Deracemisation of amines and α-amino acids using enzymes in combination with chemical reducing agents

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December 2001
Declaration

This thesis is submitted in part fulfilment of the requirements for the degree of Doctor of Philosophy at the University of Edinburgh. Unless otherwise stated, the work described here is original and has not been previously submitted, in whole or in part, for any degree at this or any other university.

Alexis Enright

The University of Edinburgh

December 2001
Abstract

Deracemisation is the conversion of a racemic mixture into a single enantiomer, with theoretical chemical yields and e.e.'s of 100%. The high yields and e.e.'s are the main advantage of deracemisation reactions over the more classical kinetic resolutions, where a 50% chemical yield is the maximum obtainable.

RS-α-amino acids bearing a β- or γ-substituent were prepared using standard methods for α-amino acid synthesis. The RS-α-amino acids where subjected to a cyclical oxidation-reduction deracemisation reaction using D-amino acid oxidase from T. variabilis and hydride reducing agents. The enzyme was found to tolerate the β- and γ-substituted α-amino acids as substrates and the use of NaCNBH3 for the deracemisation of acyclic α-amino acids has been demonstrated for the first time.

Screening of a library of organisms was undertaken to identify novel oxidase enzymes for use in the deracemisation reaction. The screen revealed five organisms that were able to oxidise L-phenylalanine to phenylpyruvic acid, indicating possible L-amino acid oxidase activity. However, when screening for monoamine oxidase the assay methods employed lacked the sensitivity to identify conversion of benzylamine to benzaldehyde.

The cyclic oxidation-reduction sequence used for the deracemisation of RS-α-amino acids has been extended to encompass racemic amines for the first time. The deracemisation of RS-α-methylbenzylamine, using an enantioselective monoamine oxidase and ammonia:borane as reducing agent, has been shown to proceed with moderate yields and high selectivity.

$\text{Me}$

$\text{MAO} \xleftrightarrow{[\text{H}]} \text{MeNH}_2 \xrightarrow{+\text{H}_2\text{O}} \text{MeNH} \xleftarrow{-\text{H}_2\text{O}} \text{MeO}$

(R)-AMBA, 77% yield, 93% e.e.
Acknowledgements

I would first of all like to thank Professor Nick Turner for all his encouragement and guidance throughout this project. Thanks also to Drs Mahmoud Mahmoudian and Mike Dawson at GlaxoSmithKline for their valuable input and support.

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To my family I am eternally thankful: for being there and for believing in me through all my decisions. Last but certainly not least thanks to Chris for everything.
Abbreviations

AAP 4-aminoantipyrine
AMBA α-methylbenzylamine
AO amine oxidase
APS ammonium persulfate
ATCC American Type Culture Collection
BDPP 2,4-bis(phospholano)pentane
BPE 1,2-bis(phospholano)ethane
BSA bovine serum albumin
Bu butyl group
BzNH₂ benzylamine
CBS Centraalbureau voor Schimmelcultures
CDCl₃ deuterochloroform
CECT Colección Española de Cultivos Tipo
CFE cell free extract
CHCl₃ chloroform
CuSO₄ copper (II) sulfate
c.v. coefficient of variation
CV column volume
d days
<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>D₂ Lamp</td>
<td>deuterium lamp</td>
</tr>
<tr>
<td>pk-DAAO</td>
<td>D-amino acid oxidase from porcine kidney</td>
</tr>
<tr>
<td>tv-DAAO</td>
<td>D-amino acid oxidase from <em>Trigonopsis variabilis</em></td>
</tr>
<tr>
<td>DCM</td>
<td>dichloromethane</td>
</tr>
<tr>
<td>d.e.</td>
<td>diastereomeric excess</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribose nucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>DuPHOS</td>
<td>1,2-bis(phospholano)benzene</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>e.e.</td>
<td>enantiomeric excess</td>
</tr>
<tr>
<td>eq.</td>
<td>equivalents</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionisation mass spectrometry</td>
</tr>
<tr>
<td>Et</td>
<td>ethyl group</td>
</tr>
<tr>
<td>Et₃N</td>
<td>triethylamine</td>
</tr>
<tr>
<td>EtOAc</td>
<td>ethyl acetate</td>
</tr>
<tr>
<td>FAD</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FPLC</td>
<td>fast protein liquid chromatography</td>
</tr>
<tr>
<td>h</td>
<td>hours</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HAT</td>
<td>hydrogen atom transfer</td>
</tr>
<tr>
<td>conc. HCl</td>
<td>concentrated hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>IFO</td>
<td>Institute for Fermentation</td>
</tr>
<tr>
<td>imac</td>
<td>immobilised metal affinity chromatography</td>
</tr>
<tr>
<td>IMI</td>
<td>International Mycological Institute</td>
</tr>
<tr>
<td>J</td>
<td>coupling constant in Hertz</td>
</tr>
<tr>
<td>kDa</td>
<td>kiloDaltons</td>
</tr>
<tr>
<td>LAAD</td>
<td>L-amino acid deaminase</td>
</tr>
<tr>
<td>LAAO</td>
<td>L-amino acid oxidase</td>
</tr>
<tr>
<td>LC/MS</td>
<td>liquid chromatography-mass spectrometry</td>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MAO-N</td>
<td>monoamine oxidase from <em>Aspergillus niger</em></td>
</tr>
<tr>
<td>Me</td>
<td>methyl group</td>
</tr>
<tr>
<td>MeCN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>min</td>
<td>minute</td>
</tr>
<tr>
<td>miniPHOS</td>
<td>methylene-bridged diphosphine ligand</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
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<td>------------</td>
</tr>
<tr>
<td>m/z</td>
<td>mass to charge ratio</td>
</tr>
<tr>
<td>NBS</td>
<td>N-bromosuccinimide</td>
</tr>
<tr>
<td>NCIMB</td>
<td>National Collection of Industrial and Marine Bacteria</td>
</tr>
<tr>
<td>NCTC</td>
<td>National Collection of Type Cultures</td>
</tr>
<tr>
<td>NCYC</td>
<td>National Collection of Yeast Cultures</td>
</tr>
<tr>
<td>NH₄CO₂</td>
<td>ammonium formate</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>ammonium sulfate</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NRRL</td>
<td>Northern Regional Research Laboratory</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>optical density at 600 nm</td>
</tr>
<tr>
<td>Ph</td>
<td>phenyl group</td>
</tr>
<tr>
<td>PhCHO</td>
<td>benzaldehyde</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
</tr>
<tr>
<td>PPA</td>
<td>phenylpyruvic acid</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>Pr</td>
<td>propyl group</td>
</tr>
<tr>
<td>PTC</td>
<td>phase transfer catalysis</td>
</tr>
<tr>
<td>PTSA</td>
<td>p-toluenesulfonic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>r.t.</td>
<td>retention time</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>rt</td>
<td>room temperature</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecylsulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SET</td>
<td>single electron transfer</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
</tr>
<tr>
<td>TBHBA</td>
<td>2,4,6-tribromo-3-hydroxybenzoic acid</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>topa</td>
<td>3-(2,4,5-trihydroxyphenyl)-1-alanine</td>
</tr>
<tr>
<td>TRAP</td>
<td>2,2''-bis(phospholano)ethyl-1,1''-biferrocene</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>UV</td>
<td>ultra-violet</td>
</tr>
<tr>
<td>w/v</td>
<td>weight to volume ratio</td>
</tr>
<tr>
<td>δH</td>
<td>chemical shift of proton</td>
</tr>
<tr>
<td>δC</td>
<td>chemical shift of carbon</td>
</tr>
<tr>
<td>ε</td>
<td>molar extinction coefficient</td>
</tr>
</tbody>
</table>
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1. Introduction

In the past few decades, the search for general methods for the synthesis of enantiomerically pure \(\alpha\)-amino acids and amines has been a major goal of synthetic organic chemists. \(\alpha\)-Amino acids are used widely in the pharmaceutical industry, as building blocks for physiologically active molecules\(^1\) and as active compounds in their own right. Non-proteinogenic \(\alpha\)-amino acids also play an important role in the investigation of enzyme mechanisms.

Enantiomerically pure amines bearing a stereogenic centre at the \(\alpha\)-position are often used in asymmetric synthesis as chiral auxiliaries or catalysts. However, they are also vital building blocks for therapeutic targets within the pharmaceutical industry.\(^2\)\(^-\)\(^4\)

In the case of pharmaceutical compounds, generally only one enantiomer of a target molecule is active, with the other showing no, or even detrimental, biological activity. Therefore the need to prepare compounds in an enantiopure form arises from both commercial and safety issues.

1.1. Synthesis of optically active \(\alpha\)-amino acids

Asymmetric synthesis is a valuable tool in the preparation of enantiomerically pure compounds. It relies on the reactive centre(s) experiencing some stereodiscriminating environment in the transition state. Often an achiral starting material is involved, with chirality introduced through a chiral auxiliary or catalyst.

There are three main strategies for the retrosynthesis of an \(\alpha\)-amino acid (figure 1.1.1): alkylation; amination and hydrogenation.\(^5\)
1.1.1. Asymmetric alkylation reactions

Various methods for asymmetric alkylation are reported in the literature, most of which are based on nucleophilic or electrophilic glycine equivalents. Some of the most established nucleophilic glycine equivalents include Schollkopf's bis-lactim ethers, Williams' oxazinones, and Seebach's imidazolidinones (figure 1.1.2). In all these cases the heterocyclic template is necessarily more rigid than the acyclic counterpart, and therefore imparts a higher degree of asymmetric induction.

Schollkopf's bis-lactim ethers have been used to prepare natural and unnatural α-amino acids with greater than 90% e.e. (figure 1.1.3). Hydrolysis of the
alkylated bis-lactim ether to the desired amino acid proceeds without significant racemisation at the α-centre.

![Chemical structure](image)

**Figure 1.1.3: alkylation of Schollkopf's bis-lactim ether**

Other methods of introducing asymmetry to a molecule have included the use of imine glycinates based on natural molecules such as camphor and hydroxypinanones (figure 1.1.4), where asymmetry is introduced through the inherent chirality of the auxiliary. The enantiomeric excesses observed range from moderate to good, depending on the size of the alkylating group, solvent, base and counter ion employed.

![Chemical structures](image)

**Figure 1.1.4: chiral imine glycinates**

As is the case with other areas of organic chemistry, the general trend in asymmetric alkylation is to employ catalytic methods as opposed to auxiliary methods. The advantages of using catalytic methods are numerous, e.g., the number of steps in the reaction is reduced by the elimination of both the attachment and the removal of the auxiliary, and recycling of the catalyst is often achievable, both contributing to a
reduction in the overall cost of the system. Phase-transfer catalysts based on the
natural alkaloids cinchonine and cinchonidine, such as the catalyst 10, have been
successfully employed in the enantioselective preparation of α-amino acids
derivatives 9a-f from the achiral imine glycinate 8 (figure 1.1.5). In recent years
these chiral PTCs have been used in solid phase synthesis with good results.

\[
\begin{align*}
\text{Ph} & \quad \text{CO}_2\text{Bu} \\
\text{Ph} & \quad \text{N} \quad \text{R} \quad \text{CO}_2\text{Bu} \\
\end{align*}
\]

\[8 \xrightarrow{10\text{ mol} \% 10} 9 \quad >94\% \text{ e.e.}\]

Figure 1.1.5: chiral phase-transfer catalysis

Many of the nucleophilic equivalents described above can be transformed into the
corresponding electrophilic equivalent, through simple halogenation followed by
displacement of the halogen by a nucleophilic species (figure 1.1.6). Such
methodology can allow access to the opposite enantiomer of an amino acid, and can
be used to introduce R groups that are not accessible via electrophilic addition.

\[
\begin{align*}
\text{Ph} & \quad \text{O} \quad \text{O} \\
\text{Ph} & \quad \text{N} \quad \text{Br} \\
\end{align*}
\]

\[2 \xrightarrow{\text{NBS}} 11 \xrightarrow{\text{RM}} 12\]

Figure 1.1.6: preparation of electrophilic glycine equivalents
1.1.2. Asymmetric amination reactions

In the synthesis of optically active α-amino acids, the asymmetric amination reaction offers a complimentary approach to asymmetric alkylation reactions. A greater variation in the nature of the R group is tolerated compared with the alkylation methodologies described in section 1.1.1. The amination reaction can occur under electrophilic or nucleophilic conditions. The type of nitrogen compound employed influences the stereospecificity of the reaction and is a useful strategy for obtaining both enantiomers of an α-amino acid in optically pure form. Some examples of electrophilic aminating reagents are di-tert-butyl-azodicarboxylate (DBAD) 15,20 benzenediazonium tetrafluorobororate 1621 and 2,4,6-tri-iso-propylbenzenesulfonyl azide (trisyl azide) 1722 (figure 1.1.7). Nucleophilic nitrogen is most commonly introduced via an azide e.g. NaN₃.

![Figure 1.1.7: electrophilic nitrogen species](image)

DBAD has found widespread use as an electrophilic aminating reagent as it shows high diastereofacial selectivity and is commercially available as a stable, crystalline solid. However, the need for reductive cleavage of the N-N bond can limit the functionality tolerated in the side chain. When introducing an amine group to a highly functionalised system, trisyl azide is often used as the source of nitrogen, e.g. in the synthesis of the natural cyclic tripeptide, K-13 (18).23
Asymmetric amination reactions are generally performed via the electrophilic amination of chiral enolates. The two most common enolate auxiliaries used are Oppolzer's sulfonamide\textsuperscript{24} and Evans' carboximides \textsuperscript{19,25} The Evans' auxiliaries are also used in nucleophilic amination reactions via bromination of the chiral enolate,\textsuperscript{26} providing access to the opposite enantiomer of the $\alpha$-amino acid without the need for a new auxiliary (figure 1.1.8).

Figure 1.1.8: amination using Evans' auxiliaries

The removal of the Evans' auxiliaries is generally performed using lithium hydroxide and in most cases occurs without significant racemisation at the $\alpha$-centre. However, when the side chain is particularly bulky, e.g. $t$-butyl group, hydrolysis using lithium peroxide is the favoured method, as LiOH often results in competing cleavage of the urethane carbonyl group.

\textbf{1.1.3. Asymmetric hydrogenation}

Catalytic asymmetric hydrogenation is potentially one of the most efficient and convenient methods for preparing $\alpha$-amino acids.\textsuperscript{27} A popular approach is to use rhodium catalysts with chiral phosphine ligands. There have been many examples of chiral phosphine ligands reported in the literature in recent years,\textsuperscript{28-30} examples of which can be seen in figure 1.1.9.
Introduction

By using the opposite enantiomeric ligand the opposite stereoselectivity can be achieved. As with many catalytic hydrogenations, optimisation is an empirical process, and sometimes even subtle differences in the ligand can produce dramatic differences in the e.e. obtained. A challenge presented to chemists in the last few years has been to prepare β,β-disubstituted α-amino acids in enantiomerically pure form. These compounds are of interest in the design of peptide and peptidomimetic therapeutics as the β-substitution introduces conformational constraints to the peptide. The DuPhos and BPE ligands developed by Burk et al. have now been shown to give e.e.'s of >98% in such reactions (figure 1.1.10), with the added advantage that hydrogenation occurs regioselectively.

Figure 1.1.10: synthesis of β,β-disubstituted α-amino acids
A complimentary approach to asymmetric hydrogenation using chiral ligands is to employ a chiral alkene followed by hydrogenation over a palladium catalyst as shown in figure 1.1.11. This type of reaction often employs the templates seen in the alkylation methodologies (§ 1.1.1).

![Chemical structure](image)

Figure 1.1.11: hydrogenation using a chiral auxiliary

### 1.2. Synthesis of optically active chiral amines

The synthesis of optically active chiral amines follows three main retrosynthetic strategies (figure 1.2.1).

![Chemical structures](image)

Figure 1.2.1: retrosynthetic strategy for chiral amines

#### 1.2.1. 1,2-Nucleophilic addition to a C=N double bond

The synthesis of chiral amines via 1,2-nucleophilic addition has been the subject of a large number of publications in recent years. The methodology is attractive in that it offers a direct and synthetically flexible route to optically active chiral amines. A brief
overview of the relevant strategies is given and further detailed information is available in a recent comprehensive review.\textsuperscript{33}

The types of C=N double bonds commonly employed in 1,2-nucleophilic additions are imines (RHC=NR'), hydrazones (RHC=NNR'R''), oxime ethers (RHC=NOR') and nitrones (RHC=N\textsuperscript{+}(O')R'). Oxime ethers are the least favoured substrates due to their lower electrophilic reactivity and their propensity to form $E/Z$ mixtures. The most attractive method of introducing chirality is through external ligand-induced stereoselectivity, as this avoids the attachment and removal of a chiral auxiliary and holds the potential for recycling the chiral ligand. There have been a number of attempts at making this methodology catalytic, however the enantioselectivity of such reactions is low (figure 1.2.2).\textsuperscript{34}

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Catalytic asymmetric addition to an imine}
\end{figure}

There have been only a few examples of these catalytic methods published. The bulk of the literature is focused on chirality built into the C=N double bond (figure 1.2.3).\textsuperscript{35,36}
1.2.2. Reduction methodologies

1.2.2.1. Asymmetric hydrogenation

The asymmetric hydrogenation of prochiral imines is similar to the hydrogenation methods described for the synthesis of α-amino acids (§ 1.1.3). Some of the more common metal catalysts employed are rhodium, iridium, ruthenium and titanium, usually complexed with chiral diphosphine ligands in situ. However, the reduction of imines is not as easily accomplished as the reduction of other functional groups, such as alkenes and ketones. A number of factors contribute to the difficulty of imine hydrogenation: orbital overlap between the imine and metal centre is less effective than in the corresponding olefin-metal centre overlap; the amine products may “poison” the catalyst and a smaller thermodynamic gain is realized from reduction of C=N bonds (-60 kJ.mol⁻¹) relative to C=C bonds (-130 kJ.mol⁻¹).

Various strategies have been developed in an attempt to improve the rate of hydrogenation and the enantioselectivity of catalytic asymmetric hydrogenations of imines. Many of these fall in the field of ligand design, for example the optimization of tridendate diphosphine ligands with $C_2$ symmetry (figure 1.2.4). Although
enantioselectivity is low, fine-tuning of the ligands should improve the selectivity of the hydrogenation.

Figure 1.2.4: optimisation of tridentate diphosphine ligands

During investigations into the diastereoselective hydrogenation of chiral imines, a group at DSM Research noted that hydrogenation of one enantiomer of a racemic imine proceeded faster than hydrogenation of its opposite enantiomer. This led to investigations into the kinetic resolution of racemic amines by metal-ligand catalysed hydrogenation (figure 1.2.5).
Figure 1.2.5: kinetic resolution by rhodium/diphosphine catalysed hydrogenation

The level of enantioselectivity was generally good but was dependent on the imine structure and the chiral ligand employed.*

The first example of transfer hydrogenation of imines was reported in 1996 as an alternative to high pressure and high temperature systems (figure 1.2.6).° The yields and enantioselectivities in these reactions were excellent, although still dependent on empirical evaluation of the catalyst. The advantage of these systems over standard hydrogenation procedures is that an increase in imine reactivity is observed and the C=N/C=O chemoselectivity is approximately 98:1. This allows for the reduction of an imine in the presence of a C=O functional group. Unfortunately, acyclic imines suffer from lower enantioselectivity, partially due to syn-anti isomerisation.

---

* The authors quote in the text the use of (S,S)-BDPP as chiral ligand, yielding the S-imine. However, the published reaction schemes show (R,R)-BDPP yielding the R-imine. Figure 1.2.5 is based on the text of the paper, rather than the published reaction scheme.
Introduction

1.2.2.2. Hydride transfer

Chemo- and stereoselective reductions of imines by hydride transfer reagents have received much attention in recent years. Most of the common metal hydride reducing agents prefer the reduction of a carbonyl group to that of an imino group due to the lower electrophilicity of the imino group. The number of imino selective, asymmetric methods reported in the literature to date is small, and only moderate enantioselectivities are observed compared to hydrogenation reactions (figure 1.2.7).

Figure 1.2.6: transfer hydrogenation of cyclic imines

Figure 1.2.7: asymmetric reduction by the chiral reducing agent 58
1.2.3. Reductive amination reactions

Asymmetric reductive amination of ketones can be achieved in one of two ways: i) addition of a prochiral ketone to a prochiral amine followed by asymmetric reduction (§ 1.2.2); or ii) addition of a prochiral ketone to a chiral amine (auxiliary) followed by reduction using a non-selective reducing agent. α-Methylbenzylamine (52, AMBA) is a cheap chiral amine that is commercially available in both enantiomeric forms and is therefore a popular choice as a chiral auxiliary. Reductive amination using AMBA as the auxiliary has been used in the synthesis of pharmaceutically important benzocyclic amines, e.g. 1-aminoindane, 62 (figure 1.2.8). Commercially available (+)- or (-)-norephedrine has also been employed as a chiral auxiliary, however lower enantioselectivities are observed.

![Chemical reaction diagram](image)

Figure 1.2.8: asymmetric reductive amination of benzocyclic compounds

1.3. Biocatalysis

An alternative approach to the conventional chemical synthesis of enantiomerically pure compounds lies in the area of biocatalysis. Enzymes are attractive catalysts for synthetic chemists in that they are inherently chiral, being produced using naturally occurring L-amino acids, they operate under mild conditions and they usually exhibit high levels of chemo- and regiospecificity.
1.3.1. Kinetic resolutions

The process of resolution whereby one enantiomer is more readily transformed into product than its mirror image is known as kinetic resolution (figure 1.3.1). \(^48\)

$$
\begin{align*}
(R)-A & \quad \xrightarrow{k_R \text{ fast}} \quad (R)-P \\
(S)-A & \quad \xrightarrow{k_S \text{ slow}} \quad (S)-P
\end{align*}
$$

\(k_R \neq k_S\)

A = substrate
P = product

Figure 1.3.1: kinetic resolution

The first kinetic resolution recorded was by Pasteur in 1858, when he discovered that ammonium tartrate fermented in the presence of *Penicillium glaucum* resulted in faster metabolism of the D-enantiomer compared to the L-enantiomer. \(^48\) Today either chemo- or biocatalysis can be used to bring about such resolutions, with reports of biocatalytic resolutions greatly outnumbering the chemocatalytic methods. The hydrolase enzymes, such as lipases and proteases, are the most popular class of enzyme in reported resolution processes. The biological function of these enzymes is the hydrolysis of ester and amide bonds respectively. Hydrolysis of \(\alpha\)-amino acid esters is a common method for the kinetic resolution of amino acids. \(^49\) However, in organic solvents these enzymes will catalyse the transfer of an acyl group from a suitable acyl donor to an acceptor alcohol or amine (figure 1.3.2).

![Figure 1.3.2: mechanism of lipase-catalysed acyl transfer to an alcohol acceptor](image)

Figure 1.3.2: mechanism of lipase-catalysed acyl transfer to an alcohol acceptor
Due to the versatility and simplicity of lipase-catalysed resolutions, a number of kinetic resolutions are now carried out on an industrial scale. One such process is the resolution of racemic primary amines using *Candida antarctica* lipase and ethyl acetate as the acyl donor. This method is used to resolve α-methylbenzylamine, with 50% recovery of the S-enantiomer and an e.e. of 90%. Chiral amines have also been kinetically resolved using an ω-transaminase (figure 1.3.3). It was found that the enzyme was inhibited by the acetophenone product, which resulted in a decrease in the yield of the reaction. The problem was overcome by using a bi-phasic system, which offered two advantages: i) the inhibitory product acetophenone can be extracted from the aqueous phase and ii) AMBA forms an imine with cyclohexanone and exists primarily in the organic phase, therefore allowing the organic phase to act as a reservoir of AMBA.

![Diagram](image)

**Figure 1.3.3:** kinetic resolution of AMBA using ω-transaminase

However, no matter how efficient the resolution, classical kinetic resolutions suffer from some major disadvantages: the maximum yield that can be obtained is only 50%; separation of the product from the unreacted starting material can be problematic; for kinetic reasons, the enantiomeric purity is depleted at 50%
1.3.2. Deracemisation

Deracemisation can be defined as a process that leads to the formation of a single enantiomer from a racemic mixture, with theoretical yields and enantiomeric excesses of 100%\(^5\). Over the years, various research groups have attempted to realise this goal, almost all relying on the high enantioselectivity offered by enzymes. A review of the biocatalytic deracemisation strategies employed will be given, along with a comparison with chemical methods where appropriate.

1.3.2.1. Dynamic Kinetic Resolution

In the past two decades it has been realised that classical kinetic resolutions could be enhanced if the substrate possessed a chirally labile stereogenic centre. In such cases the substrate can undergo in situ racemisation, usually by chemocatalysis. To indicate the non-static behaviour of the process the term 'Dynamic Kinetic Resolution' was coined (figure 1.3.4).

\[
\begin{align*}
(R)-A & \xrightarrow{k_R} (R)-P \\
(S)-A & \xrightarrow{k_S} (S)-P \\
\text{A = substrate} \\
\text{P = product}
\end{align*}
\]

Figure 1.3.4: dynamic kinetic resolution

If the rate of racemisation (\(k_{\text{rac}}\)) is much greater than the rate of product formation (\(k_R\)), then a theoretical yield of 100% is possible. A kinetic treatment of dynamic resolutions revealed the optimum conditions for efficient resolution processes.\(^5\) For maximum efficiency, product formation should be irreversible, the enantiomeric ratio
(E) should be $\geq 20$ (where $E = k_R/k_S$) and $k_{\text{rac}}$ should be approximately ten times faster than $k_R$.\textsuperscript{55}

Since the first practical reports of dynamic kinetic resolutions in the 1980s\textsuperscript{56, 57} numerous examples of dynamic kinetic resolutions have been published.\textsuperscript{48, 55, 58, 59} Racemisation of the unreacted starting material can be achieved by a variety of techniques, one of the most common being base-catalysed proton abstraction, \textit{e.g.} the ring opening of chirally labile 5(4H)-oxazolones 65 and 66 to produce amino acid derivatives 67 and 68 (figure 1.3.5).\textsuperscript{60-63}

\begin{align*}
\text{Racemisation of the unreacted starting material can be achieved by a variety of techniques, one of the most common being base-catalysed proton abstraction, \textit{e.g.} the ring opening of chirally labile 5(4H)-oxazolones 65 and 66 to produce amino acid derivatives 67 and 68 (figure 1.3.5).}\end{align*}

![Figure 1.3.5: lipase-catalysed ring opening of 5(4H)-oxazolones\textsuperscript{60}](image)

A chemical variation of this racemisation/ring opening of racemic azlactones makes use of 4-(dimethylamino)pyridine as a planar-chiral catalyst.\textsuperscript{64} Although the method suffers from low to moderate e.e.'s (50-60%), it is one of only a few examples of enantioselective ring opening by a non-enzymatic catalyst.

The selective ring opening of D-hydantoins (69) by the enzyme D-hydantoinase is the basis for a successful industrial scale preparation of D-amino acids (figure 1.3.6).\textsuperscript{65} The spontaneous racemisation of hydantoins at pH 8 or above allows a dynamic kinetic resolution to occur.
Figure 1.3.6: industrial preparation of D-amino acids by dynamic kinetic resolution

A formal chemical deracemisation for the preparation of enantiomerically pure \( \alpha \)-amino acids has been performed through a diastereoselective esterification of a protected racemic amino acid (figure 1.3.7). The yields of the reaction are very good, although the stereoselectivity was found to be R group dependent. The number of steps involved in the reaction makes this an unattractive option for the synthesis of enantiopure \( \alpha \)-amino acids compared with the elegant biocatalytic processes available.

Figure 1.3.7: formal deracemisation via diastereoselective esterification

When compounds do not possess a chirally labile centre, e.g. secondary alcohols, racemisation may be achieved by a decomposition reaction, as can be seen in the lipase-catalysed resolution of cyanohydrins (figure 1.3.8).
Figure 1.3.8: lipase-catalysed dynamic resolution of cyanohydrins

The previous examples often require conditions for racemisation that are incompatible with the enzymes used for resolution. A relatively new area in dynamic kinetic resolution uses enzymes and transition metals complexes in tandem, where the transition metals that bring about racemisation are more compatible with biological systems. The dynamic resolution of secondary alcohols can be achieved via palladium(II)-catalysed racemisation of allylic acetates (figure 1.3.9), albeit with exceedingly long reaction times.

Figure 1.3.9: palladium(II)-catalysed racemisation of allylic acetates

Benzylic α-substituted primary amines can be racemised by palladium on charcoal at elevated temperatures, allowing the preparation of enantiopure chiral amides in >60% yield (figure 1.3.10). The mechanism of racemisation is thought to occur via initial palladium insertion into an N-H bond, followed by β-elimination of PdH to give a
palladium-complexed imine (84). This is then non-selectively reduced by palladium-catalysed hydrogenation to give the racemic starting material.

![Chemical Structure](image)

Figure 1.3.10: palladium-catalysed racemisation of benzylic amines

This process has since been scaled-up by BASF Technologies, and is used for the bulk preparation of chiral amines, with an annual capacity of 1000 tonnes per annum. The lipase enzyme employed by BASF has a broad substrate specificity and accepts arylalkyl amines, alkyl amines and cyclic amines. The separation of the amine from the reactor is carried out by distillation, therefore the only major requirement of the amine to be resolved is that its boiling point falls within the accepted boiling point range for the process.

### 1.3.2.2. Enantioconvergence

Enantioconvergence processes are highly specific, as the requirements for successful deracemisations via this method are difficult to meet. In fact the only enzymes suitable for enantioconvergent processes are the microbial epoxide hydrolases. An elegant deracemisation has been described using enantiocomplimentary epoxide hydrolases from *Aspergillus niger* and *Beauveria sulfurescens*. The organisms *A. niger* and *B. sulfurescens* exhibit the opposite enantioselectivity towards the epoxide 85, therefore allowing production of either enantiomer of 85. Interestingly, the absolute configuration of the diol product 86 is the same from each organism, indicating that the epoxide hydrolases of the two organisms operate with different regioselectivities. This observation was the basis of the enantioconvergent process described in figure 1.3.11 for the production of diol 86.
1.3.2.3. Stereoinversion

Stereoinversion is a cyclical process where only one enantiomer of a racemic mixture is oxidised to form an intermediate, which is then followed by reduction of the intermediate by an enzyme with the opposite stereochemical preference.\textsuperscript{73} The net redox-balance of the system is zero and the necessary redox cofactors, \textit{e.g.} nicotinamide adenine dinucleotide, may be recycled internally.\textsuperscript{74} The success of such systems relies on at least one of the redox reactions being irreversible. Biocatalytic stereoinversions have been successfully used in the preparation of optically pure $\alpha$-amino acids\textsuperscript{75-77} and sec-alcohols.\textsuperscript{78,79} The preparation of natural and unnatural $\alpha$-amino acids \textit{via} a two-enzyme system is exemplified in figure 1.3.12.\textsuperscript{75} D-Amino acid oxidase form porcine kidney (pk-DAAO) selectively deaminates the D-amino acid from the racemic mixture. The $\alpha$-keto acid \textsuperscript{88} produced from the reaction is then converted to the corresponding L-amino acid \textsuperscript{90} using a branched-chain aminotransferase from \textit{Escherichia coli}, with L-glutamate \textsuperscript{89} as the amine donor. Although excellent yields and enantioselectivities are observed, the system suffers from a few disadvantages: i) ion-exchange chromatography is required to separate L-glutamate from the desired amino acid and ii) the first step is ultimately a kinetic resolution and therefore the rate of oxidation of the D-enantiomer slows as the reaction proceeds.
Introduction

$\text{NH}_2\overset{+}{\text{R}}\text{CO}_2^-$  \[\xrightarrow{\text{D-amino acid oxidase}}\]  $\text{NH}_3\overset{+}{\text{R}}\text{CO}_2^-$  \[\xrightarrow{\text{amino-transferase}}\]  $\text{R}^\text{+}\text{CO}_2^-$

\[\text{yield } = 61-96\%\]
\[\text{e.e. } >98\%\]

$R = -\text{CH(CH}_3\text{)}, -\text{CH}_2\text{CH(CH}_3\text{)}_2, -\text{C(CH}_3\text{)}\text{CH(CH}_3\text{)}_2, -\text{CH}_2\text{SCH}_3, -\text{CH}_2\text{Ph}, -\text{CH}_2\text{CH=CH}_2, -\text{CH}_2\text{CH}_3$

Figure 1.3.12: stereoinversion of $\alpha$-amino acids

1.3.2.4. Deracamisation by a cyclic oxidation-reduction sequence

Perhaps a more general methodology for deracamisation is the use of specific enzymatic oxidation to an achiral intermediate followed by non-specific chemical reduction to yield the racemic mixture (figure 1.3.13). At first glance, this system would appear to be of limited practical use as the first step is basically a kinetic resolution with the inherent limitation of 50% yield, and the second step bears no chiral induction at all. However, a combination of the two reactions and examination of the kinetics show this to be a potentially very versatile and economic technique, as theoretically only four oxidation-reduction cycles are required to reach an e.e. of approximately 97%.

\[k'R > k'S\]
\[k'_{\text{red}} = 2 \times k'R\]

Figure 1.3.13: cyclic oxidation-reduction sequence

23
In 1971, Hafner and Wellner demonstrated that the reaction of D-alanine with D-amino acid oxidase and sodium borohydride (NaBH\(_4\)) resulted in significant formation of L-alanine, thus establishing the principle of stereoinversion by a cyclic oxidation-reduction sequence.\(^8\) However, the yield of the reaction was low and it was not until the early 1990s that the practical feasibility of this cyclic sequence was demonstrated, when Soda \textit{et al.} prepared the cyclic \(\alpha\)-amino acids proline (92) and pipecolic acid (93) in high yield and e.e. (figure 1.3.14).\(^8\,^2\,^3\)

![Figure 1.3.14: deracemisation of cyclic amino acids](image)

High chemical and optical yields were obtained due to the stability of the cyclic imines 94 and 95 in aqueous systems and their susceptibility to chemical reduction. The major limitation of the reaction was the large excess of NaBH\(_4\) used for reduction (500 equivalents). Research carried out within our group has shown that the deracemisation of DL-proline can be successfully carried out using only 6 equivalents of sodium cyanoborohydride (NaCNBH\(_3\)),\(^8\,^4\) as NaCNBH\(_3\) has greater aqueous stability than NaBH\(_4\) at pH < 8.\(^8\,^5\) The method has since been developed to produce optically pure acyclic \(\alpha\)-amino acids in high yield (75-90%), using either NaBH\(_4\)\(^8\,^4\) or amine:boranes\(^8\,^6\) as reducing agent.

This cyclic oxidation-reduction deracemisation protocol has also received much interest by the groups of Carnell\(^8\,^7\) and Faber,\(^8\,^8\) and both groups have published examples of this type with reasonable success. However both examples require separation of the achiral intermediate before “reduction” can take place.
1.4. Oxidase enzymes

Oxidase enzymes belong to the flavoprotein family and are characterised by the ability of the reduced flavin to react rapidly with molecular oxygen to yield hydrogen peroxide and the reoxidised co-factor. Amongst these enzymes are the amino acid oxidases and the amine oxidases, which are of interest due to the possibility of using them in the deracemisation of \( \alpha \)-amino acids and amines.

1.4.1. Amino acid oxidase

Amino acid oxidases oxidatively deaminate \( \alpha \)-amino acids to produce the corresponding \( \alpha \)-keto acid, ammonia and hydrogen peroxide (figure 1.4.1). There are two classes of enzymes in this family: i) D-amino acid oxidase, which specifically oxidises D-amino acids and ii) L-amino acid oxidase, which is L-enantiomer specific.

\[
\text{R,CO}_2\text{H} \quad \text{DAAO} \quad \text{R,CO}_2\text{H} + \text{NH}_3
\]

\[
\text{O}_2 \quad \text{H}_2\text{O}_2
\]

\[
\text{R,CO}_2\text{H} \quad \text{LAAO} \quad \text{R,CO}_2\text{H} + \text{NH}_3
\]

\[
\text{O}_2 \quad \text{H}_2\text{O}_2
\]

Figure 1.4.1: oxidation of amino acids by amino acid oxidase

Amino acid oxidases use flavin adenine dinucleotide (FAD) as a cofactor, which is reoxidised by molecular oxygen, thereby eliminating the need for external cofactor regeneration. The product released from the active site of the enzyme is an \( \alpha \)-imino acid, which is non-enzymatically hydrolysed to the \( \alpha \)-keto acid.

D-Amino acid oxidases are found mainly in eukaryotic cells, e.g. *Trigonopsis variabilis*, *Rhodotorula gracilis*, *Fusarium oxysporum*, *Aspergillus* species and porcine kidney. D-amino acid oxidase from *T. variabilis* (tv-DAAO) is commercially available in bulk quantities as it is used in the production of glutaryl-7-aminocephalosporanic acid from Cephalosporin C (96). This is the first step in a two-step enzymatic route to 7-aminocephalosporanic acid (98), a valuable building block in the synthesis of many important cephalosporin antibiotics (figure 1.4.2).
L-Amino acid oxidase is commonly found in the venom of a variety of snakes. Studies have shown that the H$_2$O$_2$ produced by L-amino acid oxidation induces apoptosis. L-Amino acid oxidase has also been identified in a number of bacterial species, e.g. Cellulomonas, Bacillus, Proteus, as well as in fungi, e.g. Trichoderma, Neurospora, Penicillium and in cyanobacterium, e.g. Synechococcus species. Recently, an enzyme termed L-amino acid deaminase has been isolated from Proteus myxofaciens (pm-LAAD). As for LAAO, LAAD oxidatively deaminates L-$\alpha$-amino acids to give the corresponding $\alpha$-keto acid and ammonia. However, unlike LAAO hydrogen peroxide is not released. The pm-LAAD has been cloned and over-expressed in E. coli, and used to prepare D-$\alpha$-amino acids from their L-counterparts.

A great deal of research has been carried out on the mechanism of amino acid oxidases, mainly with D-amino acid oxidases from porcine kidney and the yeasts T. variabilis and R. gracilis due to the relatively recent discovery of L-amino acid oxidases in microbial sources. Although differences have been noted between the tv-DAAO and pk-DAAO mechanisms, these are due to the variable kinetics of each step rather than actual differences in the catalytic cycle. For instance, the rate-determining step in DAAO from R. gracilis is the reductive half-reaction, whereas the slow step in pk-DAAO catalysis is product release from the oxidised enzyme. Despite the number of studies carried out the mechanism remains controversial, and three proposals have been put forward. The “carbanion”
mechanism proposes the existence of a carbanion intermediate by removal of the 
α-proton from the substrate by an active site base (figure 1.4.3).

![Figure 1.4.3: "carbanion" mechanism](image)

However, when FAD is replaced by 5-deaza-FAD, the α-proton of the substrate is 
incorporated into the C(5) position of the 5-deazaflavin, which is consistent with a 
direct hydride transfer (figure 1.4.4).

![Figure 1.4.4: direct hydride transfer mechanism](image)

The final proposal suggests a concerted reaction in which α-proton abstraction is 
coupled to the transfer of a hydride from the amino acid nitrogen to the flavin (figure 
1.4.5). This model also fits the experimental evidence for a carbanion mechanism.

![Figure 1.4.5: direct hydride transfer mechanism](image)

The problem with clarification lies mainly in the versatility of the flavin cofactors as 
they can accept or donate redox equivalents via hydride transfer, radical steps or 
formation of covalent adducts. It is clear that until the crystal structures of the
non-reduced enzymes are published the debate on amino acid oxidase mechanism will continue. The important point with regards to deracemisation experiments is that all three proposals show release of the α-imino acid from the oxidised enzyme.

1.4.2. Monoamine oxidase

Amine oxidases oxidatively deaminate amines to give the corresponding aldehyde, hydrogen peroxide and ammonia (figure 1.4.6).

$$\text{R.\,} \text{NH}_2 \xrightarrow{\text{AO}} \text{R.\,} \text{H} + \text{NH}_3$$

$$\text{O}_2 \text{\rightarrow H}_2\text{O}_2$$

Figure 1.4.6: oxidation of amines by amine oxidase

A number of enzymes belong to the amine oxidase family, including diamine oxidase, polyamine oxidase, copper amine oxidase and monoamine oxidase. As the names suggest the first two enzymes are responsible for the oxidation of compounds containing more than one amine group, e.g. spermine. These enzymes are found in plants and microorganisms.

The copper amine oxidases are abundant in a variety of organisms, their function being to convert primary amines into products that can be utilised as a carbon source, a nitrogen source or both. Copper dependent amine oxidase requires the organic compound topa-quinone as cofactor, which is produced by the enzyme from a peptidyl tyrosine residue by a post-translational modification process. As a result of the availability of X-ray crystal structures of enzymes from a number of organisms, the catalytic mechanism of the copper amine oxidases is well understood (figure 1.4.7). The first step is formation of the imine from the amine and carbonyl group of the topa quinone. Tautomerism to the aromatic imine is followed by hydrolysis to the aldehyde. Reoxidation of the cofactor by molecular oxygen produces the hydrogen peroxide and ammonia by-products. There is evidence in the literature that the enzyme shows stereospecificity, however the mechanism reveals that copper amine oxidases are not suitable enzymes for the deracemisation of amines, as the intermediate imine is not released from the active site.
Monoamine oxidase (MAO) on the other hand is a flavin dependent enzyme, similar to the amino acid oxidases. It is found in a variety of mammals, and oxidises neurogenic amines such as dopamine and serotonin as well as a variety of xenobiotic amines. A great deal of research has been carried out on inhibitors of MAO as they are used in the treatment of mental disorders and in slowing the progress of Parkinson's disease. The enzyme exists as two isozymes in mammalian systems, MAO-A and MAO-B. Both are flavoproteins of the mitochondrial outer membrane, and attempts to crystallise the proteins have failed due to their tendency to aggregate on removal from the outer membrane. As a result the catalytic mechanism is still unclear. Two possible pathways have been proposed for the mechanism of oxidation of the MAO-B substrate 1-methyl-4-phenyl-1,2,3,6-terahydropyridine 104 (figure 1.4.8). The single electron transfer (SET) is the favoured proposal, however some experimental evidence suggests the aminyl radical cations 105 may not be obligatory intermediates. For this reason the hydrogen atom transfer (HAT) pathway has also put forward by Castagnoli et al.
This mechanism should allow for deracemisation reactions since the imine intermediate is not covalently bound to the cofactor, thereby allowing reduction back to the amine. In 1995 the first direct evidence of an imine product from the reaction with MAO-B was published.\textsuperscript{117,118} There is also some evidence that MAO-B shows a stereospecific removal of the \textit{pro-R} hydrogen for several different substrates.\textsuperscript{119} 

For commercially viable deracemisations of racemic amines, a microbial source of flavin dependent monoamine oxidase is required. A number of organisms have been reported as having flavoprotein amine oxidases, however almost all are either polyamine oxidases or have very narrow substrate specificities, \textit{e.g.} tyramine oxidase. The fungus \textit{A. niger} is the only reported source to date which has a flavin dependent monoamine oxidase (MAO-N).\textsuperscript{120} The authors have since cloned, sequenced and expressed the MAO-N gene in \textit{E. coli}.\textsuperscript{121} The enzyme has been compared with MAO-A and MAO-B and has been declared an "evolutionary precursor" to the mammalian enzymes (figure 1.4.9).\textsuperscript{122}

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
\textbf{N-terminus} & \textbf{C-terminus} \\
\hline
\textbf{MAO-A} & \begin{array}{c}
\vdots 1 \\
\vdots 26 \\
\vdots 527 \\
\end{array} \\
\hline
\textbf{MAO-N} & \begin{array}{c}
\vdots 1 \\
\vdots 495 \\
\end{array} \\
\hline
\textbf{MAO-B} & \begin{array}{c}
\vdots 1 \\
\vdots 35 \\
\vdots 31 \\
\vdots 78 \\
\vdots 520 \\
\end{array} \\
\hline
\end{tabular}
\caption{Comparison of monoamine oxidases from mammalian and fungal sources (areas of high sequence identity are shown in bold)}
\end{table}

All three N-termini have the $\beta$-$\alpha$-$\beta$ fold necessary for binding the ADP moiety of the flavin dinucleotide. However, unlike the mammalian enzymes MAO-N does not contain the highly conserved C-terminus pentapeptide, Ser-Gly-Gly-Cys-Tyr, responsible for the covalent bonding of FAD. Although the FAD is non-covalently bound, it has been shown that MAO-N behaves like a classical flavoprotein oxidase in that it has a stabilised anionic semiquinone. The MAO-A and MAO-B enzymes have an extended hydrophobic C-terminus compared to MAO-N, which is thought to be required for binding to the outer mitochondrial membrane. The lack of a membrane
binding sequence in MAO-N should provide improved solubility \textit{in vitro}, which may aid crystallisation studies. Structural information should help clarify the catalytic mechanism of the flavin dependent monoamine oxidases, however unfortunately no X-ray crystal structure has been reported to date.

\section*{1.5. General conclusions & Aims}

Research into the synthesis of optically active $\alpha$-amino acids and amines over the last few decades has been extensive. However, there is still a need for increased research and optimisation in most areas, especially for the synthesis of enantiopure chiral amines, as a generic method for their production in high yields has not yet been realised.

The recent work by Soda \textit{et al.}\textsuperscript{82,83} on the deracemisation of cyclic amino acids is a promising route to both enantiopure $\alpha$-amino acids and amines in high yields. In order to successfully employ this deracemisation as a generic method, oxidase enzymes with high enantioselectivity and substrate specificity are required. In the last few decades, work has focused on the production of unnatural D-amino acids, which are important building blocks in the pharmaceutical industry.\textsuperscript{123} Commercially available D-amino acids are generally more expensive than their L-counterparts as their production by fermentation is not often possible. Deracemisation reactions would provide an efficient route to D-amino acids, either from the racemate or from the more readily available L-enantiomer. However, L-amino acid oxidase is only commercially available from snake venom (Sigma), which raises issues with toxicity and cost. In order to successfully use the deracemisation reaction in the production of D-amino acids an alternative, microbial source of L-amino acid oxidase will be required. The deracemisation of racemic amines using this cyclic oxidation-reduction strategy is a potentially cheap and efficient method for their production, assuming an enantioselective monoamine oxidase can be found.

The aims of this work were to develop the deracemisation of $\alpha$-amino acids to incorporate unnatural $\beta$-substituted $\alpha$-amino acids as substrates and to extend the methodology to include the deracemisation of racemic amines.
2. Results & Discussion: Synthesis of unnatural α-amino acids

One of the initial aims of this project was to prepare racemic β- and γ-substituted α-amino acids for use as substrates in the deracemisation reaction. Thereafter, work would be directed towards preparation of α-amino acids containing stereochemically defined centres at the β- or γ-position and a mixture of R- and S- at the α-position.

2.1. Strecker synthesis

The Strecker synthesis was first reported in 1850 and is one of the oldest known methods for the synthesis of α-amino acids.7 The reaction is carried out at room temperature, on an equimolar scale under aqueous conditions, and is a popular choice for the large-scale synthesis of α-amino acids. In the reaction an aminonitrile 108 is formed through addition of a cyano donor to an imine 107 derived from an aldehyde 106 and ammonia. The aminonitrile is then hydrolysed to the corresponding amino acid 109 under acidic conditions (figure 2.1.1).

![Figure 2.1.1: Strecker synthesis of an α-amino acid](image)

2.1.1. Synthesis of (2RS,3R5)-2-amino-3-phenylbutanoic acid

![Synthesis of (2RS,3RS)-2-amino-3-phenylbutanoic acid](image)
A suspension of the aldehyde 110 in water was treated with ammonium chloride and potassium cyanide. The resulting aminonitrile 111 was extracted into ether and then into dilute HCl. It was found that the volume of aqueous acid used for extraction was crucial to obtaining even a reasonable yield (table 2.1.1). The low yield of 111 was probably due to the reversible nature of the imine formation reaction when carried out in water. Excess ammonia and cyanide donor may drive the reaction to completion.

<table>
<thead>
<tr>
<th>aq. HCl extraction volume</th>
<th>Yield/ %</th>
<th>d.r. a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 50 ml</td>
<td>9</td>
<td>70:30</td>
</tr>
<tr>
<td>2 165 ml</td>
<td>26</td>
<td>70:30</td>
</tr>
</tbody>
</table>

a) calculated from integration of HPLC peak areas

Hydrolysis of 111 proceeded in 70% yield to produce the amino acid 112 with an overall yield of 18%. The diastereomeric excess (40%) observed by $^{13}$C NMR and HPLC is most likely due to a preference for the (2R,3R) and (2S,3S) enantiomers (figure 2.1.2), these being the diastereomers with the least unfavourable steric interactions. HPLC analysis showed a ratio of 35:35:15:15 (L:D:L:D), where L and D are the configuration at the α-centre and assigned by their behaviour with D-amino acid oxidase. Following the above argument the elution order would be (2S,3S), (2R,3R), (2S,3R), (2R,3S).

![Figure 2.1.2: diastereoselectivity of the Strecker reaction](image-url)
2.1.2. Synthesis of (2RS,3RS)-2-amino-3-methylhexanoic acid

Following the optimised reaction conditions used in § 2.1.1, the desired amino acid 115 was recovered in 23% yield. Again HPLC analysis showed 40% d.e., but with an elution order of (2S,3R):(2S,3S):(2R,3S):(2R,3R) (15:35:15:35).

Although sufficient material for deracemisation experiments was obtained via the Strecker synthesis, it was decided that the low yield of the reaction and the high toxicity of the potassium cyanide warranted research into other methods for the synthesis of α-amino acids.

2.2. Alkylation of N-(diphenylmethylene)aminoacetonitrile

The alkylation of glycine anion equivalents has received much attention in the asymmetric synthesis of α-amino acids (§ 1.1.1). However, it is also a useful methodology for the synthesis of racemic α-amino acids. The alkylation of an imine glycinate developed in the late 1970s by O'Donnell et al. (figure 2.2.1),125,126 offers an attractive alternative to the Strecker synthesis, as the methodology uses cheap and readily available starting materials.

Figure 2.2.1: alkylation of 117, N-(diphenylmethylene)aminoacetonitrile
2.2.1. Synthesis of (2RS,3R5)-2-amino-3-phenylbutanoic acid

\[
\begin{align*}
\text{Me} & \quad + \quad \text{CN} \\
\text{Ph} & \quad \text{Br} \quad \text{base} \quad \text{Me} \\
\text{N}_{2}\text{CPh}_2 & \quad \text{c.HCl} \quad \text{Me} \\
120 & \quad 117 \quad 121 \quad 112
\end{align*}
\]

Initially, the reaction was attempted under phase-transfer catalysis (PTC) conditions, due to the attractive reaction and separation conditions this method offers, i.e. room temperature, catalytic and bi-phasic. Benzytributylammonium bromide was employed as the PT catalyst in aqueous NaOH:DCM (1:1). However, upon work-up a mixture of both starting materials and the desired iminonitrile intermediate 121 was recovered. A search of the literature revealed that refluxing in acetonitrile in the presence of potassium carbonate should be sufficient for alkylation to occur. After 24 h, excess base was removed and 121 was recovered in 78% yield. Hydrolysis proceeded quantitatively to yield the corresponding amino acid 112 as a colourless solid in 65% overall yield. The diastereomeric excess of the reaction was 40%, the same as observed in the Strecker reaction (§ 2.1.1), indicating the diastereoselectivity of the reactions was a result of thermodynamic equilibration. For successful alkylation five equivalents of base were required, less than five equivalents resulted in remaining starting material, which could be removed by chromatography. The O'Donnell alkylation methodology provided a higher yielding, less toxic route to the amino acid, 2-amino-3-phenylbutanoic acid 112.

2.2.2. Synthesis of (2RS,3RS)-2-amino-3-ethylhexanoic acid

\[
\begin{align*}
\text{Me} & \quad + \quad \text{CN} \\
\text{Me} & \quad \text{Br} \quad \text{base} \quad \text{Me} \\
\text{N}_{2}\text{CPh}_2 & \quad \text{c.HCl} \quad \text{Me} \\
122 & \quad 117 \quad 123 \quad 124
\end{align*}
\]

The preparation of 2-amino-3-ethylhexanoic acid 124 was attempted via the alkylation methodology used in § 2.2.1. The bromide 122 and imine 117 were refluxed for 24 h, and then for 48 h in acetonitrile, in the presence of potassium carbonate, which should be suitable for alkylation to occur. After 24 h, excess base was removed and 123 was recovered in 78% yield. Hydrolysis proceeded quantitatively to yield the corresponding amino acid 124 as a colourless solid in 65% overall yield. The diastereomeric excess of the reaction was 40%, the same as observed in the Strecker reaction (§ 2.1.1), indicating the diastereoselectivity of the reactions was a result of thermodynamic equilibration. For successful alkylation five equivalents of base were required, less than five equivalents resulted in remaining starting material, which could be removed by chromatography. The O'Donnell alkylation methodology provided a higher yielding, less toxic route to the amino acid, 2-amino-3-ethylhexanoic acid 124.
carbonate. After separation only the imine glycinate starting material 117 was observed by $^1$H NMR. The reaction was repeated with a reaction time of 5 days, excess base was removed and the solution concentrated under vacuum to leave an oil with a $^1$H NMR spectrum consistent with the intermediate aminonitrile 123. However, after hydrolysis the $^1$H NMR spectrum of the product showed only aromatic protons. This spectrum was consistent with benzophenone, which is the other product of hydrolysis. It is probable that the amino acid 124 was not soluble in chloroform, the solvent used for analysis, whereas benzophenone is. Regardless, the long reaction times required to drive the reaction to completion made this an unattractive method for the synthesis of $\alpha$-amino acid 124.

The reaction was next attempted using the PT catalyst cetyltrimethylammonium bromide (125) and sodium hydroxide as base. After isolation $^1$H NMR showed a spectrum consistent with 125 and benzophenone. The PTC experiments showed that the unactivated halide 122 was not effectively alkylated under the alkylation conditions attempted. However, activation of the bromide by in situ iodine exchange looked to be a viable alternative. The PT catalyst tetrabutylammonium iodide (126) was chosen as the iodine exchange reagent, but with no success. Only 126 and benzophenone were isolated from the reaction mixture.

The literature available on the O'Donnell alkylation methodology revealed that alkylation of glycine equivalents by simple alkyl halides such as methyl- and ethylbromide could be achieved by using $n$-butyllithium for the deprotonation of the imine glycinate 117. When this was attempted with bromide 122, the intermediate 123 was obtained in 67% yield, after chromatography. Hydrolysis of the intermediate proceeded with only 50% yield, to give 2-amino-3-ethylhexanoic acid 124 (32% overall yield). $^{13}$C NMR and HPLC showed no diastereomeric excess, probably due to the similar steric bulk of the two alkyl groups. The low yield of the hydrolysis step, compared with the quantitative hydrolysis of compound 121, could be a result of steric hinderance by the $\beta$-ethyl group.
2.2.3. Preparation of (2RS,4RS)-2-amino-4-ethyloctanoic acid

It was hoped that primary bromides would be more reactive under the milder PTC or potassium carbonate conditions (§ 2.2.1) compared with the secondary bromide 122. However, as in the synthesis of 2-amino-3-ethylhexanoic acid, only the starting materials were observed. Switching to the use of n-butyllithium at -78 °C yielded the desired amino acid 129, in 87% yield. Hydrolysis proceeded quantitatively and $^{13}$C NMR showed a diastereomeric ratio of ~ 55:45. The lower diastereoselectivity compared with the β-substituted α-amino acids results from the second stereogenic centre being one position further away from the reacting centre.

2.2.4. Preparation of (2RS,4RS)-2-amino-4-phenylpentanoic acid

Attempts at the synthesis of 2-amino-4-phenylpentanoic acid proved unsuccessful. Using n-butyllithium as base yielded an oil with a mass spectrum consistent with the intermediate 131 and unknown impurities. However, upon chromatography the compound decomposed and no intermediate was recovered. Attempts at alkylation using potassium carbonate as the base resulted in recovery of the starting materials only.
2.2.5. Preparation of (2RS,4S)-2-amino-4-methylhexanoic acid

One of the aims of this work was to synthesise α-amino acids with epimeric α-centres and stereochemically defined β- or γ-centres. Conceivably this could be achieved by using an enantiomerically pure bromide in the O'Donnell alkylation reaction. However, the reaction may suffer from epimerisation at the stereochemically defined centre. In an attempt to realise this goal, the commercially available enantiomerically pure bromide 132 was used in the alkylation of 117. It was hoped that a primary bromide would be more reactive than a secondary bromide and that racemisation of the bromide would be less likely to occur if the stereogenic centre was positioned beta to the reacting centre. The reaction was first attempted with n-butyllithium as previous reactions had shown most success with this base. The intermediate 133 was recovered in 67% yield after chromatography, with $\alpha_D = +14.3^\circ$ (c = 1.0, CHCl$_3$).

Hydrolysis proceeded to give α-amino acid 134 as a white solid in 65% yield, with $\alpha_D = +7.0^\circ$ (c = 1.0, H$_2$O). The $^{13}$C NMR spectrum showed diastereomeric signals, indicating that some epimerisation at the γ-centre had occurred. This was confirmed by HPLC analysis as integration of the HPLC peak area showed a d.e. of 88%.

Combining the high cost and limited availability of optically active bromides with the racemisation problems observed makes the alkylation methodology unsuitable for the preparation of β- and γ-substituted α-amino acids bearing optically active centres at the β- and γ-positions.

2.3. Conclusions & Future Work

Studies into the preparation of β- and γ-substituted α-amino acids have concentrated on the Strecker synthesis and alkylation of glycinate imines as developed by O'Donnell et al. However, both syntheses have their limitations. The Strecker synthesis suffers from low yields and highly toxic reagents. A recent paper has shown an improvement in yield for the Strecker synthesis (>90%) by using lithium
perchlorate/diethyl ether solution and trimethylsilyl cyanide. However, the synthesis still involves the use of highly toxic chemicals. The O’Donnell methodology is not effective when using certain bromides, especially non-activated bromides. However, further optimisation of the reaction by alteration of the glycine equivalent may yield the desired amino acids.

Another area that requires study is the synthesis of α-amino acids with non-stereochemically defined α-centres and stereochemically defined β-centres. To date no reports have been made on such compounds, and their synthesis will be challenging. A recent method reported in the literature may prove interesting, where allylation of azlactones is achieved under catalytic conditions (figure 2.3.1).

\[
\begin{align*}
&\text{135} + \text{136} \rightarrow 7.5 \text{ mol\% 138} \\
&2.5 \text{ mol\% Pd}^{\text{II}} \rightarrow \text{137}
\end{align*}
\]

\[
\text{R} = \text{CH}_3, \text{CH}_2\text{Ph}
\]

Figure 2.3.1: allylation of azlactones

The diastereomeric ratio in this reaction can be up to 19:1, with e.e.s of 99%. It may be possible to modify this reaction to maintain selectivity at the β-position but to have R = H, resulting in a mixture of R- and S- at the α-position.
3. Results & Discussion: Deracemisation of
\( \alpha \)-amino acids

The ultimate aim of this project was to develop a general system for the
deracemisation of racemic chiral amines and \( \alpha \)-amino acids using oxidase enzymes
(\S\ 1.4) and chemical reduction in a cyclic oxidation-reduction sequence. Soda et al.
demonstrated the practical feasibility of this method for \( \alpha \)-amino acids in the early
1990s.\textsuperscript{82,83} This was recently further developed in Edinburgh to include acyclic
natural and unnatural \( \alpha \)-amino acids.\textsuperscript{84,86} Initial work on the deracemisation strategy
focused on further modification of the method to encompass other natural \( \alpha \)-amino
acids and \( \beta \)- and \( \gamma \)-substituted \( \alpha \)-amino acids, and to examine the use of D-amino
acid oxidase from \textit{T. variabilis} in the deracemisation reaction.

3.1. Deracemisation of natural \( \alpha \)-amino acids

3.1.1. Deracemisation using pk-DAAO

In order to establish reproducibility, the reaction was first carried out using
pk-DAAO and sodium cyanoborohydride as developed in Edinburgh.\textsuperscript{84} The amino
acids proline 92 and phenylalanine 139 were used as substrates, at a concentration of
2.5 mM. Reactions were incubated with catalase and FAD in an orbital shaker, at
37 °C, until no further reaction was observed (table 3.1.1). One unit of enzyme
catalyses the oxidation of 1 \( \mu \)mol of D-alanine per minute under standard conditions.
From entries 1-3 it can be seen that the amount of pk-DAAO must be > 0.8 U to
achieve high yields, and that the reaction appears reproducible (entries 2&3).
However, the use of excess enzyme and reducing agent lowers the yield of the
reaction (entry 4). Acyclic \( \alpha \)-imino acids are not as stable in aqueous conditions as
their cyclic counterparts and as a result lower yields are observed for phenylalanine
compared with proline (entry 5). To avoid competing hydrolysis of the \( \alpha \)-imino acid
(140) to the \( \alpha \)-keto acid (141), \textit{i.e.} \( k_H > k_R \) (figure 3.1.1), sodium borohydride was
used as the reducing agent as it has a higher reactivity towards imines compared with sodium cyanoborohydride. It has been shown that in reactions with NaCNBH₃ the reduction of the imine is the rate-limiting step.

![Chemical Reaction Diagram](image)

**Figure 3.1.1: deracemisation of DL-phenylalanine**

**Table 3.1.1**

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>U</th>
<th>[H] (eq.)</th>
<th>Time/h</th>
<th>Yield/%</th>
<th>e.e./%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.2.1.1</td>
<td>92</td>
<td>0.80 NaCNBH₃ (6.4)</td>
<td>6.0</td>
<td>58</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2</td>
<td>8.2.1.1</td>
<td>92</td>
<td>1.60 NaCNBH₃ (12.8)</td>
<td>24.0</td>
<td>94</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3</td>
<td>8.2.1.1</td>
<td>92</td>
<td>1.60 NaCNBH₃ (6.4)</td>
<td>2.5</td>
<td>94</td>
<td>&gt;99</td>
</tr>
<tr>
<td>4</td>
<td>8.2.1.1</td>
<td>92</td>
<td>2.60 NaCNBH₃ (25.6)</td>
<td>2.5</td>
<td>82</td>
<td>&gt;99</td>
</tr>
<tr>
<td>5</td>
<td>8.2.1.3</td>
<td>139</td>
<td>1.60 NaBH₄ (31.6)</td>
<td>20.0</td>
<td>74</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol of D-alanine oxidised per minute

The success of the reaction with proline prompted us to try the reaction on a larger scale (§ 8.2.1.2). A 25 mM solution of DL-proline in ammonium formate buffer, pH 6.5, was incubated at 37 °C with pk-DAAO (13 U), NaCNBH₃ (10 eq.), FAD (0.4 mM) and catalase for 20 h. After this time HPLC analysis showed a yield of 94% of L-proline with an e.e. >99%.
3.1.2. Deracemisation using tv-DAAO

The use of DAAO from porcine kidney would be problematic when performing large-scale reactions. Although commercially available, the enzyme is expensive in bulk quantities, being from a mammalian source, and it also requires the addition of an external cofactor, FAD, raising the cost of the reaction further. tv-DAAO was chosen as an alternative enzyme as it is readily available in bulk quantities as a whole cell preparation, therefore eliminating the need to add the external cofactor. This commercially available enzyme has the added advantage that it is supplied in immobilised form, thereby simplifying recovery from the reaction.

For comparison purposes, the deracemisation of natural α-amino acids using tv-DAAO was initially attempted (table 3.1.2). Reactions were carried out at 30 °C as this was found to be the optimum temperature for the enzyme. One unit of enzyme catalyses the oxidation of 1 μmol of cephalosporin C per minute under standard reaction conditions.
Table 3.1.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>U a</th>
<th>[H] (eq.)</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.2.2.3</td>
<td>142</td>
<td>1.70 NaCNBH₃ (10.0)</td>
<td>22.0</td>
<td>59</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2</td>
<td>8.2.2.3</td>
<td>142</td>
<td>1.70 NaCNBH₃ (20.0)</td>
<td>22.0</td>
<td>66</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3</td>
<td>8.2.2.3</td>
<td>142</td>
<td>1.70 NaCNBH₃ (30.0)</td>
<td>22.0</td>
<td>75</td>
<td>&gt;99</td>
</tr>
<tr>
<td>4</td>
<td>8.2.2.3</td>
<td>142</td>
<td>1.70 NaCNBH₃ (40.0)</td>
<td>22.0</td>
<td>77</td>
<td>&gt;99</td>
</tr>
<tr>
<td>5</td>
<td>8.2.2.3</td>
<td>139</td>
<td>1.70 NaCNBH₃ (40.0)</td>
<td>22.0</td>
<td>66</td>
<td>&gt;99</td>
</tr>
<tr>
<td>6</td>
<td>8.2.2.3</td>
<td>139</td>
<td>3.40 NaCNBH₃ (63.5)</td>
<td>22.0</td>
<td>66</td>
<td>&gt;99</td>
</tr>
<tr>
<td>7</td>
<td>8.2.2.2</td>
<td>139</td>
<td>0.68 NaBH₄ (42.1)</td>
<td>3.5</td>
<td>64</td>
<td>&gt;99</td>
</tr>
<tr>
<td>8</td>
<td>8.2.2.2 b</td>
<td>139</td>
<td>0.68 NaBH₄ (42.1)</td>
<td>3.5</td>
<td>54</td>
<td>&gt;99</td>
</tr>
<tr>
<td>9</td>
<td>8.2.2.2 c</td>
<td>139</td>
<td>0.68 NaBH₄ (42.1)</td>
<td>3.0</td>
<td>54</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol of cephalosporin C oxidised per minute; b) 0.34 U added after 1 h; c) 5 eq. NH₄CO₂ added

The deracemisation of DL-leucine 142 was attempted with NaCNBH₃ and was optimised to yield optically pure L-leucine in 77% (entries 1-4). However, the deracemisation of DL-phenylalanine gave only low yields of L-phenylalanine, irrespective of the reducing agent used or the amount of enzyme added (entries 5-8).

It is known that the α-keto acid hydrolysis products are inhibitors of amino acid oxidases, especially when in a highly conjugated state, e.g. the enol tautomer of phenylpyruvic acid 141 (figure 3.1.3). Previously NH₄CO₂ was successfully used to increase the yield of deracemisation by reversing the hydrolysis step. However, in this case, yields remained low when NH₄CO₂ was added (entry 9).

Figure 3.1.3: tautomereism of phenylpyruvic acid 141
Reactions with DL-proline showed that it is a poor substrate for tv-DAAO. Incubation of DL-proline with tv-DAAO at 30 °C resulted in incomplete oxidation of D-proline after 6 h (e.e. = 86%). By comparison, oxidation of D-proline by pk-DAAO is complete within 80 min and oxidation of D-phenylalanine by tv-DAAO is complete within 30 min. This suggests that for the successful deracemisation of a wide range of substrates a set of enzymes with complementary and overlapping substrate specificities will be needed.

The deracemisation of DL-phenylalanine, DL-leucine and DL-methionine 143, using tv-DAAO and NaCNBH3 was attempted on a 25 mM scale (table 3.1.3). The high enantiomeric excesses (>94%) indicated that the enzyme was capable of oxidising the substrates at this concentration. However, the low yields showed that more reducing agent is required to bring about successful deracemisation.

Table 3.1.3

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>Ua</th>
<th>[H] (eq.)</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.2.2.4 139</td>
<td>17.0</td>
<td>NaCNBH3 (10.2)</td>
<td>22.0</td>
<td>46</td>
<td>94</td>
</tr>
<tr>
<td>2</td>
<td>8.2.2.4 142</td>
<td>17.0</td>
<td>NaCNBH3 (10.2)</td>
<td>22.0</td>
<td>52</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3</td>
<td>8.2.2.4 143</td>
<td>17.0</td>
<td>NaCNBH3 (10.2)</td>
<td>22.0</td>
<td>48</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 µmol of cephalosporin C oxidised per minute

3.2. Deracemisation of unnatural α-amino acids

After the success of the reactions using natural α-amino acids, our attention turned to the β- and γ-substituted α-amino acids synthesised in chapter 2. These compounds are important in the studies of peptidomimetics and enzyme inhibitors, 31,124 as they introduce conformational constraint to the secondary structure of polypeptides. It was first necessary to determine if such compounds would be accepted as substrates by tv-DAAO (table 3.2.1).
Table 3.2.1

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>Conc./ mM</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>d.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>112</td>
<td>2.5</td>
<td>0.68</td>
<td>2.0</td>
<td>48</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>115</td>
<td>2.5</td>
<td>0.68</td>
<td>3.0</td>
<td>52</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>112</td>
<td>25.0</td>
<td>6.8</td>
<td>5.0</td>
<td>61</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>112</td>
<td>25.0</td>
<td>8.5</td>
<td>22.0</td>
<td>44</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>134</td>
<td>25.0</td>
<td>8.5</td>
<td>22.0</td>
<td>47</td>
<td>&gt;99 b</td>
</tr>
</tbody>
</table>

a) 1 U = 1 µmol of cephalosporin C oxidised per minute; b) e.e. quoted

Although incomplete oxidation of the major 2R-diastereomer, i.e. 2R,3R, was observed in both cases, the β-substituted α-amino acids 2-amino-3-phenylbutanoic acid 112 and 2-amino-3-methylhexanoic acid 115 appear to be substrates for tv-DAAO, albeit with lower activity than the natural α-amino acids. The γ-substituted α-amino acid 2-amino-4-methylhexanoic acid 134 was also a substrate for tv-DAAO. However, attempts at oxidation of the amino acid 2-amino-3-ethylhexanoic acid 124 proved difficult. In the first attempt (§ 8.2.1.2), using 0.68 U of tv-DAAO, the first three chromatographic peaks were approximately 75% of their original integration, and the fourth chromatographic peak had completely disappeared. Using 1.7 U of tv-DAAO in an attempt to drive the reaction to completion, the first two peaks showed approximately 135% of their original integration, the third peak was approximately 80% and the fourth peak had disappeared. This unexpected result would appear to indicate a problem with the integration of the chromatographic peaks. Due to the negligible UV absorbance of the amino acid the peak area is very small, resulting in large errors in integration. No further reactions were attempted with this amino acid. Similar problems with HPLC analysis were observed for the amino acid (2RS,4RS)-2-amino-4-ethyloctanoic acid and no oxidation studies were attempted.

The deracemisation of the β- and γ-substituted α-amino acids was next attempted (table 3.2.2). Theoretically the four diastereoisomers should be converted to a single
pair of enantiomers in a 1:1 ratio. Figure 3.2.1 shows that after only three cycles the majority of the amino acid bears \( S \)-specificity at the \( \alpha \)-position, with an approximately equal proportion of \( R \) - and \( S \) - at the \( \beta \)-position.

![Diagram of interconversion of stereoisomers](image)

Figure 3.2.1: interconversion of stereoisomers of \( \beta \)-substituted \( \alpha \)-amino acids

Table 3.2.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>U (^a)</th>
<th>([H]) (eq.)</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.3.1.3 b</td>
<td>115</td>
<td>0.68</td>
<td>NaCNBH(_3) (25.4)</td>
<td>18.0</td>
<td>63</td>
</tr>
<tr>
<td>2</td>
<td>8.3.1.3</td>
<td>115</td>
<td>0.68</td>
<td>NaBH(_4) (42.1)</td>
<td>3.0</td>
<td>94</td>
</tr>
<tr>
<td>3</td>
<td>8.3.1.3</td>
<td>115</td>
<td>0.68</td>
<td>NaBH(_4) (42.1)</td>
<td>3.5</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>8.3.1.3</td>
<td>112</td>
<td>0.68</td>
<td>NaCNBH(_3) (25.4)</td>
<td>2.0</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>8.3.1.4</td>
<td>112</td>
<td>1.70</td>
<td>NaCNBH(_3) (38.1)</td>
<td>22.0</td>
<td>68</td>
</tr>
<tr>
<td>6</td>
<td>8.3.1.4</td>
<td>112</td>
<td>3.40</td>
<td>NaCNBH(_3) (63.5)</td>
<td>22.0</td>
<td>86</td>
</tr>
<tr>
<td>7</td>
<td>8.3.1.3</td>
<td>112</td>
<td>0.68</td>
<td>NaBH(_4) (42.1)</td>
<td>6.0</td>
<td>71</td>
</tr>
<tr>
<td>8</td>
<td>8.3.1.4</td>
<td>134</td>
<td>1.70</td>
<td>NaCNBH(_3) (38.1)</td>
<td>22.0</td>
<td>66</td>
</tr>
<tr>
<td>9</td>
<td>8.3.1.4</td>
<td>134</td>
<td>3.40</td>
<td>NaCNBH(_3) (63.5)</td>
<td>22.0</td>
<td>60</td>
</tr>
</tbody>
</table>

\(^a\) a) 1 \( U = 1 \) \( \mu \)mol of cephalosporin C oxidised per minute; \( b \) 5 eq. \( \text{NH}_4\text{CO}_2 \) added; \( c \) diastereomeric excess quoted
Not all the reactions proceeded to completion and the quoted yield is the sum of any diastereoisomers remaining at the end of the reaction. For 2-amino-3-phenylbutanoic acid, the 2R,3S isomer was completely depleted, however some 2R,3R isomer remained, i.e. incomplete oxidation of the R-enantiomer was observed. In these cases the d.e. is quoted instead of e.e. For 2-amino-3-methylhexanoic acid and 2-amino-4-methylhexanoic acid, only the stereoisomers with S-configuration at the 2-position remain at completion of the reaction.

The reactions with sodium cyanoborohydride were not efficient and in the case of 2-amino-3-phenylbutanoic acid resulted in incomplete oxidation of the R-enantiomer (entries 4-7). Addition of excess NaCNBH₃ and enzyme actually resulted in a lower yield of (2S,4S)-2-amino-4-methylhexanoic acid (entries 8&9). Replacing NaCNBH₃ with NaBH₄ allowed the amino acids 2-amino-3-methylhexanoic acid and 2-amino-3-phenylbutanoic acid to be prepared in optically pure form in good to excellent yields (entries 2,3&7). Once again the amino acid with its α-keto acid in a conjugated state resulted in a lower yield (entry 7, figure 3.2.2).

\[
\begin{align*}
\text{Me} & \quad \text{Me} \\
\text{\textbf{144}} \\
\end{align*}
\]

Figure 3.2.2: tautomerism of 2-keto-3-phenylbutanoic acid, 144

Attempts at deracemisation on a 25 mM scale resulted in only oxidation of the R-enantiomer due to insufficient reducing agent present (table 3.2.3).

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>U *</th>
<th>[H] (eq.)</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.3.1.6 112</td>
<td>25.5</td>
<td>NaCNBH₃ (5.1)</td>
<td>26.0</td>
<td>58</td>
<td>24 b</td>
</tr>
<tr>
<td>2</td>
<td>8.3.1.6 112</td>
<td>17.0</td>
<td>NaCNBH₃ (10.2)</td>
<td>42.0</td>
<td>60</td>
<td>22 b</td>
</tr>
<tr>
<td>3</td>
<td>8.3.1.6 134</td>
<td>25.5</td>
<td>NaCNBH₃ (5.1)</td>
<td>28.0</td>
<td>50</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

Table 3.2.3
### 3.3. Conclusions & Future Work

The deracemisation of β-substituted α-amino acids using tv-DAAO and borohydride reducing agents has been demonstrated. NaCNBH₃ has been successfully used in the deracemisation of acyclic α-amino acids for the first time. Certain aromatic α-amino acids are not deracemised as efficiently as aliphatic α-amino acids due to the inhibition of the enzyme by the α-keto acid by-product. The scale-up of the reaction remains to be optimised, however, the alternative reducing agents now developed for the production of D-amino acids may improve the success of the deracemisation of α-amino acids bearing two stereogenic centres.⁸⁶

In previous work the cyclic oxidation-reduction sequence has been successfully used in the stereoinversion of α-amino acids from the R- to the S-enantiomer and vice versa.⁸⁴,⁸⁶ Asymmetric hydrogenation (§ 1.1.3) is often used to prepare β-substituted α-amino acids with both centres stereochemically defined. The drawback of this approach is that, due to the nature of the catalyst, hydrogen can only be added in a syn fashion. A combination of asymmetric hydrogenation and stereoinversion should allow access to all four diastereomers of β-substituted α-amino acids (figure 3.3.1).

---

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Sub.</th>
<th>U</th>
<th>[H] (eq.)</th>
<th>Time/ h</th>
<th>Yield/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>8.3.1.6</td>
<td>134</td>
<td>17.0</td>
<td>NaCNBH₃ (10.2)</td>
<td>42.0</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>8.3.1.6</td>
<td>115</td>
<td>42.5</td>
<td>NaCNBH₃ (10.2)</td>
<td>22.0</td>
<td>58</td>
</tr>
<tr>
<td>6</td>
<td>8.3.1.6</td>
<td>115</td>
<td>17.0</td>
<td>NaBH₄ (10.0)</td>
<td>22.0</td>
<td>46</td>
</tr>
</tbody>
</table>

a) a) 1 U = 1 μmol of cephalosporin C oxidised per minute; b) d.e. quoted

---

Figure 3.3.1: chemoenzymatic synthesis of β-substituted α-amino acids
4. Results & Discussion: Screening for oxidase enzymes

In order to broaden the scope of the deracemisation reaction to include production of D-\(\alpha\)-amino acids and chiral amines, it is necessary to have access to a wider range of oxidase enzymes. The production of D-\(\alpha\)-amino acids from the racemic mixture is dependent on finding commercially viable sources of L-amino acid oxidase. To prove the concept of amine deracemisation, flavin-dependent monoamine oxidases exhibiting stereoselectivity are required. Examination of the literature revealed a variety of organisms displaying amino acid and amine oxidase activities (§ 1.4), therefore it was decided to screen randomly selected organisms in an attempt to find novel LAAO and MAO activities.

4.1. Development of high-throughput assay

Screening a large library of organisms requires an efficient assay method in order to maximise the probability of identifying a “hit”. The production of a dye via enzymatic reaction is a common method for the measurement of enzyme activity,\(^{135,136}\) as it allows quick and easy identification of possible hits and can also be adapted to quantitative methods using a spectrophotometer in conjunction with a kinetics-measuring programme. Hydrogen peroxide is a product in the oxidation of amines and L-amino acids by monoamine oxidase and L-amino acid oxidase respectively. A number of assays have been developed that allow measurement of enzymatic activity based on hydrogen peroxide production.\(^{137,138}\) Hydrogen peroxide is reduced to water in the presence of horseradish peroxidase (HRP) and a proton donor (e.g. 4-aminoantipyrine, AAP, 145). The proton donor for this peroxidase reaction is oxidised in the process and subsequently reacts with an acid, in this case vanillic acid (146), to form a soluble dye 147 (figure 4.1.1).\(^{137}\) The absorbance change at 500 nm can be measured and used to calculate the activity of the oxidase enzyme.
Figure 4.1.1: peroxidase-coupled colorimetric assay *

Activity (U.ml$^{-1}$) = initial rate(Au.s$^{-1}$) $\times$ ($1/\varepsilon$) $\times$ 60 $\times$ ($t.v./s.v.$) $\times$ $1\times10^3$.

where $\varepsilon$ = molar extinction coefficient (M$^{-1}$.cm$^{-1}$)

t.v. = total reaction volume
s.v. = sample volume

4.1.1. Validation of assay

A series of experiments were carried out using a Cobas Mira discrete data analyser (§ 9.1). The optimum reagent concentrations were determined and linearity and reproducibility of the assay proved (table 4.1.1). Benzylamine was used as the substrate, which reacts with MAO to give benzaldehyde. The HRP, AAP and vanillic acid were pre-mixed in a 1:1:1 ratio (§ 9.5.17). Plasma amine oxidase (PAO, Sigma #M4636, 1.1 U.ml$^{-1}$) was used as the standard for monoamine oxidase assay and a 3% H$_2$O$_2$ solution was prepared for use as positive control.

* exact structure of dye has not been determined.
Table 4.1.1

<table>
<thead>
<tr>
<th>T/ °C</th>
<th>[BzNH₂]</th>
<th>Sample a</th>
<th>Activity (U.ml⁻¹) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 30</td>
<td>0.2 mM</td>
<td>H₂O₂ (0.04%)</td>
<td>-0.18</td>
</tr>
<tr>
<td>2 30</td>
<td>0.2 mM</td>
<td>PAO (0.015 U)</td>
<td>0.01</td>
</tr>
<tr>
<td>3 30</td>
<td>0.4 mM</td>
<td>H₂O₂ (0.04%)</td>
<td>-0.18</td>
</tr>
<tr>
<td>4 30</td>
<td>0.4 mM</td>
<td>PAO (0.015 U)</td>
<td>0.03</td>
</tr>
<tr>
<td>5 37</td>
<td>0.4 mM</td>
<td>H₂O₂ (0.04%)</td>
<td>-0.23</td>
</tr>
<tr>
<td>6 37</td>
<td>0.4 mM</td>
<td>PAO (0.015 U)</td>
<td>0.05</td>
</tr>
<tr>
<td>7 37</td>
<td>1.6 mM</td>
<td>H₂O₂ (0.40%)</td>
<td>0.00</td>
</tr>
<tr>
<td>8 37</td>
<td>1.6 mM</td>
<td>H₂O₂ (0.08%)</td>
<td>-0.07</td>
</tr>
<tr>
<td>9 37</td>
<td>1.6 mM</td>
<td>H₂O₂ (0.04%)</td>
<td>-0.18</td>
</tr>
<tr>
<td>10 37</td>
<td>1.6 mM</td>
<td>H₂O₂ (0.02%)</td>
<td>-0.36</td>
</tr>
<tr>
<td>11 37</td>
<td>1.6 mM</td>
<td>PAO (0.015 U)</td>
<td>0.11</td>
</tr>
<tr>
<td>12 37</td>
<td>3.2 mM</td>
<td>PAO (0.015 U)</td>
<td>0.14</td>
</tr>
<tr>
<td>13 c 37</td>
<td>3.2 mM</td>
<td>PAO (0.015 U)</td>
<td>0.16</td>
</tr>
<tr>
<td>14 37</td>
<td>3.2 mM</td>
<td>PAO (0.075 U)</td>
<td>0.42</td>
</tr>
<tr>
<td>15 37</td>
<td>3.2 mM</td>
<td>PAO (0.015 U)</td>
<td>0.18</td>
</tr>
<tr>
<td>16 37</td>
<td>3.2 mM</td>
<td>PAO (0.010 U)</td>
<td>0.12</td>
</tr>
<tr>
<td>17 37</td>
<td>3.2 mM</td>
<td>PAO (0.008 U)</td>
<td>0.09</td>
</tr>
<tr>
<td>18 37</td>
<td>3.2 mM</td>
<td>PAO (0.005 U)</td>
<td>0.06</td>
</tr>
<tr>
<td>19 37</td>
<td>3.2 mM</td>
<td>PAO (0.004 U)</td>
<td>0.05</td>
</tr>
</tbody>
</table>

a) 1 U = 1 µmol of benzylamine oxidised per minute; b) negative activity is a result of non-linear change in absorbance; c) reagent concentration halved

Concentrations of hydrogen peroxide above 0.08% show a very low change in absorbance. This is probably due to denaturation of the HRP at high H₂O₂
concentrations. The concentration of benzylamine was varied until no significant variation in activity was observed (entries 2, 4, 6, 11 & 12). When the concentration of reagent was halved (entry 13) no difference in activity was observed showing that the HRP reaction or dye-formation were not rate limiting. Linearity (entries 14-19) has been proved within the range 0.025 to 0.1 (figure 4.1.2) and reproducibility experiments showed c.v. <2% (calculated by Cobas Mira), i.e. within the acceptable limits of ± 5%.

Figure 4.1.2: linearity of MAO assay

L-Amino acid oxidase purified from snake venom (Sigma #A8390, 1.0 U.ml⁻¹) was used as the standard for the L-amino acid oxidase assay. L-Phenylalanine was used as substrate, which reacts with LAAO to give phenylpyruvic acid and hydrogen peroxide. Linearity and reproducibility experiments, using optimised concentrations from monoamine oxidase assay, were within acceptable limits of ± 5%.

4.2. Screening of culture collections

4.2.1. Monoamine oxidase

Both copper and FAD dependent amine oxidases (AO) are prevalent in microbial sources. The copper dependent AO is mainly found in bacteria and yeast, whereas the flavin dependent AO is present in a number of fungi. Although copper dependent
AO is not a viable enzyme for amine deracemisation due to the covalent binding of
the imine to the active site of the enzyme, the screening of bacterial and yeast
sources was undertaken as it has been shown that the fungus *A. niger* produces both a
copper dependent AO and a flavin dependent form of MAO.\(^{120}\)

The organisms used in the screening program were chosen from the literature and
from a random selection of the GlaxoSmithKline biotransformation collection.
Organisms were selected from fungal, bacterial and yeast species, and grown until
sufficient biomass was obtained. Fungal strains were grown in FS media (§ 9.5.4),
yeast strains were grown in YEPD media (§ 9.5.5) and bacterial species were grown
in SV2 media (§ 9.5.3). The biomass was harvested and then used to inoculate
induction media (growth media + 0.1% butylamine). After incubation the cells were
harvested and disrupted by sonication. Both the media supernatant and the cleared
cell lysate were assayed for monoamine oxidase activity.

Seventy-four organisms were assayed using the colorimetric assay outlined in § 4.1
(§ 11.1, entries 1-74), including a number that were cited as monoamine oxidase
producers. However, the assay showed zero absorbance change from any of the
organisms. It was thought that production of catalase by the organisms might be
preferentially reacting with the hydrogen peroxide, or the amount of H\(_2\)O\(_2\) produced
by the organism is denaturing the horseradish peroxidase. In view of this an
alternative assay method was sought. Monoamine oxidase reacts with benzylamine to
form benzaldehyde. Benzylamine has a molar extinction coefficient (\(\epsilon\)) of zero at
250 nm, whilst benzaldehyde has \(\epsilon = 12500\) M\(^{-1}\).cm\(^{-1}\). In 1954, Tabor *et al.* used this
information to assay monoamine oxidase from beef plasma.\(^{139}\) A solution of
benzylamine with enzyme was monitored at 250 nm for 10 minutes. Activity
(U.ml\(^{-1}\)) can be calculated using the same format as before. A number of organisms
were assayed using this method (§ 11.1, entries 75-143), with some showing a
change in absorbance at 250 nm (table 4.2.1).
<table>
<thead>
<tr>
<th>Organism</th>
<th>Collection no.</th>
<th>Extra-cellular activity/ U.ml⁻¹</th>
<th>Intra-cellular activity/ U.ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Penicillium chrysogenum</td>
<td>Q176 wisco</td>
<td>0.009</td>
<td>-</td>
</tr>
<tr>
<td>2 S. carlsbergensis</td>
<td>nyc 530</td>
<td>0.028</td>
<td>-</td>
</tr>
<tr>
<td>3 Schizosaccharomyces pombe</td>
<td>nyc 380</td>
<td>-</td>
<td>0.008</td>
</tr>
<tr>
<td>4 Zygosaccharomyces fermentatai</td>
<td>cbs 67.72</td>
<td>0.009</td>
<td>-</td>
</tr>
<tr>
<td>5 Curvularia lunata</td>
<td>nrrl 2434</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td>6 Absidia cylindrospora</td>
<td>nrrl 2796</td>
<td>0.009</td>
<td>-</td>
</tr>
<tr>
<td>7 Streptomyces lavendulae</td>
<td>cbs 414.59</td>
<td>-</td>
<td>0.008</td>
</tr>
<tr>
<td>8 Streptomyces griseus</td>
<td>nrrl 3851</td>
<td>-</td>
<td>0.008</td>
</tr>
<tr>
<td>9 Streptomyces punipalis</td>
<td>nrrl 3529</td>
<td>-</td>
<td>0.012</td>
</tr>
<tr>
<td>10 Mucor racemosus</td>
<td>-</td>
<td>0.011</td>
<td>-</td>
</tr>
<tr>
<td>11 A. ochraceus</td>
<td>imi 16264</td>
<td>0.002</td>
<td>0.032</td>
</tr>
<tr>
<td>12 Septomyxa affinis</td>
<td>atcc 6737</td>
<td>-</td>
<td>0.064</td>
</tr>
<tr>
<td>13 Verticillium lecanii</td>
<td>imi 68689</td>
<td>0.044</td>
<td>0.025</td>
</tr>
<tr>
<td>14 A. terreus</td>
<td>imi 045543</td>
<td>-</td>
<td>0.068</td>
</tr>
<tr>
<td>15 A. alliaceus</td>
<td>atcc 10060</td>
<td>-</td>
<td>0.010</td>
</tr>
<tr>
<td>16 Xylaria polymorpha</td>
<td>F5755</td>
<td>0.016</td>
<td>-</td>
</tr>
<tr>
<td>17 Pichia angusta</td>
<td>atcc 76722</td>
<td>0.012</td>
<td>-</td>
</tr>
<tr>
<td>18 Providencia rustigianii</td>
<td>atcc 13159</td>
<td>0.002</td>
<td>0.008</td>
</tr>
<tr>
<td>19 Serratia marcesens</td>
<td>atcc 274</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td>20 Erwinea aroideae</td>
<td>atcc 25206</td>
<td>-</td>
<td>0.008</td>
</tr>
</tbody>
</table>
The change in absorbance at 250 nm was non-linear in all these cases, and it was not possible to say if the absorbance change was due to monoamine oxidase activity or as a result of some artefact. In an attempt to overcome any ambiguity samples were submitted for LC/MS analysis. However, this proved unsuccessful because electrospray ionisation does not ionise benzaldehyde, either in the positive or negative mode. Finally, an HPLC method was developed to separate benzylamine and benzaldehyde. This method was used to screen organisms that had shown an absorbance change in the spectrophotometric assay method (§ 9.4.1). Analysis was performed at $t = 1 \text{ h}$, $t = 24 \text{ h}$, and $t = 48 \text{ h}$. Unfortunately, none of the organisms screened in this way showed a peak assignable to benzaldehyde.

From these results it appears that the organisms screened do not produce monoamine oxidase. However, the fact that some of these organisms are known producers of MAO suggests that lack of sensitivity in the assays is the reason for the lack of hits. Further optimisation of the HPLC assay may reveal novel monoamine oxidase sources.

4.2.2. L-Amino acid oxidase

L-Amino acid oxidases documented in the literature are mainly found in bacteria and fungi. As before, the organisms used in the screening program were chosen from the literature and from a random selection of the GlaxoSmithKline biotransformation collection. Organisms were selected from fungal, bacterial and yeast species and these organisms grown as for the monoamine oxidase assay. The biomass was then used to inoculate induction media (growth media + 0.1% L-Phe). After incubation the cells were harvested and disrupted by sonication. Both the media supernatant and the cleared cell lysate were assayed for L-amino acid oxidase activity.

The organisms were first assayed based on hydrogen peroxide production (§ 11.1, entries 1-74). However, as with the MAO screen, no activity was found, most likely due to production of catalase and an alternative assay was sought. Again a spectrophotometric method was available, where the absorbance change at 320 nm
was measured over 10 minutes, *i.e.* measurement of phenylpyruvic acid production. Activity (U.ml⁻¹) was calculated as before. A number of organisms were assayed using this method (§ 11.1, entries 75-143), with some showing a change in absorbance at 320 nm (table 4.2.2).

Table 4.2.2

<table>
<thead>
<tr>
<th>Organism</th>
<th>Collection no.</th>
<th>Extra-cellular activity/ U.ml⁻¹</th>
<th>Intra-cellular activity/ U.ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Proteus vulgaris</em></td>
<td>atcc 13315</td>
<td>0.029</td>
<td>-</td>
</tr>
<tr>
<td><em>Pseudomonas putida</em></td>
<td>ncimb 12190</td>
<td>0.033</td>
<td>-</td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>ncimb 11617</td>
<td>0.029</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>atcc 9637</td>
<td>0.056</td>
<td>-</td>
</tr>
<tr>
<td><em>Klebsiella oxytoca</em></td>
<td>-</td>
<td>0.075</td>
<td>-</td>
</tr>
<tr>
<td><em>Corynebacterium equi</em></td>
<td>ifo 3730</td>
<td>-</td>
<td>0.012</td>
</tr>
<tr>
<td><em>Nocardioides simplex</em></td>
<td>atcc 6946</td>
<td>0.008</td>
<td>-</td>
</tr>
<tr>
<td><em>Mucor racemosus</em></td>
<td>-</td>
<td>-</td>
<td>0.016</td>
</tr>
<tr>
<td><em>Cylindrocarpon radicicola</em></td>
<td>atcc 11011</td>
<td>0.003</td>
<td>0.015</td>
</tr>
<tr>
<td><em>Septomyxa affinis</em></td>
<td>atcc 6737</td>
<td>-</td>
<td>0.019</td>
</tr>
<tr>
<td><em>Giberella fujikuroi</em></td>
<td>atcc 12616</td>
<td>-</td>
<td>0.016</td>
</tr>
<tr>
<td><em>Verticillium lecanii</em></td>
<td>imi 68689</td>
<td>-</td>
<td>0.007</td>
</tr>
<tr>
<td><em>P. angusta</em></td>
<td>atcc 76722</td>
<td>0.013</td>
<td>-</td>
</tr>
<tr>
<td><em>P. angusta</em></td>
<td>atcc 76723</td>
<td>0.009</td>
<td>0.006</td>
</tr>
<tr>
<td><em>Providencia rustigianii</em></td>
<td>atcc 13159</td>
<td>-</td>
<td>0.015</td>
</tr>
</tbody>
</table>

The absorbance change at 320 nm was non-linear and LC/MS in negative ionisation mode was used to confirm production of phenylpyruvic acid (§ 11.3). Five organisms were found to show an LC/MS spectrum corresponding to phenylpyruvic acid,
indicating possible L-amino acid oxidase activity (table 4.2.3). However, these organisms will need further study to determine if they indeed contain L-amino acid oxidases as the deaminase family of enzymes would also produce phenylpyruvic acid.

Table 4.2.3

<table>
<thead>
<tr>
<th>Organism</th>
<th>Collection no.</th>
<th>Extra-cellular activity a</th>
<th>Intra-cellular activity a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Septomyxa affinis</td>
<td>atcc 6737</td>
<td>10 % PPA</td>
<td>3 % PPA</td>
</tr>
<tr>
<td>2 Verticillium lecanii</td>
<td>imi 68689</td>
<td>3 % PPA</td>
<td>n</td>
</tr>
<tr>
<td>3 Proteus vulgaris</td>
<td>atcc 13315</td>
<td>5 % PPA</td>
<td>2 % PPA</td>
</tr>
<tr>
<td>4 Pseudomonas putida</td>
<td>ncinb 12190</td>
<td>n</td>
<td>1 % PPA</td>
</tr>
<tr>
<td>5 Providencia rustigianii</td>
<td>atcc 13159</td>
<td>9 % PPA</td>
<td>4 % PPA</td>
</tr>
</tbody>
</table>

a) % PPA calculated from MS intensity

4.3. Conclusions & Future Work

The random screening program adopted for identifying novel oxidase activities has proved relatively unsuccessful. In general, the assays employed lack the sensitivity required to identify activity against the background activity of the organisms. The exception appears to be the LC/MS assay used in screening for L-amino acid oxidase activity, however this could not be extended to encompass screening for monoamine oxidase activity.

The five organisms identified as having L-amino acid oxidase activity require further purification and characterisation to positively identify them as L-amino acid oxidases. The two fungi, Septomyxa affinis and Verticillium lecanii, have not been previously reported as containing L-amino acid oxidases.
5. Results & Discussion: Monoamine oxidase from *Aspergillus niger*

As no monoamine oxidase activity was found through the screening program, attention turned to the monoamine oxidase recently identified by Schilling *et al.* in the fungus *A. niger* (MAO-N).\(^{120}\) The gene has been isolated, sequenced and subcloned in pET3a to produce the expression vector pECME3.\(^{121}\) Although extensive characterisation of the enzyme had been reported, the stereospecificity of the enzyme with respect to chiral substrates had not been examined.\(^{122}\)

5.1. Oxidation of RS-AMBA by *E. coli* BLR(DE3) [pECME3]

5.1.1. Growth of *E. coli* BLR(DE3) [pECME3]

The plasmid MAO-N [pECME3] was transformed into *E. coli* BLR(DE3) competent cells and incubated in LB/amp50 media (10 x 50 ml in 250 ml flasks) at 37 °C, 250 rpm for 24 h (OD\(_{600}\) = 3.5). The saturated media was used to inoculate LB/amp50 media (10 x 400 ml in 2 L florence flasks), which were incubated at 37 °C, 200 rpm for 14.5 h (OD\(_{600}\) = 3.5). The cells were harvested (wet weight 36 g), resuspended in phosphate buffer (100 mM, 110 ml) and disrupted. The cleared cell lysate had an activity of 0.20 U.ml\(^{-1}\) towards benzylamine (§ 9.4.3).

5.1.2. Oxidation of RS-AMBA by cell free extract

\(\alpha\)-Methylbenzylamine is a valuable compound in organic synthesis and is produced on a multi-kilogram scale *via* a resolution process.\(^2\) It is often used as a model substrate in the development of new processes for the synthesis of enantiomerically pure amines.\(^2,3,39,51,70\) The cell free extract of BLR(DE3) [pECME3] was examined for activity and stereoselectivity towards RS-AMBA, using HPLC for analysis.
The extract was incubated with a solution of AMBA in 50 mM phosphate buffer, pH 7.4 at 30 °C until no further reaction was observed (table 5.1.1).

Table 5.1.1

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Conc./ mM</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.1.1</td>
<td>0.9</td>
<td>0.2</td>
<td>95.0</td>
<td>28</td>
</tr>
<tr>
<td>2</td>
<td>8.4.1.1</td>
<td>0.8</td>
<td>0.4</td>
<td>96.0</td>
<td>26</td>
</tr>
<tr>
<td>3</td>
<td>8.4.1.1</td>
<td>0.7</td>
<td>0.6</td>
<td>97.0</td>
<td>23</td>
</tr>
<tr>
<td>4</td>
<td>8.4.1.1</td>
<td>4.5</td>
<td>0.2</td>
<td>74.0</td>
<td>39</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol benzylamine oxidised per minute

The enzyme appeared to act with some stereoselectivity towards the S-enantiomer of AMBA. However, the amount of enzyme used did not significantly affect the yield or enantiomeric excess of the reaction (entries 1-3). Increasing the substrate concentration resulted in negligible stereoselectivity (entry 4). In an effort to evaluate the stereoselectivity of the reaction the lysate of BLR(DE3) [pECME3] was incubated separately with each enantiomer of AMBA at a concentration of 0.9 mM (table 5.1.2). Only a slight preference for the S-enantiomer was observed.

Table 5.1.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Substrate</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.1.2</td>
<td>R-AMBA</td>
<td>0.1</td>
<td>96.0</td>
<td>67</td>
</tr>
<tr>
<td>2</td>
<td>8.4.1.2</td>
<td>S-AMBA</td>
<td>0.1</td>
<td>96.0</td>
<td>63</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol benzylamine oxidised per minute

When using biocatalysts in organic synthesis the main advantages are that the reactions can be carried out under mild conditions, i.e. room temperature, pH 7, and that they offer an environmentally friendly alternative as the reactions are carried out in water. However, the poor solubility of many organic compounds in aqueous systems can limit the practicality of the reaction. An accepted method to overcome
this problem is to perform the reaction in either bi-phasic systems or in organic solvents with low water content. Recently it had been shown that using different amine oxidases in organic solvents is a convenient method for the oxidation of poorly soluble amines. It is also known that the MAO-B isozyme efficiently catalyses the oxidation of amines in organic solvent. It was decided to perform the oxidation of AMBA in the presence of organic solvent in an attempt to increase the efficiency of the oxidation reaction. The cell free extract (0.1 U, BzNH₂) was incubated with 1 mM RS-AMBA in 50 mM buffer containing hexane as co-solvent (table 5.1.3).

Table 5.1.3

<table>
<thead>
<tr>
<th>§ no.</th>
<th>% Hexane</th>
<th>pH</th>
<th>Time/ h</th>
<th>Yield (R)/%</th>
<th>e.e./%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>7.4</td>
<td>94</td>
<td>13</td>
<td>40</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>7.4</td>
<td>95</td>
<td>16</td>
<td>22</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>5</td>
<td>96</td>
<td>32</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>6</td>
<td>96</td>
<td>26</td>
<td>16</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>7</td>
<td>96</td>
<td>23</td>
<td>17</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>8</td>
<td>96</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>9</td>
<td>96</td>
<td>28</td>
<td>18</td>
</tr>
</tbody>
</table>

Initial reactions looked promising, with approximately four-fold increase in e.e. observed and a concomitant decrease in yield (entry 1), indicating an improvement in the rate of oxidation. However, this result was not reproducible and the best e.e. obtained thereafter was <20%. The effect of pH on the reaction was found to be negligible (entries 3-7).
5.1.3. Purification studies

*E. coli* is a known producer of monoamine oxidase\(^{142}\) and we considered that the *E. coli* enzyme may be interfering with the stereoselectivity of the reaction. In order to discount interference from the indigenous enzyme, purification of the MAO-N enzyme encoded by pECME3 was undertaken. Sablin et al. recently isolated the enzyme in pure form\(^{122}\) and stated that the *E. coli* monoamine oxidase can be removed in the first stage of the purification (DEAE-sepharose anion exchange chromatography). The cell free extract obtained in § 5.1 was dialysed against Tris/HCl buffer (25 mM, pH 7.8) containing 1 mM DTT and subjected to FPLC purification on a DEAE-sepharose column (§ 9.3.1.3). The fractions were assayed against benzylamine using the colorimetric assay 9.4.1.2. Active fractions were diluted in ammonium sulfate (1.7 M in 50 mM phosphate buffer, pH 7.2) and eluted through a phenyl-sepharose hydrophobic interaction resin (§ 9.3.2.1). The colorimetric assay showed no activity in any of the fractions collected or in the flow-through from loading the column. The absorbance at 280 nm was measured and it was discovered that approximately half the protein loaded was in the flow-through, i.e. protein had not bound to resin, and the remainder was spread throughout the fractions. This loss of activity is in contrast to the published work, which observed very little loss in the total activity (437 U c.f. 499 U at start).

The MAO-N enzyme appeared to suffer from problems of stability during and after purification, e.g. CFE activity \(t(0) = 0.20 \text{ U.mL}^{-1}\), \(t(5\text{d}, 4 \degree \text{C}) = 0.006 \text{ U.mL}^{-1}\). Thus it was decided to investigate the effect of different inhibitors on the stability of the enzyme during purification and storage. The whole cells were disrupted (5 min, 30 s) in the presence of three different inhibitor cocktails (c1, c2 & c3) and then purified on a MonoQ anion exchange column (§ 9.3.1.4). The activity of each enzyme preparation was assayed using hydrogen peroxide based method using benzylamine as substrate (table 5.1.4).
Table 5.1.4

<table>
<thead>
<tr>
<th>Temp / °C</th>
<th>Time / d</th>
<th>Activity (c1) a / U.ml(^{-1})</th>
<th>Activity (c2) a / U.ml(^{-1})</th>
<th>Activity (c3) a / U.ml(^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>CFE</td>
<td>-</td>
<td>0.241</td>
<td>0.229</td>
<td>0.199</td>
</tr>
<tr>
<td>CFE</td>
<td>4</td>
<td>0.082</td>
<td>0.106</td>
<td>-</td>
</tr>
<tr>
<td>CFE</td>
<td>4</td>
<td>0.171</td>
<td>0.144</td>
<td>0.089</td>
</tr>
<tr>
<td>CFE</td>
<td>-80</td>
<td>0.108</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Purified b</td>
<td>-</td>
<td>0.307</td>
<td>0.655</td>
<td>-</td>
</tr>
<tr>
<td>Purified</td>
<td>4</td>
<td>0.285</td>
<td>0.596</td>
<td>-</td>
</tr>
<tr>
<td>Purified</td>
<td>-80</td>
<td>0.420</td>
<td>0.618</td>
<td>-</td>
</tr>
</tbody>
</table>

a) c1 = 1 mM PMSF, 1 mM EDTA, 1 mM DTT  c2 = Sigma general use protease inhibitor cocktail  c3 = Sigma mammalian cell protease inhibitor cocktail + 1 mM EDTA; b) CFE in presence of c1 was overloaded on column, therefore purification was not as efficient as for CFE in presence of c2

The addition of a cocktail of commercial inhibitors did not offer any great advantage, and it was therefore decided to perform purification in the presence of PMSF, EDTA and DTT. The cell free extract showed a considerable drop in activity with benzylamine when stored at 4 °C, however the purified enzyme appeared more stable at 4 °C. Storage of the purified enzyme at -80 °C showed good stability. The effect of glycerol on the activity of purified MAO-N towards benzylamine was investigated (table 5.1.5) and the best storage conditions were at -80 °C, when no glycerol was present. In both investigations the hydrogen peroxide assay showed some problems with reproducibility. Loss of HRP activity was ruled out as the probable cause, therefore the fluctuations may have been to do with aeration differences between assays.
### Table 5.1.5

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Activity/ U.ml(^{-1}) (t = 0\ d)</th>
<th>Activity/ U.ml(^{-1}) (t = 7\ d)</th>
<th>Activity/ U.ml(^{-1}) (t = 19\ d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0% glycerol, 4 °C</td>
<td>0.023</td>
<td>0.030</td>
<td>-</td>
</tr>
<tr>
<td>0% glycerol, -20 °C</td>
<td>0.023</td>
<td>0.017</td>
<td>0.012</td>
</tr>
<tr>
<td>0% glycerol, -80 °C</td>
<td>0.023</td>
<td>0.044</td>
<td>0.050</td>
</tr>
<tr>
<td>20% glycerol, -20 °C</td>
<td>0.023</td>
<td>0.015</td>
<td>-</td>
</tr>
<tr>
<td>20% glycerol, -80 °C</td>
<td>0.023</td>
<td>0.011</td>
<td>negative rate</td>
</tr>
<tr>
<td>50% glycerol, -20 °C</td>
<td>0.023</td>
<td>0.010</td>
<td>-</td>
</tr>
<tr>
<td>50% glycerol, -80 °C</td>
<td>0.023</td>
<td>0.036</td>
<td>0.026</td>
</tr>
</tbody>
</table>

### 5.1.4. Oxidation of RS-AMBA by partially purified MAO-N

BLR(DE3) [pECME3] was grown in LB/amp 50 media at 37 °C and harvested to yield 5.6 g of cells. The cells were resuspended in lysis buffer (1 mM DTT, 1 mM PMSF and 1 mM EDTA), disrupted by sonication (10 min, 30 s period) and the cell free extract was subjected to purification on Q-Sepharose anion-exchange resin (§ 9.3.1.1). The fractions collected were assayed by the hydrogen peroxide based method (§ 9.4.1.2), with the most active fraction having 0.32 U.mg\(^{-1}\) activity towards benzylamine. The partially purified extract (0.46 U) was incubated at 30 °C with RS-AMBA (0.9 mM) in 100 mM buffer, pH 8 and monitored by HPLC (§ 8.6.8). After 94 h HPLC showed 37% yield of the R-enantiomer and 37% yield of the S-enantiomer, indicating that the stereoselectivity observed previously must have been due to the native *E. coli* monoamine oxidase enzyme.
5.2. Oxidation of RS-AMBA by *E. coli* BL21(DE3) [pMAO-N]

5.2.1. Growth of *E. coli* BL21(DE3) [pMAO-N]

Analysis of the protein purification by SDS-PAGE showed that the expression of MAO-N from pECME3 was poor (data not shown). Therefore, in an attempt to improve expression of the protein, the gene encoding monoamine oxidase from *A. niger* was subcloned into the plasmid pET16b by Marina Alexeeva to produce the plasmid pMAO-N.

Experiments by M. Alexeeva showed that in this new construct MAO-N acted with some stereoselectivity towards RS-AMBA.\(^{143}\) BL21(DE3) [pMAO-N] was grown in LB/amp100 media at 30 °C until OD\(_{600}\) = 3.8. Aliquots (12 × 1 ml) of the growth media were spun down (14000 g, 3 min) and the frozen pellets stored at -20 °C. The remainder of the culture media was harvested to yield 10.4 g of cells and stored at -20 °C. The activity of the whole cells towards amylamine was 0.15 U.ml\(^{-1}\) (§ 9.4.2).

5.2.2. Oxidation of RS-AMBA by whole cells and CFE

The thawed pellets of BL21(DE3) [pMAO-N] were incubated at 30 °C with AMBA in 100 mM buffer, pH 8 (table 5.2.1). The reaction was followed by HPLC analysis (§ 8.6.8). Increasing the amount of enzyme relative to the substrate did not have an appreciable effect on the yield or stereoselectivity of the reaction (entries 1-3). Increasing the reaction time showed no difference in the e.e. (entries 4&5), indicating that both enantiomers were being oxidised to the same extent. A significant effect was observed when the buffer concentration was reduced to 20 mM, with the enantiomeric excess increasing to 58% (entry 6). This result may offer an alternative explanation to the lack of selectivity observed in the oxidation of RS-AMBA by the purified fraction of BLR(DE3) [pECME3] (§ 5.1.3) as this reaction was also performed in 100 mM buffer. Increasing the amount of enzyme increased the reaction rate but lowered the selectivity of the reaction (entry 7).
Using *E. coli* BL21(DE3) [pET16b] cells showed that AMBA was oxidised by the *E. coli* cells without MAO-N present to almost the same extent as when the gene is present but without the same selectivity (entry 8). This indicates that the selectivity previously observed in the reaction was probably due to the recombinant monoamine oxidase, MAO-N.

Table 5.2.1

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Conc./ mM</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.3.1</td>
<td>0.9</td>
<td>0.10 b</td>
<td>47</td>
<td>54</td>
</tr>
<tr>
<td>2</td>
<td>8.4.3.1</td>
<td>0.7</td>
<td>0.29 b</td>
<td>47</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>8.4.3.1</td>
<td>0.5</td>
<td>0.48 b</td>
<td>47</td>
<td>46</td>
</tr>
<tr>
<td>4</td>
<td>8.4.3.1</td>
<td>0.9</td>
<td>0.10 b</td>
<td>89</td>
<td>45</td>
</tr>
<tr>
<td>5</td>
<td>8.4.3.1</td>
<td>0.9</td>
<td>0.10 b</td>
<td>94</td>
<td>42</td>
</tr>
<tr>
<td>6</td>
<td>8.4.3.1</td>
<td>0.9</td>
<td>0.08</td>
<td>99</td>
<td>78</td>
</tr>
<tr>
<td>7</td>
<td>8.4.3.1</td>
<td>1.0</td>
<td>0.16</td>
<td>97</td>
<td>34</td>
</tr>
<tr>
<td>8</td>
<td>8.4.2.1 c,d</td>
<td>0.9</td>
<td>10%</td>
<td>89</td>
<td>40</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol amylamine oxidised per minute; b) cells provided by M. Alexeeva (0.20 U.ml⁻¹); c) 20 mM buffer, pH 8; d) plain BL21(DE3) [pET16b] cells

The frozen cells of MAO-N [pET16b] (10.4 g) were resuspended in lysis buffer (1 mM PMSF) and disrupted (10 min, 30 s). The CFE was incubated with RS-AMBA in 20 mM buffer, pH 8 at 30 °C (table 5.2.2).
Table 5.2.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Conc./ mM</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.3.2</td>
<td>0.9</td>
<td>0.19</td>
<td>99</td>
<td>48</td>
</tr>
<tr>
<td>2 b</td>
<td>8.4.3.2</td>
<td>0.9</td>
<td>0.19</td>
<td>99</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>8.4.3.2</td>
<td>0.9</td>
<td>0.27</td>
<td>97</td>
<td>50</td>
</tr>
<tr>
<td>4 c</td>
<td>8.4.3.2</td>
<td>0.9</td>
<td>0.27</td>
<td>97</td>
<td>15</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol amylamine oxidised per minute; b) catalase added;  
c) reaction vial 4 x usual size

The cell free extract showed a similar reactivity and selectivity towards RS-AMBA as the whole cells (entry 1). Addition of catalase slowed the reaction but increased the selectivity (entry 2). Surprisingly, increasing the amount of MAO-N did not affect the yield of the reaction but improved the selectivity (entry 3). The most interesting effect was noted when the size of the reaction vessel was increased relative to the reaction volume (entry 4), i.e. the volume of oxygen present in the vessel was increased. The yield of the R-AMBA was much lower indicating a faster reaction rate and the reaction was almost completely enantioselective. The oxidation of amines by the isozyme MAO-B is known to be sensitive to O₂ concentration and Sablin et al. have demonstrated that, in this respect, MAO-N resembles the behaviour of MAO-B.²²

### 5.3. Production of a MAO-N mutant

In all experiments with BLR(DE3) [pECME3] or BL21(DE3) [pMAO-N], either whole cell, CFE or purified extract, the rate of AMBA oxidation was very slow and the enantioselectivity was variable. This led to the conclusion that the wild type MAO-N would not be a viable enzyme for the deracemisation of chiral amines. In recent years, the activity and selectivity of enzymes have been modified using a “directed evolution” approach.¹⁴⁴⁻¹⁴⁶ The amino acid residues in the enzyme are
randomly mutated, and the mutated enzyme library is screened for enhanced activity. Marina Alexeeva used *E. coli* XL1-Red mutator strain to randomly mutate the plasmid containing MAO-N [pET16b]. This mutator strain offers the advantage that all parts of the plasmid are subject to mutation, not only the gene of interest. A library of approximately one million variants was obtained, a fraction of which was screened against S-AMBA. The assay method was based on the hydrogen peroxide colorimetric method. However, the AAP proton donor was replaced by 3,3′-diaminobenzidine, which results in an insoluble dye more appropriate to the screening of agar plates as the colour does not diffuse from the active colony.

Twenty-four colonies were identified as having increased activity towards S-AMBA, which were subsequently grown on a small scale and one clone was shown to be superior in terms of its selectivity and activity towards S- versus R-AMBA. This clone was sequenced and one specific mutations identified: N336S. After purification of the selected mutant $k_{cat}$ for S- and R-AMBA was measured, which revealed that the activity of the mutant towards S-AMBA ($k_{cat} = 8.0 \text{ min}^{-1}$) was 47-fold higher than the wild type ($k_{cat} = 0.17 \text{ min}^{-1}$). Moreover the selectivity of the mutant for the S-enantiomer versus the R-enantiomer (ca. 100:1) was also increased relative to the wild type (ca. 17:1). Oxidation reactions of AMBA by this mutant (BL21(DE3) [pMAO-Nmut.]) were carried out and compared to the reactions with the wild type enzyme.

### 5.3.1. Investigation into background activity of *E. coli* cells

BL21(DE3) [pET16b], BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.] were grown in LB/amp100 media (300 ml) at 30 °C. Aliquots (12 × 1 ml) were spun down (14000 g, 3 min) and stored at -20 °C. The remainder of the growth media was harvested and stored at -80 °C. The BL21(DE3) [pET16b] cells had zero activity towards amylamine, BL21(DE3) [pMAO-N] had an activity of 0.15 U.ml$^{-1}$ and BL21(DE3) [pMAO-Nmut.] had an activity of 0.20 U.ml$^{-1}$. The cells were incubated at 30 °C with RS-AMBA (0.9 mM) in 20 mM buffer, pH 8 and the results shown in table 5.3.1.
Table 5.3.1

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Strain</th>
<th>U (^a)</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.2.1 BL21 [pET16b]</td>
<td>10%</td>
<td>99</td>
<td>58</td>
<td>76</td>
</tr>
<tr>
<td>2</td>
<td>8.4.3.1 BL21 [pMAO-N]</td>
<td>0.08</td>
<td>99</td>
<td>78</td>
<td>58</td>
</tr>
<tr>
<td>3</td>
<td>8.4.4.1 BL21 [pMAO-Nmut.]</td>
<td>0.10</td>
<td>99</td>
<td>86</td>
<td>74</td>
</tr>
</tbody>
</table>

\(^a\) 1 U = 1 \(\mu\)mol amylamine oxidised per minute

After 20 h the mutated enzyme showed approximately a 10-fold increase in enantioselectivity relative to the wild type MAO-N enzyme and BL21(DE3) [pET16b] control, both of which oxidised RS-AMBA to the same extent. However, as the reaction progressed, the BL21(DE3) [pET16b] cells increased in selectivity to the point that they showed the greatest selectivity relative to the MAO-N enzymes (figure 5.3.1).

Figure 5.3.1: oxidation of RS-AMBA by whole cells
5.2.1. *Purification of BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.]*

The question of interference by the native *E. coli* monoamine oxidase had obviously not yet been fully resolved. In an attempt to eliminate this background activity, we decided that BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.] needed to be purified. The plasmid encodes six histidyl residues at the C-terminus of MAO-N. This hexahistidine motif is often introduced as an aid to purification, as metal affinity chromatography can be used as a one-step purification technique (§ 9.2.8). The purification relies on the hexahistidine motif binding to Ni$^{2+}$ ions on the resin and imidazole is then used as a competitive chelator to elute the protein. However, attempts at purification of BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.] using this method were unsuccessful. On elution from the resin the protein was active (1.57 U.ml$^{-1}$ against amylamine) and partially purified (figure 5.3.2). However, the protein precipitated over time, even when stored at -80 °C, and activity towards amine substrates was lost. The precipitation may have been due to the high salt and imidazole concentration of the eluted fractions.

![SDS-PAGE analysis of His-Tag purification of MAO-N](image)

**Figure 5.3.2: SDS-PAGE analysis of His-Tag purification of MAO-N [pET16b]**

It was decided to purify the monoamine oxidase using the proven method of anion exchange chromatography (§ 9.3.1.2). The frozen cells of BL21(DE3) [pET16b], BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.] were resuspended in lysis buffer (1 mM PMSF), disrupted by sonication (5 min, 15 s) and dialysed against Tris/HCl buffer (25 mM, pH 7.8). Purification of all three cell free extracts was
performed on a ResourceQ anion exchange column (§ 11.4). The activity of the CFE and partially purified extracts was measured against amylamine, benzylamine and \( R \)- and \( S \)-AMBA (table 5.3.2).

Table 5.3.2

<table>
<thead>
<tr>
<th></th>
<th>Activity/ ( \text{U.mg}^{-1} \text{a} )</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amylamine</td>
<td>Benzylamine</td>
<td>( S )-AMBA</td>
<td>( R )-AMBA</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3) CFE</td>
<td>-</td>
<td>-</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>BL21(DE3) p.p.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MAO-N CFE</td>
<td>0.43</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>MAO-N p.p.</td>
<td>7.07</td>
<td>4.16</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>MAO-N \textit{mut.} CFE</td>
<td>0.02</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>MAO-N \textit{mut.} p.p.</td>
<td>0.97</td>
<td>1.76</td>
<td>0.14</td>
<td>0.008</td>
<td></td>
</tr>
</tbody>
</table>

a) n.d. = not determined

SDS-PAGE analysis showed a high concentration of MAO-N in the purified fractions of BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-N\textit{mut.}] (figure 5.3.3).

Figure 5.3.3: SDS-PAGE analysis of purified extracts (fractions 15&16)

The CFE of each preparation was incubated at 30 °C with \( RS \)-AMBA (0.9 mM) in 20 mM buffer, pH 8 (table 5.3.2, figure 5.3.4). After the first twenty hours,
BL21(DE3) [pMAO-Nmut.] showed approximately double the selectivity of BL21(DE3) [pMAO-N] and a four-fold increase in selectivity over BL21(DE3) [pET16b]. However, the *E. coli* monoamine oxidase acts with sufficient stereoselectivity, 26% e.e. of *R*-enantiomer, to cause interference with the deracemisation reaction. Therefore we concluded that the CFE could not be used for deracemisation studies.

Table 5.3.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Strain</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.2.2 [pET16b]</td>
<td>10%</td>
<td>20</td>
<td>54</td>
<td>26</td>
</tr>
<tr>
<td>2</td>
<td>8.4.2.2 [pET16b]</td>
<td>10%</td>
<td>96</td>
<td>46</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>8.4.3.2 [pMAO-N]</td>
<td>0.30</td>
<td>20</td>
<td>56</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>8.4.3.2 [pMAO-N]</td>
<td>0.30</td>
<td>96</td>
<td>48</td>
<td>&gt;99</td>
</tr>
<tr>
<td>5</td>
<td>8.4.4.2 [pMAO-Nmut.</td>
<td>0.02</td>
<td>20</td>
<td>45</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 µmol amylamine oxidised per minute

Figure 5.3.4: oxidation of *RS*-AMBA by cell free extracts

The partially purified extracts of the three different preparations were incubated at 30 °C with *RS*-AMBA (0.9 mM) in 20 mM buffer, pH 8 (table 5.3.3, figure 5.3.5).
After 20 h BL21(DE3) [pMAO-Nmut.] had fully oxidised only the S-enantiomer and BL21(DE3) [pET16b] and BL21(DE3) [pMAO-N] showed only 5% e.e. As the partially purified extract of BL21(DE3) [pMAO-Nmut.] showed no interference from the native *E. coli* monoamine oxidase an enzyme suitable for study of the deracemisation of amines has been obtained, the first such enzyme reported.

Table 5.3.3

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Enzyme</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.2.3 BL21(DE3)</td>
<td>10%</td>
<td>20</td>
<td>53</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>8.4.2.3 BL21(DE3)</td>
<td>10%</td>
<td>96</td>
<td>31</td>
<td>38</td>
</tr>
<tr>
<td>3</td>
<td>8.4.3.3 MAO-N w.t.</td>
<td>0.27</td>
<td>20</td>
<td>52</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>8.4.3.3 MAO-N w.t.</td>
<td>0.27</td>
<td>96</td>
<td>46</td>
<td>38</td>
</tr>
<tr>
<td>5</td>
<td>8.4.4.3 MAO-N mut.</td>
<td>0.04</td>
<td>7</td>
<td>51</td>
<td>94</td>
</tr>
<tr>
<td>6</td>
<td>8.4.4.3 MAO-N mut.</td>
<td>0.06</td>
<td>20</td>
<td>47</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol amylamine oxidised per minute

Figure 5.3.5: oxidation of RS-AMBA by partially purified extract
5.4. Oxidation of RS-AMBA by the MAO-N mutant

Optimisation of the oxidation of RS-AMBA by partially purified BL21(DE3) [pMAO-Nmut.] was undertaken. Incubation of the enzyme with RS-AMBA (0.9 mM) at three different temperatures (table 5.4.1) showed that the enzyme worked efficiently at room temperature or 30 °C but the rate of reaction was reduced at 37 °C. All future experiments were therefore performed at 30 °C.

Table 5.4.1

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Temp./ °C</th>
<th>U a</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>rt.</td>
<td>0.004</td>
<td>18</td>
<td>56</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>0.004</td>
<td>18</td>
<td>52</td>
<td>&gt;99</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>0.004</td>
<td>18</td>
<td>67</td>
<td>72</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol amylamine oxidised per minute

To continue with the oxidation and deracemisation studies fresh enzyme was required. BL21(DE3) [pMAO-Nmut.] was grown in LB/amp100 media at 30 °C, harvested, disrupted and purified as before (§ 5.3.2). The protein expression was lower than previously and consequently an increase in enzyme concentration and reaction time was required for successful oxidation of S-AMBA. The effect of pH on the reaction with RS-AMBA (0.9 mM) was studied next (table 5.4.2) and the optimum was found to be pH 8.
Table 5.4.2

<table>
<thead>
<tr>
<th>§ no.</th>
<th>pH</th>
<th>$U^a$</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.4.3</td>
<td>6.03</td>
<td>22</td>
<td>55</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>8.4.4.3</td>
<td>7.03</td>
<td>22</td>
<td>47</td>
<td>86</td>
</tr>
<tr>
<td>3</td>
<td>8.4.4.3</td>
<td>8.03</td>
<td>22</td>
<td>54</td>
<td>92</td>
</tr>
<tr>
<td>4</td>
<td>8.4.4.3</td>
<td>9.03</td>
<td>22</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>5</td>
<td>8.4.4.3</td>
<td>10.03</td>
<td>22</td>
<td>56</td>
<td>16</td>
</tr>
</tbody>
</table>

a) $1 \text{U} = 1 \mu\text{mol amylamine oxidised per minute}$

Finally, the limits of concentration for both the enzyme and substrate were studied (table 5.4.3). The enzyme has a low tolerance for $RS$-AMBA (entry 2) and it will be necessary to modify the enzyme further to accept higher substrate concentrations as, for the deracemisation to be commercially viable, a substrate concentration of 100 g.1$^{-1}$ is desirable. Reducing the enzyme concentration by a third did not affect the rate of reaction (entry 3).

Table 5.4.3

<table>
<thead>
<tr>
<th>§ no.</th>
<th>Conc./ mM</th>
<th>$U^a$</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4.4.3</td>
<td>0.90</td>
<td>0.03</td>
<td>25</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>8.4.4.3</td>
<td>4.50</td>
<td>0.03</td>
<td>25</td>
<td>56</td>
</tr>
<tr>
<td>3</td>
<td>8.4.4.3</td>
<td>0.90</td>
<td>0.01</td>
<td>24</td>
<td>61</td>
</tr>
</tbody>
</table>

a) $1 \text{U} = 1 \mu\text{mol amylamine oxidised per minute}$
5.5. Investigation into the substrate specificity of the MAO-N mutant

The mutant was assayed against a range of aromatic substrates using the hydrogen peroxide based assay (table 5.5.1). Chromogenic solution 2 (§ 9.5.18, 990 μl), substrate (6 μl) and HRP (5 μl) were placed in the spectrophotometer, which was zeroed. The solution was monitored at 500 nm for 10 min, at 3 s intervals before BL21(DE3) [pMAO-Nmut.] (10 μl) was added and the assay mixture monitored at 500 nm for a further 10 min. Addition of the substrate to the chromogenic solution sometimes resulted in an increase in absorbance at 500 nm, which was a result of oxidised amine reacting with TBHBA, i.e. impurities were present in the substrate.

Table 5.5.1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate 1/ Au.s⁻¹ a</th>
<th>Rate 2/ Au.s⁻¹ b</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{Me} ) &amp; negative &amp; ( 2.0 \times 10^{-4} ) &amp; 100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Me} ) &amp; negative &amp; negative &amp; -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Br} ) &amp; ( 3.5 \times 10^{-3} ) &amp; not measured &amp; -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{MeO} ) &amp; ( 2.0 \times 10^{-5} ) &amp; ( 6.3 \times 10^{-5} ) &amp; 32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{MeO} ) &amp; negative &amp; negative &amp; -</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Me} ) &amp; ( 5.9 \times 10^{-4} ) c &amp; ( 2.3 \times 10^{-5} ) &amp; 11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \text{Me} ) &amp; negative &amp; negative &amp; -</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a) rate without enzyme present; b) rate with enzyme present; c) rate levelled off before enzyme was added
Surprisingly aliphatic substrates showed increased activity relative to the aromatic substrates (table 5.5.2). As the mutant was evolved towards AMBA it was expected that the substrate range would be narrow. However, the mutant showed a similar specificity as the wild type, which favours the aliphatic amines such as amylamine and hexylamine. This suggests that the mutation has increased the size of the active site to allow the enzyme to accept an α-methyl group. Gratifyingly the enzyme acted with the same stereospecificity on all substrates, oxidising only the S-enantiomer.

Table 5.5.2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Rate 1/ Au.s⁻¹ a</th>
<th>Rate 2/ Au.s⁻¹ b</th>
<th>Relative rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeMeNH₂</td>
<td>5.9 ×10⁻⁶</td>
<td>2.8 ×10⁻⁴</td>
<td>140</td>
</tr>
<tr>
<td>negative</td>
<td>3.0 ×10⁻⁴</td>
<td>148</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>negative</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>7.4 ×10⁻⁵</td>
<td>37</td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>negative</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

a) rate without enzyme present; b) rate with enzyme present
5.6. Conclusions & Future Work

The activity and stereoselectivity of BLR(DE3) [pECME3] and BL21(DE3) [pMAO-N] towards α-methylbenzylamine was very low. The use of a monoamine oxidase obtained by \textit{in vitro} evolution has resulted in the enantioselective oxidation of RS-AMBA. Monoamine oxidase present in \textit{E. coli} has been shown to act stereoselectively on RS-AMBA with preference for the S-enantiomer. However, the rate of reaction is much slower than for BL21(DE3) [pMAO-N\textit{mut.}] (96 h \textit{c.f.} 20 h) and partial purification of BL21(DE3) [pMAO-N\textit{mut.}] removed any interference from the native \textit{E. coli} enzyme.

A preliminary study into the substrate specificity of the mutated MAO-N showed that the enzyme accepts both aromatic and aliphatic substrates, with preference for the S-enantiomer in all cases. Further characterisation will need to be carried out and the amine substrates purified to eliminate interference in the assay.

The success of the \textit{in vitro} evolution experiment should allow access to a wide variety of mutated enzymes with different substrate specificities. It will be especially important to evolve an enzyme with preference for the R-enantiomer in order to prepare both enantiomers of chiral amines.
6. Results & Discussion: Deracemisation of
RS-α-methylbenzylamine

6.1. Deracemisation reaction

With the availability of the mutated MAO-N enzyme, the oxidation of RS-AMBA could now be performed enantioselectively within a reasonable time frame. Therefore application of the enzyme to deracemisation of racemic amines was attempted. Initially, the reaction was performed with sodium borohydride as for the deracemisation of α-amino acids (chapter 3). Purified BL21(DE3) [pMAO-Nmut.] (0.04 U) was incubated with RS-AMBA (0.9 mM) and NaBH₄ (58.5 eq.) for 20 h, after which time HPLC analysis showed 50% yield R-AMBA and e.e. = 12%, i.e. almost zero oxidation of the S-enantiomer. It is known that a Schiff base can form between key protein amino acid residues and the ketone hydrolysis products of the amino acid oxidation by amino acid oxidase. The Schiff base formed is then irreversibly reduced by sodium borohydride, thereby inactivating the enzyme. It is conceivable that a similar inactivation is occurring with the monoamine oxidase catalysed oxidation of RS-AMBA, thereby affecting the deracemisation reaction.

The ammonia:borane reducing agent developed for the deracemisation of α-amino acids offered an attractive alternative to sodium borohydride. Ammonia:borane is stable in water at neutral or basic pH, soluble and unreactive towards a range of protic and aprotic solvents. Initial studies on the deracemisation of RS-AMBA using NH₃BH₃ were promising. After 72 h, R-AMBA was observed in 69% yield, 74% e.e. (table 6.1.1, entry 1). Further optimisation of the reaction was then undertaken. Addition of the enzyme and reducing agent in four aliquots did not improve the yield or e.e. (entry 2). Increasing the enzyme concentration resulted in optical purity after 24 h, however, the yield of R-AMBA was only 52% (entry 3).

The yield and optical purity of R-AMBA were adversely affected by increasing the NH₃BH₃ concentration (entries 4&8), probably as a result of inactivation of the enzyme. Decreasing the NH₃BH₃ concentration resulted in optical purity at the
expense of yield (entries 11-14). Addition of catalase or ammonium formate did not improve the reaction (entries 5&7). The best result was obtained with 0.04 U of enzyme and 178 eq. reducing agent, yielding R-AMBA in 79\% with e.e. = 94\% (entry 6).

Table 6.1.1

<table>
<thead>
<tr>
<th>Conc./ mM</th>
<th>NH$_3$BH$_3$/ eq.</th>
<th>U</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.9</td>
<td>44.4</td>
<td>0.04</td>
<td>72</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>44.4</td>
<td>0.01</td>
<td>45</td>
<td>59</td>
</tr>
<tr>
<td>3</td>
<td>0.9</td>
<td>88.9</td>
<td>0.04</td>
<td>24</td>
<td>52</td>
</tr>
<tr>
<td>4</td>
<td>0.9</td>
<td>177.8</td>
<td>0.02</td>
<td>96</td>
<td>58</td>
</tr>
<tr>
<td>5</td>
<td>0.9</td>
<td>177.8</td>
<td>0.04</td>
<td>23</td>
<td>53</td>
</tr>
<tr>
<td>6</td>
<td>0.8</td>
<td>177.8</td>
<td>0.04</td>
<td>24</td>
<td>79</td>
</tr>
<tr>
<td>7</td>
<td>0.8</td>
<td>177.8</td>
<td>0.04</td>
<td>17</td>
<td>63</td>
</tr>
<tr>
<td>8</td>
<td>0.8</td>
<td>177.8</td>
<td>0.02</td>
<td>48</td>
<td>74</td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>100</td>
<td>0.02</td>
<td>48</td>
<td>69</td>
</tr>
<tr>
<td>10</td>
<td>0.75</td>
<td>120</td>
<td>0.02</td>
<td>48</td>
<td>62</td>
</tr>
<tr>
<td>11</td>
<td>0.8</td>
<td>100</td>
<td>0.02</td>
<td>24</td>
<td>63</td>
</tr>
<tr>
<td>12</td>
<td>0.8</td>
<td>50</td>
<td>0.02</td>
<td>24</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>0.8</td>
<td>25</td>
<td>0.02</td>
<td>24</td>
<td>58</td>
</tr>
<tr>
<td>14</td>
<td>0.8</td>
<td>25</td>
<td>0.02</td>
<td>24</td>
<td>54</td>
</tr>
</tbody>
</table>

a) 1 U = 1 \mu mol amylamine oxidised per minute; b) 44.4 eq. added at 3 h; c) 44.4 eq. and 0.01 U added at 1.5, 3 & 19 h; d) 88.9 eq. and 0.04 U added at 4 h; e) 177.8 eq. and 0.02 U added at 2 h; f) catalase added; g) 1 M ammonium formate added; h) 177.8 eq. and 0.02 U added at 26 h; i) 100.0 eq. and 0.02 U added at 26 h; j) 133.0 eq. and 0.02 U added at 26 h
A study of the time-course of the reactions showed that in the initial stages of the reaction the “typical” deracemisation plot was observed, i.e. an increase in R-enantiomer with a concomitant decrease in S-enantiomer (figure 6.1.1). However, after a few hours the yield of R-enantiomer became variable. Analysis of the HPLC chromatograms revealed formation of a by-product at r.t. = 34 min, which was preventing the yield of R-AMBA reaching 100%. To obtain high yields and optical purity, it will be necessary to identify the unknown by-product and attempt to eliminate its formation. The obvious by-product is the hydrolysis product acetophenone, however analysis of pure acetophenone by HPLC showed this compound to have a retention time of 73 min.

Figure 6.1.1: representative time-course of deracemisation experiment (entry 9)

Although the by-product did not appear to be acetophenone, it may be Schiff base 149, the result of condensation between AMBA and acetophenone, or even the reduced form of the Schiff base 150 (figure 6.1.2).

![Chemical structure of 148, 52, 149, and 150](image)

Figure 6.1.2: condensation of α-methylbenzylamine and acetophenone
Extraction of acetophenone into an organic solvent may improve the yield of the reaction. Therefore deracemisation of 1 mM RS-AMBA using 1% organic co-solvent was attempted (table 6.1.2). Although oxidation of S-AMBA was not adversely affected, except in the case of dioxane, the addition of organic solvent did not improve the yield of the deracemisation.

Table 6.1.2

<table>
<thead>
<tr>
<th>Solvent</th>
<th>NH₃BH₃/ eq.</th>
<th>Uᵃ</th>
<th>Time/ h</th>
<th>Yield (R) %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 DMSO</td>
<td>80.0</td>
<td>0.02</td>
<td>24</td>
<td>55</td>
<td>80</td>
</tr>
<tr>
<td>2 dioxane</td>
<td>80.0</td>
<td>0.02</td>
<td>21</td>
<td>51</td>
<td>46</td>
</tr>
<tr>
<td>3 acetonitrile</td>
<td>80.0</td>
<td>0.02</td>
<td>21</td>
<td>61</td>
<td>88</td>
</tr>
<tr>
<td>4 hexane</td>
<td>80.0</td>
<td>0.02</td>
<td>21</td>
<td>57</td>
<td>96</td>
</tr>
<tr>
<td>5 hexane</td>
<td>20.0</td>
<td>0.02</td>
<td>23</td>
<td>51</td>
<td>99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 µmol amylamine oxidised per minute

6.2. Stereoinversion reaction

The definitive proof of deracemisation is to convert a single enantiomer into its antipode, i.e. stereoinversion (figure 6.2.1).

![Figure 6.2.1: stereoinversion of S-α-methylbenzylamine](image)

Figure 6.2.1: stereoinversion of S-α-methylbenzylamine

Each enantiomer of AMBA was separately incubated with BL21(DE3) [pMAO-Nmut.] and NH₃BH₃ at 30 °C (table 6.2.1, figure 6.2.2). The S-enantiomer was converted to the R-enantiomer in 18% yield, 99% e.e. whereas no conversion of
R- to S-AMBA was observed under identical conditions. As for the standard deracemisation reaction, the low yield of the reaction is due to the formation of the unknown by-product at r.t. = 34 min.

Table 6.2.1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>NH₃BH₃/ eq.</th>
<th>U ⁰</th>
<th>Time/ h</th>
<th>Yield (R)/ %</th>
<th>e.e./ %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 S-AMBA</td>
<td>20.0</td>
<td>0.02</td>
<td>23</td>
<td>18</td>
<td>&gt;99</td>
</tr>
<tr>
<td>2 R-AMBA</td>
<td>20.0</td>
<td>0.02</td>
<td>23</td>
<td>98</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

a) 1 U = 1 μmol amylamine oxidised per minute

Figure 6.2.2: time-course of stereoinversion reaction (entry 1)

6.3. Conclusions & Future Work

The deracemisation of racemic amines using a cyclic oxidation-reduction sequence has been demonstrated for the first time. The success of the reaction was dependent on the availability of an enantioselective monoamine oxidase, BL21(DE3) [pMAO-Nmut.], which was obtained by in vitro evolution. However the deracemisation reaction was not as efficient as the deracemisation of α-amino acids
One possibility for the reduced efficiency of the reaction would be decreased activity of the monoamine oxidase compared with \( \eta \)-DAAO. Presently Marina Alexeeva is carrying out further work on the directed evolution of the enzyme in an attempt to improve the catalytic efficiency of the mutated monoamine oxidase.

Decreasing the electron density of an imino bond increases the reactivity of the C=\( N \) bond towards reduction.\(^{149} \) Therefore the reactivity of the imine towards reduction is less than for the \( \alpha \)-imino acid in the amino acid deracemisation. Further optimisation of the reduction conditions will be required to achieve an efficient deracemisation. Unfortunately there are limited options for reducing imines under aqueous conditions. Other possible reducing agents include cyanoborohydride anion, palladium on charcoal and pyridine:borane. These reducing agents all bring their own problems: cyanoborohydride produces cyanide gas at low pH, therefore an alternative analysis system will be required; palladium on charcoal can be poisoned by the amine substrate and pyridine:borane is a toxic compound.

After optimisation of the deracemisation conditions the key aim of the work would be to develop the system for other racemic amines. It is hoped that from initial studies on the substrate specificity of the mutated MAO-N (§5.5) the enzyme could be used without further modification to deracemise other racemic amines. However it may be necessary to further evolve the enzyme to cope with all the amines of interest. Evolution of the enzyme to accept the \( R \)-enantiomer will definitely be required. In order to analyse the deracemisation reactions of other amines a new analysis system will be needed as many of these amines have no chromophore and cannot therefore be analysed using UV detection. Other analysis options include LC/MS, GC/MS or derivatisation followed by fluorescence detection. This last option is obviously the least attractive as another analysis step has been introduced.
7. General Discussion

The increase in demand for enantiomerically pure α-amino acids and amines has caused resurgence in the development of new methodologies for their preparation. One such growth area has been in the use of enzymes to bring about chemical transformations. Enzymes offer the advantages of high stereo- and regiospecificity, whilst being perceived as a "green" alternative to conventional organic reactions. The deracemisation of enantiomerically pure α-amino acids and amines using enzymes in combination with chemical reducing agents has the potential to provide an efficient, cost-effective route towards the preparation of these sought after compounds. Initial research into this area has shown that natural racemic α-amino acids could be transformed into their enantiomerically pure L-enantiomers using the enzyme D-amino acid oxidase in combination with a hydride reducing agent, either sodium borohydride or sodium cyanoborohydride. 84

The aim of this work was to further develop the technique to encompass the preparation of enantiomerically pure β-substituted α-amino acids and chiral amines. β-Substituted α-amino acids are currently receiving a great deal of attention in the field of peptidomimetics as well as in the study of enzyme mechanistics. Chiral amines are of interest because of their use as chiral auxiliaries in asymmetric synthesis and for their use as building blocks for drug molecules in the pharmaceutical industry. There were a number of obstacles to be overcome in order to achieve our goals. Firstly, the synthesis of β-substituted α-amino acids was required to test their suitability as substrates for the deracemisation reaction. Secondly, if enantiomerically pure D-α-amino acids were to be prepared a commercially viable L-amino acid oxidase had to be identified and, similarly, for the preparation of enantiopure chiral amines a monoamine oxidase of the flavin family was required.
Sufficient quantities of β- and γ-substituted α-amino acids were obtained using conventional synthetic methods, albeit in low overall yields. These compounds were then tested as substrates for the D-amino acid oxidase from *Trigonopsis variabilis* and found to be suitable, although longer reaction times were necessary than for the non-β-substituted amino acids. The deracemisation of these compounds was then shown to be effective using the enzyme with the hydride reducing agents. Further optimisation of the system will be needed to allow the deracemisation of all α-amino acids of interest and to reach the concentrations and yields required for industrial scale reactions. Subsequent research within our group has identified further reducing agents suitable for use in the deracemisation reaction, which may prove more effective in the deracemisation of the β- and γ-substituted α-amino acids.

The production of D-α-amino acids requires the use of an L-amino acid oxidase. As the commercially available LAAO's are prepared from snake venom there is a need for less expensive and less toxic sources. To this end a random screening program was adopted in an attempt to identify novel L-amino acid oxidases. Five organisms were found that were capable of transforming L-phenylalanine to phenylpyruvic acid. Further work on the characterisation of these organisms is required to prove the identity of an oxidase enzyme.

Research into preparation of D-α-amino acids using an L-amino acid oxidase with chemical reducing agents was also being carried out elsewhere within our group therefore it was decided to focus our attention on the preparation of enantiomerically pure chiral amines. The initial plan of work was to attempt the identification of a novel monoamine oxidase for use in the deracemisation reaction. Towards this aim a random screening of a variety of organisms was undertaken, in parallel to the LAAO screening program, with both spectrophotometric and chromatographic techniques being used for detection of products. The sensitivity of these assays proved to be insufficient and no new sources of monoamine oxidase were identified.
An existing monoamine oxidase from the fungus *Aspergillus niger* has been previously identified, sequenced and cloned by Schilling et al.\textsuperscript{120,121} The plasmid was kindly donated by Dr. B. Schilling (Givaudan-Roure Research Ltd., Switzerland) and transformed into various vectors by Marina Alexeeva (University of Edinburgh). Studies into the deracemisation using the wild type enzyme were ineffective, with any enantioselectivity observed perhaps due to either a native *E. coli* monoamine oxidase or to the production in the reaction mixture of another enzyme capable of transforming an amine, *e.g.* transaminase. To overcome these difficulties M. Alexeeva performed random mutagenesis on the plasmid and identified a mutant with increased stereospecificity towards the model substrate, *S*-α-methylbenzylamine. With this mutated enzyme in hand it was then possible to attempt the deracemisation of *RS*-α-methylbenzylamine. Experiments showed that the deracemisation system was suitable for the production of one enantiomer of a chiral amine from a racemic mixture. The conclusive proof for the deracemisation reaction is in the ability to perform the stereoinversion on one enantiomer into its antipode as shown by the conversion of *S*-AMBA to *R*-AMBA in 18% yield, 99% e.e. As with the deracemisation of α-amino acids it will be necessary to optimise the reaction further in order to compete with the industrial processes already established for the production of α-amino acids and amines. It may be that for bulk chemicals the deracemisation reaction cannot compete. However, for the production of specialist chiral compounds the deracemisation may offer an attractive alternative.
8. Experimental: Synthesis of α-amino acids

8.1. General Techniques

$^1$H and $^{13}$C NMR were recorded on Varian Gemini 200 or Brucker AC 250 instruments. Chemical shifts ($\delta_{HH}$) are reported in parts per million (ppm) downfield of tetramethylsilane and were referenced to residual undeuterated solvent present in the deuterated sample, e.g. CHCl$_3$ in CDCl$_3$. Assignments were made on the basis of chemical shift and coupling data. Abbreviations used in the descriptions of multiplicities are s (singlet), d (doublet), t (triplet), m (multiplet) and br (broad). Coupling constants (J) are quoted in hertz. Where diastereomeric signals are observed, * denotes the minor diastereomer. Chemical shifts ($\delta_C$) are quoted in parts per million (ppm) downfield of tetramethylsilane and are referenced to residual undeuterated solvent present in the deuterated sample. Assignments were made by comparison with the data obtained from similar structures. Carbon multiplicity was established by DEPT (distortionless enhancement by polarisation transfer). Where diastereomeric signals are observed, * denotes minor diastereomer.

Electrospray ionisation (ESI) mass spectrometry was carried out on a Micromass VG Platform II instrument from a mixed solvent system (MeCN:H$_2$O, 1:1) in +ve scan mode, using flow injection analysis. Mass Lynx version 2.3 software in open access mode was used for analysis of the data. m/z values are reported in Daltons and are followed by their percentage abundances in parentheses.

Thin layer chromatography was performed on Merck DC-Alufolien Kieselgel 60F$_{254}$ 0.2 mm pre-coated plates or Merck 60F$_{254}$ 0.25mm glass backed silica gel plates. Components were visualised by the quenching of u.v. fluorescence ($\lambda_{max.} = 254$ nm). Flash column chromatography was performed by the method of Still et al.$^{150}$ using silica gel (Merck 9385, particle size 0.04-0.063mm). The solvent system follows in parentheses.
All solvents and reagents for chemical synthesis were of standard laboratory grade and were used as supplied from commercial sources. All non-aqueous experiments were carried out under N$_2$ atmosphere unless specified otherwise, using oven-dried glassware (T 150 °C). Solvents and commercially available reagents were dried or purified, where appropriate, using standard procedures. THF was obtained dry and oxygen-free by distillation from sodium benzophenyl ketyl under nitrogen. In each experimental procedure the work-up was performed at room temperature unless reported otherwise. Brine refers to a saturated aqueous solution of sodium chloride, and ether refers to diethyl ether.

Optical rotations were measured on an Optical Activity AA-1000 polarimeter with a cell path length of 1 dm and concentrations (c) quoted in g.100 ml$^{-1}$ (sodium 589 nm detection).

### 8.2. Synthesis of unnatural DL-α-amino acids

#### 8.2.1. Strecker Synthesis

#### 8.2.1.1. Synthesis of (2RS,3RS)-2-amino-3-phenylbutanoic acid

\[
\text{Me} \hspace{1cm} \text{CO}_2^- \hspace{1cm} \text{NH}_2
\]

A solution of RS-2-phenylpropanal (13.3 ml, 100.0 mmol, 1.0 eq.), ammonium chloride (5.35 g, 100.0 mmol, 1.0 eq.), and potassium cyanide (6.55 g, 100.0 mmol, 1.0 eq.) in water (100 ml) was stirred at room temperature for 24 h. The mixture was extracted with ether (3 x 75 ml), and the combined organic phase washed with water (100 ml), then extracted with dilute HCl (165 ml). The aqueous phase was concentrated under vacuum to give a white solid (4.12 g, 25.8 mmol, 26%); m/z (ESI+) 201.6 (100%), 178.6 (5), 160.5 (M$^+$ 58) (required 160).
The aminobutyronitrile intermediate 111 (4.12 g, 25.8 mmol) was refluxed in conc. HCl (25 ml) for 16 h, and then concentrated under vacuum. The residue was triturred in MeCN to leave a white solid (3.18 g, 17.8 mmol, 18%); δH (200 MHz, DMSO-d6) 1.30-1.38 (3H, m, CH₃CH), 3.30-3.37 (1H, m, CH₃CH), 3.94-3.97 (1H, m, CHCH), 7.25-7.30 (5H, m, Ph), 8.53 (3H, br s, NH₃); δC (63 MHz, DMSO-d6) 16.2 (q*), 17.7 (q), 40.6 (d), 57.7 (d), 57.9 (d*), 127.4 (d), 128.1 (2 × d), 128.6 (2 × d), 140.3 (s), 140.5 (s*), 169.9 (s); d.e. = 40%, from the integration of HPLC peak area; m/z (ESI+) 220.6 (66%) 179.5 (M⁺ 100), 142.6 (70), 134.7 (18) (required 179).

8.2.1.2. Synthesis of (2RS,3RS)-2-amino-3-methylhexanoic acid

A solution of RS-2-methylpentenal (10.0 g, 100.0 mmol, 1.0 eq.), ammonium chloride (5.36 g, 100.0 mmol, 1.0 eq.), and potassium cyanide (6.57 g, 100.0 mmol, 1.0 eq.) in water (100 ml) was stirred at room temperature for 24 h. The mixture was extracted with ether (3 × 75 ml), and the combined organic phase washed with water (100 ml), then extracted with dilute HCl (165 ml). The solution was concentrated under vacuum, and the residue triturred in ether to leave an off-white solid (6.35 g, 50.4 mmol, 50%); m/z (ESI+) 167.6 (100%), 126.4 (M⁺ 28) (required 126).

The resultant solid (6.35 g, 50.4 mmol) was reflexed in conc. HCl (25 ml) for 18 h, concentrated under vacuum, and the residue triturred in MeCN to leave a white solid (3.29 g, 22.7 mmol, 23%); δH (200 MHz, DMSO-d6) 0.84-0.92 (2 × 3H, m, CH₃CH₂ + CH₃CH), 1.15-1.39 (2 × 2H, m, CH₃CH₂CH₂), 2.01 (1H, br s, CH₃CH), 3.73 (1H, br s, CH₃CHCH), 8.37 (3H, br s, NH₃); δC (63 MHz, DMSO-d6) 14.0 (q), 14.9 (q), 19.8 (t), 33.6 (d), 34.6 (t), 56.3 (d), 170.2 (s); d.e. = 40%, from the
Experimental: Synthesis

integration of HPLC peak area; \( m/z \) (ESI+) 186.5 (100%), 145.7 (M+ 92) (required 145).

8.2.2. Alkylation of N-(diphenylmethylene)aminoacetonitrile

8.2.2.1. Synthesis of (2RS,3RS)-2-amino-3-phenylbutanoic acid

\[
\begin{align*}
\text{Me} & \quad \text{Ph} \\
\text{NH}_3 & \quad \text{CO}_2^-
\end{align*}
\]

\( N-\text{(Diphenylmethylene)aminoacetonitrile} \) (0.50 g, 2.3 mmol, 1.0 eq.), potassium carbonate (1.60 g, 11.6 mmol, 5.0 eq.) and 1-bromo-1-phenylethane (0.41 ml, 3.0 mmol, 1.3 eq.) were refluxed in acetonitrile (10 ml) for 24 h. Excess base was removed by filtration, and the mother liquor dried over anhydrous MgSO\(_4\) then concentrated under vacuum to leave an orange oil. (0.58 g, 1.8 mmol, 78%);

\( \delta_H \) (250 MHz, CDCl\(_3\)) 1.51 (3H, d, J 7.0, CH\(^*\)3CH), 1.55 (3H, d, J 7.0, CH\(_2\)CH), 3.37 (1H, q, J 7.0, CH\(_3\)CH), 4.25 (1H, d, J 7.5, CH\(_2\)CHCH), 4.28 (1H, d, J 7.5, CH\(_3\)CHCH\(^*\)), 7.12-7.66 (15H, m, Ph); \( \delta_C \) (63 MHz, DMSO-d\(_6\)) 16.4 (q\(*\)), 17.0 (q), 44.2 (d\(*\)), 44.3 (d), 59.0 (d\(*\)), 60.0 (d), 126.1-129.1 (15 \times d), 130.9 (d), 131.0 (d\(*\)), 135.0 (s), 138.4 (s), 140.9 (s), 172.9 (s\(*\)), 173.4 (s).

The resultant solid (0.48g, 1.5 mmol) was refluxed in conc. HCl (15 ml) for 6 h, concentrated under vacuum, and the residue triturated in MeCN to leave a white solid (0.26 g, 1.5 mmol, 65%); \( \delta_H \) (250 MHz, DMSO-d\(_6\)) 1.36 (3H, d, J 7.0, CH\(_2\)CH), 1.38 (3H, d, J 7.0, CH\(^*\)3CH), 3.39 (1H, br s, CH\(_3\)CH), 3.96 (1H, br s, CH\(_3\)CHCH), 7.22-7.33 (5H, m, Ph), 8.55 (3H, br s, NH\(_3\)); \( \delta_C \) (63 MHz, DMSO-d\(_6\)) 16.1 (q), 17.8 (q\(*\)), 40.6 (d), 57.7 (d\(*\)), 58.0 (d), 127.3 (d), 127.4 (d\(*\)), 128.1 (2 \times d), 128.6 (2 \times d), 140.3 (s\(*\)), 140.6 (s), 169.7 (s), 169.8 (s\(*\)); d.e. = 40%, from the integration of HPLC peak area.
8.2.2.2. Synthesis of (2RS,3RS)-2-amino-3-ethylhexanoic acid

N-(Diphenylmethylene)aminoacetonitrile (1.00 g, 4.6 mmol, 1.0 eq.) in anhydrous THF (15 ml) was cooled to -78 °C under a nitrogen atmosphere. n-Butyllithium (3.20 ml of 1.6 M solution, 5.1 mmol, 1.1 eq.) was added over 5 minutes and the suspension stirred at -78 °C for 1.5 h, after which 3-bromohexane (0.80 ml, 5.7 mmol, 1.2 eq.) was added and the reaction stirred at room temperature for 20 h. The reaction was quenched with THF (1 ml) and concentrated under vacuum to leave an orange-brown oil which was resuspended in water (20 ml), then extracted with ether (3 x 25 ml). The combined organic phase was washed with brine (30 ml), then dried over anhydrous MgSO₄ and concentrated under vacuum to leave an orange oil (1.32 g).

The oil was chromatographed on silica (5% EtOAc/hexane). The relevant fractions were combined and reduced to leave a colourless oil (0.95 g, 3.1 mmol, 67%), which was refluxed in conc. HCl (30 ml) for 17 h. The solution was concentrated under vacuum and the residue triturated in ether to leave a white solid (0.23 g, 1.5 mmol, 32%); δH (250 MHz, MeOH-d₄) 1.01-1.16 (6H, m, 2 × CH₃), 1.43-1.62 (6H, m, 3 × CH₂), 1.98-2.04 (1H, m, CH), 4.11 (1H, m, CH); δC (63 MHz, MeOH-d₄) 10.1 (q), 12.4 (q), 19.4 (t), 21.8 (t), 30.8 (t), 40.3 (d), 54.0 (d), 169.7 (s); m/z (ESI+) 200.9 (100%), 159.8 (M⁺ 67) (required 159).
8.2.2.3. Synthesis of \((2RS,4RS)-2\)-amino-4-ethyloctanoic acid

\[
\text{Me} \begin{array}{c}
\text{Me} \\
\end{array}
\text{CO}_2\text{H}
\]

\(N\)-(Diphenylmethylene)aminoacetonitrile (1.00 g, 4.6 mmol, 1.0 eq.) in anhydrous THF (15 ml) was cooled to -78 °C under a nitrogen atmosphere. \(n\)-Butyllithium (3.20 ml of 1.6 M solution, 5.1 mmol, 1.1 eq.) was added over 5 minutes and the suspension stirred at -78 °C for 1 hour, after which 3-bromomethylheptane (1.00 ml, 5.6 mmol, 1.2 eq.) was added and the reaction stirred at room temperature for 18 h. The solution was concentrated under vacuum to leave a brown oil, which was resuspended in water (20 ml) and extracted with ether (4 x 25 ml). The combined organic phase was washed with brine (25 ml), then dried over anhydrous MgSO\(_4\) and concentrated under vacuum to leave an orange oil (1.71 g); \(m/z\) (ESI+) 332.5 (M\(^+\) 18%), 250.9 (75), 209.4 (100) (required 332).

The resultant oil was purified by chromatography on silica (5% EtOAc/hexane). The relevant fractions were combined and reduced to leave a colourless oil (1.21 g, 3.6 mmol, 78%); \(\delta_H\) (200 MHz, CDCl\(_3\)) 0.62-1.90 (17H, m, 2 x CH\(_3\), 5 x CH\(_2\), CH), 4.21 (1H, t, J 7.5, CH\(_{\text{Ar}}\)), 7.12-7.59 (10H, m, 2 x Ph).

The oil (1.21 g, 3.6 mmol) was refluxed in conc. HCl (30 ml) for 17 h, then concentrated under vacuum and triturated in ether to leave a white solid (0.74 g, 4.0 mmol, 87%); \(\delta_H\) (250 MHz, MeOH-d\(_4\)) 0.99-1.05 (6H, m, 2 x CH\(_3\)), 1.41-1.51 (8H, m, 4 x CH\(_2\)), 1.82-2.02 (2H, m, CH\(_2\)), 3.38-3.42 (1H, m, CH), 3.99-4.07 (1H, m, CH\(_{\text{Ar}}\)), \(\delta_C\) (63 MHz, MeOH-d\(_4\)) 8.3 (q*), 8.7 (q), 12.5 (q), 22.1 (t), 24.1 (t), 24.5 (t*), 27.3 (t), 27.6 (t*), 31.2 (t), 31.4 (t*), 34.2 (d), 34.3 (t), 50.5 (d), 170.4 (s); d.e. ~ 10%, from the integration of \(^{13}\)C NMR signals; \(m/z\) (ESI+) 229.0 (100%), 187.9 (M\(^+\) 87) (required 187).
8.2.2.4. Synthesis of 2-[N-(diphenylmethylene)]-4-phenylpentanenitrile

\[ \text{Ph} \cdot \text{CN} \]
\[ \text{CPh}_2 \]

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\( N-(\text{Diphenylmethylene})\text{aminoacetonitrile} (0.10 \text{ g}, 0.5 \text{ mmol}, 1.0 \text{ eq.}) \) in anhydrous THF (2 ml) was cooled to -78 °C under a nitrogen atmosphere. \( n\)-Butyllithium (0.30 ml of 1.6 M solution, 0.5 mmol, 1.1 eq.) was added over 5 minutes and the suspension stirred at -78 °C for 1.5 h, after which 2-bromo-1-phenylpropane (1.00 ml, 0.6 mmol, 1.2 eq.) was added and the reaction stirred at room temperature for 16 h. The solution was concentrated under vacuum to leave an orange residue, which was resuspended in water and extracted with ether (3 × 5 ml). The combined organic phase was washed with brine (7 ml), then dried over anhydrous MgSO\(_4\) and concentrated under vacuum to leave an orange oil (0.32 g); \( m/z \) (ESI+) 339.0 (M\(^+\) 100%), 215.7 (29), 181.5 (36), 174.6 (26) (required 339). Chromatography (5% EtOAc/hexane) did not yield the desired product.

\( N-(\text{Diphenylmethylene})\text{aminoacetonitrile} (0.10 \text{ g}, 0.5 \text{ mmol}, 1.0 \text{ eq.}), \) potassium carbonate (0.35 g, 2.5 mmol, 5.0 eq.) and 2-bromo-1-phenylpropane (0.38 ml, 2.5 mmol, 5.0 eq.) were refluxed in acetonitrile (5 ml) for 28 h. Further potassium carbonate (0.10 g, 0.7 mmol, 1.5 eq.) and 2-bromo-1-phenylpropane (0.08 ml, 0.5 mmol, 1.0 eq.) were added and the suspension refluxed for 18 h. Excess base was removed by filtration, and the mother liquor concentrated under vacuum to leave an orange oil, consistent with starting material and benzophenone.
8.2.2.5. Synthesis of (2RS,4S)-2-amino-4-methylhexanoic acid

\[ \text{Me} \overset{\text{Me}}{\text{NH}} \]

\[ \text{CO}_2 \]

\[ \text{134} \]

\( N\)-(Diphenylmethylene)aminoacetonitrile (1.21 g, 5.5 mmol, 1.0 eq.) in anhydrous THF (20 ml) was cooled to -78 °C under a nitrogen atmosphere. \( n\)-Butyllithium (4.00 ml of 1.6 M solution, 6.1 mmol, 1.1 eq.) was added over 5 minutes and the suspension stirred at -78 °C for 1 hour, after which (2S)-1-bromo-2-methylbutane (1.00 g, 6.6 mmol, 1.2 eq.) was added and the reaction stirred at room temperature for 18 h. The solution was concentrated under vacuum to leave a red-brown oil (2.80 g), which was resuspended in water (30 ml) and extracted with ether (4 x 35 ml). The combined organic phase was washed with brine (50 ml), then dried over anhydrous MgSO\(_4\) and concentrated under vacuum to leave an orange-red oil (1.45 g); \( m/z \) (ESI+) 336.1 (65%), 309.0 (37), 290.9 (M\(^\text{+} \) 100), 236.9 (30), 211.9 (33) (required 290).

The resultant oil was purified by chromatography (5% EtOAc/hexane). The relevant fractions were combined and reduced to leave a pale-yellow oil (1.07 g, 3.7 mmol, 67%); \( \delta_H \) (200 MHz, CDCl\(_3\)) 0.65-0.90 (6H, m, 2 x CH\(_3\)), 0.95-2.15 (5H, m, 2 x CH\(_2\), 1 x CH), 4.20-4.40 (1H, m, CH), 7.15-7.70 (10H, m, 2 x Ph); \( \alpha_0 \) +14.3 ° (c = 1.0, CHCl\(_3\)).

The oil (0.98 g, 3.4 mmol) was refluxed in conc. HCl (30 ml) for 22 h, then concentrated under vacuum and triturated in ether to leave a white solid (0.65 g, 3.6 mmol, 65%); \( \delta_H \) (250 MHz, DMSO-d\(_6\)) 0.78-0.86 (6H, m, 2 x CH\(_3\)), 1.07-1.79 (5H, m, 2 x CH\(_2\), CH), 3.75 (1H, br s, CH), 8.52 (3H, br s, NH\(_3\)); \( \delta_C \) (63 MHz, DMSO-d\(_6\)) 11.0 (q), 18.7 (q*), 19.0 (q), 28.7 (t), 28.8 (t*), 29.9 (d*), 30.0 (d), 37.3 (t), 37.3 (t*), 50.5 (d), 171.4 (s), 171.5 (s*); d.e. = 88%, from the integration of HPLC area; \( m/z \) (ESI+) 186.6 (45%), 145.5 (M\(^\text{+} \) 100) (required 145); \( \alpha_0 \) +7.0 ° (c = 1.0, H\(_2\)O).
9. Experimental: Deracemisation

9.1. General Techniques

HPLC analysis was carried out using a Waters 486 Tuneable Absorbance Detector and a Waters 600E Pump and Controller. Injection was through either a Rheodyne 7522i valve or Waters 717 Autosampler. Waters Millennium Chromatography Manager software was used for analysis of the data. Chiral HPLC analysis was performed on one of three different stationary phases: i) Chirex 3126 column (D-penicillamine based stationary phase) which was obtained from Phenomenex; ii) Chiralcel OD-H (cellulose based stationary phase) which was obtained from Merck Chromatography; iii) Crownpak CR(+) (crown ether based stationary phase) also obtained from Merck Chromatography.

Solvents for HPLC analysis were of the highest grade obtainable, and were filtered before use. α-Amino acids and reagents for deracemisation reactions were of standard laboratory grade and were used as supplied from commercial sources, except for the unnatural α-amino acids, which were synthesised as described in chapter 2. Commercial enzymes were used as supplied from Sigma, except for D-aminoacid oxidase from *Trigonopsis variabilis*, which was received as a gift from Boehringer Mannheim. The gene encoding monoamine oxidase from *Aspergillus niger*, which was integrated into the plasmid pECME3, was received as a gift from Dr. B. Schilling (Givaudan-Roure Research Ltd., Switzerland). The gene was subsequently subcloned into pET16b and subjected to random mutagenesis by Marina Alexeeva at the University of Edinburgh.

An Innova 4430 orbital shaker or Stuart Scientific SI 50 orbital incubator, set at the desired temperature, was used for incubation of the biotransformations.
9.2. Deracemisation of natural DL-α-amino acids

9.2.1. Reactions with porcine kidney D-amino acid oxidase

9.2.1.1. General Procedure I: Deracemisation on 2.5 mM scale, pH 8

A solution of DL-amino acid (100 μl of 25 mM solution, final concentration = 2.5 mM), FAD (100 μl of 0.8 mM solution, final concentration = 80 μM), catalase (1 mg of 21,000 U.mg⁻¹, 21,000 U), pk-DAAO and NaCNBH₃ in phosphate buffer (800 μl, 50 mM, pH 8) was incubated at 37 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.2.1.1.1. Deracemisation of DL-proline

Following general procedure I, using pk-DAAO (1 mg of 0.8 U.mg⁻¹ = 0.8 U) and NaCNBH₃ (1 mg, 15.9 mM, 6.4 eq.), and analysed by HPLC method 1. R.t. (L-92) = 7.5 min, r.t. (D-92) = 13.8 min. At t = 6 h: yield L = 1.45 mM, 58%; e.e. >99%.

Following general procedure I, using pk-DAAO (2 mg of 0.8 U.mg⁻¹ = 1.6 U) and NaCNBH₃ (1 mg, 15.9 mM, 6.4 eq.), and analysed by HPLC method 1. R.t. (L-92) = 7.8 min, r.t. (D-92) = 14.4 min. At t = 2.5 h: yield L = 2.36 mM, 94%; e.e. >99%.

Following general procedure I, using pk-DAAO (2 mg of 0.8 U.mg⁻¹ = 1.6 U) and NaCNBH₃ (2 mg, 31.8 mM, 12.8 eq.), and analysed by HPLC method 1. R.t. (L-92) = 8.6 min, r.t. (D-92) = 16.0 min. At t = 24 h: yield L = 2.36 mM, 94%; e.e. >99%.

Following general procedure I, using pk-DAAO (2 mg of 1.3 U.mg⁻¹ = 2.6 U) and NaCNBH₃ (4 mg, 63.6 mM, 25.6 eq.), and analysed by HPLC method 1. R.t. (L-92) = 5.5 min, r.t. (D-92) = 9.1 min. At t = 2.5 h: yield L = 2.06 mM, 82%; e.e. >99%.
9.2.1.2. General Procedure II: Deracemisation on 25 mM scale, pH 6.5

A solution of DL-amino acid (3 ml of 25 mM solution, final concentration = 25 mM), FAD (1 mg, 1.2 µmol, final concentration = 0.4 mM), catalase (3 mg of 21000 U.mg⁻¹, 63000 U), pk-DAAO (10 mg of 1.3 U.mg⁻¹ = 13.0 U) and NaCNBH₃ (50 mg, 264.6 mM, 10.6 eq.) in ammonium formate buffer (50 ml, pH 6.5) was incubated at 37 °C. Reactions were monitored by HPLC analysis, with aliquots filtered and injected directly.

9.2.1.2.1. Deracemisation of DL-proline

Analysis by HPLC method 1 (254 nm). R.t. (L-92) = 10.1 min, r.t. (D-92) = 21.0 min. At t = 3 h: yield L = 23.5 mM, 94%; e.e. >99%.

9.2.1.3. General Procedure III: Deracemisation on 2.5 mM scale with ammonium formate, pH 8

A solution of DL-amino acid (100 µl of 25 mM solution, final concentration = 2.5 mM), FAD (100 µl of 0.8 mM solution, final concentration = 80 µM), catalase (100 µl of 21000 U.ml⁻¹ solution, 2100 U), ammonium formate (100 µl of 124 mM solution, final concentration = 12.4 mM, 5.0 eq.), pk-DAAO (2 mg of 0.8 U.mg⁻¹ = 1.6 U) and NaBH₄ (3 mg, 79.0 mM, 31.6 eq.) in phosphate buffer (600 µl, 50 mM, pH 8) was incubated at 37 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.2.1.3.1. Deracemisation of DL-phenylalanine

Following general procedure III, and analysed by HPLC method 2. R.t. (L-139) = 15.0 min, r.t. (D-139) = 19.2 min. At t = 20 h: yield L = 1.86 mM, 74%; e.e. >99%.
9.2.2. Reactions with D-aminoacid oxidase from T. variabilis

9.2.2.1. General Procedure IV: Oxidation on 2.5 mM scale, pH 8

A solution of DL-amino acid (10 µl of 250 mM solution, final concentration = 2.5 mM), catalase (1 mg of 21000 U.mg⁻¹, 21000 U), and tv-DAAO (20 mg of 34 U.g⁻¹ = 0.68 U) in phosphate buffer (990 µl, 50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.2.2.1.1. Oxidation of DL-proline

Analysis by HPLC method 1. R.t. (L-92) = 5.0 min, r.t. (D-92) = 8.1 min. At t = 6 h: yield L = 1.20 mM, 48%; yield D = 0.10 mM, 4%, e.e. = 86%.

9.2.2.2. General Procedure V: Deracemisation on 2.5 mM scale, pH 8

A solution of DL-amino acid (100 µl of 25 mM solution, final concentration = 2.5 mM), catalase (100 µl of 21000 U.ml⁻¹ solution, 2100 U), tv-DAAO (20 mg of 34 U.g⁻¹ = 0.68 U) and NaBH₄ (4 mg, 105.3 mM, 42.1 eq.) in phosphate buffer (800 µl, 50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

9.2.2.2.1. Deracemisation of DL-phenylalanine

Following general procedure V and analysed by HPLC method 3. R.t. (L-139) = 18.5 min, r.t. (D-139) = 24.1 min. At t = 3.5 h: yield L = 1.59 mM, 64%; e.e. >99%.

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Experimental: Deracemisation

Following general procedure V, using ammonium formate (100 µl of 124 mM solution, final concentration = 12.4 mM, 5.0 eq.) and analysed by HPLC method 3 (1 ml.min⁻¹). R.t. (L-139) = 29.5 min, r.t. (D-139) = 38.2 min. At t = 3 h: yield L = 1.36 mM, 54%; e.e. >99.

Following general procedure V and analysed by HPLC method 3 (1 ml.min⁻¹). tv-DAAO (10 mg of 34 U.g⁻¹ = 0.34U) added after 1 hour. R.t. (L-139) = 29.6 min, r.t. (D-139) = 38.1 min. At t = 3.5 h: yield L = 1.36 mM, 54%; e.e. >99%.

9.2.2.3. General Procedure VI: Deracemisation on 2.5 mM scale, pH 8

A solution of DL-amino acid (2 ml of 2.5 mM solution, final concentration = 2.5 mM), catalase (1 mg of 21000 U.mg⁻¹, 21000 U), tv-DAAO and reducing agent in phosphate buffer (50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were centrifuged (14000 rpm, 1 min), and analysed by HPLC at regular intervals until no further reaction was observed.

9.2.2.3.1. Deracemisation of DL-leucine

Following general procedure VI, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U) and NaCNBH₃ (3 mg, 25.0 mM, 10.0 eq.), and analysed by HPLC method 4. R.t. (L-142) = 20.0 min, r.t. (D-142) = 30.1 min. At t = 22 h: yield L = 1.46 mM, 59%; e.e. >99%.

Following general procedure VI, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U) and NaCNBH₃ (6 mg, 50.0 mM, 20.0 eq.), and analysed by HPLC method 4. R.t. (L-142) = 20.0 min, r.t. (D-142) = 30.1 min. At t = 22 h: yield L = 1.65 mM, 66%; e.e. >99%.

Following general procedure VI, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U) and NaCNBH₃ (9 mg, 75.0 mM, 30.0 eq.), and analysed by HPLC method 4. R.t. (L-142) = 20.0 min, r.t. (D-142) = 30.1 min. At t = 22 h: yield L = 1.88 mM, 75%; e.e. >99%.
Experimental: Deracemisation

Following general procedure VI, using \( t \nu \)-DAAO (50 mg of 34 U.g\(^{-1} \) = 1.7 U) and NaCNBH\(_3\) (12 mg, 100.0 mM, 40.0 eq.), and analysed by HPLC method 4. R.t. (L-142) = 20.0 min, r.t. (D-142) = 30.1 min. At \( t = 22 \) h: yield L = 1.91 mM, 77%; e.e. >99%.

9.2.2.3.2. Deracemisation of DL-phenylalanine

Following general procedure VI, using \( t \nu \)-DAAO (50 mg of 34 U.g\(^{-1} \) = 1.7 U) and NaCNBH\(_3\) (12 mg, 100.0 mM, 40.0 eq.), and analysed by HPLC method 5. R.t. (L-139) = 18.8 min, r.t. (D-139) = 26.0 min. At \( t = 22 \) h: yield L = 1.65 mM, 66%; e.e. >99%.

Following general procedure VI, using \( t \nu \)-DAAO (100 mg of 34 U.g\(^{-1} \) = 3.4 U) and NaCNBH\(_3\) (20 mg, 158.7 mM, 63.5 eq.), and analysed by HPLC method 5. R.t. (L-139) = 20.4 min, r.t. (D-139) = 28.2 min. At \( t = 22 \) h: yield L = 1.64 mM, 66%; e.e. >99%.

9.2.2.4. General Procedure VII: Deracemisation on 25 mM scale, pH 8

A solution of DL-amino acid (2 ml of 25 mM solution, final concentration = 25 mM), catalase (2 mg of 21000 U.mg\(^{-1} \), 42000 U), \( t \nu \)-DAAO (500 mg of 34 U.g\(^{-1} \) = 17.0 U) and NaCNBH\(_3\) (32 mg, 254.0 mM, 10.2 eq.) in phosphate buffer (50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were centrifuged (14000 rpm, 1 min), and analysed by HPLC at regular intervals until no further reaction was observed.

9.2.2.4.1. Deracemisation of DL-phenylalanine

Following general procedure VII and analysed by HPLC method 5. R.t. (L-139) = 18.1 min, r.t. (D-139) = 25.0 min. At \( t = 22 \) h: yield L = 11.38 mM, 46%; e.e. = 94%, at \( t = 42 \) h: yield L = 12.13 mM, 49%; e.e. >99%.
9.2.2.4.2. Deracemisation of DL-leucine

Following general procedure VII and analysed by HPLC method 4. R.t. (L-142) = 17.2 min, r.t. (D-142) = 26.1 min. At t = 22 h: yield L = 12.88 mM, 52%; e.e. >99%.

9.2.2.4.3. Deracemisation of DL-methionine

Following general procedure VII and analysed by HPLC method 4. R.t. (L-143) = 13.5 min, r.t. (D-143) = 17.4 min. At t = 22 h: yield L = 12.00 mM, 48%; e.e. >99%.

9.3. Deracemisation of unnatural DL-α-amino acids

9.3.1. Reactions with D-aminoacid oxidase from T. variabilis

9.3.1.1. General Procedure VIII: Oxidation on 2.5 mM scale, pH 8

A solution of RS-amino acid (100 µl of 25 mM solution, final concentration = 2.5 mM), catalase (100 µl of 21000 U.ml⁻¹ solution, 2100 U), and rv-DAAO (20 mg of 34 U.g⁻¹ = 0.68 U) in phosphate buffer (800 µl, 50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.3.1.1.1. Oxidation of (2RS,3RS)-2-amino-3-phenylbutanoic acid, 112

Following general procedure VIII and analysed by HPLC method 2 (2 mM CuSO₄).

R.t. (2S,3S) = 9.4 min, r.t. (2R,3R) = 11.2 min, r.t. (2S,3R) = 16.7 min, r.t. (2R,3S) = 23.1 min. At t = 2 h: yield 2S,3S = 0.81 mM, 32%; yield 2R,3R = 0.05 mM, 2%; yield 2S,3R = 0.36 mM, 14%. 
9.3.1.1.2. Oxidation of (2RS,3RS)-2-amino-3-methylhexanoic acid, 115

Following general procedure VIII and analysed by HPLC method 4 (2 mM CuSO₄).
R.t. (2S,3R) = 20.3 min, r.t. (2S,3S) = 26.7 min, r.t. (2R,3S) = 29.0 min, r.t. (2R,3R) = 40.9 min. At t = 3 h: yield 2S,3R = 0.45 mM, 18%; yield 2S,3S = 0.83 mM, 33%; yield 2R,3R = 0.03 mM, 1%.

9.3.1.2. General Procedure IX: Oxidation on 2.5 mM scale, pH 6.5

A solution of RS-amino acid (100 μl of 25 mM solution, final concentration = 2.5 mM), catalase (1 mg of 21000 U.mg⁻¹, 21000 U), and tv-DAAO in ammonium formate buffer (900 μl, 50 mM, pH 6.5) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.3.1.2.1. Oxidation of (2RS,3RS)-2-amino-3-ethylhexanoic acid, 124

Following general procedure IX, using tv-DAAO (20 mg of 34 U.g⁻¹ = 0.68 U), and analysed by HPLC method 5. R.t. (1) = 31.5 min, r.t. (2) = 37.0 min, r.t. (3) = 41.0 min, r.t. (4) = 54.4 min. At t = 3 h: % (1) = 76; % (2) = 78; % (3) = 73; % (4) = 0.

Following general procedure IX, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U), and analysed by HPLC method 5. R.t. (1) = 29.9 min, r.t. (2) = 35.3 min, r.t. (3) = 39.3 min, r.t. (4) = 51.9 min. At t = 17 h: % (1) = 137; % (2) = 136; % (3) = 81; % (4) = 0.

9.3.1.3. General Procedure X: Deracemisation on 2.5 mM scale, pH 8

A solution of RS-amino acid (100 μl of 25 mM solution, final concentration = 2.5 mM), catalase (100 μl of 21000 U.ml⁻¹ solution, 2100 U), tv-DAAO (20 mg of 34 U.g⁻¹ = 0.68 U) and reducing agent in phosphate buffer (800 μl, 50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.
Reactions were carried out according to the general procedure, with deviations indicated in italics.

9.3.1.3.1. Deracemisation of \((2RS,3RS)-2\text{-amino-3-phenylbutanoic acid}\), 112

Following general procedure X, using NaCNBH\(_3\) (4 mg, 63.5 mM, 25.4 eq.) and ammonium formate (\(100 \mu\text{l of 124 mM solution, final concentration = 12.4 mM, 5.0 eq.}\)), and analysed by HPLC method 2 (2 mM CuSO\(_4\)). R.t. (2S,3S) = 9.5 min, r.t. (2R,3R) = 11.3 min, r.t. (2S,3R) = 17.1 min, r.t. (2R,3S) = 23.5 min. At t = 2 h: yield 2S,3S = 0.93 mM, 37%; yield 2R,3R = 0.36 mM, 14%; yield 2S,3R = 0.43 mM, 17%; yield 2R,3S = 0.04 mM, 1%.

Following general procedure X, using NaBH\(_4\) (4 mg, 105.3 mM, 42.1 eq.), and analysed by HPLC method 2 (1 ml.min\(^{-1}\), 2 mM CuSO\(_4\)). R.t. (2S,3S) = 14.6 min, r.t. (2R,3R) = 17.4 min, r.t. (2S,3R) = 26.2 min, r.t. (2R,3S) = 36.1 min. At t = 6 h: yield (2S,3S) = 1.04 mM, 42%; yield 2S,3R = 0.72 mM, 29%; total yield S-112 = 1.76 mM, 71%; e.e. >99%.

9.3.1.3.2. Deracemisation of \((2RS,3RS)-2\text{-amino-3-methylhexanoic acid}\), 115

Following general procedure X, using NaCNBH\(_3\) (4 mg, 63.5 mM, 25.4 eq.) and ammonium formate (\(100 \mu\text{l of 124 mM solution, final concentration = 12.4 mM, 5.0 eq.}\)), and analysed by HPLC method 4 (2 mM CuSO\(_4\)). R.t. (2S,3S) = 19.3 min, r.t. (2S,3R) = 24.9 min, r.t. (2R,3S) = 27.2 min, r.t. (2R,3R) = 38.5 min. At t = 18 h: yield 2S,3R = 0.69 mM, 28%; yield (2S,3S) = 0.88 mM, 35%; total yield S-115 = 1.57 mM, 63%; e.e. >99%.

Following general procedure X, using NaBH\(_4\) (4 mg, 105.3 mM, 42.1 eq.), and analysed by HPLC method 4 (2 mM CuSO\(_4\)). R.t. (2S,3R) = 19.6 min, r.t. (2S,3S) = 25.6 min, r.t. (2R,3S) = 27.9 min, r.t. (2R,3R) = 39.6 min. At t = 3 h: yield 2S,3R = 1.20 mM, 48%; yield (2S,3S) = 1.16 mM, 46%; total yield S-115 = 2.36 mM, 94%; e.e. >99%.
Following general procedure X, using NaBH₄ (4 mg, 105.3 mM, 42.1 eq.), and analysed by HPLC method 4 (2 mM CuSO₄). R.t. (2S,3R) = 18.9 min, r.t. (2S,3S) = 25.4 min, r.t. (2R,3S) = 27.7 min, r.t. (2R,3R) = 39.1 min. At t = 3.5 h: yield 2S,3R = 1.28 mM, 51%; yield 2S,3S = 1.21 mM, 48%; total yield S-115 = 2.49 mM, 99%; e.e. >99%.

9.3.1.4. General Procedure XI: Deracemisation on 2.5 mM scale with ammonium formate, pH 8

A solution of RS-amino acid (2 ml of 2.5 mM solution, final concentration = 2.5 mM), catalase (1 mg of 21000 U.mg⁻¹, 21000 U), tv-DAAO and reducing agent in ammonium formate buffer (50 mM, pH 8) was incubated at 30 °C. Aliquots of the reaction mixture were centrifuged, and analysed by HPLC at regular intervals until no further reaction was observed.

9.3.1.4.1. Deracemisation of (2RS,3RS)-2-amino-3-phenylbutanoic acid, 112

Following general procedure XI, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U) and NaCNBH₃ (12 mg, 95.2 mM, 38.1 eq.), and analysed by HPLC method 5. R.t. (2S,3S) = 20.2 min, r.t. (2R,3R) = 24.2 min, r.t. (2S,3R) = 41.2 min, r.t. (2R,3S) = 57.3 min. At t = 22 h: yield 2S,3S = 9.75 mM, 39%; yield 2R,3R = 4.63 mM, 19%; yield 2S,3R = 2.56 mM, 10%.

Following general procedure XI, using tv-DAAO (100 mg of 34 U.g⁻¹ = 3.4 U) and NaCNBH₃ (20 mg, 158.7 mM, 63.5.eq.), and analysed by HPLC method 5. R.t. (2S,3S) = 23.0 min, r.t. (2R,3R) = 27.5 min, r.t. (2S,3R) = 47.8 min, r.t. (2R,3S) = 65.9 min. At t = 22 h: yield 2S,3S = 10.79 mM, 43%; yield 2R,3R = 6.21 mM, 25%; yield 2S,3R = 4.42 mM, 18%.

9.3.1.4.2. Deracemisation of (2RS,4S)-2-amino-4-methylhexanoic acid, 134

Following general procedure XI, using tv-DAAO (50 mg of 34 U.g⁻¹ = 1.7 U) and NaCNBH₃ (12 mg, 95.2 mM, 38.1 eq.), and analysed by HPLC method 5. R.t. (L) = 19.4 min, r.t. (D) = 25.3 min. At t = 22 h: yield S-134 = 16.56 mM, 66%; e.e. >99%.
Following general procedure XI, using $\tau$-DAAO (100 mg of 34 U.g$^{-1}$ = 3.4 U) and NaCNBH$_3$ (20 mg, 158.7 mM, 63.5 eq.), and analysed by HPLC method 5. R.t. (L) = 23.0 min, r.t. (D) = 29.3 min. At t = 22 h: yield $S$-134 = 14.96 mM, 60%; e.e. >99%.

9.3.1.5. General Procedure XII: Oxidation on 25 mM scale, pH 6.5

A solution of RS-amino acid (1 ml of 25 mM solution, final concentration = 25 mM), catalase (1 mg of 21000 U.mg$^{-1}$, 21000 U) and $\tau$-DAAO in ammonium formate buffer (50 mM, pH 6.5) was incubated at 30 °C. Aliquots of the reaction mixture were filtered, and analysed by HPLC at regular intervals until no further reaction was observed.

9.3.1.5.1. Oxidation of (2RS,3RS)-2-amino-3-phenylbutanoic acid, 112

Following general procedure XII, using $\tau$-DAAO (200 mg of 34 U.g$^{-1}$ = 6.8 U), and analysed by HPLC method 5. R.t. (2S,3S) = 24.5 min, r.t. (2R,3R) = 29.7 min, r.t. (2S,3R) = 50.9 min, r.t. (2R,3S) = 71.5 min. At t = 5 h: yield 2S,3S = 7.84 mM, 31%; yield 2R,3R = 3.89 mM, 16%; yield 2S,3R = 3.59 mM, 14%.

Following general procedure XII, using $\tau$-DAAO (250 mg of 34 U.g$^{-1}$ = 8.5 U), and analysed by HPLC method 5. R.t. (2S,3S) = 20.5 min, r.t. (2R,3R) = 24.3 min, r.t. (2S,3R) = 40.1 min, r.t. (2R,3S) = 56.8 min. At t = 22 h: yield 2S,3S = 7.24 mM, 29%; yield 2R,3R = 0.85 mM, 3%; yield 2S,3R = 2.95 mM, 12%.

9.3.1.5.2. Oxidation of (2RS,4S)-2-amino-4-methylhexanoic acid, 134

Following general procedure XII, using $\tau$-DAAO (250 mg of 34 U.g$^{-1}$ = 8.5 U), and analysed by HPLC method 5. R.t. (2S,3R) = 11.0 min, r.t. (2R,3S) = 17.0 min, r.t. (2S,3S) = 20.3 min, r.t. (2R,3R) = 25.8 min. At t = 22 h: yield 2S,3R = 1.19 mM, 5%; yield 2S,3S = 10.53 mM, 42%; total yield S-134 = 11.72 mM, 47%; e.e. >99%.
9.3.1.6. General Procedure XIII: Deracemisation on 25 mM scale, pH 6.5

A solution of RS-amino acid (3 ml of 25 mM solution, final concentration = 25 mM), catalase (3 mg of 21000 U.mg⁻¹, 63000 U), \( \nu \)-DAAO and reducing agent in ammonium formate buffer (50 mM, pH 6.5) was incubated at 30 °C. Aliquots of the reaction mixture were centrifuged (14000 rpm, 1 min), and analysed by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

9.3.1.6.1. Deracemisation of (2RS,3RS)-2-amino-3-phenylbutanoic acid, 112

Following general procedure XIII, using \( \nu \)-DAAO (750 mg of 34 U.g⁻¹ = 25.5 U) and NaCNBH₃ (24 mg, 127.0 mM, 5.1 eq.), and analysed by HPLC method 5. R.t. \((2S,3S) = 20.3 \text{ min}, \ t.t. \ (2R,3R) = 24.3 \text{ min}, \ t.t. \ (2S,3R) = 40.4 \text{ min}, \ t.t. \ (2R,3S) = 56.8 \text{ min}. \) At \( t = 26 \text{ h} \): yield \( 2S,3S = 8.44 \text{ mM}, 34\%; \) yield \( 2R,3R = 1.77 \text{ mM}, 7\%; \) yield \( 2S,3R = 4.30 \text{ mM}, 17\% \).

Following general procedure XIII (2 ml amino acid solution, 2 mg catalase), using \( \nu \)-DAAO (500 mg of 34 U.g⁻¹ = 17.0 U) and NaCNBH₃ (32 mg, 254.0 mM, 10.2 eq.), and analysed by HPLC method 5. R.t. \((2S,3S) = 20.7 \text{ min}, \ t.t. \ (2R,3R) = 24.7 \text{ min}, \ t.t. \ (2S,3R) = 41.0 \text{ min}, \ t.t. \ (2R,3S) = 57.7 \text{ min}. \) At \( t = 42 \text{ h} \): yield \( 2S,3S = 8.10 \text{ mM}, 32\%; \) yield \( 2R,3R = 2.14 \text{ mM}, 9\%; \) yield \( 2S,3R = 4.65 \text{ mM}, 19\% \).

9.3.1.6.2. Deracemisation of (2RS,3RS)-2-amino-3-methylhexanoic acid, 115

Following general procedure XIII (5 ml amino acid solution, 5 mg catalase), using \( \nu \)-DAAO (1.25 g of 34 U.g⁻¹ = 42.5 U) and NaCNBH₃ (80 mg, 254.0 mM, 10.2 eq.), and analysed by HPLC method 6. R.t. \((2S,3R) = 48.4 \text{ min}, \ t.t. \ (2S,3S) = 68.6 \text{ min}, \ t.t. \ (2R,3S) = 74.4 \text{ min}, \ t.t. \ (2R,3R) = 114.7 \text{ min}. \) At \( t = 22 \text{ h} \): yield \( 2S,3R = 7.07 \text{ mM}, 28\%; \) yield \( 2S,3S = 7.51 \text{ mM}, 30\%; \) total yield \( S-115 = 14.58 \text{ mM}, 58\%; \) e.e. >99%.
Experimental: Deracemisation

Following general procedure XIII (2 ml amino acid solution, 2 mg catalase), using tv-DAAO (500 mg of 34 U.g\(^{-1}\) = 17.0 U) and NaBH\(_4\) (19 mg, 250.0 mM, 10.0 eq.), and analysed by HPLC method 6. R.t. (2S,3R) = 44.8 min, r.t. (2S,3S) = 63.1 min, r.t. (2R,3S) = 68.1 min, r.t. (2R,3R) = 103.1 min. At t = 22 h: yield 2S,3R = 3.70 mM, 15%; yield 2S,3S = 7.76 mM, 31%; total yield \(S-\)115 = 11.46 mM, 46%; e.e. >99%.

9.3.1.6.3. Deracemisation of (2RS,4S)-2-amino-4-methylhexanoic acid, 134

Following general procedure XIII, using tv-DAAO (750 mg of 34 U.g\(^{-1}\) = 25.5 U) and NaCNBH\(_3\) (24 mg, 127.0 mM, 5.1 eq.), and analysed by HPLC method 5. R.t. (2S,3R) = 10.3 min, r.t. (2R,3S) = 15.8 min, r.t. (2S,3S) = 19.0 min, r.t. (2R,3R) = 24.3 min. At t = 28 h: yield 2S,3R = 0.91 mM, 4%; yield 2S,3S = 11.56 mM, 46%; total yield \(S-\)134 = 12.47 mM, 50%; e.e. >99%.

Following general procedure XIII (2 ml amino acid solution, 2 mg catalase), using tv-DAAO (500 mg of 34 U.g\(^{-1}\) = 17.0 U) and NaCNBH\(_3\) (32 mg, 254.0 mM, 10.2 eq.), and analysed by HPLC method 5. R.t. (2S,3R) = 10.9 min, r.t. (2R,3S) = 16.8 min, r.t. (2S,3S) = 19.9 min, r.t. (2R,3R) = 25.6 min. At t = 42 h: yield 2S,3R = 0.68 mM, 3%; yield 2S,3S = 10.37 mM, 42%; total yield \(S-\)134 = 11.05 mM, 45%; e.e. >99%.

9.4. Oxidation of RS-\(\alpha\)-methylbenzylamine

9.4.1. Oxidation by E. coli BLR(DE3) [pECME3]

9.4.1.1. General Procedure XIV: Oxidation by cell free extract

BLR(DE3) [pECME3] cell free extract was added to a solution of RS-AMBA in ammonium formate buffer (50 mM, pH 7.4). A 300 \(\mu\)l aliquot was mixed with 300 \(\mu\)l NaOH (50 mM) and 300 \(\mu\)l hexane, and the hexane layer analysed by HPLC method 7. The reaction mixture was incubated at 30 °C and monitored at regular intervals by HPLC until no further reaction was observed.
Experimental: Deracemisation

Following general procedure XIV, using CFE (§ 5.1.1, 1 ml of 0.20 U.ml⁻¹ = 0.20 U) and RS-AMBA (9 ml of 1 mM solution, final concentration = 0.9 mM). R.t. (R-AMBA) = 21.2 min, r.t. (S-AMBA) = 29.4 min. At t = 95 h: yield R-AMBA = 0.25 mM, 28%; e.e. = 16%.

Following general procedure XIV, using CFE (§ 5.1.1, 2 ml of 0.20 U.ml⁻¹ = 0.40 U) and RS-AMBA (8 ml of 1 mM solution, final concentration = 0.8 mM). R.t. (R-AMBA) = 21.2 min, r.t. (S-AMBA) = 29.4 min. At t = 96 h: yield R-AMBA = 0.21 mM, 26%; e.e. = 8%.

Following general procedure XIV, using CFE (§ 5.1.1, 3 ml of 0.20 U.ml⁻¹ = 0.60 U) and RS-AMBA (7 ml of 1 mM solution, final concentration = 0.7 mM). R.t. (R-AMBA) = 21.2 min, r.t. (S-AMBA) = 29.4 min. At t = 97 h: yield R-AMBA = 0.16 mM, 23%; e.e. = 10%.

Following general procedure XIV, using CFE (§ 5.1.1, 1 ml of 0.20 U.ml⁻¹ = 0.20 U) and RS-AMBA (9 ml of 5 mM solution, final concentration = 4.5 mM). R.t. (R-AMBA) = 20.8 min, r.t. (S-AMBA) = 29.0 min. At t = 74 h: yield R-AMBA = 1.77 mM, 39%; e.e. = 2%.

9.4.1.2. General Procedure XV: Oxidation of single enantiomer

BLR(DE3) [pECME3] cell free extract (§ 5.1.1, 500 µl of 0.20 U.ml⁻¹ = 0.10 U) was added to a solution of AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM) in ammonium formate buffer (50 mM, pH 7.4) and the reaction mixture was incubated at 30 °C. After 4 days, 5 ml NaOH (50 mM) and 5 ml hexane was added and the hexane layer was analysed by HPLC method 7.

Following general procedure XV, R-AMBA. R.t. (R-AMBA) = 23.9 min, yield R-AMBA = 0.60 mM, 67%.

Following general procedure XV, S-AMBA. R.t. (S-AMBA) = 33.1 min, yield S-AMBA = 0.57 mM, 63%.
9.4.1.3. General Procedure XVI: Oxidation with hexane as co-solvent

BLR(DE3) [pECME3] cell free extract (§ 5.1.1, 500 µl of 0.20 U.ml\(^{-1}\) = 0.10 U) was added to a solution of RS-AMBA (50 µl of 100 mM solution in hexane, final concentration = 1 mM) in hexane and ammonium formate buffer (50 mM, pH 7.4). A 300 µl aliquot was mixed with 300 µl NaOH (50 mM) and 300 µl hexane and the hexane layer was analysed by HPLC method 7. The reaction mixture was incubated at 30 °C and monitored at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XVI, using hexane (450 µl) and buffer (4 ml). R.t. (\(R\)-AMBA) = 25.2 min, r.t. (\(S\)-AMBA) = 33.1 min. At t = 94 h: yield \(R\)-AMBA = 0.13 mM, 13%; e.e. = 40%.

Following general procedure XVI, using hexane (700 µl) and buffer (3.75 ml). R.t. (\(R\)-AMBA) = 24.5 min, r.t. (\(S\)-AMBA) = 33.7 min. At t = 95 h: yield \(R\)-AMBA = 0.16 mM, 16%; e.e. = 22%.

Following general procedure XVI, using hexane (450 µl) and buffer (4 ml, pH 5). R.t. (\(R\)-AMBA) = 20.6 min, r.t. (\(S\)-AMBA) = 27.1 min. At t = 96 h: yield \(R\)-AMBA = 0.32 mM, 32%; e.e. = 19%.

Following general procedure XVI, using hexane (450 µl) and buffer (4 ml, pH 6). R.t. (\(R\)-AMBA) = 20.8 min, r.t. (\(S\)-AMBA) = 27.2 min. At t = 96 h: yield \(R\)-AMBA = 0.26 mM, 26%; e.e. = 16%.

Following general procedure XVI, using hexane (450 µl) and buffer (4 ml, pH 7). R.t. (\(R\)-AMBA) = 20.8 min, r.t. (\(S\)-AMBA) = 27.2 min. At t = 96 h: yield \(R\)-AMBA = 0.23 mM, 23%; e.e. = 17%.

Following general procedure XVI, using hexane (450 µl) and buffer (4 ml, pH 8). R.t. (\(R\)-AMBA) = 21.1 min, r.t. (\(S\)-AMBA) = 27.6 min. At t = 96 h: yield \(R\)-AMBA = 0.29 mM, 29%; e.e. = 14%.
Following general procedure XVI, using hexane (450 µl) and buffer (4 ml, pH 9).
R.t. (R-AMBA) = 20.5 min, r.t. (S-AMBA) = 27.0 min. At t = 96 h: yield R-AMBA = 0.28 mM, 28%; e.e. = 18%.

9.4.1.4. General Procedure XVII: Oxidation by partially purified MAO-N

Partially purified BL21(DE3) [pECME3] (§ 5.1.3, 500 µl of 0.92 U.ml⁻¹ = 0.46 U) was added to a solution of RS-AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM) in phosphate buffer (100 mM, pH 8). A 100 µl aliquot was diluted in 900 µl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

R.t. (S-AMBA) = 14.9 min, r.t. (R-AMBA) = 19.1 min. At t = 94 h: yield R-AMBA = 0.33 mM, 37%; e.e. = 0%.

9.4.2. Oxidation by E. coli BL21(DE3) [pET 16b]

9.4.2.1. General Procedure XVIII: Oxidation by whole cells of E. coli BL21(DE3) [pET16b]

Freeze-thawed whole cells of BL21(DE3) [pET16b] (§ 5.3.1, 500 µl) was added to a solution of RS-AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM) in phosphate buffer (100 mM, pH 8). A 100 µl aliquot was diluted in 900 µl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XVIII. R.t. (S-AMBA) = 14.3 min, r.t. (R-AMBA) = 18.3 min. At t = 89 h: yield R-AMBA = 0.36 mM, 40%; e.e. = 8%.
Following general procedure XVIII (buffer = 20 mM). R.t. (S-AMBA) = 14.8 min, r.t. (R-AMBA) = 19.2 min. At t = 99 h: yield R-AMBA = 0.52 mM, 58%; e.e. = 76%.

9.4.2.2. General Procedure XIX: Oxidation by cell free extract of BL21(DE3) [pET16b]

BL21(DE3) [pET16b] cell free extract (§ 5.3.2, 100 μl) was added to a solution of RS-AMBA (900 μl of 1 mM solution, final concentration = 0.9 mM) in phosphate buffer (20 mM, pH 8). A 10 μl aliquot was diluted in 990 μl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At t = 20 h: yield R-AMBA = 0.49 mM, 54%; e.e. = 26%. At t = 96 h: yield R-AMBA = 0.41 mM, 46%; e.e. = 98%.

9.4.2.3. General Procedure XX: Oxidation by purified BL21(DE3) [pET16b]

Partially purified BL21(DE3) [pET16b] (§ 5.3.2, 100 μl) was added to a solution of RS-AMBA (900 μl of 1 mM solution, final concentration = 0.9 mM) in phosphate buffer (20 mM, pH 8). A 10 μl aliquot was diluted in 990 μl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At t = 20 h: yield R-AMBA = 0.48 mM, 53%; e.e. = 4%. At t = 96 h: yield R-AMBA = 0.46 mM, 51%; e.e. = 38%.
9.4.3. Oxidation by *E. coli* BL21(DE3) [pMAO-N]

9.4.3.1. General Procedure XXI: Oxidation by whole cells of BL21(DE3) [pMAO-N]

Freeze-thawed whole cells of BL21(DE3) [pMAO-N] was added to a solution of RS-AMBA in phosphate buffer (100 mM, pH 8). A 100 µl aliquot was diluted in 900 µl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 500 µl of 0.19 U.ml⁻¹ = 0.10 U) and RS-AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 14.9 min, r.t. (R-AMBA) = 19.2 min. At t = 94 h: yield R-AMBA = 0.38 mM, 42%; e.e. = 14%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 500 µl of 0.19 U.ml⁻¹ = 0.10 U) and RS-AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 14.3 min, r.t. (R-AMBA) = 18.3 min. At t = 89 h: yield R-AMBA = 0.41 mM, 45%; e.e. = 10%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 2.5 ml of 0.19 U.ml⁻¹ = 0.48 U) and RS-AMBA (2.5 ml of 1 mM solution, final concentration = 0.5 mM). R.t. (S-AMBA) = 15.2 min, r.t. (R-AMBA) = 19.6 min. At t = 47 h: yield R-AMBA = 0.23 mM, 46%; e.e. = 10%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 1.5 ml of 0.19 U.ml⁻¹ = 0.29 U) and RS-AMBA (3.5 ml of 1 mM solution, final concentration = 0.7 mM). R.t. (S-AMBA) = 15.1 min, r.t. (R-AMBA) = 19.5 min. At t = 47 h: yield R-AMBA = 0.39 mM, 56%; e.e. = 10%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 500 µl of 0.19 U.ml⁻¹ = 0.10 U) and RS-AMBA (4.5 ml of 1 mM solution, final concentration
Experimental: Deracemisation

= 0.9 mM). R.t. (S-AMBA) = 15.1 min, r.t. (R-AMBA) = 19.4 min. At t = 47 h: yield R-AMBA = 0.49 mM, 54%; e.e. = 10%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.3.1, 500 µl of 0.16 U.ml⁻¹ = 0.08 U), RS-AMBA (4.5 ml of 1 mM solution, final concentration = 0.9 mM) and buffer = 20 mM. R.t. (S-AMBA) = 14.7 min, r.t. (R-AMBA) = 19.0 min. At t = 99 h: yield R-AMBA = 0.70 mM, 78%; e.e. = 58%.

Following general procedure XXI, using BL21(DE3) [pMAO-N] (§ 5.2.1, 1 ml of 0.16 U.ml⁻¹ = 0.16 U), RS-AMBA (0.13 µl, final concentration = 1.0 mM) and buffer = 20 mM. Analysis: 10 µl aliquot in 990 µl HClO₄. R.t. (S-AMBA) = 14.8 min, r.t. (R-AMBA) = 19.3 min. At t = 97 h: yield R-AMBA = 0.34 mM, 34%; e.e. = 26%.

9.4.3.2. General Procedure XXII: Oxidation by cell free extract of BL21(DE3) [pMAO-N]

BL21(DE3) [pMAO-N] cell free extract was added to a solution of RS-AMBA in phosphate buffer (20 mM, pH 8). A 10 µl aliquot was diluted in 990 µl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XXII, using BL21(DE3) [pMAO-N] CFE (§ 5.2.1, 100 µl of 1.90 U.ml⁻¹ = 0.19 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM). Analysis: 100 µl in 900 µl HClO₄. R.t. (S-AMBA) = 14.8 min, r.t. (R-AMBA) = 19.2 min. At t = 99 h: yield R-AMBA = 0.43 mM, 48%; e.e. = 24%.

Following general procedure XXII, using BL21(DE3) [pMAO-N] CFE (§ 5.2.1, 100 µl of 1.90 U.ml⁻¹ = 0.19 U), RS-AMBA (900 µl of 1 mM solution, final
Experimental: Deracemisation

clearance = 0.9 mM) and catalase (1 mg of 21000 U.mg⁻¹ = 21000 U). Analysis:
100 µl in 900 µl HClO₄. R.t. (S-AMBA) = 14.8 min, r.t. (R-AMBA) = 19.2 min. At t
= 99 h: yield R-AMBA = 0.63 mM, 70%; e.e. = 50%.

Following general procedure XXII, using BL21(DE3) [pMAO-N] CFE (§ 5.2.1,
100 µl of 2.68 U.ml⁻¹ = 0.27 U) and RS-AMBA (900 µl of 1 mM solution, final
concentration = 0.9 mM). R.t. (S-AMBA) = 14.5 min, r.t. (R-AMBA) = 18.8 min. At
t = 97 h: yield R-AMBA = 0.45 mM, 50%; e.e. = 46%.

Following general procedure XXII, using BL21(DE3) [pMAO-N] CFE (§ 5.3.2,
100 µl of 3.0 U.ml⁻¹ = 0.30 U) and RS-AMBA (900 µl of 1 mM solution, final
concentration = 0.9 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At
t = 20 h: yield R-AMBA = 0.50 mM, 56%; e.e. = 44%. At t = 96 h: yield R-AMBA =
0.43 mM, 48%; e.e. > 99%.

9.4.3.3. General Procedure XXIII: Oxidation by purified BL21(DE3)
[pMAO-N]

Partially purified BL21(DE3) [pMAO-N] was added to a solution of RS-AMBA in
phosphate buffer (20 mM, pH 8). A 10 µl aliquot was diluted in 990 µl perchloric
acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at
30 °C and the reaction monitored by HPLC at regular intervals until no further
reaction was observed.

Reactions were carried out according to the general procedure, with deviations
indicated in italics.
Following general procedure XXIII, using BL21(DE3) [pMAO-N] (§ 5.3.2, 100 µl of 1.60 U.ml⁻¹ = 0.16 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM). Further MAO-N (100 µl of 1.60 U.ml⁻¹ = 0.16 U) was added at t = 68 h and 91 h. R.t. (S-AMBA) = 14.0 min, r.t. (R-AMBA) = 17.9 min. At t = 96 h: yield R-AMBA = 0.35 mM, 39%; e.e. = 8%.

Following general procedure XXIII, using BL21(DE3) [pMAO-N] (§ 5.3.2, 100 µl of 1.60 U.ml⁻¹ = 0.16 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) at room temperature. Further MAO-N (100 µl of 1.60 U.ml⁻¹ = 0.16 U) was added at t = 68 h and 91 h. R.t. (S-AMBA) = 14.4 min, r.t. (R-AMBA) = 18.6 min. At t = 96 h: yield R-AMBA = 0.45 mM, 50%; e.e. = 10%.

Following general procedure XXIII, using BL21(DE3) [pMAO-N] (§ 5.3.2, 100 µl of 2.69 U.ml⁻¹ = 0.27 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At t = 20 h: yield R-AMBA = 0.47 mM, 52%; e.e. = 6%. At t = 96 h: yield R-AMBA = 0.41 mM, 46%; e.e. = 38%.

### 9.4.4. Oxidation by E. coli BL21(DE3) [pMAO-Nmut.]

#### 9.4.4.1. General Procedure XXIV: Oxidation by whole cells of BL21(DE3) [pMAO-Nmut.]

Freeze-thawed whole cells of BL21(DE3) [pMAO-Nmut.] was added to a solution of RS-AMBA in phosphate buffer (20 mM, pH 8). A 100 µl aliquot was diluted in 900 µl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

Following general procedure XXII, using BL21(DE3) [pMAO-Nmut.] (§ 5.3.1, 500 µl of 0.20 U.ml⁻¹ = 0.10 U) and RS-AMBA (4.5 ml of 1 mM solution, final
Experimental: Deracemisation

concentration = 0.9 mM). R.t. (S-AMBA) = 14.7 mm, r.t. (R-AMBA) = 19.1 mm. At t = 99 h: yield R-AMBA = 0.77 mM, 86%; e.e. = 74%.

9.4.4.2. General Procedure XXV: Oxidation by cell free extract of BL21(DE3) [pMAO-Nmut.]

BL21(DE3) [pMAO-Nmut.] cell free extract was added to a solution of RS-AMBA in phosphate buffer (20 mM, pH 8). A 10 μl aliquot was diluted in 990 μl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

Following general procedure XXV, using BL21(DE3) [pMAO-Nmut.] CFE (§ 5.3.2, 100 μl of 0.18 U.ml⁻¹ = 0.02 U) and RS-AMBA (900 μl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At t = 20 h: yield R-AMBA = 0.41 mM, 45%; e.e. > 99%.

9.4.4.3. General Procedure XXVI: Oxidation by purified BL21(DE3) [pMAO-Nmut.]

Partially purified BL21(DE3) [pMAO-Nmut.] was added to a solution of RS-AMBA in phosphate buffer (20 mM, pH 8). A 10 μl aliquot was diluted in 990 μl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.3.2, 100 μl of 0.62 U.ml⁻¹ = 0.06 U) and RS-AMBA (900 μl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.4 min. At t = 20 h: yield R-AMBA = 0.42 mM, 47%; e.e. > 99%.
Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.3.2, 100 µl of 0.35 U.ml\(^{-1}\) = 0.04 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.9 min, r.t. (R-AMBA) = 18.0 min. At t = 7 h: yield R-AMBA = 0.46 mM, 51%; e.e. = 94%. At t = 20 h: yield R-AMBA = 0.43 mM, 48%; e.e. > 99%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 10 µl of 0.35 U.ml\(^{-1}\) = 0.004 U) and RS-AMBA (90 µl of 1 mM solution, final concentration = 0.9 mM) at room temperature. R.t. (S-AMBA) = 13.8 min, r.t. (R-AMBA) = 17.6 min. At t = 18 h: yield R-AMBA = 0.50 mM, 56%; e.e. > 99%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 10 µl of 0.35 U.ml\(^{-1}\) = 0.004 U) and RS-AMBA (90 µl of 1 mM solution, final concentration = 0.9 mM) at 37 °C. R.t. (S-AMBA) = 13.8 min, r.t. (R-AMBA) = 17.6 min. At t = 18 h: yield R-AMBA = 0.47 mM, 52%; e.e. > 99%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml\(^{-1}\) = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) at pH 6. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.4 min. At t = 22 h: yield R-AMBA = 0.50 mM, 55%; e.e. = 2%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml\(^{-1}\) = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) at pH 7. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.4 min. At t = 22 h: yield R-AMBA = 0.42 mM, 47%; e.e. = 86%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml\(^{-1}\) = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) at pH 7. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.4 min. At t = 22 h: yield R-AMBA = 0.42 mM, 47%; e.e. = 86%.
Experimental: Deracemisation

concentration = 0.9 mM) at pH 8. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.5 min. At t = 22 h: yield R-AMBA = 0.49 mM, 54%; e.e. = 92%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml⁻¹ = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) at pH 9. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.4 min. At t = 22 h: yield R-AMBA = 0.43 mM, 48%; e.e. = 74%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml⁻¹ = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM) in Tris/HCl buffer pH 10. R.t. (S-AMBA) = 13.5 min, r.t. (R-AMBA) = 17.5 min. At t = 22 h: yield R-AMBA = 0.50 mM, 56%; e.e. = 16%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml⁻¹ = 0.03 U) and RS-AMBA (900 µl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.7 min. At t = 25 h: yield R-AMBA = 0.53 mM, 59%; e.e. = 96%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.25 U.ml⁻¹ = 0.03 U) and RS-AMBA (900 µl of 5 mM solution, final concentration = 4.5 mM). R.t. (S-AMBA) = 13.6 min, r.t. (R-AMBA) = 17.5 min. At t = 25 h: yield R-AMBA = 0.50 mM, 56%; e.e. = 2%.

Following general procedure XXVI, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 50 µl of 0.20 U.ml⁻¹ = 0.01 U) and RS-AMBA (450 µl of 1 mM solution, final concentration = 0.9 mM). R.t. (S-AMBA) = 13.3 min, r.t. (R-AMBA) = 17.2 min. At t = 24 h: yield R-AMBA = 0.55 mM, 61%; e.e. = 96%.
9.5. Deracemisation of $RS$-$\alpha$-methylbenzylamine

9.5.1. Deracemisation by *E. coli* BL21(DE3) [pMAO-Nmut.]

9.5.1.1. General Procedure XXVII: Deracemisation using NaBH$_4$

Partially purified BL21(DE3) [pMAO-Nmut.] ($\S$ 5.4, 100 $\mu$L of 0.35 U.ml$^{-1}$ = 0.04 U) was added to a solution of $RS$-AMBA (900 $\mu$L of 1 mM solution, final concentration = 0.9 mM) and NaBH$_4$ (2 mg, 52.7 mM, 58.5 eq.) in phosphate buffer (20 mM, pH8). A 10 $\mu$L aliquot was diluted in 990 $\mu$L perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

R.t. ($S$-AMBA) = 14.0 min, r.t. ($R$-AMBA) = 18.1 min, e.e. = 11.5%. At $t = 24$ h: yield $R$-AMBA = 0.45 mM, 50%; e.e. = 23%.

9.5.1.2. General Procedure XXVIII: Deracemisation using NH$_3$:BH$_3$

Partially purified BL21(DE3) [pMAO-Nmut.] was added to a solution of $RS$-AMBA and ammonia-borane complex in phosphate buffer (20 mM, pH8). A 10 $\mu$L aliquot was diluted in 990 $\mu$L perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

Reactions were carried out according to the general procedure, with deviations indicated in italics.

Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] ($\S$ 5.4, 100 $\mu$L of 0.35 U.ml$^{-1}$ = 0.04 U), $RS$-AMBA (900 $\mu$L of 1 mM solution, final concentration = 0.9 mM) and NH$_3$BH$_3$ (10 $\mu$L of 4M solution, final concentration = 40 mM, 44.4 eq). Further NH$_3$BH$_3$ (10 $\mu$L of 4M solution, final concentration = 40 mM, 44.4 eq) was added at 3 h. R.t. ($S$-AMBA) = 14.0 min, r.t. ($R$-AMBA) = 18.0 min. At $t = 2$ h: yield $R$-AMBA = 0.59 mM, 65%; e.e. = 47%. At $t = 4$ h: yield
Experimental: Deracemisation

\[ R-\text{AMBA} = 0.50 \text{ mM, 56%; e.e. = 66}. \] At t = 72 h: yield \( R-\text{AMBA} = 0.62 \text{ mM, 69%; e.e. = 74} \). 

Following general procedure XXVIII, using BL21(DE3) [pMAO-N\text{mut.}] (§ 5.4, 25 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.01 U), \( R S-\text{AMBA} \) (900 \( \mu \text{l} \) of 1 mM solution, final concentration = 0.9 mM) and \( \text{NH}_3\text{BH}_3 \) (10 \( \mu \text{l} \) of 4M solution, final concentration = 40 mM, 44.4 eq). Further MAO-N mutant (25 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.009 U) and \( \text{NH}_3\text{BH}_3 \) (10 \( \mu \text{l} \) of 4M solution, final concentration = 40 mM, 44.4 eq) were added at 1.5, 3 and 19 hours. R.t. (\( S-\text{AMBA} \)) = 12.4 min, r.t. (\( R-\text{AMBA} \)) = 15.8 min. At t = 2 h: yield \( R-\text{AMBA} = 0.52 \text{ mM, 58%; e.e. = 22}. \) At t = 3 h: yield \( R-\text{AMBA} = 0.59 \text{ mM, 66%; e.e. = 30}. \) At t = 45 h: yield \( R-\text{AMBA} = 0.53 \text{ mM, 59%; e.e. = 72}. \) 

Following general procedure XXVII, using BL21(DE3) [pMAO-N\text{mut.}] (§ 5.4, 100 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.04 U), \( RS-\text{AMBA} \) (900 \( \mu \text{l} \) of 1 mM solution, final concentration = 0.9 mM) and \( \text{NH}_3\text{BH}_3 \) (20 \( \mu \text{l} \) of 4M solution, final concentration = 80 mM, 88.9 eq). Further MAO-N mutant (100 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.04 U) and \( \text{NH}_3\text{BH}_3 \) (20 \( \mu \text{l} \) of 4M solution, final concentration = 80 mM, 88.9 eq) was added at 4 h. R.t. (\( S-\text{AMBA} \)) = 13.3 min, r.t. (\( R-\text{AMBA} \)) = 17.0 min. At t = 3 h: yield \( R-\text{AMBA} = 0.70 \text{ mM, 78%; e.e. = 52}. \) At t = 24 h: yield \( R-\text{AMBA} = 0.47 \text{ mM, 52%; e.e. > 99}. \) 

Following general procedure XXVIII, using BL21(DE3) [pMAO-N\text{mut.}] (§ 5.4, 50 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.02 U), \( RS-\text{AMBA} \) (450 \( \mu \text{l} \) of 1 mM solution, final concentration = 0.9 mM) and \( \text{NH}_3\text{BH}_3 \) (20 \( \mu \text{l} \) of 4M solution, final concentration = 160 mM, 177.8 eq). Further MAO-N mutant (50 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.02 U) and \( \text{NH}_3\text{BH}_3 \) (20 \( \mu \text{l} \) of 4M solution, final concentration = 160 mM, 177.8 eq) was added at 2 h. R.t. (\( S-\text{AMBA} \)) = 13.8 min, r.t. (\( R-\text{AMBA} \)) = 17.9 min. At t = 3 h: yield \( R-\text{AMBA} = 0.55 \text{ mM, 61%; e.e. = 34}. \) At t = 96 h: yield \( R-\text{AMBA} = 0.52 \text{ mM, 58%; e.e. = 68}. \) 

Following general procedure XXVIII, using BL21(DE3) [pMAO-N\text{mut.}] (§ 5.4, 100 \( \mu \text{l} \) of 0.35 U.m\(^{-1}\) = 0.04 U), \( RS-\text{AMBA} \) (900 \( \mu \text{l} \) of 1 mM solution, final
Experimental: Deracemisation

concentration = 0.9 mM), NH$_3$BH$_3$ (40 µl of 4M solution, final concentration = 160 mM, 177.8 eq) and catalase (1 mg of 2800 U.mg$^{-1}$ = 2800 U). R.t. (S-AMBA) = 13.9 min, r.t. (R-AMBA) = 18.0 min. At t = 3 h: yield R-AMBA = 0.43 mM, 48%; e.e. = 26%. At t = 23 h: yield R-AMBA = 0.48 mM, 53%; e.e. = 44%.

Following general procedure XXVII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.35 U.ml$^{-1}$ = 0.04 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH$_3$BH$_3$ (20 µl of 4M solution, final concentration = 160 mM, 177.8 eq). R.t. (S-AMBA) = 14.0 min, r.t. (R-AMBA) = 18.2 min. At t = 3 h: yield R-AMBA = 0.57 mM, 71%; e.e. = 60%. At t = 24 h: yield R-AMBA = 0.63 mM, 79%; e.e. = 94%.

Following general procedure XXVII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.35 U.ml$^{-1}$ = 0.04 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM), NH$_3$BH$_3$ (20 µl of 4M solution, final concentration = 160 mM, 177.8 eq) and ammonium formate (32 mg, 1 M, 1111.1 eq.). R.t. (S-AMBA) = 13.4 min, r.t. (R-AMBA) = 17.3 min. At t = 17 h: yield R-AMBA = 0.50 mM, 63%; e.e. = 12%.

Following general procedure XXVII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml$^{-1}$ = 0.02 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH$_3$BH$_3$ (20 µl of 4M solution, final concentration = 160 mM, 177.8 eq). Further MAO-N mutant (100 µl of 0.20 U.ml$^{-1}$ = 0.02 U) and NH$_3$BH$_3$ (20 µl of 4M solution, final concentration = 160 mM, 177.8 eq) was added at 26 h. R.t. (S-AMBA) = 13.3 min, r.t. (R-AMBA) = 17.1 min. At t = 5 h: yield R-AMBA = 0.64 mM, 80%; e.e. = 36%. At t = 24 h: yield R-AMBA = 0.72 mM, 90%; e.e. = 52%. At t = 48 h: yield R-AMBA = 0.59 mM, 74%; e.e. = 78%.

Following general procedure XXVII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml$^{-1}$ = 0.02 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH$_3$BH$_3$ (10 µl of 4M solution, final concentration = 80 mM, 100.0 eq). Further MAO-N mutant (100 µl of 0.20 U.ml$^{-1}$ = 0.02 U) and NH$_3$BH$_3$ (10 µl of 4M solution, final concentration = 80 mM, 100.0 eq) was added at
Experimental: Deracemisation

26 h. R.t. (S-AMBA) = 13.2 min, r.t. (R-AMBA) = 17.0 min. At t = 5 h: yield R-AMBA = 0.62 mM, 77%; e.e. = 58%. At t = 24 h: yield R-AMBA = 0.62 mM, 77%; e.e. = 86%. At t = 48 h: yield R-AMBA = 0.55 mM, 69%; e.e. = 98%.

Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml⁻¹ = 0.02 U), RS-AMBA (300 µl of 1 mM solution, final concentration = 0.75 mM) and NH₃BH₃ (9 µl of 4M solution, final concentration = 90 mM, 120 eq). Further MAO-N mutant (100 µl of 0.20 U.ml⁻¹ = 0.02 U) and NH₃BH₃ (10 µl of 4M solution, final concentration = 100 mM, 133.3 eq) was added at 26 h R.t. (S-AMBA) = 13.2 min, r.t. (R-AMBA) = 17.0 min. At t = 5 h: yield R-AMBA = 0.57 mM, 76%; e.e. = 64%. At t = 24 h: yield R-AMBA = 0.60 mM, 80%; e.e. = 90%. At t = 48 h: yield R-AMBA = 0.47 mM, 62%; e.e. > 99%.

Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml⁻¹ = 0.02 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH₃BH₃ (9 µl of 4M solution, final concentration = 80 mM, 100.0 eq). R.t. (S-AMBA) = 12.8 min, r.t. (R-AMBA) = 16.5 min. At t = 5 h: yield R-AMBA = 0.50 mM, 63%; e.e. = 64%. At t = 24 h: yield R-AMBA = 0.50 mM, 63%; e.e. = 88%.

Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml⁻¹ = 0.02 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH₃BH₃ (5 µl of 4M solution, final concentration = 40 mM, 50.0 eq). R.t. (S-AMBA) = 12.7 min, r.t. (R-AMBA) = 16.4 min. At t = 5 h: yield R-AMBA = 0.43 mM, 54%; e.e. = 78%. At t = 24 h: yield R-AMBA = 0.46 mM, 58%; e.e. > 99%.

Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 µl of 0.20 U.ml⁻¹ = 0.02 U), RS-AMBA (400 µl of 1 mM solution, final concentration = 0.8 mM) and NH₃BH₃ (2.5 µl of 4M solution, final concentration = 20 mM, 25.0 eq). R.t. (S-AMBA) = 12.7 min, r.t. (R-AMBA) = 16.3 min. At t = 5 h: yield R-AMBA = 0.37 mM, 46%; e.e. = 84%. At t = 24 h: yield R-AMBA = 0.46 mM, 58%; e.e. > 99%.
Following general procedure XXVIII, using BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 μl of 0.20 U.ml⁻¹ = 0.02 U), RS-AMBA (400 μl of 1 mM solution, final concentration = 0.8 mM) and NH₃BH₃ (2.5 μl of 4M solution, final concentration = 20 mM, 25.0 eq). R.t. (S-AMBA) = 13.0 min, r.t. (R-AMBA) = 16.8 min. At t = 5 h: yield R-AMBA = 0.45 mM, 56%; e.e. = 80%. At t = 24 h: yield R-AMBA = 0.43 mM, 54%; e.e. > 99%.

9.5.1.3. General Procedure XXIX: Deracemisation with organic co-solvent

Partially purified BL21(DE3) [pMAO-Nmut.] (§ 5.4, 100 μl of 0.20 U.ml⁻¹ = 0.02 U) was added to a solution of RS-AMBA (100 mM solution in organic co-solvent) and ammonia-borane complex in phosphate buffer (20 mM, pH8). A 10 μl aliquot was diluted in 990 μl perchloric acid, pH 1.5 and analysed by HPLC method 8. The reaction mixture was incubated at 30 °C and the reaction was monitored by HPLC at regular intervals until no further reaction was observed.

Following general procedure XXIX, using RS-AMBA (5 μl in DMSO, final concentration = 1.0 mM), buffer (395 μl) and NH₃BH₃ (10 μl of 4M solution, final concentration = 80 mM, 80.0 eq). R.t. (S-AMBA) = 12.8 min, r.t. (R-AMBA) = 16.5 min. At t = 5 h: yield R-AMBA = 0.49 mM, 49%; e.e. = 34%. At t = 24 h: yield R-AMBA = 0.55 mM, 55%; e.e. = 80%.

Following general procedure XXIX, using RS-AMIBA (5 μl in dioxane, final concentration = 1.0 mM), buffer (395 μl) and NH₃BH₃ (10 μl of 4M solution, final concentration = 80 mM, 80.0 eq). R.t. (S-AMBA) = 12.6 min, r.t. (R-AMBA) = 16.2 min. At t = 21 h: yield R-AMBA = 0.51 mM, 51%; e.e. = 46%.

Following general procedure XXIX, using RS-AMIBA (5 μl in hexane, final concentration = 1.0 mM), buffer (395 μl) and NH₃BH₃ (10 μl of 4M solution, final concentration = 80 mM, 80.0 eq). R.t. (S-AMBA) = 12.6 min, r.t. (R-AMBA) = 16.2 min. At t = 21 h: yield R-AMBA = 0.57 mM, 57%; e.e. = 96%.
Following general procedure XXIX, using $RS$-AMBA ($5 \mu l$ in acetonitrile, final concentration $= 1.0 \text{ mM}$), buffer ($395 \mu l$) and $NH_3BH_3$ ($10 \mu l$ of 4M solution, final concentration $= 80 \text{ mM, } 80.0 \text{ eq}$). R.t. ($S$-AMBA) $= 12.6 \text{ min}$, r.t. ($R$-AMBA) $= 16.2 \text{ min}$. At $t = 21 \text{ h}$: yield $R$-AMBA $= 0.61 \text{ mM, } 61\%$; e.e. $= 88\%$.

Following general procedure XXIX, using $RS$-AMBA ($5 \mu l$ in hexane, final concentration $= 1.0 \text{ mM}$), buffer ($395 \mu l$) and $NH_3BH_3$ ($2.5 \mu l$ of 4M solution, final concentration $= 20 \text{ mM, } 20.0 \text{ eq}$). R.t. ($S$-AMBA) $= 13.0 \text{ min}$, r.t. ($R$-AMBA) $= 16.8 \text{ min}$. At $t = 5 \text{ h}$: yield $R$-AMBA $= 0.52 \text{ mM, } 52\%$; e.e. $= 86\%$. At $t = 23 \text{ h}$: yield $R$-AMBA $= 0.51 \text{ mM, } 51\%$; e.e. $> 99\%$.

9.5.2. Stereoinversion by *E. coli* BL21(DE3) [pMAO-Nmut.]

9.5.2.1. General Procedure XXX: Stereoinversion by purified BL21(DE3) [pMAO-Nmut.]

Partially purified BL21(DE3) [pMAO-Nmut.] ($\S$ 5.4, $100 \mu l$ of $0.20 \text{ U.ml}^{-1} = 0.02 \text{ U}$) was added to a solution of AMBA ($400 \mu l$ of $0.5 \text{ mM solution, final concentration } = 0.4 \text{ mM}$) and ammonia-borane complex ($2.5 \mu l$ of 4M solution, final concentration $= 20 \text{ mM, } 20 \text{ eq}$) in phosphate buffer ($20 \text{ mM, pH8}$). A $10 \mu l$ aliquot was diluted in $990 \mu l$ perchloric acid, pH 1.5 and analysed by HPLC. The reaction mixture was incubated at $30^\circ\text{C}$ and the reaction monitored by HPLC at regular intervals until no further reaction was observed.

Following general procedure XXX, using $S$-AMBA. R.t. ($S$-AMBA) $= 12.9 \text{ min}$, r.t. ($R$-AMBA) $= 16.6 \text{ min}$. At $t = 5 \text{ h}$: yield $R$-AMBA $= 0.06 \text{ mM, } 15\%$; e.e. $= 22\%$. At $t = 23 \text{ h}$: yield $R$-AMBA $= 0.07 \text{ mM, } 18\%$; e.e. $> 99\%$.

Following general procedure XXX, using $R$-AMBA. R.t. ($R$-AMBA) $= 16.6 \text{ min}$. At $t = 5 \text{ h}$: yield $R$-AMBA $= 0.39 \text{ mM, } 98\%$; e.e. $> 99\%$. At $t = 23 \text{ h}$: yield $R$-AMBA $= 0.39 \text{ mM, } 98\%$; e.e. $> 99\%$. 

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9.6. Analysis conditions

9.6.1. HPLC method 1
Phenomenex Chirex 3126 stationary phase, 100% CuSO₄ (1.5 mM), 1.5 ml.min⁻¹, 242 nm.

9.6.2. HPLC method 2
Chirex 3126, 70% CuSO₄ (1.5 mM), 30% MeOH, 1.5 ml.min⁻¹, 254 nm.

9.6.3. HPLC method 3
Chirex 3126, 85% CuSO₄ (2.0 mM), 15% MeOH, 1.5 ml.min⁻¹, 254 nm.

9.6.4. HPLC method 4
Chirex 3126, 90% CuSO₄ (1.5 mM), 10% MeOH, 1.5 ml.min⁻¹, 254 nm.

9.6.5. HPLC method 5
Chirex 3126, 70% CuSO₄ (1.5 mM), 30% MeOH, 1.3 ml.min⁻¹, 254 nm.

9.6.6. HPLC method 6
Chirex 3126, 94% CuSO₄ (1.5 mM), 6% MeOH, 1.5 ml.min⁻¹, 254 nm.

9.6.7. HPLC method 7
Chiralcel OD-H stationary phase, 95% hexane, 5% propan-2-ol, 0.5 ml.min⁻¹, 215 nm, 5 °C.

9.6.8. HPLC method 8
Crownpak CR(+) stationary phase, 100% perchloric acid (pH 1.5), 0.8 ml.min⁻¹, 200 nm, 25 °C.
10. Experimental: Enzyme production

10.1. General Techniques

Reagents for buffers, assay mixtures and culture media were of standard laboratory grade and were used as supplied from commercial sources. Commercial enzymes were used as supplied from Sigma. Bacterial growth in liquid culture was performed in an orbital shaker (Innova 4430 or Innova 4000), set at the desired temperature. For static incubation an oven, a dry block heater (Anachem HBS-130) or a water bath set at the desired temperature were used.

Centrifugation of culture media and lysed cells was carried out in a Sorvall RC5C plus centrifuge. Speed and rotor size follow in parentheses. Small-scale (< 1.5 ml) centrifugation was performed in either an Eppendorf 5G15C microfuge or a Hereaeus Biofuge pico.

Sterile procedures were performed either on the bench under flame, or in a SterilGard Class II Type A/B3 biological safety cabinet. Equipment and media were sterilised using a Monarch 745rh autoclave (121 °C, 20 min) or an Astell Scientific autoclave (121 °C, 15 min).

Optical density measurements and Bradford assay measurements were performed on a Cecil CE1020s spectrophotometer (D_2 lamp). Kinetic measurements were carried out on a Hewlett Packard 8453 spectrophotometer (D_2 lamp), using HP 845x UV-visible system software for analysis or a Unicam UV4 UV-spectrophotometer with integrated kinetics software. Automated assays were performed using Cobas Mira S, a Roche-Diagnostic discrete analytical analyser. HPLC analysis was performed on a Hewlett Packard HP1100. LC/MS analysis was performed on a Hewlett Packard HP1100 coupled to a Finnigan LCQ ion trap in electrospray ionisation mode.

FPLC purification was performed on an Amersham pharmacia biotech Åkta FPLC system, using Unicorn v. 3.21 software for analysis. Columns were obtained pre-packed from Pharmacia.
Three different gels were used for SDS-PAGE analysis; i) Biorad Ready gels 10-20% Tris/HCl, 10 wells, 30 μl, ii) Biorad Criterion™ precast gel 10-20% Tris/HCl, 18 wells, 30 μl, iii) self-poured 15% acrylamide gels (§ 9.5.9 & 9.5.10).

10.2. General Procedures

10.2.1. Growth of organisms for screening

The appropriate media (§ 9.5.3-5, 50 ml in 250 ml flasks) were inoculated in duplicate, from bead. The flasks were incubated for 3 days (28 °C, 250 rpm), or until sufficient biomass was obtained. The biomass was harvested by centrifugation (2500 rpm, aseptically) and used for inoculation of induction media (§ 9.5.6-7, 50 ml in 250 ml flasks). The media was incubated for 18 h (28 °C, 250 rpm) then the cells were harvested by centrifugation (2500 rpm), resuspended in minimum buffer (phosphate, 100 mM, pH 7.5) and disrupted by sonication (1 ml, 3 x 15 s, 4 °C).

10.2.2. DNA Plasmid Purification

Plasmid DNA was purified from 10 ml bacterial culture using QIAprep Spin Miniprep kits (QIAGEN) following the supplied protocol. Purified plasmids were eluted from the spin columns in 30 μl of Tris/HCl buffer (25 mM, pH 7.8).

10.2.3. Transformation of E. coli with plasmid DNA by heat shock

Plasmid DNA (1 μl) was mixed with chemically competent cells (20 μl, Novagen BL21(DE3) or BLR(DE3)), placed in ice for 5 minutes, heated at 42 °C for 30 s and returned to ice for 2 minutes. Growth medium (LB or SOC, 80 μl) was added and the culture was incubated at 37 °C for 2-4 h before plating on LB agar plates containing ampicillin at the appropriate concentration. Plates were incubated at 37 °C overnight.
10.2.4. **Growth of monoamine oxidase from a starting culture**

LB media containing ampicillin (50 μg.ml⁻¹) (10 ml) in 30 ml tube was inoculated with a single colony of *E. coli* BL21(DE3) [pECME3] and was incubated at 37 °C (250 rpm) overnight. The resulting saturated culture was used as a 10% inoculum for fresh LB/amp50 and was incubated at 37 °C (250 rpm) for 24 hours when OD₆₀₀ ≈ 3.5.

10.2.5. **Growth of monoamine oxidase from a single colony**

LB media (300 ml), containing ampicillin at the appropriate concentration, in 1 L baffled flasks was inoculated with a single colony of BL21(DE3) [pMAO-N], and incubated at the desired temperature overnight (250 rpm) until OD₆₀₀ ≈ 4. Cells were harvested by centrifugation at 4 °C (7000 rpm, Sorvall SLA 3000 rotor) and washed with buffer (20 mM, pH 8) before being stored at -20 °C.

10.2.6. **Cell Lysis**

Lysis was performed as close to 4 °C as possible. Frozen biomass from growth media was thawed and resuspended in Tris/HCl buffer (25 mM, pH 7.8) containing the appropriate protease inhibitors. The suspension was sonicated (Soniprep 150) on ice following an on-off protocol, time and period follows in parentheses. The viscous mixture was centrifuged (20000 rpm, Sorvall SS-34 rotor) at 4 °C until a clear supernatant was obtained. Cell free extracts were stored at -80 °C.
10.2.7. **Purification of proteins by FPLC**

Cell free extract (as prepared in § 9.2.6) was dialysed against starting buffer appropriate to column. The cell free extract was filtered through 0.45 μm sterile membrane prior to loading on the column using a peristaltic pump (Pharmacia P1). Fractions were assayed using a colorimetric hydrogen peroxide based assay (§ 9.4.2) and active fractions were stored at -80 °C.

10.2.8. **Purification of proteins by imac**

A disposable column was packed with Novagen HisBind® resin according to supplied protocol, and charged with Ni\(^{2+}\) ions. Cell free extract containing 0.5 M NaCl was loaded onto the column under gravity and the protein eluted with an imidazole gradient (0 M to 1 M). Fractions were assayed using colorimetric hydrogen peroxide based assay (§ 9.4.2).

10.2.9. **Bradford Assay**

Protein concentrations were measured by mixing Bradford reagent (5 ml, § 9.5.8) with enzyme solution (100 μl) and standing for 2 minutes. The spectrophotometer was zeroed against Bradford reagent (5 ml) and water (100 μl), and then the absorbance of the protein sample at 595 nm was measured. If the absorbance exceeded the calibration range then the enzyme was diluted accordingly and the assay repeated. The protein concentration was obtained by comparison with a pre-determined calibration curve derived from known dilutions of BSA.
10.2.10. **SDS-PAGE protein analysis**

Protein samples were prepared in SDS-reducing buffer (§ 9.5.12) and loaded on acrylamide gel and run at a constant voltage of 200 V (Powerpac 200). The gel was placed in staining solution (§ 9.5.15) and agitated on a rocking platform until the gel was dark blue in colour. The gel was de-stained in the same way using fresh de-stain solution (§ 9.5.16) until protein bands were visible and the background gel was clear.

10.3. **FPLC purification protocol**

10.3.1. **Anion exchange chromatography**

10.3.1.1. **Q-Sepharose**

- Column = HiFlow QSepharose 26/10 (bed volume = 50 ml)
- Buffer A = Tris/HCl (25 mM, pH 7.8)
- Buffer B = Tris/HCl (25 mM, pH 7.8) + 1 M NaCl
- Flow rate = 4 ml.min$^{-1}$
- Fraction collect = 10 ml
- Column wash = 2 CV 100% buffer A
- Elution = 10 CV 100% buffer A to 100% buffer B
- Column clean = 4 CV 100% buffer B

10.3.1.2. **ResourceQ**

- Column = ResourceQ (bed volume = 6 ml)
- Buffer A = Tris/HCl (25 mM, pH 7.8)
- Buffer B = Tris/HCl (25 mM, pH 7.8) + 1 M NaCl
- Flow rate = 3 ml.min$^{-1}$
- Fraction collect = 2 ml
Experimental: Enzyme production

Column wash = 2 CV 100% buffer A
Elution = 20 CV 100% buffer A to 100% buffer B
Column clean = 4 CV 100% buffer B

10.3.1.3. DEAE-Sepharose

Column = HiFlow DEAE-Sepharose 26/10 (bed volume = 50 ml)
Buffer A = Tris/HCl (25 mM, pH 7.8)
Buffer B = Tris/HCl (25 mM, pH 7.8) + 1 M NaCl
Flow rate = 8 ml.min⁻¹
Fraction collect = 5 ml
Column wash = 2 CV 100% buffer A
Elution = 5 CV 100% buffer A to 100% buffer B
Column clean = 2 CV 100% buffer B

10.3.1.4. MonoQ

Column = MonoQ (bed volume = 6 ml)
Buffer A = Tris/HCl (25 mM, pH 7.8)
Buffer B = Tris/HCl (25 mM, pH 7.8) + 1 M NaCl
Flow rate = 1 ml.min⁻¹
Fraction collect = 1 ml
Column wash = 3 CV 100% buffer A
Elution = 20 CV 100% buffer A to 100% buffer B
Column clean = 5 CV 100% buffer B
10.3.2. **Hydrophobic interaction chromatography**

10.3.2.1. Phenyl-Sepharose

Column = HiFlow Phenyl-Sepharose 26/10 (bed volume = 25 ml)

Buffer A = \((\text{NH}_4)_2\text{SO}_4\) (0.85 M in K. Phos. buffer, 50 mM, pH 7.2)

Buffer B = \((\text{NH}_4)_2\text{SO}_4\) (1.7 M in K. Phos. buffer, 50 mM, pH 7.2)

Flow rate = 4 ml.min\(^{-1}\)

Fraction collect = 5 ml

Column wash = 2 CV 100% buffer A

Elution = 10 CV 100% buffer A to 100% buffer B

Column clean = 4 CV 100% buffer B

10.4. **Assay methods**

10.4.1. **Hydrogen peroxide coupled assay using vanillic acid**

10.4.1.1. Automated assay – Cobas Mira robot

The enzymatic sample (40 µl), water (15 µl) and chromogenic solution 1 (§ 9.5.17, 200 µl, \(\varepsilon_{500} = 4650\ \text{M}^{-1}.\text{cm}^{-1}\)) were mixed at 37 °C and the absorbance at 500 nm measured for 4 minutes. After this time substrate (20 µl, 48 mM, final concentration = 3.3 mM) and water (15 µl) were added and the absorbance (500 nm) measured for a further 13 minutes. Cobas mira calculates activity (U.ml\(^{-1}\)) from initial rate.

*Activity calculation:*

\[
\text{Activity (U.ml}^{-1}\text{)} = \text{initial rate (Au.s}^{-1}\text{)} \times \left(\frac{1}{\varepsilon}\right) \times 60 \times \left(\frac{\text{t.v.}}{\text{s.v.}}\right) \times 1 \times 10^6.
\]

where \(\varepsilon\) = molar extinction coefficient (M\(^{-1}\).cm\(^{-1}\))

\(\text{t.v.}\) = total reaction volume

\(\text{s.v.}\) = sample volume
10.4.1.2. Non-automated assay

The spectrophotometer was zeroed against chromogenic solution 1 (690 µl) and water (310 µl). Chromogenic solution 1 (§ 9.5.17, 690 µl), water (100 µl), amine (70 µl, 48 mM, final concentration = 3.4 mM) and enzyme (140 µl) were mixed and the absorbance at 500 nm measured over 10 minutes at 5 s intervals. HP 845x UV-visible software was used to calculate the initial rate and the activity was calculated from this.

10.4.2. Hydrogen peroxide coupled assay using 2,4,6-tribromo-3-hydroxybenzoic acid

The spectrophotometer was blanked against chromogenic solution 2 (§ 9.5.18, 990 µl, $\varepsilon_{510} = 29500 \text{ M}^{-1}.\text{cm}^{-1}$) and HRP (5 µl of 12000 U.ml$^{-1}$ solution, 60 U). Enzyme (10 µl) was added and the absorbance at 500 nm was measured at 3 s intervals for a 10 minute period. The initial rate was measured and the activity was calculated from this (activity calculation as for 9.4.1).

10.4.3. Product formation assay – monoamine oxidase

The spectrophotometer was zeroed against water (900 µl) and enzyme (100 µl). Benzylamine (900 µl, 2 mM, final concentration = 1.8 mM) and enzyme (100 µl) were mixed in a quartz cuvette at room temperature and the absorbance at 250 nm was measured over 3 minutes at 5 s intervals. The initial rate was measured and the activity was calculated from this (activity calculation as for method 9.4.1). $\varepsilon(\text{PhCHO})_{250} = 12500 \text{ M}^{-1}.\text{cm}^{-1}$. 
10.4.4. *Product formation assay – L-amino acid oxidase*

L-Phenylalanine (700 µl, 1 mM, final concentration = 0.7 mM) and enzymatic sample (300 µl) were mixed in a quartz cuvette at room temperature and the absorbance at 320 nm was measured for 10 minutes at 20 s intervals. The initial rate was measured and the activity was calculated from this (activity calculation as for method 9.4.1). 

\[ \varepsilon_{320}^{\text{PPA}} = 1017 \text{ M}^{-1}.\text{cm}^{-1}. \]

Deviations from assay methods are indicated in italics.

10.4.5. *HPLC assay for benzaldehyde production*

Benzylamine (900 µl, 1 mM) and enzymatic sample (100 µl) were left at room temperature. An aliquot (100 µl) was removed at regular time intervals and diluted in acetonitrile (900 µl). The mixture was analysed by HPLC.

HPLC conditions: Zorbax Eclipse XDB C₈ column (150 x 4.6)

- 34% Ammonium acetate (50 mM, pH 6), 66% acetonitrile
- 1 ml.min⁻¹, 254 nm, 5 µl injection loop
- R.t. (BzNH₂) = 1.50 min, r.t. (BzCHO) = 2.23 min

10.4.6. *LC/MS assay for phenylpyruvic acid production*

L-Phenylalanine (700 µl, 1 mM) and enzymatic sample (300 µl) were left at room temperature. An aliquot was submitted for LC/MS analysis.

MS conditions: electrospray ionisation (es) 50-350 mass units

- scan time ~1 s, alternate positive/negative switching

HPLC conditions: Hypersil HyPurity Elite C₁₈ (50 x 2.1, 3µ)

- Solvent A - 0.1% formic acid in ammonium acetate (10 mM)
- Solvent B - 90% acetonitrile, 10% A
Experimental: Enzyme production

1 ml.min⁻¹

Gradient:  
- t = 0 min  100% A  
- t = 11 min  40% A, 60% B  
- t = 15 min  30% A, 70% B  
- t = 15.1 min  100% A  
- t = 20 min  100% A

10.5. Stock recipes

10.5.1. \textit{LB medium}

Per litre:  
- Tryptone peptone  10 g  
- Sodium chloride  10 g  
- Yeast extract  5 g  

pH adjusted to 7 with NaOH

10.5.2. \textit{LB agar medium}

- LB medium  100 ml  
- Agar  1.5 g

10.5.3. \textit{SV2 medium}

Per litre:  
- Glucose  15 g  
- Glycerol  15 g  
- Soypeptone  15 g  
- NaCl  3 g  
- CaCO₃  1 g  

adjusted to pH 7
10.5.4. **FS medium**

Per litre:
- Peptone 10 g
- Malt extract 21 g
- Glycerol solution 40 g
- Junlon 110 1 g
- glass beads (x 2)

10.5.5. **YEPD medium**

Per litre:
- Yeast extract 10 g
- Peptone 20 g
- Glucose 20 g
- natural pH

10.5.6. **Monoamine oxidase induction media**

Per litre growth medium:  
- n-butylamine 1 ml

10.5.7. **L-Amino acid oxidase induction media**

Per litre growth medium:  
- L-phenylalanine 1 g

10.5.8. **Bradford reagent**

- Coomassie Brilliant Blue G-250 50 mg
- Ethanol (95%) 25 ml
- Phosphoric acid (85%) 50 ml
- Water 425 ml
Experimental: Enzyme production

### 10.5.9. SDS stacking gel

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<td>10% APS</td>
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TEMED and 10% APS added immediately before pouring gel

### 10.5.10. SDS separating gel

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<td>TEMED</td>
<td>10 μl</td>
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<tr>
<td>10% APS</td>
<td>100 μl</td>
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TEMED and 10% APS added immediately before pouring gel

### 10.5.11. Acrylamide solution

30% (w/v) acrylamide/\textit{bis}-acrylamide (Severn Biotech)
**10.5.12. SDS reducing buffer**

- Tris/HCl (0.5 M, pH 6.8) 2.0 ml
- Glycerol 1.6-2.0 ml
- 10% (w/v) SDS 3.2 ml
- 2-β-Mercaptoethanol 0.8 ml
- 0.05 (w/v) Bromophenol blue 0.4 ml

**10.5.13. 0.05 (w/v) Bromophenol blue**

0.04% in 20% Methanol, pH 2.8-4.6

**10.5.14. 5 x SDS running buffer**

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<th>Per litre</th>
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<tr>
<td></td>
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<td></td>
<td>10% (w/v) SDS</td>
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**10.5.15. Coomassie Blue staining solution**

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<td>Coomassie blue G-250</td>
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10.5.16. **Coomassie Blue de-staining solution**

- Methanol 450 ml
- Acetic acid 100 ml
- Water 450 ml

10.5.17. **Chromogenic solution 1**

- Vanillic acid 3 mM
- 4-AAP 1.5 mM
- HRP 12 U.ml⁻¹

mixed in a 1:1:1 ratio

10.5.18. **Chromogenic solution 2**

- Potassium phosphate buffer (1 M, pH 7.6) 5 ml
- THBHA (2% in DMSO) 500 μl
- 4-AAP (1.5 M) 37.5 μl
- Amine substrate 30 μl
- Water 44.4 ml
11. Bibliography


Bibliography


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Bibliography


151 in 'Nova gen catalogue', 2000, p TB009 005/000.

12. Appendices

12.1. Organisms screened for both MAO and LAAO activity

<table>
<thead>
<tr>
<th>Organism</th>
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<th>Intra MAOᵈ</th>
<th>Extra LAAOᶜ</th>
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<td>n</td>
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<td>Time b</td>
<td>Extra MAO c</td>
<td>Intra MAO d</td>
<td>Extra LAAO e</td>
<td>Intra LAAO e</td>
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<td>-------------</td>
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<td>n</td>
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<td>UV</td>
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<td>140 Rhodopseudomonas sphaeroides</td>
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<tr>
<td>141 Erwinea aroideae</td>
<td>atcc 25206</td>
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<tr>
<td>142 Trichoderma viride</td>
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<td>143 Neurospora crassa</td>
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<td>3(1)</td>
<td>n</td>
<td>0.002</td>
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</tbody>
</table>

a) commercial culture collection no.; b) growth in days (induction time in days); c) extra-cellular activity (U.ml\(^{-1}\)); d) intra-cellular activity (U.ml\(^{-1}\)).
### 12.2. Organisms screened for MAO activity only

<table>
<thead>
<tr>
<th>Organism</th>
<th>C.C. no.(^a)</th>
<th>Time(^b)</th>
<th>Extra MAO(^c)</th>
<th>Intra MAO(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1  <em>A. ochraceus</em></td>
<td>imi 16264</td>
<td>5(1)</td>
<td>n</td>
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</tr>
<tr>
<td>2  <em>A. itaconicus</em></td>
<td>atcc 10021</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>3  <em>Penicillium chrysogenum</em></td>
<td>Q176 wisco</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
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<tr>
<td>4  <em>Curvularia lunata</em></td>
<td>nrrl 2434</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
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<tr>
<td>5  <em>Absidia cylindrospora</em></td>
<td>nrrl 2796</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>6  <em>Septomyxa affinis</em></td>
<td>atcc 6737</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>7  <em>Verticillium lecanii</em></td>
<td>imi 68689</td>
<td>5(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>8  <em>Fusarium monoliforme</em></td>
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<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9  <em>A. terreus</em></td>
<td>imi 045543</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>10 <em>A. alliaceus</em></td>
<td>atcc 10060</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>11 <em>Xylaria polymorpha</em></td>
<td>F5755</td>
<td>5(1)</td>
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<td>n</td>
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<td>12 <em>Streptomyces lavendulae</em></td>
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<td>13 <em>Streptomyces griseus</em></td>
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<tr>
<td>14 <em>Streptomyces punipalis</em></td>
<td>nrrl 3529</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>15 <em>Serratia marcesens</em></td>
<td>atcc 274</td>
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<td>n</td>
<td>n</td>
</tr>
<tr>
<td>16 <em>Erwinea aroideae</em></td>
<td>atcc 25206</td>
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<td>n</td>
<td>n</td>
</tr>
<tr>
<td>17 <em>Providencia rustigianii</em></td>
<td>atcc 13159</td>
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<td>n</td>
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<tr>
<td>18 <em>Rhodospiridium toruloides</em></td>
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<tr>
<td>19 <em>S. carlsbergensis</em></td>
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<tr>
<td>20 <em>Schizosaccharomyces pombe</em></td>
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<tr>
<td>21 <em>Zygosaccharomyces fermentatia</em></td>
<td>cbs 67.72</td>
<td>3(1)</td>
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</table>
## Appendices

### Organism Table

<table>
<thead>
<tr>
<th>Organism</th>
<th>C.C no.</th>
<th>Time</th>
<th>Extra MAO</th>
<th>Intra MAO</th>
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</thead>
<tbody>
<tr>
<td>22 P. angusta</td>
<td>atcc 76722</td>
<td>3(1)</td>
<td>n</td>
<td>n</td>
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<tr>
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<td>atcc 76723</td>
<td>3(1)</td>
<td>n</td>
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</tbody>
</table>

a) commercial culture collection no.; b) growth in days (induction time in days);

### 12.3. Organisms screened for LAAO activity only

<table>
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<th>C.C. no.</th>
<th>Time</th>
<th>Extra LAAO</th>
<th>Intra LAAO</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Rhizopus stolonifer</td>
<td>atcc 6227B</td>
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<td>n</td>
</tr>
<tr>
<td>2 Septomyxa affinis</td>
<td>atcc 6737</td>
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<td>y</td>
<td>y</td>
</tr>
<tr>
<td>3 Verticillium lecanii</td>
<td>imi 68689</td>
<td>5(1)</td>
<td>y</td>
<td>n</td>
</tr>
<tr>
<td>4 Proteus vulgaris</td>
<td>atcc 13315</td>
<td>3(1)</td>
<td>y</td>
<td>y</td>
</tr>
<tr>
<td>5 Pseudomonas putida</td>
<td>ncimb 12190</td>
<td>3(1)</td>
<td>n</td>
<td>y</td>
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<tr>
<td>6 Bacillus cereus</td>
<td>ncimb 11617</td>
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<tr>
<td>7 E. coli</td>
<td>atcc 9637</td>
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<td>8 Klebsiella oxytoca</td>
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<td>9 Providencia rustigianii</td>
<td>atcc 13159</td>
<td>3(1)</td>
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<td>y</td>
</tr>
</tbody>
</table>

a) commercial culture collection no.; b) growth in days (induction time in days);

"y" indicates PPA observed in LC/MS (negative ionisation mode)
11.4. Purification of BL21(DE3) [pET16b], BL21(DE3) [pMAO-N] and BL21(DE3) [pMAO-Nmut.]

Alexis resourceQ288 – BL21(DE3) [pMAO-Nmut.]

Alexis resourceQ286 – BL21(DE3) [pET16b]

Alexis resourceQ287 – BL21(DE3) [pMAO-N]