Biotransformations of hydroxylamine derivatives

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Declaration

I declare that this thesis is based on results obtained from investigations which I have personally carried out, and that the entire thesis is my own composition. Any work other than my own is clearly acknowledged with reference to the relevant investigators or contributors. This thesis has not been published previously, in whole or in part, for the award of any degree.

Tom Eve March 2007

Portions of the researched contained within have previously been presented at scientific meetings and conferences both in oral and written form. These meetings include; ProBio Faraday meetings, AstraZeneca CASE Award internal review meetings and University of Edinburgh School of Chemistry internal presentations.

Abstract

The results presented herein represent a concerted experimental programme focussed on producing a deracemisation system for hydroxlyamines. A number of different avenues have been explored. An attempt to engineer the monoamine oxidase N enzyme (MAO-N) which displayed no activity towards the substrates of interest was made. The rationale behind this was the close relationship between the amines known to be substrates and their hydroxylamine analogues.

Following the initial engineering efforts two different courses of action were undertaken. Modification of the substrates being studied led to success in discovering novel substrates for the MAO-N enzyme. The enantioselective oxidation of hydroxylamine ethers by MAO-N is a novel discovery. A further development is the engineering of a MAO-N which displayed improved catalytic activity for a model hydroxylamine ether substrate compared with the parental enzyme.

A second research programme lead to the discovery of the applicability of a laccase:mediator system to the oxidation of hydroxylamines. This represents a further novel discovery.

An experimental programme investigating the reduction of oximes under conditions suitable for the biocatalysts described above was also performed.

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Abbreviations

Abs	absorbance
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Alloc	allyloxycarbonyl
bp	base pairs
BSA	bovine serum albumin
CAL-B	*Candida antarctica* lipase B
CE	capillary electrophoresis
CFE	cell free extract
CHARMM	Chemistry at HARvard Molecular Mechanics
CTH	catalytic transfer hydrogenation
DAB	3,3'-diaminobenzidine
DCM	dichloromethane
DKR	dynamic kinetic resolution
DMD	dimethyldioxirane
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	any deoxyribonucleoside triphosphate
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
e.e.	enantiomeric excess
EI	electron impact
EtOAc	ethyl acetate
EtOH	ethanol
epPCR	error prone polymerase chain reaction
eq.	equivalents
ES	electrospray
ET	electron transfer
FACS	fluorescence activated cell sorting
FAB	fast atom bombardment
FAD	flavin adenine dinucleotide (oxidised state)

$FADH_2$	flavin adenine dinucleotide (reduced state)
FDA	Food and Drug Administration
FPLC	fast protein liquid chromatography
FT	fourier transform
GC	gas chromatography
GC-MS	gas chromatography - mass spectrometry
GFP	green fluorescent protein
HAT	hydrogen atom transfer
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IPTG	isopropylthio-β-D-galactoside
ITCHY	incremental truncation for the creation of hybrid enzymes
kDa	kilodalton
kbp	kilobase pairs
L	path length
LB	Luria-Bertani broth
LC-MS	liquid chromatography – mass spectroscopy
lit.	literature
LM	laccase:mediator
MAO	monoamine oxidase
MAO-A	type A monoamine oxidase from *homo sapiens*
MAO-B	type B monoamine oxidase from *homo sapiens*
MAO-N	monoamine oxidase from *Aspergillus niger*
m-CPBA	*meta*-chloroperbenzoic acid
MeCN	acetonitrile
Ni-NTA	nickel-nitrilotriacetic acid
NMR	nuclear magnetic resonance
OD	optical density
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride

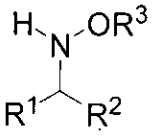
PROXYL	2,2,5,5-tetramethyl-1-pyrrolidinyloxyl
rac	racemic
RACHITT	random chimeragenesis on transient templates
rpm	revolutions per minute
r.t.	room temperature
sat.	saturated
SDS	sodium dodecylsulfate
SDS PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SET	single electron transfer
TCF	totally chlorine-free
TEMPO	2,2',6,6'-tetramethylpiperidine- *N*-oxyl
Teoc	trimethylsilylethoxycarbonyl
THF	tetrahydrofuran
TPQ	topaquinone
Tris	tris(hydroxymethyl)aminomethane
Troc	trichloroethoxycarbonyl
U	units
UV	ultraviolet
Vis	visible
v/v	volume by volume
w/v	weight by volume
w/w	weight by weight
Z	benzyloxycarbonyl
α_D	optical rotation
ε	molar extinction coefficient

Contents

1 Hydroxylamines

1.1 Hydroxylamines

Hydroxylamines, of the general structure **1**, are a class of compounds found in many different areas of chemistry, from drugs[1] to pesticides[2], narcotic metabolites[3] to heavy metal sequestration.[4] In addition they are precursors to hydroxamic acids[5,6] and may also be regarded as masked amines. For biological studies to be performed and synthetic routes devised hydroxylamines have been synthesised and a relatively small but significant body of literature exists reporting the methods available to the synthetic chemist.



1

R^1, R^2, R^3 = alkyl, aryl

Figure 1: General structure of hydroxylamines.

1.2 Synthesis of hydroxylamines and derivatives

1.2.1 Racemic synthetic methods

A number of avenues have been explored for the synthesis of hydroxylamines. Inspection of the literature shows that the standard method is a two step synthesis starting from the ketone **2** to the hydroxylamine **4**, via the reduction of oxime intermediate **3** (Figure 2).[7]

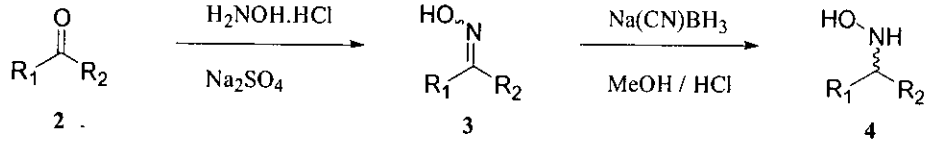


Figure 2: Standard hydroxylamine synthetic route.

The nature of the reducing agent, solvent and reaction conditions vary across the literature.[8,9] Good to excellent yields are generally achieved and a wide variety of parental ketones have been employed.[10,11] It is for these reasons that this route is found across the literature. The limitation inherent in this synthetic route is the racemic nature of the final product. For pharmaceutical and agriculture applications enantiopurity may be an essential factor.

Amines **5** may also be used as the starting materials for hydroxylamine synthesis. Direct oxidation of the amine to the hydroxylamine **4** using oxone / SiO_2[12] or 2,2-dimethyldioxirane (DMD)[13] has been reported with varying results. The DMD oxidation has also been reported to oxidise amines to the corresponding nitrone **6**, which was hydrolysed to the hydroxylamine **4**. Amines may also be converted to aryl imines **7**, thence to hydroxylamines via oxaziridines **8**.[14,15] A further method is the alkylation of amines, followed by *m*-CPBA oxidation to the nitrone derivative **9** and subsequent treatment with hydroxylamine hydrochloride (Figure 3).[16]

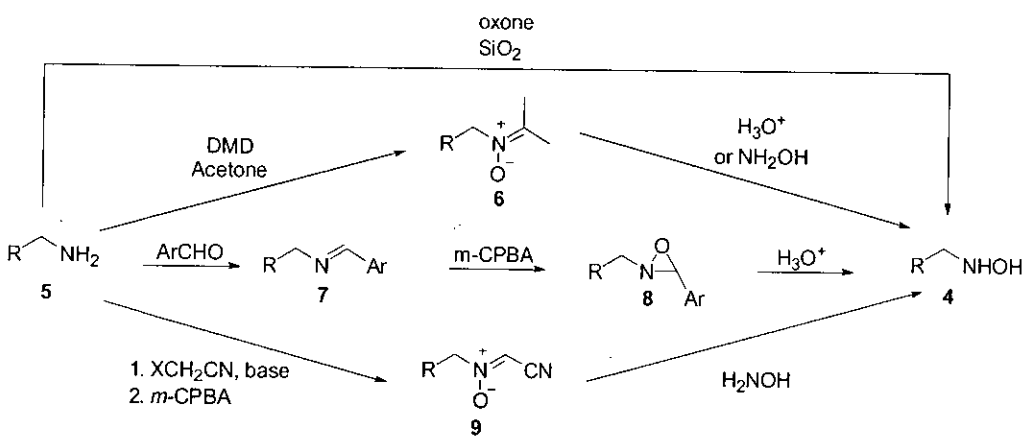


Figure 3: Hydroxylamine synthesis from amine starting material.

Generally, the oxidation methods described used primary amine starting materials and stereoselectivity was not addressed in any of the reports.

1.2.2 Stereoselective synthetic methods

Chirality is a key issue concerning the production of a number of classes of fine chemicals. In 1992 the FDA and European Committee for Proprietary Medicinal Products confirmed the requirement for manufacturers to research and characterise all stereoisomers in chiral drugs proposed to be marketed as a mixture.[17] Thus the necessity to synthesise enantiomerically pure compounds was magnified. Sales of chiral drugs in single enantiomer dosage continue to grow at more than 11.4% annually with the market estimated to be worth $14.94 billion by the end of 2009.[18]

An adjunct to the possibility of using hydroxylamine derivatives as drug substances is the role hydroxylamines may play as drug substance metabolites. As an example, it has been reported that oxidation of amphetamines to a hydroxylamine derivatives is a crucial step on the pathway of amphetamine detoxification.[3] When considering the roles that hydroxylamine metabolites may play in biological systems, once again the different reactivities of enantiomers may be crucial to the understanding of the metabolic pathways involved. The need for enantiopure synthetic metabolites is thus obvious.

As with pharmaceuticals, chiral agrochemicals are increasingly sold as single enantiomer formulations. Enantiomerically enriched hydroxylamine derivatives have also been reported in the literature as useful as chiral auxiliaries in organic synthesis. The production of these fine chemicals provides a further rationale for developing enantioselective synthetic routes to this class of compounds.

The mechanisms by which enantiopurity may be introduced to a synthetic route comprise: enantiomerically pure precursors, synthesis or resolution of a racemic

mixture. The methods mentioned above have appeared to one extent or another numerous times in the literature.

The modification of enantiomerically pure starting materials has been exploited by Wovkulich and Uskoković to produce a simple synthesis of enantiomerically pure hydroxylamines (*S*)- and (*R*)-**4** from the corresponding amines (Figure 4).[19]

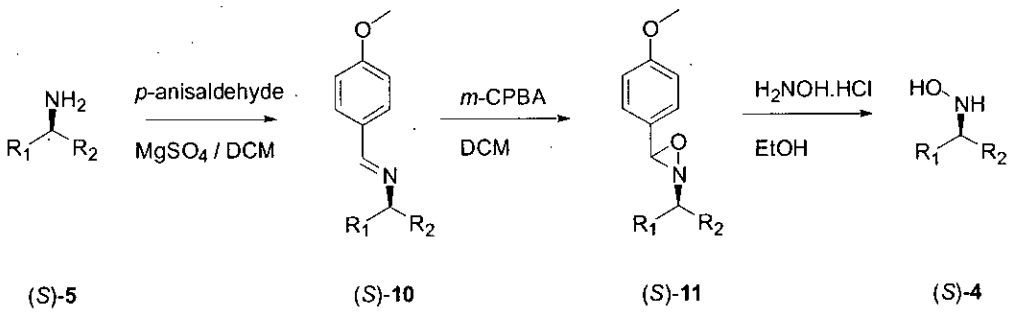


Figure 4: Synthesis of enantiopure hydroxylamines from corresponding amines.

This synthetic route has a number of attractive features but the foundation is the availability of enantiomerically pure amines, thus the route merely defers the difficulties of accessing enantiopure materials. The cost and availability of the starting materials is a key limitation of this route. The same limitation applies to another synthetic route which could employ single enantiomer alcohols as the starting material. A Mitsunobu reaction, leading to inversion of stereochemistry, has been performed using a number of different alcohols and *N,O*-doubly protected hydroxylamines. Z, Aloc, Troc and Teoc functionalities have been used to protect the hydroxylamine and the hydroxylamine afforded in good to high yield (Figure 5).[20] Racemic alcohols were employed in this case but it may be predicted that the use of single enantiomer alcohols could allow access to single enantiomer hydroxylamines of the opposite stereoconfiguration.

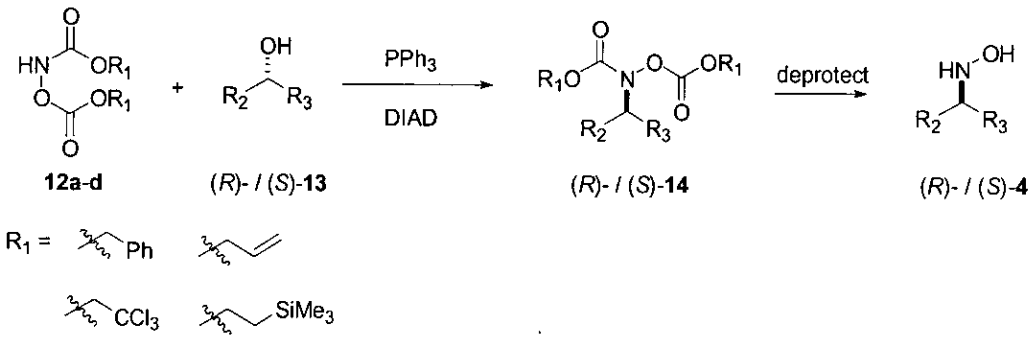


Figure 5: Synthesis of hydroxylamines via the Mitsunobu reaction.

The resolution of hydroxylamines has also been described in the literature. The use of enantiomerically pure tartaric or mandelic acid to form a diastereomeric mix of the hydroxylamine:acid followed by selective crystallisation afforded enantiomerically pure hydroxylamines.[21] The disadvantage of this technique is the inherent limitation to the reaction yield. No greater than 50% yield can be achieved for the desired enantiomer. The reaction is poorly atom-efficient and yields high quantities of waste material (Figure 6).

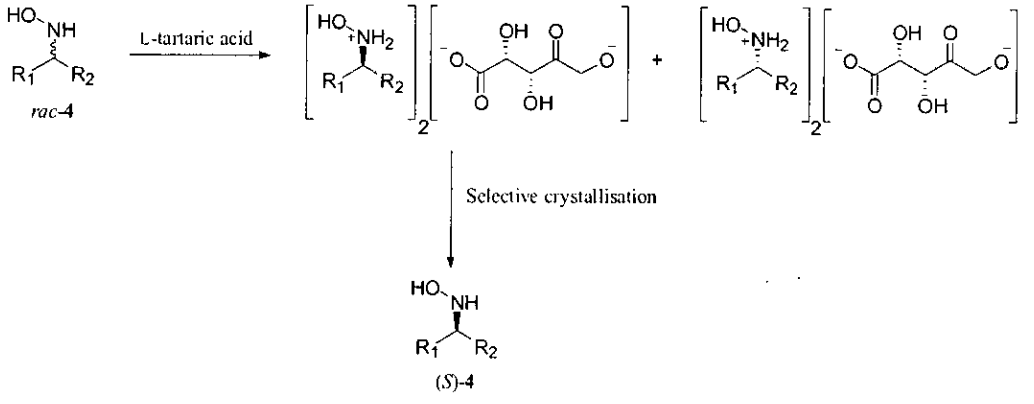


Figure 6: Selective crystallisation to access single enantiomer hydroxylamines.

Perhaps the most elegant, but often the most expensive, solution to the problems of synthesising enantiomerically pure products is that of asymmetric synthesis. A reaction that modifies an achiral moiety to produce an enantiomerically enriched chiral centre opens the possibility for an efficient synthetic route. The asymmetric

reduction of an oxime **15** to the corresponding hydroxylamine derivative **16** is just such a reaction (Figure 7).

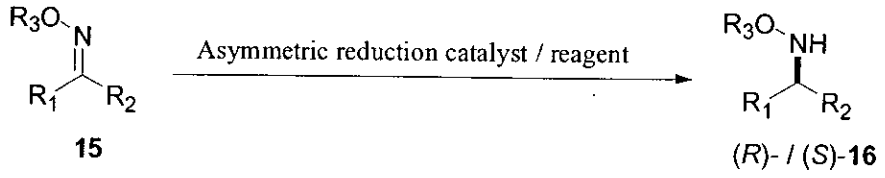


Figure 7: Asymmetric reduction of oximes.

A number of authors have reported reactions of this nature in the literature. The results are variable with the enantiomeric excesses reported ranging from poor to good. There are a number of disadvantages associated with the published methods for asymmetric reduction of oximes. A critical issue is the regularly observed over-reduction to the amine product,[22] leading to low chemoselectivities and yields. Enantioselectivities also are often poor to moderate.[23] Additionally, there is a requirement for either chiral auxiliaries or reagents, for example borane-oxazaborolidine adducts.[22] Chiral auxiliaries often require lengthy synthetic routes, as do the asymmetric reducing agents reported. The limitation when reagents are acquired from the chiral pool is that the enantiomer desired is not always available. If synthesised, then the expense and difficulty in acquiring the species employed to induce an enantioselective reduction often over-shadows the success in producing the enantioenriched hydroxylamine products.

An overall assessment of the literature regarding the synthesis of enantiomerically enriched hydroxylamine derivatives indicates that the area is significantly under-developed when compared to methods to make chiral amines and alcohols. It may be argued that this is due to the relative importance attached to these moieties in the fields of organic synthesis, drug discovery and chemical manufacture. Nevertheless there clearly exists an opportunity for the investigation and exploitation of novel techniques to access enantiomerically enriched hydroxylamine derivatives. The limited nature of the literature reported starkly illustrates the fact that the use of

biocatalysis, a rapidly expanding are of interest for enantioselective synthesis, has not been reported to any significant extent for hydroxylamines.[24]

1.3 Biocatalysts

Biocatalysis describes any chemical conversion of substances that is mediated by living organisms or enzyme preparations derived there from. Biocatalysis has become an increasingly important tool in the industrial synthesis of pharmaceutical and agrochemical intermediates and speciality bulk chemicals. A number of attractive features are associated with the use of a biocatalyst and the growth in the exploitation of biocatalysts can be traced to a combination of driving and enabling factors.

1.3.1 Factors driving the development of biocatalysis

Factors that have driven the development of biocatalytic synthetic routes include the high enantioselectivities regularly exhibited by a wide variety of systems.[25] This has been one of the most crucial driving forces. On occasions there has been no rival system that can offer comparable enantioselectivity. A further key factor has been the drive towards 'greener' chemical processes.[26] The reduction in volume and hazardousness of waste streams and the lowering of energy requirements top the list of methods to make a process more environmentally benign. Whilst biocatalytic systems are no panacea to the problems of synthesis, they may offer significant advantages over rival systems on occasions. Biocatalytic processes generally require moderate temperatures and pressures,[27] thus lower energy input compared with traditional processes. In addition, bulk solvents are often reasonably 'green', water and/or non-chlorinated solvent mixtures being most commonly used. Finally, biocatalysis may be the only method to perform the reaction in question, in other words, no viable alternative methods are available.

1.3.2 Factors enabling the development of biocatalysis

Factors that have enabled the development and exploitation of biocatalytic processes include the advances in both technological and biological knowledge of recent times. A crucial area is that of genomics which has allowed the unravelling of many of the secrets of biocatalytic processes. It has also fed into the science of directed enzyme evolution. The use of the directed evolution[28], saturation mutagenesis[29] and chemical modification of enzymes[30] has provided researchers with the opportunity to tune, improve and alter biocatalysts much as has been done with traditional chemocatalysts. A second enabling factor has been the sourcing of biocatalysts from a far broader range of biodiversity. Organisms and the biocatalysts produced by them are now known from a wide range of environments; deep-sea vents[31], African soda lakes[32] and the Antarctic[33] to name but a few. This in turn leads to both novel reactions and novel conditions at which reactions may be performed being discovered. A third factor is the commercialisation of many biocatalyst preparations which has allowed 'off-the-shelf' access for researchers. A final, intangible, factor is the increasing acceptance of biocatalytic routes as viable and acceptable synthetic routes within the community of synthetic chemists. Clearly this builds from the factors outlined previously, along greater knowledge transfer[34] and inter-disciplinary interactions. The realisation that biocatalysis may offer great benefits and the acceptance that they may perform stable, reproducible and understandable reactions has led to a recognition that biocatalysis must be taken seriously.

1.3.3 Biocatalytic routes to enantioenriched products

Asymmetric synthesis

The transformation of an achiral substrate to an enantioenriched chiral product is an asymmetric synthetic process. Biocatalysts have proved to have many applications in this area and the literature covering this area is broad and extensive. Biocatalysts employed in asymmetric transformations include transaminases which have been

used in the production of optically active amines **18** from a carbonyl compound such as a ketone **17** or alternatively an α-keto acid (Figure 8).[35]

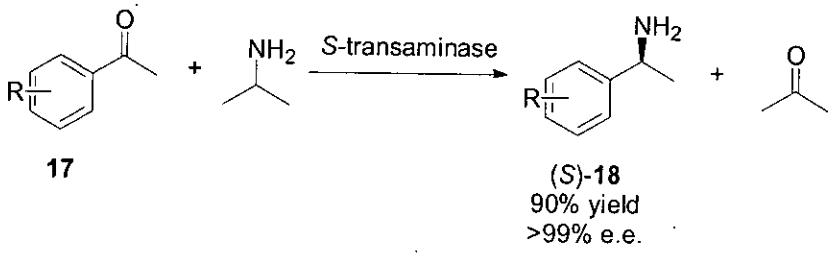


Figure 8: Synthesis of optically active amines using transaminase enzymes.

Enantiopure lactones have been synthesised using Baeyer-Villigerase enzymes[36] and many other biocatalysts have been described to catalyse asymmetric transformations including epoxide hydrolases,[37] alcohol dehydrogenases and oxidases[38] and reductases[39] to name but a few. It is also noteworthy that the use of biocatalysts is not restricted to simple single step transformations; complex cascade reactions may be performed.[40]

Enantioconvergent synthesis

An enantioconvergent transformation describes a process by which a racemic mixture is converted to a single enantiomer of a product via two different reactions for the two substrate enantiomers. One enantiomer is converted with retention of configuration and the other enantiomer is converted with inversion of configuration, producing a single enantiomer product with 100% theoretical maximum yield. An enantioconvergent system often relies on the use of two different biocatalysts, with opposite stereoselectivity, in a one-pot system, for example the combination of two epoxide hydrolases with opposite hydrolysis specificities.[41] Interestingly a single biocatalyst can perform an enantioconvergent reaction, if it displays opposite selectivities on the two different enantiomers of a racemic mixture. Epoxide hydrolase from *Novacardia* species hydrolyses both enantiomers of *cis*-2,3-disubstituted epoxyalkanes however, crucially, hydrolysis is performed at the (*S*)-

configured oxirane carbon with inversion of configuration to afford diol (*R*,*R*)-**20**. (Figure 9).[42]

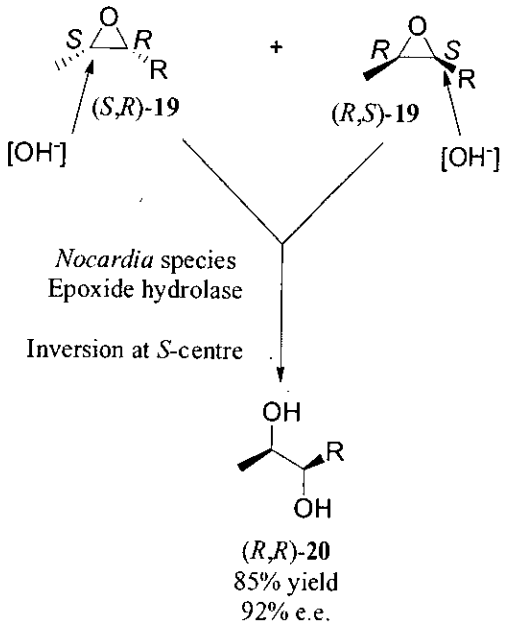


Figure 9: Enantioconvergent synthesis of epoxyalkanes.

Kinetic resolution

A kinetic resolution is the achievement of partial or complete resolution, by virtue of unequal reaction rates, of the enantiomers of a racemate with a chiral agent. Thus enantioselection is based on the different reaction rates of the transformation of substrates S_R and S_S by a chiral catalyst to P_R and P_S. Recovery of the product P_R and the unreacted enantiomer S_S in a non-racemic form constitutes a kinetic resolution. Clearly the reaction may progress with opposite enantioselectivity, dependent upon the nature of the catalyst (Figure 10).

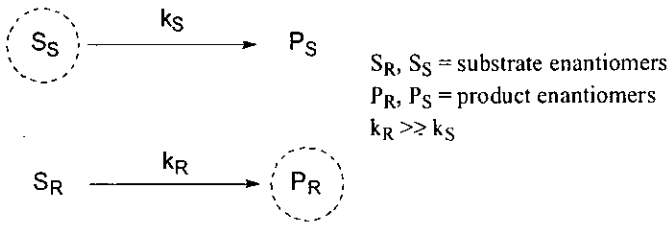


Figure 10: Theoretical basis of a kinetic resolution.

Biocatalytic kinetic resolutions are extremely common in the literature.[43] The bulk of the resolutions presented employ a lipase as the enantioselective catalyst. Most commonly the lipase is employed to selectively acylate a hydroxyl moiety on one enantiomer using an acyl donor in a transesterification reaction, vinyl acetate being the most common choice.[44] The reasons for this include the fact that a number of lipases are fully commercialised products, available in bulk quantities. This means that they are a catalyst that may be considered for scale-up and they are readily available to researchers worldwide. In addition to simple lipases a number of solid-supported lipase preparations are also widely available including Novozyme 435 and Lipolase,[45] increasing the scope for the lipase-catalysed reactions. The specificity and enantioselectivity of lipases has been studied and is predictable,[46] along with the stereochemical preference for ester and alcohol substrates, generally found to follow Kazlauskas' rule (Figure 11).[47]

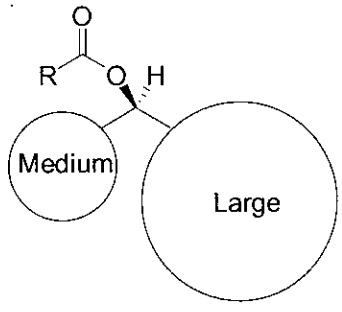


Figure 11: Pictorial representation of preferred lipase substrate for Kazlauskas' rule.

As with any empirical rule, exceptions may be found but as a general guide to the stereoselectivity of lipases this rule allows for predictable results to be achieved. The

factors outlined above contribute to a 'snowball' effect, in which increasing literature reports of lipase-catalysed kinetic resolutions stimulate further work. The practical requirements[48] and techniques needed to perform a biocatalysed kinetic resolution have entered the mainstream synthetic chemist's arena.

A number of different substrate classes, with different chiral functionalities have been resolved using a lipase catalysed kinetic resolution system. These include a wide diversity of compounds with a hydroxyl moiety but also amines.[49] The reverse reaction of the transesterification, hydrolysis of one enantiomer of a racemic ester, is also regularly reported. Lipases are not the only catalysts successfully used in kinetic resolutions; enzymes including epoxide hydrolases[50] and esterases[51] have also been reported to catalyse the resolution of chiral substrates. Kinetic resolution is a powerful technique to access enantioenriched products and, as demonstrated above, has been widely adopted and reported in the literature. The key limitation of the process is the inherent maximum yield of the desired product. As a racemate contains only 50% of the desired enantiomer, a maximum yield of 50% can be achieved. This restriction will always apply and only in the rare case when the desired enantiomer of the product and the remaining enantiomer of the substrate are both required will the reaction be theoretically high yielding. Recognising this crucial deficiency research has been directed towards overcoming the maximum yield restriction. The recycling or racemisation of the unwanted enantiomer is often the focus for research, particularly by process chemists.

Dynamic kinetic resolution

A kinetic resolution may be modified by the addition of a second process in which the two substrate enantiomers are interconverted (Figure 12). This is named a dynamic kinetic resolution (DKR). This leads to the possibility of a maximum theoretical yield of 100%.

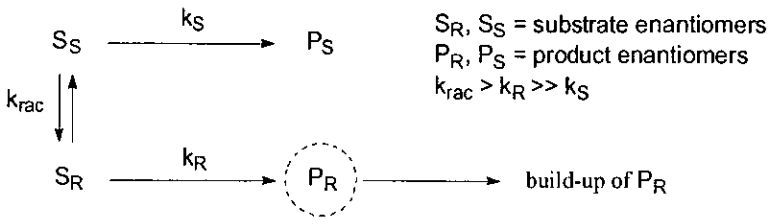


Figure 12: Theoretical basis of a dynamic kinetic resolution.

The key to a successful DKR is finding conditions which allow an *in situ* racemisation whilst maintaining the activity of the resolution catalyst. Both the conditions and catalysts used for the two processes must be mutually compatible. Broadly, two techniques have been used for the racemisation process; chemocatalysis or a second biocatalytic process. Simple chemocatalysis, such as acid/base catalysed enolisation can be used to racemise labile chiral centres. However it is advantageous to operate with stable chiral centres, to ensure a stable, non-labile product is formed. Alcohols and amines can be racemised by an oxidation and reduction sequence with a transition metal catalyst such as Ru, Rh, Ir, Al, or Pd.[52] Bäckvall and co-workers have developed a process based on the use of *p*-chloroacetate as the acyl donor and a ruthenium complex as the racemisation catalyst.[53] This was found to be compatible with *Candida antartica* lipase B catalysed resolution, allowing the construction of a DKR (Figure 13).

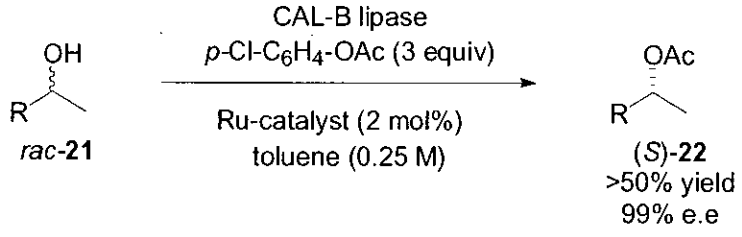


Figure 13: Dynamic kinetic resolution of secondary alcohols.

Deracemisation

Formally, a deracemisation process is one in which a racemate is converted into a chiral, non-racemic product with a theoretical maximum e.e. of 100% and with the theoretical possibility of 100% yield. No intermediate separation of materials occurs.

Dynamic kinetic resolutions (DKR), dynamic thermodynamic resolution, stereoinversion and enantioconvergent transformations of a racemate are all classified as deracemisation processes. However, in the context of this report, a deracemisation describes a subset of these processes, specifically the conversion of a racemate to a single enantiomer of a substrate in a maximum theoretical yield of 100% (Figure 14).

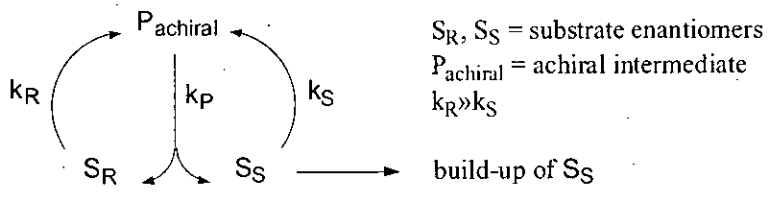


Figure 14: Theoretical basis of a deracemisation.

As demonstrated in Figure 14, the requirements for the deracemisation process are a stereoselective transformation of one enantiomer of the substrate to an achiral intermediate. Secondly, a non-stereoselective transformation of the achiral intermediate back to the substrate is needed. Critically, these two processes need to be mutually compatible in a one-pot situation. Overall a cyclic transformation system is set-up and repeated progression of the transformed enantiomer through the cyclic leads to an enantioenrichment of the substrate. Depending on the nature of the racemic substrate the stereoselective portion may be an oxidation or a reduction. The use of a deracemisation process to access enantioenriched products has been reported in the literature,[54,55] Soda *et al.* employed this method for the deracemisation of *DL*-proline[56] and *DL*-pipecolic acid[57] using *D*-amino acid oxidase to perform the stereoselective oxidation and sodium borohydride to non-stereoselectively reduce the achiral imine intermediate (Figure 15).

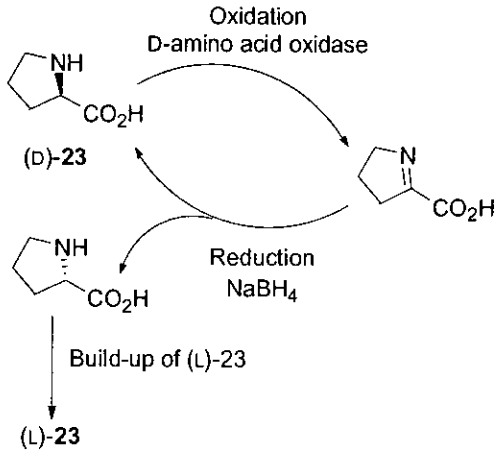
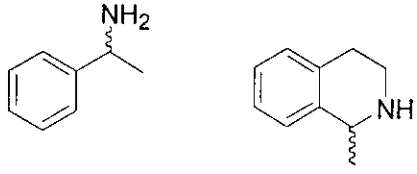


Figure 15: Deracemisation of *DL*-proline.

The use of these conditions has been expanded to encompass the deracemisation of acyclic substrates. The use of sodium cyanoborohydride as the reducing agent has also been reported, with some advantages over sodium borohydride.[58] Catalytic transfer hydrogenation has also shown to be effective as the reducing agent in the deracemisation of amino acids.[59] The stereoselective biocatalytic oxidation coupled with borohydride reagent reduction process has more recently been applied to the deracemisation of amines. The deracemisation of α-methylbenzylamine **24** in 77% yield and 93% e.e. has been reported,[60] as has the deracemisation of 1-methyl-1,2,3,4-tetrahydroisoquinoline **25**.[61] In the case of the acyclic substrate the yield was sub-optimum as a result of intermediate imine hydrolysis. However it has been conclusively demonstrated that the deracemisation of amines, as with amino acids, is a viable proposition.



24 **25**

Figure 16: Previously demonstrated amine deracemisation substrates.

Stereoinversion

A stereoinversion process consists of one enantiomer of a substrate being selectively converted to an achiral intermediate. The achiral intermediate is in turn converted to the opposite enantiomer with a second step. An example of this process is the stereoinversion of a chiral secondary alcohol via an achiral ketone. This process has been reported in the literature, employing two different enzymes; a dehydrogenase which stereoselectively oxidises one enantiomer of the secondary alcohol and a redox enzyme which stereoselectivity reduces the ketone with opposite selectivity to the dehydrogenase.[62] Crucially the stereoselective reduction of the ketone is irreversible, thus a build-up of a single enantiomer occurs. This is important as the conversion of the racemate to a single enantiomer in this process is energetically disfavoured in thermodynamic terms.

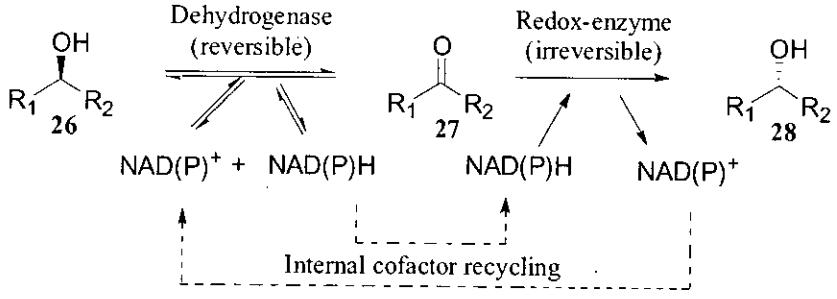


Figure 17: Stereoinversion of secondary alcohols.

A stereoinversion can be regarded as a more refined version of a deracemisation, described above, replacing the second non-stereoselective step of a deracemisation with a stereoselective transformation. The combination of two stereoselective steps means that the completion of a number of cycles of conversion, as with a deracemisation, is unnecessary.

1.4 Conclusion

The reasons detailed above illustrate the rationale for conducting an investigation into possible biocatalytic routes for the synthesis of hydroxylamine derivatives. The area is not developed significantly in the literature and both the synthetic products and procedures warrant a detailed survey of possible biocatalytic routes that may be exploited. Due to the prior success of a deracemisation system in producing enantiomerically enriched amine enantiomers from the corresponding racemates[61,63] the development of an analogous system for hydroxylamines was the focus for the project as a whole. The development of the various components necessary for this are described further in the chapters that follow. As an alternative to the deracemisation, an asymmetric reduction of the achiral oxime substrates to the analogous hydroxylamines employing a biocatalyst could be envisaged, and has also been explored to some extent.

2 Laccase

As outlined in Chapter 1 a successful construction of a deracemisation system requires a stereoselective conversion, in the case of this report, a stereoselective oxidation of a hydroxylamine substrate. With a focus on biocatalytic methods a survey of the literature was performed in an effort to identify suitable biocatalyst. One such catalyst identified was the laccase family of enzymes.

2.1 Laccase enzymes

Laccases (benzenediol:oxygen oxidoreductases, E.C. 1.10.13.2) are a class of oxidoreductase enzymes that participate in the a variety of enzymatic processes.[64] They have been found in various plant species, in bacterium[65], and in a number of studied insects.[66] Laccases have been implicated in a number of processes which include lignin biosynthesis[67], plant pathogenicity[68], the degradation of plant cell walls[69] and insect sclerotisation.[70] Laccases are also widespread in numerous fungi.[71] Of relevance to this study is the involvement of white-rot fungi laccases in the multi-enzymatic process of lignocellulose degradation.[72] The lignocellulose degradation process is a crucial step in carbon recycling in terrestrial ecosystems and a number of fungi demonstrate the ability to break down the lignin present in the wood they grow on, this degradation is achieved via the oxidation of the lignin catalysed by laccase, using atmospheric oxygen as the ultimate oxidant.

Crystal structures for a number of laccases have been published and indicate the enzyme contains 4 copper atoms.[64,73] Laccases are described as being type 1, type 2 and coupled binuclear type 3 centres. These assignments are based on the EPR properties exhibited by the Cu atoms. The type 2 Cu atom and the type 3 binucleur Cu atoms form the trinucleur cluster which is thought to represent the active site for the binding and reduction of O_2 and for the release of water. Mechanistic studies indicate that a single-electron transfer mechanism is involved.[74] The depolymerisation of lignin requires the oxidation of two general moieties: electron-rich phenolic fragments and non-phenolic fragments. Studies have showed that the

substrate specificity range of these enzymes is relatively narrow, with a specificity limited to the phenolic fragments of lignin. The reaction mechanism proceeds through the generation of the phenoxyl radical, after which a variety of reaction pathways have been proposed (Figure 18).[75]

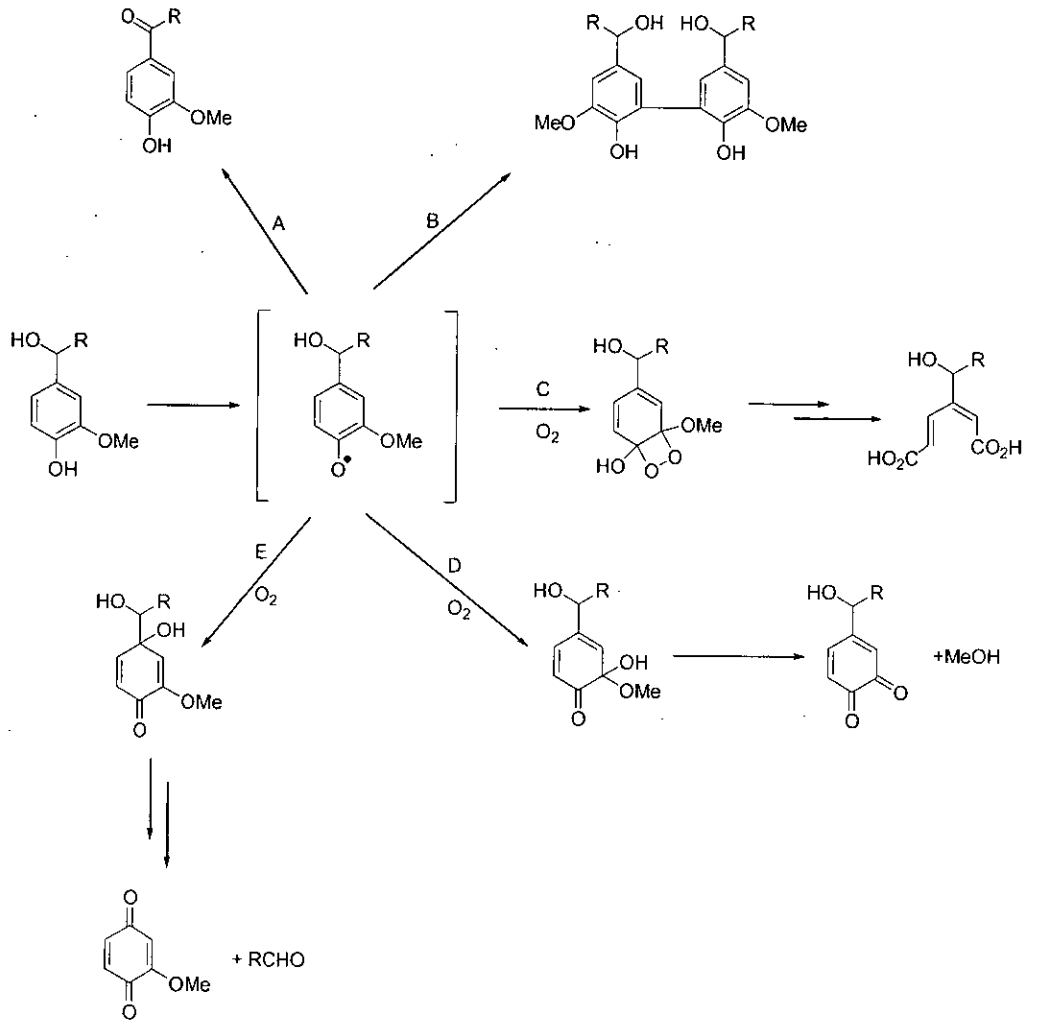
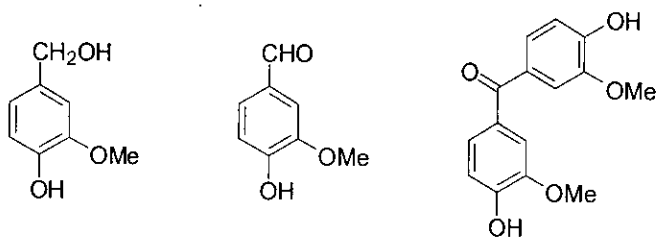


Figure 18: Possible reactions of the phenoxyl radical derived from a lignin subunit; A – side-chain oxidation, B – radical coupling condensation, C – aromatic ring cleavage via dioxetane intermediate, D – further oxidation / oxygen addition at C-3 to yield *o*-quinones, E – further oxidation at C-1 to yield *p*-quinones.

It has been shown that the distribution of products from the laccase mediated oxidation of vanillyl alcohol, **29**, a standard lignin model, is very narrow. Traces of

vanillin **30**, the product of side-chain oxidation (pathway A) and **31**, the radical coupling (pathway B) derived product are the only observed products. Much of the starting material remained. However, work in a number of publications has illustrated how the scope of laccase catalysed oxidations may be expanded significantly from the limited wild-type activity reported.[76]



29 **30** **31**

Figure 19: Vanillyl alcohol **29** and observed oxidation products **30** and **31**.

The interest and research regarding laccases has been driven chiefly by the technological applications that these enzymes have in the wood processing industry. Lignin removal, the major step in the degradation of lignocellulose, is the key step in the conversion of wood to paper pulp. In addition, the bleaching of paper pulp fibres requires further lignin removal. In targeting a totally chlorine-free (TCF) manufacturing sequence industrial and academic research groups have exploited a number of enzymes, including laccases, as oxidising agents. The use of atmospheric oxygen as the stoichiometric oxidant ensures a cheap and unrestricted bulk reagent supply. Environmental concerns are reduced with TCF processes and economic drivers are high for developing these enzymatic processes.

2.2 Laccase:mediator systems

The use of laccases in paper-processing has exploited the wild type substrate of the enzyme, as have a number of other bioremedial and synthetic systems[77], however developments of pulp processing systems[78] have demonstrated that the substrate range of laccase can be expanded greatly by the incorporation of a mediator in the oxidation system, the so called laccase:mediator system (LM system).[79] An extra

oxidation/reduction cycle is inserted into the reaction sequence which has the effect of decoupling the direct oxidation of the substrate from the laccase active site (Figure 20).

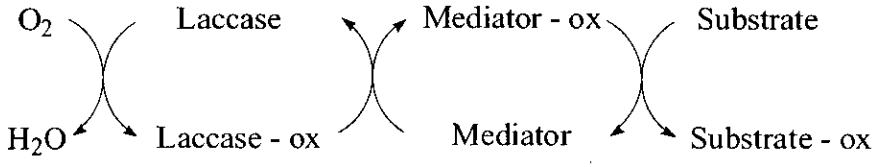
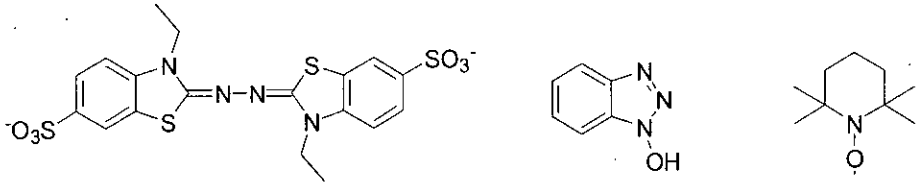


Figure 20: Laccase-mediator system.

After being oxidised the mediator exits the enzyme active site and in turn oxidises the substrate, thus the mediator acts as an 'electron shuttle'. This would expand the possible substrates to those unable to access the laccase active site. Alternatively, the mediator could oxidise the substrate through a mechanism unavailable to the laccase, again expanding the substrates able to be oxidised.[80] A combination of both of the factors outlined above is possible.

Research in the area of LM systems has led to a reassessment of the natural lignin degradation process. Naturally incorporated mediators would expand the substrate range of the laccases and allow an oxidation of the non-phenolic lignin fragments, giving the expressed laccase broad spectrum lignin degradation activity. A number of compounds, including 3-hydroxyanthranilic acid, have been postulated as a naturally occurring mediator of the laccase activity in fungi. The area is still under investigation and conflicting results have been reported.[81,82] A key advantage that the use of a LM system has allowed is that the range of substrates that may be oxidised is broadened significantly. This has been manifested in a number of ways; the modification of a paper bleaching system to afford a much improved process or product[83], water treatment[84] and in the decolourisation of recalcitrant dyes[85], including a fully commercialised process for denim decolourisation sold under the trade name *Denilite* and its reformulated successor *Denilite II S.*[86]

A number of different mediators have been described in the literature.[87] The essential feature is the ability of the small molecule mediators to undertake redox cycles. Whilst many molecules could be suggested that have this feature, prior work has generally focused on three categories of mediator; 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) **32**, *N*-hydroxy compounds such as 1-hydroxybenzotriazole (HOBt) **33** and species based on the nitroxyl radical containing 2,2',6,6'-tetramethylpiperidine- *N*-oxyl (TEMPO) **34**.



32 **33** **34**

Figure 21: Mediators previously reported for laccase:mediator systems.

2.3 Laccase:mediator reaction mechanisms

The mechanism for ABTS mediated oxidation has been postulated to be an electron transfer (ET) route (Figure 22). It was shown that the reactivity of the mediation reaction correlated with the oxidation potentials of a series of analogous substrates, based on substituted benzyl alcohols.[88]

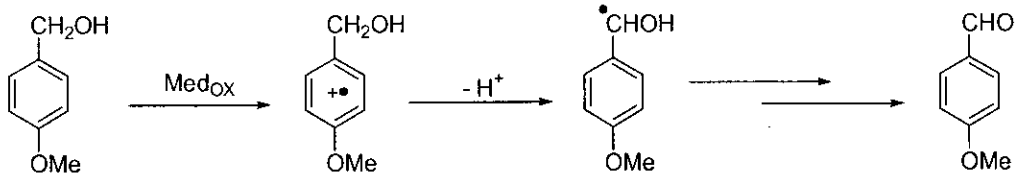


Figure 22: ET route for ABTS mediated reactions.

The mechanism for *N*-hydroxy mediators has been extensively investigated and, in the case of *N*-hydroxyphthalimide and 1-hydroxybenzotriazole, a radical hydrogen atom transfer (HAT) model has been demonstrated to be the most appropriate

explanation.[88] Hydrogen atom abstraction from the benzylic C-H bond was shown to be the rate determining step, differing from the ET route for ABTS (Figure 23).

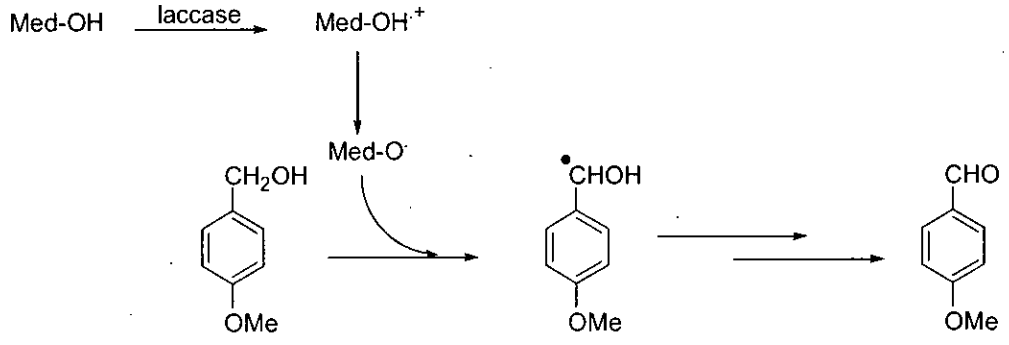


Figure 23: HAT route for *N*-hydroxyl compound mediated reactions.

The fact that the *N*-hydroxy compounds are believed to operate through a nitroxyl radical intermediate points to the use of stable nitroxyl compounds as possible mediators. The mode of action of these compounds, such as TEMPO, is not, however, a simple case. The stable nitroxyl compounds are not, in themselves, active oxidising agents; in other words, the nitroxyl is not the active oxidising species in these systems. This is easily demonstrated by the fact that laccase is required for the system to work, TEMPO alone is not sufficient for an oxidising system. The mechanism is proposed to go through the key oxoammonium ion (Figure 24).

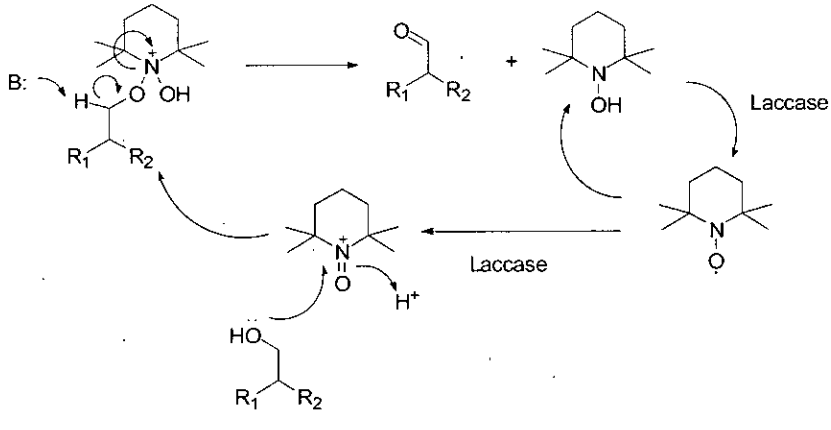


Figure 24: Proposed TEMPO mediated laccase oxidation mechanism for alcohols.[89]

Of greatest relevance to the studies presented herein is the work of Galli and co-workers.[89] Over the course of a number of publications this group has shown that the use of a LM system can be applied to the oxidation of a number of simple alcohols and related substrates. A comprehensive survey of possible mediators and the substrate range with respect to substituted alcohols has been reported. The regular use of *N*-hydroxy compounds as mediators suggested that hydroxylamine derivatives could be viable targets as the ultimately oxidised substrate in a LM system. In an attempt to define a system suitable for the efficient and reproducible oxidation of hydroxylamine derivatives to the corresponding oxime derivatives a programme investigating the application of a LM system to this transformation was undertaken.

A number a key variables were identified and a systematic investigation performed in an attempt to firstly; assess whether a LM system could be applied to the oxidation of hydroxylamine derivatives, and secondly; optimise reaction conditions to construct a viable reaction system. The source of the laccase, reaction conditions in terms of substrate concentration, solvent and reaction time were key parameters. The nature of the mediator was predicted to be a crucial and the experimental program reflected this.

2.4 Laccase results and discussion

2.4.1 Laccase source

A number of sources of laccase are available, both wild-type and recombinant. These enzymes have been use in a number of different preparations including immobilised on silica and Amberlite IRA-400 resin.[90,91] Commercial sources were considered as the preferred option for reasons of expediency and price and were prioritised for investigation and assessment. Two sources were employed for initial testing, laccase derived from *Rhus vernificera* (the Japanese lacquer tree) and *Trametes versicolor* (the turkey-tail fungus) and both were supplied as off-white to brown powders. These preparations were analysed using SDS-PAGE chromatography and were found to

contain multiple bands (Figure 25). The preparations were used in this crude formulation, because it was desirable to construct an oxidation system that was convenient and transferable. The use of commercially available reagents without further purification is hugely advantageous if the process is to be used at large scale. If the oxidation was found to be ineffective than re-examination of the system with purified laccase would be possible.

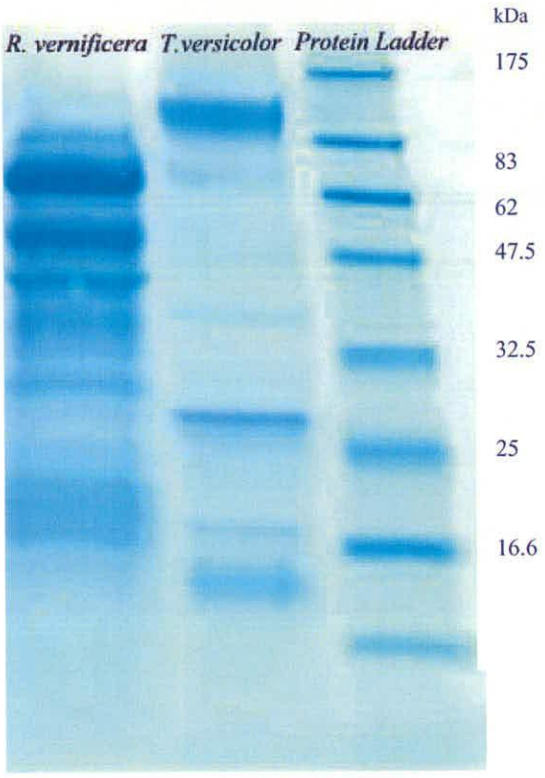
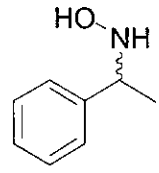


Figure 25: SDS-PAGE gel of *Rhus vernificera* and *Trametes versicolor* laccase preparations.

Initial experiments were focused on a single substrate, 1-phenylethylhydroxylamine **35**, to assess the suitability of applying a LM system to hydroxylamine systems and in an attempt to define optimised reaction conditions.



35

Figure 26: 1-phenylethylhydroxylamine **35**.

Analysis was performed using GC-MS. This technique provided a number of advantages; rapidity, the use of an automated injection system and the development of a short run time lead to a high-throughput screen. Also, if the reaction produced undesired or unexpected products in the reaction an initial identification of these could be achieved from the mass spectrum acquired.

Initial experiments performed were designed to explore whether laccase would directly catalyse the oxidation of hydroxylamines without requiring a mediator, thus the laccases were first tested without any mediator added. No conversion of the starting material was observed with either *Rhus vernificera* or *Trametes versicolor* derived laccase.

Having demonstrated that a non-mediated system was completely ineffectual in the attempted oxidation of a hydroxylamine derivative the investigation of possible mediators was commenced.

2.4.2 Mediator

A large number of possible mediators for use in a LM system have been described by previously published work. A comprehensive survey of these possible mediators was undertaken. Commercial availably appeared to have driven selection of these possible mediators in the literature and this focus was followed in the selection of the initial mediator panel to be tested. The target of this experimental programme was to

define a simple and practical oxidation system, so the use of mediators that required significant synthetic steps to access or were available only from niche sources would be a significant limitation. A diverse selection of possible mediators was assembled **32-34, 36-39** (Figure 27) and challenged against **35**. The laccase preparation from *Trametes versicolor* was selected for the first mediator survey. This was due to observations from the initial laccase survey; the laccase preparation derived from *Rhus vernificera* contained a noticeable amount of insoluble particulates.

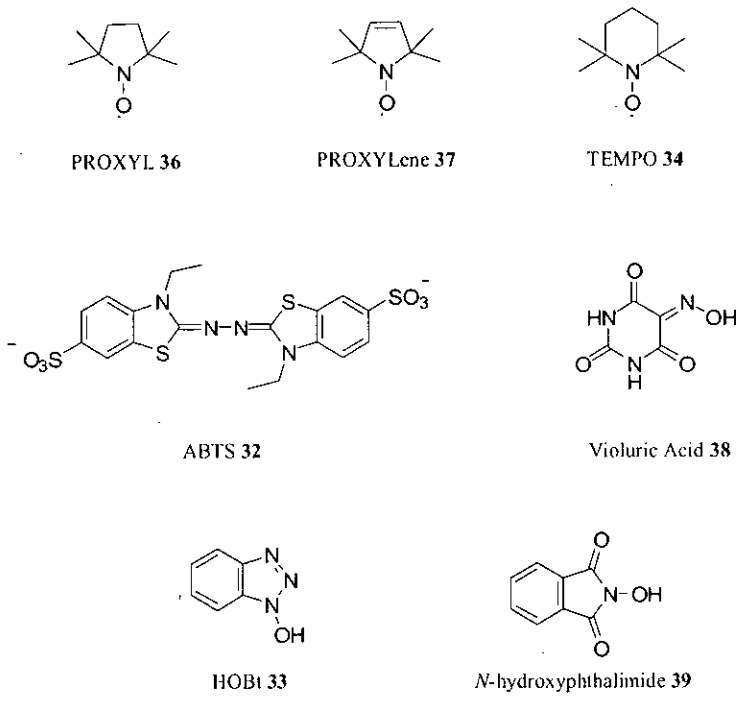


Figure 27: Possible LM system mediators.

Mediator / 0.3eq.	Conversion at t = 180mins / %
ABTS (**32**)	>99
HOBt (**33**)	11
TEMPO (**34**)	>99
PROXYL (**36**)	36
PROXYLene (**37**)	45
Violuric Acid (**38**)	>99
N-hydroxyphthalimide (**39**)	5

Table 2: Survey of possible mediators for LM system.

Due to the excellent conversion observed, coupled with reasons of economy and ease of supply, TEMPO was selected as the mediator of choice for further experiments. One might expect the oxidation mechanism for this process to be closely analogous to the proposed mechanism for alcohol oxidation using the same system (Figure 28).

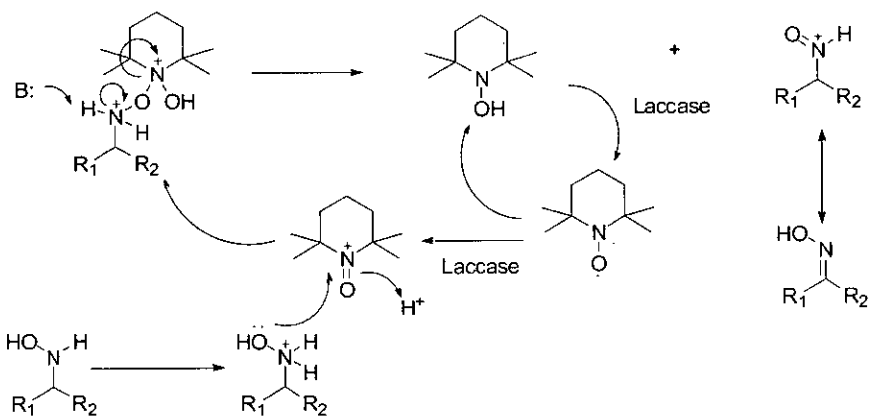
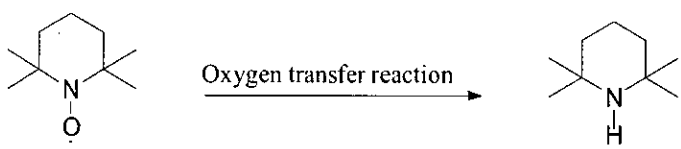


Figure 28: Proposed mechanism for TEMPO mediator laccase oxidation of hydroxylamines.

To confirm the choice of laccase from *Trametes versicolor* as the preferred enzyme preparation a TEMPO- *Rhus vernificera* LM system was tested and found to produce 0% conversion. The reason for this is unclear, however the experiment was repeated with a new supply of *Rhus vernificera* laccase and the same negative result obtained.

Thus TEMPO / *Trametes versicolor* laccase was defined as the LM system of choice for further work. The test system that was defined was: 3mM (0.3eq.) TEMPO, 100U laccase, 10mM substrate in pH 4.5 citrate buffer. 10% v/v MeCN was used for systems in which the substrate was not fully soluble in aqueous buffer. The reaction mixture was shaken at 200 rpm at 25 °C. The use of 0.3 eq. TEMPO is regularly reported in the literature and suggests a very inefficient catalyst system. A possible explanation is that the TEMPO undertakes an oxygen transfer reaction to enzyme residues, which results in the corresponding secondary amine being formed (Figure 29).



TEMPO **34** 2,2,6,6-Tetramethyl-piperidine **40**

Figure 29: Possible TEMPO degradation reaction.

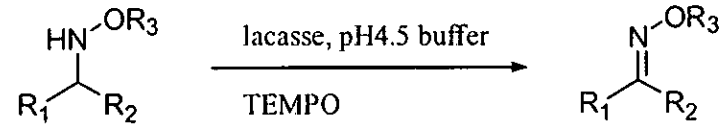
Evidence for this is that TEMPO concentration in these reactions can be observed to rapidly fall initially, before reaching an equilibrium concentration. Addition of further portions of TEMPO results in a rising of this equilibrium concentration.[92] This suggests TEMPO undergoes a fixed number of degradation reactions after which it is solely involved in the redox cycle of the substrate oxidation.

It was decide to continue with the system defined above to probe the substrate specificity of the reaction.

2.4.3 Substrate specificity

An assessment of the substrate specificity of the LM system detailed above was performed in an attempt to define its utility and generality. The members of the substrate panel to be tested were selected with a view to challenging the system with substrates of varying steric properties. Challenging the LM system with these

substrates would provide an opportunity to gauge the usefulness of this reaction and address concerns regarding the possible lack of generality of the system. Substrates **35**, **41** - **50** were tested and the results detailed in Table 3 observed.



35,41-49 **50-59**

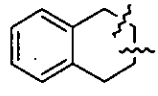
Hydroxylamine starting material	R_1	R_2	R_3	Oxime product	Conversion / % (GC-MS)
35	Ph	Me	H	**50**	>99%
41	Ph	Et	H	**51**	>99%
42	Ph	Me	Me	**52**	>99%
43	*p*OH-Ph	Me	H	**53**	14%
44	*p*OH-Ph	Me	Me	**54**	>99%
45	*p*Br-Ph	Ph	H	**55**	51%
46	Cyclohexyl	Me	H	**56**	25%
47	Cyclohexyl	Me	Me	**57**	79%
48			Me	**58**	>99%
49			H	**59**	>99%

Table 3: Screen to establish substrate specificity of LM system.

As Table 3 indicates, the results of the substrate specificity experiments suggest that the reaction system has a broad scope, with a wide range of substrates tolerated. The reaction is not limited to hydroxylamines but shows hydroxylamine methyl ethers may be oxidised. Interesting, no evidence for the oxidation of the phenolic functionality of compounds **43** and **44** is observed although the wild-type activity of

laccase would suggest they may be vulnerable to oxidation. Analysis of the initial survey was performed by GC-MS. Standard samples allowed for the development of definitive analytical methods and an indication of the nature of by-product was possible. Clearly a fuller characterisation of a number of the reactions, to ensure conclusive identification of the reaction product would be desirable and was performed for a selection of the reactions detailed in Table 4.

Hydroxylamine Starting material	Oxime product	Oxime identification
35	50	GC-MS, LC-MS, NMR
44	54	GC-MS, LC-MS, NMR
46	56	GC-MS, LC-MS, NMR

Table 4: LC-MS and NMR analysis of oxidation product.

Whilst confidence in the LM system developed for the oxidation of hydroxylamine derivatives had been established a further level of development was considered crucial. A preparative scale reaction, with full characterisation of the reaction products, was performed to test the utility of this reaction for the preparation of viable quantities of oxime (Figure 30).

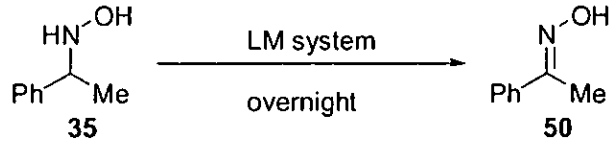
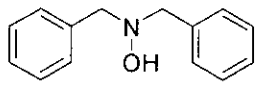


Figure 30: Preparative scale product of oxime **50** using the LM system.

0.3g of fully characterised **50** was obtained in 89% yield from the LM system reaction (see 7.13, page 147).

The LM system detailed above represents a novel reaction for the oxidation of hydroxylamine derivatives, an area which has received little attention in the

literature. The reaction conditions are relatively mild and inexpensive and the substrate specificity has been shown to have no major limitations. To probe whether an even broader range of functionality may be successfully oxidised using the LM system compound **60** was tested. The reaction was found to be compatible with a tertiary nitrogen centre and the corresponding nitrone **61** was observed via GC-MS.



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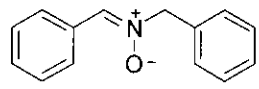


61

Figure 31: *N,N*-Dibenzylhydroxylamine and nitrone analogue.

With the LM system for hydroxylamine derivative oxidation in hand, further expansion of the range of the reaction was envisaged. A large proportion of the hydroxylamine derivatives employed were chiral and thus an enantioselective oxidation would be not only possible but highly attractive. A large number of enzymatically catalysed reactions exhibit excellent enantioselectivity. The chiral environment of the majority of enzyme active sites, derived from the single enantiomer nature of most amino acids, explains this phenomenon. However, as reported in the literature and the substrates tested above, this is not the case for the LM system. To confirm this fact the enantioselectivity of the oxidation with respect to compound **47** was investigated and found, as expected, to be completely non-enantioselective.

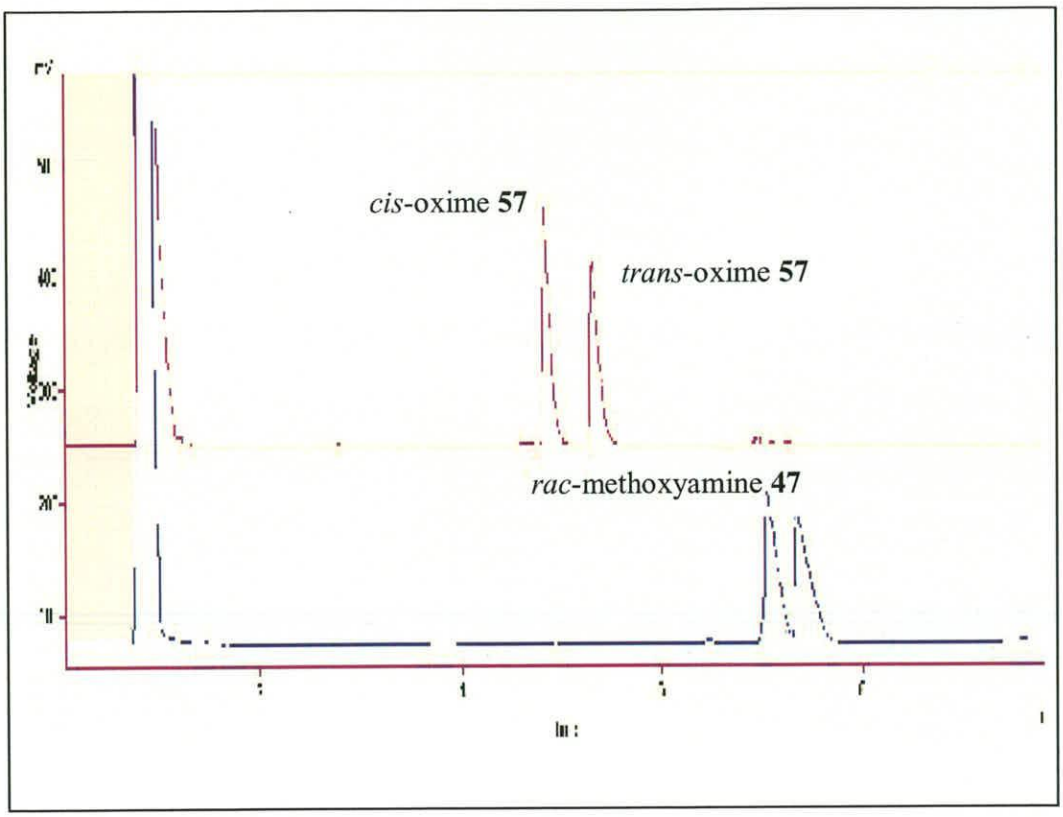


Figure 32: GC trace of oxidation reaction of **47**. Non-enantioselective oxidation of **47** to a mixture of geometric isomers of the corresponding oxime **57** is illustrated (GC Method 1).

This result is most simply explained by the fact that the substrates do not interact with the laccase directly but via the mediator. The mediators do not offer the possibility of enantio-discrimination when oxidising the substrates. In extending this theory it is obvious that if an enantioselective oxidation is desired using the LM system the mediator must oxidise the substrate in an enantioselective fashion. By analogy to the enzyme active site it may be postulated that a single enantiomer chiral mediator may oxidise the hydroxylamine derivative in an enantioselective manner.

2.4.4 Further mediator investigations

In an attempt to further improve the rate of oxidation the identity of the optimum mediator was further probed. With a view not only to rate improvement but also to

future specialisation of the LM system, a number of TEMPO analogues **62** - **64** were tested to investigate whether substituted TEMPO mediators would exhibit comparable activity to the parent mediator. These experiments would provide an indication as to whether more complex nitroxyl radical mediators could be viable mediators. This was of interest as elaboration of a TEMPO core could afford chiral mediators, and therefore possibly, enantioselective oxidation mediators. Table 5 displays the results of testing a number of commercially available substituted TEMPO analogues.

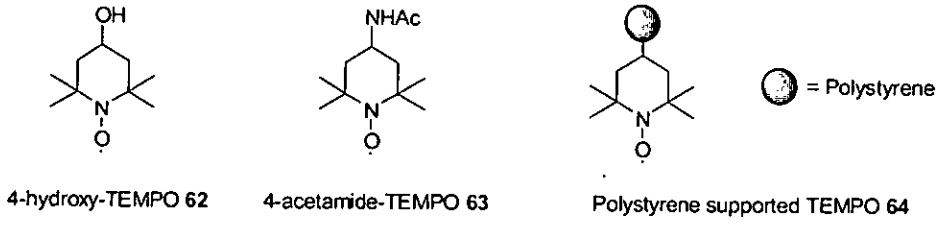


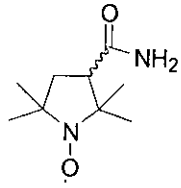
Figure 33: Substituted TEMPO analogues.

Mediator	Conversion at t=3h / %
62	>99
63	22
64	39

Table 5: Survey of substituted TEMPO analogues.

With a view on cost and availability, none of the substituted TEMPO analogues presented decisive reasons for replacing TEMPO, although the fact that these mediators were active was an indication that substituted TEMPO analogues could be viable mediators. A further experiment was performed to investigate whether enantioselective oxidations employing homochiral mediators would be viable. Clearly the 4-substitued ring structures could not afford mediators with single enantiomer configuration close to the nitroxyl radical. Any chirality could only be practically introduced by further elaboration of the 4-positon, distant from the active oxidising species at position 1. It was felt that, to maximise the chances of transfer of

chirality, single enantiomer chirality should be located close to the active oxidising moiety in the mediator molecule. However this focus was balanced with an attempt to test a single enantiomer mediator rapidly. To address this issue a substituted analogue of PROXYL, 3-carbomoyl-PROXYL **65**, was also investigated, as a resolution of the racemic mediator could provide single enantiomer mediator in short order. Unfortunately this substituted PROXYL analogue proved to be inactive as a mediator.



3-Carbomoyl-PROXYL **65**

Figure 34: 3-carbomoyl-PROXYL **65**.

A number of literature preparations of single enantiomer TEMPO analogues have been published.[93,94] All involve lengthy synthetic routes or extensive recrystallisations, implying low yields and were thus unattractive. The use of single enantiomer mediators to attempt to affect the enantioselective oxidation of a single hydroxylamine enantiomer was not pursued not only for this reason but the intrinsic flaws suffered by the laccase:mediator system in the context of a deracemisation, the ultimate goal of the project. As detailed in Chapter 5, a key issue is the fact that the oxidising mediator and any reducing agent will both be in the bulk solution and therefore interact with each other in a redox cycle.

2.5 Laccase conclusions

Consideration of the inherent limitations of the laccase:mediator system with regard to a deracemisation system led to a change of focus in terms of the stereoselective oxidation portion of the research. Whilst the laccase:mediator system was ultimately unsuited to a role in a deracemisation system, a novel oxidation system for hydroxylamines was defined. This system was demonstrated to be reproducible,

effective and, in the use of atmospheric oxygen as the bulk oxidant, environmentally friendly.

3 Monoamine oxidase

In an attempt to identity a highly stereoselective hydroxylamine oxidation catalyst attention turned to the amine oxidase class of enzymes. Work within the laboratory had identified an amine oxidase with highly stereoselective oxidation activity for secondary amines.[63] The concept was to test, and if necessary, modify and optimise this enzyme in an attempt to identify a viable stereoselective hydroxylamine oxidation catalyst.

3.1 Amine oxidases

Amine oxidases catalyse the oxidation of amines to the corresponding ketones or aldehyde, ammonia and hydrogen peroxide. An imine intermediate is produced (Figure 35). As is readily apparent this reaction can be regarded as being analogous to the desired reaction with a hydroxylamine, the oxime being the achiral intermediate produced as opposed to an imine.

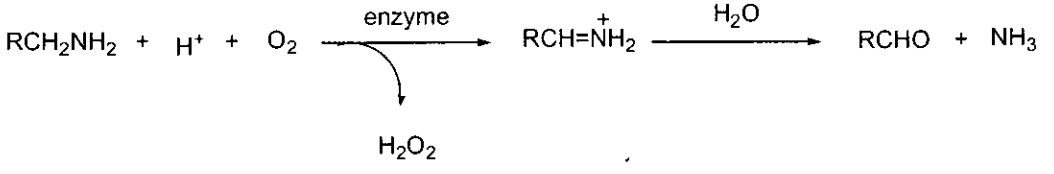


Figure 35: Amine oxidase general wild-type activity.

Amine oxidases may be divided into two general groups based on the cofactor they possess. Type I amine oxidases are copper dependent and use 2,4,5-trihydroxyphenylalanine quinine (TPQ) as a cofactor. The type II amine oxidases, of which monoamine oxidases (MAO) are the most abundant, use flavin as the co-factor. There is a significant body of literature associated with MAO enzymes[95] due to the fact that they are implicated in human oxidative deamination pathways, especially of certain neurotransmitters. This has led to great interest in the MAO enzymes and inhibitors from a medical treatment standpoint.[96,97] MAO inhibitors have been used clinically both for the treatment of Parkinson's disease[98] and as

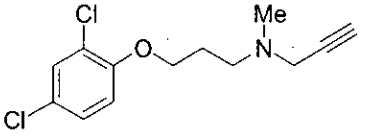
antidepressants[99], and continue to be important targets for pharmaceutical research, development and sales.[100]

Due to the collective experience gained within the Turner Group[101,102] the research into hydroxylamine activity and optimisation was concentrated solely on a flavin-containing monooxidase.

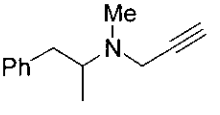
3.2 Flavin containing amine oxidases

The utility of MAO inhibitors, and the reason for much of the research into them, is based on the fact that MAO regulates neurotransmitter levels. This fact was first discovered when tuberculosis patients dosed with isoniazid showed improvements in mood. As this was an unforeseen, albeit fortunate, side-effect further research was initiated that discovered isoniazid was inhibiting MAO.[103]

Subsequent work has investigated the nature of MAO enzymes in humans. Two different MAO enzymes are present in humans, type A (MAO-A) and type B (MAO-B). A number of experimental results lead to the unravelling of this fact. Key to the understanding that there are two different MAO enzymes in humans was the observation that MAO-A is inhibited by clorgyline **66** whilst MAO-B was inhibited by deprenyl **67**.[104] Conclusive evidence of the presence of two different MAO enzymes was obtained from cDNA cloning experiments, which allowed the isolation of MAO-A and MAO-B from human liver.[105]



66
clorgyine



67
deprenyl

Figure 36: MAO-A inhibitors.

The MAO-A and -B enzymes exhibit high amino acid sequence identity and the subunit molecular weights differ by only 1.7 kDa, 59.7 kDa and 58 kDa respectively. Both enzymes contained the pentapeptide Ser-Gly-Gly-Cys-Tyr.[106] This is known to be critical in FAD binding; it was found that the FAD is bound by Cys-406 in MAO-A and Cys-397 in MAO-B. Research on MAO-A and MAO-B has established that they are integral proteins of the outer membrane of mitochondria and this fact has led to difficulty in preparing soluble, functional membrane proteins.

The three-dimensional structure of MAO-B has been solved.[107] This success has also led to the solving of the MAO-A structure through a single crystal isomorphous replacement technique.[108] The crystal structure showed the enzyme is dimeric. The proposed structure is postulated to be the quaternary structure *in vivo*, as the dimer was observed in two different geometries, triclinic and orthorhombic.

An irreversible inhibitor was successfully co-crystallised in the active site of MAO-B and this has provided an insight into the active site location and geometry.[109] The inhibitor was an analogue of the MAO-B inhibitor **67**, and was bound covalently to the *N*5 of flavin on the *re*-face in a solvent inaccessible position. The binding area is a cavity of volume 420Å^3 and is a hydrophobic environment due to aromatic and aliphatic amino acids that line it. The recognition site for the substrate amino group is an aromatic cage formed by two tyrosine residues, 398 and 435. The tyrosine aromatic rings are seen to be rotated slightly towards the flavin. These aromatic rings are clearly important to the function as it has been shown that mutating one or other of the tyrosines to serine resulted in inactivation.[110] On the other hand mutations to phenylalanine retained activity with an increase in K_M.

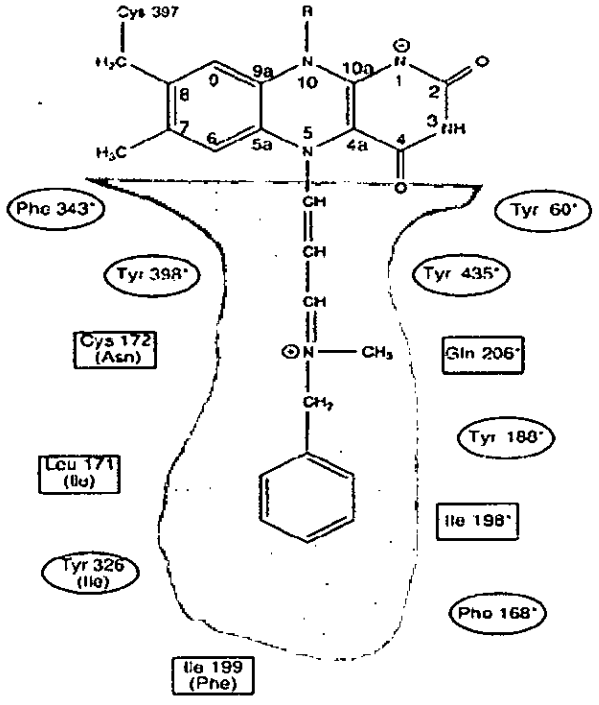


Figure 37: Representation of pargyline suicide inhibitor covalently attached to FAD and bound in the active site of MAO-B.[109] MAO-B active site residues are shown in black (aromatic side chains in ellipsoidal frame and others in rectangular boxes).

A separate cavity, named the entrance cavity, is present in MAO-B, between the protein's exterior surface and the substrate binding cavity. This is lined with hydrophobic and aromatic residues, much as the substrate binding cavity is. Access to the entrance cavity from the outer surface of the protein is postulated to be controlled by a loop of 13 amino acids. The suggestion is that movement of this loop allows for substrate binding in the entrance cavity. Subsequently a transient movement of the residues which separates the entrance and substrate cavities occurs. This second step allows diffusion of the substrate into the substrate cavity.

A further interesting attribute of the MAO-B crystal structures is that the normally planar FAD ring is bent around the *N*5-*N*10 axis. Additionally two aromatic amino acids form an aromatic sandwich about 8Å apart, perpendicular in orientation to FAD on the *re*-side.[107] Structural models of substrates bound in the active site of

MAO-B suggested that the aromatic sandwich and flavin combine in amino recognition of the substrate.

3.2.1 FAD oxidation mechanism

The overall catalytic cycle of amine oxidation in MAO has been proposed to occur in three steps (Figure 38). The first of these steps is the oxidation of the substrate. This occurs with concurrent reduction of FAD to $FADH_2$. This is followed by the deamination of the imine intermediate by water to produce the corresponding ketone or aldehyde and ammonia. This step is not enzyme catalysed, it occurs spontaneously in water. Lastly, the reoxidation of $FADH_2$ to FAD occurs. This is performed with molecular oxygen and releases hydrogen peroxide.

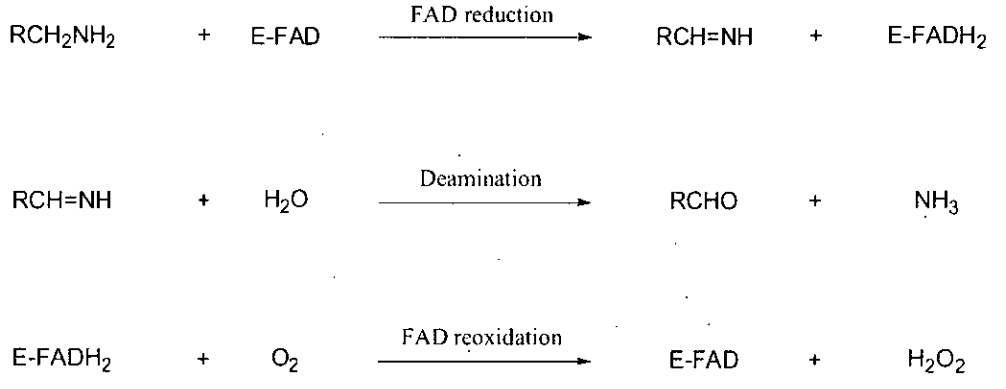


Figure 38: The steps in the catalytic cycle of amine oxidation catalysed by MAO.

The exact mechanism of FAD reduction and amine oxidation has been the subject of debate and three pathways have been proposed: a single electron transfer (SET) pathway[111], a nucleophilic pathway[112] and a direct hydrogen atom transfer (HAT) pathway.[113] Conflicting evidence has been presented but the SET pathway has been the generally accepted version for MAO catalysed α-carbon oxidation of amines and *N*-cyclopropylamines inactivators have been used to probe this.[114] It was demonstrated that an amine radical cation is formed which is proposed to undergo rapid ring opening to form a highly reactive primary carbon centred radical. The

carbon centred radical is thought to be responsible for inactivation of the enzyme and this was probed by the use of ^{14}C labelled *N*-cyclopropylamine. This was demonstrated to react with MAO in a 1:1 stoichiometric ratio at the flavin or at an active site cysteine residue.[115]

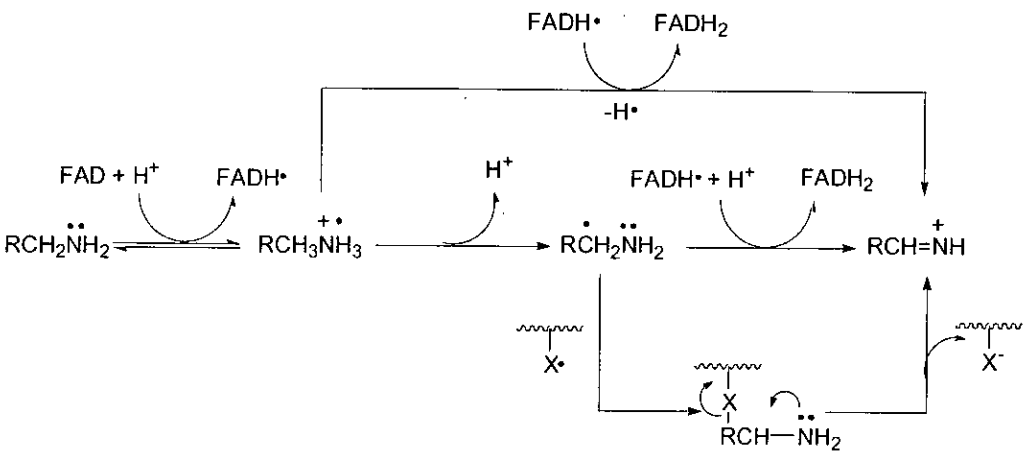


Figure 39: The possible SET mechanisms for amine oxidation catalysed by a FAD dependent MAO.

Evidence for the existence of radical species during the amine oxidation step has been provided by EPR experiments, with clear evidence for a tyrosyl radical observed.[116] This is believed to be located on a tyrosine residue close to the active site and the tyrosyl radical is thought to be in equilibrium with the flavosemiquinoine of FAD.

More recently support for the polar nucleophilic mechanism has been forthcoming.[117] Support for this mechanism has been based on structural data which indicates that no amino acid residues that could act as acids or bases are in close proximity to the active site. Further computational studies have supported this contention.[118]

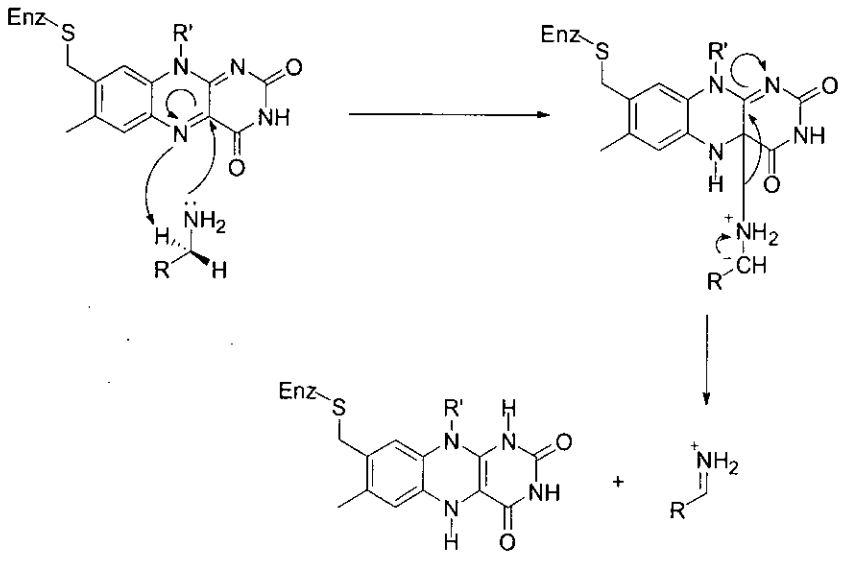


Figure 40: Nucleophilic (polar) mechanism.

Using stopped flow spectroscopy experiments it has been shown that the substrate is influential in the $FADH_2$ reoxidation step.[115] A binary or ternary complex has been shown to be present in the catalytic cycle. This complex can consist of reduced enzyme, oxygen and product or reduced enzyme, oxygen and substrate.[119]

The release of the intermediate imine from the enzyme complex, as opposed to hydrolysis within the active site, has been demonstrated.[120] Experiments with *N*-methylbenzylamine confirmed the release of the imine, the amine was oxidised in 1% v/v water in benzene and oxidised products were examined by GC. The release of an imine rather than the hydrolysed product is of course essential if a MAO enzyme is to be employed in a deracemisation system. The extensive work in the Turner group with amine deracemisation systems clearly confirms the generality of the imine release.

3.3 MAO-N

A number of amine oxidases have been discovered in microorganisms[121] and a type II amine oxidase enzyme (MAO-N) which oxidises mono-amine substrates compared to the previously reported di- or polyamines has been identified.[122] This enzyme was

discovered from butylamine-induced cultures of *Aspergillus niger* and further research has allowed purified recombinant MAO-N expressed in *E. coli* to be obtained.[123] This allows for relatively simple expression of large amounts of the enzyme and the substrate specificity indicates that the MAO-N enzyme is useful in transformations of small molecule, chiral amines.[61] The human monoamine oxidase MAO-B is the most closely related enzyme to MAO-N for which a three-dimensional structure is available. There is 24% sequence identity and 43% sequence similarity between the two amino acid sequences. Previously a sequence alignment of MAO-B and MAO-N had been carried out by M. Alexeeva[102] and this has provided some insight into some of the key residues in the MAO-N enzyme.

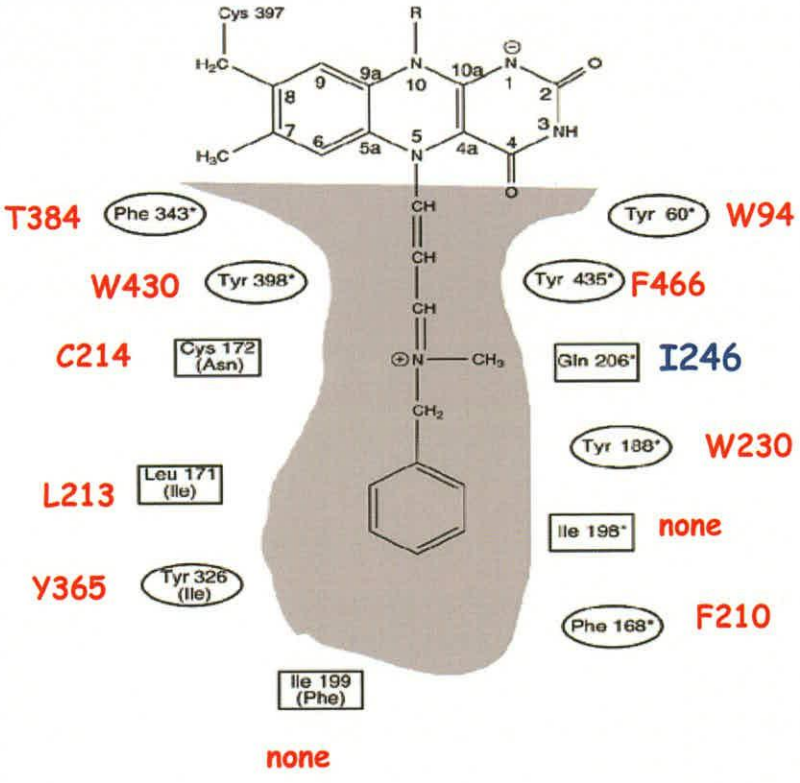


Figure 41: Representation of pargyline suicide inhibitor covalently attached to FAD and bound in the active site of MAO-B.[109] MAO-B active site residues are shown in black (aromatic side chains in ellipsoidal frame and others in rectangular boxes). Aligned amino acids in MAO-N are shown in red and the 246 aligned residue is shown in blue.[102]

3.3.1 Homology model of MAO-N

This alignment data has led to investigations into the active site of the MAO-N enzyme and confirmation that the 246 residue is at the heart of the active site. The use of an alignment strategy between MAO-B and MAO-N has been taken a step further. A three dimensional homology model of MAO-N has been developed using the three dimensional structure of MAO-B of which the crystal structure has been solved. The related MAO-N sequence was overlaid over this model and the potential energy of the system minimised using the CHARMM (Chemistry at HARvard Molecular Mechanics) force field system.[124]

As a result of the previous work in the group a good deal of information is available regarding the MAO-N variants and the handling of them. A number of researchers, within the Turner group and industrial partners, have studied the MAO-N enzyme and undertaken significant development work on it. Programs of research have been focused on fully characterising the enzyme, improving the expression system, evolving novel activities[125] and probing the effects of residue changes. In addition, using the enzyme as a stereoselective catalyst in deracemisation systems has been achieved for a variety of structurally diverse amines.[63] This presents a good basis to commence investigation of the enzymes in terms of their ability to accept hydroxylamines as substrates and utility in a hydroxylamine deracemisation. The research program described herein has concentrated on expanding the utility of the MAO-N enzyme by employing it in deracemisations of hydroxylamines and hydroxylamine derivatives.

3.4 MAO-N Hydroxylamine Results and Discussion

Previous work by other researchers had resulted in a number of MAO-N variants being available in the laboratory (Table 6). These variants had been created in a number of research programs designed to produce variants with higher activity towards chiral amines and had also resulted in variants with broader substrate specificity. The enzyme variants and the genes encoding for them were generously made available for this research program.

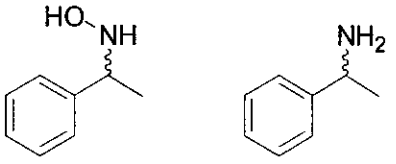
Enzyme name	Encoding gene name	Mutations from wild-type	Source
MAO-Nwt	*mao-Nwt*	n/a	M.Alexeva
MAO-N1	*mao-N1*	N336S M348K	M.Alexeva
MAO-N2	*mao-N2*	*mao-N1* + I246M	R. Carr
MAO-N5I	*mao-N5I*	*mao-N2* + T384S D385N	R. Carr
MAO-N5D	*mao-N5D*	*mao-N2* + T384N D385S	R. Carr

Table 6: MAO-N variants available.

The genes were inserted into a pET16b expression vector and an expression system in *E. coli* BL21 (DE3) was in place. The pET16b vector inserts a 6 x His tag at the N terminus end of the protein, resulting in the ability to purify the MAO-N enzymes using Ni chelation chromatography.

3.4.1 Hydroxylamine activity investigation

The stereoselective oxidation of hydroxylamine **35** was selected to be the reaction that would be attempted initially. There were a number of reasons for this choice; **35** was easily synthesised from acetophenone and was easily obtained in high purity. The key rationale was that this substrate was the hydroxylamine analogue of α-methylbenzylamine **24**. The MAO-N enzymes had been demonstrated to turnover this substrate and it was felt that the structural similarity meant that this substrate was a good foundation to base the research upon.



35 24

Figure 42: Initial test substrates for MAO work.

A programme was envisaged in which the ability of the enzyme to turnover the substrate and the associated rates, enzyme stability and necessary purification would be characterised and optimised. The group of available MAO-N enzymes was rationalised to reduce the workload and ensure optimum use of resources. MAO-N5D, MAO-N1 and MAO-Nwt were selected as the three enzymes to take forward into this investigation. MAO-N5D had been demonstrated to be highly active towards secondary amines. MAO-N1 had been evolved specifically to have high activity for α-methylbenzylamine. MAO-Nwt was tested as all the other variants had been engineered to have improved activity towards secondary amines. There was no guarantee that this engineering process had not caused the mutants to become inactive towards hydroxylamines. It was therefore decided to test the wild-type with a view to conducting engineering experiments on this if necessary.

3.4.2 Screening for hydroxylamine activity

A plate-based colony screen for oxidation activity was employed to gain an overview of the activity of the variants towards the substrate. This process allowed for a rapid screening system as purification of the MAO-N enzyme was not required; colonies expressing the enzyme were screened directly. The output of the screen is colorimetric and thus analysis is performed by eye. The solid phase assay relies on an indirect screening approach in that the hydrogen peroxide by-product from the amine oxidation catalysed by MAO-N is used as a reagent in a second step. Horseradish

peroxidase (HRP) is used to catalyse the reaction of the hydrogen peroxide with another substrate and the product of this reaction is coloured (Figure 43).

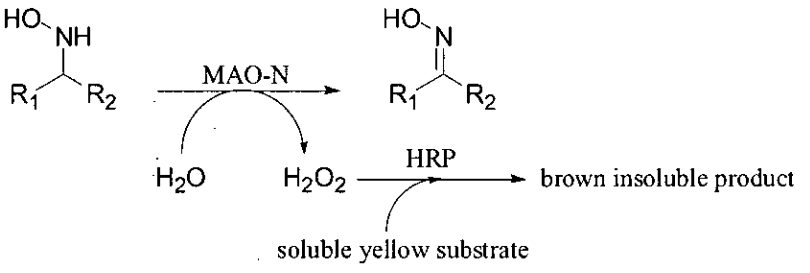


Figure 43: Plate-based screen concept.

The 3,3'-diaminobenzidine (DAB) reagent **68** was found to be optimum for screening MAO-N variant activity against amines and was employed for this screen also, Figure 44 illustrates the reaction.

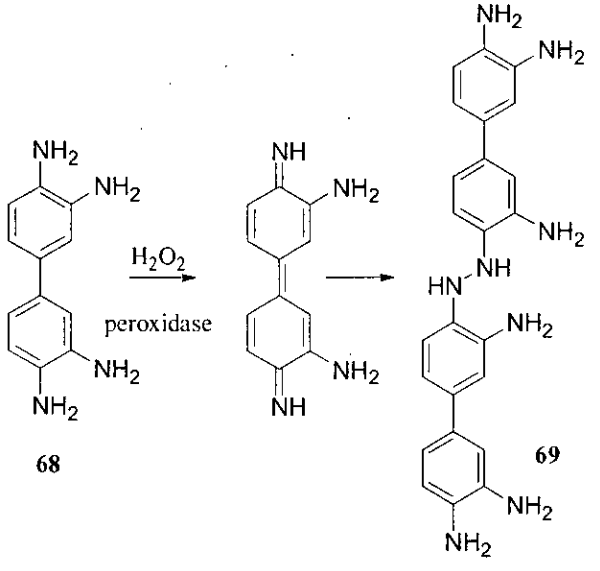


Figure 44: Dye formation reaction in plate-based screen.

The polymeric final product **69** is insoluble in the assay medium and thus does not diffuse from the site of production. This is crucial so that individual colonies producing the colour can be identified. The DAB and its product do not inhibit the

action of MAO-N nor does it react with the amine substrates. The DAB screen does suffer form a general background oxidation which results in an orange to brown darkening of the whole plate over an extended period of days (Figure 44). Colonies that darken extremely slowly and are obscured by this background may be regarded as having so little activity as to be classifiable as negative anyway.

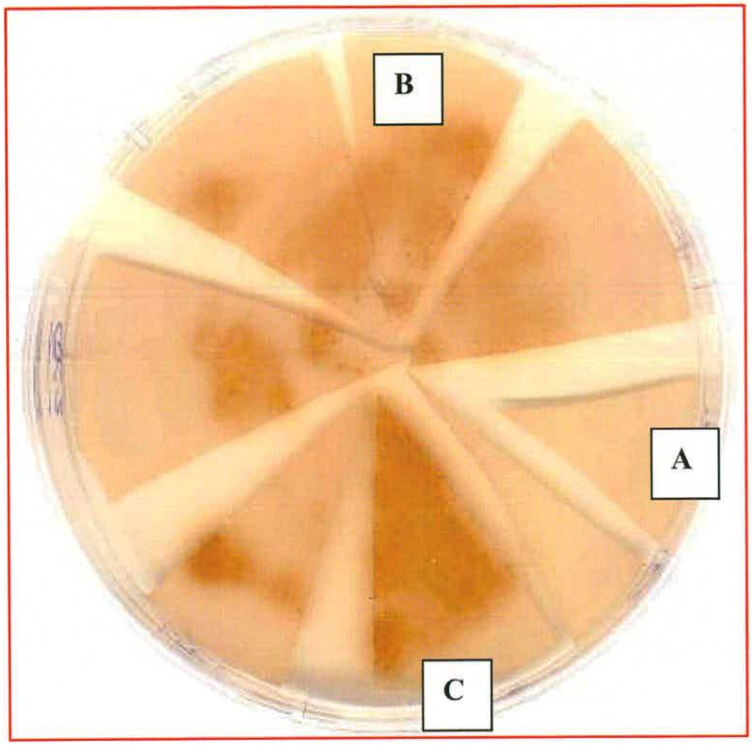


Figure 45: Example of solid-phase assay plate darkening. Sector A) has inactive colonies, B) moderately active colonies and C) active colonies present.

To test whether the DAB would be suitable a plate was produced containing only hydroxylamine **35** and the assay components. No colorimetric reaction occurred apart from the slow background darkening observed with the screen in all applications. Screening of MAO-N5D, MAO-N1 and MAO-Nwt expressing colonies was carried out. Negative control plates, with colonies transformed with pET16b plasmid without an insert and positive control plates, with α-methylbenzylamine **24** as the substrate were employed for comparison (Table 7).

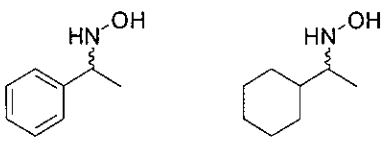
Variant	Substrate	Activity
MAO-N5D	**24**	High
MAO-N5D	**35**	None
MAO-N1	**24**	High
MAO-N1	**35**	None
MAO-Nwt	**24**	Very low
MAO-Nwt	**35**	None
pET16b control	**24**	None
pET16b control	**35**	None

Table 7: Plate-based screen results for hydroxylamine **35** activity screen.

It is apparent from Table 7 that no activity is observed for either MAO-N variant against hydroxylamine **35**. Two different courses of action were followed in an attempt to address this difficulty. Firstly, the use of a second, more sensitive screen was attempted. The rationale behind this was that very low levels of activity may have been missed due to the limitations of the plate-based screen. Previous work in the group had demonstrated that low level activity could be the foundation of a program of enzyme engineering which led to novel variants exhibiting high levels of desired activity. Thus identifying a variant with very low activity could be valuable. Secondly, a different hydroxylamine substrate was tested. The reason for this was that dismissing the ability of an enzyme to turnover a class of substrates on the basis of the failure of a single example would be premature.

A liquid-phase assay, performed in a 96-well microtitre plate was employed to re-assay the MAO-N variants for activity against hydroxylamine **35**. This assay employed the same concept as the solid-phase assay, in terms of producing a colorimetric product via HRP catalysed reaction with H_2O_2. The difference is that the substrate is soluble in the aqueous buffer and thus is suitable for a spectrophotometer based screen. The 96-well plate coupled with an UV/Vis spectrophotometer allow for time-point data collection and thus rate data to be produced. The sensitivity of the instrument allows for lower activities to be observed and recorded compared with the

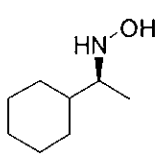
plate-based screen. This screen was used to test both hydroxylamine **35** and **46**. Hydroxylamine **46** is the analogue of the amine which was found to be most active in the work of Carr in the group previously. It was thus chosen as a second substrate to test, however no activity was observed.



rac-**35** *rac*-**46**

Figure 46: Hydroxylamines tested initial MAO screening.

At this juncture it appeared that the possibility that the MAO-N variants would display activity against hydroxylamine substrates had to be dismissed. In a final attempt to confirm this (*S*)-**35** was tested for activity. By analogy with all the amine substrates, this is the enantiomer that the MAO-N variants would be expected to be highly active towards. No activity was observed.



(*S*)-**35**

Figure 47: Hydroxylamine analogous to most active amine.

Two routes forward presented themselves. Firstly; to attempt to engineer a MAO-N variant that did display the desired properties. Secondly; to attempt to modify the substrates to some extent to allow them to be turned over by the MAO-N enzyme.

3.4.3 Inhibitory action of hydroxylamines

Before embarking on a process of enzyme engineering of a MAO-N variant in an attempt to access activity toward hydroxylamines the theoretical possibility of activity had to be established. A key consideration was whether MAO-N was

intrinsically unsuited to a role of hydroxylamine oxidation. A number of experiments established that it was.

To check whether hydroxylamines had an inhibitory effect upon MAO-N variants a simple experiment was performed. A plate-based screen of MAO-N1 expressing colonies against a mixture of 10mM α-methylbenzylamine **24** and 10mM 1-phenylethylhydroxylamine **35** was performed. No activity was observed. This confirmed that the hydroxylamine had an inhibitory effect. The experiment was repeated in a liquid-phase assay and the result confirmed. The mechanism of inhibition was not fully established however some information was elucidated.

The inhibition appeared to be irreversible. MAO-N1 enzyme solution was incubated with 10mM hydroxylamine **35** and then purified via Ni chelation to the His tag. The purified enzyme was then incubated with 10mM α-methylbenzylamine **24** and no activity observed. A variation of this reaction was performed in which MAO-N1, purified and subsequently supported on Eupergit resin, was incubated with 10 mM hydroxylamine **35**. The resin was filtered off, washed with copious amounts of pH 7.4 phosphate buffer and then tested for activity against α-methylbenzylamine. No activity was observed.

A number of possibilities arise for the mechanism of inhibition including covalently binding in the active site pocket, possibly to the *N*5 of flavin, as with the inhibitor pargyline (Figure 41). Analysis of the flavin post incubation with hydroxylamine was carried out. The flavin UV curves were found to be very similar with no discernable differences (Figure 48). LC-MS analysis of the flavin solution was inconclusive with a wide distribution of poorly resolved peaks.

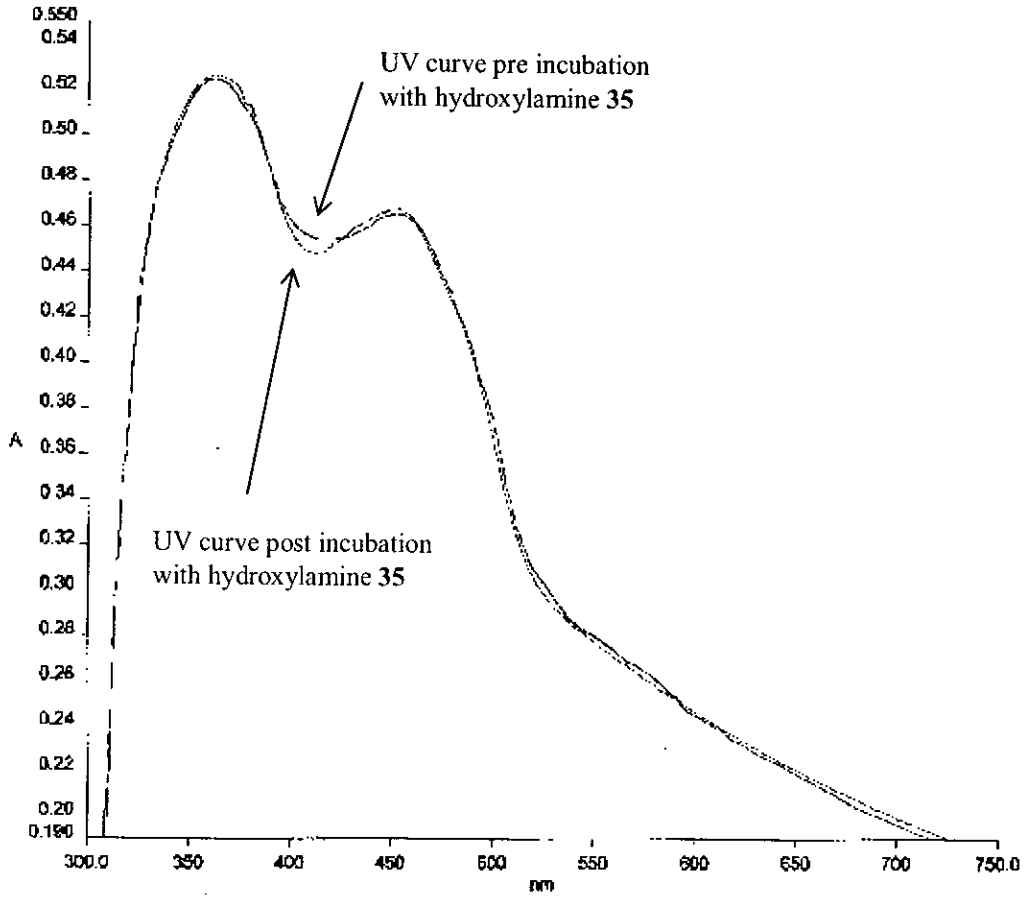


Figure 48: UV curves of MAO-N per- and post- incubation with hydroxylamine **35**.

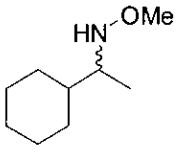
As a result of the experiments described above it was decided to abandon attempts to engineer a MAO-N variant that exhibited activity towards a hydroxylamine substrate. The results above, coupled with literature data uncovered indicating that hydroxylamine acts as a MAO inhibitor[126], suggested that it would be futile to pursue this line of investigation.

3.5 MAO-N Hydroxylamine ether Results and Discussion

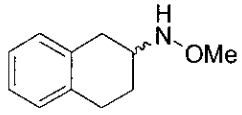
As detailed above, a second approach to be problems described would be to attempt to modify the substrates and assess whether they were viable as MAO-N substrates. To this end it was decided to investigate whether 'capping' the hydroxyl functionality would alleviate the problems found thus far. Two hydroxylamine

ethers, **47** and **48**, were thus synthesised[127] and assayed against MAO-N5D colonies (Table 8). It was decided to concentrate on a single MAO-N variant, MAO-N5D for these experiments.

3.5.1 Screening for hydroxylamine ether activity



47



48

Figure 49: Hydroxylamine ether substrates screened.

Variant	Substrate	Activity
MAO-N5D	**24**	Very high
MAO-N5D	**47**	High
MAO-N5D	**48**	High

Table 8: results of plate-based screen of hydroxylamine ether substrates.

As Table 8 illustrates the concept of capping the hydroxyl functionality appears to have successful to an extent. It was decided to further probe the possibility of hydroxylamine ether turnover by using a liquid phase assay screen for greater sensitivity and accuracy and to broaden the panel of substrates to be tested (Figure 50).

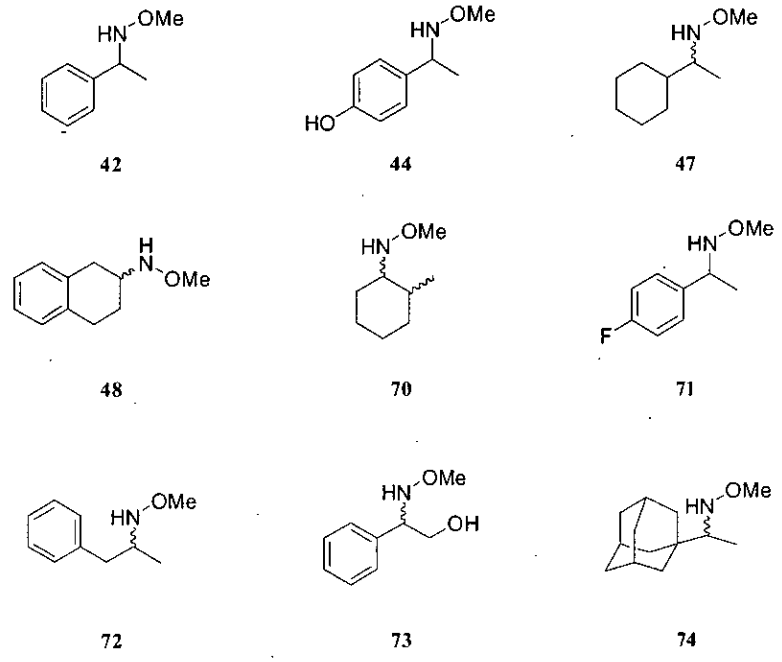


Figure 50: Expanded panel of hydroxylamine ether substrates for screening.

Assessment of the rate data produced was based on relative rates referenced to a standard substrate, α-methylbenzylamine **24** (Table 9). This was to allow comparability and standardisation between any subsequent studies. The various CFE preparations used throughout the project would be very unlikely to contain the same amount of enzyme; however relative rates will always be comparable.

Substrate / 10mM	MAO-5D activity
24	100[1]
48	17.4
47	15.6
70	13.1
74	3.5
71	3.4
42	1.2
72	0.6
44	<0.5
73	<0.5

[1] Standardised to 100%

Table 9: Relative rate of substrate turnover using MAO-N5D.

A number of substrates are turned-over by MAO-N5D at rates which are moderate but rival the rates observed for a number of amines. The substrates tested were analogous to those amines which were most active. This means that there is a different profile to the structure of active amine as hydroxylamine ethers. Hydroxylamine **47** was selected to be investigated to a fuller extent and Michealis-Menten kinetic calculations were performed using GraphPad Prism 4 software.[128] This was to gain accurate kinetic data for a hydroxylamine substrate. Once again α-methylbenzylamine **24** was used as a comparison (Table 10).

Substrate **24**	V_{Max} (µmol/min/mg)	k_{cat} (min^{-1})	K_M (mM)	Catalytic efficiency k_{cat} / K_m (min^{-1} mM^{-1})
MAO-5D Published data[101]	$0.81\pm1\times10^{-2}$	45.3±0.5	3.55±0.12	12.76
MAO-5D	$0.78\pm8\times10^{-2}$	43.4±0.4	3.52±0.19	12.33
Substrate **47**				
MAO-5D	$0.18\pm3\times10^{-2}$	10.2±0.8	5.66±0.24	1.80

Table 10: Michealis-Menten kinetic data for hydroxylamine ether **47**.

The data shows that the hydroxylamine ether **47** is a reasonable substrate for the MAO-N5D, when compared with a substrate known to be suitable for deracemisation experiments.

The kinetic data demonstrates that the MAO-5D enzyme is a poorer system for the oxidation of the model hydroxylamine ether than for the model amine. The K_M, the concentration of substrate at half V_{max}, has risen. This indicates a lower affinity for the substrate from the enzyme. This change in K_M means that the reaction will decelerate sooner in the case of the hydroxylamine ether **47** than the amine. The lowering of the k_{cat} indicates that the turnover is slower compared with the amine substrate. When these two factors are combined it is apparent that the catalytic efficiency of the enzyme towards the hydroxylamine ether model substrate is an order of magnitude lower than for the model amine. This data confirms the un-quantified observations that the assay systems provided as described above. Although the enzyme was demonstrably slower at affecting the oxidation of the hydroxylamine ether model compound than the amines tested it retained enough activity to still be potentially viable as a deracemisation catalyst.

3.5.2 Kinetic resolution of a hydroxylamine ether

Attention now turned to whether the MAO-N5D could be a viable catalyst for the deracemisation of hydroxylamine ethers. This is a very different question from whether it turns over the substrate. Critically, is the oxidation stereoselective? If not then the novel MAO-N oxidation discovered is of little value. In the case of the oxidation of hydroxylamines ethers to oximes a further detail must be considered which is not relevant for the amine to imine or hydroxylamine to oxime analogues. The oxime product may exist in both *cis* and *trans* geometric isomers, therefore investigation of the stereoselectivity of the oxidation must be coupled with investigation of geometry of the product.

A kinetic resolution of hydroxylamine ether **47** was performed using the MAO-N5D enzyme variant. This experiment was designed to answer a number of questions. Firstly, was the MAO-N5D enzyme, in a whole-cell system, a viable catalyst for a gram scale resolution of *rac*-**45**? Secondly, was the reaction high yielding and did it produce highly an enantioenriched product? Thirdly, what enantiomer of **47** remained at the completion of the reaction? Finally, what geometric isomer of the oxime product **57** was formed?

The resolution was performed on 1.2g scale and proceeded smoothly overnight (Figure 51). Gratifyingly the use of a whole-cell preparation was found to be effective with little of the work-up difficulties that can occur. This allowed for a simple preparative procedure to be used, with no enzyme purification step required. The two products of the resolution were isolated by column chromatography and analysed.

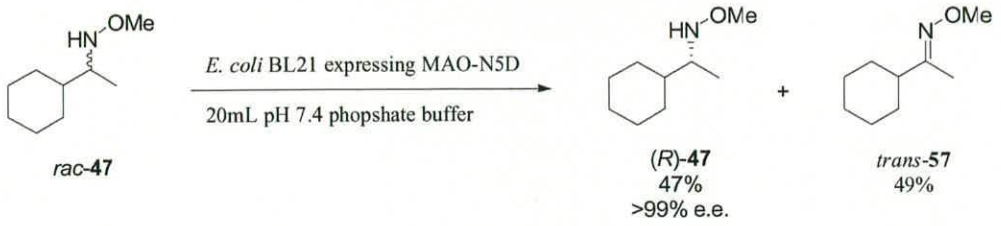


Figure 51: Kinetic resolution of *N*-methoxycyclohexylethylamine **47**.

The geometry of the oxime was determined by ^{1}H NMR NOSEY spectroscopy and found to be *trans*. A NOE interaction between the OMe and Me protons was observed. This was confirmed by GC of authentic *cis*, *trans* and reaction samples (Figure 52).

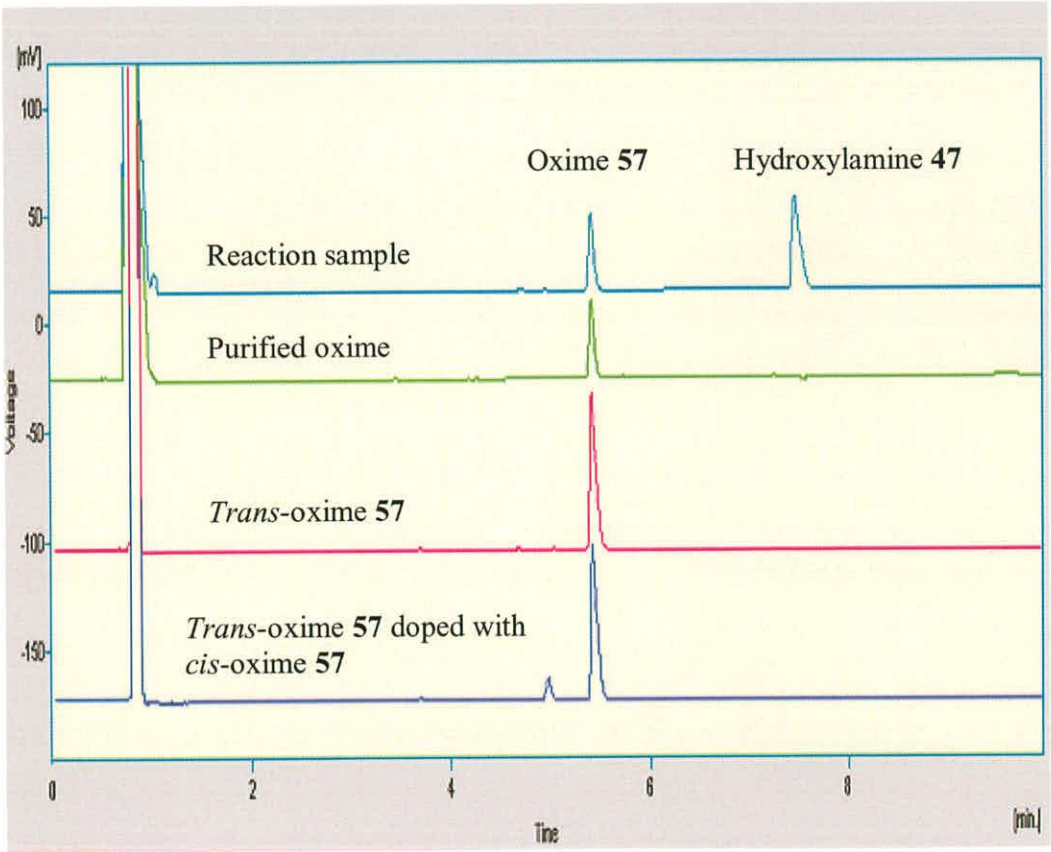


Figure 52: GC confirmation of oxime **57** geometry.

The remaining enantiomer of hydroxylamine ether **47** was found to be (*R*)-configuration. This was determined by GC analysis of a derivative. The remaining

hydroxylamine ether **47** was hydrogenated with H_2 gas and Pd/C catalyst overnight. The resulting amine was derivatised with triflic anhydride and the resulting mono-triflate analysed by chiral GC. Comparison with an authentic sample indicated that it was (*R*)-configuration (Figure 53). This is as expected as it is in agreement with the fact the MAO-N is known to oxidise amines in a highly (*S*)-selective manner.

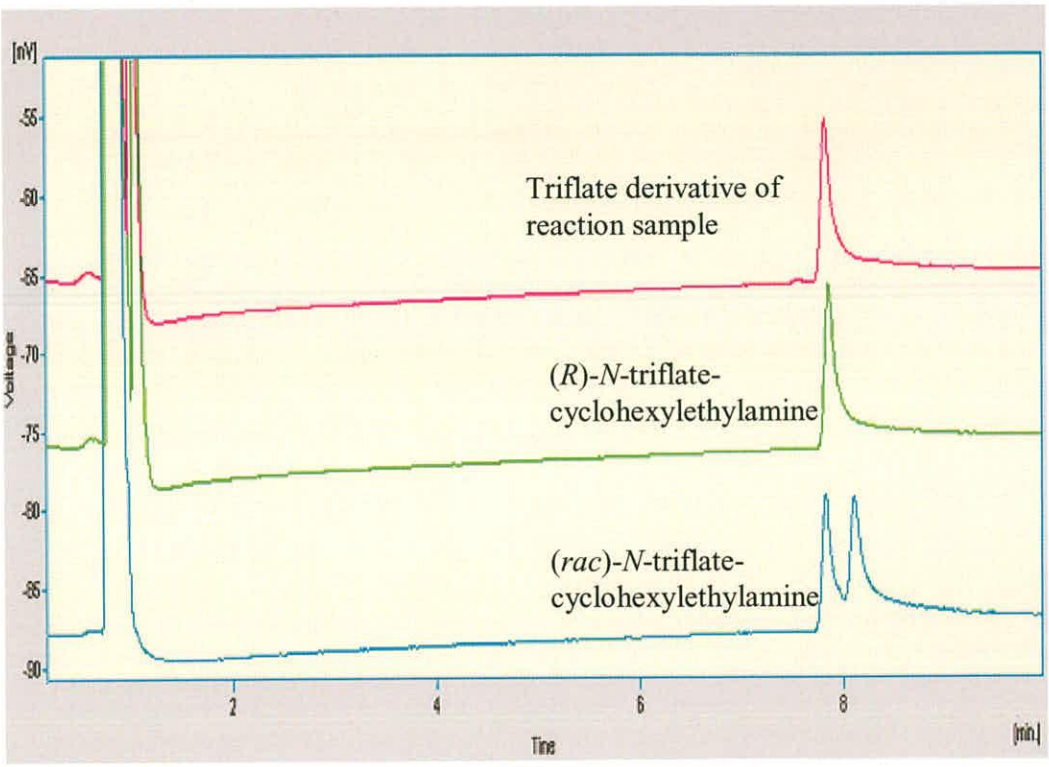


Figure 53: Chiral GC analysis of hydroxylamine ether **47** enantiopurity.

The reaction detailed above represents a novel biotransformation of a hydroxylamine ether. The use of a MAO enzyme to transform a non-amine substrate is an interesting development, expanding the utility of the MAO-N enzyme. The resulting chiral product is produced in excellent e.e. and the oxime product is produced as a single geometric isomer, both in high yield.

3.5.3 Molecular modelling of active species in MAO-N

With the information regarding the geometry of the oxime product in hand, an investigation of the active site of the enzyme was conducted using a 3D computer homology model.[129] The (*S*)-hydroxylamine substrate was docked into the active site and an energy minimisation protocol applied (Figure 54). The basis for the modelling experiments is the three dimensional homology model of MAO-N described in section 3.3.1. Into the active site of this model was docked the substrate **47** and the resulting active site configuration is presented.

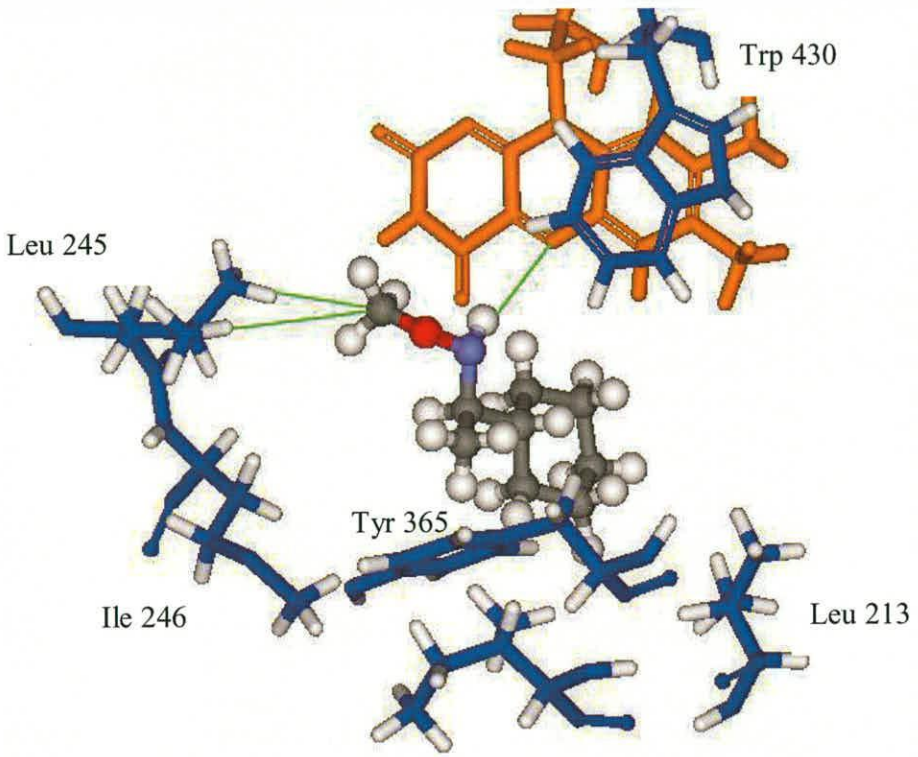


Figure 54: Hydroxylamine ether (*S*)-**47** docked in MAO-N active site model. Green lines indicate closest contacts.

The illustration of the active site indicates a number of key features. The nitrogen atom of the substrate is located close in space to the *N*5 of the flavin, with the C-N bond aligned perpendicular to the flavin ring plane. This is a result which closely

mimics that observed for similar modelling experiments performed with amine substrates, without having been fixed as part of the model parameters.

The model indicates that a void is available in the active site into which the methoxy ether moiety may be located. Clearly the need to position this subsituent is a factor that differs completely from the experiments with amine substrates. Residue 245 appears to be close contacting to the methoxy methyl in this model. Whilst a model derived at some length from a solved crystal structure clearly has limitations in terms of accuracy, it is pleasing to note that the substrate appears to fit the active site and be located in a fashion analogous to amine substrates modelled previously. Additionally, attempts to successfully dock the (*R*) analogue were unsuccessful with major steric hindrances being displayed.

Turning to the configuration of the oxime products, further molecular modelling was undertaken. This was in order to try and gain an insight into whether the oxime product configuration (*cis* or *trans*) was likely to be determined by the active site geometry. It was decided that the configuration of the active site was to be maintained from the previous experiment and the two possible oxime substrates docked separately into this active site. Clearly it is possible that the active site geometry alters as the oxidation reaction occurs, however it was felt that an indication as to whether the geometry of the active site would be crucial could be achieved by simply docking each oxime into the geometry derived from the previous experiments. Figures 55 and 56 illustrate the results of docking the two oxime geometries into the active site model.

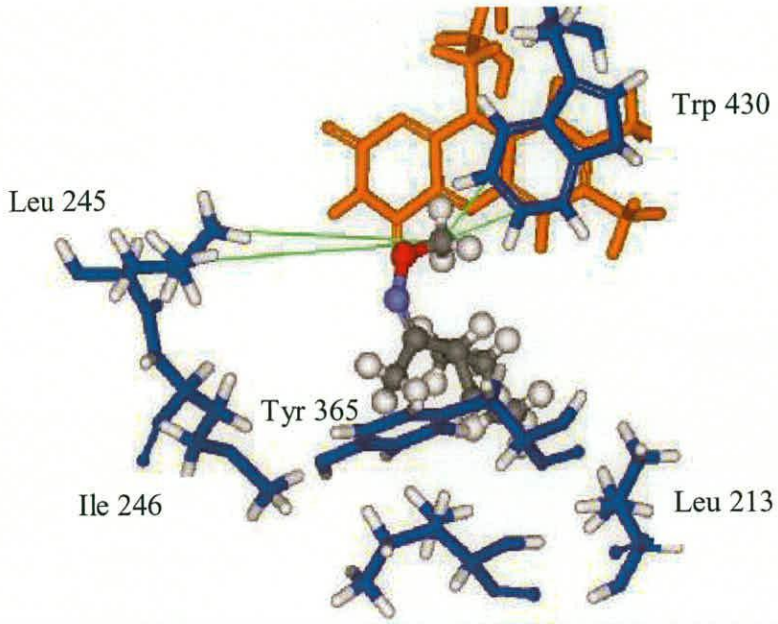


Figure 55: *cis*-**57** oxime docked into active site model. Green lines indicate closest contacts.

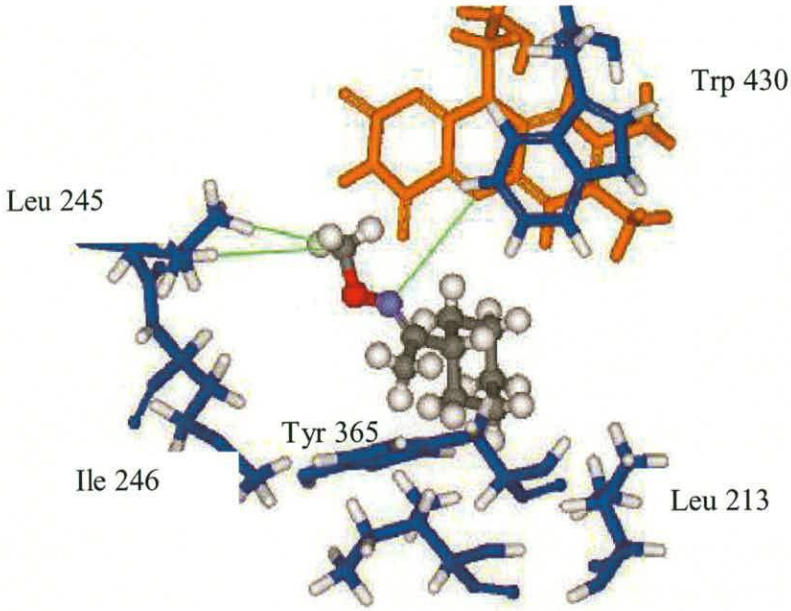


Figure 56: *trans*-**57** oxime docked into active site model. Green lines indicate closest contacts.

As can readily be seen, the production of solely *trans* product can understood with respect to the active site geometry. The *cis* product must be heavily disfavoured for a

number of reasons. The methoxy methyl closely contacts the W430 residue and is sterically hindered due to this. Additionally the *cis* configuration leads to the location of the methoxy methyl group directly between the *N*5 of flavin and the substituent nitrogen atom undergoing oxidation. This would seem unlikely to occur as hydrogen atom transfer is postulated to occur between these sites (see Figure 40).

Conversely, the *trans* product fits the active site in a fashion that allows for unimpeded access between the flavin and substrate reactive sites and the position of the non-reactive portions of the molecule closely match those of the hydroxylamine ether substrate before oxidation. Gratifyingly, the data from the molecular modelling experiments is in close agreement with the data derived from laboratory experiments.

3.6 MAO-N Conclusion

In terms of establishing a deracemisation system for hydroxylamine the MAO-N variants are not viable as stereoselective oxidation catalysts. However, by capping the hydroxyl functionality as a methyl ether activity has been demonstrated. This activity has been shown to be of such a level that the construction of a deracemisation system is possible, as the activity is within an order of magnitude of amine substrates that have been deracemised previously. Crucially, the high enantioselectivity of the oxidation has been demonstrated; a prerequisite to a successful deracemisation. The whole-cell system has been shown to be effective and this allows for a simple experimental procedure. Common problems associated with whole-cell systems such as work-up difficulties have not been observed to any great extent. The experimental program was now taken forward in two different directions; firstly, improving the biocatalyst through enzyme engineering and secondly, development of a complementary reduction system to allow for a deracemisation.

4 Biocatalyst modification

With a naturally derived biocatalyst in hand it is possible to perform a wide variety of synthetic reactions successfully, however, industrially relevant applications for biocatalysts often involve substrates or conditions which do not correspond to the wild-type environment of the biocatalyst. This may be because the specific substrate is not encountered by the wild-type enzyme, the substrate is not turned over by the wild-type enzyme or the reaction conditions do not correspond to the conditions that the wild-type enzyme operates in. The problems identified above can lead to difficulties in employing a biocatalyst. Modification of the biocatalyst may be attempted to overcome the factors which prevent successful exploitation of a biocatalyst system.

Broadly, there are two different techniques to produce an effective biocatalyst from one which is ineffective. One may leave the active enzymatic components of the biocatalyst unchanged but modify the method by which they are delivered to the bulk reaction medium. Secondly, one may modify the enzyme(s).

4.1 Modifying biocatalyst delivery form

Modifying the delivery of the enzyme to the bulk reaction solution may solve difficulties associated with the use of the purified enzyme as the biocatalyst. The use of purified enzyme is, of course, perfectly possible. The positive factors associated with this method include the fact that the catalyst is known to be pure, is fully characterised and should be consistent batch-to-batch. The amount of catalyst added can be known to a high degree of accuracy and downstream purification is not normally difficult. There are a number of significant disadvantages with employing purified enzymes in a reaction. When considering a simple transformation, the limiting factor in terms of time and the highest costs are often associated with enzyme purification. If a biocatalysis system comprises more than one enzyme in the same pot then this problem is only exacerbated. The stability of many enzymes can be compromised when they are purified, and are thus no longer exposed to the

mixture of biomolecules present in the whole-cell. This may manifest itself in degradation of the enzyme or a lack of solubility in standard solution media.

Rather than purified protein being employed, the enzyme may be delivered within the wild-type host or by addition of the organism which was used as the expression host – a whole-cell system. This has been reported as successful on numerous occasions.[130] As is often the case, the use of a whole-cell system is a balance of factors, positive and negative. A key benefit of using the whole-cell is the removal of the enzyme purification step. A further advantageous element that whole-cell systems may have is the ease at which the cells can be removed form the reaction medium due to there relatively large size and stability.[131] A clear advantage with the whole-cell system becomes evident if the biocatalysis system to be used consists of more than one enzyme. If all the required enzymes can be expressed in a single organism then the use of a whole-cell can be very efficient and impressive.[132] An additional benefit is that whole-cell systems are often active in non-conventional solvents such as organic solvents or ionic liquids.[133] This is in contrast to most purified enzymes. Clearly there are also a number of difficulties that can be found when employing a whole-cell system. Downstream processing can be lengthy and difficult. Whole-cells have a complex mixture of cellular products which can leach into the bulk solution and disrupt purification of the desired products.[134] Another factor is that whole-cell systems may contain alternative metabolic pathways which process the reaction substrate to undesired products. The fact that batches of whole-cells may differ also leads to the possibility of inconsistence across reaction batches.

The use of a solid support matrix of some nature to stabilise the enzyme is an alternative to whole-cells. Many commercialised enzyme systems involve the use of a solid-support for the active enzyme species. Purified or semi-purified enzymes may be placed on a solid support. This technique often increases the stability of the enzyme with respect to solvent and temperature. It also aids in the recovery and reuse of the enzyme as removal from the bulk solution is simplified. It has been found that the substrate profile of an enzyme can be altered by the use of a solid support[135]. A range of supports have been reported including hollow fibres[136], ceramics[137],

resins[138], glass[139], nanocomposites[140] and within monolithic reactors.[141] The concept of a solid supported enzyme system has been extended to other solids such as cross-linked enzyme crystals (CLECS)[142] and cross-linked enzyme aggregates (CLEAS).[143]

The modification of the method of delivery of the biocatalyst offers much scope for the development of a viable biocatalysts reaction. On occasions where the enzyme in question does not intrinsically exhibit the desired properties then modification of the enzyme itself may be either necessary or desirable.

4.2 Modifying the structure of the biocatalyst

The second area that may be explored is the modification of the enzyme itself, at the amino acid level. This may be achieved by making changes at the genetic level which, in turn, alter the enzyme. Alternatively direct chemical modification of the enzyme may be performed. At this level the substrate specificity, substrate turnover rates and the stability of the enzyme may all be affected. Whilst only experimentation will show whether the effects are positive or negative in terms of the desired characteristics of the enzyme, efficient experimental design can maximise the possible benefits to be realised for this fundamental alteration of the enzyme.

Direct chemical modification may be broadly divided into two, specific and non-specific side chain-modification. Non-specific modification refers to the covalent modification of the side-chain of a number of amino acid residues. The modification may be limited to a single amino acid type or residue but crucially there is no control as to the specific site residue that is modified. It may be one, some or all at varying positions on each enzyme molecule, thus a heterogeneous enzyme mixture is often formed. Non-specific side-chain modification has resulted in altered properties of a ribonuclease, glucose oxidase and lipases amongst others.[30] Specific site chemical modification exploits the fact that each amino acid residue is located in a different environment along the protein chain, thus with care a specific residue site may be covalently modified. A serine protease, fatty acid binding protein and papain have been modified in this more controlled fashion.[30]

The approach to modifying enzymes at the genetic level can be from two directions. One may modify residues in a random way and select for desired characteristics. Subsequently one may attempt to deconvolute the modification in residues that lead to the change in properties, if this knowledge is desired.[144] The term 'directed evolution' has been coined for an experimental program following this course. Alternatively one may modify known residues and observe the results, in a process known as 'rational design'.[145] It is of course possible to marry together elements from both techniques.[146] Both methods require the modification to be performed at the DNA level and thus having the enzyme gene in hand is pre-requisite for the use of either of the methods. The limitation of the vast majority of these genetically based techniques is that the only variation is between the 20 commonly naturally occurring amino acids. Elegant techniques have been described for the introduction of non-natural amino acids[147] but the pool of naturally occurring amino acids has provided enough diversity for nearly all successful protein engineering programs thus far.

4.2.1 Directed evolution

Directed evolution is a term used to describe the use of Darwinian evolutionary concepts to alter the properties of biocatalysts.[148] It comprises mutation of the gene encoding the potential enzyme and selection on the basis of the desired functional changes. The directed evolution experiment thus comprises the generation of a library of mutant genes and the subsequent screening and selection for desired characteristics. A number of iterations of this cycle may be followed. The two distinct parts of this evolutionary process are both under a degree of experimental control. Firstly, random mutagenesis of the encoding gene. The frequency of mutation, whilst not precisely controlled using the techniques which have become standard, may be calibrated. In turn the rate has been found to be relatively reproducible. This means that the researcher may be confident of the approximate rate of mutation.[149] The crucial factor allowing a high degree of control is that the selection pressure place upon the mutants is determined by the researcher. This

means that the researcher may direct the evolutionary course of the enzyme in question; hence the name 'directed evolution'. The great benefit of directed evolution with respect to rational design experiments is that no prior knowledge of either the 3-dimensional protein structure or the mode of catalytic action is required.

4.2.1.1 Generation of libraries of variants

The initial step of the directed evolution protocol is the generation of a library of variants. This library of genes will contain all the different variants to be exposed to the selection pressure in the second stage of the experiment. As the library of variants is constructed in a pseudo-random fashion a large number of variants are required. This is because many of the mutations will produce variants that are unlikely to survive the selection pressure. All variant genes that do not encode for proteins with improved properties being screened for, with respect to the wild-type, will be rejected at the screening stage. The random nature of the mutagenesis means that this is likely to be the majority of variants, as many mutations will not affect the catalytic properties of the enzyme or be deleterious. A number of techniques for constructing the libraries of variant genes are available including PCR based techniques[150], gene shuffling[151], chemical modification[152] and mutator-strain *E.coli*.[153]

Error prone polymerase chain reaction (epPCR) is a modification of the standard PCR procedure in which the fidelity of the PCR reaction is deliberately undermined.[150] The PCR conditions are tuned to ensure that during each replication cycle the products are not faithfully copied. The mutated PCR products are then cloned into an expression vector and the resulting library can be screened for the desired change in the function of the protein. epPCR employs the early generation of PCR DNA polymerases which have a significant propensity for misincorporation of nucleotides. These have been superseded by high fidelity enzymes for most PCR applications. Increasing the concentration of magnesium chloride or manganese chloride or varying the relative concentrations of the four dNTPs also leads to lower fidelity. The presence of Mn^{2+} along with an over representation of dGTP and dTTP in the amplification reaction leads to error rates of 1 to 10nt/kb.[154] The epPCR

technology has been refined to the extent that a number of commercial suppliers supply kits including detailed instructions to achieve defined mutagenesis rates. The ease by which mutations can be generated by epPCR and the control of the mutation rate makes this method powerful; however there are some disadvantages to the techniques.

The amplification nature of the PCR means that a mutation introduced early in the reaction will dominate. The mutated daughter molecules are amplified during the remaining PCR cycles and will be over represented in the final library compared with a mutation that is introduced in the final PCR cycle. This problem can be reduced by a combination of pooling multiple reactions and reducing the number of amplification cycles per reaction.

A further significant problem is that of mutational bias. epPCR is not random, in the true unbiased sense, and must be regarded as pseudo-random. Transitions (purine :purine or pyrimidine:pyrimidine) mutations are more common than transversion (purine:pyrimidine interchanges). Secondly, epPCR only introduces single nucleotide mutations which can lead to a bias in the amino acid that the mutant DNA encodes. The degeneracy of the genetic code means that single point mutations in codons can result in only a limited number of alternative amino acids. The result of this bias is that some amino acid changes are much more likely to occur than others.

A further disadvantage is the fact that after an epPCR is complete the mutant DNA products must be cloned into a vector prior to screening. This is disadvantages from a practical standpoint, increasing the time required to construct a library.

DNA shuffling (Figure 57) allows the recombination of genes from different sources or genes containing different point mutations.[151] This recombination method mimics naturally occurring evolutionary processes in which mutations are incorporated. The foundation of the DNA shuffling process is the digestion of genes and their subsequent recombination. The overall effect of DNA shuffling, and its variations, is to combine fragments of different genes into a single DNA molecule. The digested

pool of DNA fragments is subjected to repeated cycles of PCR. By careful control of the extent to which the initial genes are digested, the number of possible 'crossover points' at which DNA sequence from one parental gene changes to DNA sequence from another, may be controlled.[155] This basic DNA shuffling technique has been refined and developed into more efficient DNA shuffling protocols.[156]

Random chimeragenesis on transient templates (RACHITT) is a variation of DNA shuffling (Figure 57).[157] The fragments are created and then reassembled by annealing together along a parental template strand. Following this step the mismatched sections are removed then fragments extended and ligated together resulting in a recombined gene. The benefit of the RACHITT technique is that many crossovers are possible, however it is necessary for the overlapping regions between fragments to be removed by exonuclease digestion. This can add both time and unwanted variables to the reaction. In addition the RACHITT method is limited to incorporation of sequence elements that are similar to the template as they must anneal to this parental template.

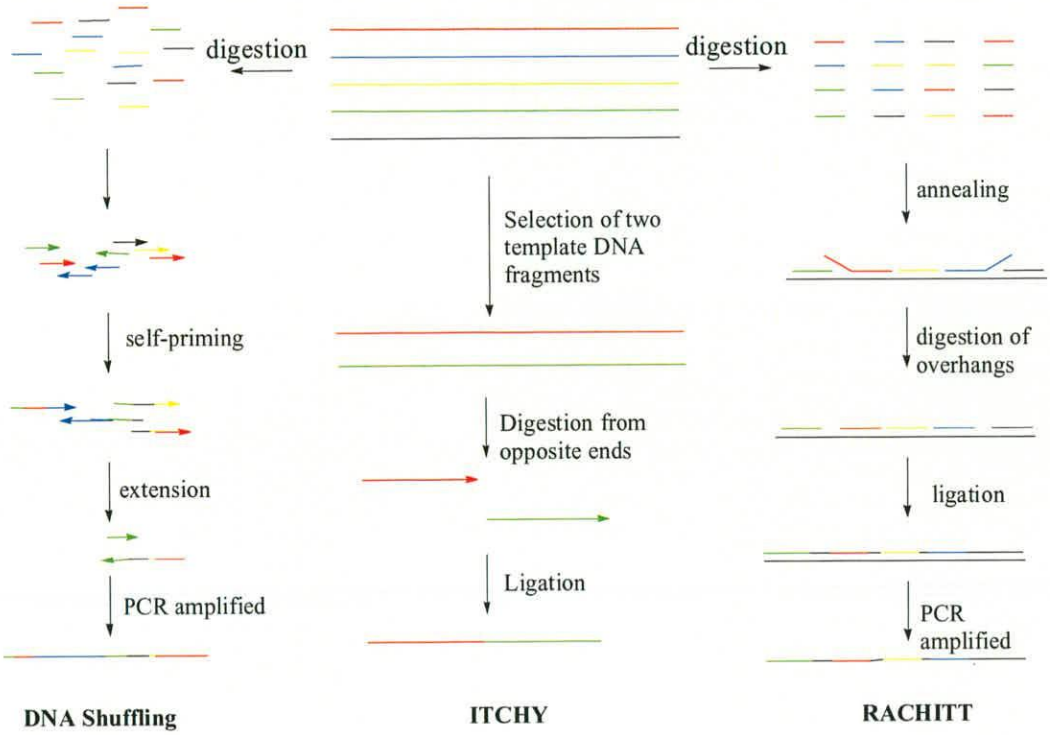


Figure 57: DNA shuffling techniques.

Incremental truncation for the creation of hybrid enzymes (ITCHY) is a technique developed for shuffling parental genes with low sequence homology.[158] The difficulty in this case is that the low sequence homology leads to severe difficulties in strand extension or annealing to a parental template. ITCHY overcomes this as it is based on the direct ligation of libraries of fragments generated from two DNA template sequences. The two fragments are processed differently; one fragment is digested with exonuclease III and S1 nuclease from the 5'-end of the gene. These fragments are ligated to fragments of a second template that have been digested from the opposite, 3'-end. The ligation step occurs in after digestion at arbitrary sites. The control of the digestion is poor and this has lead to the ITCHY method being modified to overcome the lack of control difficulties with the process. The phosphates of the DNA are replaced with phosphothioate bonds in a process named thio-ITCHY.[159] Incorporation of random, exonuclease resistant, phosphorothioate linkages into the DNA determine the length of the truncated fragments that are ligated. The other benefit with the thio-ITCHY method is the inherent problem of

out-of-frame shift products can be addressed as the phosphorothioate linkages can be used to ensure that the digestion fragments remain in frame when ligated.[160] Further developments in combining portions of genes with little homology have been reported such as Diversa Corporation's Tunable GeneReassembly™ technology.[161]

As with all PCR based mutagenesis techniques a final cloning step, into an expression vector, is required for DNA shuffling experiments. This can lead to a bottleneck in the experiment.

A mutator-strain of *E.coli*, XL1-Red, has been developed as a commercial product and has been shown to be a reproducible method for introducing random mutations into a cloned gene.[162] XL1-Red is deficient in three of the primary DNA repair pathways in *E.coli*, namely *mutS*, *mutD* and *mutT*.[163] These genes are knocked out in the XL1-Red strain and thus the repair pathways dependent upon them are inoperable. *MutS* is one of the genes responsible for correcting mismatched DNA base-pairs after replication has occurred; its role is to recognise a base pair mismatch and to recruit other proteins involved in the mismatch repair pathway.[164] *MutD* gene encodes for a subunit of DNA polymerase III, subunit ε, which is responsible for proof-reading (3'-5' exonuclease activity),[165] thus DNA polymerase III is still operating but without proof-reading capability. M*utT* encodes for 8-oxodGTPase which ensures that no 8-oxoGTP is available for incorporation into DNA.[166] These three deficiencies ensure that XL1-Red is an efficient mutator of cloned DNA. However the fundamental disadvantage of the XL1-Red strain is that DNA mutation is not confined to the cloned DNA of interest but the entirety of the genetic material in the host strain. This includes the remainder of the plasmid in which the parental gene is cloned and the genomic DNA of the *E. coli*. The indiscriminate mutation can lead to a number of difficulties for the directed evolution process. Firstly, enzyme variants may be lost as the bacterial host becomes too sickly to survive. Thus these variants are lost before the screening stage without having been subjected to a selection pressure based on the enzyme properties. In addition mutations of the expression vector can lead to viable enzyme variants not being expressed and thus failing the selection pressure. Alternatively mutations to the promoter region can lead

to an increase in enzyme expression levels leading to a positive hit at the selection stage. On inspection these are found to be expression mutants and not mutant enzymes with improved characteristics.

4.2.1.2 Selection of mutants with desired characteristics

After the creation of a large library of variants a robust and rapid method of screening is required. The random nature of the mutants formed in the first stage of a directed evolution experiment means that for the system to be effective at identifying mutants with the desired properties a large number of variants need to be screened. This in turn necessitates the development of high-throughput screening methods to allow the experiment to be conducted over a realistic time-scale. Initial high-throughput screening, followed by more accurate and intensive analysis of putative positive hits is the most effective method of identifying mutants of interest. A number of techniques are available to achieve this high-throughput screening.[167,168]

Crucial to the high-throughput nature of the screen is the fact that the enzyme variants are not isolated and purified before screening. It is therefore the case that what is screened is the ability of colonies to perform the conversion being studied. Careful use of negative and positive controls should ensure that the reason that positive hits display the desired characteristic is due to the fact that an enzyme variant of interest has been created. There are three main methods to screen the colonies; direct analysis of products, life-or-death and colour/fluorometric output.

A life or death selection system is constructed so that only clones which perform the transformation of interest are viable. No active enzyme and the cells die. Thus, after a suitable period of time all the viable colonies should perform the transformation of interest and all other, non-viable mutants are dead.[169] This should, theoretically, reduce false positives to zero. A number of disadvantages have been identified with respect to life-or-death screens. The development of the screen can be lengthy and cumbersome. Due to the multiple metabolic pathways present within standard screening organisms, such as *E. coli*, it can be complex to develop a strain that isn't

viable without the enzyme activity being studied. Knocking out multiple genes is often necessary and this can be difficult. A further problem can manifest itself during the screening process itself. Knock-out strains may mutate and recover knocked-out gene products or develop replacement pathways. This leads to false positives and will only be discovered when analysis of the mutant enzyme, or its encoding DNA, is performed.

The direct analysis of products involves determining if a colony produces the desired chemical product of the transformation being studied. This screening method may be regarded as the most direct method of screening, because one is detecting the presence of a desired product. There are numerous methods of detecting and analysing a chemical product, however for the purposes of high-throughput screening chromatography such as HPLC , CE or GC[170,171] and mass spectrometry / GC-MS[172] are the most viable, although techniques such as ^{1}H-NMR[173] and FT-IR[174] have been employed. The reason for this is two-fold. These methods may be developed to have short run times, so that each sample does not take too much time to analyse. Secondly, the equipment used for chromatography and mass spectrometry is amenable to automation. This allows a large number of samples to be processed in a relatively short time.

The analysis of reaction products can be extended to investigating the enantioselectivity of a reaction. Isotopically labelled compounds have been used to determine enantioselectivity of a panel of nitrilase enzymes.[175] The reaction of ^{15}N-3-hydroxyglutaronitrile **75** pseudo-prochiral enantiomers can be used to assay the enantioselectivity of nitrilases using mass spectrometry (Figure 58). Isotopically labelled pseudo racemic substrates have also been used in high throughput screening using ^{1}H-NMR[176] and FT-IR.[177]

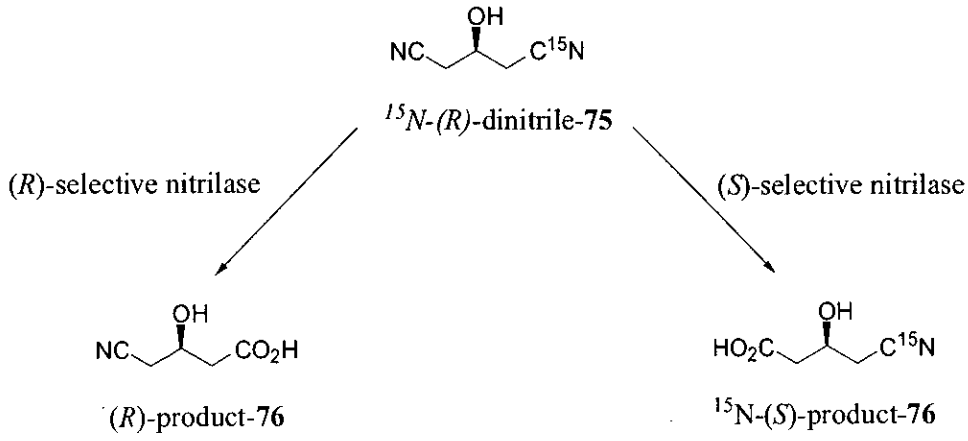


Figure 58: Determination of the enantioselectivity of nitrilase enzymes using isotopically labelled pseudo-enantiomers of a prochiral substrate.

Difficulties with direct analysis of products are found. Samples can require preparation before they are suitable for delivering to the analytical equipment, involving intervention by the researcher. Although systems can be developed which do allow rapid sample turnover, this method is not as efficient in sample throughput with respect to the other screens described and generally direct product analysis is the most time-consuming method to screen large libraries of variants. In addition all samples must be analysed, thus there is not a method for dismissing negative hits before analysis on the instruments. This contrasts with life-or-death screens.

A colorimetric screen offers many advantages and for this reason many high throughput assays are based on the use of chromogenic (or fluorogenic) substrates. A high quality colorimetric screen combines many advantages; the equipment employed means a high numbers of variants may be screened, the development of the analytical method is usually simple, no organism engineering is required and the system is often transferable to other chemical transformations of interest.

The colorimetric output of a screen may be linked to a number of different events. It may be proportional to the disappearance of a substrate, if the substrate of interest has a colorimetric or fluorometric output. The appearance of a desired colorimetric product may also occur. Most commonly the generation of a by-product of the

reaction of interest is detected.[174] The formation of a detectable product may also require further reagents or catalysts in the assay mixture.

The colorimetric output of a screen may be detected by a number of different methods, of varying complexity, cost and speed. The simplest, and often most efficient in terms cost and development time, is the use of the human eye. Plates of colonies with varying colour output may be surveyed and those exhibiting the desired output selected. The downsides associated with this method of hit selection are that no accurate numerical data is associated with the hit, in terms of colour density, rate of colour formation, etc. Secondly the problem of human fallibility, most especially the possible lack of objectivity, can be manifested. Bias, in terms of 'wanting something to work' and fallibility, in terms of problems such as tiredness, can influence selection adversely. The use of basic imaging technology, such as filtered digital cameras can allow for more accurate screening.[178]

A recent development in colorimetric screening is the release of a digital imaging spectrophotometer called Kcat Technology.[179,180] Kcat Technology allows the high throughput solid phase assay of microbial libraries by the analysis of each microcolony on a plate. Each microcolony is analysed simultaneously at a very high resolution (75μm/pixel). The benefit of this technology is that for each pixel it is possible to plot a kinetic profile and thereby rank in order the relative performance of hits identified in the screen, which is not facile with a regular plate based screen. The technology also allows for highly confluent plates to be screened, and thus more variants per unit time. Clearly the manual selection of a specific microcolony from a highly confluent plate must be considered if this experimental path is to be followed.

To overcome the bottleneck of manual selection and picking a number of packages are available combining robotic arm pickers, cameras and control software.[181] Calibration and experimental design can be lengthy but once the experiments are running automation of the process offers significant opportunities for high-throughput screening.

Most fluorogenic and chromogenic screens have been designed to be performed in parallel on microtitre or agar plates. The implication of this is that these screening methods are useful and applicable for screening several hundred thousand variants. This is a significant number; however it is still small with respect to the total number of possible variants that library creation techniques can produce. A key technique that has been developed to address the desire to screen many millions of variants is fluorescence activated cell sorting (FACS) (Figure 59).[182] Using flow cytometry, up to 10^9 variants can be analysed in a practical time period.[183] A screen is developed in which an optical output occurs for a desired functionality of a cell. This may, for instance, be the conversion of a substrate or alteration in the expression levels of a protein. The use of Green Fluorescent Protein (GFP) coupled is one method described to produce an optical output for a FACS system.[184] The mass of cells is forced to travel past a sensing point one cell at a time. As the cells move in suspension past a sensing point the optical property is measured and any cells that fall within predefined parameters may be collected. This is achieved by the cytometer machine which charges the steam of cells at the point when the flow has become a droplet. The sorted cell droplet now carries a charge that is different to the bulk stream and may then be deflected into a collection hopper by the application of a controlled electrical field. As a further refinement the physical properties of each cell can be analysed. This allows dead cells to be discarded and prevents attempts to grow-up unviable cells. The equipment to perform FACS is relatively expensive and specialist however today they are a mainstream instrument and with an optimised sorting rate of 30,000 cells per second can provide a system that is ultra high throughput.

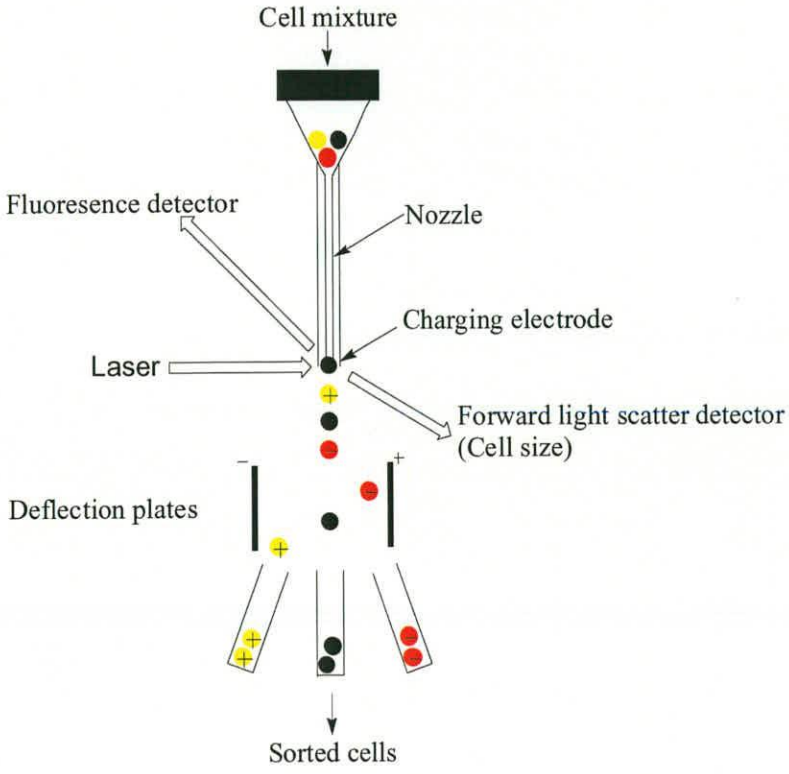


Figure 59: FACS system.

In conclusion, there are various technologies available that can be used in a screen. Each method has benefits and drawbacks and the most appropriate method should be selected individually for each case to best suit the conditions of the screen. Furthermore it is imperative that the method chosen is high throughput and is sensitive enough to monitor the production of product or products to maximise the numbers of candidates that can be examined.

4.2.1.3 Directed evolution - Conclusion

The combination of a well-defined procedure to create a library of variants coupled with a selection procedure which effectively discriminates between the variants is crucial for a successful directed evolution experiment. The technology and techniques exist for this and, as such, a directed evolution program is a viable method for the production of novel enzyme variants. The crucial factor associated with direct evolution is that it is a 'results-orientated' technique. This means that a successful outcome, in terms of improving a property of an enzyme, is the sole focus.

Naturally, this may lead to negative consequences. If a positive hit is found containing multiple mutations, with respect to the parental variant, then deconvoluting the effect of each mutation is not possible without further work. If no information about the enzyme structure is available then rationalising why mutations have altered a property is also very difficult. It must be remembered there can be no guarantee that improved variants identified are the optimum variant possible, merely that they exhibit the characteristics screened for.

4.2.2 Rational design

Rational design is the term used to describe the creation of variants in a non-random fashion. Whilst the name rather unfairly suggests that directed evolution is an irrational approach to the question of producing enzymes with improved proprieties, the term rational design does emphasise that the make up of the variant library is entirely dictated by the researcher. The fact that mutations are produced at defined positions on the gene, and thus the enzyme, means that variant libraries are generally much smaller. At the extreme the library may contain only a single variant. This, in turn, means that lower-throughput screening systems are required. The benefit of a rational design process is that one may investigate the effect that a specific residue change has, additionally if directed evolution produces a variant with a mutation at a specific residue, then investigation as to whether the optimum residue at that position has been found can be performed. A specific variation of a rational design experiment which can prove to be very powerful is saturation mutagenesis.

4.2.2.1 Saturation mutagenesis

To ensure a library of variants had the maximum possible diversity it is necessary to fully randomise each nucleotide within the encoding gene. This equates to 4^n variants where n = the number of nucleotides in the gene. This number is incomprehensively large and is simply impossible to achieve, as is even attempting to fully randomise at each amino acid residue. The effect of this is that if an improved variant is discovered it is incredibly unlikely that any experimental procedure will have

ensured that all possible amino acids at that particular residue will have been screened and assessed. Saturation mutagenesis is a technique which allows the researcher to investigate all possible amino acids at a defined residue position. The technique may also be used to investigate whether putatively important residues, perhaps predicted by a homology model or structural data, have an effect on the properties of the enzyme variants.

The 3 nucleotides that encode the position of interest are substituted for NNS, where N represents any of the 4 DNA bases and S either G or C. This achieves the situation of having all 20 amino acids represented whilst reducing the occurrence of a stop codon. Clearly a stop codon is undesirable at any residue on the gene, as it will result in a truncated product. The use of a NNS codon does alter the degeneracy of the genetic code; however production of all 20 amino acid variants is theoretically still achieved. A statistical analysis of the resulting possible gene products (Equation 1)[101] demonstrates that 147 colonies are required to ensure a 99% probability of all possible mutations.

$$P = 1\text{-}e^{-N/V}$$

P = % probability (defined as 99%)
N= number of colonies required
V = total possible number of combination

Equation 1: Required library size for saturation mutagenesis screening.

Clearly, a saturation mutagenesis experiment can be expanded to comprise the saturation mutagenesis at a number of positions simultaneously, with the corresponding increase in the library size and thus required number of colonies.

The standard technique for performing saturation mutagenesis is the use of a PCR protocol. Two oligonucleotide primers are designed to encode the NNS sequence along with flanking regions of complementary sequence to allow hybridisation of the primer to the template. These primers are in the region of 30 nucleotide bases long. A

PCR amplification is carried out. The PCR products are then ligated into a transformation vector. After transformation and growing up of the host the genes vectors are recovered, digested and the gene pool cloned into an expression vector.

Site directed saturation mutagenesis may also be performed using a commercial system, Stratagene's Quickchange kit.[185] The advantage with this method is there is no need to purify the mutated fragment of DNA from the PCR reaction and has the added benefit of no requirement for ligating PCR products into a plasmid. Once again two oligonucleotide primers are designed to encode the NNS sequence along with flanking regions of complementary sequence to allow hybridisation of the primer to the template. These primers are of the region of 30 nucleotide bases long. A PCR is performed with these primers, the cycle times and temperatures designed to allow the primers to extend completely around the plasmid. The PCR product mixture is exposed to *Dpn* I endonuclease. *Dpn I* specifically digests the methylated DNA of hemimethylated DNA. The template DNA used the PCR is methylated by the dam gene from *E. coli* whilst PCR products are unmethylated so only the template DNA is degraded. The final step is to address the fact that the PCR products are nicked and not completely circularised, transformation into a specific *E. coli* strain ensures the 'nick' is repaired (Figure 60).

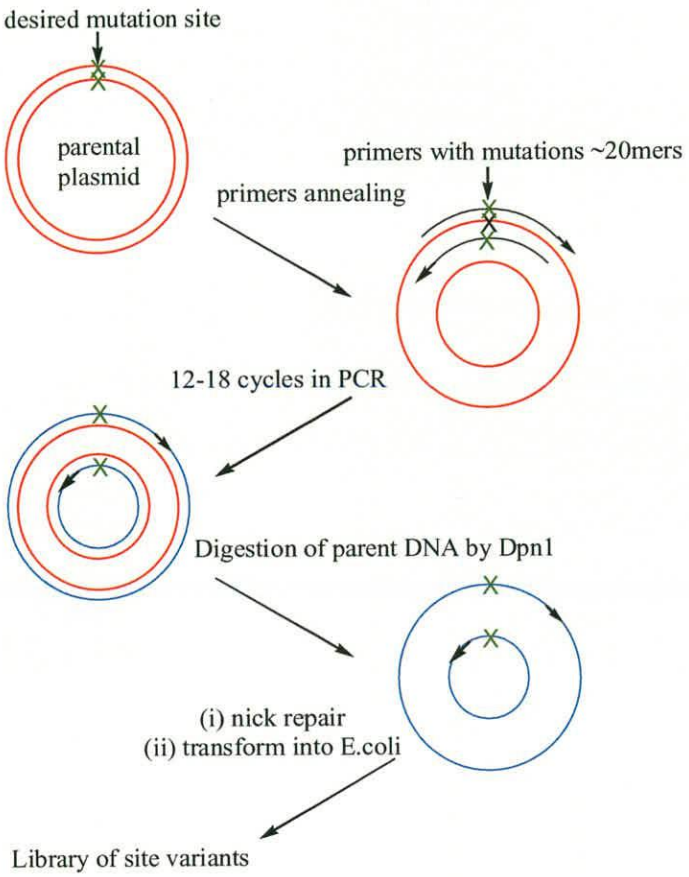


Figure 60: Stratagene's Quikchange system.

A development of the saturation mutagenesis techniques outlined above is the Gene Site Saturation Mutagenesis (GSSM) technology of Diversa Corporation.[186] This is a system which, to some extent, bridges the gap between the directed evolution and rational design concepts. The GSSM system allows each codon in the parental gene to be mutated to all other possible codons in turn. This system is very powerful in terms of identifying residues key to the property of interest, so called 'hot spots'. As with directed evolution, knowledge of important residues before the experiment is unnecessary. The difficulties associated with deconvoluting which mutations have been effective within the multiple mutations sometimes observed in a directed evolution experiment are overcome. The proprietary nature[187] of the technology does mean that the opportunity to exploit this technique is limited.

4.2.2.2 Screening

As demonstrated above, the library size for the simplest saturation mutagenesis experiment need not necessarily be large. This means that the need for high-throughput screening is less urgent, however methods detailed for directed evolution experiments can often be employed for saturation mutagenesis experiments also. A smaller number of variants to screen does lend itself to more detailed and labour intensive analysis. Screening of purified enzymes becomes viable, as does direct product screening using lower throughput methods.

4.2.2.3 Rational Design - Conclusion

Rational design is a technique which allows a much greater degree of control in terms of the variants produced compared with directed evolution. Probing of active site residues and assessing the effect of exact residue changes becomes possible. The negative side of this is that prior knowledge of the mode of action and structure of the parental enzyme is necessary to make any experiments worthwhile, this is in contrast to directed evolution. In addition, as such a narrow range of mutation is studied, a large amount of resources can be dedicated to experiments that ultimately do not alter the enzyme properties in the desired fashion.

4.3 Biocatalyst modification - Conclusion

Biocatalyst modification techniques most be considered as complementary to each other. A successful program may well involve a directed evolution program, further optimisation using rational design procedures and optimisation of the actual biocatalyst form used in scaled-up reactions. It is crucial to define what is required of the biocatalyst and then focus on employing the most relevant methods to achieve this; these will vary on a case by case basis.

4.4 Directed evolution of MAO

In an attempt to improve the catalytic properties of the MAO-N variants towards the hydroxylamine and hydroxylamine ether substrates of interest a programme of directed evolution was undertaken. The investigation would focus on trying to identify mutants with higher activity towards the substrates. Drawing from the experience of directed evolution programs that had been performed on the MAO-N enzyme previously, serendipitous benefits such as a broadening of substrate specificity or an increase in enzyme stability may be observed. Monitoring of these is an important factor.

4.4.1 Library creation

As described above, a number of different protocols exist by which a mutant library can be generated. The *E. coli* mutator strain XL1-Red was selected as the agent to create a *mao5D* mutant gene library for a number of reasons. Previous experience in the laboratory meant that the use of this technique was well understood. An advantage of the method over others is that it is not resource intensive, in terms of time, money and labour. Perhaps the strongest driving force was the fact that the previous use of the XL1-Red system in our laboratory[101,102] had lead to successful isolation of MAO mutants with improved properties.

As a baseline, to assist with designing the mutation experiment, it has been experimentally measured that in XL1-Red the mutation rate is typically 1 mutation/$5x10^6$ bp/generation/cell.[153] In addition it has been experimentally measured that with a pBluescript the mutation rate was 1 mutation per 2kb after 30 generations.[163] Whilst these figures provide a general guide to mutation rates it must be noted that it is based on a standard plasmid control experiment. This does not reflect the actual circumstances, in terms of growth cycles, growth conditions and plasmid to be used in the series of experiments described herein. There is no substitute for experimentally determining the mutation rate under the conditions that are proposed to be used. The conditions employed for the construction of random

mutation libraries in the experiments presented in this report are chiefly based on work previously performed in our laboratory with various mao gene libraries.

The mao gene is 1489 base pairs in length, so assuming an error rate comparable with the rate observed with pBluescript then it would take just over 22 generations for a single base mutation to the mao gene to be introduced. Experimentally, however, the optimum conditions were found to be 4 cycles of transformation and inoculation, corresponding to approximately 36 – 48 generations.

It was decided that a number of mutant libraries were to be created and screened, the essential difference between them was the parental mao gene employed at the start. The libraries created were;

1. RL*mao-N5D* 1

The gene *mao-N5D* encoding for the MAO enzyme which demonstrated activity towards the hydroxylamine substrates **47** and **48** (Chapter 3) was used as the parental gene for the construction of this library, for the obvious reason that it demonstrated the activity desired and so was the ideal start point for a program of directed evolution.

2. RL*mao-N5D* 2

As above, a repeat in all respects.

3. *RLmao-N*

The mutant mao genes available in the laboratory had been subjected to various mutation protocols in an attempt to improve the activity towards amine substrates. It may be the case that the mutations introduced were deleterious with respect to activity towards hydroxylamine substrates. For this reason, the wild-type mao gene, *maoN*, was used as the starting point for the construction of this mutant library.

4. RL*mao-N2*

This parental mutant contained two of the five mutations present in the *mao-N5D*, N336S and M348K. This mutant was a product of the directed evolution experiments that ultimately lead to the *mao-N5D* mutant. This mutant library was created by, and acquired from, Dr R. Speight, Ingenza Ltd.

The RL*mao-N*, RL*mao-N5D*1 and RL*mao-N5D*2 mutant libraries were constructed in the same fashion. XL1-Red competent cells were transformed with the parental *mao* gene in pET-16b plasmid using a modified version of the manufacturer's protocol. The transformed cells were used to inoculate 10ml of LB containing 100µg/ml ampicillin. The cells were grown for 18 hours after which 1ml of culture was removed for isolation of the plasmid DNA. This procedure gave the first generation library of mutants. The plasmid DNA was then re-submitted to the same procedure a total of four times. This protocol was designed to balance two critical parameters; the number of mutations per mao gene and the mutation to an inefficient mutator strain.

Clearly, the more generations of XL1-Red allowed to form, the greater the likelihood of a mutation in the mao gene. This must be balanced with the fact that too many XL1-Red generations and one may find multiple mutations in the mao gene. Alternatively, but even more problematically, genomic mutation of the XL1-Red may lead to the re-emergence of, or the formation of alternative, DNA repair pathways. This in turn leads to cells which do not introduce mutations to the parental mao gene and reproduce far more efficiently than those that do. They out-compete the mutator cells with the result that the non-mutating strain dominates the cultures.

An investigation into whether the protocol above did produce mutations at an acceptable rate was performed. Plasmid DNA from the RL*mao-N5D* 1 library generated by XL1-Red was used to transform BL21 (DE3) star *E.* coli competent cells and grow-up on 100µg/ml ampicillin plates. 10 colonies were selected and the plasmid DNA isolated and analysed by DNA sequencing (Table 11).

Clone – RL*mao-N5D* 1.	Mutation in gene	Effect of mutation
1[1]	n/a	n/a
2	None	None
3	A408G	Thr136Thr
4	G167A	Arg56Lys
5	A337 deletion	frame shift
6[1]	n/a	n/a
7	G845, T846 deletion	frame shift
8[1]	n/a	n/a
9	C1345T	His449Tyr
10	T61G	Ser21Ala

[1] Sequencing failed

Table 11: Mutations introduced into *mao-N5D* in 10 random clones of RL*mao-N5D1* library.

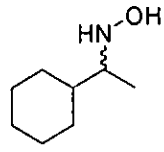
As illustrated in Table 11 the library creation protocol outlined above produces a wide variety of mutations. Translations, transversions and deletions are present. In addition it most be noted that a number of clones had no mutations in the mao gene. No other portion of the plasmid was sequenced.

The protocol described above represents the optimisation of the difficult balance between the various parameters when using a mutator strain library creation system and has produced libraries in which most plasmids contain a single base-pair mutation in the parental mao gene.

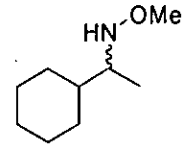
4.4.2 Library screening

The variant libraries created were separately screened against two substrates, hydroxylamine **46** and its methyl ether derivatives **47**. These two substrates were chosen with different goals in mind. Hydroxylamine ether **47** was a reasonable substrate for MAO-N5D, as demonstrated in Chapter 3. The purpose for screening the libraries against this substrate was to investigate whether a mutant with enhanced

rate could be isolated. The screening against hydroxylamine **46** was a far more ambitious program, designed to investigate whether any of the inactive MAO-N variants could be successfully evolved to exhibit activity against a hydroxylamine. It should be noted that inhibitory action had been demonstrated for hydroxylamines against MAO-N5D, (Chapter 3), and thus this program had the target of fundamentally altering the relationship between the MAO-N enzymes and the substrate.



46



47

Figure 61: Initial screening substrates for directed evolution experimental programme.

Plasmid DNA from the libraries generated by XL1-Red was used to transform BL21 (DE3) star *E. coli* competent cells which were then subjected to the plate-based assay described in Chapter 3. Control plates, consisting of *E. coli* transformed with the parental mao gene of each particular library were assayed in parallel against **47**. This was to have a comparison against which possible hits could be compared to assess whether darkening rates were quicker.

Library	No. of Plates	No. of colonies on a representative plate[1]	Approximate no. of colonies screened	Hits
RL*mao-N5D* 1	20	1992	39800	0
RL*mao-N5D* 2	18	2140	38500	0
RL*mao-N*	18	1664	30000	0
RL*mao-N2*	18	912	16400	0

[1]All colonies on ¼ of the plate counted, result multiplied by 4

Table 12: Results of screening random libraries against hydroxylamine ether **47**.

As displayed in Table 12, the use of a directed evolution protocol was wholly unsuccessful in identifying a MAO-N variant that had improved activity towards **47** compared with the original MAO-N5D mutant. Activity was retained by approximately 5% of colonies, indicating that experimental method was sound. None of the active colonies produced activity greater than the control plates. The possible reasons for this are manifold. To take an optimistic, but unrealistic, view it could be claimed that the reason no improved variants were found was because the enzyme is fully optimised towards the substrate – in other words every possible mutation to the mao genes leads to a less active enzyme product. This fact could only be ascertained by producing every single mutant individually and assaying them but is unlikely compared with other possible explanations.

Flawed experimental procedure may have led to the difficulties in a isolating an improved variant. Although the sequencing of a small sample of clones (Table 11) indicated that a range of mutations had been introduced into the parental mao genes, perhaps this was an unrepresentative sample and a large number of unmutated parental genes remained in the library DNA pool. As described in Chapter 3, there are also known limitations with the plate-based assay procedure, specifically regarding the homogeneity of the assay across the plate. It is entirely possible that a more active variant was located in a region that was not subjected to the optimum assay conditions compared with the control plate and thus was missed. The final consideration is that the libraries happened not to contain the variants that would have higher activity towards the substrate **47**, simply a case of bad luck. The design of the experiment and the number of variants screened represent a viable and worthwhile attempt to isolate variants displaying higher activity towards hydroxylamine ether **47**, however the results were disappointing. Further generation of new libraries and screening them was not attempted as other avenues were available to explore.

The same libraries were screened against hydroxylamine **46** (Table 13). This was in an attempt to isolate variants which turned over a substrate that had proven to be an

inhibitor of the parental enzyme variants. This goal was extremely ambitious and, as such, the risk of it failing was large and obvious.

Library	No. of Plates	No. of colonies on a representative plate[1]	Approximate no. of colonies screened	Hits
RL*mao-N5D* 1	18	2440	43900	1
RL*mao-N5D* 2	18	476	8600	2
RL*mao-N*	18	2012	36200	0
RL*mao-N2*	10	1204	12000	0

[1]All colonies on ¼ of the plate counted, result multiplied by 4

Table 13: Results of screening random libraries against hydroxylamine **46**.

The plasmid DNA from the 3 hits isolated was separately retransformed and re-assayed, 1 hit, RL*mao-N5D* 2HB appeared to darken significantly faster than the other two, which on re-assaying did not appear to darken significantly. The one possible hit was taken forward and the CFE from a 10mL culture used to perform a liquid phase assay experiment as per Chapter 3 (Table 14).

Sample	OD / 510 at t= 60 mins
RL*mao-N5D* 2HB CFE sample 1	Baseline
RL*mao-N5D* 2HB CFE sample 2	Baseline
RL*mao-N5D* 2HB CFE sample 3	Baseline

Table 14: Liquid-phase assay results for positive hit RL*mao-N5D* 2HB.

A SDS-PAGE gel confirmed that protein of the correct mass was being over-expressed and thus the possible activity proved to be illusionary when more detailed examination of the variant was undertaken. This starkly highlights the usefulness of a control plate of the parental variant produced and assayed in parallel with the screening experiment. If the inactive parental variant is observed to gradually darken over time, inactive variants which darken at the same rate can and will be ignored.

The fact that an active variant was not isolated is not terribly surprising and no further libraries were generated or screened.

4.4.3 Directed evolution conclusion

The use of a directed evolution protocol to discover improved MAO-N enzyme variants did not prove to be successful. Whilst the system had proved very effective within the laboratory for altering and improving the characteristics of the MAO-N enzymes with respect to various classes of amines, this was not the case for the hydroxylamine derivatives of interest. Simply put, the MAO enzyme is a monoamine oxidase, not a hydroxylamine oxidase, and so the enzyme is already operating outside of its normal parameters – further improvement of the activity was not achievable.

In an attempt to investigate whether improvement of the MAO-N5D activity towards hydroxylamine derivatives could be made, attention was turned to the other main method of engineering an enzyme; site specific mutagenesis.

4.5 Site specific mutagenesis

In an attempt to improve the catalytic properties of the MAO-N variants towards the hydroxylamine and hydroxylamine ether substrate of interest a programme of site specific saturation mutagenesis was undertaken. The investigation would focus on trying to identify mutants with higher activity towards the substrates. The location of residues that may alter the MAO-N activity towards hydroxylamines and hydroxylamine ethers were investigated using the computer homology model described earlier (Chapter 3).

4.5.1 Selection of residue 245

As described previously, molecular modelling studies suggested that residue 245 of MAO-5D may have an influence on the catalytic activity of MAO-5D with respect to

substrate **47**. Residue 245 was a close-contacting residue when the substrate was docked into the active site. The decision was made to perform site specific mutagenesis upon this residue. Previous work within the group had shown, as expected, that the close contacting residues in the active site region could be very influential with regards to catalytic activity. Indeed the MAO-5D variant included a mutation at position 246.

4.5.2 Residue 245 saturation mutagenesis library creation

Site directed saturation mutagenesis at position 245 was performed using Stratagene Quickchange protocol described above. The two oligonucleotide primers were designed to introduce an NNS codon in the *mao5D* gene, encoding for amino acid position 245 in the MAO-5D enzyme (Figure 62).

Forward primer: 5'-GGATGCATGGACTGCNNSATGAGTTATAAGTTCA-3'

Reverse primer: 5'-TGAACTTATAACTCATSNNGCAGTCCATGCATCC-3'

Figure 62: Oligonucleotide primers designed for the NNS saturation mutagenesis library at amino acid residue 245 in MAO-5D.

Completion of the Quickchange protocol led to approximately 500 colonies on 4 plates being produced. Before screening of the saturation mutagenesis library commenced the distribution of the library had to be ascertained. The library must be shown to be representative in terms of codons present and, more importantly, it must be shown the experiment had actually worked and that a library of mutants had been created.

10 colonies were selected at random (clones TESAT1.1 – TESAT1.10) and the nucleotide sequence at the 245 position analysed by DNA sequencing. The two key points of this sequencing were to ascertain that firstly, there was not an over-representation of the parental TTG codon and secondly, that a range of alternative codons were present. This was the case, as illustrated in Table 15.

Clone – TESAT1.	245 Codon	Amino acid
1	GTC	Val
2	ACC	Thr
3	GTC	Val
4	AAG	Lys
5	TCG	Ser
6	ATG	Met
7	CGG	Arg
8	ACG	Thr
9	TTG	Leu
10	TGC	Cys

Table 15: Sequencing of 10 clones (TESAT1.1 – TESAT1.10) of 245 saturation mutagenesis library generated by the Quickchange kit.

4.5.3 Screening of residue 245 saturation mutagenesis library

The plasmid DNA library was used in the transformation of *E. coli* BL21 (DE3) competent cells giving and grown-up on plates as per Chapter 7, Experimental Section. 155 colonies and 120 colonies grew on plates TESAT1P1 and TESAT1P2 respectively. As 147 colonies were required to have a >99% probability of each possible mutant being represented, 275 colonies represents an acceptable sampling number. The membranes were then assayed against (*rac*)-**47**. After 19 hours, 6 colonies were significantly deeper brown-coloured than the remainder of the colonies. In addition all colonies on a plate of solely the parental clone appeared paler. This control was employed to ensure large amounts of time and resources were not employed on re-selecting the parental clone if it turned out to be the optimum codon. The 6 colonies were picked and separately grown in LB containing 100μg/ml ampicillin. The plasmid DNA of the 6 positive colonies was separately and simultaneously taken forward to two experiments.

Firstly, the plasmid DNA of each positive clone was re-transformed into *E. coli* BL21 (DE3) competent cells and re-grown on plates for a second assay test. Each

plate consisted of colonies with the same, initially positive, plasmid transformed in them. Thus all colonies on the plate should darken. When re-assayed and compared with the parental control plate, 5 plates were assigned as positive, the sixth was unclear and indeterminate, and thus work was discontinued on this clone. Secondly the plasmid DNA was sequenced at the 245 site. All six plasmids were found to have ATG, encoding for methionine, at the NNS site. The ATG codon has a 1 in 32 chance of occurring amongst the NNS library; therefore in the library size screened here it should be expected to occur on average 8.6 times ((1/32) x 275). It may be expected that on average a further 2 hits would be identified, however the screen is necessarily non-precise and a colony 'slipping through the net' is not unexpected or unreasonable. The close match of the predicted and observed numbers of optimum colonies is pleasing.

The optimum residue at position 245 would appear to be methionine as described above. This represents a single base change from the TTG parental leucine codon and the enzyme is named MAO-N6, the gene *mao6*. The leucine to methonine change is not a dramatic alteration of the environment at this amino acid position.

Leucine Methionine

Figure 63: Leucine to methonine change

Both are uncharged residues under standard physiological conditions and both are hydrophobic. The key side-chain difference is that leucine has a larger side chain volume than methionine (Figure 63). The linearity of the side chain of methonine differs from the branched nature of leucine. The change from leucine to methionine results in removal of a γ-methyl branch in the amino acid side group. It may be postulated that the enzyme active site is affected by hydrophobic exclusion and Van der Vaals interactions between the enzyme's active site and the substrate, thus the steric nature of the side chain may be crucial.

In addition to selecting clones which appeared to have the fastest reaction rates a number of inactive clones were selected and investigated. There were a number of reason for this; as a re-check that the library contained different codons at the 245 site, to investigate whether inactive clones consistently contained certain residues and to ensure that the putative optimal residue, methonine, did not appear. This would give increased confidence in the screen and that the ATG mutant was indeed worthy of further investigation (Table 16).

Clone – TESAT1NA.	245 Codon	Amino acid
1	TTC	Phe
2	CCG	Pro
3	TAC	Tyr
4[1]	n/a	n/a
5	TTC	Phe
6[1]	n/a	n/a

[1] Sequencing reaction failed

Table 16: Inactive mutants from 245 saturation mutagenesis experiment.

Pleasingly, the inactive mutants selected showed a consistency, in that they may all be regarded as residues that would sterically hinder the 245 site, as compared with the 245 methonine and leucine mutants which have been demonstrated to be active. Clearly, this rationale is not the only explanation as to why these mutants may be inactive. The enzyme may not fold into the active form with these residues present, there may have been a problem associated with protein expression for these particular clones that is wholly unrelated to the 245 residue change. Additionally, it is possible that the assay was faulty in some aspect and these clones were in fact active, and misidentified as inactive. No system can be devised that is infallible, however the assay and mutagenesis system has been proven to be robust and the methonine mutant, MAO-6, was taken forward for further investigation.

4.5.4 Effect of methonine mutation

In an attempt to gain an insight into what effect the L245M mutation may have had upon the MAO enzyme a further round of molecular modelling was undertaken. MAO-6 structure was subjected to the same modelling conditions as for the MAO-5D experiment, with the same substrate, (*S*)-**47**, docked in the active site. The active site structure determined by this process is shown below (Figure 64).

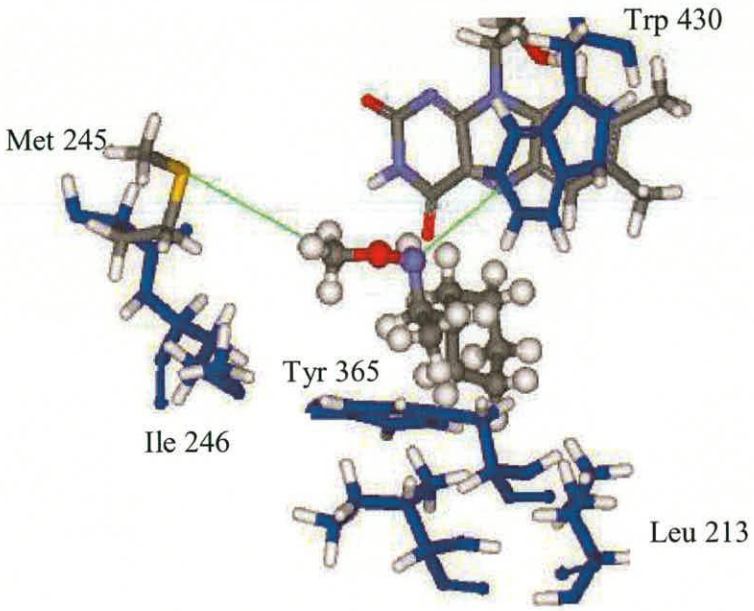


Figure 64: Hydroxylamine ether (*S*)-**47** docked in MAO-N6 active site model. Green lines indicate closest contacts.

It can be seen that the modification of the leucine 245 residue to methonine has caused the pocket into which the methoxy ether moiety is located to increase in volume (cf. Figure 55). It would appear from these studies that a modification of the active site configuration occurs with the L245M mutation. It may be speculated that the alteration of the active site volume allows for the more rapid entry and exit to the active site by the substrate. Clearly this contention is speculation to some degree. The suggested configuration of the active site relies on a number of assumptions. The original model is merely a homology model of MAO-N, derived from alignment with the known crystal structure of MAO-B. In turn this model has been subjected to

energy minimisation protocols which, whilst well described and extensively used, are necessarily predictions rather than fact. A crystal structure of the MAO-6 mutant is required to conclusively understand the structure and mechanism both of the parental MAO-5D and mutant MAO-6 enzymes. Acquiring a crystal structure of any of the MAO mutants is outside the scope of this investigation.

The MAO-6 was subjected to a further level of investigation and screened against a number of hydroxylamine substrates, in parallel with the MAO-5D, to assess the extent to which improvements in the catalytic properties of the enzyme had been achieved (Table 17). The plate-based screen necessarily lacks fine detail in terms of catalytic rates and does not provide any sort of data output that may be used for accurate comparison.

Substrate / 10mM	MAO-5D activity / %	MAO-6 activity / %
24	100	98
48	17.4	19.9
47	15.6	18.4
70	13.1	15.8
74	3.5	3.6
72	3.4	3.7
42	1.2	1.6
72	0.6	<0.5
44	<0.5	<0.5
46	<0.5	<0.5
73	<0.5	<0.5

Table 17: Substrate conversion rate, relative to MAO-5D with α-methylbenzylamine **24** at 100%. Analytical threshold 0.5%.

As Table 17 indicates the MAO-6 mutant shows a slightly modified profile with respect to MAO-5D in terms of relative activities. There remains no free hydroxylamine activity, as discussed in Chapter 3 this is unsurprising, however an

alteration in the relative rates for a number of substrates is observed. The key point to note is that there are no major differences between the activities of the two mutants; substrates remain in the same order of relative activity and the absolute values do not different greatly. This indicates that the effect of replacing the leucine with a methonine is not dramatic; rather it subtly alters the active site, and provides a degree of rate enhancement for substrates already proven to be active with the parental MAO-5D mutant. The alteration to a methonine residue does not allow for any of the panel **44**, **46** and **73** that were not substrates to become so; specificity does not appear to be altered. One may speculate that a small increase in the rate at which the substrate may enter and leave the active site is achieved with the leucine to methonine substitution. As specificity is not altered the 245 residue does not appear to have a crucial role in the active site configuration and catalytic action. An analysis of the kinetic properties of MAO-6, compared to MAO-5D, turning-over a model substrate may assist in elucidating this, and is presented below. These results also indicate that the plate-based assay is perhaps more sensitive than thought, as a rate improvement of 18% was successfully detected. This provides extra confidence in the use of the plate-based screen at the top of a hierarchical screening system.

The kinetics of the enzyme reactions were probed using Michealis-Menten studies as per Chapter 3. The experimental procedure was exactly the same and the data for the MAO-5D mutant presented previously is replicated for clarity (Table 18).

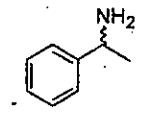
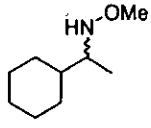
 24	V_{Max} (µmol/min/mg)	k_{cat} (min^{-1})	K_M (mM)	Catalytic efficiency k_{cat} / K_m (min^{-1} mM^{-1})
MAO-5D	$0.78\pm8\times10^{-2}$	43.4±0.4	3.52±0.19	12.33
MAO-6	$0.74\pm2\times10^{-2}$	41.1±0.3	3.49±0.11	11.78
 47				
MAO-5D	$0.18\pm3\times10^{-2}$	10.2±0.8	5.66±0.24	1.80
MAO-6	$0.20\pm2\times10^{-2}$	11.1±0.4	4.23±0.12	2.92

Table 18: Comparison of kinetic properties of MAO-5D and MAO-6 mutants.

Interestingly the parental gene (*mao5D*) for this portion of work was constructed in an attempt to optimise the MAO-N for a wide range of secondary amines. Saturation mutagenesis of position 246 was undertaken and methionine was found to be the optimum residue, using a similar protocol to that described above. The mutated residue was originally isoleucine and so the more active mutant and the mutation that occurred followed a pattern very similar to the results described above.

4.5.5 Residue 246 saturation mutagenesis library creation

The fact that the parental gene for this optimisation experiment has already incorporated a mutation derived from a previous experimental program, focusing on secondary amine substrates, leads to the possibility that the MAO-N6 contains a deleterious mutation at 246, the methonine residue that was introduced, replacing the isoleucine. Returning the 246 residue to the original, wild-type isoleucine and comparing the result to the double methionine product would both be possible and interesting. The resources, in terms of time and money, involved for this however are comparable to performing a saturation mutagenesis experiment. Therefore it was decided to perform a further saturation mutagenesis experiment. With residue 245 as

methionine, residue 246 was subjected to a saturation mutagenesis protocol. The experiment was performed as above

The two oligonucleotide primers were designed to introduce an NNS codon in the *mao6* gene, encoding for amino acid position 246 in the MAO-N6 enzyme (Figure 65).

Forward primer: 5'-GGATGCATGGACTGCATGNNSAGTTATAAGTTCA-3'

Reverse primer: 5'-TGAACTTATAACTSNNCATGCAGTCCATGCATCC-3'

Figure 65: Oligonucleotide primers designed for the NNS saturation mutagenesis library at amino acid residue 246 in MAO-6

Completion of the Quickchange protocol led to approximately 430 colonies on 4 plates being produced. Before screening of the saturation mutagenesis library commenced the distribution of the library had to be ascertained.

10 colonies were selected at random (clones TESAT3.1 – TESAT3.10) and the nucleotide sequence at the 246 position analysed by DNA sequencing. The two key points of this sequencing were to ascertain that firstly, there was not an over-representation of the parental ATG codon and secondly that a range of alternative codons were present. This was the case, as illustrated in Table 19.

Clone – TESAT3.	246 Codon	Amino acid
1	AAG	Lys
2	ACC	Thr
3	ACG	Thr
4	TGC	Cys
5	TCG	Ser
6	GTC	Val
7[1]	n/a	n/a
8	CCC	Pro
9	TTG	Leu
10	GTC	Val

[1] Sequencing reaction failed

Table 19: Sequencing of 10 clones (TESAT3.1 – TESAT3.10) of 246 saturation mutagenesis library generated by the Quickchange kit.

4.5.6 Screening of residue 246 saturation mutagenesis library

The plasmid DNA library was used in the transformation of *E. coli* BL21 (DE3) competent cells giving and grown-up on plates as per Chapter 7, Experimental Section. 100 colonies and 195 colonies grew on plates TESAT3P1 and TESAT3P2 respectively. The membranes were then assayed against (*rac*)-**45**. After 18 hours, 3 colonies were significantly deeper brown-coloured than the remainder of the colonies. In addition all colonies on a plate of solely the parental clone appeared paler. This control was employed to ensure large amounts of time and resources were not employed on re-selecting the parental clone if it turned out to be the optimum codon. The 3 colonies were picked and separately grown in LB containing 100μg/ml ampicillin. The plasmid DNA of the 3 positive colonies was re-transformed and re-assayed. All three appeared as positive hits once more, however 2 hits turned dark noticeably faster than the other, additionally none of the three hits appeared top be darkening appreciably quicker than the parental control plate.

When sequenced the DNA of the three plasmids were found to contain two different sequences, corresponding to the two different darkening rates observed, as shown in Table 20.

Clone – TESAT3HIT.	246 Codon	Amino acid
1	ATG	Met
2	ATG	Met
3[1]	TTG	Leu

[1] Darkened appreciably slower than both hits 1 & 2 and parental gene *mao6* control plate

Table 20: Codon for position 246 in 3 saturation mutagenesis hits.

ATG, encoding for methionine at the 246 site, appears to be optimum and this explains why the positive hits did not out-perform the control plate – the parental codon encodes for the optimum amino acid at this residue.

To fully explore the 245/246 pair of residues a double saturation mutagenesis experiment is required. This is because the two experiments above only investigate 40 of the possible 400 residue pairings possible. A single Quickchange reaction is all that is necessary to perform a double residue saturation mutagenesis library if the residues are adjacent. A NNS / NNS library is constructed using primers just as for the single site saturation mutagenesis, with two NNS codons present instead of one. Screening is more challenging as V (total possible number of combinations) is increased from 32 for a single amino acid change to 1024 (32^2) for a double amino acid change, thus a 99% probability of representing all possible combinations requires 4695 clones. Unfortunately it was not viable to perform the experiment due to time constraints.

4.5.7 Saturation mutagenesis - Conclusion

The use of a site saturation mutagenesis protocol has lead to the discovery of an enzyme mutant, MAO-6, which has improved catalytic performance against the model hydroxylamine ether **47**. A conservative amino acid change, with respect to

the charge and hydrophobicity properties of the residue, has led to a modest rate enhancement. The improved properties should not be overstated, only a moderate rate enhancement is observed and no broadening of specificity was achieved. The experimental program detailed above does illustrate that the combination of *in silico* modelling and subsequent site directed mutagenesis is a viable and valuable method for addressing enzyme engineering problems.

4.6 Biocatalyst modification – Conclusion

The enzyme engineering program described above has resulted in the identification of a MAO-N variant with moderately improved catalytic properties. A number of hydroxylamine ether substrates are now turned over faster than with the MAO-N5D variant which formed the starting point of the investigation. The substrate specificity has not broadened and activity against hydroxylamines has not been achieved. The MAO-N6 variant does provide a possible catalyst for the deracemisation of hydroxylamine ethers when used in combination with a compatible reduction system.

5 Reduction of oximes

As detailed in Chapter 1, for a successful deracemisation process to be formulated an *in situ*, non-stereoselective counterpart to the stereoselective transformation is required. In most cases this will be a reduction to complement a stereoselective oxidation, although the reverse is possible, for instance in the case of sulfoxide deracemisation.

A number of systems for the reduction of oximes have been described in the literature, and these may be broadly classified into three groups, namely hydride reductions, hydrogenations and others. The reduction reactions result in the formation of one, or a mixture of two, products. The C=N double bond may be reduced to form the hydroxylamine. This is the reaction course necessary for the reduction method to have utility in the hydroxylamine deracemisation systems. The N-O bond may be cleaved, in addition to reducing the C=N bond. This results in an amine as the product of the reaction. Clearly this result renders the oxime reduction system useless, with respect to a deracemisation, thus careful tuning and optimisation of the reduction reaction is critical

5.1 Hydride reductions of oximes

The use of sodium cyanoborohydride to reduce a wide variety of oximes to the corresponding hydroxylamine has been demonstrated in the literature. The yields of these reactions are generally excellent and this method may legitimately be regarded as the standard method of synthesising racemic substituted hydroxylamines.[8] Related borohydrides display a spectrum of activity; sodium triacetoxyborohydride has been reported to be ineffective at reducing oximes[188] whilst sodium borohydride has been reported in the literature to produce the amine as the major product.[9]

Amine:borane complexes have been employed to reduce oximes to hydroxylamines. Pyridine:borane has been used to reduce oximes and *O*-acyl oximes to the corresponding hydroxylamines[10], avoiding over-reduction to amines. It is reported

that a crucial factor is the use of acidic conditions, 10% aqueous hydrochloric acid or 20% ethanolic hydrogen chloride solution being used as the reaction solvent. A number of papers have reported the use of borane:oxazaborolidine adducts to access hydroxylamine products.[189] The product distribution of these reactions demonstrates that careful optimisation is required as a mixture of amine and hydroxylamine products are often observed. A report from Kremiński & Zaidlewicz indicated hydroxylamine to amine ratios ranging from 87:13 to 3:97, dependent on the borane adduct employed.[22]

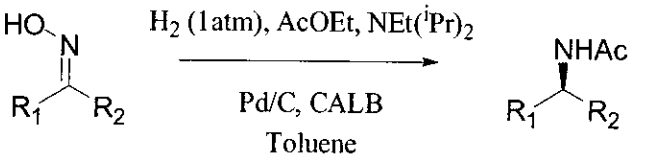
The reduction of oximes to the corresponding amines using hydride reagents has been described in a number of reports. The reagents used include sodium borohydride, lithium aluminium hydride and Superhydride.[9] The scope of the reaction has been extended to include the selective reduction of an oxime to afford single isomer products. The majority of hydride reagents produce amine products and are thus unsuitable for the production of hydroxylamines in high yield.

As demonstrated above, the use of hydride reagents offers a viable route to the reduction of oximes to hydroxylamines. The danger of further reduction to the analogous amines means that a wide survey of possible hydride reduction systems may be necessary to isolate an efficient system.

5.2 Hydrogenation

The use of catalytic hydrogenation to reduce oximes has been reported. The systems employed a metal catalyst and H_2 gas under pressure. PtO_2 catalyst was used to afford a wide range of hydroxylamines in good yield.[11] HCl in ethanol was the solvent system of choice and the products isolated as their oxalate salts. Other reports of the use of hydrogenation reactions suggest that the amine product may be formed. The use of Pd/C, Raney nickel and PtO_2 catalysts to synthesise the amine has been reported.[9] The asymmetric reduction of prochiral oximes has been reported using a lipase/palladium catalysed system. The dynamic kinetic resolution (DKR) of a

number of oximes **77** to the corresponding *N*-acyl amines **78** via the amine intermediate was reported (Figure 66).[190]



77 **78**

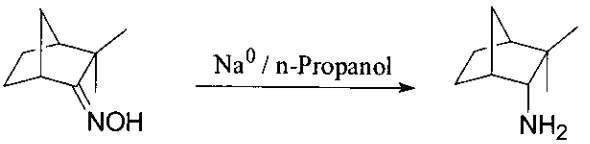
Figure 66: Asymmetric transformation of oximes to *N*-acyl amines.

The Pd/C catalysed reduction formed amines in good yield, no hydroxylamine products were reported. The high reaction yields suggest that the Pd/C catalysed hydrogenation yields amines cleanly.

The literature to date clearly suggests that the catalytic hydrogenation of oximes is possible but the reaction is sensitive, thus formulating a robust system to produce hydroxylamine products and avoid N-O bond cleavage may be difficult.

5.3 Other oxime reduction methods

The use of a dissolving metal reduction has been reported for the reduction of an oxime. Sodium in *n*-propanol reduction of **79** produced **80** in 78% yield, no hydroxylamine product was observed. Literature reports also present the sodium dithionite reduction of oximes.[191] This system results in good yield of the parental carbonyl compound, postulated to occur via the hydrolysis of the N-O cleavage product. This system has little utility for a program requiring the production of hydroxylamine products.



79 endo-80

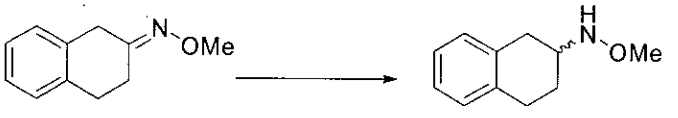
Figure 67: Sodium in *n*-propanol reduction of **79**.

Of more relevance to a program focussing on the synthesis of hydroxylamines is the hydrosilane/H^+ reduction system, reported to reduce a number of oximes to the corresponding hydroxylamines in good yield.[192] Forcing conditions were required for a number of oximes but no significant amine by-product production was reported.

5.4 Results and Discussion

5.4.1 Chemical reductions

A program investigating the reduction of a model oxime, β-tetralone oxime (Scheme 68), was undertaken due to the essential nature of developing a reduction system to compliment the stereoselective oxidation, and thus produce a deracemisation. The crucial factor was to ensure that the reaction conditions would not be detrimental to the oxidation. As the oxidation was envisaged to be enzymatically catalysed the use of reaction conditions that would be tolerated by the biocatalyst was crucial and this was the major focus of the investigation.



58 48

Figure 68: Model oxime reduction.

As demonstrated both in this report and the literature, the use of sodium cyanoborohydride as a reducing agent was found to be very effective in the reduction of oximes. The reaction was typically performed in a methanolic hydrogen chloride

solution, operating at as pH of between 3 and 4. Whilst it would not be expected that the MAO whole-cell system defined as optimum for the biotransformation would be viable in a strongly acidic medium this fact was confirmed before further development work was attempted. A whole-cell resolution as per Chapter 3 was performed in a number of different buffers (Table 21).

Buffer pH	4.1	6.5	7.0	7.5
Conversion at t = 18h[1] / %	0	2	43	48

[1] Maximum conversion for the kinetic resolution is 50%

Table 21: Whole-cell resolution of **48** with varying buffer pH.

As illustrated in Table 21, the whole-cell reaction was inactive at pH 4.1 and the activity displayed at pH 6.5 was not adequate for performing a viable deracemisation as the reaction rate was too slow. It would be expected that the whole-cell system would be the most robust and the most likely to operate under non-optimum pH, however this was confirmed by testing two alternatives, purified MAO-5D and MAO-5D on a Eupergit resin solid-support (Table 22). As predicted neither of these two preparations showed any activity at either pH 4.1 or pH 6. After defining the pH at which the enzymatic portion of the reaction operated, work turned to the nature of the reducing agent.

Buffer pH	4.1		6.5	
Enzyme form	Isolated	Eupergit	Isolated	Eupergit
Conversion at t = 18h / %	0	0	0	0

Table 22: Test of enzyme preparations other than whole-cell.

Four commercially available borohydride reagents were the initial panel of reducing agents tested. Along with sodium cyanoborohydride, sodium borohydride, tributylammonium cyanoborohydride and sodium triacetoxyborohydride were

included in the initial screen, to provide a comprehensive coverage of borohydrides with varying reactivity.

0.1M phosphate buffer pH / **Reducing agent**[2]	8.0	7.0	6.0	5.5	5.0	4.5[1]	4.1[1]
$Na(CN)H_3$	0%	0%	0%	0%	6%	18%	82%
$NaBH_4$	0%	0%	0%	x	x	x	x
$Na(OAc)_3H$	0%	0%	0%	x	x	x	x
$^tBu_3NHB(CN)H_3$	0%	0%	0%	x	x	x	x

[1] 0.1M citrate buffer, [2] 20eq., x = not performed

Table 23: Conversion of oxime **58** using borohydride reducing agents screened in various pH 0.1M phosphate buffers.

As illustrated in Table 23, the use of an aqueous system does not intrinsically prevent the successful reduction of the oxime. Whilst sodium borohydride, known to be unstable in water, was ineffective both sodium cyanoborohydride and triacetoxyborohydride are known in the literature to be active in aqueous solutions. In addition, oximes are also known to be stable in aqueous solution. Thus with both reagent and substrate amenable to use in water it is not surprising that the use of aqueous buffer as the bulk solution is not an impediment. Table 23 also clearly illustrates the fact that the bulk pH is a critical parameter of the reaction. Comparing Table 21 and Table 23 shows a mismatch in the buffer pH conditions for the two components of the deracemisation reaction.

The requirement for a low pH medium for successful reduction of the oxime to hydroxylamine is postulated to be due to the fact that the nitrogen atom needs to be protonated to facilitate the hydride addition. The crucial parameter is thus the pK_a of the nitrogen protonation step and thus the bulk pH required to ensure that protonation occurs; this need not be complete protonation of all the oxime due to the irreversible nature of the reduction. Literature data is available for a limited number of oximes and pKa values range from 0.44 to 1.75 for the protonation of the oxime on the

nitrogen atom.[193] These values clearly indicate that in a solution with a pH compatible with MAO-N activity, close to pH 7, the amount of oxime protonated will be zero.

The contradictory nature of the optimal conditions required for the two 'half reactions' of the putative deracemisation resulted in the requirement that a further survey of possible reduction conditions was required. It was decide that the bulk solvent system of the reduction systems to be tested would be fixed at 0.1M pH 7.0 phosphate buffer. All further investigations were directed at developing a reduction system active under these conditions.

The fact that successful reduction systems were achieved under conditions in which the nitrogen atom is postulated to be protonated, suggested that attempting to develop a pseudo-positive charge on the nitrogen atom may be an avenue to investigate. Coordinating metals to the nitrogen centre may draw electron density from the nitrogen and allow for reduction at neutral pH. A number of metal salts were added to the reaction mixture. It should be noted that the metal salt additives were soluble to varying degrees.

Salt[1]	LiCl	$Ca(OAc)_2$	$MgCl_2.6H_2O$	$In_2(SO_3)_4$	$Ce_2(SO_4)_3$	$Ln_2(SO_4)_3$
Conversion of oxime / %	0	0	0	0	67	0

[1] 5eq.

Table 24: Use of metal salt additives with $Na(CN)H_3$ in attempted reduction of **58**.

As illustrated in Table 24, $Ce_2(SO_4)_3$ appeared to facilitate the successful reduction of oxime **58** to the corresponding hydroxylamine. Close investigation of the reaction solutions, however, revealed that the effect of the Ce salt was to lower the pH of the bulk solution. The buffering capacity of the phosphate buffer had been overcome and an acidic solution formed. This fact was confirmed when the reaction was repeated in

a StatTitrino apparatus. This allowed for pH control and, when the pH was constantly maintained at pH 7.0, no reduction was observed.

The concept of coordination of the oxime to a metal centre to promote reduction at neutral pH was not abandoned after the disappointing results above but developed to attempt to remove the possibility of affecting the bulk pH. In pursuit of this a number of solid supported Ce complexes were tested in an attempt to access a viable reduction system. The rationale was that selected metal sites would be available for coordination to the oxime, allowing the reduction to take place, whilst being fixed on a solid support. This would prevent the bulk solution pH being altered and would allow it to be maintained at pH7. The complexes in question were Ce phosphonates on a silica support.

Name	Ce ion	Loading of Ce ion / mmol /g	Surface area / m^2/g	Particle size / μm
P-12-014c	III	0.8	246	45-125
P-09-014	III	1.1	n/a[1]	25-125
P-04-014b	III	2.5	22	10-45
P-12-004c	IV	0.8	247	45-125
P-09-004X	IV	1.2	495	25-125
P-09-004L	IV	0.8	475	25-125

[1] data not supplied

Table 25: Properties of cerium phosphonate on silica additives.

The catalysts are prepared from cerium salts and disodium ethyl/butyl phosphonate in water, to produce a catalyst containing a mixture of ethyl phosphonate and butyl phosphonate coordinated cerium centres on silica.[194] An initial screen was performed on the range of catalysts available. The preparations were selected to ensure a range of surface areas and Ce loadings were surveyed in case either of these parameters proved critical. The route to prepare these catalysts contains a final stage of washing the solid with water so prior to testing the catalysts they were rigorously washed with distilled water. This additional washing step was performed in an attempt to ensure

that only cerium firmly bound to the solid surface was introduced into the reaction mixture. Table 26 illustrates the results of this initial screen.

Name	Conversion at t=19h
P-12-014c	0%
P-09-014c	0%
P-04-014b	0%
P-12-004c	>99%
P-09-004X	>99%
P-09-004L	0%

Table 26: Cerium phosphonate on silica additives used in attempted reduction of **58**.

The results for both P-12-004c and P-09-004X look promising upon first inspection. Mindful of the erroneous conclusions that the use of the cerium salt had lead to earlier, an inspection of the bulk solvent pH at the conclusion of the reaction was performed. The solution appeared acidic when analysed by universal indicator pH paper. The use of a StatTitrino system, as above, demonstrated that by maintaining the pH at 7.0 the reaction was suppressed and no conversion to the hydroxylamine was observed. It would appear that, even with rigorous water washing, the bulk pH is altered with the addition of the cerium phosphonate - silica catalysts.

At this juncture it was decided to attempt a different solid-phase strategy. Two resins containing acidic sites, Dowex-MAC and Dowex 50WX2-400, were separately added to a 0.1M pH7 phopshate buffer solution of the substrate **58** and the pH of the bulk solvent was monitored. Initially this experiment was performed in the absence of reducing agent to assess whether it would be a viable method of providing acidic coordination sites in the experiment without affecting the bulk pH. The bulk pH was found to become acidic, even after rigorous washing of the resins before use. This concept was therefore not used to attempt a reduction reaction.

The results outlined above suggested that the pursuit of a reduction system using sodium cyanoborohydride as the reducing agent would not be effective. Alternative methods of reducing the model oxime **58** were investigated. The next avenue followed was to consider whether a hydrogenation system may be effective. The use of H_2 gas was dismissed early in the investigation. The reason for this was the focus of the project upon constructing a deracemisation protocol. As demonstrated earlier, the use of a MAO expressing whole-cell system was found to be optimal for the stereoselective oxidation portion of the deracemisation. The requirement that this system has for O_2 means that it would be incompatible with a system requiring gaseous hydrogen. Major safety issues arise when a reaction vessel requires both O_2 and H_2 gas simultaneously supplied and no firm basis of safety can be achieved. An alternative to the use of gaseous hydrogen in a catalytic hydrogenation reaction is to employ a catalytic transfer hydrogenation (CTH) system (Figure 69). This alleviates the danger posed by gaseous hydrogen and has been shown to be effective for the catalytic hydrogenation of a number of species in the literature. A crucial factor is that transfer hydrogenation systems have been reported which employ water as the bulk solvent, one of the core requirements for success in this case.[195]

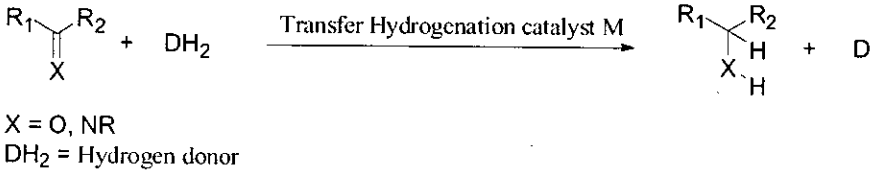


Figure 69: General scheme for Catalytic Transfer Hydrogenation reactions.

The hydrogenation catalysts tested were of the metal on carbon type. These metal catalysts have a long history of use in catalytic hydrogenation reactions and are easily available.[196] They exhibit good stability on the shelf and are easily handled in the laboratory. They have also been employed in transfer hydrogenation reactions to good effect.[197] In addition to considering the catalyst, the second key component of a transfer hydrogenation reaction is the hydrogen source. A number of different hydrogen sources are available. The classic reagent used is an azeotropic mixture of triethylamine and formic acid, found to be a 5:2 ratio.[198] This mixture has been

reported to be effective for the catalytic transfer hydrogenation of amines amongst other substrates. Wu *et al.* reported that in some circumstances formic acid alone is more effective than the triethylamine:formic acid mixture.[195] Both these hydrogen sources have been tested. Ammonium formate is a variation on the use of a formate compound and has also been used successfully as a hydrogen source.[199] Finally sodium hypophosphite was tested, a hydrogen source that was specifically chosen not to be formate based, to provide variety to the screen.[200]

Catalyst / **Hydrogen source**	Pd / C JM117 paste	Pt / C JM87L powder	Ru / C JM619 powder
HCO_2H	Amine	Amine	Amine
$Et_3N : HCO_2H$	Amine	Amine	Amine
NH_4CO_2H	Oxime	Oxime	Amine
NaH_2PO_3	Oxime	amine: oxime (50/50)	Oxime

Table 27: Major product of attempted reduction of **58** via transfer hydrogenation reactions.

As illustrated in Table 27, two different results were observed with the transfer hydrogenation reaction. The fact that the amine product was observed in a number of cases indicated that a reduction occurs, but N-O bond cleavage is the effect observed. On other occasions starting material is recovered. At this juncture it was decided to pursue this line of enquiring a little further with the discovery that a Pt / C based catalyst was available which was specifically advertised as being suitable for the hydrogenation of oximes to hydroxylamines, without N-O bond cleavage to the amine, albeit in a gaseous hydrogen system.[201] This catalyst was a sulfur poisoned version of a standard Pt/C catalyst. Once again a number of different hydrogen sources were used but no conversion was observed.

A number of other metal / carbon catalyst were available, however they differed only in terms of loading and surface area with respect to the catalysts previously tested. No indication of the desired selectivity had been observed and so this area of investigation was discontinued.

The use of an amine:borane system was investigated to establish whether this may be a viable method for the reduction of the model oxime under the desired conditions. A number of amine:borane complexes, both commercially available and synthesised within the research group[202] were tested (Figure 70).

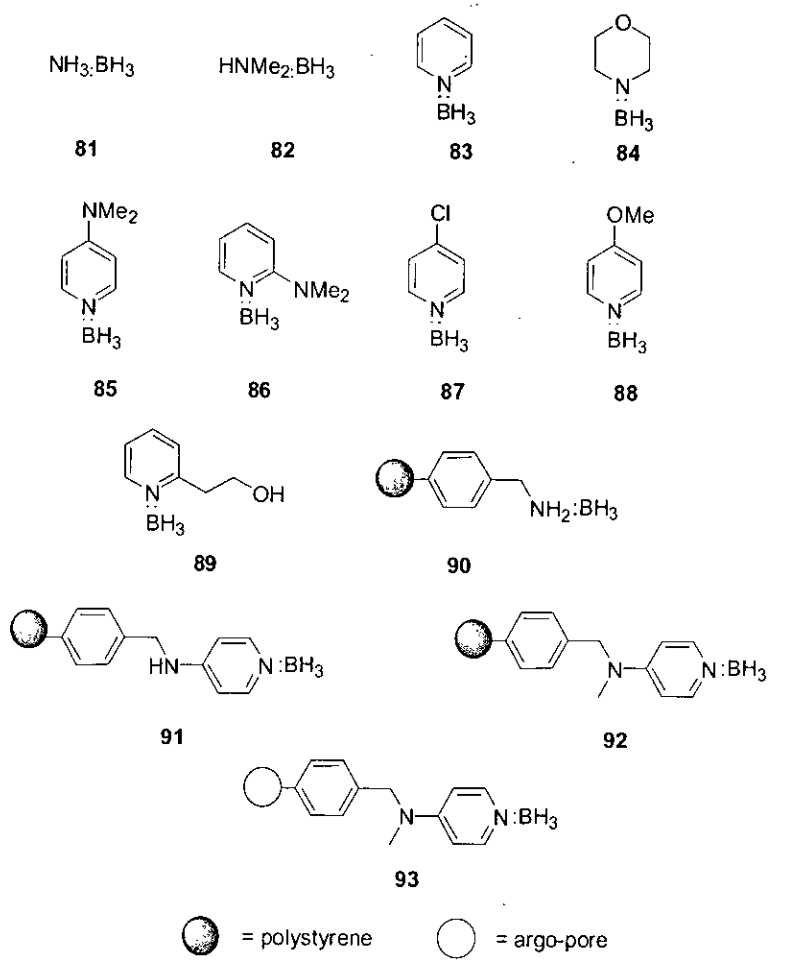


Figure 70: Amine:borane reducing agents employed in attempted reduction of **58**.

Unfortunately the use of an amine:borane complex was found to be completely unviable for the reduction of the model oxime to the corresponding hydroxylamine in pH 7.0 aqueous buffer. No indication of any reduction products was observed upon analysis by GC-MS and NMR and starting material was recovered. This was a disappointment but, considering the previous work and the conditions employed in literature reports, not a surprise.

In conjunction with the research program studying laccases (Chapter 2) a number of hydroxylamine reductions were tested using conditions amenable to laccase activity. In the context of a deracemisation, what was considered was that an enantioselective oxidising mediator could be combined with a non-selective reduction. The coupling of the two 'half-reactions' in one-pot would not be viable if both reactions were in the solution phase. This is because a 'short circuit' could occur, with the oxidising agent and reducing agent forming a redox cycle between themselves, excluding the substrate. This is the result of the fact that the substrate oxidation catalyst is not located within the enzyme active site but is in solution (Figure 71).

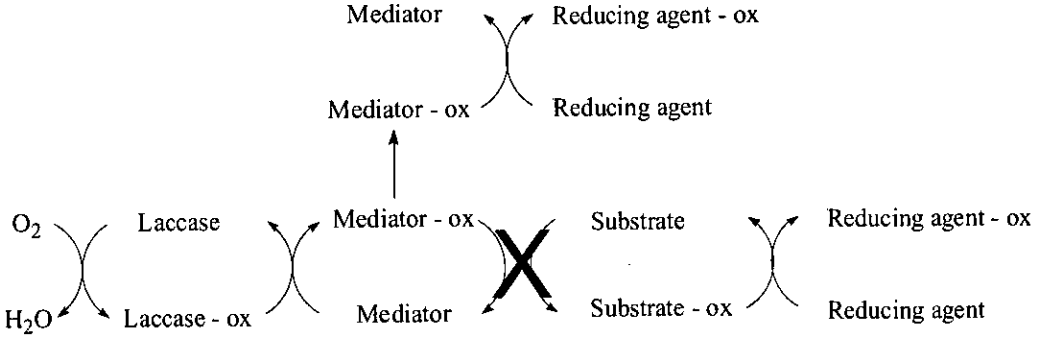


Figure 71: Possible 'short-circuit' cycle excluding substrate.

A manner of addressing the 'short circuit' problem would be to mount both reducing and oxidising agent on a solid-phase. If a solid-phase reducing agent could be found to reduce the oximes derivatives to hydroxylamine derivatives under the same conditions then it may be possible to construct a deracemisation system. The solid-supported amine:boranes tested above were also tested for activity under the conditions suitable for laccase / TEMPO oxidations. Unfortunately the reducing

agents in question were not active. No suitable solid-phase reducing agent could be discovered. Whilst sodium cyanoborohydride was found to be effective for the reduction of **60** under the same conditions as required for the oxidation, the commercially available solid-phase analogue was found to be inactive. A number of solid supported amine:boranes **96-99** were available within the group[202] and these were also tested (Figure 72). They were found to be ineffective as reducing agents upon testing.

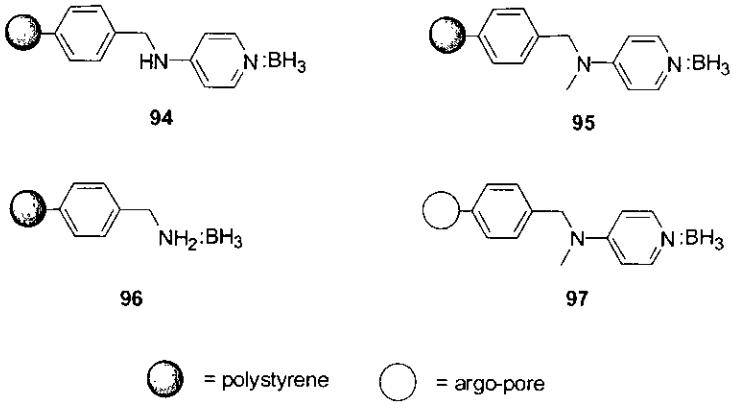


Figure 72: Solid-supported amine:boranes tested under laccase compatible conditions.

The lack of a suitable solid-supported reducing agent coupled with the anticipated difficulty in accessing single enantiomer solid-supported mediator led to the decision to discontinue this line of investigation.

At this stage a thorough assessment of oxime reduction systems and their limitations was conducted. The results described above indicate that standard oxime reduction systems are not active in pH neutral aqueous conditions and that this appears to be the crucial limiting factor. It may be speculated that systems that would operate successfully in these conditions are most likely to be biocatalytic systems. The investigative program was thus modified to focus on biocatalytic systems, in particular the use of baker's yeast.

5.5 Biocatalytic reduction of oximes

5.5.1 Baker's yeast

The use of a biocatalytic system has been reported for the reduction of oximes. In the majority of cases these experiments have been associated with investigations into drug metabolism within the human body. The reports regarding these hepatic enzymes suggest that reduction to the amine product is the pathway observed.[203] Aside from this area of exploration little has been reported in the area of hydroxylamine synthesis via biocatalytic reduction of oximes. An intriguing area which has received a little attention is the use of Baker's yeast preparations to affect the reduction of oximes.

Baker's yeast (*Saccharomyces cerevisiae*) has of course been used for culinary purposes for thousands of years. The use of Baker's yeast as a synthetic tool within the chemistry laboratory also has a long and well documented history.[204] Baker's yeast has been reported to be a useful reagent in the reduction of a number of ketones, β-ketoesters and α,β-unsaturated systems.[205,206] The synthetic utility of Baker's yeast is enhanced by its ease of use. Baker's yeast preparations are easily available, robust and are simple to store and handle. In the context of this report Baker's yeast represents an attractive option due to the fact that reported reaction conditions are usually similar to those found to be optimum for the MAO whole-cell preparation (Chapter 3) in addition to the reasons detailed above.

Baker's yeast reduction of a limited number of oximes through to amines has been reported in a short paper.[24] Fermenting baker's yeast was used to produce a small number of enantioenriched amines in moderate enantiomeric excess. The use of baker's yeast to reduce oximes was further developed in a paper from Chimni & Singh.[207] This contains a report of a single oxime substrate being reduced to the corresponding hydroxylamine. A range of reaction conditions were described, only one of which produced the hydroxylamine product and no yields were presented in the paper. The use of a baker's yeast reduction system for oxime substrates is not

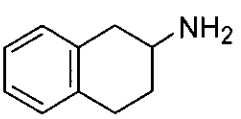
well described; however it has the attractive feature of being most likely to require conditions complementary to the use of an enzymatically catalysed stereoselective oxidation.

5.5.2 Baker's Yeast Results and Discussion

The series of reactions performed with Baker's yeast were performed under strict pH control to ensure that the solutions were maintained at pH 7.0. The reduction of oxime **58** was used as a model experiment. Baker's yeast (Sigma Chemicals) was tested under both fermenting and non-fermenting conditions (Table 28).

Conditions	Time	Reaction products observed
Glucose 1/1 Baker's yeast (w/w)	1 day	Oxime **58**
Glucose 1/1 Baker's yeast (w/w)	5 days	Amine **98** 85%:15% hydroxylamine **48**
Glucose 0.1/1 Baker's yeast (w/w)	1 day	**58**
Glucose 0.1/1 Baker's yeast (w/w)	5 days	**58**
No glucose	1day	**58**
No glucose	5 days	**58**

Table 28: Baker's yeast reduction of oxime **58**.



98

Figure 73: Amine product observed in fermenting conditions.

As can be seen it would appear that Baker's yeast under fermenting conditions does possess an oxime reduction activity. In an attempt to define conditions under which

the production of the desired hydroxylamine would be optimised, further experiments were performed.

Repeated attempts to isolate hydroxylamine product were frustrated by work-up difficulties. Two courses of action were followed to address this fact. Firstly; an attempted deracemisation experiment was performed, in an attempt to produce single enantiomer product and address the work-up issues of the final constructed deracemisation system. The MAO whole-cell system (Chapter 3) was coupled with the Baker's yeast conditions and an inspection of the reaction products performed. No desired hydroxylamine product **48** was observed.

The difficulties in work-up were exacerbated when the two biocatalytic systems were combined. Filtration of the biomass was extremely difficult and lengthy. Attempts to remove the solid biomass via centrifugation were only marginally more successful, with interfacial separation of the extractive organic solvent and aqueous buffer being poor. In an attempt to address these difficulties both the *E.coli* expressing MAO and the Baker's yeast were subjected to a protocol to entrap them within a calcium alginate bead. This modification had been reported to alleviate work-up difficulties without removing reductive activity of Baker's yeast.[208] The attempted reduction of oxime **60** with the calcium alginate entrapped Baker's yeast was then performed. No reduction was observed.

Disappointingly the discovery and optimisation of a biocatalytic reduction method to complement the biocatalytic stereoselective oxidation was not achieved.

5.6 Conclusion

The attempted reduction of oximes proved to be unsuccessful. This was wholly due to the stringent requirements placed upon the development of such reactions by the biocatalyst system previously developed (Chapter 3). Critically, the requirement for a aqueous pH7 bulk reaction medium prevented a successful reduction reaction. This,

in turn, signals the failure of the attempt to define and optimise a deracemisation protocol for hydroxylamines and hydroxylamine ethers.

6 Conclusion and Further Work

The results presented herein represent a concerted and lengthy experimental programme focussed towards producing a deracemisation system for hydroxylamines. A number of different avenues have been explored. An ambitious attempt to engineer the MAO-N enzyme which displayed no activity towards the substrates of interest was made. The rationale behind this was the close relationship between the amines known to be substrates and their hydroxylamine analogues.

To address the difficulties posed by the unfavourable results of initial experiments two separate courses of action were undertaken. The modification of the substrates being studied led to success in discovering novel substrates for the MAO-N enzyme. The enantioselective oxidation of hydroxylamine ethers by MAO-N is a novel discovery. A further development is the engineering of a MAO-N variant which displayed improved catalytic activity for a model hydroxylamine ether substrate compared with the parental enzyme.

A second research programme lead to the discovery of the applicability of the well documented laccase:mediator system to the oxidation of hydroxylamines. This represents a further novel discovery. A robust and simple biocatalysed process has been defined.

Research into the reduction of oximes in a pH neutral, aqueous environment has also been performed. This area has had no success. A number of possible techniques and conditions have been attempted but the problem remained intractable.

The difficulties in discovering and defining two systems, a stereoselective oxidation and non-selective reduction, which operate efficiently and effectively with the same bulk reaction conditions, has resulted in a disappointing failure to perform a deracemisation of a hydroxylamine substrate.

Possible future courses of action to develop a successful deracemisation include the discovery and optimisation of novel enzymes which stereoselectively convert hydroxylamines. Enzymes have been reported that accept hydroxylamines as substrates, however the synthetic utility of them remains to be investigated[3]. These, or new, enzymes may exhibit characteristics which are better suited to the task than those currently available, perhaps having greater stability in low pH solutions. The increased access to extremophile derived enzymes may provide opportunities in the future[209]. The discovery of oxime reducing systems which use pH neutral conditions would also herald a better chance of a breakthrough.

7 Experimental

Buffers and growth media are made up as per Molecular Cloning: A laboratory manual, 2nd Ed., J. Sambrook, E.F. Fritsch and T. Maniatis, 1989, Cold Spring Harbor, Cold Spring Harbor Press.

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC250 instruments. Chemical shifts are reported in parts per million (ppm) and coupling constants in Hertz (Hz). Residual protic solvent present in the deuterated solvent was used as the internal standard in NMR experiments.

Electron impact (EI) nominal mass spectra were recorded on a Kratos Profile mass spectrometer. EI accurate mass and fast atom bombardment (FAB) nominal and accurate mass spectra were recorded on a Kratos MS50 spectrometer.

IR spectra were obtaining using a Jasco FT-IR 460Plus spectrophotometer. Frequencies are reported in wavenumbers (cm^{-1}). Compounds were measured as neat thin films or as Nujol mulls

Analytical t.l.c. was performed on aluminium backed Merck 254nm active M_{60} silica plates. Ultraviolet light at 254nm or permanganate solution were used as developing agents. Column chromatography was performed on M60 silica, size 20 – 40.

Experiments performed with maintenance of the bulk pH were performed in a Metrohm 718 STAT Titrino system.

Acetophenone oxime **50** and *N,N*-dibenzylhydroxylamine **60** were purchased from Sigma Aldrich. All reagents were purchased from Aldrich Chemical Company and used as supplied unless otherwise noted. Solvents were HPLC grade, purchased from Fisher Scientific and used as supplied unless otherwise noted. Laccase preparations were purchased from Sigma Life Sciences.

7.1 Buffer and reagent recipes

LB medium

a) In 1 litre of ultrapure water: 20g of pre-formulated LB medium powder.

b) In 1 litre of ultrapure water: tryptone (10g), NaCl (10g) and yeast extract (5g).

LB agar medium

a) LB medium (100ml) and agar (1.5g).

SOC media

In 1 litre of ultrapure water: yeast extract (5g), tryptone (20g), 5M NaCl (2ml), KCl (0.19g), 1M $MgCl_2$ (10ml), 1M $MgSO_4$ (10ml) and *D*-glucose (3.6g).

SDS loading buffer

0.5M Tris/HCl pH 6.8 (2ml), glycerol (2ml), 10% (w/v) SDS (3.2ml), β-mercaptoethanol (0.8ml) and 0.05% (v/v) bromophenol blue (0.4ml).

SDS gel running buffer 5x

In 50ml: Tris (15.1g), glycine (94g) and 10% w/v SDS.

SDS gel staining solution

Methanol (450ml), acetic acid (100ml), water (450ml) and Coomassie blue G-250 (500mg).

SDS gel destaining solution

Methanol (450ml), acetic acid (100ml) and water (450ml).

Bradford reagent

Coomassie blue G-250 (100mg), ethanol (50ml), 85% phosphoric acid (100ml) and ultrapure water (850ml).

TAE (Tris acetate EDTA buffer) 50x

Tris base (242g), acetic acid (57.1ml) and 0.5M EDTA pH 8.0 (100ml).

7.2 Transformation and fermentation

Transformation of *E. coli* TOP10 chemically competent cells

Plasmid DNA and a single shot of *E. coli* TOP10 chemically competent cells (Invitrogen C4040-10) were thawed on ice. 1µl of the plasmid DNA was added to the shot and the DNA mixed by gentle tapping before returning the tube to ice for 30 minutes. The sample was then placed in a 42°C water bath for 30 seconds and quickly placed back on ice. After 2 minutes on ice 500µl of pre-warmed SOC media was added and the sample was incubated at 37°C, 225rpm for 1 hour. 100µl of the transformation mixture was spread onto a LB agar plate containing 100µg/ml ampicillin and the plate left to grow overnight at 37°C.

Transformation of *E. coli* BL21 (DE3) chemically competent cells

Plasmid DNA and a single shot of *E. coli* BL21 (DE3) chemically competent cells (Invitrogen C6000-03) were thawed on ice. The procedure for TOP10 cells was then followed.

Transformation of *E. coli* XL1-red chemically competent cells

Plasmid DNA and a single shot of *E. coli* XL1-red chemically competent cells (Stratagene 200129) were thawed on ice. 1.7µl of β-mercaptoethanol was added to each shot and mixed by gentle tapping. 1µl of the plasmid DNA was then added and mixed by gentle tapping. The tube was returned to ice for 30 minutes. The sample was then placed in a 42°C water bath for 45 seconds and quickly placed back on ice. After 2 minutes on ice back on ice the sample had 940µl of pre-warmed SOC media added the sample incubated at 37°C, 225rpm for 1 hour. 100µl of the transformation

mixture was spread onto a LB agar plate containing 100µg/ml ampicillin and the plate left to grow overnight at 37°C.

Creation of *E. coli* XL1-red random mutation libraries

The remaining 900µl of *E. coli* XL1-red transformation mixture (above) was added to 9.1ml of LB containing 100µg/ml ampicillin and grown at 37°C, 225rpm overnight. 1ml was removed and the plasmid DNA purified. This DNA constituted the first cycle in preparing the random mutageneisis DNA library. This 1st cycle DNA was retransformed into *E. coli* XL1-red and the process repeated giving the second cycle of variants. This protocol was repeated four times to give a DNA library generated from four cycles. This pool of DNA constituted a random mutagenesis library.

Fermentation of *E. coli* BL21 (DE3) expressing MAO-N

A number of different protocols were employed depending on the scale of fermentation required. Interestingly, in all cases IPTG induction was found to be unnecessary, although the pET 16b plasmid used is designed to require IPTG expression.

Small scale expression

A single colony from a fresh transformation was picked and added to 10ml of LB medium containing 100µg/ml ampicillin in a 50ml falcon tube. The culture was left to grow overnight at 30°C. The cells were harvested by centrifugation at 10,000rpm for 15 minutes. The resultant supernatant was discarded and the cell pellet stored at -20°C. A typical fermentation yielded 0.5g of cells.

Medium scale expression

A single colony from a fresh transformation was picked and added to 10ml of LB containing 100µg/ml ampicillin in a 50ml falcon tube. The culture was left to grow at 30°C until OD_{600nm} was between 0.6 and 1.0. A number of 1 litre baffled flasks containing 200ml of LB medium containing 100µg/ml ampicillin were inoculated with 200µl of the starter culture. The fermentation was left to grow for 24 hours at 30°C, 225rpm. The cells were harvested by centrifugation at 6000rpm for 15 minutes at 4°C. The resultant supernatant was discarded and the cell pellet stored at -20°C. A typical fermentation yielded 15g of cells.

Large scale expression

A single colony from a fresh transformation was picked and added to 100ml of LB medium containing 100µg/ml ampicillin. The cells were left to grow at 30°C, 225rpm until OD_{600nm} was between 0.5 and 0.9. At this point the 100mL fermentation was added into a 10 litre batch fermentor containing 10 litres of LB medium containing 100µg/ml ampicillin. The cells were grown at 30°C, 225rpm paddle stirring overnight. The cells were harvested by centrifugation at 6000rpm for 15 minutes at 4°C in a Sorval RC5C refrigerated centrifuge. The resultant supernatant was discarded and the cell pellet stored at -20°C. A typical fermentation yielded 100g of cells

Preparation of CFE

The cell pellet was thawed on ice and resuspended in the desired buffer at 5g/mL concentration of cells in buffer. The sample was sonicated at 10 micron amplitude on ice using a 30secs on 30secs off cycle. This cycle was repeated 5 times. The resulting homogenate was centrifuged at 20,000rpm for 1 hour at 4°C. The resultant supernatant was retained and the cell pellet discarded. The CFE was stored on ice prior to use.

7.3 DNA purification and manipulation

Plasmid purification

1ml of fermentation broth was spun using a microcentrifuge at 12,000 rpm for 10 minutes and the resultant supernatant discarded. The manufacturer's instructions were then followed (Qiagen, QIA prep Spin Miniprep kit protocol). The purified plasmid DNA was stored at -80°C.

DNA agarose gel

DNA agarose gels were prepared by addition of agarose (0.4g) to TAE x 1 buffer (50ml). The mixture was melted and the molten agarose was left to cool by stirring in a 50C water bath. Ethidium bromide (10mg/ml) was added (2.5µl). The gel was poured into a gel tray and a loading comb inserted.

To each of the DNA samples (18µl) to be run was added Blue Juice x 10 (2µl) (New England Biolabs). Each sample was loaded into a lane, as was a pre-stained DNA ladder (New England Biolabs) for reference purposes. The gel was run and visualised under UV irradiation.

DNA gel extraction

The desired DNA band as visualised by UV irradiation, was cut from the rest of the gel and weighed. The DNA was recovered from the gel following the manufacturer's instructions (Qiagen QIAquick gel extraction kit protocol). The recovered DNA was stored at -80°C.

DNA sequencing

Plasmid DNA was prepared as below in a suitable PCR tube:

Oligonucleotide primer (3.2pmol)	1µl
ABI Prism BigDye Terminator v3.1 cycle sequencing reaction kit	4µl
Plasmid DNA template	250ng
Ultrapure water to make up to final volume of:	20µl

To sequence the MAO-N gene five primers were employed. A forward and a reverse primer designed to sequence from each end of the gene within the pET 16b plasmid (Novagen). Three internal primers were also employed (design by T. Fleming, University of Edinburgh). These five separate sequencing reactions ensured coverage of the entire gene.

Novagen T7 promoter primer #69348-3
Start site
5' taatacgactcactataggg 3'

mao-N(169)
Start site at MAO-N 169 nucleotide
5' gacttgactgtagcaggcttc 3' (21 mer 52%GC)

mao-N(512)
Start site at the 512 nucleotide
5' cgcatgacatgttctatgttcctgagt 3' (32 mer 46%GC)

mao-N(830)
Start site at the 830 nucleotide
5' gtccggttaggagtgttgttaatgagagag 3' (30 mer 46% GC)

Novagen T7 terminator primer #69337-3

3'-end of the gene

5' gctagttattgctcagcgg 3'

The PCR was carried out on an Eppendorf mastercycler using the program below

1. 95°C	1 minute
2. HOLD	
3. 96°C	30 seconds
4. 50°C	30 seconds
5. 60°C	4 minutes
6. Repeat steps 3-5,	30 cycles
7. 4°C	HOLD

Samples were made up to a final volume of 20µl with ultrapure water and the sequenced by the ICMB automated sequencing service, University of Edinburgh.

Saturation mutagenesis library creation

The two oligonucleotide primers containing the NNS site were made up to 100pmol/µl with water and the reaction mixture below prepared:

Reaction buffer 10x (Stratagene 600153-82)	5µl
Plasmid DNA containing *mao5D* gene (33ng/µl)	1µl
Oligonucleotide primer – forward NNS	1µl
Oligonucleotide primer – reverse NNS	1µl
dNTP mix (Sigma D-7295)	1µl
Water	20µl

Pfu Turbo DNA Polymerase (1µl) (2.5U/µl) (Stratagene 600250-52) was added as the final step. The PCR was carried out in the following manner using an Eppendorf mastercycler:

1. 95°C	1 minute
2. 95°C	1 minute
3. 55°C	1 minute
4. 68°C	7 minutes
5. Repeat steps 2-4,	16 cycles
6. 4°C	HOLD

The PCR product was taken forward to the next stage of nick repair.

The crude PCR product had DpnI restriction enzyme (1µl) (New England Biolabs R0176S) added and the reaction mixture gently pipetted. The mixture was then spun for 1 minute at 12,000 rpm in a microcentrifuge. The reaction mixture was then incubated at 37°C for 1 hour.

The digested PCR product (1µl) was then added to *E. coli* XL1-blue chemically competent cells (Stratagene) on ice. The tube was gently tapped and returned to ice for 30 minutes. The tube was then placed in a water bath at 42°C for 45 seconds and then returned to the ice. Pre-warmed SOC media (500µl) was added to each tube and the samples were placed at 37°C, 225 rpm for 1 hour. 100µl of *E. coli* saturation library transformation was spread onto LB agar plates containing 100µg/ml ampicillin. And the plates incubated at 30°C overnight. After ensuring that there was a high enough number of transformants to ensure that all library members were fully represented a random selection of colonies were picked. They were grown overnight in LB medium (10mL) containing 100µg/ml ampicillin at 37°C, 225 rpm and the plasmid DNA isolated. The plasmid DNA was sequenced with the appropriate primer as above.

7.4 Assay methods

Solid phase assay

The DNA sample to be screened was transformed into *E. coli* BL21 (DE3) competent cells. Unlike a standard transformation, a nitrocellulose membrane (Amersham) was placed on top of the agar in the pertri dish. The transformation was spread over the membrane and the plate incubated at 37°C overnight to allow the colonies to develop. The membrane was then removed and stored at -20°C. The optimal storage time at -20°C was 48 hours. The membrane was allowed to warm up to ambient room temperature before performing the assay.

The following chromogenic solution was prepared; 2 DAB tablets (Sigma, D4418), 0.1M potassium phosphate pH 7.6 (80ml), HRP 1mg/ml (24µl) and 10mM of screening substrate. 2% (w/v) agarose in ultrapure water was melted and then cooled to 50°C in a water bath. 10ml of the chromogenic solution was rapidly mixed with 10ml of the molten agarose and immediately poured over the thawed nitrocellulose membrane. The assay was then left to develop at room temperature.

Liquid phase assay

The following chromogenic solution was prepared; 1M aminoantipurine (37.5µl), 2% (w/v) TBHBA in DMSO (500µl), 10mM of screening substrate, 1M potassium phosphate buffer pH 7.6 (5ml) and ultrapure water to give a total volume of 50ml.

5µl of 1mg/ml HRP was added to 10µl of the purified MAO-N protein sample in a well of a microtitre plate. To this was added 185µl of the chromogenic solution giving a total volume of 200µl. The colourimetric response was monitored by the 510_{nm} absorbance with a plate reader (Molecular Devices, Versa Max tunable microplate reader) at 30°C. The activity of the MAO-N sample towards the substrate was calculated from rate of change of the 510_{nm} absorbance.

The procedure above can also be performed employing CFE in place of purified protein. The relative rates of substrates to the control substrate **33** were obtained by performing the assay above in parallel on the same plate using the same protein sample.

The specific activity was determined by dividing the activity measured on the assay by the measured soluble protein concentration from a Bradford assay of the same protein sample, using the equation below

$[(\text{Activity}/(\varepsilon \times L)) \times 20 \times 60 \times 1\times10^{3}]/P = \text{Specific Activity } \mu\text{mol min}^{-1}\text{mg}^{-1}$

Activity	Measured rate (OD/sec)
ε	Extinction coefficient of colourimetric product, 29,400 M^{-1} cm^{-1}
L	Path length (cm)
P	Protein concentration based on Bradford assay (mg/ml)

The path length for a 200µl volume in a the U-shaped 96-well microtitre plate employed for all the experiments above was measured to be 0.53cm.[101]

Michaelis-Menten kinetic calculations

A chromogenic solution was prepared with a final substrate concentration of 50mM substrate and serially diluted to produce solutions with substrate concentrations of 20, 10, 8, 6, 4, 2, 1, 0.6, 0.2, 0.1 mM and 50, 20µM. Each solution was assayed as above using freshly prepared protein samples to ensure any loss of activity on storage of the protein was minimal.

The calculated specific activities were entered into the GraphPad Prism 4 program. Using the Michaelis-Menten function V_{max} (µmol/min/mg) and K_M (mM) were obtained. The k_{cat} was subsequently calculated by multiplication of V_{max} by the molecular weight of MAO-N (55.6 kDa).

7. 5 MAO-N purification techniques

Nickel affinity purification

Gravity flow system

A cell pellet (1g) from a MAO-N fermentation was thawed on ice and resuspended in 10mL of Ni-NTA resuspension buffer (25mM Tris/HCl pH 7.8, 300mM NaCl, 1mM PMSF and 1mM β-mercaptoethanol). The sample was subjected to sonication and centrifugation as per CFE preparation (Section 6.3) to produce the CFE. Ni-NTA slurry (Qiagen 30210, 10ml) was applied to a sintered resin column and equilibrated with Ni-NTA resuspension buffer (30ml) at 4°C. The CFE was applied to the top of the column and passed through the column under gravity. The column was washed with wash buffer (Ni-NTA resuspension buffer with 60mM imidazole added, 30ml). The protein was then eluted with elution buffer (Ni-NTA resuspension buffer with 200mM imidazole added, 10ml). The eluent was collected in 1mL fractions and the MAO-N activity for each assayed. The active fractions were pooled and then stored as 1ml aliquot at -80°C.

FPLC system

A cell pellet (1g) from a MAO-N fermentation was thawed on ice and resuspended in 10mL of Ni-NTA resuspension buffer (25mM Tris/HCl pH 7.8, 300mM NaCl, 1mM PMSF and 1mM β-mercaptoethanol). The sample was subjected to sonication and centrifugation as per CFE preparation (Section 6.3) to produce the CFE. A 25cm Ni affinity column operated on an UPC-900 Amersham pharmica biotech FPLC and the column equilibrated with Ni-NTA resuspension buffer (5 column volumes) at 6°C. The CFE was injected onto the column. The column was washed with wash buffer (Ni-NTA resuspension buffer with 60mM imidazole added, 10 column volumes). The protein was then eluted with elution buffer (Ni-NTA resuspension buffer with 200mM imidazole added, 10 column volumes). The eluent was automatically

collected in 1mL fractions and the MAO-N activity for each assayed. The active fractions were pooled and then stored as 1ml aliquot at -80°C.

PD10 desalting column - removal of imidazole

An Amersham Bioscience PD-10 desalting column was equilibrated with 25ml of 0.1M potassium phosphate buffer pH 7.0. 2.5ml of the sample from nickel affinity purification was loaded onto the top of the column and the flow through discarded. 3.5ml of the phosphate storage buffer was applied to the top of the column and the flow through collected in 1mL fractions.

Ammonium sulfate precipitation – removal of imidazole

A sample from nickel affinity purification was placed onto ice on a stirrer plate. Ammonium sulfate was added to the stirred solution in a stepwise fashion until 60% ammonium sulfate saturation (36.6g/100ml). The sample was centrifuged at 4000 rpm for 20 minutes affording a yellow pellet. The supernatant was discarded and the MAO-N protein pellet was resuspended in 0.1M potassium phosphate buffer pH 7.0.

SDS PAGE protein gel

Analysis of the purity of protein and MAO-N expression samples was done using SDS PAGE. Precast gels were purchased (SDS Biorad 10-15% polyacrylamide gel) and New England Biolabs markers (P77085) were used as with the molecular weights of the standard proteins being 175, 83, 62, 47.5, 32.5, 25, 16.5 and 6.5 kDa. 20µl of the protein sample was mixed with 20µl of the loading buffer, boiled for 1 minute and centrifuged at 13,000 rpm for 1 minute. Each sample was loaded in a lane of the SDS PAGE gel and the gel was placed into the gel apparatus (Biorad) and submerged in SDS gel running buffer. The electrophoresis was carried out at 200V for approximately 75 minutes. The gel was placed into staining solution for 60 mins and then destained overnight to develop the bands.

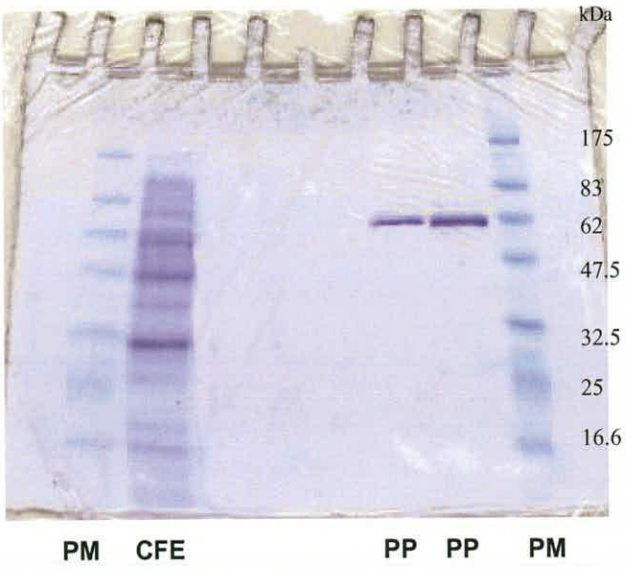


Figure 74: Example of a SDS polyacrylamide gel of fractions from nickel affinity chromatography purification; molecular weight protein markers (PM), Cell-free extract (CFE) and fractions from nickel affinity chromatography (PP).

The Bradford assay

Protein concentrations were determined using the Bradford assay. The protein sample (20µl) had pre-mixed Bradford reagent (980µl) added. The sample was left to stand for 2 minutes and then the OD_{595nm} was measured in triplicate on a 96-well microtitre plate using a plate reader. The protein concentration was obtained by comparison with a calibration curve derived from known concentrations of BSA. Bradford reagent (980µl) was added to a known BSA concentration sample (20µl) (BSA standard concentrations; 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/ml). The sample was left to stand for 2 minutes and then the OD_{595nm} measured in triplicate (200µl) on a 96-well microtitre plate using a plate reader. A straight line of best fit was fitted to the plot of BSA concentration against OD_{595nm}.

Estimation of error in the Bradford assay

A purified protein sample was measured six times by the Bradford assay method. The mean OD_{595nm} for the six measured samples was found and the standard deviation of the data calculated. The 95% confidence bounds were calculated from the mean ± 2 standard deviations from the mean. Using the upper limit and lower limits, a V_{Max} range was determined.

UV-visible spectrum of MAO-N

The purified MAO-N sample was placed in a 1ml quartz cuvette and the UV-visible spectrum measured in a Perkin Elmer instrument, Lambda 900, UV/VIS/NIR spectrometer. The spectrometer was zeroed against 0.1M potassium phosphate buffer pH 7.0 and then the UV-visible spectrum recorded from 300-750nm.

7.6 Formation of Calcium alginate beads

Sodium alginate (9g) is added to growth medium (300mL) portion wise to prevent clumping and stirred until completely dissolved. Wet cells (250g wet weight) are then suspended in the alginate solution prepared in the previous step. The cell - alginate mixture is then added dropwise, from a height of 20 cm, into 1000 ml of cross-linking solution. This is achieved by using a syringe, 20 gauge needle and a syringe pump. The cross-linking solution is a 0.05M solution of $CaCl_2$ in the growth media and is agitated on a magnetic stirrer. A bead diameter of 0.5-2 mm is achieved under these conditions. The resultant beads are left for 1 hour in the cross-linking solution. The beads are then washed with fresh calcium cross-linking solution.

7.7 Synthesis of oximes

General procedure for the synthesis of oximes

To a mixture of the corresponding aldehyde or ketone (1eq.) in THF (80mL) and hydroxylamine hydrochloride (1.2eq.) in water (30mL) was added Na_2CO_3 (0.6eq.) in water (30mL). The reaction mixture was rapidly stirred for 18h. To the reaction mixture was added EtOAc (50mL) and the organic layer separated. The aqueous layer was extracted with EtOAc (2 x 50mL) and the combined organic layers dried with $MgSO_4$. The solvent was removed *in vacuo* and the residue purified to afford the oxime.

1-Phenyl-propan-1-one oxime **51**

The general procedure was followed employing phenylacetaldehyde (5.0g) to afford 5.39g of the title compound (96% yield) as a *cis*:*trans* mixture of white crystals. Analytical data matched published values.[209]

m.p. 49-51°C, **lit** 50-51°C; **^{1}H NMR** δ_H (250 MHz, $CDCl_3$) 1.11 (t, 3H, J=7.6Hz, CH_3), 2.76 (q, 2H, J= 7.6Hz, CH_2), 7.30-7.34 (m, 3H, ArH), 7.52-7.56 (m, 2H, ArH); **GC-MS** (Method A) 10.1mins, >99.5% purity, ES (m/z) 149 (M), 148 (base peak) 132, 117, 104.

1-(4-hydroxyphenyl)-ethanone oxime **53**

The general procedure was followed employing 4-hydroxyacetophenone (5.0g) to afford 4.84g of the title compound (87% yield). Analytical data matched published values.[210]

m.p. 142-144°C, **lit** 141-143°C; **^{1}NMR** δ_H (250 MHz, $CDCl_3$) 2.10 (s, 3H, CH_3), 6.79 (d, 2H, J= 8.9Hz, ArH), 7.50 (d, 2H, J=8.9Hz, ArH), 9.48 (bs, 1H, N-OH), 10.89 (bs, 1H, *p*-OH); **GC-MS** (Method A) 15.3mins, >99.5% purity, ES (m/z) 151 (M), 134, 119 (base peak), 110, 94.

1-(4-Bromo-phenyl)-phenyl-methanone oxime **56**

The general procedure was followed employing 1-(4-Bromo-phenyl)-phenyl-methanone (5.0g) to afford 4.46g of the title compound (84% yield) as white crystals after recrystalisation from ether:*n*-hexane. Analytical data matched published values.[211]

m.p. 169-171 C, lit. 170-172; **^{1}H NMR** δ_H (250 MHz, $CDCl_3$) 7.21-7.66 (m, 7H, ArH), 7.88-7.94 (m, 2H, ArH); **GC-MS** (Method A) 26.5mins, >99.5% purity, ES (m/z) 185, 183 (base peak), 165.

1-Cyclohexylethanone oxime **56**

The general procedure was followed employing 1-cyclohexylethanone (10.0g) to afford 8.45g of the title compound (76% yield) as a colourless oil after Kugelrohr purification. Analytical data matched published values.[212]

GC-MS (Method A) 15.4mins, >99.5% purity, ES (m/z) 141 (M), 124, 109, 86 (base peak).

Cyclopentanone oxime **59**

The general procedure was followed employing 2-methylcyclohexanone (2.0g) to afford 2.15g of the title compound (91% yield) as a white crystals after recrystalisation form *n*-hexane. Analytical data matched published values.[213]

m.p. 58-60°C, lit. 57-58°C; **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 24.2 (CH_2CH_2C=N), 24.9 (CH_2CH_2C=N), 26.8 (CH_2C=N), 30.4 (CH_2C=N), 166.9 (C=N); **GC-MS** (Method A) 12.0mins, >99.5% purity, ES (m/z) 99 (M, base peak), 82, 67.

7.8 Synthesis of *O*-methyloximes

General procedure for the synthesis of *O*-methyloximes

To a mixture of the corresponding aldehyde or ketone (1eq.) in THF (80mL) and *O*-methylhydroxylamine hydrochloride (1.2eq.) in water (30mL) was added Na_2CO_3 (0.6eq.) in water (30mL). The reaction mixture was rapidly stirred for 18h. To the reaction mixture was added EtOAc (50mL) and the organic layer separated. The aqueous layer was extracted with EtOAc (2 x 50mL) and the combined organic layers dried with $MgSO_4$. The solvent was removed *in vacuo* and the residue purified to afford the *O*-methyl oxime.

1-Phenyl-ethanone-*O*-methyloxime **52**

The general procedure was followed employing acetophenone (2.0g) to afford 2.26g of the title compound (91% yield) as a colourless liquid after Kugelrohr purification. Analytical data matched published values.[214]

b.p. 87°C/5mmHg, lit. 89°C/5mmHg; **^{1}H NMR** δ_H (250 MHz, $CDCl_3$) 2.24 (s, 3H, CH_3), 4.02 (s, 3H, OCH_3), 7.18-7.50 (m, 5H, ArH); **GC-MS** (Method A) 5.8mins, >99.5% purity, ES (m/z) 149 (M), 118, 103, 77 (base peak).

1-(4-hydroxyphenyl)-ethanone-*O*-methyl-oxime **54**

The general procedure was followed employing 4-hydroxyacetophenone (2.0g) to afford 2.17g of the title compound (89% yield) as a white solid after recrystalisation from ether. Analytical data matched published values.[215]

m.p. 63-64°C, lit 64°C; **^{1}H NMR** δ_H (250 MHz, $CDCl_3$) 2.21 (s, 3H, CH_3), 3.98 (s, 3H, OCH_3), 6.75 (d, 2H, J=8.9Hz, ArH), 7.46 (d, 2H, J=8.8Hz, ArH); **GC-MS** (Method A) 14.7mins, >99.5% purity, ES (m/z) 165 (M), 134 (base peak), 119.

1-Cyclohexylethanone-*O*-methyl-oxime **57**

The general procedure was followed employing 1-cyclohexylethanone (5.0g) to afford 4.45g of the title compound (72% yield) as a yellow oil after Kugelrhor purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.26-1.52 (m, 7H, cyclohexyl ring), 1.77-1.99 (m, 4H, cyclohexyl ring) 1.91 (s, 3H, cis/trans CH_3), 2.27 (s, 3H, cis/trans CH_3), 3.94 (s, 3H, cis/trans OCH_3), 3.96 (s, 3H, cis/trans OCH_3); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 11.5 (cis/trans CH_3), 16.3 (cis/trans CH_3), 25.8 (cyclohexyl CH_2) 29.6 (cyclohexyl CH_2), 36.7 (cyclohexyl CH_2), 44.3 (cyclohexyl CH), 51.3 (cis/trans OCH_3), 60.9 (cis/trans OCH_3), 161.4 (cis/trans C=N), 162.0 (cis/trans C=N); **FT-IR** (cm^{-1}) 1633, 1447, 1376, 1051; **MS** EI+ (m/z) 155 (M^+), 124, 100 (base peak), 87; **Acc. Mass**: found 155.13104, calc. 155.13101; **GC-MS** (Method A) Purity >99.5%, ES (m/z) 155.10 (M^+), 100.08 (base peak).

3,4-Dihydro-1*H*-naphthalene-2-one-*O*-methyl-oxime **58**

The general procedure was followed employing β-tetralone (5.0g) to afford 5.67g of the title compound (95% yield) as a colourless oil after Kugelrohr purification.

^{1}NMR δ_H (250 MHz, $CDCl_3$); 2.55-2.60 (dd, 1H, J=6.2Hz, 3.3Hz, CH_2CH_2C=N), 2.64-2.69 (dd, 1H, J=6.2Hz, 3.3Hz, CH_2CH_2C=N), 2.82-2.93 (m, 2H, CH_2CH_2C=N), 3.53 (s, 1H, CH_2C=N), 3.78 (s, 1H, CH_2C=N), 3.86 (s, 3H, cis/trans OCH_3), 3.92 (s, 3H, cis/trans OCH_3), 7.16-7.20 (m, 4H, ArH); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 24.5 (cis/trans CH_2CH_2C=N), 27.5 (cis/trans CH_2CH_2C=N), 28.9 (cis/trans CH_2CH_2C=N), 29.0 (cis/trans CH_2CH_2C=N), 34.9 (ArCH_2C=N), 61.2 (OCH_3), 126.0 (cis/trans Ar), 126.5 (cis/trans Ar), 127.1 (cis/trans Ar), 127.4 (cis/trans Ar), 128.0 (cis/trans Ar), 128.7 (cis/trans Ar), 133.1 (cis/trans CCH_2CH_2C=N), 134.7 (cis/trans CCH_2CH_2C=N), 137.0 (cis/trans CCH_2C=N), 138.2 (cis/trans CCH_2C=N), 157.7 (cis/trans C=N), 158.2 (cis/trans C=N); **FT-IR** (cm^{-1}) 1636, 1493, 1457, 1052; **MS** EI+ (m/z) 175 (M^+), 143, 129, 116 (base peak), 104; **Acc. Mass**: found 175.09928, calc. 175.09971; **GC-MS** (Method A) Purity >99.5%, ES (m/z) 175.02 (M^+), 116.00 (base peak).

7.9 Synthesis of racemic hydroxylamines

General procedure for the synthesis of racemic hydroxylamines

Method A – formation of hydroxylamine from isolated or commercially available oxime

To a solution of the oxime in MeOH (60mL), containing a trace of methyl orange, $NaB(CN)H_3$ (5eq.) was added; and then 2M HCl-ether was added dropwise with stirring to maintain the strong red colour for 30 mins. The reaction mixture was stirred overnight, and the solvent removed *in vacuo*. The residue was suspended in water (60mL) and brought to pH 10 using 6M NaOH aq. and sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification of the crude product afforded the hydroxylamine.

Method B – direct formation of hydroxylamine from ketone / aldehyde

To a mixture of the corresponding aldehyde or ketone (1eq.) in THF (80mL) and hydroxylamine hydrochloride (1.2eq.) in water (30mL) was added Na_2CO_3 (0.6eq.) in water (30mL). The reaction mixture was rapidly stirred for 18h. To the reaction mixture was added EtOAc (50mL) and the organic layer separated. The aqueous layer was extracted with EtOAc (2 x 50mL) and the combined organic layers dried with $MgSO_4$. The solvent was removed *in vacuo* to give the crude oxime. To a solution of the crude oxime in MeOH (60mL), containing a trace of methyl orange, $NaB(CN)H_3$ (5eq.) was added; and then 2M HCl-Ether was added dropwise with stirring to maintain the strong red colour for 30 mins. The reaction mixture was stirred overnight, and the solvent removed *in vacuo*. The residue was suspended in water (60mL) and brought to pH 10 using 6M NaOH aq. and sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification of the crude product afforded the hydroxylamine.

N-(1-Phenylethyl)-hydroxylamine **35**

Acetophenone oxime (2.00g) was treated as per Method A to afford 1.98g of the title compound (98% yield) as a white solid after recrystallisation from ether. Analytical data matched published values.[10,22]

m.p. 71-73°C, lit. 71-72°C; **^{1}H NMR** δ_H (250 MHz, CD_3OD) 1.67 (q, 3H, J=6.9Hz, CH_3), 4.50 (q, 1H, J=6.9Hz, CH-N), 7.44–7.47 (m, 5H, ArH).

N-(1-Phenylpropyl)-hydroxylamine **41**

1-Phenyl-propan-1-one oxime **51** (2.0g) was treated as per Method A to afford 1.40g of the title compound (69% yield) as white crystals after recrystalisation of the crude product from petroleum ether 40/60:hexanes. Analytical data matched published values.[216]

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.07 (t, 3H, J=7.4Hz, CH_3), 1.80-1.97 (m, 1H, CH_2), 2.05-2.22 (m, 1H, CH_2), 4.11 (dd, 1H, J=8.7, 5.5Hz, CH-N), 7.52-7.61 (m, 5H, Ar).

4-(1-Hydroxyaminoethyl)-phenol **43**

1-(4-hydroxyphenyl)-ethanone oxime **53** (0.57g) was treated as per Method C to afford 0.41g of the title compound (71% yield) as a pale yellow oil after Kugelrohr purification. Analytical data matched published values.[217]

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.59 (d, 3H, J=6.4Hz, CH_3), 4.19 (q, 1H, J=6.4Hz, CH-N), 6.88 (d, 2H, J=8.2Hz, ArH), 7.19 (d, 2H, J=8.2Hz, ArH).

N-[(4-Bromophenyl)-phenylmethyl]-hydroxylamine **45**

1-(4-Bromo-phenyl)-phenyl-methanone oxime **55** (1.0g) was treated as per Method C to afford 0.90g of the title compound (89% yield) as a cream solid after recrystalisation from ether.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 5.29 (m, 1H, CH-N); 6.92-7.33 (m, 9H, ArH); **FT-IR** (cm^{-1}) 1440, 1397, 1136; **MS** EI+ (m/z) 279 (M^+), 277, 248, 245(base peak); **Acc. Mass**: found 277.01018, calc 277.01022.

N-(1-Cyclohexylethyl)-hydroxylamine **46**

The general procedure was followed employing 1-cyclohexylethanone oxime **56** (2.0g) to afford 1.70g of the title compound (84% yield) as white crystals after recrystallisation from petroleum ether:hexane.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.10-1.44 (m, 5H, ring CH_2 & ring CH), 1.31 (d, 3H, J=6.8Hz, CH_3) 1.70-1.89 (m, 6H, ring CH_2), 3.23-3.32 (m, 1H, CH-N); **^{13}C NMR** δ_C (60 MHz, CD_3OD); 12.4 (CH_3), 27.7 (ring CH_2), 27.9 (ring CH_2), 28.9 (ring CH_2), 31.6 (ring CH_2), 40.1 (ring CH), 64.3 (CH-N); **FT-IR** (cm^{-1}) 1617, 1450, 1294; **MS** EI+ (m/z) 143 (M^+), 128, 69, 60 (base peak); **Acc. Mass**: found 143.13095, calc. 143.13101.

N-Cyclopentyl-hydroxylamine **49**

Cyclopentanone oxime **59** (2.0g) was treated as per Method A to afford 1.77g of the title compound (87% yield) as a pale yellow oil after Kugelrohr purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$) 1.42-1.78 (m, 8H, CH_2), 3.45-3.50 (m, 1H, CH-N); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 24.3 (CH_2CH_2CH-N), 29.9 (CH_2CH_2CH-N), 62.8 (CH-N); **FT-IR** (cm^{-1}) 1519, 1443, 1351, 1192, 1049; **MS** EI+ (m/z) 101 (M^+), 84, 72 (base peak), 69; **Acc. Mass**: found 101.08396, calc. 101.08406.

7.10 General procedure for the synthesis of racemic *O*-methyl hydroxylamines

Method C – formation of hydroxylamine from isolated or commercially available oxime

To a solution of the *O*-methyl oxime in MeOH (60mL), containing a trace of methyl orange, $NaB(CN)H_3$ (5eq.) was added; and then 2M HCl-ether was added dropwise

with stirring to maintain the strong red colour for 30 mins. The reaction mixture was stirred overnight, and the solvent removed *in vacuo*. The residue was suspended in water (60mL) and brought to pH 10 using 6M NaOH aq. and sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification of the crude product afforded the *O*-methyl hydroxylamine.

Method D – direct formation of *O*-methyl hydroxylamine from ketone / aldehyde

To a mixture of the corresponding aldehyde or ketone (1eq.) in THF (80mL) and *O*-methylhydroxylamine hydrochloride (1.2eq.) in water (30mL) was added Na_2CO_3 (0.6eq.) in water (30mL). The reaction mixture was rapidly stirred for 18h. To the reaction mixture was added EtOAc (50mL) and the organic layer separated. The aqueous layer was extracted with EtOAc (2 x 50mL) and the combined organic layers dried with $MgSO_4$. The solvent was removed *in vacuo* to give the crude oxime. To a solution of the crude oxime in MeOH (60mL), containing a trace of methyl orange, $NaB(CN)H_3$ (5eq.) was added; and then 2M HCl-Ether was added dropwise with stirring to maintain the strong red colour for 30 mins. The reaction mixture was stirred overnight, and the solvent removed *in vacuo*. The residue was suspended in water (60mL) and brought to pH 10 using 6M NaOH aq. and sat. $NaHCO_3$ and extracted with EtOAc. The organic layer was dried over $MgSO_4$ and the solvent removed *in vacuo*. Purification of the crude product afforded the *O*-methylhydroxylamine.

N-(1-phenylethyl)-*O*-Methyl-hydroxylamine **42**

1-Phenyl-ethanone-*O*-methyloxime **52** (1.0g) was treated as per Method C to afford 0.90g of the title compound (89% yield) as a colourless oil after Kugelrohr purification. Analytical data matched published values.[214]

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.37 (d, 3H, J=7.3 Hz, CH_3), 3.44 (s, 3H, OCH_3), 4.11 (q, 1H, J=7.3Hz, CH-N), 7.22-7.41 (m, 5H, ArH).

4-(1-Methoxyaminoethyl)-phenol **44**

1-(4-hydroxyphenyl)-ethanone-*O*-methyl-oxime **54** (2.0g) was treated as per Method C to afford 1.94g of the title compound (96% yield) as pale yellow crystals after recrystalisation of the crude product for ether:hexane.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.56 (d, 3H, J=6.7Hz, CH_3), 3.71 (s, 3H, OCH_3), 4.30 (q, 1H, J=6.7Hz, CH-N), 6.92 (d, 2H, J=8.5Hz, ArH), 7.37 (d, 2H, J=8.5Hz, ArH); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 19.1 (CH_3), 59.6 (CH-N), 62.0 (OCH_3), 115.3 (ArCH), 128.3 (ArCH), 133.4 (ArC), 155.3 (ArC-OH); **FT-IR** (cm^{-1}) 1614, 1516, 1454, 1374, 1242; **MS** EI+ (m/z) 167 (M^+), 135, 121 (base peak), 107; **Acc. Mass**: found 167.09469, calc. 167.09463.

N-(1-Cyclohexylethyl)-*O*-methyl-hydroxylamine **47**

1-cyclohexylethanone-*O*-methyl-oxime **57** (2.0g) was treated as per Method C to afford 1.51g of the title compound (75% yield) as a colourless oil after Kugekrohr purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.01 (d, 3H, J=6.5Hz, CH_3), 0.88-1.09 (m, 4H, ring CH_2), 1.10-1.34 (2H, ring CH_2),1.37-1.53 (m, 1H, ring CH), 1.66-1.78 (m, 4H, ring CH_2), 2.83 (dt, 1H, J=, 1H, CH-N), 3.54 (s, 3H, OCH_3); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$); 14.4 (CH_3), 26.2 (ring CH_2), 26.4 (ring CH_2), 26.5 (ring CH_2), 29.1 (ring CH_2), 29.8 (ring CH_2), 39.9 (ring CH), 60.4 (CH-N), 62.1 (OCH_3); **FT-IR** (cm^{-1}) 2371, 1725, 1660, 1608, 1453, 1375, 1220, 1156; **MS** EI+ (m/z) 157 (M^+), 149, 136, 127, 111 (base peak); **Acc. Mass**: found 157.14666, calc. 157.14666; **GC-MS** (Method A) >99.5% Purity, ES (m/z) 157.10 (M^+), 74.07 (base peak).

N-(1,2,3,4-Tetrahydronaphthalen-2-yl)-*O*-methyl-hydroxylamine **48**

3,4-Dihydro-1*H*-naphthalene-2-one-*O*-methyl-oxime **58** (5g) was treated as per Method C to afford 4.07g of the title compound (81% yield) as a colourless oil after distillation of the crude product.

^{1}H NMR δ_H (250 MHz, $CDCl_3$); 1.47-1.62 (m, 1H, CH_2CH_2CH-N), 1.92-2.03 (m, 1H, CH_2CH_2CH-N), 2.50-2.60 (m, 1H, $ArCH_2CH$-N), 2.72-2.80 (m, 2H, CH_2CH_2CH-N), 2.82-2.95 (m, 1H, $ArCH_2CH$-N), 3.21-3.32 (m, 1H, CH-N), 3.51 (s, 3H, OCH_3), 7.01 (m, 4H, ArH); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 26.2 (CH_2CH_2C-N), 26.9 ($ArCH_2C$-N), 32.8 (CH_2CH_2C-N), 55.3 (C-NH), 61.9 (OCH_3), 125.1 (Ar), 125.2 (Ar), 127.9 (Ar), 128.7 (Ar), 134.0 (Ar), 135.5 (Ar); **FT-IR** (cm^{-1}) 1580, 1494, 1453; **MS** EI+ (m/z) 177 (M^+), 146 (base peak), 131, 115; **Acc. Mass**: found 177.11549, calc. 177.11536; **GC-MS** (Method A) Purity >99.5%, ES (m/z) 177.10 (M^+), 129.10 (base peak).

N-(2-Methylcyclohexyl)-*O*-methyl-hydroxylamine **70**

2-Methylcyclohexanone (4.0g) was treated as per Method D to afford 3.11g of the title compound (61% yield) as a colourless oil after Kugelrohr purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$) 1.14 (d, 1H, J=7.2Hz, diastereomer A CH_3), 1.19 (d, 1H J=7.2Hz, diastereomer B CH_3), 1.27-1.71 (m, 6H, ring CH_2), 1.84-2.00 (m, 2H, CH_2CH-N), 2.22-2.29 (m, 1H $CHCH_3$), 2.65-2.56 (m, 1H, CH-N), 3.76 (s, 3H, OCH_3); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 13.8 (diastereomer A CH_3), 19.0 (diastereomer B CH_3), 22.03 (diastereomer A CH_2CHCH_3), 23.32 (diastereomer B CH_2CHCH_3), 25.1 (diastereomer A $CHCH_3$), 25.7 (diastereomer B $CHCH_3$), 30.8 (ring CH_2), 31.3 (ring CH_2), 34.3 (ring CH_2), 60.6 (diastereomer A OCH_3), 62.0 (diastereomer A C-N), 62.4 (diastereomer B OCH_3), 65.6 (diastereomer B C-N); **FT-IR** (cm^{-1}) 1463, 1375, 1062, 1044; **MS** EI+ (m/z) 143 (M^+, base peak), 137, 129, 125; **Acc. Mass**: found 143.13115, calc. 143.13101; **GC-MS** (Method A) purity >99.5%, ES (m/z) 143.10 (M^+), 86.00 (base peak).

N-[1-(4-Fluorophenyl)-ethyl]-*O*-methyl-hydroxylamine **71**

1-(4-Fluorophenyl)-ethanone (2.2g) was treated as per Method D to afford 1.62g of the title compound (60% yield) as a white solid after recrystalisation from EtOAc:hexanes. Analytical data matched published values.[218]

MS EI+ 169 (M+), 123 (base peak), 103; **Acc. Mass.** found 169.09012, calc.169.09029.

N-(1-methyl-2-phenylethyl)-*O*-methyl-hydroxylamine **72**

1-Phenylpropan-2-one (4.0g) was treated as per Method D to afford 3.28g of the title compound (67% yield) as a cream solid after recrystallisation from *n*-hexane.

m.p. 127-129°C, lit. 129-130°C; **^{1}H NMR** δ_H (250 MHz, $CDCl_3$) 1.06 (d, 3H, J=6.4Hz, CH_3), 2.58 (dd, 1H, J=6.8,6.6Hz, CH_2), 2.81 (dd, 1H, J=6.8,6.6Hz, CH_2), 3.15-3.28 (m, 1H, CH-N), 3.54 (s, 3H, OCH_3), 7.16-7.31 (m, 5H, ArH); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 17.7 (CH_3), 40.1 (CH_2), 57.2 (CH-N), 62.4 (OCH_3), 126.2 (ArCH), 128.3 (ArCH), 129.2 (ArCH), 138.6 (ArC); **FT-IR** (cm^{-1}) 1733, 1616, 1454, 1375, 1031; **MS** EI+ (m/z) 166 (MH^+), 134, 119, 91, 74 (base peak); **Acc. Mass**: found 165.11515, calc. 165.11536.

2-Methoxyamino-2-phenylethanol **75**

2-Hydroxy-1-phenyl-ethanone (0.97g) was treated as per Method D to afford 0.89g of the title compound (75% yield) as a yellow oil after Kugelrohr purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$) 3.46 (s, 3H, J=7.4Hz, OCH_3), 4.19 (dd, 1H, J=8.7, 5.5Hz, CH-N), 4.46 (m, 2H, CH_2), 5.28 (bs, 1H, OH), 7.31-7.58 (m, 5H, Ar); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 57.4 (CH-N), 60.8 (OCH_3), 71.8 (CH_2), 127.8 (ArCH), 128.0 (ArCH), 143.4 (ArC); **FT-IR** (cm^{-1}) 2550, 1484, 1249; **MS** EI+ 167 (M+), (base peak); **Acc. Mass.** found 167.09452, calc.167.09463.

N-(1-Adamantan-2-ylethyl)-*O*-methyl-hydroxylamine **76**

1-Adamantan-1-yl-ethanone (1.1g) was treated as per Method D to afford 0.53g of the title compound (41% yield) as a pale yellow oil after Kugelrohr purification.

^{1}H NMR δ_H (250 MHz, $CDCl_3$) 1.07 (d, 3H, J=6.8Hz, CH_3), 1.49-1.71 (m, 12H, Ad CH_2), 1.97 (s, 3H, Ad CH), 2.56 (m, 1H, CH-N), 3.32 (s, 3H, OCH_3); **^{13}C NMR** δ_C (60 MHz, $CDCl_3$) 18.8 (CH), 29.2 (Ad CH), 29.4 (Ad CH), 39.4 (Ad C), 41.1 (Ad

CH_2), 41.5 (Ad CH_2), 61.6 (OCH_3), 67.2 (C-N); **FT-IR** (cm^{-1}) 2480, 1634, 1459; **MS** EI (m/z) 195 (M), 163, 148 (base peak); **Acc. Mass.** found 209.17801, calc. 209.17796.

7.11 Synthesis of enatiopure hydroxylamines

Synthesis of enatiopure hydroxylamines

Anhydrous magnesium sulfate (25g) was added to a solution of (*R*)-amine (1eq.) and *p*-anisaldehyde (1.1eq) in dry DCM (150mL) under an atmosphere of argon and stirred for 24 hours. The mixture was then filtered througha pad of anhydrous magnesium sulfate and washing with DCM (300mL). The filtrate was cooled under argon to 0°C and a slurry of 70-75% *m*-CPBA (1.2eq.) in DCM (100mL) added in several portions. After stirring for 1.5 hours the cooling was removed and stirring continued at r.t. for 2.5 hours. The mixture was then filtered, the solid residue washed with DCM (100mL) and the combined filtrates washed successively with 0.5M aqueous Na_2SO_3 (150mL), 0.5M K_2CO_3 (150mL) and water (50mL) then dried over anhydrous magnesium sulfate. The solvent was removed *in vacuo*, maintaining the water bath temperature at r.t. and the oxaziridine residue dissolved in EtOH (200mL). This solution was cooled to 0°C under an atmosphere of argon and hydroxylamine hydrochloride (1.3eq) was added portion wise. The mixture was stirred for 24 hours, allowing the cooling bath to warm to r.t.. Chloroform (300mL) was added and stirring continued for 2 hours. The mixture was filtered and the solvent removed in *vacuo*. The oily residue was taken up on water (50mL) and washed with ether (2x50mL). The aqueous phase was then treated with sat. aq. $NaHCO_3$ (50mL) and re-extracted with ether (3x50mL). The combined organic extracts were dried over anhydrous magnesium sulphate and the filtered into a flask containing anhydrous oxalic acid (1.2eq) in ether (200mL). The precipitated oxalate salt was collected by filtration. Drying in a desiccator overnight afforded the enantiopure (*R*)-hydroxylamine as its oxalate salt.

(*R*)-*N*-(1-Phenylethyl)-hydroxylamine oxalate salt (***R*)-35**

The general procedure was followed using crude (*R*)-α-methylbenzylamine (1.12g) to afford 0.65g of the title compound (52% yield) as a white crystals. Analytical data matched published values.[19, 22]

Oxalate salt $[\alpha]_D^{20}$ -2.7(c 1.03, MeOH), lit. -2.9 (c 1.0, MeOH); Free base $[\alpha]_D^{20}$ -29.3 (c 4.00, $CHCl_3$), lit. -30.1 (c 4.25, $CHCl_3$); spectral data as for *rac*-**35**.

7.13 Laccase mediator preparative scale oxidation

N-(1-Phenylethyl)-hydroxylamine oxalate salt **35** (0.343g), laccase (*Trametes versicolor*, 0.624g) and TEMPO free radical (0.118g) were dissolved in pH 4.5 citrate buffer (90mL) and MeCN (10mL). The reaction mixture was shaken at 200rpm at 25°C in a sponge stoppered vessel. After 3 hours the reaction mixture was centrifuged at 4000rpm for 2mins. The reaction mixture was then decanted and washed with ether (2x100mL).The combined organic layers were dried ($NaSO_4$) and the solvent removed in vacuo to afford a cream solid. This was found to be substantially pure acetophenone oxime **50**. The solid was washed with hexane (2 x 5mL) to afford acetophenone oxime **50** (0.301g, 89%) as a white solid.

7.13 Kinetic resolution of *N*-methoxycyclohexylethylamine 47

rac-*N*-Methoxycyclohexylethylamine **47** (1.21g) was dissolved in pH 7.4 0.1M potassium phosphate buffer (20mL). To the solution was added a pellet of *E. coli* BL21 expressing MAO-N5D (2g wet weight). The pellets had been stored at -20°C from fermentation until use. The reaction soloution was sponge stoppered and then incubated at 25°C overnight with shaking (200rpm). The reaction mixture was centrifuged (3000rpm) for 10mins and the supernatant decanted. The cell pellet was resuspended in pH 7.4 0.1M phosphate buffer (10mL) and then centrifuged (3000rpm) for 5 mins. The combined aquoues extracts were washed with EtOAc (3x25mL) and the combined organic layters dried over anhydrous magnesium sulphate. The solvent was removed in vaccuo to yield a mixture of the product oxime

and unconverted hydroxylamine. The residue was prificed using column chromotagraphy (*n*-hexane 70:30 EtOAc to EtOAc 30:70 *n*-hexane) to yield *trans* 1-cyclohexylethanone-*O*-methyl-oxime **57** (R_f 0.59 (*n*-hexane 50:50 EtOAc), 0.55g, 46% yield) and *(R)-N*-Methoxycyclohexylethylamine **47** (R_f 0.64 (*n*-hexane 50:50 EtOAc), 0.53g, 44% yield).

7.14 Analytical methods

GC-MS Method A

GC-MS analysis was performed using an Agilent 6890N Gas Chromatograph interfaced to an Agilent 7683 auto-injector and sample tray. ChemStation software was used to record and analyse data.

CP-Sil 5 CB Low Bleed/MS column, 25m x 0.25mm, df = 0.25 µm, He 180kPa, splitless, Inj 275 C, Temp 50°C – 150°C, 5°C/min, 150°C 5min, 150– 250°C, 10°C/min, 250°C 5min.
Agilent 5973 EI Mass Selective Detector, cone voltage 25V, Data was acquired in the full scan mode over the range m/z 50-250.

GC Method A

GC analysis was performed using a Shimadzu GC-8 Gas Chromatography with manula injection. Chrompack software was used to record and analyse data.

CP-Chirasil-DEX CB column, 25m x 0.25mm, df = 0.25 µm, He 200kPa, 100mL/min split, Inj 250°C, FID Det 250°C, Temp 50°C – 180°C, 10°C/min, 180°C – 200°C, 20°C/min, 200°C 10min, *cis*-oxime **57** (5.00 mins), *trans*-oxime **57** (5.46 mins), (1-Phenylethyl)-hydroxylamine **47** (7.52 mins).

GC Method B

CP-Chirasil-DEX CB column, 25m x 0.25mm, df = 0.25 μm, He 200kPa, 100mL/min split, Inj 250°C, FID Det 250°C, Temp 50°C – 180°C, 10°C/min, 180°C – 200°C, 20°C/min, 200°C 10min, *(R)*-*N*-triflate-(1-Phenylethyl)-hydroxylamine (7.86 mins), *(S)*-*N*-triflate-(1-Phenylethyl)-hydroxylamine (8.14 mins).

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Appendix 1

MAO-N wild type sequence:

1 atg acc tcc cga gac gga tac cag tgg aca ccc gag aca ggg ctc 45
1 Met Thr Ser Arg Asp Gly Tyr Gln Trp Thr Pro Glu Thr Gly Leu 15

46 acg cag ggc gtc ccc tct cta gga gtc atc tcc ccg ccc act aat 90
16 Thr Gln Gly Val Pro Ser Leu Gly Val Ile Ser Pro Pro Thr Asn 30

91 atc gaa gac acg gac aaa gat ggt cca tgg gac gtg att gtc att 135
31 Ile Glu Asp Thr Asp Lys Asp Gly Pro Trp Asp Val Ile Val Ile 45

136 ggt gga ggg tac tgc ggg ttg act gcc act agg gac ttg act gta 180
46 Gly Gly Gly Tyr Cys Gly Leu Thr Ala Thr Arg Asp Leu Thr Val 60

181 gca ggc ttc aaa acc ctt ctc ctc gaa gcc cga gac cgc ata ggc 225
61 Ala Gly Phe Lys Thr Leu Leu Leu Glu Ala Arg Asp Arg Ile Gly 75

226 ggc cgc tcc tgg tcc tct aac atc gac ggc tat cct tac gag atg 270
76 Gly Arg Ser Trp Ser Ser Asn Ile Asp Gly Tyr Pro Tyr Glu Met 90

271 ggc ggc aca tgg gtc cac tgg cac caa tcg cac gta tgg cgc gaa 315
91 Gly Gly Thr Trp Val His Trp His Gln Ser His Val Trp Arg Glu 105

316 atc acg cgc tac aag atg cac aac gcc cta tca ccc tcc ttc aac 360
106 Ile Thr Arg Tyr Lys Met His Asn Ala Leu Ser Pro Ser Phe Asn 120

361 ttc tcc cgc ggc gtg aat cac ttc cag cta cgg acc aac ccc acc 405
121 Phe Ser Arg Gly Val Asn His Phe Gln Leu Arg Thr Asn Pro Thr 135

406 aca tca acc tac atg act cac gaa gcc gag gac gag ctc ctc cgc 450
136 Thr Ser Thr Tyr Met Thr His Glu Ala Glu Asp Glu Leu Leu Arg 150

451 tcc gca ttg cac aag ttc acc aac gtg gat ggc acc aac ggc cgt 495
151 Ser Ala Leu His Lys Phe Thr Asn Val Asp Gly Thr Asn Gly Arg 165

496 act gtc ctg ccc ttc ccg cat gac atg ttc tat gtt cct gag ttc 540
166 Thr Val Leu Pro Phe Pro His Asp Met Phe Tyr Val Pro Glu Phe 180

541 agg aag tat gat gag atg tca tac tcg gag cgg att gat caa atc 585
181 Arg Lys Tyr Asp Glu Met Ser Tyr Ser Glu Arg Ile Asp Gln Ile 195

586 cgg gat gag ttg agc ctt aat gaa cgg agt tct ctg gaa gcg ttt 630
196 Arg Asp Glu Leu Ser Leu Asn Glu Arg Ser Ser Leu Glu Ala Phe 210

631 ata ttg ctt tgc tct ggc gga acg ctg gag aat agc tca ttt gga 675
211 Ile Leu Leu Cys Ser Gly Gly Thr Leu Glu Asn Ser Ser Phe Gly 225

676 gaa ttc ctg cat tgg tgg gcg atg agc gga tat acg tat cag gga 720
226 Glu Phe Leu His Trp Trp Ala Met Ser Gly Tyr Thr Tyr Gln Gly 240

721 tgc atg gac tgc ttg ata agt tat aag ttc aag gat ggg cag tct 765
241 Cys Met Asp Cys Leu Ile Ser Tyr Lys Phe Lys Asp Gly Gln Ser 255

766 gca ttt gcg agg agg ttt tgg gag gag gcg gcc ggg acg ggg agg 810
256 Ala Phe Ala Arg Arg Phe Trp Glu Glu Ala Ala Gly Thr Gly Arg 270

811 ttg ggg tat gtg ttt ggg tgt ccg gtt agg agt gtt gtt aat gag 855
271 Leu Gly Tyr Val Phe Gly Cys Pro Val Arg Ser Val Val Asn Glu 285

856 aga gat gcg gcg aga gtg acg gcg agg gat ggg agg gag ttc gtt 900
286 Arg Asp Ala Ala Arg Val Thr Ala Arg Asp Gly Arg Glu Phe Val 300

901 gcg aag cgg gtg gtt tgc act att ccc ctc aat gtc ttg tcc acg 945
301 Ala Lys Arg Val Val Cys Thr Ile Pro Leu Asn Val Leu Ser Thr 315

946 atc cag ttc tca cct gcg ctg tcg acg gag agg atc tct gct atg 990
316 Ile Gln Phe Ser Pro Ala Leu Ser Thr Glu Arg Ile Ser Ala Met 330

991 cag gca ggt cat gtg aat atg tgc acg aag gtg cat gcc gaa gtg 1035
331 Gln Ala Gly His Val Asn Met Cys Thr Lys Val His Ala Glu Val 345

1036 gac aat atg gat atg cgg tcg tgg acg ggc att gcg tac cct ttc 1080
346 Asp Asn Met Asp Met Arg Ser Trp Thr Gly Ile Ala Tyr Pro Phe 360

1081 aat aaa ctg tgc tat gct att ggt gat ggg acg act ccc gcg gga 1125
361 Asn Lys Leu Cys Tyr Ala Ile Gly Asp Gly Thr Thr Pro Ala Gly 315

1126 aac acg cat ctg gtg tgt ttc ggg acg gat gcg aat cat atc cag 1170
376 Asn Thr His Leu Val Cys Phe Gly Thr Asp Ala Asn His Ile Gln 390

1171 ccg gat gag gac gtg cgg gag acg ttg aag gcg gtt ggg cag tta 1215
391 Pro Asp Glu Asp Val Arg Glu Thr Leu Lys Ala Val Gly Gln Leu 405

1216 gcg cct ggg aca ttt gga gtg aag cgg ttg gtg ttt cac aat tgg 1260
406 Ala Pro Gly Thr Phe Gly Val Lys Arg Leu Val Phe His Asn Trp 420

1261 gtg aag gat gag ttt gcg aag ggc gcg tgg ttc ttc tct agg cct 1305
421 Val Lys Asp Glu Phe Ala Lys Gly Ala Trp Phe Phe Ser Arg Pro 435

1306 ggg atg gtg agt gag tgt ttg cag ggg ttg agg gag aag cat ggg 1350
436 Gly Met Val Ser Glu Cys Leu Gln Gly Leu Arg Glu Lys His Gly 450

1351 ggt gtg gtg ttt gcg aat tca gat tgg gcg ttg ggg tgg agg agc 1395
451 Gly Val Val Phe Ala Asn Ser Asp Trp Ala Leu Gly Trp Arg Ser 465

1396 ttt att gat ggg gcg att gag gag ggg acg aga gct gct agg gtg 1440
466 Phe Ile Asp Gly Ala Ile Glu Glu Gly Thr Arg Ala Ala Arg Val 480

1441 gtg ttg gag gaa ttg gga acg aag agg gag gtg aag gct cgt ttg 1485
481 Val Leu Glu Glu Leu Gly Thr Lys Arg Glu Val Lys Ala Arg Leu 495

1486 tga 1488
496 End

MAO-5D sequence:

as wild-type except Ile246 to Met, Asn336 to Ser, Met348 to Lys, Thr384 to Asn, Asp385 to Ser.

MAO-6 sequence:

as MAO-5D except Leu246 to Met.

The Genetic code

	T	C	A	G
T	TTT Phe TTC Phe TTA Leu TTG Leu	TCT Ser TCC Ser TCA Ser TCG Ser	TAT Tyr TAC Tyr TAA Ter TAG Ter	TGT Cys TGC Cys TGA Ter TGG Trp
C	CTT Leu CTC Leu CTA Leu CTG Leu	CCT Pro CCC Pro CCA Pro CCG Pro	CAT His CAC His CAA Gln CAG Gln	CGT Arg CGC Arg CGA Arg CGG Arg
A	ATT Ile ATC Ile ATA Ile ATG Met	ACT Thr ACC Thr ACA Thr ACG Thr	AAT Asn AAC Asn AAA Lys AAG Lys	AGT Ser AGC Ser AGA Arg AGG Arg
G	GTT Val GTC Val GTA Val GTG Val	GCT Ala GCC Ala GCA Ala GCG Ala	GAT Asp GAC Asp GAA Glu GAG Glu	GGT Gly GGC Gly GGA Gly GGG Gly

Amino acid symbols and abbreviations

Letter	Code	Amino acid	Letter	Code	Amino acid
A	Ala	Alanine	M	Met	Methionine
C	Cys	Cysteine	N	Asn	Asparagine
D	Asp	Aspartic acid	P	Pro	Proline
E	Glu	Glutamic acid	Q	Gln	Glutamine
F	Phe	Phenylalanine	R	Arg	Arginine
G	Gly	Glycine	S	Ser	Serine
H	His	Histidine	T	Thr	Threonine
I	Ile	Isoleucine	V	Val	Valine
K	Lys	Lysine	W	Trp	Tryptophan
L	Leu	Leucine	Y	Tyr	Tyrosine