ZERO Blunt TOPO PCR cloning vector (K2830-20) following the manufacture's protocol.

The *yqhD* gene was subcloned into pET-28b expression vector containing a *N*-terminal His₆-tag with a thrombin linker. The inserted gene was in frame and downstream from the ribosome binding site as confirmed *via* DNA sequencing (Eurofins) and gave plasmid pET-28b-yqhD.

The pET-28b-yqhD plasmid was transformed in *E.coli* BL21 (DE3) and grown on 50 µg/mL kanamycin LB agar plates.

4.7.3 Protein expression and purification of yqhD gene

The N-His₆-tag recombinant SpuC proteins Pp-spuC, Pf-spuC and Pc-spuC were expressed and purified as described elsewhere⁵. The freshly prepared pET-28b-yqhD strains were cultivated in 600 mL of LB medium supplemented with 50 µg/mL kanamycin in 1L baffled flask at a rotary shaking rate of 220 rpm at 37 °C.

The recombinant protein expression was induced by adding isopropyl β-d-1-thiogalactopyranoside (IPTG) (0.2 mM, final) when OD₆₀₀ reached 0.6–0.8. The cell cultures were incubated at 18 °C for 16 h and the cell pellets were harvested by centrifugation and lysed by ultra-sonication (Soniprep 150, 20 cycles of 20 s on/20 s off).

The His₆-tag recombinant proteins were purified manually on a Ni-NTA agarose column using 50 mM HEPES, 1 mM PLP, 30 mM imidazole at pH 8.0 and an elution buffer of 50 mM HEPES, 1 mM PLP, 300 mM imidazole at pH 8.0.

The collected fractions were washed twice with 50 mM HEPES containing 1 mM PLP and concentrated on an Amicon® Ultra-15 10 K centrifugal filter device. The purity was analysed by SDS-PAGE with isolated protein more than 95% pure. The purified enzymes were flash-frozen and stored at –80 °C.

4.7.4 Biotransformations using aldehyde reductase (AlRed)/phosphite dehydrogenase (PtxD) cascade

The reductive amination of ketones using the AlRed/PtxD cascade system were carried out in 2 mL Eppendorf tubes in a final volume of 0.5 mL. To a mixture containing 50 mM HEPES buffer and 1 mM PLP was added 7.5 mM *n*-butylamine and 5 mM ketone in DMSO, 1 mM NAD(P)⁺, 100 mM Na₂HPO₃·5H₂O, with Ec-YghD (1 mg/mL), PtxD (1 mg/mL) and 2 mg/mL of purified SpuC enzyme. The reactions were placed in a shaking incubator at 37 °C and 250 rpm for 16 h. The reactions were quenched by addition of 2 M NaOH (50 µL), followed by extraction with methyl *tert*-butyl ether (300 µL). The organic phase was dried on MgSO₄ and analysed on normal phase chiral HPLC, using analytical methods previously described⁵.

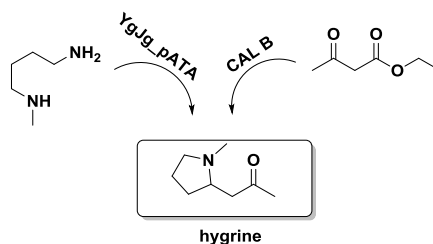
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Chapter 5. Biomimetic synthesis of pyrrolidine and piperidine alkaloids

Iustina Slabu¹, James L. Galman¹, Richard C. Lloyd^{2,3}, Nicholas J. Turner³



5.1 Foreword

One of the main outcomes of the biogenic diamines transamination, performed by the *ygjG* putrescine transaminase (described in Chapter 2), is represented by the synthesis of the reactive cyclic imine intermediates. These reactive imines are essential intermediates in the biosynthesis of a large range of pyrrolidine and piperidine alkaloids, so we envisaged the possibility of incorporating them to a biomimetic cascade for the synthesis of simple hygrine- and pelleterine-type alkaloids from simple 1,4- and 1,5-diamines.

This chapter consists of a manuscript describing a research project into the synthesis of pyrrolidine and piperidine alkaloids. It has been written in preparation for submission for publication, pending additional experimental work.

5.2 Acknowledgements

The doctoral candidate has written the manuscript and has performed all the experimental work described herein, which includes protein expression and isolation; analytical and preparative scale biocatalysed reactions; isolation, purification, analysis and spectral data collection and interpretation for compounds **4-18** and **26**, under the supervision of James L. Galman¹.

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5.3 Abstract

Pyrrolidine and piperidine alkaloids are attractive targets for total synthesis, due to their diverse biological activity. Numerous synthetic strategies have been explored ever since their discovery and biocatalytic methods add to the ever expanding chiral synthetic toolbox. Here we propose a biomimetic synthetic pathway which enables the synthesis of this class of simple alkaloids in good recovered yields, from simple aliphatic diamines and β -keto esters *via* a putrescine transaminase/lipase cascade.

5.4 Introduction

Pyrrolidine and piperidine alkaloids are broadly distributed in nature and constitute a highly diverse class of secondary metabolites. This class of heterocyclic compounds are predominantly found in higher plants (*e.g. Erythroxylaceae, Lobelia, Sedum, Solanacea*, etc.), but also in some microorganisms and animals^{1,2}. Alkaloids and their chemistry have been fascinating topics since ancient times, due to their extraordinary biological activity, making them useful as both drugs and poisons³. They usually incorporate at least one nitrogen atom and their biological activity is based on the ability of the amine group to be protonated at physiological pH. The structure and location of other functional groups in the molecules affect the degree of basicity of these compounds, which enables their isolation and purification.

2-substituted pyrrolidine and piperidine alkaloids (**Figure 1**) display an extensive range of biological activities, such as antibacterial and anticancer effects³, although they are very often eclipsed by the more active tropane alkaloids.

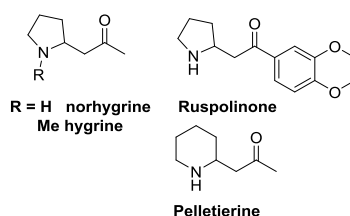


Figure 1. Naturally occurring pyrrolidine and piperidine alkaloids

The high occurrence of this structural motif in a large number of pharmaceutical products makes these compounds remarkably attractive targets for total synthesis.

Alkaloids originate from very distinct biosynthetic pathways and precursors and considerable studies have been carried out for elucidation of these intracellular processes. The piperidine core of these alkaloids is derived from L-lysine, while the non-proteinogenic amino acid L-ornithine is the precursor of the pyrrolidine ring in hygrine-type alkaloids. The L-ornithine is not coded for by DNA and it derives from L-glutamic acid in plants and from L-arginine in animals².

Since their first reported isolations, hygrine⁴, pelletierine⁵ and their analogues have been targeted by sustained efforts for their chemical total synthesis. The most common synthetic approaches involve asymmetric alkylation and subsequent functionalization of N-protected heterocycles⁶, combinatorial Henry-Nef protocol using chiral pool methods^{7,8}, oxidative coupling of cyclic tertiary amines⁹, biomimetic asymmetric Mannich strategies^{10,11,12} and intramolecular aza-Michael addition^{13,14}.

Biocatalysis provides numerous advantages to the traditional chemical synthetic methods, allowing for “greener” processes, catalysed by stable biocatalysts, very often displaying excellent stereo- and regioselectivity. The expanding toolbox of enzymes available for the synthesis of chiral amines opens new synthetic routes and disconnections and enables asymmetric synthesis methods catalysed by ω -transaminases¹⁵, reductive aminases¹⁶ or ammonia lyases¹⁷, while dynamic kinetic resolution processes use monoamine oxidases and hydrolases¹⁸.

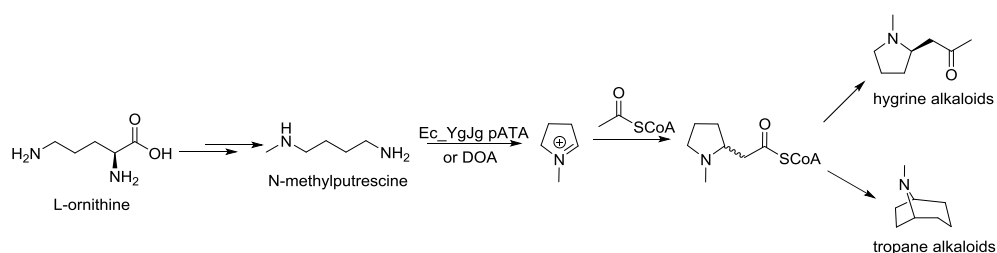
Biocatalytic strategies for the synthesis of these alkaloids rely on biomimetic approaches¹⁹, using 1,4-diaminobutane (putrescine) or 1,5-diaminopentane (cadaverine) as substrates to be oxidised by pea-seedling diamine oxidase, followed by a Mannich addition and subsequent decarboxylation with β -keto acids²⁰. Recently, diastereomerically pure 2,6-disubstituted analogues of pelletierine have been synthesised *via* an ω -transaminase mediated aza-Michael reaction, in an one-pot reaction from diketone precursors²¹.

Herein we describe our approach to the synthesis of hygrine- and pelletierine-type alkaloids, which mimics the natural biosynthetic pathways, by assembling a putrescine transaminase/ lipase cascade and using simple diamines and β -keto esters as substrate precursors. Furthermore, we show the biocatalysed preparation of these compounds on a preparative scale, which allows isolation, purification and characterisation of several members of this class of compounds.

5.5 Results and discussion

We decided to apply the principles of biocatalytic retrosynthesis²² to this type of compounds. Biocatalysis offers an unequalled advantage in enabling synthetic disconnections that are not immediately obvious or possible in traditional organic synthesis.

Acting as a connection between the primary and secondary metabolism, putrescine is an essential precursor of structurally diverse alkaloid classes, including pyrrolidine, pyrrolizidine and tropane alkaloids¹. Following the *N*-methylation process catalysed by the SAM-dependent putrescine *N*-methyltransferase, a subsequent transamination process²³ or oxidative deamination²⁰ yield the reactive 4-methyl-aminobutanal, which spontaneously cyclizes to form an *N*-methyl- Δ^1 -pyrrolinium cation. This reactive intermediate is then subjected to a series of intra- and intermolecular Mannich reactions and Claisen condensations with the enolate of acetyl coenzyme A, resulting in a rich class of pyrrolidine and tropane alkaloids². (Scheme 1)

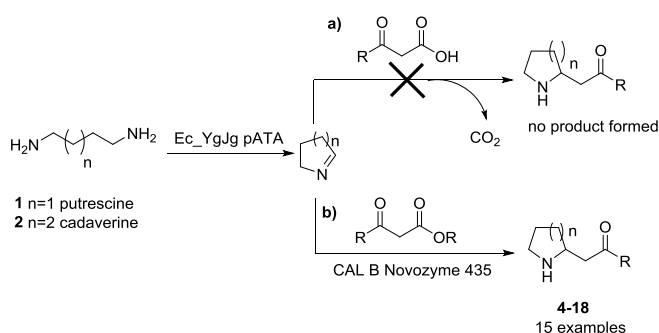


Scheme 1. Biosynthetic pathway to pyrrolidine alkaloids

By studying the natural biosynthetic pathway of ornithine and lysine-derived alkaloids, a biomimetic cascade was proposed, in which the heterocyclic core can be accessed from simple aliphatic diamines, while the ketone moiety is donated by a β -keto acid.

The aliphatic diamines, 1,4-diaminobutane and 1,5-diaminopentane precursors were subjected to a transamination reaction catalysed by the putrescine transaminase Ec_ygjG pATA²³, using pyruvate as an amino acceptor. A subsequent Mannich reaction with a β -keto acid should yield an unstable adduct (1), which would spontaneously undergo a decarboxylation process, resulting in the formation of the desired alkaloid (Scheme 2a)

Our attempts at performing this reaction using the Ec_ygjG pATA and the β -keto acid were unsuccessful; the alkaloid product was not observed by common analytical methods. We decided to investigate β -keto esters precursors as more stable alternative to the β -keto acids. The β -keto acid could be synthesized *in situ via* a hydrolysis process catalysed by lipases (Scheme 2b).



Scheme 2. Transaminase/lipase cascade for the synthesis of pyrrolidine and piperidine alkaloids

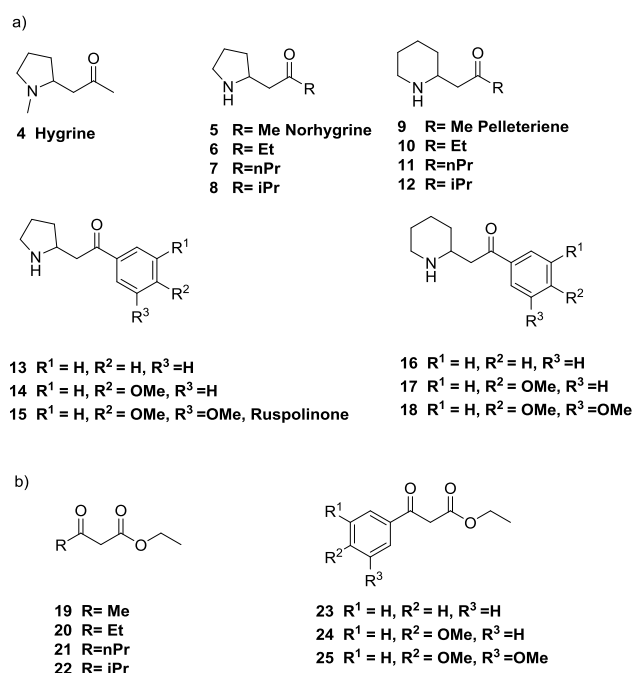
The synthesis of the 1-pyrroline or 1-piperidine scaffolds was achieved by the transamination reaction from putrescine and cadaverine, respectively. We chose the Ec_ygjG pATA as biocatalyst for this reaction because of its strict preference for small linear diamines and pyruvate. Although the Spuc transaminases are more active and display a larger substrate spectrum¹⁵, both for amines and ketones, they would lead to unwanted side products by potential transamination of the β -keto esters or β -keto acids, thus lowering the efficiency of the reaction. In contrast, the Ec_ygjG pATA have shown no

effect on these types of compounds. This consideration is important as the two-step cascade was designed as one-pot cascade with simultaneous addition of all the reagents. Sequential addition was avoided because of the tendency of the reactive imine intermediates to polymerize to complex and stable structures²³ in aqueous media.

A series of β -keto acids were chosen for this cascade, based on the most frequent keto moieties found in this type of alkaloids (Figure 2b). Due to their instability, they were accessed from their β -keto esters precursors *via* lipase-catalysed hydrolysis. The CAL B lipase (Novozyme 435) was selected for this cascade, as it proved to accept very well all the β -keto esters as substrates. To enable the isolation, purification and complete characterisation of the products, all the reactions were performed on a preparative scale.

A total of 14 alkaloids, both natural products and analogues, were prepared in good to excellent yields using this method (Figure 2a).

Figure 2. a) Pyrrolidine and piperidine alkaloids and analogues synthesised using the transaminase/lipase cascade; b) panel of beta-keto esters used in this study

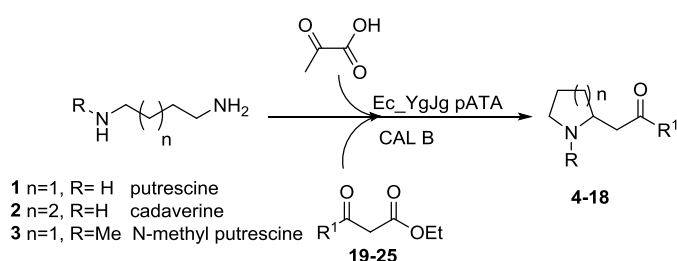


Following an acid-base work up, the compounds were obtained in sufficient purity.(see supporting information for full spectral details) (Table 1). Interestingly, pelletierine **9**

proved difficult to obtain in sufficient purity and further attempts to purify it lead to significant losses of product. Racemic hygrine **4** was obtained using a comparable synthetic strategy, from the commercially available *N*-methyl putrescine **3**.

Without exception, the compounds were isolated as racemates, so a deracemization method was sought, by involving the well-established monoamine oxidase MAO¹⁸ or 6-hydroxy-D-nicotine oxidase HDNO²⁴ dynamic kinetic resolution protocol. The deracemization method proved to be unsuccessful, only compounds **14**, **15**, **17** and **18** gave a very modest enantiomeric excess (< 10 %).

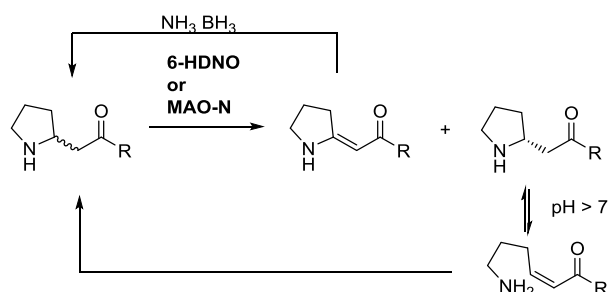
Table 1. Substrate scope of the Ec_ygjG pATA/lipase cascade. Synthesis of 2-substituted pyrrolidine and piperidine alkaloids



Product	Diamine	β -keto ester	Recovered yield %
4	3	15	75 %
5	1	15	50%
6	1	20	50 %
7	1	21	78 %
8	1	22	74 %
9	2	15	N/A
10	2	20	55 %
11	2	21	59 %
12	2	22	67 %
13	1	23	64 %
14	1	24	81 %
15	1	25	71 %
16	2	23	60 %
17	2	24	73 %
18	2	25	69 %

Reaction conditions: 25 mM diamine **1-3**; 25 mM β -keto ester **19-25**; 40 mM pyruvate; 5 mg/mL Ec_ygjG pATA (fresh cell-free lysate), 5 mg/mL CAL B Novozyme 435, 50 mM HEPES buffer, 1mM PLP, pH 8, 37 °C, 18 h.

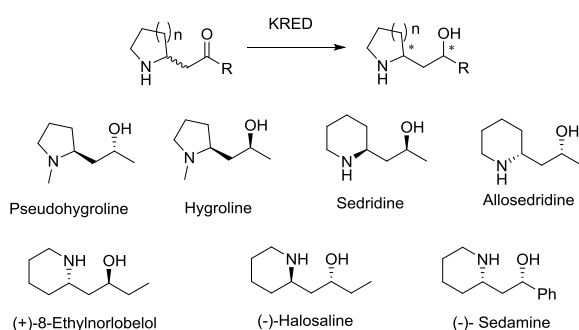
This result was not completely unexpected, as it is known that β -amino ketones have the tendency to racemise easily at basic pH, with hygrine **4** and pelletierine **9** being known to racemise²⁵ *via* a retro aza-Michael reaction (Scheme 3).



Scheme 3. Deracemization approaches using monoamine oxidase MAO-N or 6-hydroxy-D-nicotine oxidase HDNO. Also shown the alkaloid racemisation mechanism *via* retro aza-Michael reaction

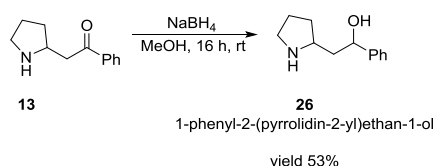
One method to avoid the racemisation at C4 is to protect the amino group of these compounds; however, this would be only a transient solution, as racemisation would occur again following deprotection.

We decided to extend the product scope by converting these alkaloids to their corresponding β -amino alcohols, *via* a biocatalytic reduction of the keto group. This would generate diastereomerically pure β -amino alcohol analogues, which constitute another rich class of natural products, the *Sedum* alkaloids²⁶ (Scheme 4). In addition, the β -amino alcohols will not undergo racemisation as above and it should be possible to obtain diastereomerically pure compounds, *via* a dynamic kinetic resolution process.



Scheme 4. Proposed biocatalytic reduction of the ketone group to yield *Sedum* alkaloids. Representative examples of *Sedum* alkaloids also shown.

For this purpose, racemic amino alcohol alkaloid 1-phenyl-2-(pyrrolidin-2-yl)ethan-1-ol **26** was prepared by chemical reduction of alkaloid **13** (**Scheme 5**). The racemic alkaloid **26** thus obtained was used in the screening of a panel of keto reductases from ProZomix (kREDy-to-go plates). Several positive hits were identified, showing various degrees of activity (**Figure 3**); however, due to shortness of time, this process could not be investigated further.



Scheme 5. Synthesis of racemic alkaloid **26** *via* chemical reduction



Figure 3. Screening of kREDy-to-go plates using racemic sedamine **26** as substrate. Positive hits are identified in red colour.

The biocatalytic reduction of these alkaloids will be investigated in the future and this step will be added to the previously developed transaminase/lipase cascade for the synthesis of γ -amino alcohols with interesting biological activities.

5.6 Conclusions

2-substituted pyrrolidine and piperidine alkaloids are a rich class of natural products which constitute attractive targets for total synthesis. We proposed and developed a transaminase/lipase cascade for the synthesis of this type of compounds starting from low-value, achiral materials. The resulting alkaloids were obtained as racemates and further deracemization methods proved unsuccessful due to their tendency to racemise at basic pH. An alternative was also suggested, by including an extra step in the cascade, a

biocatalytic reduction of the ketone group, which enable the formation of high-value β -amino alcohol alkaloids.

5.7 Experimental section

5.7.1 Materials and methods

Commercially available reagents were used throughout without further purification. Putrescine, cadaverine and the β -keto esters were purchased from Sigma Aldrich (St Louis, MO, USA), Alfa Aesar or Fluorochem. Spectra from ^1H and ^{13}C NMR were recorded on a Bruker Advance 400 instrument (400 MHz for ^1H and 100 MHz for ^{13}C) in CDCl_3 , using residual protic solvent as internal standard. Chemical shifts (δ) are reported in parts per million (ppm) and they are relative to the residual protic solvent signal (CHCl_3 in CDCl_3 , $^1\text{H}= 7.26$; $^{13}\text{C}= 77.0$).

High-resolution mass spectrometry (HRMS) was recorded using a Waters LCT time-of-flight mass spectrometer, connected to a Waters Alliance LC (Waters, Milford, MA, USA), with data processed using Waters Masslynx software.

Chiral normal phase HPLC was performed on an Agilent system (Santa Clara, CA, USA) using a CHIRALCEL[®]OD-H Analytical (Daicel, Osaka, Japan), 250 mm length, 4.6 mm diameter, 5 μm particle size.

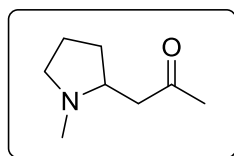
5.7.2 General method for the synthesis of alkaloids on preparative scale

The diamine dihydrochloride (25 mM) and pyruvate (40 mM) were dissolved in 30 mL buffer (50 mM HEPES, 1 mM PLP, pH 9). The β -keto ester (25 mM) was dissolved in 5 mL MeOH (10% v/v) and added to the mixture and the pH was adjusted to 9. Lipase Novozyme 435 (5 mg/mL) and transaminase clarified cell lysate (15 mL from 1.5 g of fresh cell pellet) were added and the reaction was incubated at 37 °C, 250 rpm for 24 hours. The reaction mixture was filtered through Celite and the β -keto amines were isolated *via* acid-base work up (pH of solution adjusted to 2 with HCl 1 M, wash with Et_2O 2 x 10 mL,

adjust pH to 10 with NaOH 1 M, extract with DCM 3 x 20 mL, dry on MgSO₄ and concentrate in vacuo to afford alkaloids as oils or powders.

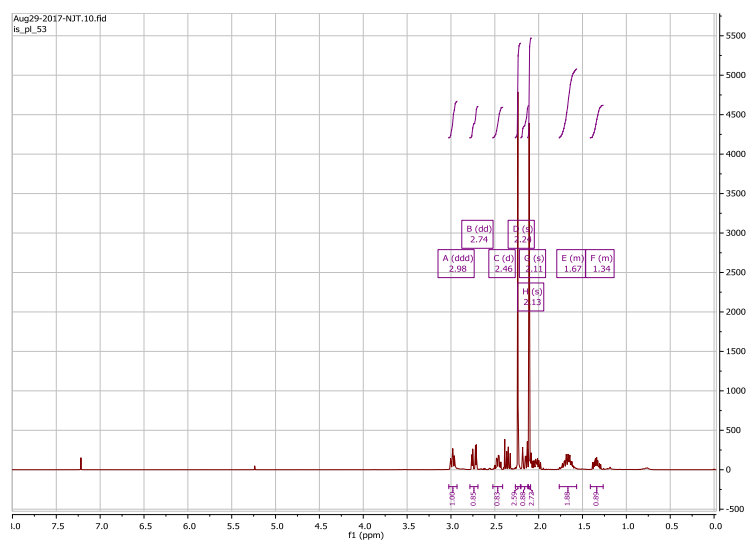
5.7.3 Characterisation of compounds 4-18

1-(1-methylpyrrolidin-2-yl)propan-2-one, hygrine, **4**

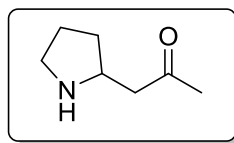


Following the general procedure for the pATA/lipase cascade, racemic hygrine **4** was obtained as pale yellow oil, 102 mg, 75 % yield.

¹H NMR (400 MHz, Chloroform-*d*) δ 2.98 (ddd, *J* = 9.7, 7.5, 2.6 Hz, 1H), 2.74 (dd, *J* = 16.1, 3.8 Hz, 1H), 2.46 (d, *J* = 3.9 Hz, 1H), 2.24 (s, 3H), 2.13 (s, 1H), 2.11 (s, 3H), 1.77 – 1.57 (m, 2H), 1.41 – 1.26 (m, 1H). HRMS calcd. for C₈H₁₅N⁺O 142.1232 [M+H]⁺, found 142.12270.

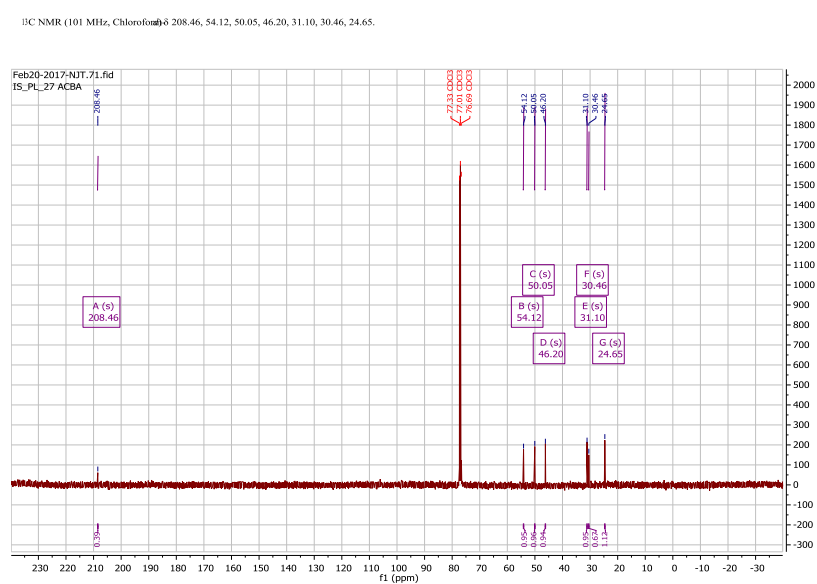
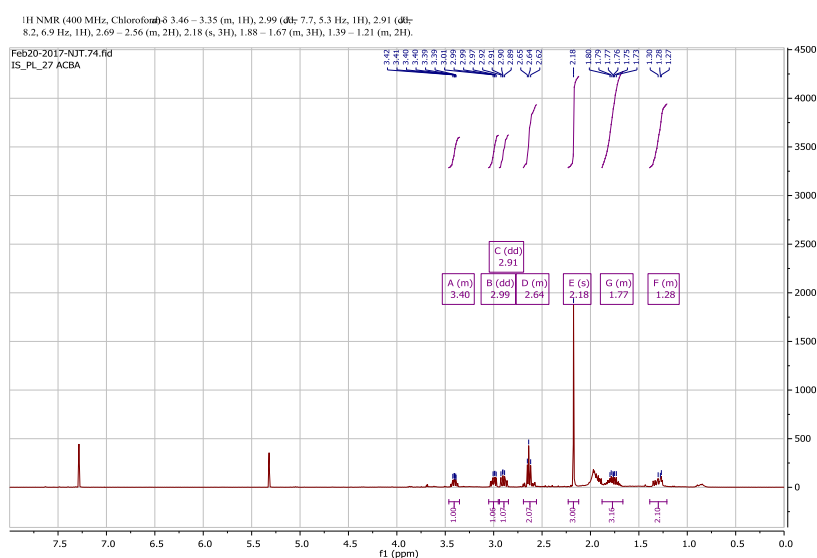


1-(pyrrolidin-2-yl)propan-2-one, 5

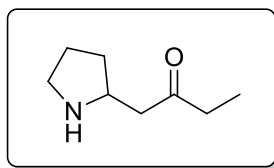


Following the general procedure for the pATA/lipase cascade, compound **5** was obtained as yellow oil, 65 mg, 50 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 3.46 – 3.35 (m, 1H), 2.99 (dd, $J = 7.7, 5.3$ Hz, 1H), 2.91 (dd, $J = 8.2, 6.9$ Hz, 1H), 2.69 – 2.56 (m, 2H), 2.18 (s, 3H), 1.88 – 1.67 (m, 3H), 1.39 – 1.21 (m, 2H). $^{13}\text{C NMR}$ (100 MHz, Chloroform-*d*) δ 208.46, 54.12, 50.05, 46.20, 31.10, 30.46, 24.65. HRMS calcd. for $\text{C}_7\text{H}_{14}\text{N}^+\text{O}$ 128.1075 $[\text{M}+\text{H}]^+$, found 128.1057.

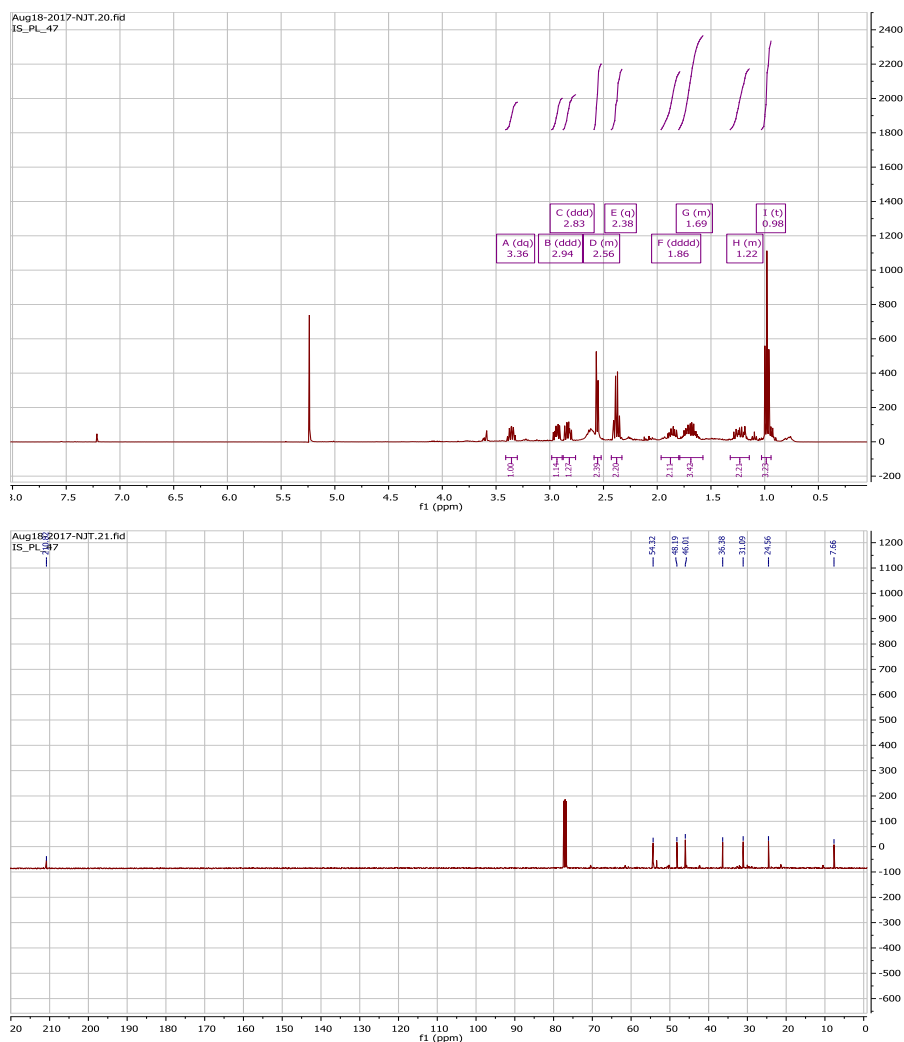


1-(pyrrolidin-2-yl)butan-2-one, **6**

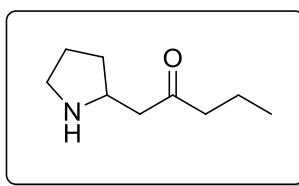


Following the general procedure for the pATA/lipase cascade, compound **6** was obtained as pale yellow oil, 89 mg, 50 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 3.36 (dq, $J = 8.5, 6.7$ Hz, 1H), 2.94 (ddd, $J = 10.2, 7.7, 5.4$ Hz, 1H), 2.83 (ddd, $J = 10.3, 8.2, 6.8$ Hz, 1H), 2.59 – 2.52 (m, 2H), 2.38 (q, $J = 7.3$ Hz, 2H), 1.86 (dddd, $J = 11.6, 8.3, 6.8, 4.5$ Hz, 2H), 1.80 – 1.57 (m, 3H), 1.32 – 1.14 (m, 2H), 0.98 (t, $J = 7.3$ Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 210.82, 54.32, 48.19, 46.01, 36.38, 31.09, 24.56, 7.66. **HRMS** calcd. for $\text{C}_8\text{H}_{16}\text{N}^+\text{O}$ 142.1232 $[\text{M}+\text{H}]^+$, found 142.1223.

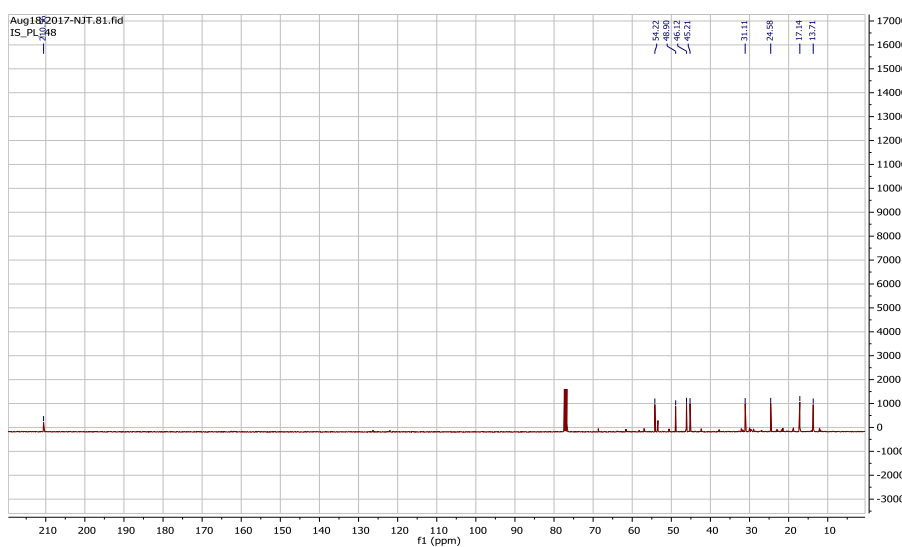
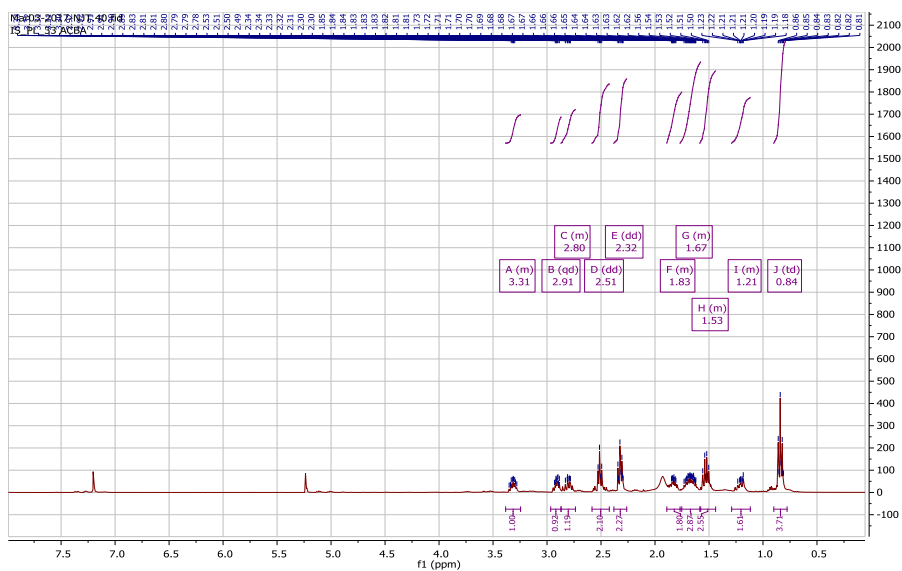


1-(pyrrolidin-2-yl)pentan-2-one, **7**

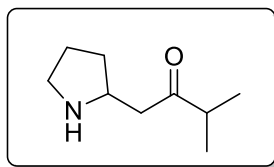


Following the general procedure for the pATA/lipase cascade, compound **7** was obtained as yellow oil, 153 mg, 78 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 3.34 (dtd, $J = 8.6, 7.1, 5.7$ Hz, 1H), 2.93 (ddd, $J = 10.1, 7.7, 5.3$ Hz, 1H), 2.82 (ddd, $J = 10.1, 8.3, 6.8$ Hz, 1H), 2.54 (dd, $J = 6.5, 1.7$ Hz, 2H), 2.33 (t, $J = 7.4$ Hz, 2H), 1.91 – 1.78 (m, 1H), 1.78 – 1.59 (m, 3H), 1.53 (q, $J = 7.4$ Hz, 3H), 0.84 (t, $J = 7.4$ Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 210.56, 54.22, 48.90, 46.12, 45.21, 31.11, 24.58, 17.14, 13.71. **HRMS** calcd. for $\text{C}_9\text{H}_{18}\text{N}^+\text{O}$ 156.1388 $[\text{M}+\text{H}]^+$, found 156.1380.

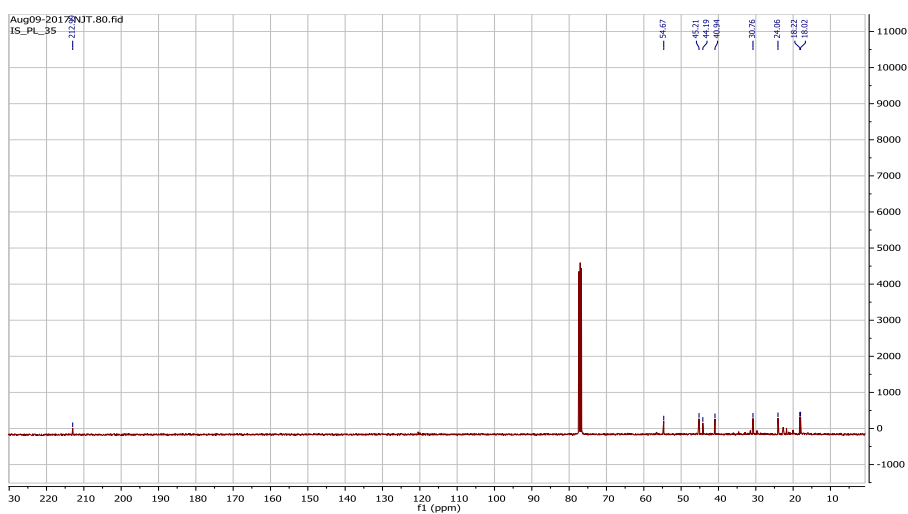
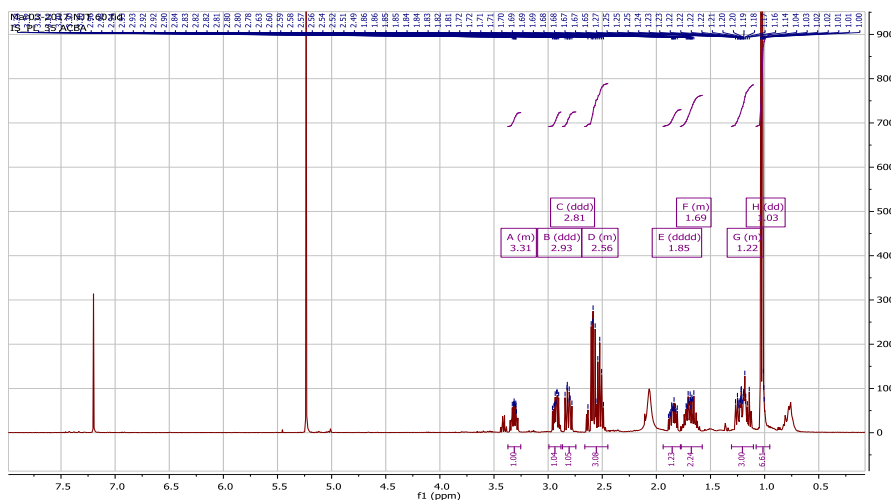


3-methyl-1-(pyrrolidin-2-yl)butan-2-one, **8**

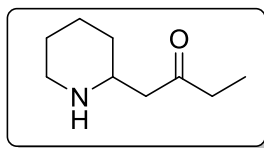


Following the general procedure for the pATA/lipase cascade, compound **8** was obtained as yellow oil, 89 mg, 50 % yield.

^1H NMR (400 MHz, Chloroform-*d*) δ 3.37 – 3.25 (m, 1H), 2.93 (ddd, $J = 10.0, 7.7, 5.1$ Hz, 1H), 2.81 (ddd, $J = 10.1, 8.3, 7.0$ Hz, 1H), 2.66 – 2.45 (m, 3H), 1.85 (dddd, $J = 11.8, 8.8, 6.9, 4.6$ Hz, 1H), 1.78 – 1.58 (m, 2H), 1.31 – 1.10 (m, 3H), 1.03 (dd, $J = 6.9, 1.1$ Hz, 7H). ^{13}C NMR (101 MHz, CDCl_3) δ 212.99, 54.67, 45.21, 44.19, 40.94, 30.76, 24.06, 18.22, 18.02. HRMS calcd. for $\text{C}_9\text{H}_{18}\text{N}^+\text{O}$ 156.1388 $[\text{M}+\text{H}]^+$, found 156.1376.

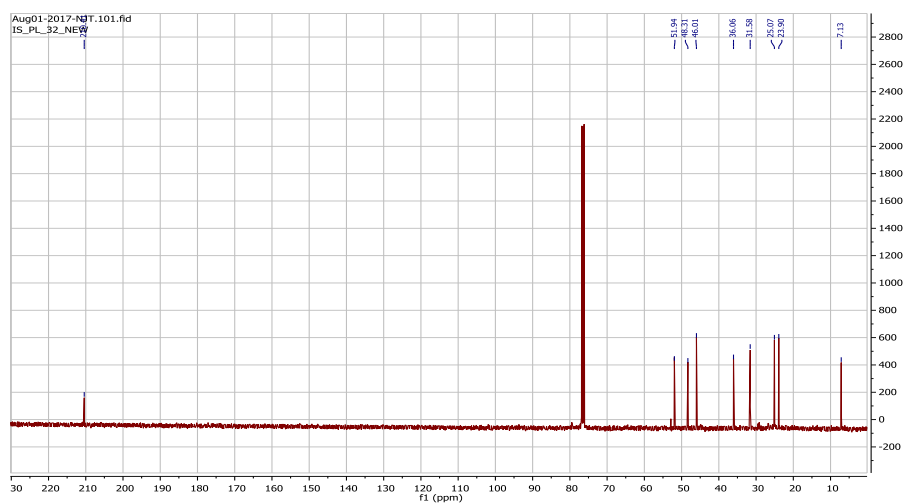
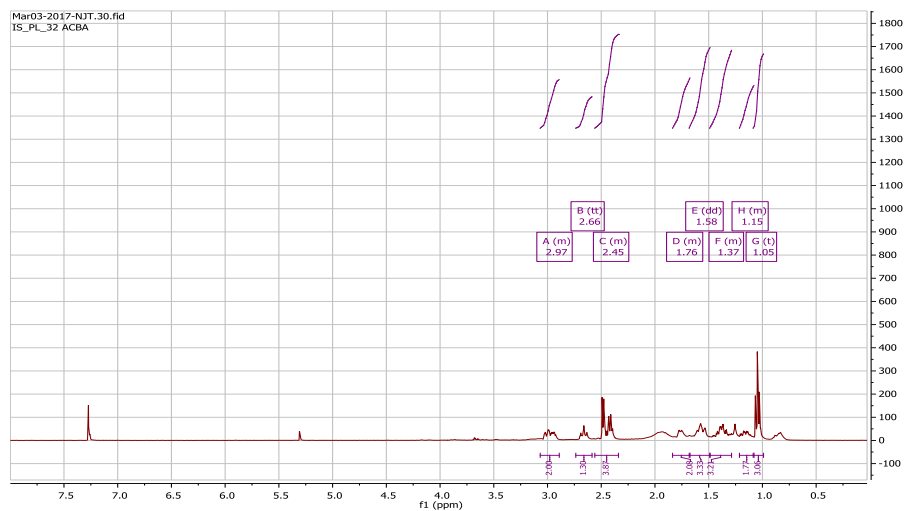


1-(piperidin-2-yl)butan-2-one, **10**

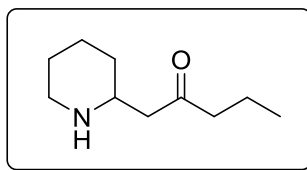


Following the general procedure for the pATA/lipase cascade, compound **10** was obtained as pale yellow oil, 107 mg, 55 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform- d) δ 3.08 – 2.90 (m, 2H), 2.68 (tt, $J = 10.0, 2.9$ Hz, 1H), 2.57 – 2.35 (m, 4H), 1.85 – 1.69 (m, 2H), 1.59 (dd, $J = 4.5, 2.3$ Hz, 3H), 1.50 – 1.30 (m, 3H), 1.23 – 1.09 (m, 2H), 1.06 (t, $J = 7.3$ Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 210.41, 51.94, 48.31, 46.01, 36.06, 31.58, 25.07, 23.90, 7.13. **HRMS** calcd. for $\text{C}_9\text{H}_{18}\text{N}^+\text{O}$ 156.1388 $[\text{M}+\text{H}]^+$, found 156.1380.

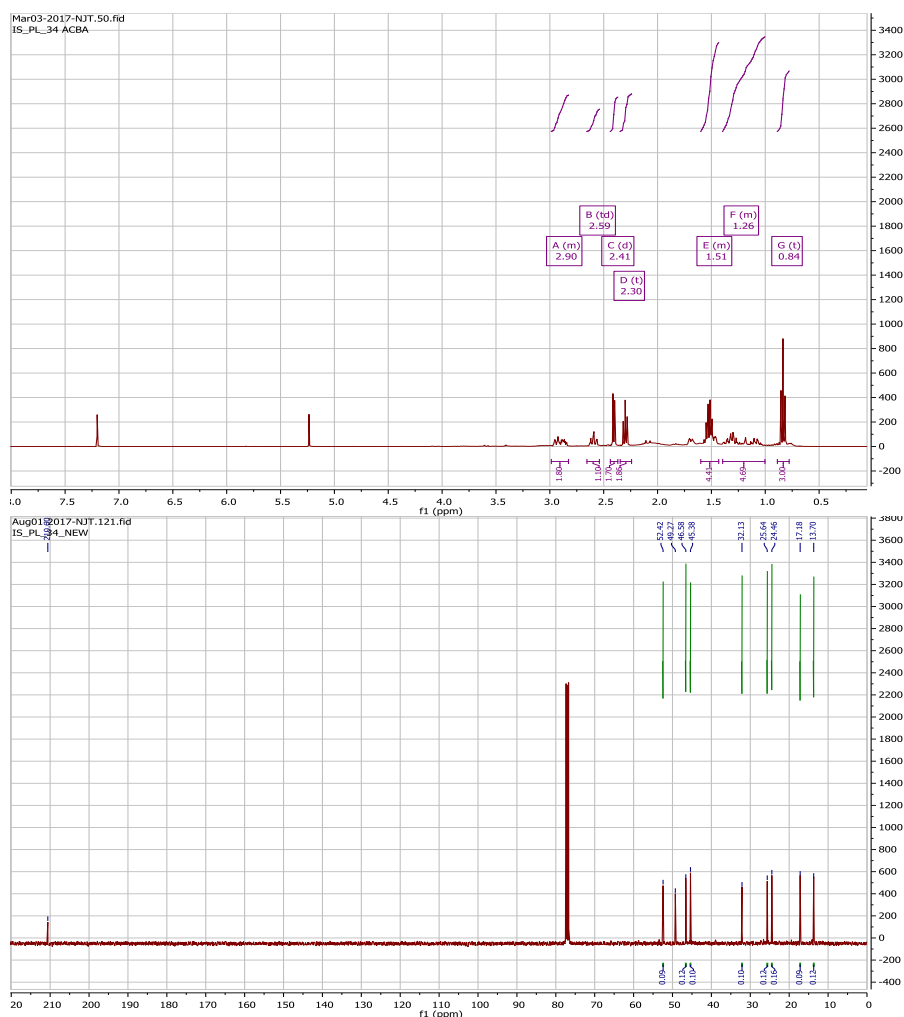


1-(piperidin-2-yl)pentan-2-one, **11**

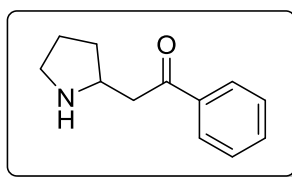


Following the general procedure for the pATA/lipase cascade, compound **11** was obtained as yellow oil, 124 mg, 59 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 2.99 – 2.83 (m, 2H), 2.59 (td, $J = 11.7, 2.8$ Hz, 1H), 2.41 (d, $J = 6.3$ Hz, 2H), 2.30 (t, $J = 7.4$ Hz, 2H), 1.60 – 1.43 (m, 4H), 1.40 – 1.00 (m, 5H), 0.84 (t, $J = 7.4$ Hz, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 210.60, 52.42, 49.27, 46.58, 45.38, 32.13, 25.64, 24.46, 17.18, 13.70. **HRMS** calcd. for $\text{C}_{10}\text{H}_{20}\text{N}^+\text{O}$ 170.1545 $[\text{M}+\text{H}]^+$, found 170.1543.

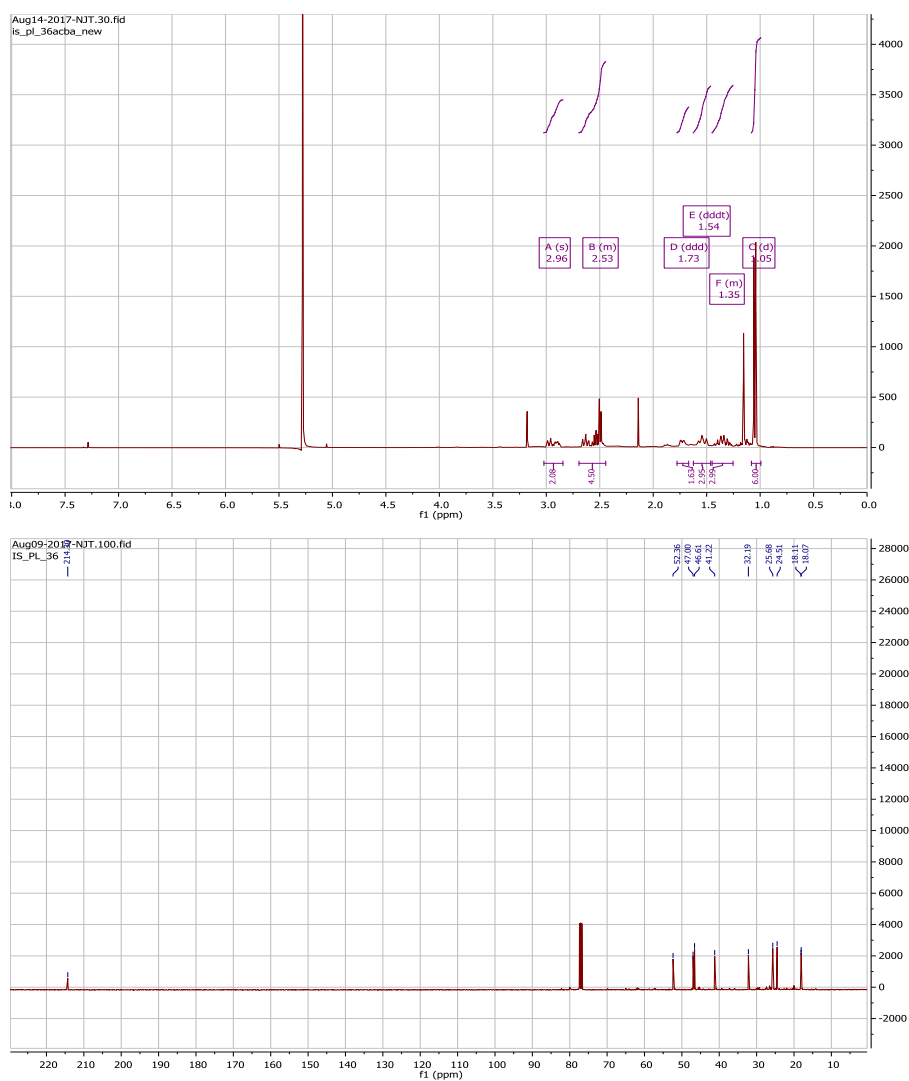


3-methyl-1-(piperidin-2-yl)butan-2-one, **12**

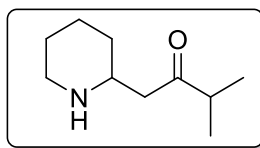


Following the general procedure for the pATA/lipase cascade, compound **12** was obtained as yellow oil, 142 mg, 67 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 2.96 (s, 2H), 2.69 – 2.44 (m, 5H), 1.73 (ddd, $J = 11.1, 4.8, 2.7$ Hz, 2H), 1.54 (dddt, $J = 16.8, 15.2, 4.4, 2.1$ Hz, 3H), 1.45 – 1.25 (m, 3H), 1.05 (d, $J = 7.0$ Hz, 6H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 214.30, 52.36, 47.00, 46.61, 41.22, 32.19, 25.68, 24.51, 18.11, 18.07. **HRMS** calcd. for $\text{C}_{10}\text{H}_{20}\text{N}^+\text{O}$ 170.1545 $[\text{M}+\text{H}]^+$, found 170.1539.

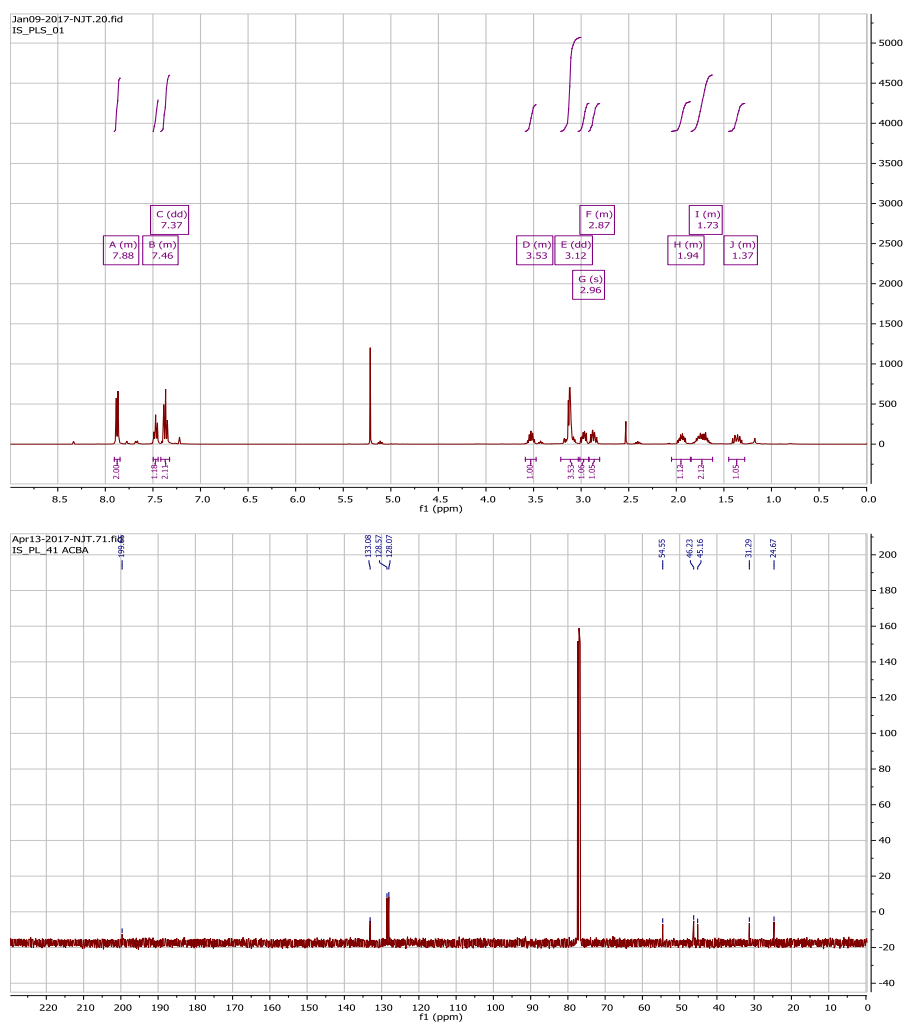


1-phenyl-2-(pyrrolidin-2-yl)ethan-1-one, **13**

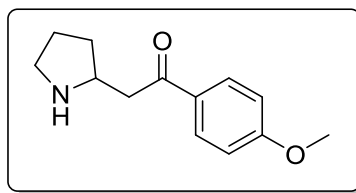


Following the general procedure for the pATA/lipase cascade, compound **13** was obtained as yellow oil, 149 mg, 64 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 7.91 – 7.85 (m, 2H), 7.50 – 7.45 (m, 1H), 7.37 (dd, J = 8.2, 6.9 Hz, 2H), 3.59 – 3.47 (m, 1H), 3.12 (dd, J = 6.4, 3.8 Hz, 4H), 2.96 (s, 1H), 2.92 – 2.81 (m, 1H), 2.05 – 1.85 (m, 1H), 1.84 – 1.62 (m, 2H), 1.45 – 1.28 (m, 1H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 199.65, 133.08, 128.57, 128.07, 54.55, 46.23, 45.16, 31.29, 24.67. **HRMS** calcd. for $\text{C}_{12}\text{H}_{16}\text{N}^+\text{O}$ 190.1232 $[\text{M}+\text{H}]^+$, found 190.1213.

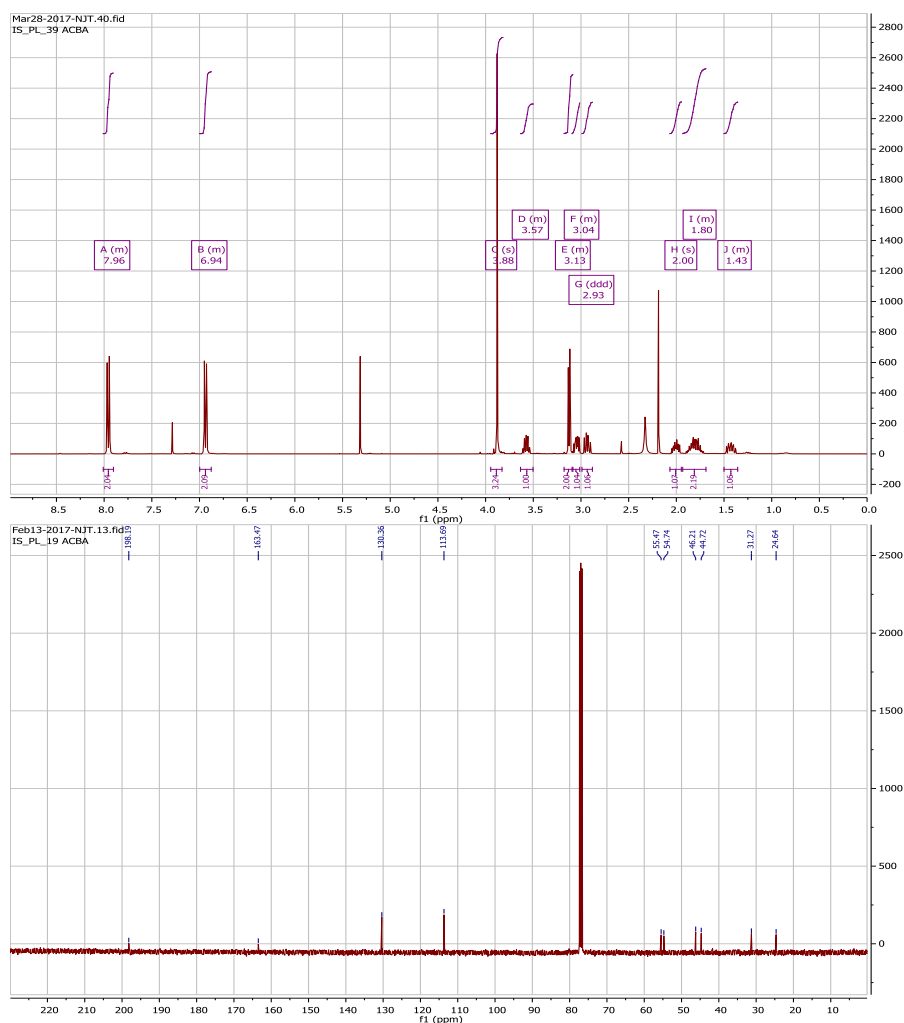


1-(4-methoxyphenyl)-2-(pyrrolidin-2-yl)ethan-1-one, **14**

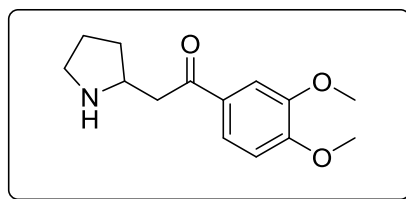


Following the general procedure for the pATA/lipase cascade, compound **14** was obtained as yellow solid, 220 mg, 81 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 8.01 – 7.90 (m, 2H), 7.00 – 6.88 (m, 2H), 3.88 (s, 3H), 3.63 – 3.50 (m, 1H), 3.18 – 3.08 (m, 2H), 3.09 – 3.01 (m, 1H), 2.93 (ddd, $J = 10.0, 8.2, 6.8$ Hz, 1H), 2.00 (s, 1H), 1.93 – 1.69 (m, 2H), 1.50 – 1.36 (m, 1H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 198.19, 163.47, 130.36, 113.69, 55.47, 54.74, 46.21, 44.72, 31.27, 24.64. **HRMS** calcd. for $\text{C}_{13}\text{H}_{18}\text{N}^+\text{O}_2$ 220.1338 $[\text{M}+\text{H}]^+$, found 220.1323.

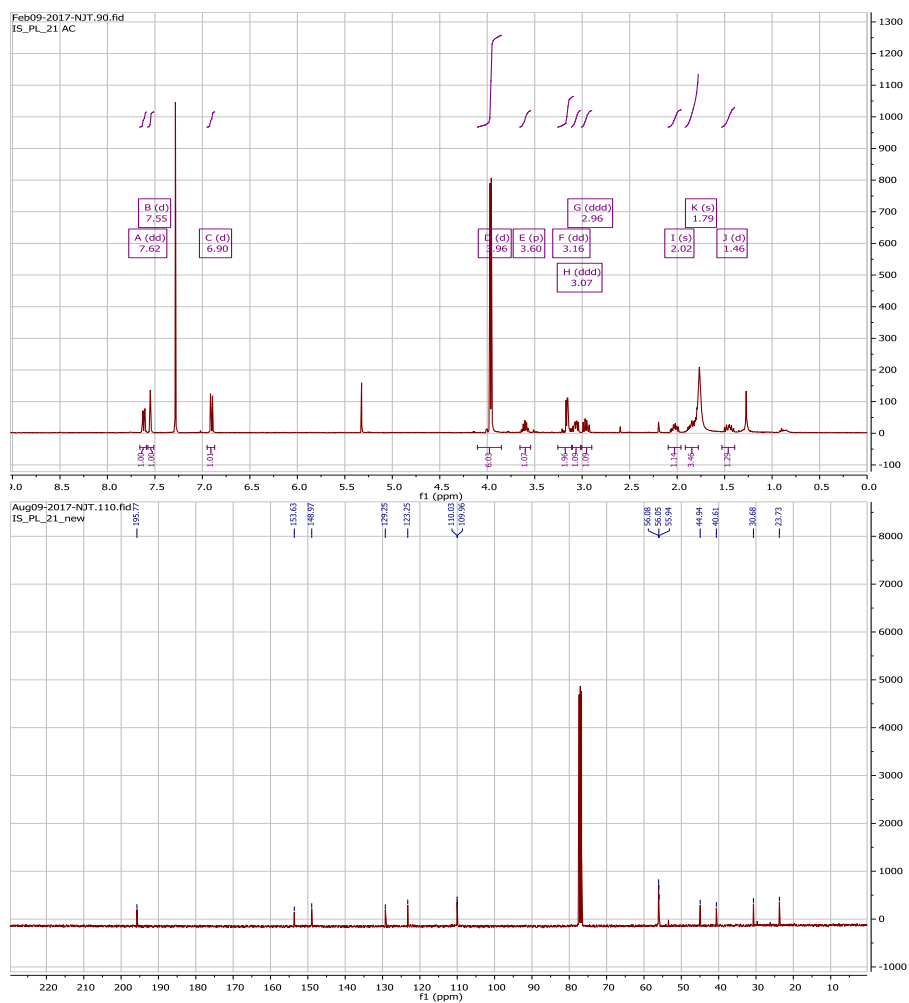


1-(3,4-dimethoxyphenyl)-2-(pyrrolidin-2-yl)ethan-1-one, **15**

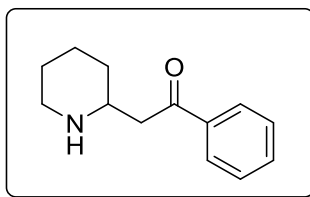


Following the general procedure for the pATA/lipase cascade, compound **15** was obtained as yellow solid, 221 mg, 71 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 8.04 – 7.86 (m, 2H), 7.02 – 6.87 (m, 2H), 3.88 (s, 3H), 3.17 – 3.02 (m, 2H), 3.00 (dd, $J = 6.2, 1.8$ Hz, 2H), 2.78 – 2.68 (m, 1H), 1.87 – 1.54 (m, 4H), 1.53 – 1.15 (m, 9H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 198.09, 163.55, 130.35, 130.22, 113.72, 55.47, 46.90, 45.27, 32.77, 24.77. **HRMS** calcd. for $\text{C}_{14}\text{H}_{20}\text{N}^+\text{O}_3$ 250.1443 $[\text{M}+\text{H}]^+$, found 250.1429.

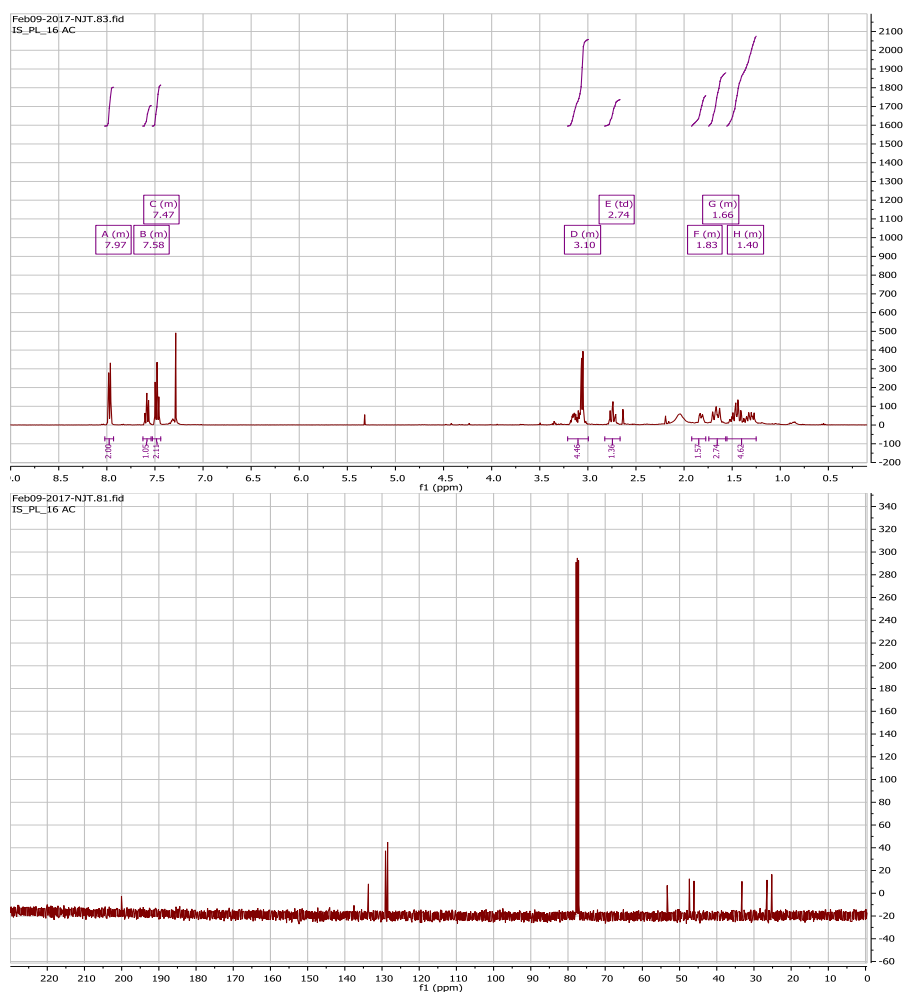


1-phenyl-2-(piperidin-2-yl)ethan-1-one, **16**

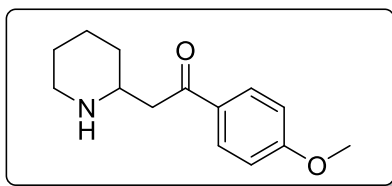


Following the general procedure for the pATA/lipase cascade, compound **16** was obtained as yellow solid, 154 mg, 60 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 8.02 – 7.93 (m, 2H), 7.62 – 7.54 (m, 1H), 7.53 – 7.44 (m, 2H), 3.21 – 3.00 (m, 4H), 2.74 (td, $J = 11.7, 2.8$ Hz, 1H), 1.92 – 1.77 (m, 2H), 1.74 – 1.57 (m, 3H), 1.55 – 1.25 (m, 5H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 200.05, 133.65, 129.06, 128.50, 53.32, 47.36, 46.13, 33.23, 26.48, 25.21. **HRMS** calcd. for $\text{C}_{13}\text{H}_{18}\text{N}^+\text{O}$ 204.1388 $[\text{M}+\text{H}]^+$, found 204.1385

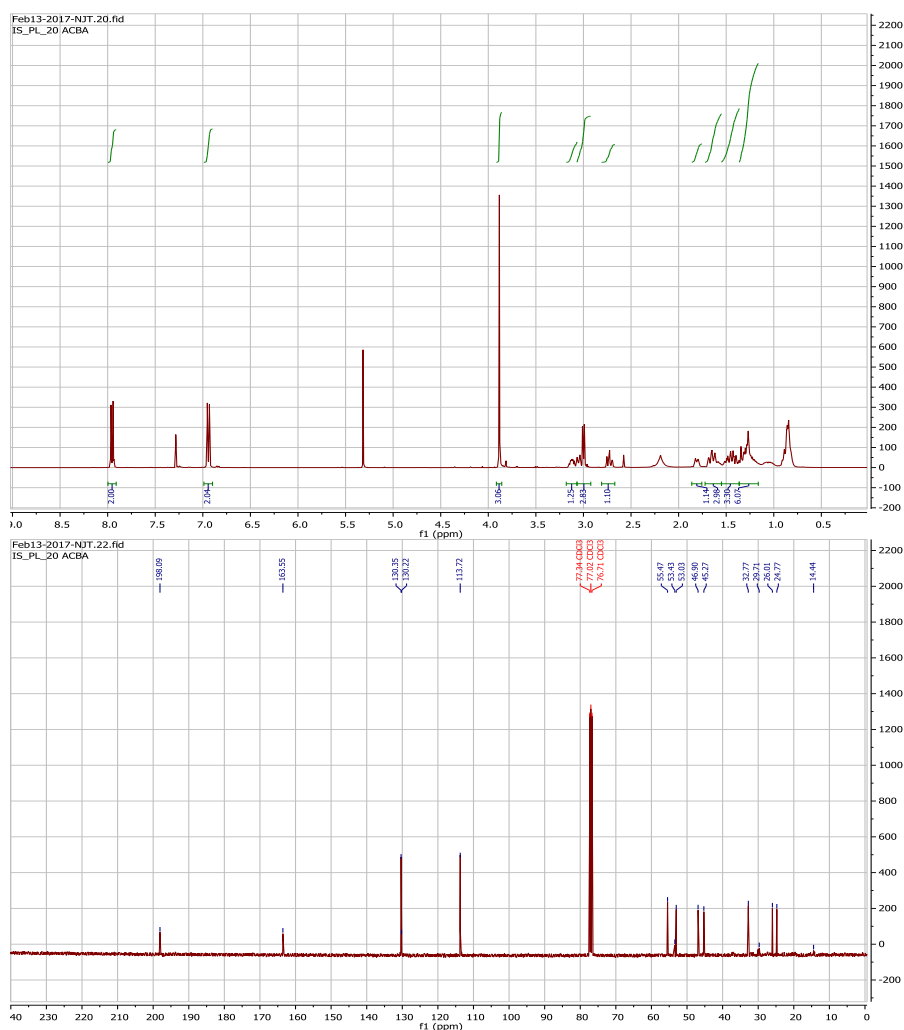


1-(4-methoxyphenyl)-2-(piperidin-2-yl)ethan-1-one, **17**

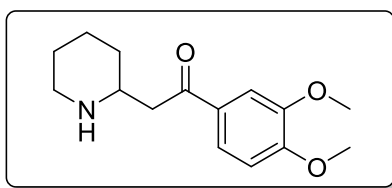


Following the general procedure for the pATA/lipase cascade, compound **17** was obtained as yellow solid, 213 mg, 73 % yield.

$^1\text{H NMR}$ (400 MHz, Chloroform-*d*) δ 8.04 – 7.86 (m, 2H), 7.02 – 6.87 (m, 2H), 3.88 (s, 3H), 3.17 – 3.02 (m, 2H), 3.00 (dd, $J = 6.2, 1.8$ Hz, 2H), 2.78 – 2.68 (m, 1H), 1.87 – 1.54 (m, 4H), 1.53 – 1.15 (m, 9H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 198.09, 163.55, 130.32, 113.72, , 55.47, 53.43, 46.90, 45.27, 32.77, 26.01, 24.77. **HRMS** calcd. for $\text{C}_{14}\text{H}_{20}\text{N}^+\text{O}_2$ 234.3190 $[\text{M}+\text{H}]^+$, found 234.1505.

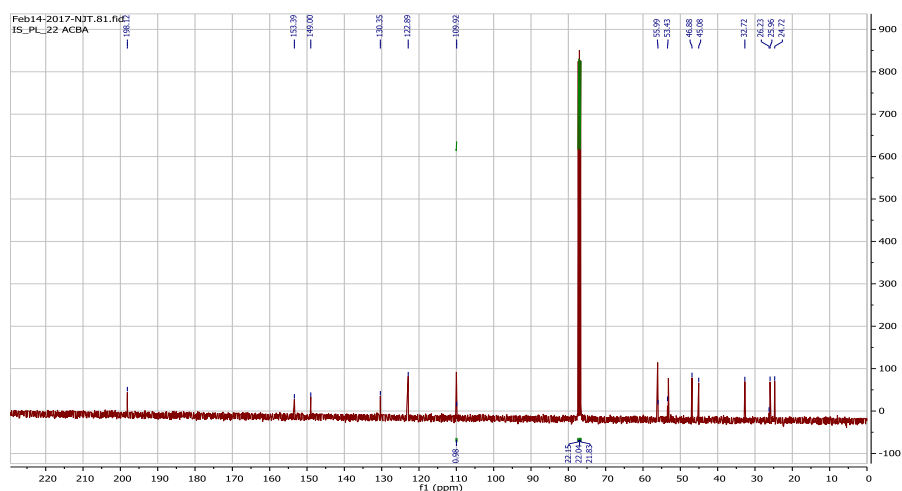
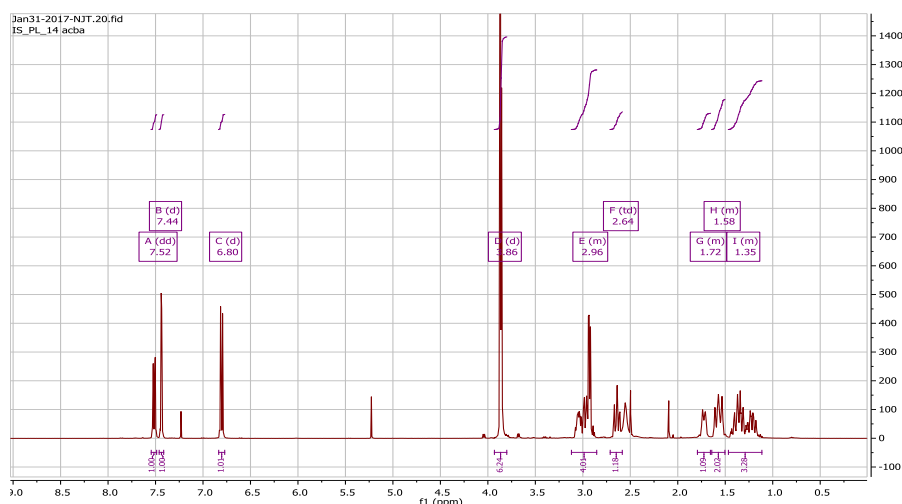


1-(3,4-dimethoxyphenyl)-2-(piperidin-2-yl)ethan-1-one, **18**

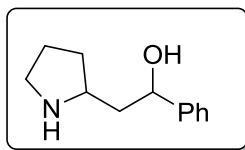


Following the general procedure for the pATA/lipase cascade, compound **18** was obtained as yellow solid, 226 mg, 69 % yield

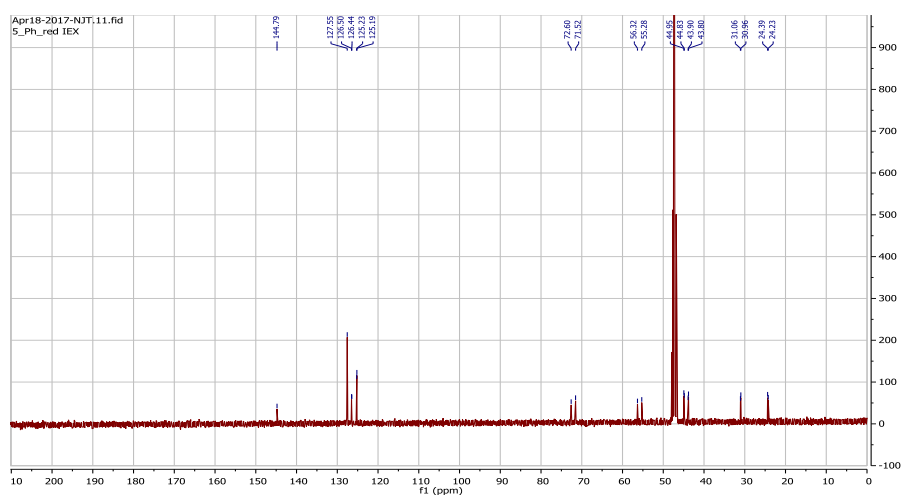
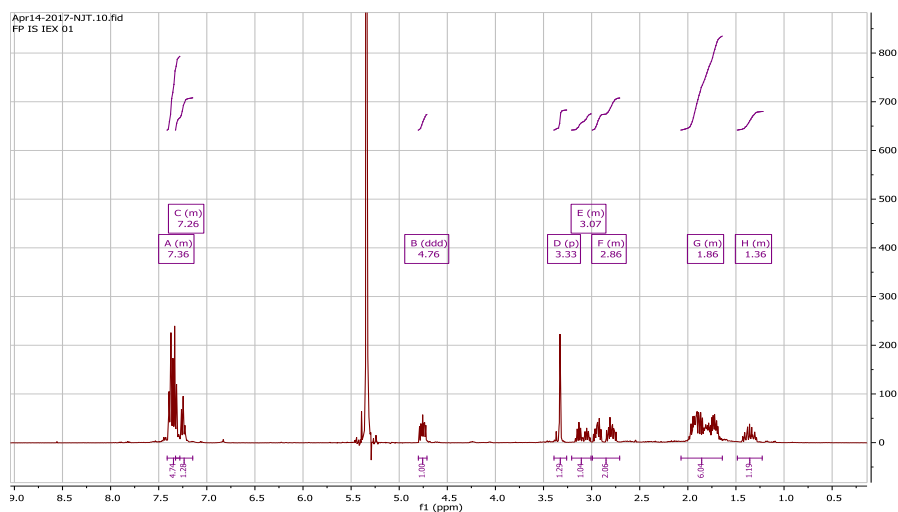
$^1\text{H NMR}$ (400 MHz, Chloroform- d) δ 7.52 (dd, $J = 8.4, 2.1$ Hz, 1H), 7.44 (d, $J = 2.0$ Hz, 1H), 6.80 (d, $J = 8.4$ Hz, 1H), 3.86 (d, $J = 5.1$ Hz, 6H), 3.12 – 2.85 (m, 4H), 2.64 (td, $J = 11.7, 2.8$ Hz, 1H), 1.79 – 1.66 (m, 1H), 1.64 – 1.50 (m, 2H), 1.47 – 1.11 (m, 3H). $^{13}\text{C NMR}$ (101 MHz, CDCl_3) δ 198.12, 153.39, 149.00, 130.35, 122.89, 109.92, 55.99, 53.43, 46.88, 45.08, 32.72, 26.23, 25.96, 24.72. **HRMS** calcd. for $\text{C}_{15}\text{H}_{22}\text{N}^+\text{O}_3$ 264.1600 $[\text{M}+\text{H}]^+$, found 264.1611.



1-phenyl-2-(pyrrolidin-2-yl)ethan-1-ol, 26



$^1\text{H NMR}$ (400 MHz, Methanol- d_4) δ 7.41 – 7.28 (m, 5H), 7.33 – 7.15 (m, 1H), 4.76 (ddd, $J = 13.0, 8.4, 5.1$ Hz, 1H), 3.33 (p, $J = 1.6$ Hz, 1H), 3.21 – 3.01 (m, 1H), 2.99 – 2.71 (m, 2H), 2.07 – 1.64 (m, 6H), 1.49 – 1.23 (m, 1H). $^{13}\text{C NMR}$ (101 MHz, MeOD) δ 144.79, 127.55, 126.50, 126.44, 125.23, 125.19, 72.60, 71.52, 56.32, 55.28, 44.95, 44.83, 43.90, 43.80, 31.06, 24.39.



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