Engineering of *Candida antarctica* lipase B for the kinetic resolution of α-halo esters

A thesis submitted to the University of Manchester for the degree of Doctor of Philosophy in the Faculty of Engineering and Physical Sciences

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This thesis contains 33163 words.
Abstract

Engineering of *Candida antarctica* lipase B for the kinetic resolution of α-halo esters

Shu-Ling Tang
PhD
University of Manchester

Retrosynthetic analyses of several therapeutic targets lead to the possibility of using enantiopure α-bromopropionic acid as a starting material. Therapeutic targets include (S)-Keppra, an anti-epileptic drug, and (R)-Tiopronin, used to prevent kidney stones. Enantiopure α-bromo acids also provide an efficient way for the synthesis of numerous other chiral starting materials. α-Fluoropropionic acid was also proposed as a substrate for the synthesis of enantiopure agrochemicals.

Kinetic resolution via enzymatic methods was proposed and development of a scalable biocatalytic process to access 2-bromopropionic acid, 2-fluoropropionic acid and their esters in a single enantiomeric form with a high enantiomeric excess was undertaken.

Initial screening results with a commercially available lipase kit indicated that *Candida antarctica* lipase B (Cal B) was active towards kinetically resolving both the substrates, 2-bromopropionic acid and 2-fluoropropionic acid. The Cal B gene was amplified from its pPIC9 construct and molecular biology techniques were used to generate two new constructs, pET16b and pET SUMO, suitable for the expression of Cal B from *Escherichia coli*. Various conditions such as temperature and incubation time were optimised for the production of soluble and active Cal B in two strains of *E. coli*. Three optimised conditions were found and the different preparations of Cal B were characterised using the p-nitrophenyl butyrate assay. Molecular modelling studies of the docking of both substrates into a known Cal B X-ray crystallographic structure for the enhancement of enantioselectivity were completed. Several residues namely Thr40, Asp134, Thr138, Gln157, Ile189, Val190, Leu278 and Ile285 were identified for the rational design of mutants of Cal B with enhanced activity or enantioselectivity. Site directed mutagenesis was used to generate a focused library of mutants with single point mutations. Mutants with higher specific activity and opposite enantiopreference were identified.
Declaration

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Finally I would like to dedicate this thesis to my partner, Mr. Nadir Iqbal, and my family, Anthony, Nancy, Lawrence, Emily, Hayley, Olivia, Charlotte and “Baby Tang” but in particular to my mother, Wong Kiu Tang, and to my father, Kwok On Tang, whose sacrifices for me over the years have not gone unnoticed.

Dei gratia
### Abbreviations

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<th>Description</th>
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<tr>
<td>AEDs</td>
<td>Anti-epileptic drugs</td>
</tr>
<tr>
<td>amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>AU</td>
<td>Absorbance units</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
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<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cal B</td>
<td><em>Candida antarctica</em> lipase B</td>
</tr>
<tr>
<td>CAST</td>
<td>Combinatorial active-site saturation test</td>
</tr>
<tr>
<td>CBS reagent</td>
<td>Corey, Bakshi, Shibata reagent</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’-Diaminobenzidine</td>
</tr>
<tr>
<td>DAST</td>
<td>Diethylaminosulfur trifluoride</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>DMF</td>
<td>(N,N)-Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DOPA</td>
<td>3,4-Dihydroxyphenylalanine</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double stranded DNA</td>
</tr>
<tr>
<td>E</td>
<td>Enantioselectivity</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>epPCR</td>
<td>Error-prone polymerase chain reaction</td>
</tr>
<tr>
<td>Eq.</td>
<td>Equation</td>
</tr>
<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<td>hr</td>
<td>Hour</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HTPB</td>
<td>Hexadecyltributylphosphonium bromide</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-(\beta)-D-1-thiogalactopyranoside</td>
</tr>
<tr>
<td>ISM</td>
<td>Iterative saturation mutagenesis</td>
</tr>
<tr>
<td>kan</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>MCD</td>
<td>Monochlorodimedone</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>Optical density at 600nm</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PIC</td>
<td>Protein inhibitor cocktail</td>
</tr>
<tr>
<td>pNPA</td>
<td>para-Nitrophenyl acetate</td>
</tr>
<tr>
<td>pNPB</td>
<td>para-Nitrophenyl butyrate</td>
</tr>
<tr>
<td>P. pastoris</td>
<td><em>Pichia pastoris</em></td>
</tr>
<tr>
<td>PPL</td>
<td>Porcine pancreatic lipase</td>
</tr>
<tr>
<td>PrnA</td>
<td>Tryptophan-7-halogenase</td>
</tr>
<tr>
<td>PrnC</td>
<td>Monodechloroaminopyrrolynitrin-3-halogenase</td>
</tr>
<tr>
<td>R</td>
<td>Rectus</td>
</tr>
<tr>
<td>S</td>
<td>Sinister</td>
</tr>
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<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SOC</td>
<td>Super Optimal Broth plus Carbon source</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris acetate EDTA</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em> DNA polymerase</td>
</tr>
<tr>
<td>TBME</td>
<td>tert-Butyl methyl ether</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
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1 Introduction

1.1 Chirality in nature

Organisms in nature have evolved primarily to use \( \text{L-} \)amino acids and \( \text{D-} \)sugars.\(^{1a}\) The inherent chirality of these simple building blocks of life means that their asymmetry is passed on to proteins and carbohydrates. As a result, we have evolved to incorporate asymmetry in all aspects of our life from our hands to the receptors in our noses. Our noses are able to distinguish between the enantiomers \((R)-(\text{--})\text{-carvone} (R)\text{-1}\) which has the smell of caraway seeds and \((S)-(\text{+})\text{-carvone} (S)\text{-1}\), which smells like spearmint (Figure 1.1).\(^{1b,2}\)

![Figure 1.1: \((R)\text{-Carvone} (R)\text{-1} \text{ and } (S)\text{-carvone} (S)\text{-1}.\)](image)

1.2 Enantiomers of drugs

The absolute stereochemistry of a molecule is biologically relevant as enantiomers are able to initiate different effects by occupying the three dimensional space of a receptor differently. Consider the two enantiomers of Thalidomide 2, a sedative hypnotic and an anti-emetic, and their effects (Figure 1.2).\(^3\) Whilst \((R)\text{-Thalidomide} (R)\text{-2}\) is harmless and has various beneficial pharmacological properties, \((S)\text{-Thalidomide} (S)\text{-2}\) is teratogenic.\(^4\) The devastating effects of the \((S)\text{-enantiomer} (S)\text{-2} \text{ and the racemisation of } (R)\text{-Thalidomide} (R)\text{-2}\) under physiological conditions were only found after the birth of abnormal children.\(^4\text{-}6\)

Enantiomers of a compound have the same physical properties except for their optical activity when in an achiral environment, but in a chiral environment they will have different properties including pharmacokinetics and pharmacodynamics. Binding sites on
proteins are three dimensional and the way an enantiomer fits into that site will depend on its orientation in space. Until recently the anaesthetic and analgesic, Ketamine 3, was used in its racemic form.7,8 Even though both enantiomers of ketamine have anaesthesia properties, the (S)-enantiomer (S)-3 is significantly more potent than the (R)-enantiomer (R)-3 (Figure 1.3).8,9

![Figure 1.2: Enantiomers of Thalidomide 2.](image)

![Figure 1.3: Enantiomers of Ketamine 3.](image)

In 1987 the U.S. Food and Drug administration (FDA) released a policy statement for the recommendation of the synthesis of chiral drugs and both the FDA and European Committee for Proprietary Medicinal Products have specified that both enantiomers of a drug need to be physiologically assessed.5 Previously the physiological effects of different enantiomers may have been ignored but now the distinct advantages of single enantiomer drugs are being recognised. Lower dosages, reducing adverse drug interactions and improved efficacy are advantages of single enantiomer drugs as well as better economical and environmental efficiency.10-12 Enantiopure agrochemicals are also important as an inactive enantiomer may interact adversely with the ecological system. Both in the pharmaceutical and agrochemical industries, the inactive enantiomer can be considered an
impurity or pollutant and regulatory agencies increasingly want toxicological reports for impurities constituting more than 1% of the content.\textsuperscript{13,14}

1.2.1 Chiral pool asymmetric synthesis

The chiral pool is a source of natural products that are used as starting materials for the synthesis of target molecules, chiral auxiliaries and chiral ligands. Homochiral starting materials that are needed can sometimes be immediately apparent in a target molecule like aspartame 4. The dipeptide is synthesised from (S)-aspartic acid (S)-5 and (S)-phenylalanine (S)-6 (Scheme 1.1).\textsuperscript{1c,15}

\[
\text{Ph} - \text{CH}_2\text{NH} - \text{CO}_2\text{H} \quad \rightleftharpoons \quad \text{HO}_2\text{C} - \text{CH}_2\text{NH}_2 - \text{CO}_2\text{H} \quad \rightleftharpoons \quad \text{Ph} - \text{CH}_2\text{NH} - \text{CO}_2\text{Me}
\]

Scheme 1.1: Retrosynthesis of aspartame 4.\textsuperscript{1c,15}

Many steps may also be required to convert the chiral pool compound to the desired target as in the case of (S)-(-)-ipsenol (S)-16, a component of the pheromone of male bark beetles of the genus Ips. A total of seven steps were needed to convert the chiral pool starting material, (S)-leucine (S)-7 to (S)-(-)-ipsenol (S)-16 (Scheme 1.2).\textsuperscript{1c,15}

Although in general extraction of chiral starting materials from nature is economical, on some occasions only one enantiomer of the desired starting material is available from the chiral pool or the difference in cost is substantial.
Scheme 1.2: Synthesis of (S)-(−)-ipsenol (S)-16 from (S)-leucine (S)-7.\textsuperscript{1c,15}

Reagents and conditions: (i) (a) NaNO\textsubscript{2}, H\textsubscript{2}O, H\textsubscript{2}SO\textsubscript{4}, 0 °C, 2 h, (b) rt, overnight (ii) (a) LiAlH\textsubscript{4}, THF, 0 °C, 1 h, (b) rt, overnight (iii) TsCl, 0 °C, (iv) KOH, H\textsubscript{2}O, ethylene glycol, (v) (a) diethyl malonate, NaOEt, EtOH, Na\textsubscript{2}CH(CO\textsubscript{2}Et)\textsubscript{2}, reflux, 5 h, (b) KOH, reflux, 1 h, (c) H\textsubscript{2}SO\textsubscript{4} (aq.), (vi) CH\textsubscript{2}O aq., Et\textsubscript{2}NH, 80-90 °C, 0.5 h, (vii) NaBH\textsubscript{4}, C\textsubscript{6}H\textsubscript{5}SeH, EtOH, rt, 2 h, (viii) i-Bu\textsubscript{2}AlH, dry THF, - 60 – 50 °C, 1 h, (ix) triphenylmethylphosphonium bromide, Na\textsubscript{2}CH\textsubscript{2}SOMe, dry DMSO, rt, 3 h.

1.2.2 Resolution

Resolution is the separation of a racemic mixture into its constituent enantiomers by using a chiral resolving agent. The reaction of a racemate with a chiral compound affords diastereoisomers which can be separated by their different physical properties i.e. chromatography or recrystallisation. This technique was used for the resolution of racemic 3-(N,N-dimethylamino)-1-(2-thienyl)propan-1-ol rac-17 in the industrial scale synthesis of Duloxetine (S)-20, an antidepressant. A saturated solution of racemic 3-(N,N-dimethylamino)-1-(2-thienyl)propan-1-ol rac-17 was seeded with (S)-mandelic acid (S)-18.
in which the diastereoisomeric salt (S)-19 precipitated. The filtrate, containing the (R)-
enantiomer (R)-17, was racemised to circumvent the disadvantage of 50% yield. Once the
crystallised (S)-19 was filtered, it was hydrolysed back to the alcohol (S)-17. (Scheme
1.3). Resolution using diastereoisomeric salts allow the formation of ionic compounds
that can be separated by their solubility. Resolution can also be done using the weakest of
forces like hydrogen bonds and van der Waals which are used in chromatography.

Scheme 1.3: Resolution of racemic alcohol rac-17.\(^{16}\)
Reagents and conditions: (i) (a) PhMe, MeOH, rt, (b) 80 °C, 0.5 h, (c) cool to rt, 1 h, (ii) NaOH, H\(_2\)O.
1.2.3 Auxiliary controlled stereoselectivity

A chiral auxiliary is an enantiomerically pure compound that is covalently attached to the substrate to impart stereoselectivity in a reaction. As with a resolution, the reaction of a chiral compound with an achiral compound results in a diastereoselective reaction and then the components can be separated. An advantage of chiral auxiliaries is that they can be recycled. Some common auxiliaries that are used are the Evans auxiliary 21 and the norephedrine derived auxiliary 22 (Figure 1.4).\textsuperscript{1c,17}

![Evans auxiliary 21 and the norephedrine derived auxiliary 22.](image)

The Evans chiral auxiliary and its many derivatives have been utilised in asymmetric reactions such as Michael additions\textsuperscript{18,19} and Diels–Alder reactions\textsuperscript{20,21}. An example of a chiral auxiliary being used in a Diels–Alder reaction allowed the synthesis of the single enantiomer 28 (Scheme 1.4). The synthesis involves the \textit{in situ} generation of the chiral auxiliary 24 from (1S,2R)-1-amino indan-2-ol 23. Reaction of the chiral auxiliary 24 with the acrylic acid dienophile 25 affords the auxiliary–dienophile 26. The Lewis acid coordinates the auxiliary–dienophile complex 26 and piperylene 27 is positioned above the complex as the auxiliary 24 blocks the diene from approaching the bottom face of the dienophile thus making the reaction diastereoselective.\textsuperscript{21}
Scheme 1.4: Diels Alder reaction using the norephedrine derived auxiliary.\textsuperscript{1c,21}

Reagents and conditions: (i) triphosgene, (ii) triethylamine, LiBr, acryloyl chloride, EtOAc, 4-6 h, (iii) CH\textsubscript{3}Cl\textsubscript{2}, Et\textsubscript{2}ACl.

\textbf{1.2.4 Reagent controlled stereoselectivity}

An alternative to attaching a chiral unit to the starting material is to use a chiral reagent to impart stereoselectivity. For example, a chiral reducing agent such as the Corey, Bakshi and Shibata (CBS) reagent 29 can be used to reduce acetophenone 30 selectively to (R)-1-phenylethanol (R)-31 (Scheme 1.5).\textsuperscript{22}

Scheme 1.5: Reduction of acetophenone 30 to 1-phenylethanol 31 selectively using the CBS reagent 29.\textsuperscript{22}

Reagents and conditions: THF, 25 °C, 1 minute.
Catalysts that result in a stereoselective reaction such as Sharpless’ titanium tetraisopropoxide and Jacobsen’s catalyst are expensive and although they are only required in small quantities they are still only available for a few reactions such as epoxidation and dihydroxylation.

1.3 Pharmacologically active compounds

1.3.1 Retrosynthetic analyses of (S)-Keppra (S)-32 and (R)-Tiopronin (R)-34

Retrosynthetic analysis of several therapeutic targets leads to the possibility of using (R)-2-bromobutyric acid (R)-33 and (S)-2-bromopropionic acid (S)-35 as starting materials. Therapeutic targets include (S)-keppra (S)-32, an anti epileptic drug and (R)-tiopronin (R)-34, used to prevent kidney stones (Scheme 1.6).

Scheme 1.6: Retrosynthetic analyses of several therapeutic targets.

1.3.2 (S)-Keppra (S)-32

Epilepsy is a neurological disorder characterised by periodic seizures. Epilepsy affects people of all ages and around 50 million people worldwide have been diagnosed with the disorder. The spontaneous seizures are symptoms of abnormal, excessive or synchronous
neuronal activity in the brain. In most diagnosed cases of epilepsy there is no known cause and treatment consists of controlling the seizures with anti-epileptic drugs (AEDs). (S)-Keppra (S)-32 is an AED, which was found to have a different mode of action; acting on the synaptic vesicle protein SV2A in the brain rather than on the ion channels or postsynaptic receptors. As traditional AEDs do not always control seizures in all patients, identification of this new mechanism of action further adds to the number of possible successful therapeutic strategies.

There are many asymmetric syntheses of (S)-Keppra (S)-32 reported including a chiral pool synthesis and one involving a kinetic resolution. The chiral pool synthesis uses L-methionine or (S)-2-aminobutyramide hydrochloride (S)-36 (Scheme1.7).

![Scheme 1.7: Chiral pool synthesis of (S)-Keppra (S)-32 from (S)-2-aminobutyramide hydrochloride (S)-36.](image)

Reagents and conditions: (a) CICO(CH₂)₂Cl, K₂CO₃, MeCN, (b) TBAB, DCM, KOH, 0 °C.

Another synthesis of (S)-Keppra (S)-32 reported is the chromatographic resolution of the racemate Etiracetam rac-32 (Figure 1.5) by employing silica gel supporting amylose tris-(3,5-dimethylphenylcarbamate) (Daicel, Chiralpak® AD) as the stationary phase.

![Figure 1.5: Etiracetam rac-32.](image)
A synthesis from a common intermediate allows facile development of analogues and one such method was reported for the synthesis of (S)-Keppra (S)-32. A chiral auxiliary (S)-37 was used for the deracemisation of 2-bromobutanoic acid rac-33. The synthesis had an overall yield of 65% and involved the coupling of the chiral auxiliary, (S)-N-phenylpantolactam (S)-37, to racemic 2-bromobutanoic acid rac-33 (Scheme 1.8).

Scheme 1.8: Coupling of the (S)-N-phenylpantolactam chiral auxiliary (S)-37 to racemic 2-bromobutanoic acid rac-33.

Reagents and conditions: (a) Cl₂SO, (b) (S)-37, CH₂Cl₂, Et₃N, -20 °C, 4 h.

Cleavage of the chiral auxiliary from intermediate (α,R,3S)-38 affords enantiopure 2-bromobutanoic acid (R)-33. Nucleophilic substitution of 2-bromobutanoic acid (R)-33 with 2-pyrrolidinone via an SN2 mechanism would afford the (S)-2-(2-oxopyrrolidin-1-yl)-butanoic acid (S)-39 which upon amidation yields (S)-Keppra (S)-32. Only a slight loss in enantiopurity under the amidation conditions was observed (Scheme 1.9).32,34

The next generation of AEDs, Brivaracetam 40 and Seletracetam 41, have already been developed and are analogues of (S)-Keppra (S)-32. They can by synthesised from the common intermediate (R)-2-bromobutanoic acid (R)-33. Brivaracetam 40 exhibited a 13-fold increase in affinity over (S)-Keppra (S)-32 but Seletracetam 41 was the most potent of the three AEDs (Figure 1.6).35-37
Scheme 1.9: Synthesis of (S)-Keppra (S)-32 from (R)-2-bromobutanoic acid (R)-33 after cleavage of the chiral auxiliary from intermediate (α,R,3S)-38.32-34
Reagents and conditions: (i) LiOH, H$_2$O$_2$, THF, 0 °C, 5 h, (ii) NaH, 2-pyrrolidinone, THF, rt, overnight, (iii) (a) ClCO$_2$Et, CH$_2$Cl$_2$, 0 °C, 0.5 h, (b) NH$_4$OH, rt, 16 h.

Figure 1.6: Next generation of anti-epileptic drugs, Brivaracetam 40 and Seletracetam 41.
1.3.3  \((R)\)-Tiopronin \((R)\)-34

Tiopronin 34 is a pharmaceutically important thiol compound with a range of biological properties. It is used to treat hepatic disorders\(^{38,39}\), cystinuria\(^{40,41}\), rheumatoid arthritis\(^{42-44}\) and can be used as a mucolytic in respiratory disorders\(^{44,45}\) and is also effective against radiation-induced damages, even at low doses\(^{46}\). The sulfhydryl side chain is responsible for its many actions. Tiopronin 34 is capable of liberating its SH group, which has an important physiological function in the body as a detoxifying agent against various exogenous and endogenous toxins.\(^{47}\) Tiopronin 34 acts as a potent free radical scavenger\(^{45}\) and that is why it is used as a very effective treatment of cystinuria.

Cystinuria is an autosomal recessive genetic defect, which results in a breakdown of the transepithelial transport of cysteine in the kidney. This causes a build up of cysteine in the urine and once a high concentration is reached, cysteine crystallises and forms stones in the kidneys. Tiopronin 34 transforms cysteine into a mixed disulfide, 50 times more soluble than cysteine itself allowing it to be excreted with urine.\(^{40}\)

Severe adverse reactions to oral thiol-drugs have been described in patients which include ageusia or dysgeusia rash, pemphigus, thrombocytopenia, myasthenia gravis, agranulocytosis, polymyositis, proteinuria, or hypersensitivity nephritic syndrome.\(^{40,48,49}\) Tiopronin 34 is a racemate and each enantiomer was found to have different pharmacological and therapeutic effects. Selected preclinical pharmacological studies showed that \((R)\)-Tiopronin \((R)\)-34 had a much greater therapeutic effect on perchlormethane-induced acute liver injury in rats than the \((S)\)-enantiomer.\(^{50}\) Currently, patients on Tiopronin 34 are recommended to have their peripheral blood count, direct platelet count, liver functioning tests as well as a whole list of other examinations to be done if they display any of the side effects.\(^{51}\) The analysis of each enantiomer of Tiopronin 34 on a pharmacological basis will allow absolute confirmation whether the \((R)\)-enantiomer \((R)\)-34 does have a greater therapeutic effect but also in the same instance it may be possible to eliminate some of the adverse reactions to the drug.

Although the Tiopronin 34 racemate is commercially available from several sources in China, the single enantiomers are not widely available. Currently there are a few
asymmetric syntheses of (S)-Tiopronin (S)-34, requiring five steps and ten stages\textsuperscript{52} but there are even less reported syntheses for (R)-Tiopronin (R)-34.

There are various methods for the synthesis of optically active thiols by nucleophilic substitution but they are racemisation prone\textsuperscript{53-60} and often restricted to certain substrates. A synthesis by nucleophilic substitution using caesium thiocarboxylates was reported\textsuperscript{61} with high yields and moderate to good enantiomeric excess (70–98\%, 47–\textgreater{}98\% e.e.).

Alternatively, optically active thiols (R)-42 can theoretically be synthesised by nucleophilic substitution using optically active bromoacids (S)-35 (Scheme 1.10).

![Scheme 1.10: Synthesis of (R)-Tiopronin (R)-34.](image)

1.3.4 Synthesis of other chiral starting materials from 2-bromoalkanoic acid

In addition to therapeutic targets, chiral \(\alpha\)-bromoacids can also be used to form other chiral starting materials such as \(\alpha\)-hydroxy and \(\alpha\)-amino groups (Scheme 1.11). The use of optically pure chiral starting materials at the beginning of a synthesis eliminates the need for resolution at the end and also eliminates the use of expensive chiral catalysts.
Scheme 1.11: Other chiral starting materials from 2-bromoalkanoic acid.

Reagents and conditions: (i) NH$_3$ in H$_2$O$^{62}$, (ii) caesium thiobenzoate, DMF$^{61}$, (iii) NaH, THF, BnOCH$_2$CH$_2$OH$^{63}$ or NaH, DMF, BnOCH$_2$CH$_2$OH$^{64}$ (iv) NaOMe, MeOH, (v) PbOH, MeNH$_2$, H$_2$O, (vi) naphth-1-ol, NaOH, H$_2$O, EtOH; (vii) dil. KOH (aq.).

1.3.5 2-Fluoropropionic acid 83

The fluorine atom has a van der Waal radius of 1.35 Å comparable to that of the hydrogen atom which is 1.20 Å.$^{13}$ Fluorine is regularly used as an isostere for hydrogen in bioactive molecules because of the similarity in steric bulk.$^{65}$ A carbon-fluorine bond is 14 kcal/mole stronger than a carbon-hydrogen bond and is able to resist metabolic transformations such as oxidative degradation.$^{66,67}$ Fluorine’s influence in a bioactive molecule can affect any of a number of parameters, such as transportation of the bioactive molecule to the target site and binding to a target receptor or enzyme. Fluorine is also able to mimic other functional groups. The carbon-fluorine bond length is comparable to a carbon-oxygen bond length, 1.39 Å versus 1.43 Å and is also a hydrogen bond acceptor and can be used to replace hydroxyl groups.$^{13}$ Fluorine is also the most electronegative atom and incorporating it into bioactive molecules can have profound effects on the overall properties including electron density, basicity and acidity of neighbouring groups.$^{13}$ Molecular recognition relies on
factors such as steric hindrance, electronegativity, dipole interactions and lipophilicity and fluorine is able to influence all of these aspects. The importance of chirality in pharmaceutical and agrochemical compounds indicates the need to synthesise enantiomerically pure fluorinated compounds.

1.4 Chemical synthesis of α-haloacids

1.4.1 Chemical synthesis of 2-bromopropionic acid 35

Currently the industrial bromination reagents are molecular bromine 56 or hydrogen bromide 53. Synthesis of alkyl bromides 54 and α-bromoacids and esters 57 are conducted on a scale of kilograms to hundreds of tonnes with yields in the range 80-95% (Scheme 1.12 and 1.13).

\[
\begin{align*}
\text{R} \backslash \text{OH} & \quad \text{HBr} \\
\text{R} & = \text{alkyl} \quad 52 & 53 & 54
\end{align*}
\]

Scheme 1.12: Synthesis of alkyl bromides 54 using hydrogen bromide 53.
Reagents and conditions: Reflux.

\[
\begin{align*}
\text{R} \backslash \text{OH} & \quad \text{Br}_2 \\
\text{R} & = \text{alkyl} \quad 55 & 56 & 57
\end{align*}
\]

Scheme 1.13: Synthesis of α-bromoesters 57 using molecular bromine 56.
Reagents and conditions: ROH, 50-110 °C depending on the alcohol.
1.4.2 Chemical synthesis of enantiopure 2-bromopropionic acid 35

Asymmetric bromination of acid chloride 58 has resulted in α-bromoester (S)-64 with high enantiomeric excess but this was not conveyable upon scale up (Scheme 1.14).^69^

Scheme 1.14: Asymmetric bromination of acid chlorides.\(^6^9\)

Reagents and conditions: Cat. Nu, 15-crown-5, NaH, THF, LG-Br, -78 °C, 5 h.
1.4.3 Chemical synthesis of 2-fluoropropionic acid 83

Currently there are several methods for the synthesis of fluorinated compounds and these include nucleophilic fluorination and electrophilic fluorination, but methodology for asymmetric fluorination is not common and generally involves reagent controlled selective fluorinations. Additionally singularly fluorinated compounds are very difficult to synthesise due to the high reactivity of fluorinating reagents and this is reflected in the commercial availability and value of singularly fluorinated compounds compared to multifluorinated compounds. The starting material 2-fluoropropionic acid 83 is sold by Fluorochem Ltd. for £85/g. 

1.4.3.1 Nucleophilic fluorination

Nucleophilic fluorinating reagents can be covalent or ionic in nature. Covalent nucleophilic fluorinating reagents such as diethylaminosulfur trifluoride 65 (DAST) generate fluoride in situ and reaction with alcohol 66 generates the reactive species 68, an alkoxydiethylaminosulfur difluoride intermediate. The fluoride that is generated attacks the intermediate 68 to afford the alkyl fluoride 70 (Scheme 1.15).

```
\begin{center}
\includegraphics[width=0.8\textwidth]{scheme15.png}
\end{center}
```

Scheme 1.15: DAST mechanism. Reagents and conditions: CH₂Cl₂, -50 – -78 °C.
Ionic nucleophilic fluorinating agents include silver fluoride, potassium fluoride and hydrogen fluoride. Silver fluoride has been used to fluorinate methyl-α-bromoisobutyrate 71 to afford methyl-α-fluoroisobutyrate 72 (Scheme 1.16). 

![Scheme 1.16](image)

Scheme 1.16: Fluorination of methyl-α-bromoisobutyrate 71 with silver fluoride. 
Reagents and conditions: 140-145 °C, 3h.

Although hydrogen fluoride is highly toxic, it has often been used as a fluorinating reagent. Its use as a fluorinating agent, however, is limited by its low boiling point (19.6 °C), which requires reactions to be conducted under pressure. Pyridinium poly-(hydrogen fluoride), synthesised from pyridine and hydrogen fluoride, is a less volatile fluorinating reagent and allows reactions to be conducted at atmospheric pressure. Many substrates have been fluorinated with this complex including α-amino acids 73, aminoarenes and carbamates to yield 2-fluorocarboxylic acids 75, fluoroarenes and fluoroformates respectively (Scheme 1.17).

![Scheme 1.17](image)

Scheme 1.17: Fluorination of α-amino acids 73 with pyridinium poly(hydrogen fluoride).
Reagents and conditions: pyridine, anhydrous HF, NaNO₂, rt, 4 h.
1.4.3.2 Electrophilic fluorination

Elemental fluorine can be considered as an electrophilic fluorinating agent and it has been used to fluorinate L-cysteine 76 to afford L-3-fluoroalanine 77 as the major product, with a yield of 33%, and L-3,3-difluoroalanine 78 as a minor product. Multifluorinations arise due to the high reactivity of elemental fluorine (Scheme 1.18). 76

![Scheme 1.18: Fluorination of L-cysteine (S)-76 using highly reactive elemental fluorine. Reagents and conditions: (i) -78 °C, HF, HBF₄, (ii) 0 °C, F₂/He (1:4 v/v), 3 h.](image)

Elemental fluorine is diluted in nitrogen and has been used to fluorinate silyl enol ethers in FCCl₃ at -78 °C to afford α-fluoro ketones, α-fluoro aldehydes and α-fluoro carboxylic acids. Difluoro compounds were also formed and efforts to minimise this were unsuccessful. 77-79

Other electrophilic fluorinating reagents have arisen in the last decade, which are more selective and don’t result in multifluorination, including fluoroxy-trifluoromethane, an RO-F reagent 80, and Selectfluor® 79 81, a N-F reagent (Figure 1.7).

![Figure 1.7: Selectfluor® 79 81](image)
1.4.4 Chemical synthesis of enantiopure 2-fluoropropionic acid 83

Generally, asymmetric fluorinations involve chiral starting materials. Methyl-\((S)\)-lactate \((S)-80\) was used to synthesise methyl \((S)\)-2-methanesulfonyloxypropanoate \((S)-81\) with retention of stereochemistry. Fluoridation with potassium fluoride in formamide results in inversion of stereochemistry to afford \((R)\)-2-fluorocarboxylic ester \((R)-82\), which was hydrolysed to the \((R)\)-2-fluorocarboxylic acid \((R)-83\). Moderate to high yields (33-83%) and good to excellent enantioselectivity (55.5-98.7%) were reported for the synthesis of optically active 2-fluoropropionic acid \((R)-83\) and analogues (Scheme 1.19).\textsuperscript{82}

![Scheme 1.19: Asymmetric fluorination with chiral substrates.\textsuperscript{82}](image)

Reagents and conditions: (i) triethylamine, methanesulfonyl chloride, 4-\(N,N\)-dimethylaminopyridine, TBME, 60 °C, 6 h, (ii) formamide, KF, 60 °C, 20 torr, 4 h, (iii) formic acid, distillation.

1.5 Biocatalysis

Biocatalysis is the use of enzymes as catalysts to perform chemical transformations on organic compounds. These transformations encompass the advantages associated with catalysts but with the selectivity of enzymes. There are numerous applications of biocatalysts reaching back to the oldest chemical transformations known to man, which include production of vinegar, fermentation of beer and wine and baking bread.
Biocatalysts are increasingly being used in a multitude of industries ranging from pharmaceutical, medicine and fine chemicals to food, energy production and mining. The use of biocatalysts is an environmentally friendly and cost effective option due to the mild conditions (low biotransformation temperatures, aqueous or biphasic solvents) they are used when compared to the energy demanding temperatures and pressures required for some chemical transformations. The use of biocatalysts allows the exploitation of their efficiency and selectivity. Under the mild conditions in which they are used, undesired side reactions such as rearrangements are minimised, which assist downstream purification.

### 1.5.1 Biocatalysts

Biocatalysts are able to catalyse reactions with high regio-, chemo- and stereospecificity and it is these properties of an enzyme that have attracted the attention of synthetic organic chemists. Although the substrate specificity of some biocatalysts is somewhat limited, when compared to conventional homogenous or heterogeneous catalysts, the ability of biocatalysts to differentiate between enantiomers of a substrate resulting in high yields and high enantioselectivities of >99% e.e. on a routine basis have lead to their increasing use.

### 1.5.2 Enzyme classes

To categorise the immense number of enzymes, each enzyme has an identification number consisting of four numbers in the form EC A.B.C.D, where EC stands for ‘Enzyme Commission’. Each letter codes for a property of the enzyme where A codes for the main type of reaction the enzyme catalyses, B codes for the subtype of reaction including information concerning the substrate, C codes for the nature of the co-substrate and D codes for the individual enzyme number (Table 1.1).
<table>
<thead>
<tr>
<th>Enzyme class</th>
<th>Reaction type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Oxidoreductases</td>
<td>Oxidation-reduction: oxygenation of C-H, C-C, C=C bonds, or overall removal or addition of hydrogen atom equivalents</td>
</tr>
<tr>
<td>2 Transferases</td>
<td>Transfer of groups: aldehydic, ketonic, acyl, sugar, phosphoryl or methyl</td>
</tr>
<tr>
<td>3 Hydrolases</td>
<td>Hydrolysis-formation of esters, amides, lactones, lactams, epoxides, nitriles, anhydrides, glycosides, organohalides</td>
</tr>
<tr>
<td>4 Lyases</td>
<td>Addition-elimination of small molecules on C=C, C=N, C=O bonds</td>
</tr>
<tr>
<td>5 Isomerases</td>
<td>Isomerisation such as racemisation, epimerisation, rearrangements</td>
</tr>
<tr>
<td>6 Ligases</td>
<td>Formation-cleavage of C-O, C-S, C-N, C-C bonds with concomitant triphosphate cleavage</td>
</tr>
</tbody>
</table>

Table 1.1: Classification of enzymes.83

1.5.3 **Hydrolases (EC 3.X.X.X)**

Hydrolases are a large class of enzymes which include glycosylases, peptidases and esterases. Lipases (EC 3.1.1) are a sub-class of esterases which act on the ester bonds of carboxylic acids. Lipases in particular are ideal for industrial applications. Since they have evolved in nature to operate at an oil-water interface they have a propensity to be compatible with organic solvents. Also their broad substrate specificity results in their ability to catalyse a variety of reactions. Lipases are used in biocatalysis for kinetic resolutions, biofuel synthesis and organic synthesis. They are widely found in microorganisms, plants and mammals. Isolation of hydrolases from microbial sources is advantageous due to well-established production technologies on small and large scales. Many lipases are produced in vast quantities and at low costs and together with their wide applications, has resulted in hydrolases being the most widely used class of enzymes in industry.84
1.6 Biosynthesis of halogenated compounds

Halometabolites of marine organisms tend to incorporate the halogen bromine unlike terrestrial organisms, that have mainly chlorinated halometabolites. This is because there is a high bromine concentration in sea water. The enzymes that incorporate halogens into these metabolites are appealing as an alternative synthetic route to halogenated compounds. Research into this class of enzymes has revealed interesting results concerning their substrate specificity and regio- and stereoselectivity.

There are currently five types of enzymes known to catalyse the formation of carbon-halogen bonds:

1. Haloperoxidases and perhydrolases
2. Flavin dependent halogenases
3. α-Ketoglutarate dependent halogenases
4. Fluorinase
5. Methyl transferases

1.6.1 Haloperoxidases and perhydrolases

The haloperoxidases are further categorised into two types: those that contain a haem group (H type) and those that do not contain a haem iron prosthetic group (NH type).

The most studied H type enzyme of the haloperoxidases is the chloroperoxidase from *Caldariomyces fumago* and its substrates have also been well documented. They include β-keto acids, cyclic β-diketones, steroids, substituted phenols, alkenes, alkynes, α,β-unsaturated carboxylic acids and heterocycles.

One of the first NH type enzymes that was isolated was a vanadium containing bromoperoxidase from *Ascophyllum nodosum*. Many vanadium containing haloperoxidases were isolated from other algae and also from lichen and fungi but not from bacteria. The vanadium containing bromoperoxidase was found to be non-substrate specific too.
The wide range of substrates that are suitable for halogenation with *Caldariomyces fumago* and *Ascophyllum nodosum* give indications about the mechanism of these enzymes. The haloperoxidases, both H type and NH type, employ hydrogen peroxide for peroxidation of chloride and bromide anions into halide cations. The halide is then transferred to the substrate in an electrophilic substitution mechanism. The halogenating species was later confirmed to be hypohalous acid. The halogenating species is released from the enzymes active site before halogenation occurs. This explains the wide range of substrates that the enzymes are able to catalyse and the non-stereoselectivity of the enzymes. The three dimensional structures of the haloperoxidases from *Caldariomyces fumago* and *Ascophyllum nodosum* supported the proposed mechanism. Both the H and NH types of haloperoxidase therefore catalyse the reaction in a mechanistically related manner and because the halogenation step is not enzyme catalysed, the overall process is non-regio- and non-stereospecific (Scheme 1.20).

\[
\text{Enzymatic reaction:} \\
\text{H}_2\text{O}_2 + X^- + H^+ \xrightarrow{\text{Haloperoxidase}} \text{HOX} + \text{H}_2\text{O} \\
\text{Non-enzymatic reaction:} \\
\text{HOX} + \text{AH} \rightarrow \text{A}--X + \text{H}_2\text{O} \\
X = \text{Cl}, \text{Br} \text{ or I}
\]

Scheme 1.20: Haloperoxidase reaction.

One of the first perhydrolases isolated was by Wiesner *et al.* from *Burkholderia pyrrocinia* formerly known as *Pseudomonas*. This perhydrolase does not contain any metal ions or require a co-factor but still used hydrogen peroxide to generate the halogenating species, hence they were classed as haloperoxidases. As more of these perhydrolase enzymes were discovered, a comparison of their structures revealed an \(\alpha,\beta\)-hydrolase fold. Perhydrolases catalyse the formation of peracids which when released from the enzyme active site react with halide ions to form the corresponding hypohalous acid. Subsequent reaction of the hypohalous acid with the substrate is spontaneous resulting in no regio- or stereoselectivity in the reaction.
1.6.2 Flavin dependent halogenases

The discovery of new haloperoxidases and perhydrolases was facilitated by the use of the monochlorodimedone (MCD) assay developed by Hager et al. MCD is not a naturally occurring halometabolite and therefore may have little resemblance to the natural substrates of the enzymes responsible for stereoselective halogenation reactions in nature.

To discover a new class of enzymes that were able to synthesise these naturally occurring halometabolites required a different approach. By cloning the gene cluster for the biosynthesis of 7-chlorotetracycline in Streptomyces aureofaciens, Dairi et al. were able to deduce the gene responsible for carbon-halogen bond formation. This approach was also used to identify the gene cluster responsible for the biosynthesis of pyrrolnitrin from tryptophan. Tryptophan was used as a screen for halogenases within the gene cluster, as it was suggested that 7-chlorotryptophan was the first intermediate species. Two genes and their halogenating functions were identified: tryptophan-7-halogenase (PrnA) and monodechloroaminopyrrolnitrin-3-halogenase (PrnC). It was found that NADH or FADH$_2$, FAD$^+$, oxygen and a flavin reductase were needed for these enzymes to function. Both PrnA and PrnC were found to have a flavin-binding site.

It has been proposed that these enzymes generate hypohalous acid which is not released from the enzyme, unlike the haloperoxidases, leading to high selectivity. Instead the hypohalous acid travels along a “tunnel” lined with amino acid side chain residues to the tryptophan where it is activated by Lys79 (Scheme 1.2). The hypohalous acid is positioned spatially within the active site leading to high regioselectivity of the enzyme catalysed reaction.

Understanding the role of halogenases involved in the biosynthesis of halometabolites is a challenging problem, as is deducing the halogenating step. It has also been observed, whilst studying the halogenases responsible for the production of other halometabolites, that the free substrate is not always accepted by the enzymes. Instead the substrate may need to be bound to an acyl or to a peptidyl carrier protein. Few flavin dependent halogenases use free forms of their substrates, but a few examples include tryptophan, monodechloroaminopyrrolnitrin and 2-(3,5-dibromo)-phenylpyrrole.
Scheme 1.21: Proposed regioselective reaction mechanism of flavin-dependant halogenases.

1.6.3 Fluorinase

The fluorinase is so named because it is a rare enzyme that catalyses formation of a carbon-fluorine bond. Sanada et al. noticed that *Streptomyces cattleya* produced fluoroacetic acid and 4-fluorothreonine. The substrate for fluorinase is (S)-adenosyl-L-methionine 90 and L-methionine is replaced by fluoride to make 5'-fluoro-5'-deoxyadenosine 91, which undergoes metabolism to 4-fluorothreonine 92 and fluoroacetate 93 (Scheme 1.22). Fluorinase has been found recently to also be a chlorinase.114
Scheme 1.22: Fluorination of (S)-adenosyl-L-methionine 90 by Fluorinase.

The use of halogenases to synthesise carbon-halogen bonds in a preparative manner would represent a major accomplishment. Both haloperoxidases and perhydrolases display no regioselectivity or stereoselectivity since the halogenating species is free hypohalous acid. The flavin-dependent halogenases are difficult to work with due to the bound substrates. The substrate specificity of the fluorinase enzyme is currently limited. However, novel halogenated metabolites have been synthesised from flavin-dependent halogenases and the future of using halogenases in mainstream chemistry shows potential.

1.7 **Kinetic resolution of α-haloacids and esters using lipases**

Resolution of α-haloacids and -esters using enzymatic methods allows for the high regio-, chemo- and stereoselectivity associated with enzymes. Kinetic resolution also affords both enantiomers of the α-haloacids/esters, which enables the synthesis of target molecules of both enantiomeric series thereby avoiding wasting 50% of the starting material.
1.7.1 Kinetic resolution of &alpha;-haloacids

Kinetic resolution of &alpha;-haloacids *rac*-94 allows the separation of the enantiomers *via* enzymatic conversion of one of the enantiomers to the ester *(R)*-95, which can be hydrolysed back to the acid *(R)*-94 (Scheme 1.23).

![Diagram of kinetic resolution of racemic &alpha;-haloacids](image.png)

Scheme 1.23: Kinetic resolution of racemic &alpha;-haloacids *rac*-94 leads to enantiopure &alpha;-haloacids *(S)*-94 and *(R)*-94 *via* the ester *(R)*-95.

Several lipases including *Candida rugosa*, formally known as *Candida cylindracea*, lipase and porcine pancreatic lipase (PPL), have been reported to be active towards &alpha;-haloacids as substrates in organic solvents. Both of these lipases are commercially available, require no co-factor for activity and have broad substrate specificity. The reactions were conducted in hexane and although the lipases were insoluble, *Candida rugosa* retained activity whilst PPL had low activity. High enantiomeric excesses were achieved when racemic acids were esterified with an excess of *n*-butyl alcohol using *Candida rugosa* and the lipase was *(R)*-selective. The substrate specificity was investigated and results showed that an electron withdrawing group on the acid was necessary for activity.\(^{116}\)
1.7.2 Kinetic resolution of α-haloesters

Another route to enantiomerically pure α-haloacids (R)- and (S)-94 is via hydrolysis of α-haloesters rac-96 (Scheme 1.24).

Scheme 1.24: Kinetic resolution of racemic α-haloesters rac-96 to enantiopure α-haloacids (R)- and (S)-94.

In this study, more hydrolases were identified as being active towards α-haloesters in aqueous buffer than towards α-haloacids in organic solvents. Several hydrolases have been reported as having activity in the kinetic resolution of α-haloesters including Carlsberg’s subtilisin, Bacillus subtilis and Pseudomonas fluorescens. Both Bacillus subtilis and Pseudomonas fluorescens lipase have shown stereoselectivity towards (S)-α-haloesters with varying enantiomeric excesses depending on the substrate used (Scheme 1.24 and Table 1.2).\textsuperscript{117,118}
Table 1.2: Kinetic resolution of α-haloesters using whole-cells and isolated enzymes.\textsuperscript{117,118}

<table>
<thead>
<tr>
<th>Biocatalyst</th>
<th>Substrate</th>
<th>Conversion</th>
<th>e.e. of (R)</th>
<th>e.e. of (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X R</td>
<td>%</td>
<td>ester %</td>
<td>acid %</td>
</tr>
<tr>
<td>\textit{Bacillus subtilis}\textsuperscript{a}</td>
<td>Br Me</td>
<td>22</td>
<td>-</td>
<td>89\textsuperscript{b}</td>
</tr>
<tr>
<td>\textit{Bacillus subtilis}\textsuperscript{a}</td>
<td>Br Et</td>
<td>33</td>
<td>-</td>
<td>5\textsuperscript{b}</td>
</tr>
<tr>
<td>\textit{Bacillus subtilis}\textsuperscript{a}</td>
<td>Br Bu</td>
<td>34</td>
<td>-</td>
<td>8\textsuperscript{b}</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>F Et</td>
<td>50\textsuperscript{c}</td>
<td>84\textsuperscript{d}</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>F Et</td>
<td>60\textsuperscript{c}</td>
<td>99.9\textsuperscript{d}</td>
<td>68.5\textsuperscript{d}</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>Br Me</td>
<td>60\textsuperscript{c}</td>
<td>93.5\textsuperscript{d}</td>
<td>93\textsuperscript{d}</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>Br n-Bu</td>
<td>50\textsuperscript{c}</td>
<td>72\textsuperscript{d}</td>
<td>-</td>
</tr>
<tr>
<td>\textit{Pseudomonas fluorescens}</td>
<td>Br Et</td>
<td>50\textsuperscript{c}</td>
<td>72.5\textsuperscript{d}</td>
<td>69.4\textsuperscript{d}</td>
</tr>
</tbody>
</table>

\textsuperscript{a} wet whole cells as enzyme, \textsuperscript{b} e.e. % determined by optical rotation values, \textsuperscript{c} % conversion determined by the amount of base added, \textsuperscript{d} e.e. % determined by GC of the diastereomeric Mosher’s ester derivatives, - indicates data not given.

Table 1.2: Kinetic resolution of α-haloesters using whole-cells and isolated enzymes.\textsuperscript{117,118}

Reagents and conditions: (a) \textit{Bacillus subtilis}, phosphate buffer, pH 8.0, 25 °C, 4 h, (b) \textit{Pseudomonas fluorescens}, buffer, pH 6.5, 30 °C.

1.8 Directed Evolution

Many enzymes have evolved to catalyse specific reactions in certain biochemical pathways. To fine tune the properties of an enzyme to a different application requires alteration and optimisation of the enzyme activity. This can be done by medium engineering, immobilisation of the enzyme or even change of reaction conditions. One example showed that upon addition of Tween-80 to a \textit{Candida antarctica} lipase B (Cal B) catalysed kinetic resolution of \(N\)-(2-ethyl-6-methylphenyl) alanine methyl ester, the enantioselectivity
increased from 11.3 to 60.1. A complimentary approach to changing enzyme activity is by protein engineering. This method potentially allows changes in substrate specificity, catalytic activity, solubility and even enhancement of regio-, chemo- or stereospecificity. Evolutionary techniques are required to evolve the gene that codes for the enzyme. Directed evolution involves mutations to the gene, expression of the mutant enzymes and a high throughput screening method for the desired characteristic. The mutant that possesses the desired improvement is selected and then subjected to further rounds of evolution until the required level of improvement is achieved (Figure 1.8).

Currently, there are several ways to generate libraries of variant genes in the laboratory, including error-prone polymerase chain reaction (epPCR), DNA shuffling and iterative saturation mutagenesis (ISM).

Once a library has been generated, consisting of thousands of variants, screening allows for the identification and isolation of positive clones. Screening is generally seen to be the bottleneck in the process of directed evolution since each variant requires expression before screening. The ideal assay would allow rapid and reliable determination of the
property of an enzyme with high accuracy and reproducibility. Additionally, an assay should convey true information about enzyme kinetics, activities and selectivities.

1.8.1 Mutagenesis techniques

1.8.1.1 Error prone PCR (epPCR)

The polymerase chain reaction is a technique to exponentially replicate DNA; however with epPCR the reliability of the genetic replication process is compromised in order to incorporate ‘errors’. *Thermus aquaticus* (*Taq*) DNA polymerase is the enzyme used to incorporate random nucleotide mutations, which result in amino acid substitutions. These mutations arise due to the lack of proof reading using the *Taq* DNA polymerase alone. Variation of the reaction conditions are also used to generate mutations including addition of Mn$^{2+}$ as an additional co-factor, having a biased ratio of nucleotides and/or an increased concentration of nucleotides.$^{123-125}$ The mutated genes are then re-cloned into an expression vector and then transformed into a suitable host, where the mutants can be screened for the desired characteristic.$^{129}$

1.8.1.2 Iterative saturation mutagenesis (ISM)

Iterative saturation mutagenesis is a recently developed method which minimises the screening effort required to locate the mutant with the desired characteristic. ISM involves initially the analysis of the protein structure for regions which may significantly affect the catalytic activity. Once these regions have been identified they are targeted for rounds of saturation mutagenesis. The amino acids in these regions or ‘hot spots’ are substituted for all other 19 natural amino acids.$^{130,131}$ If four regions of a protein are submitted to saturation mutagenesis, then the best hit from each of those libraries is used as a template for the next round of saturation mutagenesis (Figure 1.9).$^{128}$
Furthermore not all pathways need to be explored. For instance, if library A has the best hit after the first round of saturation mutagenesis then this library can be focussed on. The other libraries can be reviewed once options in library A have been exhausted.\textsuperscript{128}

### 1.8.2 Screening

There are several high throughput screening methods that are used in laboratories including colorimetric/fluorometric assays or chromatographic assays. The chromatographic assay is the least high throughput as preparation of samples for common analytical techniques such as gas chromatography are required. The most commonly used screens are colorimetric/fluorometric screens, where even small fluctuations in activity can be observed. Also this screen is a high throughput method with samples being screened in microtiterplates simultaneously.\textsuperscript{132,133}

#### 1.8.2.1 Solid phase tributyrin agar screen

The tributrin agar screen is a preliminary screen used to identify mutants of hydrolytic enzymes that are not able to hydrolyse the substrate tributyrin.\textsuperscript{134} Tributyrin is insoluble when added to agar and the agar takes on a milky appearance (Figure 1.10).
The products from hydrolysis of tributyrin are soluble and so in the case of an active mutant, clear spots appear around the mutant colony (Figure 1.11).

The active mutants highlighted by the clearance halos can be picked for further investigation thereby reducing the number of mutants that need to be grown in liquid phase for further analysis.

1.8.2.2 para-Nitrophenyl acetate screen (pNPA)

The para-nitrophenyl acetate assay is a high throughput assay, that can be used to determine the activity of hydrolytic enzymes. The pNPA assay makes use of a chromogenic substrate, para-nitrophenyl acetate and represents a facile and
quantitative method to monitor hydrolytic reactions, because for every mole of substrate hydrolysed, one mole of para-nitrophenol 98 is formed (Scheme 1.25).

![Scheme 1.25: pNPA assay reaction.](image)

The product, para-nitrophenol 98, is colourless when protonated, but becomes yellow in aqueous alkaline solutions and hence can be detected at 410 nm allowing directed measurement of enzyme activity. The assay can be conducted in 96-well plates allowing for high throughput screening of libraries of variants. The screen takes only a few minutes to conduct and together with multichannel pipettes permits the screening of hundreds to thousands of mutants a day. However, a disadvantage of the pNPA assay is the use of a surrogate substrate. This assay is mostly used to further reduce screening efforts by eliminating inactive mutants.

1.8.2.3 High throughput enantioselective screen

To date, very few high throughput enantioselective screens have been reported. The first such screen was described by Reetz et al., in 1997\textsuperscript{134} and is based on an adaptation of the pNPA assay.\textsuperscript{136} In place of using a racemic chromogenic substrate, which would only give kinetic information about the mutants, the rate of hydrolysis of enantiopure substrates, tested in separate wells, is measured to assess the enantioselectivity. Comparison of the rate of reaction for each enantiomer shows the apparent enantioselectivities of the mutants. True enantioselectivities then need to be calculated by using more conventional chiral analytical techniques.

1.9 Candida antarctica lipase B (Cal B)

Cal B is a well known lipase used in industry and has been the subject of a large amount of research in academia. There are numerous publications dealing with Cal B showing that it
is an efficient enzyme with applications in regio- and enantioselective synthesis. Cal B is highly robust and although its optimal pH for catalysis is 7, it can operate in a pH range of 3.5–9.5 and in organic solvents. Its high denaturation temperature and the fact that it can be easily immobilised, which further increases thermostability\textsuperscript{137,138}, has allowed it to find its way into many industrial applications.

1.9.1 Cal B structure and mechanism of hydrolysis of an ester

The X-ray crystallographic structure of Cal B was solved in 1994 by Uppenburg \textit{et al.}\textsuperscript{139,140} allowing prediction of selectivity and providing an additional tool for molecular modelling studies. Cal B is 317 amino acids in length and possesses an α/β- hydrolase fold.\textsuperscript{141} A characteristic of lipases is the catalytic triad at the active site. Cal B has a catalytic active site consisting of the residues serine 105 (Ser105), aspartic acid 187 (Asp187) and histidine 224 (His224). The active site is situated at the bottom of a narrow and deep hydrophobic tunnel approximately 10 x 4 Å wide and 12 Å deep.\textsuperscript{140,142} The active site consists of two channels; one which accommodates the acyl moiety and the other the alcohol moiety of an ester substrate.\textsuperscript{142}

The substrate ethyl butyrate \textbf{100} is hydrolysed to butanoic acid \textbf{105} by Cal B. The mechanism involves the binding of the substrate \textbf{100} to the active site of Cal B. The oxygen of the hydroxyl side chain on the Ser105 residue is positioned to be the catalytic nucleophile. The hydroxyl group attacks the carbonyl carbon of the ester substrate \textbf{100}. The His224 residue acts as a hydrogen acceptor and accepts a proton from Ser105 (A, Scheme 1.26). An electron pair moves from the carbonyl bond onto the oxygen to form a tetrahedral intermediate. The oxyanion intermediate is stabilised by a hydrogen bonding network composed of the residues Thr40 and Gln106. The negative oxygen reforms the carbonyl bond and the carbon-oxygen bond of the alcohol moiety is broken, with His224 now acting as a hydrogen donor (B, Scheme 1.26). The ethanol that is produced is released from the active site and water enters to re-establish the active site. The water molecule is activated by His224 and acts as a nucleophile to attack the carbonyl of the acyl-enzyme intermediate (C, Scheme 1.26). The newly generated negative oxygen reforms the carbonyl bond and breaks the C–O bond between the substrate and the enzyme which accepts a proton from His224 (D, Scheme 1.26). The product, butanoic acid \textbf{105}, is released from the active site and the active site is reinstated for further catalysis (E, Scheme 1.26). The
Asp187 catalytic triad residue serves to stabilise the changing nature of His224 via hydrogen bonding with the carbonyl group in Asp187 (Scheme 1.26).

Scheme 1.26: Ester hydrolysis mechanism by Cal B.
1.9.2 Biosynthesis of biodiesel using Cal B

Cal B has many industrial applications including the biosynthesis of biodiesel. Biodiesel, which is an alternative to conventional fossil fuel based diesel, can be prepared from locally generated waste products such as spent cooking oil and animal fats and a catalyst such as Cal B. The use of biocatalysts are advantageous over traditional chemical alcoholysis as the enzymatic route tolerates more diverse feedstock, conserves energy and has minimised side reactions, which alleviates downstream purification. The blend of triglycerides and fatty acids undergo transesterification and esterification with short chain alcohols to fatty acid alkyl esters to be used in conventional diesel engines. Cal B used in conjunction with lypoyme is the first reported lipase catalysed biodiesel production on an industrial scale worldwide. In China this technology is used to produce 20,000 tons per year of biodiesel.

1.9.3 Enantioselective synthesis of (±)-harzia lactone A, 56 using Cal B

Cal B has been applied to the enantioselective synthesis of (±)-harzia lactone A 106. Many natural products, such as (±)-harzia lactone A 106, have been found to harbour a wealth of new pharmacological properties. The problem arises when attempting to discover which enantiomer is the active compound for large scale synthesis which requires access to both enantiomers of (±)-harzia lactone A 106. Cal B was used for the synthesis of the secondary metabolite, (±)-harzia lactone A 106, which showed moderate biological activity against infective agents (Figure 1.12).

![Figure 1.12: (±)-Harzia lactone A, 106.](image)

An investigation into whether the (–)-stereoisomer had increased levels of cytotoxicity over the (+)-stereoisomer was conducted. The absolute configuration of (±)-harzia lactone
A 106 was determined as (3R,5R)-106 by chemical synthesis from D-glucose146 and D-xylose147, each in seven steps. To access the (−)-stereoisomer, a synthesis was undertaken from L-malic acid to give the (3S,5S)-enantiomer. A more simplified approach would be a kinetic resolution of an intermediate from which both stereoisomers can be obtained. Cal B was used for the kinetic resolution of homoallylic alcohol 107 to give selective acylation of the (R)-enantiomer (R)-107 with a yield of 47% and an enantiomeric excess of 97%. The (S)-enantiomer (S)-107 was recovered in 44% yield and an enantiomeric excess of 87%. From these chiral intermediates the first chemoenzymatic route to all stereoisomers of harzia lactone A was developed (Scheme 1.2).148

Scheme 1.27: Kinetic resolution of homoallylic alcohol 107 using Cal B.
Reagents and conditions: Cal B, vinyl acetate, α-iso-propyl ether, 25 °C, 26 h.

1.9.4 Enhancing the enantioselectivity of Cal B towards secondary alcohols by directed evolution

There are numerous reports of the use of Cal B to kinetically resolve secondary alcohols yielding enantiomerically pure products in high enantiomeric excess.148 Cal B is often included in screening processes due to its broad substrate specificity.

Cal B was used for the kinetic resolution of secondary alcohols bearing keto or acetoxy groups in the δ-position from the carbinol.149 Cal B has a small enantiorecognition pocket for secondary alcohols defined by threonine 42 (Thr42), serine 47 (Ser47) and tryptophan 104 (Trp104).150-152 Poor enantioselectivity (E_R = 9) was initially reported for the kinetic
resolution of secondary alcohols bearing keto or acetoxy groups. The kinetic resolution of simpler secondary alcohols, such as those bearing a hydroxy group, using Cal B has been reported with an $E_R > 200$. The difference in $E_R$ was attributed to the interaction of the $\delta$-position functional group with the surrounding active-site residues. Electrostatic interactions between the substrate and enzyme induced by a small polar environment close to the oxyanion hole created by threonine 40’s carbonyl group was found to be especially important. The mutant Ala281Ser was designed to probe this model and was produced by site-directed mutagenesis. This variant was found to have a 13-fold higher enantioselectivity ($E_R = 120$). This example demonstrates that the enantioselectivity of Cal B can be enhanced significantly by introducing a single amino acid change.

1.9.5 Enhancing the enantioselectivity of Cal B by engineered substrate-assisted catalysis

To enhance the enantioselectivity of lipases, strategies such as site-directed mutagenesis\textsuperscript{153,154} and directed evolution\textsuperscript{155,156} have been used. A complementary approach is to employ substrate engineering. The kinetic resolution of ethyl 2-hydroxypropanoate using Cal B proceeded with very poor enantioselectivity ($E_R = 1.6$).\textsuperscript{157} It was proposed that the hydroxyl group of the substrate ethyl 2-hydroxypropanoate could substitute for an active site residue namely Thr40 (Figure 1.13). As discussed in the sections 1.91 and 1.9.4, the Thr40 residue is part of a network of hydrogen bonds that stabilises the intermediate. Specifically it is the hydroxyl side chain and the amide backbone of Thr40 that constitute two of the three hydrogen bonds in this network.\textsuperscript{158} The mutants Thr40Val and Thr40Ala were engineered using site directed mutagenesis. The kinetic parameters of these mutants were compared to the wild-type and it was found that the Thr40Val and Thr40Ala mutants had $k_{cat}/k_M$ values three orders of magnitude lower than the wild-type. Screening of the mutants Thr40Val and Thr40Ala with the substrate ethyl 2-hydroxypropanoate showed an increase of enantioselectivity to 22 and 9.8 respectively. Through engineering of the enzyme or the substrate, it has been shown that properties such as enantioselectivity can be manipulated.
Figure 1.13: Cal B active site.

A = transition-state stabilisation in wild-type enzyme. B = Substrate-assisted transition state stabilisation in a Thr40Val mutant.

1.9.6 Enhancing the enantioselectivity of Cal B using a genetic selection system

Section 1.8 discusses the combination of gene mutagenesis, expression and high throughput screening of libraries of enzyme variants. In place of screening, selection has also been reported in which cell survival is linked to an enzyme-catalysed reaction.\textsuperscript{159-161}

This selection strategy has been applied to the hydrolytic kinetic resolution of esters of chiral isopropylidene glycol \textsuperscript{109}.\textsuperscript{162} Cal B displays an $E_R$ of 1.9 for the kinetic resolution of racemic isopropylidene glycerol acetate \textsuperscript{109} in which Cal B is (R)-selective. (S)-isopropylidene glycerol acetate (S)-\textsuperscript{109} and (R)-isopropylidene glycerol fluoroacetate (R)-\textsuperscript{112} were synthesised as pseudo-enantiomers for the selection strategy. Upon hydrolysis of (S)-isopropylidene glycerol acetate (S)-\textsuperscript{109}, acetic acid \textsuperscript{111} and (R)-isopropylidene glycol (R)-\textsuperscript{110} are formed. The expression host, \textit{Pichia pastoris}, can use acetic acid \textsuperscript{111} as a carbon source (Scheme 1.28). However, upon hydrolysis of (R)-isopropylidene glycerol fluoroacetate (R)-\textsuperscript{112} the product formed is fluoroacetic acid \textsuperscript{113}, which is toxic to the expression host (Scheme 1.29).
Scheme 1.28: Hydrolysis of (S)-isopropylidene glycerol acetate (S)-109 by Cal B to form acetic acid 111 and (R)-isopropylidene glycol (R)-110.

Scheme 1.29: Hydrolysis of (R)-isopropylidene glycerol acetate (R)-112 by Cal B to form acetic acid 113 and (S)-isopropylidene glycol (S)-110.

Mutagenesis of Cal B using the Combinatorial Active-Site Saturation Test (CAST), which involves systematic saturation mutagenesis at relevant residues, was carried out. Variants of Cal B were generated and screened using the selection assay. Selection plates with 0.3% of (S)-109 and 0.003% of (R)-112 were used for the assay. Variants of Cal B with an \( E_R \) of 3-8 were isolated and they had a reversal of enantioselectivity to the wild-type.

There are many uses of Cal B as demonstrated in the examples in section 1.9. Additionally the use of molecular dynamics and directed evolution together creates a very powerful tool for the rational design of mutants. Novel screening systems for enantioselectivity are also being developed and have been applied to Cal B. With these tools the activity and enantioselectivity of Cal B can be enhanced for many industrial applications.
2 Results and Discussion

2.1 Objectives

The overall objective was to develop a scaleable biocatalytic process to access 2-bromopropionic acid \(35\), 2-fluoropropionic acid \(83\) and their esters in a single enantiomeric form with a high enantiomeric excess. Since kinetic resolution based upon the hydrolysis or esterification of an appropriate substrate would allow access to both enantiomers, kinetic resolution of racemic 2-bromopropionic acid \(35\), 2-fluoropropionic acid \(83\) and their ester derivatives using hydrolases was proposed.

In order to develop a biocatalytic process, several key objectives need to be met:

a) Identification of a suitable hydrolase that would furnish 2-bromopropionic acid \(35\), 2-fluoropropionic acid \(83\) and their ester derivatives at high conversion rates with reasonable optical purity.

b) Once a hydrolase had been identified, isolation of the gene and insertion into a suitable vector for production of soluble and active protein.

c) Purification and characterisation of the protein to be used as a baseline for genetic manipulation.

d) Development of a strategy to identify mutants of the hydrolase with enhanced enantioselectivity.

This approach would allow the synthesis of both enantiomers of a small range of chiral starting materials and some pharmacologically active target compounds.

2.2 Chiral analytical techniques

In order to screen for active lipases against the substrates 2-bromopropionic acid \(35\) and 2-fluoropropionic acid \(83\) (Table 2.1), a chiral high-performance liquid chromatography (HPLC) method was developed which allowed analysis of the biotransformation reactions. The separation of the enantiomers of the substrate (acid) and the product (ester) was achieved on a chiral Daicel Chiralpak® AD-H column using an eluent combination. The separated substrate and product were then detected by UV. Using the developed HPLC
methods, retention times of the standards were recorded (Table 2.2) with assignment of the HPLC peaks being achieved by comparison with authentic samples.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Column Temp. (°C)</th>
<th>Flow rate (ml/ min)</th>
<th>Mobile Phase</th>
<th>Hexane</th>
<th>Ethanol</th>
<th>TFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid 35</td>
<td>10</td>
<td>0.3</td>
<td>95</td>
<td>4.9</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>2-fluoropropionic acid 83</td>
<td>22</td>
<td>0.3</td>
<td>95</td>
<td>4.9</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>n-butyl-2-bromopropionate 114</td>
<td>0</td>
<td>0.2</td>
<td>98</td>
<td>1.9</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Column – Daicel Chiralpak® AD-H - 5 µm particle size, 4.6 mm internal diameter, 250 mm column length

Table 2.1: Chiral HPLC conditions for the separation of 2-bromopropionic acid 35, 2-fluoropropionic acid 83 and n-butyl-2-bromopropionate 114 using a Daicel Chiralpak® AD-H column.

An HPLC method was developed to analyse the progress of the biotransformation reactions. This method allowed both the disappearance of the substrate, 2-bromopropionic acid 35 and the appearance of n-butyl-2-bromopropionate 114 from the esterification reaction of 2-bromopropionic acid 35 to be observed in a single analysis run. Chiral HPLC conditions with shorter retention times, providing baseline separation of the enantiomers of 2-bromopropionic acid 35 and the appearance of n-butyl-2-bromopropionate 114, were also developed (Table 2.3 and Table 2.4). In the enzymatic esterification of 2-fluoropropionic acid 83 only the disappearance of the substrate was monitored as the n-butyl ester of 2-fluoropropionic acid 115 was not available.
### Table 2.2: Retention times of the authentic purchased standards using the method outlined in Table 2.1.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Retention time (min)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid 35</td>
<td>28.7</td>
<td>(S)-2-bromopropionic acid¹</td>
</tr>
<tr>
<td>2-fluoropropionic acid 83</td>
<td>21.6</td>
<td>first acid enantiomer</td>
</tr>
<tr>
<td>n-butyl-2-bromopropionoate 114</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.4</td>
<td>acrylic acid¹,²</td>
</tr>
<tr>
<td></td>
<td>23.7</td>
<td>first ester enantiomer</td>
</tr>
<tr>
<td></td>
<td>24.3</td>
<td>second ester enantiomer</td>
</tr>
</tbody>
</table>

¹ verified with authentic purchased samples, ² product from degradation of substrate

### Table 2.3: HPLC conditions for the analysis of the biotransformation reactions on a Daicel Chiralpak® AD-H column.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Column Temp. (°C)</th>
<th>Flow rate (ml/ min)</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid 35</td>
<td>40</td>
<td>1.5</td>
<td>95 4.9 0.1</td>
</tr>
<tr>
<td>2-fluoropropionic acid 83</td>
<td>40</td>
<td>1.0</td>
<td>95 4.9 0.1</td>
</tr>
</tbody>
</table>

Table 2.3: HPLC conditions for the analysis of the biotransformation reactions on a Daicel Chiralpak® AD-H column.
<table>
<thead>
<tr>
<th>Substrate</th>
<th>Retention time (min)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid</td>
<td>5.0</td>
<td>(S)-2-bromopropionic acid¹</td>
</tr>
<tr>
<td></td>
<td>5.3</td>
<td>(R)-2-bromopropionic acid¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ester</td>
</tr>
<tr>
<td>2-fluoropropionic acid</td>
<td>5.8</td>
<td>acrylic acid¹</td>
</tr>
<tr>
<td></td>
<td></td>
<td>first acid enantiomer</td>
</tr>
<tr>
<td></td>
<td></td>
<td>second acid enantiomer</td>
</tr>
</tbody>
</table>

Table 2.4: Retention times of the substrates and products of the biotransformation reactions using the method described in Table 2.3.

The degree of conversion of the biotransformation reactions was measured by taking samples of the biotransformation reactions and analysing them using the HPLC method developed (Table 2.3). Samples of the substrate and product of known concentrations were also analysed using the HPLC method and the molar extinction coefficient (ε) was calculated. The degree of conversion was calculated using the ratio between the produced peak areas of *n*-butyl-2-bromopropanoate 114 (A<sub>prod</sub>) and both enantiomers of 2-bromopropionic acid 35 (A<sub>sub</sub>). This ratio is shown in Equation 1. The HPLC data was also used to calculate the enantiomeric excess of the substrate for the biotransformation reactions using Equation 2. The selectivity or enantiomeric ratio, E<sub>R</sub>, was calculated using conversion (c) and enantiomeric excess of the substrate (ee(S)) according to Chen et al. (Equation 3)<sup>163</sup>

\[
\text{Conversion} = \frac{A_{\text{prod}}/\varepsilon}{(A_{\text{sub}}/\varepsilon) + (A_{\text{prod}}/\varepsilon)} \quad (1)
\]
\[ \text{ee} = \text{conc. of the (S)-enantiomer} - \text{conc. of the (R)-enantiomer} \] \[ \text{conc. of the (S)-enantiomer} + \text{conc. of the (R)-enantiomer} \] \[ E_R = \ln[(1-c)(1-\text{ee}(S))] \div \ln[(1-c)(1+\text{ee}(S))] \] 

2.3 Screening of hydrolases for enantioselective esterification of \( \alpha \)-bromo- and \( \alpha \)-fluoropropionic acid

A preliminary screen of a panel of hydrolases for the enantioselective esterification of the two substrates, 2-bromopropionic acid 35 and 2-fluoropropionic acid 83, with \( n \)-butanol 117 was undertaken. A commercially available lipase kit from Alphamerix, Cambridge, UK containing 15 various lipases was used for the screening together with \textit{Candida rugosa} esterase powder (Dr. Andrew Carnell, University of Liverpool, U.K.) which had been shown to be active in previous studies.\textsuperscript{164} The conditions chosen for the screening process were not optimised for a particular lipase and were the general conditions chosen for the initial screen.

2.3.1 Screening of 2-bromopropionic acid 35 using a lipase kit

The HPLC method developed for the analysis of the biotransformation reactions was used to monitor and analyse the reactions (Table 2.3). Samples were taken initially after 30 minutes and 1 hour and they were analysed using the HPLC method described in Table 2.3. Based on those initial results, active enzymes were monitored more frequently (Table 2.5).

![Scheme 2.1: Kinetic resolution of 2-bromopropionic acid 35 using a commercially available lipase kit.](image_url)

Reagents and conditions: 2-bromopropionic acid (0.5g/L), enzyme (0.5g/L), \( iso \)-hexane (2mL), 1-butanol (5 equiv.), incubated in a 30 °C shaker.
<table>
<thead>
<tr>
<th>Species</th>
<th>Preparation</th>
<th>Time (h)</th>
<th>Degree of conversion (%)</th>
<th>Acid ee (%)</th>
<th>E&lt;sub&gt;R&lt;/sub&gt; (R/S)</th>
<th>Concentration of protein μg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas cepacia lipase</td>
<td>Alphamerix P1 AE 06</td>
<td>48</td>
<td>0</td>
<td>-</td>
<td>-</td>
<td>134.9</td>
</tr>
<tr>
<td>Alcaligenes spp lipase</td>
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<td>E&lt;sub&gt;R&lt;/sub&gt;&lt;sup&gt;4&lt;/sup&gt; (R/S)</td>
<td>Concentration of protein µg/mL</td>
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<sup>1</sup> Commercial supplier number  
<sup>2</sup> Calculated from HPLC data using Eq. 1 (Appendix 1).  
<sup>3</sup> Calculated from HPLC data using Eq. 2 (Appendix 2).  
<sup>4</sup> Calculated from HPLC data using Eq. 3 (Appendix 3).

Table 2.5: Results from the kinetic resolution of 2-bromopropionic acid 35 using commercially available lipases.
The enzymes identified from this screen that were active towards 2-bromopropionic acid 35 included both preparations of *Candida cylindracea* (*rugosa*), *Candida antarctica* A lipase, *Mucor miehei* lipase, Cal B and *Candida rugosa* esterase, however, the selectivity of these enzymes was not high. The most selective enzymes were *Candida cylindracea* (*rugosa*), the Alphamerix C2 AE 02 preparation, and Cal B with an $E_R$ of 2.4-2.5 and 2.5-2.6 respectively. Very few enzymes were able to accept 2-bromopropionic acid 35 as a substrate in all probability due to the bulky bromine atom and the size of the active site in these enzymes. Interestingly *Mucor miehei* lipase and Cal B exhibited opposite stereopreference for the substrate 2-bromopropionic acid 35 to the other active enzymes.

### 2.3.2 Screening of 2-fluoropropionic acid 83 using a lipase kit

The lipase kit was also used to screen for activity against the substrate 2-fluoropropionic acid 83. The samples were analysed using the HPLC method outlined in Table 2.3. Samples were taken at regular intervals (Scheme 2.2 and Table 2.6).

![Reaction Scheme](image)

Scheme 2.2: Kinetic resolution of 2-fluoropropionic acid 83 using a commercially available lipase kit.

Reagents and conditions: 2-fluoropropionic acid (5g/L), enzyme (0.5g/L), iso-hexane (2mL), 1-butanol (5 equiv.), incubated in a 30 °C shaker.

When considering the steric hindrance caused by the bromine atom in 2-bromopropionic acid 35 in the active site of Cal B, in contrast to the comparatively small fluorine atom in 2-fluoropropionic acid 83, one would expect more enzymes to be able to accept the substrate 2-fluoropropionic acid 83. However, the results show that fewer enzymes were able to process 2-fluoropropionic acid 83. Only two enzymes were found to be active towards 2-fluoropropionic acid 83, namely *Alcaligenes* spp lipase (Alphamerix A2 AE 011 preparation) and Cal B. Once more the selectivity of these active enzymes was not high with $E_R$ values of 1.4 and 2.5 respectively. It was observed during HPLC method...
development that 2-fluoropropionic acid 83 was not a stable substrate and liberated hydrogen fluoride to produce acrylic acid. Since the biotransformations were conducted in \( \text{iso} \)-hexane, with no buffering capacity available, it is possible that some of these reactions failed due to the highly acidic products from the degradation of the substrate. Cal B was able to process 2-fluoropropionic acid 83 because it can operate in a pH range of 3.5-9.5. Both active enzymes were selective for the first eluting enantiomer of 2-fluoropropionic acid 83.

<table>
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<th>Hydrolase</th>
<th>Species</th>
<th>Preparation</th>
<th>Time (h)</th>
<th>Degree of conversion (%)</th>
<th>Acid 83 ee (%)</th>
<th>( E_R ) (1/2)</th>
<th>Concentration of protein μg/mL</th>
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<table>
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<td>Time (h)</td>
<td>Degree of conversion (%)</td>
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<td>$E_R$ (1/2)</td>
<td>Concentration of protein (µg/mL)</td>
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1 Commercial supplier number  
2 Calculated from HPLC data using Eq. 4 (Appendix 4).  
3 Calculated from HPLC data using Eq. 2 (Appendix 2).  
4 Calculated from HPLC data using Eq. 3 (Appendix 3).

Table 2.6: Results from the kinetic resolution of 2-fluoropropionic 83 acid using lipases.

### 2.3.3 Bicinchoninic acid assay

To provide an indication of how much protein was present in each biotransformation, the total amount of protein was determined using a bicinchoninic acid (BCA) protein assay. It should be noted that the preparations of enzyme used were not pure. The assay works by combining the biuret reaction with colorimetric detection of the cuprous cation produced. In the biuret reaction, $\text{Cu}^{2+}$ is reduced to $\text{Cu}^{1+}$ by the protein in an alkaline medium and then the $\text{Cu}^{1+}$ cation is detected by chelating with two molecules of BCA 116 (Scheme 2.3).

By preparing known concentrations of bovine serum albumin (BSA), the concentrations of protein in the biotransformations were determined. A standard curve, generated by plotting the average blank-corrected measurements for each BSA standard against concentration (µg/mL), was used to determine the protein concentration of each of the unknown samples. The BCA assay was conducted on a microplate scale and the end-point was measured at 584 nm on a plate reader.
The enzymes that were active towards the substrate 2-bromopropionic acid were *Candida cylindracea* (rugosa) Alphamerix C2 AE 02 preparation (131.6 µg/mL), *Candida cylindracea* (rugosa) Alphamerix C1 AE 01 preparation (64.3 µg/mL), *Candida antarctica* A lipase (287.4 µg/mL), *Mucor miehei* lipase (66.3 µg/mL), Cal B (39.6 µg/mL) and *Candida rugosa* esterase (29.9 µg/mL). The enzymes that were active towards the substrate 2-fluoropropionic acid were *Alcaligenes* spp lipase Alphamerix A2 AE 011 preparation (194.3 µg/mL) and Cal B (39.6 µg/mL). There was no correlation between the amount of protein present in the biotransformations and the activity of the enzymes towards the substrates. The Cal B lipase, which was found to be active towards both 2-bromopropionic acid and 2-fluoropropionic acid, had a very low protein concentration when compared to some of the other preparations.

### 2.3.4 Results of the initial screen of hydrolase enzymes

Enantioselectivity arises from a difference in rates of reaction of one enantiomer over the other, which is in turn derived from the difference in activation energies and this difference is due to the way each enantiomer fits into the active site. Chiral discrimination occurs when one enantiomer fits into the active site preferentially over the other. As the initial screen showed, none of the enzymes tested was truly enantioselective and the enzymes
were able to accept both enantiomers. Highly enantioselective enzymes reach a conversion
of ca. 50 % whilst enzymes with low enantioselectivity are able to proceed to 100 %
conversion. This is because after one enantiomer has been converted, it will proceed to
react with the slower enantiomer resulting in a racemic mixture of the product. As Cal B
demonstrated activity towards both 2-bromopropionic acid \( \text{35} \) and 2-fluoropropionic acid
\( \text{83} \), it was decided to select this lipase for further study, particularly the application of
protein engineering methods to improve the \( E_R \) of the reaction.

2.4 Cloning of the Cal B gene for protein production

The next phase of the project involved the development of a suitable expression system for
the over-production of soluble and active Cal B. Prior to 2006, the production of Cal B was
prepared in \textit{Aspergillus oryzae}, for large scale industrial purposes\(^{166}\), and in \textit{Pichia pastoris}
(\( P.\ pastoris \))\(^{167}\) and \textit{Saccharomyces cerevisiae}, for research applications\(^ {168}\). The first
functional expression of Cal B in the cytoplasm of \textit{Escherichia coli} (\( E.\ coli \)) was in 2006
by Liu et al.\(^ {169}\) The expression of Cal B from several different plasmids and strains of \( E.\ coli \)
was reported. The expression system in which Cal B was most efficiently expressed
was from a pColdIII construct with coexpression of pGro7, a molecular chaperone (61 U
\( \text{mg}^{-1} \)).

The Cal B gene in a pPIC9 vector for expression in \( P.\ pastoris \) was kindly provided by
Professor Stefan Lutz (Emory University, U.S.A.) (Figure 2.1).

Eukaryotic systems allow secretion of the protein into the medium, which facilitates
subsequent purification. Eukaryotic systems are also capable of post-translational
modifications such as glycosylation and disulfide bond formation, which results in higher
levels of correctly folded protein. However, a disadvantage of the eukaryotic systems is
that their optimal induction times are in the order of days whereas for prokaryotic systems
it is in the order of hours. Additionally, \( P.\ pastoris \) cells need to be produced prior to use
whereas competent \( E.\ coli \) cells required for transformation with plasmid DNA can be
stored frozen. A decision to express Cal B in \( E.\ coli \) was undertaken due to the ease of
downstream genetic modification in prokaryotic systems.\(^ {170}\) Additionally prokaryotic
systems are fast growing and have good protein production rates.
2.4.1 Amplification of the pPIC9 Cal B construct in One Shot® Top10 competent cells

The Cal B gene was cloned on the pPIC9 plasmid between the unique XhoI and NotI restriction sites. To sub-clone the Cal B gene into a vector for expression in prokaryotic systems, the pPIC9 Cal B construct (0.5 µL, 200 ng/µL) was amplified in One Shot® Top10 competent cells, according to the manufacturer’s protocol. The transformation of One Shot® Top10 competent cells with the pPIC9 Cal B plasmid was plated onto Luria-Bertani (LB) agar plates containing 100 µg/mL ampicillin (LB amp) for selection. Single colonies were picked off the LB amp plates and inoculated overnight in LB amp media. The overnight cultures were incubated at 37 °C for 18 hours. Glycerol stocks (25%) were made with the cultures for long term storage at – 80 °C. The plasmid DNA was extracted and purified from the remaining bacterial cells and the concentration of the double stranded DNA was measured.

Restriction digest analysis, using the restriction enzymes SphI and XbaI, was used to identify colonies with the gene incorporated. The restriction digest analysis, by agarose gel electrophoresis, and subsequent DNA sequencing of the Cal B gene showed that the Cal B samples still had the gene incorporated (Figure 2.2). All the plasmid DNA samples were sequenced to verify the restriction digest analysis results.
Lane 1: DNA marker, Lanes 2 – 8: eight colony samples that were subjected to restriction digest analysis showing the gene was still incorporated in the plasmid and in the correct orientation.

Figure 2.2: Agarose gel analysis of the restriction digest.

2.4.2 Amplification of the Cal B gene

The Cal B gene was amplified using primers designed for the vectors pET16b (Novagen) and Champion™ pET SUMO (Invitrogen) (Table 2.7). The pET16b vector was chosen as a general expression vector for *E. coli* and the Champion™ pET SUMO vector was chosen because the vector incorporates fusion of the SUMO protein, which aids solubility of the fusion protein produced. The Cal B was amplified by PCR, purified and sub-cloned into the appropriate vectors.

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<tr>
<td>pET SUMO forward sequence</td>
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</tr>
<tr>
<td>pET SUMO backward sequence</td>
<td>TTA GGG GGT GAC GAT GC</td>
</tr>
</tbody>
</table>

Table 2.7: Primers for cloning Cal B into the vectors pET16b and Champion™ pET SUMO.
2.4.3 Sub-cloning the Cal B gene into pET16b and Champion™ pET SUMO vectors

2.4.3.1 Sub-cloning the Cal B gene into the pET16b plasmid

There are several features of the pET16b Cal B construct that include the Cal B gene, the ampicillin resistant gene, lacI gene, restriction enzyme sites and the T7 lac promoter (Figure 2.3).

The Cal B gene is the gene of interest and the ampicillin resistant gene allows selection of only the *E. coli* cells that harbour the pET16b Cal B construct by simply growing the bacteria on an agar plate containing ampicillin. At the N-terminus of the Cal B gene is also a short sequence of histidine residues (HIS tag) that allows for identification and purification of the protein. The restriction enzyme sites are sites which contain a specific sequence of bases that a restriction enzyme recognises and is able to cut. Restriction enzymes can be used to cut the plasmid to incorporate a gene of interest or can be used to cut a plasmid, a circular piece of DNA, into a linear strand for analysis. Most importantly of all, the pET16b Cal B construct is under the control of the T7 lac promoter system, which also includes the lacI gene.
The lacI gene encodes for the lac repressor which is continuously expressed in the host chromosome as well as in the plasmid. The lac repressor binds to the T7 lac operator on the plasmid and prevents the basal level production of T7 RNA polymerase, the enzyme that transcribes the Cal B gene. The lac repressor also binds to the host chromosome blocking the lacUV5 promoter, which is the promoter for the T7 RNA polymerase gene. Isopropyl-β-D-1-thiogalactopyranoside (IPTG), a lactose analogue, is added to induce the expression of the Cal B gene. IPTG binds to the lac repressor which changes its conformation preventing it from binding to the T7 lac operator and the lacUV5 promoter. As a result, T7 RNA polymerase is produced and is able to bind to the T7 lac promoter and transcribe the Cal B gene.

To sub-clone the Cal B gene into the pET16b vector between the unique NdeI and XhoI restriction sites, a double digestion reaction of the vector and gene was prepared. The restriction enzymes used were NdeI and XhoI. The restriction digest reaction was incubated at 37 °C for 3 hours. After 3 hours, a DNA analytical agarose gel was run to check if the reaction was complete. After it was complete, the reaction mixtures were run on an agarose gel to separate the vector from the multiple cloning sites and the gene with the restriction sites. The DNA was then extracted from the gel under a blue light transilluminator and purified using a QIAquick Gel Extraction Kit.

Ligation reactions with different ratios of insert to vector, 1:1 and 3:1, were prepared. The amount of insert to vector to use was calculated from Eq. 5 (Appendix 5). The ligation reactions were incubated at 4 °C overnight.

A transformation of both the ligation reactions, 1:1 and 3:1 ratio of insert to vector, into One Shot® BL21 competent cells following the manufacturer’s protocol was prepared. Two volumes of the transformation reaction were plated, 50 µL and 100 µL, onto LB amp plates. Both transformation reactions were successful but the 3:1 ratio of insert to vector produced more colonies.

To obtain plasmid DNA, LB amp media was inoculated with a single colony picked from the transformation reaction and incubated at 37 °C for 18 hours. From the overnight cultures, glycerol stocks were made (25%) and the plasmid DNA was harvested and purified using a QIAGEN Plasmid Prep Kit.
A restriction digest analysis of the pET16b Cal B plasmid DNA was prepared to ensure that the gene was incorporated in the correct orientation. The restriction enzymes used for the analysis were SacII and MluI. When the Cal B gene is incorporated in the correct orientation, the fragment sizes expected are 1.7 kb and 4.9 kb but if the gene is incorporated incorrectly then the fragment sizes expected are 1.0 kb and 5.6 kb. The samples were also sequenced to confirm the results of the restriction digest analysis. Standard T7 forward and reverse primers were used for sequencing. Both the results of the restriction digest analysis and sequencing showed the gene was incorporated in the correct orientation in the pET16b plasmid.

2.4.3.2 Sub-cloning the Cal B gene into the Champion™ pET SUMO plasmid

The Champion™ pET SUMO expression system was designed to possibly increase expression of recombinant proteins in E. coli and enhance the solubility of partially insoluble protein. The pET SUMO Cal B construct has similar features to the pET16b Cal B construct including the Cal B gene, the lacI gene, the T7 lac promoter, the HIS tag and restriction enzyme sites (Figure 2.4). In addition to the HIS tag, in pET SUMO Cal B there is a SUMO tag on the N-terminus which enhances the solubility of the protein of interest. In pET SUMO Cal B, a kanamycin resistant gene is used rather than ampicillin which again allows for selection of the E. coli cells that have the pET SUMO Cal B plasmid incorporated. The SUMO gene is fused to the N-terminus and can be cleaved using SUMO protease to produce our native protein, Cal B.

To sub-clone the Cal B gene into the Champion™ pET SUMO Cal B vector, two ligation reactions were set up with a 1:1 and 3:1 ratio of insert to vector. The ligation reactions were incubated at 4 °C overnight. The Champion™ pET SUMO Cal B plasmid was transformed into Mach1 T1-R competent cells. The transformation reactions were plated onto kanamycin inoculated agar plates (LB kan). The plates were incubated at 37 °C overnight. The ligation reaction with a 1:1 ratio of insert to vector provided the best conditions and the colonies were subsequently used for overnight cultures.
Figure 2.4: Plasmid map of Cal B gene in the *E. coli* Champion® pET SUMO expression vector with a kanamycin resistant gene.

From the overnight cultures, glycerol stocks (25%) were prepared and the plasmid DNA was harvested and purified. To ensure the Cal B gene had been incorporated in the correct orientation, a restriction digest analysis was prepared using the restriction enzymes *Sph*I and *Bam*HI. The fragment sizes expected if the Cal B gene had been incorporated with the correct orientation were 0.8 kb and 5.8 kb. If the orientation had been incorrect, the fragment sizes would have been 1.5 kb and 5.1 kb. The analysis showed that only samples 1, 3, 5, 8, 11-13 and 19-20 had the Cal B gene incorporated with the correct orientation. These samples were also submitted for sequencing, using the standard T7 forward and reverse primers, which confirmed the results of the restriction digest analysis.

After identification of a construct with a gene of the correct orientation, the Champion™ pET SUMO Cal B vector was then transformed into One Shot BL21(DH5) competent cells. Overnight cultures of 20 colonies were prepared and glycerol stocks (25%) were prepared from the overnight cultures. The plasmid DNA was harvested and purified from the remaining bacterial cells.
2.5 Expression of Cal B from pET16b and pET SUMO in E. coli competent cells

Having amplified and sub-cloned the Cal B gene into two E. coli expression vectors, pET16b and pET SUMO, various expression and protein production conditions were examined for the synthesis of soluble and active Cal B.

2.5.1 Initial expression studies of Cal B from pET16b and pET SUMO in BL21(DE3) competent cells at 37 °C with IPTG

To determine whether the pET16b Cal B and pET SUMO Cal B constructs would yield over-produced protein, an initial expression study was conducted. Previously prepared glycerol stocks (25 %) of BL21(DE3) E. coli competent cells transformed with the pET16b, pET16b Cal B and pET SUMO Cal B constructs were streaked onto LB plates with the appropriate antibiotic for selection. The LB plates were incubated at 37 °C overnight. The growth of single colonies on all of the LB plates was successful. Overnight cultures were prepared by inoculating LB media with the appropriate antibiotic and then with a single colony from the corresponding LB plates. The cultures were incubated in a 37 °C shaking incubator overnight. All the overnight cultures grew successfully and were used to inoculate LB media (1 in 100 dilution factor) possessing the correct antibiotic for retention of the appropriate plasmid. The cultures were first grown at 37 °C to an optical density (OD\textsubscript{600}) of ~ 0.6, where the cell culture growth is in the exponential phase. The cell cultures were then induced with IPTG and the temperature was reduced to 30 °C, where the cells were left to grow for 18 hr. In order to determine which expression system produced the most soluble and active Cal B as a function of time, time point protein samples were taken at t\textsubscript{0}, t\textsubscript{2}, t\textsubscript{3} and t\textsubscript{18} hours post induction. The samples were centrifuged to obtain the bacterial cell pellet. Samples were taken at t\textsubscript{0} to examine the basal level of expression prior to induction with IPTG. The samples were chemically lysed using BugBuster\textsuperscript{®} Protein Extraction Reagent, according to the manufacturer’s instruction. The insoluble and soluble fractions of cell free extract were then analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Figure 2.5).
Figure 2.5: SDS-PAGE of the soluble (top) and insoluble fractions (bottom) of the samples taken during the initial expression studies.

Top SDS gel: Soluble fractions, Bottom SDS gel: Insoluble fractions, L = protein ladder, **Lane 1** = pET16b Cal B t₀, **Lane 2** = pET16b Cal B t₂, **Lane 3** = pET16b Cal B t₃, **Lane 4** = pET16b Cal B t₁₈, **Lane 5** = pET16b t₀, **Lane 6** = pET16b t₂, **Lane 7** = pET16b t₃, **Lane 8** = pET16b t₁₈, **Lane 9** = pET SUMO Cal B t₀, **Lane 10** = pET SUMO Cal B t₂, **Lane 11** = pET SUMO Cal B t₃ and **Lane 12** = pET SUMO Cal B t₁₈.

The SDS-PAGE of the soluble fractions show that there was no over-produced band of protein that corresponds to Cal B (33 kDa) or the Cal B SUMO fusion protein (46 kDa). In the insoluble fractions, it is more evident that there are bands of over-produced protein in both the pET16b Cal B (lanes 2-4) and pET SUMO Cal B (lanes 10-11) systems. The over-produced band of protein in the pET SUMO Cal B expression was larger in size (46 kDa) due to the SUMO fusion protein and 6xHis-tag, which in total is 13 kDa in size. The results of the SDS-PAGE also indicate that Cal B was produced about 2 hours after addition of IPTG in both the pET16b Cal B and pET SUMO Cal B systems (lanes 2 and 10). The observed inclusion body formation may be due to several factors, as there are three stages in which expression problems can occur: the transcription of the Cal B gene,
the translation of the mRNA and post-translational events such as the folding of the peptide chains. As there is over-production of Cal B both from the pET16b and pET SUMO systems, although largely as inclusion bodies, it can be assumed that transcription and translation of the Cal B gene was successful. The results indicate that incorrect folding of the protein, in particular the three disulfide bonds of Cal B, may be the problem. At this point, a decision to focus initially on the pET16b plasmid was made.

2.5.2 Expression of Cal B from pET16b in BL21(DE3) competent cells at 37 °C without IPTG and induction at 26 °C

In order to obtain increased levels of soluble Cal B, another set of expression conditions was investigated. It has previously been reported that a basal level of T7 RNA polymerase exists when under the control of the lacUV5 promoter in DE3 lysogens such as BL21(DE3). Although our plasmid is also under the influence of the lac operator, the pET16b plasmid originating from the Turner lab had been successfully used previously for the production of protein without addition of an inducer such as IPTG. This uninduced method of protein production may be due to incomplete binding of the lac repressor to the operator. This results in a basal level of T7 RNA polymerase which can bind to the lac operator and allow transcription of the Cal B gene. Additionally, although an external inducing source was not used, a lowering of the temperature from 30 °C to 26 °C at induction was proposed to aid folding of the protein. A reduction in induction temperature has been found to be important in the prevention of the formation of inclusion bodies or insoluble aggregates. The rate of protein folding was not directly affected by reduced expression temperatures, however, the rate of transcription and translation are directly affected by low expression temperatures. The increase in functional protein expression was a result of sufficient time for protein refolding.

A culture containing a single colony of pET16b Cal B or pET16b was grown at 37 °C. The cultures were then induced by incubating at 26 °C overnight. The bacterial cell pellets were harvested by centrifugation and then mechanically lysed to obtain the soluble and insoluble fractions. The soluble fractions were analysed by SDS-PAGE and Western blot (Figure 2.6). A Western blot is an analytical technique used for identification of specific proteins in an extract. Initially, the proteins were separated by size using SDS-PAGE and then blotted onto polyvinylidene fluoride membrane where proteins can be identified using specific
antibodies to the target protein. The Cal B protein has a HIS-tag which can be used for identification with the monoclonal anti-polyhistidine antibody. Staining of the membrane to display histidine tagged protein is carried out by using a second anti-body coupled with a staining agent.

![Western blot and SDS-PAGE gel](image)

Figure 2.6: Western blot (left) and SDS-PAGE gel (right) of small scale non-induced production of Cal B from pET16b.

L = protein ladder. **Lanes 1-2 = Cal B expressed from pET16b Cal B without addition of IPTG.**

From the Western blot and SDS-PAGE gel, it is evident that the Cal B gene is over-produced in a soluble form compared to the previous study discussed in section 2.5.1. Additionally, the Western blot illustrates the production of truncated HIS-tagged proteins resulting from either incomplete translation or degradation products arising from protease activity on the Cal B protein.

### 2.5.2.1 Determining activity of the recombinant Cal B

The activity of the soluble recombinant Cal B was tested using the assay described in section 2.3. The biotransformation of 2-bromopropionic acid **35** into 2-butyl-2-bromopropionoate **114** was carried out using the soluble fraction of recombinant Cal B. The pET16b vector was also expressed under the same conditions. Samples were taken and analysed by chiral HPLC, however after 24 h no product was observed. After 48 h very little product was observed so the reactions were repeated and monitored for 4 days. Even after 4 days, very little product was observed and hence calculation of enantiomeric excess and enantioselectivity was not possible. Additionally, there was little difference in activity between the recombinant Cal B reaction and the control.
2.5.2.2 *para*-Nitrophenyl acetate high-throughput assay

As the results to the HPLC assay were inconclusive, a faster and easier way to determine the activity of the recombinant Cal B was needed. The *p*NPA assay, as described in section 1.8.2.2, can be used to determine the hydrolysis activity of the recombinant Cal B (Scheme 1.23). The assay was conducted in buffer at pH 7.4 containing recombinant Cal B and initiated by addition of the *p*NPA substrate as a solution in DMSO (50 mM). A graph was plotted of absorption *versus* time (Figure 2.7). The gradient of the curve showed that the recombinant Cal B had little difference in activity when compared to the pET16b negative control.

![Graph of pNPA assay](image)

**Red** = soluble fraction of recombinant Cal B, **Blue** = soluble fraction of pET16b

Figure 2.7: *p*NPA assay graph of time *vs.* absorbance for pET16b Cal B.

2.5.2.3 Purification of pET16b Cal B

To characterise the specific activity of Cal B, the recombinant Cal B expressed from BL21(DE3) *E. coli* cells grown at 37 °C with induction at 26 °C was purified using affinity chromatography. The HIS-tagged protein was easily separated from the non HIS-tagged proteins by using a HiTrap Chelating HP affinity column. The column was supplied free of
metal ions and once it was charged with NiSO$_4^{2-}$ it was used to purify the protein. A single exposed histidine residue may result in adsorption of the protein onto Cu$^{2+}$, while two vicinal histidine residues are needed for absorption on Zn$^{2+}$ and NiSO$_4^{2-}$ ions and therefore NiSO$_4^{2-}$ ions was used for the purification of the recombinant HIS-tagged Cal B. The HIS-tagged Cal B can be separated from the truncated HIS-tagged proteins and other proteins that contain histidine residues by using a gradient solvent profile. Increasing the concentration of imidazole leads to separation of different proteins.

A fresh cell free extract was prepared by resuspending the bacterial cell pellet in potassium phosphate buffer and then subjecting the suspension to mechanical lysing (sonication with protein inhibitor cocktail and lysozyme). The cell free extract of BL21(DE3) pET16b Cal B was purified initially on a linear gradient solvent profile (Figure 2.8).

The UV chromatogram showed a large peak of over 4000 mAU, consisting of protein that does not bind to the column at all (A1), followed by a second peak, eluting at 0.5 M imidazole concentration (B1). Fractions collected from the purification were analysed by SDS-PAGE to see which fractions contain Cal B (Figure 2.9).
From the SDS-PAGE, it can be seen fraction A1 contains proteins of various sizes that have not bound to the column. A protein of 33 kDa, the correct size for Cal B, is present in fractions B3 - B11.

To separate the tailing peak in fractions B5-B9, some of which contain Cal B, a step gradient method was developed which consisted of holding the gradient at 10%, 20%, 30% and 100% salt concentration for 10 mL. The purification was repeated using this method and analysis of some of the fractions by SDS-PAGE showed that there was very little Cal B in the fractions as a significant amount was lost through purification. The activity of the recombinant Cal B was not recorded at this time.

Now that a basic purification method had been established, attention was turned to the expression of significantly more soluble and active Cal B.

### 2.5.3 Optimisation of expression of Cal B in BL21(DE3) E. coli cells

A number of parameters were investigated to optimise production of soluble and active protein, including temperature of induction and the final concentration of IPTG, both of which affect transcription, translation and post-translational events. A set of expression trials on a 250 mL scale using three different induction temperatures, 30 °C, 26 °C and 16 °C and four different final IPTG concentrations, 1 mM, 0.5 mM, 0.025 mM and 0 mM, was set up to establish the optimum appropriate conditions for maximum production of soluble and active Cal B protein.
BL21(DE3) E. coli competent cells transformed with pET16b Cal B were used for the expression study. The expression was conducted in LB amp media for plasmid selection. Once the twelve cultures had grown to an OD$_{600}$ of ~ 0.6, they were each induced under different conditions. Samples (2 mL) of the cultures were taken at t$_0$-t$_6$, t$_8$ and t$_{24}$ hrs post-induction for analysis by SDS-PAGE (Figure 2.10 – 2.12).

Figure 2.10: SDS-PAGE analysis of the time point protein samples from the induction at 30 °C.

Figure 2.11: SDS-PAGE analysis of the time point protein samples from the induction at 26 °C.
Figure 2.12: SDS-PAGE analysis of the time point protein samples from the induction at 16 °C.

The SDS-PAGE gels in Figure 2.10-2.12 showed overexpression of Cal B at 30°C and 26°C from about 1-2 h post-induction. The 16 °C expression studies revealed that Cal B was only produced 4-6 h post-induction. The SDS-PAGE gels also show that each expression system yielded different quantities of recombinant Cal B.

From the SDS-PAGE gels in Figure 2.10-2.12, it is evident that Cal B is overproduced but it is not known whether this Cal B is soluble or active. To determine the solubility of the Cal B, the bacterial cell pellets were obtained by centrifugation and chemically lysed. The soluble and insoluble fractions were then analysed by SDS-PAGE. From the SDS-PAGE gels in Figure 2.10-2.12, twenty two promising time-point samples were selected for further study (Figure 2.13).
Figure 2.13: SDS-PAGE analysis of the soluble and insoluble fractions from chemical lysing of the bacterial cell pellets at some chosen time points.

L = protein ladder, s = soluble fraction, i = insoluble fraction, Lane 1 = 16 °C 1 mM 24 hr, Lane 2 = 16 °C 0.5 mM 24 hr, Lane 3 = 16 °C 0.025 mM 24 hr, Lane 4 = 16 °C 0 mM 24 hr, Lane 5 = 26 °C 1 mM 4 hr, Lane 6 = 26 °C 1 mM 24 hr, Lane 7 = 26 °C 0.5 mM 5 hr, Lane 8 = 26 °C 0.5 mM 24 hr, Lane 9 = 26 °C 0.025 mM 4 hr, Lane 10 = 26 °C 0.025 mM 8 hr, Lane 11 = 26 °C 0.025 mM 24 hr, Lane 12 = 26 °C 0 mM 5 hr, Lane 13 = 26 °C 0 mM 24 hr, Lane 14 = 30 °C 1 mM 3 hr, Lane 15 = 30 °C 1 mM 8 hr, Lane 16 = 30 °C 0.5 mM 5 hr, Lane 17 = 30 °C 0.5 mM 24 hr, Lane 18 = 30 °C 0.025 mM 3 hr, Lane 19 = 30 °C 0.025 mM 4 hr, Lane 20 = 30 °C 0.025 mM 24 hr, Lane 21 = 30 °C 0 mM 4 hr, Lane 22 = 30 °C 0 mM 24 hr.

From the SDS-PAGE gels in Figure 2.13, it is apparent that many of the protein samples yielded insoluble Cal B such as can be seen in lanes 1-3. These lanes correspond to the expression studies conducted at 16 °C with 0.025-1 mM IPTG. However, in lane 4 this expression conditions (16 °C with 0 mM IPTG) provided Cal B that was partially soluble. The production of partially soluble Cal B can also be seen in lanes 6, 8, 11-13, 15-17 and
The expression conditions that furnished partially soluble Cal B are summarised in Table 2.8.

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Final IPTG conc. (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
</tr>
<tr>
<td>26</td>
<td>0.5</td>
</tr>
<tr>
<td>26</td>
<td>0.025</td>
</tr>
<tr>
<td>26</td>
<td>0</td>
</tr>
<tr>
<td>30</td>
<td>1</td>
</tr>
<tr>
<td>30</td>
<td>0.5</td>
</tr>
<tr>
<td>30</td>
<td>0.025</td>
</tr>
<tr>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.8: A summary of the expression conditions that yielded partially soluble Cal B.

Measurement of the activity of the soluble fractions from the chemical lysis of the bacterial cell pellets using the pNPA assay showed that there were several expression conditions that yielded soluble and active Cal B. Two of the conditions, 30 °C 0 mM IPTG and 16 °C 0 mM IPTG, yielded significantly more active enzyme (Figure 2.14). These two expression conditions were repeated on a larger scale. Samples were taken at t₀ and t₂₄ hrs after induction and analysed by SDS-PAGE (Figure 2.15) and centrifuged to obtain cell pellets. These cell pellets were chemically lysed and the soluble and insoluble fractions were also analysed by SDS-PAGE (Figure 2.16-2.17).

The SDS-PAGE gels in Figure 2.15 show a basal level production of Cal B in lane 2. This is from the presence of basal levels of T7 RNA polymerase in BL21(DE3) competent cells. Interestingly, there was no basal level of production in lane 1. In lanes 3 and 4, it is evident that Cal B is overproduced in both the expression conditions. The SDS-gels of the soluble and insoluble fractions of protein (Figure 2.16-2.17), show that although the majority of the expressed Cal B is insoluble there is also a significant amount of soluble Cal B. Now that the solubility of the recombinant Cal B had been established, the activity of the soluble
Cal B was investigated. The pNPA assay was used to test the activity of the soluble fractions. The pNPA assay again showed that the soluble Cal B was active from both the expression conditions. However, the soluble Cal B from the 16 °C 0 mM IPTG was more active than the soluble fraction from the 30 °C 0 mM IPTG expression. The soluble and purified Cal B were also used to test the stability of Cal B when stored at – 4 °C and the data showed that Cal B did not lose activity after 1 week (discussed in detail in section 2.6).

**Figure 2.14:** pNPA assay of the soluble fractions of recombinant Cal B produced using various conditions conducted in potassium phosphate buffer at pH 7.4 and 25 °C.
Figure 2.15: SDS-PAGE gel of the samples taken at $t_0$ and $t_{24}$ hrs after induction. L = protein ladder. **Lane 1** = 30 °C $t_0$, **Lane 2** = 16 °C $t_0$, **Lane 3** = 30 °C $t_{24}$, **Lane 4** = 16 °C $t_{24}$.

Figure 2.16: SDS-PAGE gel of the soluble fractions from the chemically lysed protein samples. L = protein ladder. **Lane 1** = 30 °C $t_0$, **Lane 2** = 16 °C $t_0$, **Lane 3** = 30 °C $t_{24}$, **Lane 4** = 16 °C $t_{24}$.

Figure 2.17: SDS-PAGE gel of the insoluble fractions from the chemically lysed protein samples. L = protein ladder. **Lane 1** = 30 °C $t_0$, **Lane 2** = 16 °C $t_0$, **Lane 3** = 30 °C $t_{24}$, **Lane 4** = 16 °C $t_{24}$.
As the 16 °C 0 mM IPTG sample demonstrated the higher activity, the protein was purified on a HiTrap chelating column to investigate the specific activity of Cal B. The basic purification protocol described in 2.5.2.3 was used to purify the Cal B. The fractions from the purification were analysed by SDS-PAGE and they showed very little Cal B protein post-purification. Purification of HIS-tagged Cal B from the 30 °C 0 mM IPTG expression under the same column conditions and methods also resulted in no Cal B being visible by SDS-PAGE.

At this point a decision was taken to change the strain of *E. coli* cells under which Cal B was expressed to establish whether increased levels of recombinant Cal B could be produced.

### 2.5.4 Expression of pET16b Cal B from Origami 2(DE3) *E. coli* cells

Cal B contains 3 disulfide bonds which need to be formed in the reductive atmosphere of the cytoplasm in the BL21(DE3) *E. coli* competent cells. Research into the development of new competent *E. coli* cells that aid the correct folding of disulfide bonds was carried out. One paper, published in 2006, has been reported to express Cal B in Origami 2(DE3) *E. coli* competent cells. The strain of *E. coli* is characterised by its thioredoxin reductase and glutathione reductase deficiency. These cells have a more oxidative cytoplasm to aid the formation of disulfide bonds. The expression in these cells is under the lac promoter as in the BL21(DE3) *E. coli* competent cells. The pET16b Cal B construct and Origami 2(DE3) cells were therefore compatible. Origami 2(DE3) cells were slow growing and require 24 hrs growth. The transformation efficiency was also very low, yielding only 3-100 colonies unlike the BL21(DE3) cells which require only 16-18 hrs to form hundreds of colonies. Due to the low number of colonies, a single clone was picked and grown in an overnight culture before preparation of the DNA for sequencing. Sequencing results confirmed that the transformation of Origami 2(DE3) cells with the pET16b Cal B plasmid was successful.

The conditions used in the expression trials described in section 2.5.3 were used for the expression of pET16b Cal B from Origami 2(DE3) cells. Samples were initially taken at t1- t6, t8 and t24 hrs post-induction for analysis by SDS-PAGE. The cell pellets, obtained by centrifugation, were also chemically lysed to determine which conditions yielded the most
soluble Cal B. The soluble and insoluble fractions were analysed by SDS-PAGE and Western blot. One particular set of conditions, 16 °C 1 mM IPTG, yielded a strong band of soluble Cal B at t₈ (Figure 2.18).

The expression condition, 16 °C with 1 mM of IPTG, was repeated on a large scale and sampled hourly over 24 hrs to discover when the maximum soluble Cal B was produced. Analysis by SDS-PAGE and Western blot showed that maximum production of soluble Cal B was reached 16 hr post-induction.
Now that an expression system had been established, purification of the recombinant Cal B was carried out using a HiTrap chelating column. The basic purification system established in section 2.5.2.3 was used for the purification. The fractions from the purification were analysed by SDS-PAGE and Western blot which showed elution of Cal B at 0.4 M imidazole concentration (Figure 2.19).

![Figure 2.19: Western blot of the purification fractions show Cal B eluted in fractions B9-C2.](image)

Initial experiments to determine the activity of the purified recombinant Cal B from Origami 2(DE3) E. coli were carried out using the pNPA assay. To remove the eluting buffer used in the purification, the fractions containing Cal B were concentrated by applying them to a Vivaspin concentrating column with a molecular weight cut off of 30,000. The concentrated recombinant Cal B was then desalted using a PD10 column. The desalted and purified recombinant Cal B was used in the pNPA assay to test for hydrolysis activity (Figure 2.20). The pNPA activity test showed that the recombinant Cal B, produced in Origami 2(DE3) E. coli cells, was active.
Characterisation of wild-type Cal B

An assessment of the various expression experiments using both the BL21(DE3) and Origami 2(DE3) E. coli competent cells in the production of soluble and active Cal B resulted in a decision to repeat three of the optimised expression conditions to provide direct comparisons (Table 2.9). Kinetic characterisation of purified Cal B obtained from the three experiments was carried out using the para-nitrophenyl butyrate (pNPB) assay in triplicate and an average of the results are presented in Table 2.10. The kinetic experiments were carried out at 25 °C. Initial rates of activity were recorded for each purified sample of Cal B across a range of substrate concentrations (0-0.1 M). The Michaelis-Menten equation was used to calculate the kinetic parameters for each sample. Comparison of the data obtained with a published purification of Cal B from E. coli is shown in Table 2.10.

The purified samples of Cal B produced in our laboratories gave comparable data to the published literature although there was some difference in the $k_{cat}$ and $k_M$ values. Interestingly the Cal B produced from Origami 2 cells appeared more active than that prepared from BL21 cells. The difference in kinetic parameters may be due to the correct
folding of the protein. As previously mentioned, Origami 2 cells promote disulfide bond formation to yield functional protein. The lower activity observed with BL21 cells maybe due to partially folded protein. It has also been reported that non-native partially folded protein can also have biological function, although the mechanism behind this is not understood.\textsuperscript{180}

<table>
<thead>
<tr>
<th>Method</th>
<th>Cells</th>
<th>Induction temperature</th>
<th>Final concentration of IPTG</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Origami 2(DE3)</td>
<td>16 °C</td>
<td>1 mM</td>
</tr>
<tr>
<td>2</td>
<td>BL21(DE3)</td>
<td>30 °C</td>
<td>0 mM</td>
</tr>
<tr>
<td>3</td>
<td>BL21(DE3)</td>
<td>16 °C</td>
<td>0 mM</td>
</tr>
</tbody>
</table>

Table 2.9: Three optimised expression conditions for the preparation of recombinant Cal B.

In addition to characterising the different samples of Cal B, the stability of the purified Cal B was monitored over ten days. The \( p\)NPA assay was used to record the activity 1, 2, 7 and 10 days post-purification (Figure 2.21 and Figure 2.22).

Figure 2.21 shows that the activity of the Origami 2(DE3) sample declined quickly over ten days with only 20% of the original activity observed after ten days. The BL21(DE3) sample (Figure 2.22) maintained 100% activity over the first seven days and only showing a 23% reduction in activity when monitored ten days after it was first purified. Although Cal B produced from BL21(DE3) cells was of higher stability, higher activity was more important, so for future productions of Cal B, wild-type or variants, the Origami 2(DE3) expression conditions were used.
<table>
<thead>
<tr>
<th>E. coli cells used for expression</th>
<th>Induction temperature (°C)</th>
<th>$v_{\text{max}}$ (μM min$^{-1}$)</th>
<th>$k_M$ (μM)</th>
<th>$k_{\text{cat}}$ (min$^{-1}$)</th>
<th>$k_{\text{cat}}/k_M$ (min$^{-1}$ μM$^{-1}$)</th>
<th>Specific activity (80 mM substrate μmol mg$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Origami 2(DE3)</td>
<td>16</td>
<td>438 ± 18</td>
<td>27682 ± 2901</td>
<td>4716 ± 27</td>
<td>0.170 ± 0.018</td>
<td>96.2</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>30</td>
<td>189 ± 13</td>
<td>24930 ± 2833</td>
<td>1921 ± 38</td>
<td>0.077 ± 0.0095</td>
<td>32.4</td>
</tr>
<tr>
<td>BL21(DE3)</td>
<td>16</td>
<td>355 ± 41</td>
<td>23291 ± 3296</td>
<td>2466 ± 53</td>
<td>0.106 ± 0.015</td>
<td>53.1</td>
</tr>
<tr>
<td>K12$^{179}$</td>
<td>25</td>
<td>237</td>
<td>5492</td>
<td>2378</td>
<td>0.433</td>
<td>10.57 (20 mM)</td>
</tr>
</tbody>
</table>

Table 2.10: Steady state kinetics of the different samples of Cal B calculated from the pNPA assay.

![Graph showing absorbance over time](image)

**Pink** = 0 days post-purification, **Yellow** = 1 day post-purification, **Green** = 2 days post-purification, **Red** = 7 days post-purification, **Blue** = 10 days post-purification

Figure 2.21: The stability of Origami 2 Cal B monitored over ten days using the pNPA assay.
Pink = 0 days post-purification, Yellow = 1 day post-purification, Green = 2 days post-purification, Red = 7 days post-purification, Blue = 10 days post-purification

Figure 2.22: The stability of BL21 16 °C Cal B monitored over ten days using the pNPA assay.

2.7 Synthesis of fluorinated substrates

Most of the synthetic approaches to 2-fluoropropionic acid 83 discussed in section 1.4.3 involve the use of hazardous and toxic reagents such as elemental fluorine, hydrogen fluoride and DAST and as such were not deemed suitable for use in industrial research and downstream scale up reactions. In addition, many of these reagents would require expensive and specialist equipment to be put in place with elaborate safety regimes. Reagents that were identified as being suitable for subsequent downstream scale up reaction limited the studies to the synthesis of the fluorinated substrates, 2-fluoropropionic acid 83 and ester derivatives. Their chemical synthesis was required for use as substrates in subsequent biotransformation reactions and due to the lack of commercial availability of the monofluorinated esters and the high cost of 2-fluoropropionic acid 83 (£85/g).
2.7.1 Synthesis of $n$-butyl-2-bromopropionate 114

$n$-Butyl-2-bromopropionate 114 was successfully prepared from 2-bromopropionic acid 35 and $n$-butanol 117. The reaction was monitored by gas chromatography which showed the reaction was complete after 5 hours providing $n$-butyl-2-bromopropionate 114 in a yield of 81%. This method was intended to be applied to the synthesis of $n$-butyl-2-fluoropropionate 115 but due to the high cost of 2-fluoropropionic acid 83, this approach was not viable (Scheme 2.4). A more cost effective preparation of 2-fluoropropionic acid 83 or an alternative method for the synthesis of $n$-butyl-2-fluoropropionate 115, not using 2-fluoropropionic acid 83 as the starting material was needed.

![Scheme 2.4: Proposed synthesis of $n$-butyl-2-fluoropropionate 115 from 2-fluoropropionic acid 83 and $n$-butanol 117. Reagents and conditions: Toluene, conc. H$_2$SO$_4$, 130 °C, 5 h.](image)

The synthesis of the esters of 2-fluoropropionic acid using commercially available potassium fluoride (KF) as the fluorinating reagent has been reported.$^{181,182}$ To generate the active fluorinating species, a mixture of hexadecyltributylphosphonium bromide (HTPB) and potassium fluoride was heated to 60 °C to form a semi-molten mass. HTPB acts as a phase transfer catalyst for the potassium fluoride making the fluoride ion more available. The substrate to be fluorinated was then added to give products with yields varying from 60-89 %, depending upon the substrate to be fluorinated. This procedure was applied to the synthesis of $n$-butyl-2-fluoropropionate 115 from $n$-butyl-2-bromopropionate 114 with direct distillation of the product from the reaction mixture (Scheme 2.5). The distillate was analysed by $^{19}$F NMR, which revealed that the fluorination had not been successful and instead the distillate contained unreacted $n$-butyl-2-bromopropionate 114.
Scheme 2.5: Synthesis of n-butyl-2-fluoropropionate 115 using potassium fluoride as a fluorinating reagent.

Reagents and conditions: a) potassium fluoride, HTPB, 60 °C.

2.8 Development of an enantioselective screen for assaying Cal B mutants

2.8.1 Development of a high throughput enantioselective screen for assaying Cal B mutants

In order to screen the Cal B mutants, it was decided to adapt the existing pNPA screen. In place of the pNPA substrate, 4-nitrophenyl 2-bromopropionate 118 and 4-nitrophenyl 2-fluoropropionate 119 was used (Figure 2.23). Hydrolysis of 4-nitrophenyl 2-bromopropionate 118 and 4-nitrophenyl 2-fluoropropionate 119 by an active Cal B mutant resulted in production of 4-nitrophenol 98. The product 4-nitrophenol 98 was deprotonated under the alkaline assay conditions and is yellow in colour. The reaction was monitored by the increase in absorbance at 410 nm.

Figure 2.23: High throughput enantioselective screen substrates 4-nitrophenyl 2-bromopropionate 118 and 4-nitrophenyl 2-fluoropropionate 119.
The synthesis of enantiopure 4-nitrophenyl 2-halopropionate 118 and 119 from the sodium salt of 4-nitrophenol 120 and (R)- or (S)-2-halopropionyl chloride 121 or 122 was proposed (Scheme 2.6).\(^{183}\)

Scheme 2.6: Synthesis of single enantiomers of (R)- and (S)-4-nitrophenyl 2-halopropionate 118 and 119.\(^{183}\)

Reagents and conditions: 0 °C, NaOH (1 N), H\(_2\)O.

In theory when enantiopure 2-halopropionyl chloride 121 or 122 is used, enantiopure 4-nitrophenyl 2-halopropionate 118 and 119 can be synthesised (Scheme 2.6). Each enantiomer can be assayed in separate wells in a high throughput enantioselective screen, from which an apparent enantioselectivity can be deduced. For true enantioselectivity values, conventional analysis methods would need to be used. Additionally, a chromogenic surrogate substrate is used and substrate competition is also not taken into consideration but if enantiopure 4-nitrophenyl 2-halopropionate 118 and 119 were available, this screen would reduce the need to grow each mutant up on a large scale and analyse by biotransformation reactions.

A solution of sodium 4-nitrophenoxide 120 in sodium hydroxide (1N) and water was cooled in an ice bath before addition of racemic 2-bromopropionyl chloride 121. The mixture was allowed to stir in an ice bath for 5 minutes before precipitation of a white solid occurred. The crude 4-nitrophenyl 2-bromopropionate 118 was recrystallised from iso-hexane. The reaction was successfully completed using racemic 2-bromopropionyl chloride rac-121, resulting in racemic 4-nitrophenyl 2-bromopropionate rac-118. When enantiopure 2-bromopropionyl chloride 121 was used, racemisation of the chiral centre (confirmed using chiral HPLC) under the reaction conditions occurred resulting in the synthesis of racemic 4-nitrophenyl 2-bromopropionate rac-118. Application of this method
for the synthesis of enantiopure 4-nitrophenyl 2-halopropionate 118 and 119 was therefore abandoned.

Other syntheses to produce enantiopure 4-nitrophenyl 2-halopropionate 118 and 119 were not proposed due to time constraints.

2.8.2 HPLC assay of Cal B mutants

In the absence of a high-throughput enantioselective assay, traditional methods for measuring the enantioselectivity of mutants generated in our studies were proposed. An HPLC assay that monitors the hydrolysis of ethyl-2-bromopropionate 123 and ethyl-2-fluoropropionate 124 was proposed. The hydrolysis of ethyl-2-bromopropionate 123 and ethyl-2-fluoropropionate 124 was chosen over the esterification of 2-bromopropionic acid 35 and 2-fluoropropionic acid 83 due to the environmentally friendly and cost effectiveness of using buffer over organic solvents (Scheme 2.7).

![Scheme 2.7: Kinetic resolution of ethyl-2-bromopropionic acid 123 and ethyl-2-fluoropropionate 124 using recombinant Cal B and its mutants.](image)

Reaction conditions: ethyl-2-halopropionic acid (5g/L), enzyme (2000 mg/reaction), potassium phosphate buffer (100 mM, pH 7.0, 4mL), ethanol (0.36 v/v%), incubated in a 30 °C shaker.

2.8.2.1 Chiral HPLC method development

In order to calculate the enantioselectivity of Cal B and its variants, HPLC methods to determine enantiomeric excess and conversion were required.

Prior to normal phase chiral HPLC method development, as the assay was conducted in buffer, a suitable extraction method to obtain the products for analysis was required. To
this end, several organic solvents were tested for their ability to extract 2-bromopropionic acid 35 and 2-fluoropropionic acid 83 from buffer. Organic solvents such as ethyl acetate, dichloromethane and tert-butyl methyl ether (TBME) were used for this investigation. After extraction, the organic solvents were removed by evaporation before resuspending in iso-hexane and analysing the solution using the chiral HPLC method outlined in Table 2.11. TBME was found to extract 2-bromopropionic acid 35 from buffer the most efficiently. A sample of ethyl-2-fluoropropionate 124 left in buffer overnight was found to degrade to unknown products. It was at this point that a decision to screen the Cal B mutants with ethyl-2-bromopropionate 123 only was decided. The retention times of 2-bromopropionic acid 35 and ethyl-2-bromopropionate 123 were recorded (Table 2.12)

<table>
<thead>
<tr>
<th>HPLC</th>
<th>Column Temp. (°C)</th>
<th>Flow rate (ml/ min)</th>
<th>Mobile Phase</th>
<th>Hexane %</th>
<th>Ethanol %</th>
<th>TFA %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal phase</td>
<td>40</td>
<td>1.3</td>
<td>98</td>
<td>1.9</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

Column – Daicel Chiralpak® AD-H - 5 μm particle size, 4.6 mm internal diameter, 250 mm column length

Table 2.11: Normal phase HPLC method for the analysis of the enantiomeric excess of 2-bromopropionic acid 35.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Retention time (min)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid 35</td>
<td>9.5</td>
<td>(S)-2-bromopropionic acid&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10.3</td>
<td>(R)-2-bromopropionic acid&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>ethyl-2-bromopropionate 123</td>
<td>3.4</td>
<td>first eluting enantiomer</td>
</tr>
<tr>
<td></td>
<td>3.5</td>
<td>second eluting enantiomer</td>
</tr>
</tbody>
</table>

<sup>1</sup> baseline separated.

Table 2.12: Retention times of 2-bromopropionic acid 35 and ethyl-2-bromopropionate 123 using the method outlined in Table 2.11.
In order to calculate the enantioselectivity of the Cal B mutants, a method on reverse phase HPLC was developed to measure the degree of conversion. A sample of 2-bromopropionic acid 35 and ethyl-2-bromopropionate 123 in buffer (100 μL) was added to acetonitrile (300 μL) and subjected to analysis according to the method developed (Table 2.13).

<table>
<thead>
<tr>
<th>HPLC</th>
<th>Column Temp. (°C)</th>
<th>Flow rate (ml/min)</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse phase</td>
<td>25</td>
<td>1</td>
<td>Acetonitrile + TFA (0.1%) %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanol + TFA (0.1%) %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2.13: Reverse phase HPLC method to analyse the conversion of the biotransformation reactions.

When this protocol was tested with wild-type Cal B, it was found that 2-bromopropionic acid 35 did not extract sufficiently from the buffer so a decision to develop a new HPLC method to analyse enantiomeric excess and conversion in a single analysis run on a reverse phase chiral column was made.

The reverse phase chiral column, Astec Chirobiotic™ T, was deemed suitable for the resolution of 2-bromopropionic acid 35. Following extensive method development, chiral HPLC conditions to provide baseline separation of 2-bromopropionic acid 35 were determined (Table 2.14). The retention times of ethyl-2-bromopropionate 123 and 2-bromopropionic acid 35 were recorded according to the method developed (Table 2.15).

<table>
<thead>
<tr>
<th>HPLC</th>
<th>Column Temp. (°C)</th>
<th>Flow rate (ml/min)</th>
<th>Mobile Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reverse phase</td>
<td>5</td>
<td>0.5</td>
<td>Ammonium acetate buffer 20mM pH 4.5 %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Methanol %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>90</td>
</tr>
</tbody>
</table>

Table 2.14: Reverse phase chiral method developed on an Astec Chirobiotic™ T column for the baseline resolution of 2-bromopropionic acid 35.
### Table 2.15: Retention times of 2-bromopropionic acid 35 and ethyl-2-bromopropionate 123 on the reverse phase chiral method described in Table 2.14.

<table>
<thead>
<tr>
<th>Racemate</th>
<th>Retention time (min)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-bromopropionic acid 35</td>
<td>5.4</td>
<td>(S)-2-bromopropionic acid$^1$</td>
</tr>
<tr>
<td>ethyl-2-bromopropionate 123</td>
<td>6.9</td>
<td>ethyl-2-bromopropionate</td>
</tr>
<tr>
<td>1-bromopropionic acid</td>
<td>6.0</td>
<td>(R)-2-bromopropionic acid$^1$</td>
</tr>
</tbody>
</table>

$^1$ baseline separated.

For the analysis of biotransformations the reverse-phase chiral column, Astec Chirobiotic™ T, was used under the method conditions outlined in Table 2.14.

#### 2.8.2.2 Mechanical lysis of bacterial cell pellets

Due to the sensitive nature of the Astec Chirobiotic™ T column, chemical lysis of bacterial cell pellet was no longer viable due to the surfactants in the chemical lysis agents. Alternative methods for lysis of bacterial cell pellets were considered. Mechanical lysis was determined to be the best alternative and a method was developed. A method was generated which initially had the bacterial cell pellets stored at -20 °C to aid the breakdown of the bacterial cell walls. The thawed cell pellets were then resuspended in buffer and lysozyme and protein inhibitor cocktail (PIC) were added to the suspension. The suspension was incubated at 37 °C for 20 minutes before sonication to generate the cell-free extract.

#### 2.8.2.3 Protein extraction from buffer

Removal of protein from samples before applying to an HPLC system prolongs the life and resolution of chiral HPLC columns. The protein was removed from the buffer by denaturisation at 95 °C for 5 minutes followed by centrifugation. This method allowed the majority of protein to be removed from a sample before analysis. The stability of 2-
bromopropionic acid 35 and ethyl-2-bromopropionate 123 at 95 °C was also investigated. Ethyl-2-bromopropionate 123 was stable and did not hydrolyse within 25 minutes at 95 °C. Enantiopure 2-bromopropionic acid 35 also did not racemise at 95 °C over a period of 25 minutes.

2.9 Molecular modelling studies

The main objective of this project was to identify a hydrolase with high enantioselectivity that was able to kinetically resolve the substrates 2-bromopropionic acid 35 and 2-fluoropropionic acid 83. The screen of commercially available lipases afforded Cal B as a suitable candidate for both substrates, although with low enantioselectivity. Engineering of a protein has long been used as powerful tool to create novel enzymes capable of meeting the needs of industry. In order to obtain an enzyme with the required properties, directed evolution strategies are used. Different variant libraries of Cal B were generated using rational design and molecular biology methods. Each library was then screened using the reverse phase chiral HPLC assay described in section 2.8.2. The generation and screening of each library will be discussed in the following section.

2.9.1 Rational design studies

The published X-ray structure of Cal B was used for our molecular modelling studies (Figure 2.24, structure reference in Protein Data Bank 1TCA).140 When the DNA and protein sequence of our Cal B was aligned with the protein and DNA sequence of 1TCA, 2 mutations were observed at Ala57Thr and Thr89Ala. These mutations have been noted before and were attributed to natural variations of the lipase gene as a consequence of evolution.169

The Cal B active site consists of two pockets, one accommodating the acyl moiety and the other the alcohol moiety of the substrate. To enhance enantioselectivity for the kinetic resolution of secondary alcohols, engineering of the alcohol pocket has been reported. Mutagenesis of the alcohol pocket has resulted in many mutants with enhanced enantioselectivity.159,184 With the multitude of examples published concerning kinetic resolution of secondary alcohols, empirical rules were formed that summarised earlier results. The substituents of a substrate can be labelled small or large, relative to each other.
With the large substituent residing in the larger pocket and the smaller substituent residing in the smaller pocket of a lipase, prediction of the faster reacting enantiomer in a lipase catalysed kinetic resolution of secondary alcohols has been reported.\textsuperscript{185} This empirical rule was successfully applied to Cal B, when X-ray structures also showed two pockets of medium and large size.\textsuperscript{186,187} The stereogenic centre of our substrates, 2-bromopropionic acid 35 and 2-fluoropropionic acid 83, reside on the acyl moiety and engineering of the acyl pocket of Cal B has seldom been reported.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure224.png}
\caption{left – flat ribbon structure of Cal B: right – Surface view of Cal B.}
\end{figure}

2.9.2 Enzyme-Substrate docking models and proposed sites for mutagenesis

A computational study was carried out aimed at elucidating the enzyme-substrate interactions that are responsible for the activity and $E_R$ observed. Accelrys Discovery Studio 1.6 and 2.0 were used to dock the substrates, 2-bromopropionic acid 35 and 2-fluoropropionic acid 83, into the active site of Cal B using the known mechanism and the hydrogen bonding network to establish orientation. The energy of the protein was minimised and this structure was used for insight into residues that influence activity and enantioselectivity.

The enantioselectivity of Cal B for the esterification of 2-bromopropionic acid 35 with $n$-butanol 117 is only moderately in favour of the (S)-enantiomer, $E_R = 2.5$. This can be explained by modelling the (S)-enantiomer of 2-bromopropionic acid (S)-35 into the active
site of Cal B (Figure 2.25). The bromine of (S)-2-bromopropionic acid (S)-35 sits in the larger pocket of the active site (Figure 2.26). In comparison, when (R)-2-bromopropionic acid (R)-35 is docked into the active site of Cal B, the bromine atom sterically clashes with the narrow channel leading to the active site (Figure 2.27).

Figure 2.25: The substrate (S)-2-bromopropionic acid (S)-35 modelled into the active site of Cal B with the large acyl pocket accommodating the bromine.

Figure 2.26: CPK view of (S)-2-bromopropionic acid (S)-35 sits comfortably in the large acyl pocket of active site of Cal B.
As the mechanism of Cal B is known\textsuperscript{140}, 2-bromopropionic acid \textbf{35} must be orientated with the carbon of the carbonyl group in close proximity to the nucleophilic serine (Ser105) of the catalytic triad. As 2-bromopropionic acid \textbf{35} contains a sterically demanding bromine atom, several models were generated with the bromine in the large pocket of the active site of Cal B. The results were used to inform subsequent focused mutagenesis at several positions namely Thr40, Ile189 and Ile285. These positions were chosen as they line the larger acyl pocket which accommodates the bromine atom. We hypothesised that by increasing the size of the acyl pocket this would facilitate the movement of the substrate in and out of the active site and thereby increase activity. It was proposed that replacement of larger amino acids such as Ile at position 189 with smaller amino acids like glycine and leucine would further reduce steric clashing between the bromine atom and the wall of the active site (Appendix 6). It is difficult to predict with any degree of certainty which residues will influence enantioselectivity. It was proposed that saturation mutagenesis at the positions identified would allow us some insight into this matter. By conducting saturation mutagenesis, investigation into interactions that govern enantioselectivity was probed by the different nature of the 20 natural amino acids.

Figure 2.27: The model of \textit{(R)}-2-bromopropionic acid \textit{(R)}-\textbf{35} docked into the active site of Cal B with the bromine orientated towards the active site tunnel showed steric clashing with the wall of the narrow channel.
The biotransformations of commercially available Cal B with 2-fluoropropionic acid 83 revealed that the enzyme was selective towards the first eluting enantiomer. As fluorine is small in comparison to bromine, a rationale based upon steric demand is not sufficient to explain the enantioselectivity observed in our studies (Figure 2.28 and 2.29). Fluorine however is the most electronegative element and electronic factors could explain the enantioselectivity of Cal B towards 2-fluoropropionic acid 83. Some of the amino acid residues near the fluorine of (S)-2-fluoropropionic acid (S)-83 include Ser105, 3.34 Å away, Asp134, 3.99 Å away and Gln157, 3.66 Å away. Interactions that are possible with the fluorine of (S)-2-fluoropropionic acid (S)-83 include F-O electrostatic interactions. By conducting saturation mutagenesis at positions 134 and 157, a fluorine-enzyme interaction that stabilises the intermediate could be investigated.

Figure 2.28: (S)-2-Fluoropropionic acid (S)-83 modelled into the active site of Cal B with the fluorine atom positioned in the acyl pocket.
In addition to the molecular modelling studies described above, reports by other research groups highlighted other potential sites for mutagenesis.\textsuperscript{188,189} These residues were Thr138, Val190 and Leu278. It was also encouraging to see other research groups also targeting the same residues namely Asp134, Gln157 and Ile 189. Some of these groups were also undertaking mutagenesis of Cal B to increase $E_R$, although our substrates differed, whilst others wanted to increase the hydrolysis activity of bulky substrates. The single mutant Leu278Ala was reported to have increased hydrolysis activity for bulky substrates and was included in our studies for this reason.\textsuperscript{190} The positions and mutations targeted in Cal B have been summarised in Table 2.16.
<table>
<thead>
<tr>
<th>Targeted sites in Cal B</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr40</td>
<td>saturation library</td>
</tr>
<tr>
<td>Asp134</td>
<td>Ala, Ser, Thr, Asn</td>
</tr>
<tr>
<td>Thr138</td>
<td>saturation library</td>
</tr>
<tr>
<td>Gln157</td>
<td>Ala, Val, Leu, Ile, Ser, Thr, Asn</td>
</tr>
<tr>
<td>Ile189</td>
<td>saturation library</td>
</tr>
<tr>
<td>Val190</td>
<td>saturation library</td>
</tr>
<tr>
<td>Leu278</td>
<td>Ala</td>
</tr>
<tr>
<td>Ile285</td>
<td>saturation library</td>
</tr>
</tbody>
</table>

Table 2.16: A summary of the positions within Cal B targeted for mutation.

2.10 Protein engineering and assaying of each Cal B mutant

2.10.1 Protein engineering

The Cal B variants discussed in section 2.9 were constructed using the wild-type Cal B gene as a template and the QuikChange® Site-directed Mutagenesis Kit from Stratagene (Figure 2.30). Two strategies were used to generate the mutations outlined in Table 2.16. Codon optimised oligomers were used for the introduction of mutations at position 40, 134, 157, 189, 278 and 285 and the NNK (N: adenine, cytosine, guanine and thymine; K: guanine and thymine) degenerate codon was used to generate saturation libraries at positions 138 and 190. The NNK degenerate codon involves 32 codons and all 20 amino acids as building blocks.
After PCR, a DpnI digestion of the template DNA was carried out at 37 °C for 1 h. *E. coli* XL1-Blue cells were transformed with the mutated DNA, according to the manufacturer’s protocol. The transformed cells were cultivated on LB amp agar plates and grown overnight in a 37 °C static incubator. Oversampling of the colonies from each transformation were prepared for overnight cultures. For the mutants generated using the NNK degenerate codon, 94 transformants are needed for 95% coverage of the 32 codons in a single position. The plasmid DNA of each culture was isolated and sequenced to verify that each mutation had been incorporated. In total 89 mutants were successfully synthesised and they are summarised in Table 2.17. Once sequencing had confirmed each mutation, the mutant DNA was used for the transformation of Origami 2(DE3) *E. coli* competent cells. Each mutant was grown up on a large scale (600 mL), according to the final protocol set out in section 2.5.4. The gene expression levels of each mutant were not recorded and the protein production levels were assumed to be comparable. The bacterial cell pellet of each mutant was stored at – 20 °C until needed for the HPLC assay.
Targeted sites in Cal B

<table>
<thead>
<tr>
<th>Mutants of Cal B successfully synthesised</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr40</td>
</tr>
<tr>
<td>Asp134</td>
</tr>
<tr>
<td>Thr138</td>
</tr>
<tr>
<td>Gln157</td>
</tr>
<tr>
<td>Ile189</td>
</tr>
<tr>
<td>Val190</td>
</tr>
<tr>
<td>Leu278</td>
</tr>
<tr>
<td>Ile285</td>
</tr>
</tbody>
</table>

Table 2.17: A summary of all the mutants synthesised using protein engineering.

### 2.10.2 Screening of the Cal B mutants using the HPLC assay

The reverse phase chiral HPLC assay was used to determine the effects of the mutations on the enantioselectivity of Cal B. The bacterial cell pellets of each mutant were mechanically lysed according to the protocol described in section 2.8.2.2 and the total protein content of each sample was measured using the BCA assay. The crude cell free extracts from the lysis of the cell pellets were used in the biotransformation of ethyl-2-bromopropionate 123 (Scheme 2.9). The substrate, ethyl-2-bromopropionate 123 (5 g/L), was added to potassium phosphate buffer (0.1 M, pH 7.0, 4 mL) containing ethanol (0.36 v/v%) to aid solubility of the substrate (Scheme 2.7). To start the reaction, 200 mg of protein (cell free extract) of
each mutant was added to the biotransformation reactions. The biotransformations were conducted at 30 °C and in total eight to ten samples of each biotransformation were taken at various time points for analysis using the reverse phase chiral HPLC method developed in section 2.8.2.1 (Table 2.14). The protein from each sample was removed by heat denaturation at 95 °C for 5 minutes and then the degree of conversion and the e.e. of the supernatant recorded using the reverse phase chiral HPLC method outlined in Table 2.14. The conversion and e.e. of each sample were used to calculate the $E_R$ of the Cal B variants.

Scheme 2.9: Biotransformations of the hydrolysis of ethyl-2-bromopropionate 123 by the mutants of Cal B.

Reaction conditions: ethyl-2-bromopropionate 123 (5 g/L), enzyme (200 mg/reaction), potassium phosphate buffer (100 mM, pH 7.0, 4 mL), ethanol (0.36% v/v), incubated in a 30 °C shaker.

2.10.3 Enantioselectivity of Cal B mutants with single point mutations

The enantioselectivity of each Cal B variant was calculated using the enantiomeric excess of the product (ee(P)), 2-bromopropionic acid 123, the degree of conversion (c) and Equation 6 (Appendix 7), as described by Chen et al.\textsuperscript{163} The $E_R$ of each sample was used to calculate an average $E_R$ of each mutant and the results are collated in Table 2.18.

$$E_R = \frac{\ln[(1-c)(1+\text{ee}(P))]}{\ln[(1-c)(1-\text{ee}(P))]}$$  \hspace{1cm} (6)
Table 2.18: The enantioselectivity of mutants of Cal B towards the kinetic resolution of the substrate ethyl-2-bromopropionate 123.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Thr40</th>
<th>Asp134</th>
<th>Thr138</th>
<th>Gln157</th>
<th>Ile189</th>
<th>Val190</th>
<th>Leu278</th>
<th>Ile285</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.1</td>
<td>1.2</td>
<td>1.8</td>
<td>1.2</td>
<td>1.0</td>
<td>1.1</td>
<td>1.3</td>
<td>2.0</td>
</tr>
<tr>
<td>C</td>
<td>1.0</td>
<td>2.0</td>
<td>1.2</td>
<td>1.1</td>
<td>1.1</td>
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<td>1.1</td>
<td>1.1</td>
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<tr>
<td>D</td>
<td>1.1</td>
<td>2.0</td>
<td>1.6</td>
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<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
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<td>G</td>
<td>1.1</td>
<td>1.7</td>
<td>1.2</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
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<td>H</td>
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<td>1.1</td>
<td>1.1</td>
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Yellow = Wild-type, Blue = (R)-selective, White = (S)-selective, Grey = not synthesised

2.10.3.1 Thr40 mutants

The transition state of Cal B catalysed hydrolysis of esters involves generation of an oxyanion that is stabilised by a network of hydrogen bonds formed from the side chain of Thr40 and the amide backbone of Thr40 and Gln106. The stabilisation of the oxyanion hole has been attributed for the lowering of the free energy of the transition state. A hypothesis was proposed that mutations at position 40 would lead to decrease in rate of reaction and the results confirm this (Figure 2.31).
The effect of the mutations at this position on enantioselectivity is also pronounced with all mutants synthesised having lost all enantioselectivity, although whether this is as a result of a loss in rate of reaction and therefore conversion or due to loss of specific interactions between the enantiomers and the active site, has not been determined. The rates of reaction relative to the wild-type Cal B were significantly lower. Less than 40% of the original activity was retained in the mutants at position 40. This confirms the importance of Thr40 as part of the hydrogen network that stabilises the oxyanion intermediate. When Thr40 was substituted for another nucleophilic amino acid, serine and cysteine, the rate of reaction and enantioselectivity could not be replicated. This result suggests that Cal B has evolved to incorporate threonine in position 40 and was necessary for activity for the kinetic resolution of ethyl-2-bromopropionate 123.

2.10.3.2 Asp134 mutants

Specific mutations at position 134 were chosen to reduce the steric clash between the active site wall and the large bromine atom in the (S)-enantiomer of the substrate ethyl-2-bromopropionate 123. To this end, mutants with substitution of the Asp amino acid for smaller amino acids such as Ala were generated. All of the variants at position 134 showed
a decrease in enantioselectivity combined with a decrease in the rate of reaction, even for the mutant Asp134Ala (Table 2.18 and Figure 2.32)

![Bar chart comparing activity of different Cal B mutants](image)

**Figure 2.32:** The rate of reaction of Asp134 mutants compared to wild-type Cal B in the kinetic resolution of ethyl-2-bromopropionate 123.

### 2.10.3.3 Thr138 mutants

From the HPLC assay, all the Thr138 variants of Cal B were found to have a higher rate of reaction than wild-type Cal B (Figure 2.33). One variant, Thr138Met, was found to have a higher rate of reaction and a higher enantioselectivity (Table 2.18).

The higher rate of reaction in the Thr138Ala mutant can be attributed to a reduction in the size of the amino acid from a threonine to an alanine. The enlarged space created in the Thr138Ala mutant can allow the substrate to move in and out of the active site readily thereby increasing activity.
Figure 2.33: Variants of Thr138 with a higher rate of reaction than the wild-type.

2.10.3.4 Gln157 mutants

The variants generated by mutation at position 157 gave rise to a library with opposite stereopreference to the wild-type. The specific mutations were chosen to increase space to accommodate the bromine atom of the (S)-enantiomer of ethyl-2-bromopropionate 123 and therefore activity towards this enantiomer. The results showed that the mutants exhibited a slower rate of reaction than the wild-type (Figure 2.34).

To account for the opposite stereopreference, models of the mutants of Gln157 were generated with both enantiomers of ethyl-2-bromopropionate 123 which showed that the space was increased, as was intended, but they could not show conclusively how enantioselectivity was affected. Although several mutants of Gln157 demonstrated opposite stereopreference, as a saturation library was not made of Gln157, it may not apply to the remaining variants of the library. Synthesis of the remaining variants will show whether this is a trend and models will aid the rationalisation of the trend.
Figure 2.34: Rate of reaction of mutants of position 157 with opposite stereopreference than the wild-type Cal B.

### 2.10.3.5 Ile189 and Val190 mutants

The Ile189 and Val190 libraries did not yield any mutants of significant interest. All the mutants generated in these 2 libraries had a lower rate of reaction and equal or lower enantioselectivity than the wild-type. When models of the mutants of position 189 were investigated, a large gap was demonstrated when compared to the wild-type. Although this fulfilled our original criteria of creating more space to accommodate the bromine atom of the (S)-enantiomer, creation of this larger void led to the loss of stereoselectivity due to the free movement of both enantiomers of the substrate ethyl-2-bromopropionate 123 in and out of the active site. Originally the large bromine of the (S)-enantiomer was restricted to a pocket of the active site, however enlarging the void has allowed the bromine of both enantiomers to fit in freely, thereby losing stereoselectivity (Figure 2.35).
2.10.3.6 Leu278Ala mutant

The mutant Leu278Ala was included in this study as it was reported to have an increased rate of reaction for the hydrolysis of bulky substrates. Our results show this did not extend to our substrate ethyl-2-bromopropionate 123 as less than 40% of the original activity was observed in the Leu278Ala mutant (Figure 2.36).
2.10.3.7 Ile285 mutants

Ile285 is the furthest amino acid residue from the active site and is positioned near the entrance to the active site. Surprisingly its effect has been the most pronounced with the identification of two mutants (Ile285Ala and Ile285Lys) with a faster rate of reaction and one mutant (Ile285Leu) with a higher enantioselectivity than the wild-type. When models of the mutants of position 285 were generated, the entrance to the active site changed in shape and in terms of electronic character. It was proposed that this affected the access of the substrate into the active site.

2.10.4 Comparison of enantioselectivity of Ile285Leu, Thr138Ala and Thr138Met

From the initial enantioselectivity screen, three mutants, Ile285Leu, Thr138Ala and Thr138Met, were chosen for further investigation due to the high rates of reaction or enantioselectivity they displayed. For a true comparison with the wild type, the biotransformations were repeated with 5 mg of protein (cell free extract) per mL added to
the reaction. Samples taken at specific time points were analysed using the method described in Table 2.14 (Figure 2.37 and 2.38).

The results confirmed that all three mutants had a higher enantioselectivity than the wild-type but the Thr138Met mutant had a significantly higher enantioselectivity. A model of the Thr138Met mutant was made with the (R)- and (S)-enantiomers of the intermediate docked into the active site. It showed that the bromine of the (R)-enantiomer sterically clashed with the Thr138Met mutation, thus making the (S)-enantiomer more favourable. The Thr138Met mutant positions the smaller methyl group next to the Thr138Met mutation (Figure 2.38).

![Graph showing product accumulation as a function of time for WT Cal B and the mutants Ile285Leu, Thr138Ala and Thr138Met.]

**Pink** = Wild-type Cal B, **Yellow** = No enzyme, **Green** = T138M mutant, **Red** = T138A mutant, **Blue** = I285L mutant

Figure 2.37: Product accumulation as a function of time for WT Cal B and the mutants Ile285Leu, Thr138Ala and Thr138Met.
Figure 2.38: Comparison of enantioselectivity of Ile285Leu, Thr138Ala and Thr138Met relative to wild-type Cal B.

Figure 2.39: Molecular model of the Thr138Met mutant with the (S)-enantiomer of the intermediate docked into the active site. Model shows the Thr138Met mutant with the smaller methyl group of (S)-2-bromopropionic acid next to the Thr138Met mutation to reduce steric clashing.
3 Conclusion

At the onset of this research programme, we hoped to identify commercially available lipases for the kinetic resolution of 2-bromopropionic acid 35 and 2-fluoropropionic acid 83 for the synthesis of a number of enantiomerically pure chiral pharmacologically active compounds. Retrosynthetic analysis identified 2-bromopropionic acid 35 and 2-fluoropropionic acid 83 as common intermediates. Additionally, optically active 2-bromopropionic acid 35 could be used for the synthesis of other chiral starting materials.

An initial screen of 16 lipases identified only one lipase, Cal B, able to kinetically resolve both 2-bromopropionic acid 35 and 2-fluoropropionic acid 83. The gene of Cal B was sourced and subcloned into two vectors, pET16b and pET SUMO, for expression in E. coli. After various investigations, Cal B was expressed from the pET16b vector in 2 types of E. coli competent cells, BL21 and Origami 2, the latter of which promotes disulfide bond formation in the cytoplasm of E. coli. The Cal B produced from the Origami 2 competent cells gave a Cal B with higher specific activity and was therefore used for future productions of Cal B.

As the enantioselectivity of Cal B towards both substrates was poor, directed evolution was used for rational design of mutants with increased activity and higher enantioselectivity towards the substrates of interest. Problems with the stability of ethyl-2-fluoropropionate 124 hampered further investigation into this substrate but Cal B mutants identified from structure-driven modelling were screened with the substrate ethyl-2-bromopropionate 123. A total of 89 mutants with single amino acid changes were generated with several mutants exhibiting higher rates of reaction than the wild type and with one mutant, Thr138Met, exhibiting a moderate increase in enantioselectivity. Finally, in addition to these, several variants of the 157 library were identified to having opposite enantiopreference.
4 Future work

This project has laid the foundations for identifying variants of Cal B for the kinetic resolution of α-haloacids and esters. With the initial results from the single amino acid mutations, researchers in future should consider probing cooperative effects of mutations and utilising the iterative saturation mutagenesis strategy demonstrated by Reetz et al.\textsuperscript{128} Additionally having demonstrated sensitive residues relevant for this project, further work into probing adjacent amino acids should be included in future work. If mutants generated using rational design does not generate variants with the desired properties, other mutagenesis strategies should be utilised such as epPCR.

Additional to using mutagenesis techniques for improving the properties of an enzyme, they can also be used to improve the expression of enzymes. The expression systems we presented in this thesis yield Cal B that is partially soluble. Cal B has many hydrophobic residues on its surface and this may increase the formation of inclusion bodies in the cytoplasm or block translocation during its secretion to the periplasm.\textsuperscript{193} Mutagenesis to introduce hydrophilic residues onto the surface of Cal B to increase the yield of functional expression of Cal B in \textit{E. coli} can also be considered. To this end, substitution of residues Leu147, Leu199, Leu219, Leu261 and Ile255 with aspartate was carried out by Park \textit{et al.}\textsuperscript{194} These substitutions resulted in an increase in the expression yield of functional Cal B by a factor of three. The effect of these mutations on hydrolysis activity and enantioselectivity was also investigated and no significant differences to the wild-type were observed.

Having successfully generated mutants of Cal B, the lack of a high throughput assay severely limited the numbers of mutants screened in this project. Addition of a solid-phase screen on tributyrin agar plates would eliminate inactive mutants generated when strategies such as epPCR are used. Further synthetic approaches towards enantiopure 4-nitrophenyl 2-bromopropionate \textsuperscript{118} and 4-nitrophenyl 2-fluoropropionate and \textsuperscript{119} would also further reduce the number of mutants assayed using the HPLC assay described in this thesis. With these two pre-screens in place, the number of mutants needed to be grown up on a large scale for biotransformations and analysed by conventional HPLC would be manageable.
With these new strategies proposed, identification of a variant of Cal B with enhanced enantioselectivity properties should be forthcoming and then utilisation of the enantiopure α-haloacids and esters for the synthesis of pharmacologically active compounds such as (S)-Keppra (S)-32 and (R)-Tiopronin (R)-34, as well as other enantiopure starting materials, would complete the goals initially intended for this project.
5 Experimental

5.1 General Procedure

All HPLC chromatograms were recorded on an Agilent 1100 series instrument with an integrated Agilent 1200 series column temperature regulation compartment. The samples were run on a Daicel Chiralpak® AD-H chiral column (19325) or an Astec Chirobiotic™ T chiral column (12024AST).

PCR reactions were performed in an Eppendorf Mastercycler® Gradient. The concentrations of double stranded DNA (dsDNA) and protein to DNA ratio were recorded using an Eppendorf BioPhotometer with an 8.5 mm light centre height in RNase-/DNA-/Protein-free Eppendorf disposable, single sealed cuvettes. Bacterial cells were harvested by centrifuge in an Eppendorf 5810R centrifuge and a table-top Eppendorf 5415R centrifuge was used for PCR and plasmid DNA purification. All 0.8% analytical agarose gels were run at 80 V for 45 minutes. Gel extraction was performed using an Invitrogen Safe Imager™ transilluminator and SYBR Safe DNA gel stain. Incubation of reactions was performed in an Infors-HT Multitron II incubation shaker.

Sterile technique was used through-out all molecular biology procedures where necessary.

5.2 Chemicals and Reagents

Standard chemicals for the preparation of buffers and media, as well as standard laboratory consumables were purchased either from BioRad (Hertfordshire, U.K.), Eppendorf U.K. Ltd. (Cambridge, U.K.), Eurofins MWG Operon (Ebersberg, Germany), Fisher Scientific U.K. Ltd. (Loughborough, U.K.), Fluorochem Ltd. (Derbyshire, U.K.), GE Healthcare (Buckinghamshire, U.K.), Invitrogen Ltd. (Paisley, U.K.), Merck Biochemicals Ltd. (Nottingham, U.K.), New England Biolabs Ltd. (Hertfordshire, U.K.), Pierce (Northumberland, U.K.), Qiagen Ltd. (West Sussex, U.K.), Romil Ltd. (Cambridge, U.K.), Sigma-Aldrich Company Ltd. (Gillingham, U.K.) or Stratagene (Texas, USA) and used as supplied unless stated otherwise.
5.2.1 Growth Media

LB (Luria-Bertani)

Broth medium was composed of tryptone (10g), NaCl (10g) and yeast extract (5g) dissolved in 1 L of distilled H₂O (dH₂O). Sterile LB media was supplied by the in-house media preparation service.

LB Agar

Medium was prepared according to the method outlined above but with the addition of agarose (15 g) in 1 L of dH₂O. Sterile LB agar was supplied by the in-house media preparation service.

SOC medium

SOC medium (S1797) was purchased from Sigma-Aldrich Company Ltd and used as bought.

5.2.2 LB agar plates

LB agar plates containing ampicillin (LB amp) were prepared by melting a bottle of LB agar and cooling it to 55°C in a water bath. Ampicillin (1 µL per mL) was added and the plates were prepared by pouring into petri dishes. The LB amp plates were inverted and stored at 4°C.

LB agar plates containing kanamycin (LB kan) were prepared by melting a bottle of LB agar and cooling it to 55°C in a water bath. Kanamycin (1 µL per mL) was added and the plates were prepared by pouring into petri dishes. The LB kan plates were inverted and stored at 4°C.
5.2.3 Buffers

50x TAE Buffer

TAE buffer was composed of 242 g tris base, 57.1 mL glacial acetic acid and 10 mL 0.5 M EDTA (pH 8.0) made up to a total volume of 1 L with dH$_2$O.

DNA loading buffer for electrophoresis

DNA loading buffer was composed of 30% glycerol (v/v), 0.2% bromophenol blue, 25 mM EDTA and was diluted with TAE.

10x Tris-Glycine-SDS buffer for electrophoresis

10x Tris-Glycine-SDS buffer (161-0772), purchased from Bio-Rad Laboratories UK, was composed of tris(hydroxymethyl)aminomethane (25 mM), glycine (192 mM) and SDS (0.1%) and 100 mL was diluted with dH$_2$O (900 mL).

2x Laemmli sample buffer for electrophoresis

2x Laemmli sample buffer (S3401) was purchased from Sigma-Aldrich Company Ltd.

Potassium phosphate buffer 0.1 M pH 7.4

Potassium phosphate buffer (0.1 M, pH 7.4) was prepared from dipotassium hydrogen phosphate buffer solution (1 M, 80.2 mL), potassium dihydrogen phosphate buffer solution (1 M, 19.8 mL) and dH$_2$O (950 mL).
**Western blotting buffers**

**Phosphate buffered saline**

Phosphate buffered saline tablets (PBS, P4417) were purchased from Sigma-Aldrich Company Ltd. The tablets of PBS were composed of phosphate buffer (0.01 M), potassium chloride (0.0027 M) and sodium chloride (0.137 M) and were dissolved in dH$_2$O (200 mL).

**Phosphate buffered saline solution with Tween 20 (0.05%)**

PBS with Tween 20 was composed of PBS (100 mL) and Tween 20 (50 µL, P7949) purchased from Sigma-Aldrich Company Ltd was added.

**Blocking buffer**

Blocking buffer was composed of casein protein (10 g) in PBS (200 mL).

**Blocking buffer with monoclonal Anti-polyHistidine–Peroxidase antibody**

The blocking buffer with monoclonal Anti-polyHistidine–Peroxidase antibody was composed of casein protein (1.25 g), PBS (25 mL) and monoclonal Anti-polyHistidine–Peroxidase antibody produced in mouse (25 µL, A7058-1VL). The monoclonal Anti-polyHistidine–Peroxidase antibody produced in mouse (25 µL, A7058-1VL) was purchased from Sigma-Aldrich Company Ltd.

**Towbin buffer**

Towbin buffer was prepared from Tris (3.03 g), glycine (14.4 g), methanol (100 mL) and dH$_2$O (900 mL).
Purification buffers

Binding buffer

To sodium phosphate buffer (0.1 M, pH 8.0), NaCl (17.55 g/L) and imidazole (0.01 M, 0.68 g/L) were added and the solution was filtered before use.

Eluting buffer

To sodium phosphate buffer (0.1 M, pH 8.0), NaCl (17.55g/L) and imidazole (1 M, 68.08 g/L) were added and the solution was filtered before use.

5.2.4 Stock solutions

Ampicillin

Stock solution of ampicillin sodium salt (1000 x) was prepared by dissolving 100 mg/mL of ampicillin in dH2O and sterilised by passage through a 0.22 µM syringe filter. These stock solutions were stored in 1 mL aliquots at –20 °C until required.

Kanamycin

Stock solution of kanamycin (1000 x) was prepared by dissolving 50 mg/mL of kanamycin in dH2O and sterilised by passage through a 0.22 µM syringe filter. These stock solutions were stored in 1 mL aliquots at –20 °C until required.

Lysozyme

Lysozyme solutions were prepared prior to use. Lysozyme (10 mg, L6876), purchased from Sigma-Aldrich Company Ltd., was dissolved in dH2O (1 mL)
**para-Nitrophenyl acetate**

*para*-Nitrophenyl acetate (0.36 g, 20 mM) in dimethyl sulfoxide (100 mL) was prepared for use in the *p*NPA assay.

**para-Nitrophenyl butyrate**

*para*-Nitrophenyl butyrate (0.21 g, 20 mM) in dimethyl sulfoxide (100 mL) was prepared for use in the *p*NPB assay.

**3,3’-Diaminobenzidine (DAB) solution**

SigmaFast DAB (D4418) solution was purchased from Sigma-Aldrich Company Ltd. and used as described in protocol.

**Isopropyl-β-D-1-thiogalactopyranoside (IPTG) solution**

IPTG (11.92 g) in dH₂O (50 mL) was prepared for use as stock solutions for the induction of cultures and sterilised by passage through a 0.22 µM syringe filter.

**5.2.5 Strains**

The bacterial strains used during this project are listed below and were stored at – 80 °C until required:

*E. coli* Top10  
*E. coli* Mach1 T1-R  
*E. coli* BL21(*DE3*)  
*E. coli* Origami 2(*DE3*)  
*E. coli* XL1-Blue
5.2.6 Oligonucleotide primers

Oligodeoxynucleotide primers for PCR-mediated cloning and sequencing were synthesised by Eurofins MWG Operon (Germany).

5.2.7 Other

DNA size markers for electrophoresis

DNA size markers, 100 base pair (N3231S) and 1 kb DNA Ladder (N3232L), were purchased from New England Biolabs UK.

PageRuler™ Prestained protein ladder for electrophoresis

PageRuler™ Prestained protein ladder (SM0671), with a range of molecular weights from 10 to 170 kDa, was purchased from Fermentas Life Sciences UK.

5.3 Experimental details

5.3.1 Initial hydrolase screen

General procedure for the esterification of 2-bromopropionic acid and 2-fluoropropionic acid

The lipases (0.5 g/L) were weighed out into 7 mL glass vials with screw on caps containing a single molecular sieve (4 Å, 10 – 18 mesh). A stock solution was prepared containing iso-hexane (2 mL per reaction), substrate (5g per L) and n-butanol (5 equiv.). The stock solution was distributed equally per reaction and the reactions were incubated at 30°C and shaken at 225 rpm for the length of the biotransformation.
General procedure for the BCA assay

Stock solutions of the enzyme that were to be assayed were prepared and from the enzyme stock solution, 3 dilutions were made; 10:1, 100:1 and 1000:1. The BCA assay was prepared according to the manual (23225, Pierce BCA™ protein assay kit). The BCA assay was completed in a microplate and 3 replicates of each assay were done. The endpoints of the standards and assay were measured at 584 nm on a plate reader.

5.3.2 Cloning

Transformation of the pPIC9 Cal B gene into One Shot Top10 competent cells

A vial of One Shot® Top10 competent cells (50 µL) was thawed on ice prior to addition of 0.5 µL pPIC9 Cal B DNA (200 ng/µL). The vial was incubated on ice for 30 minutes and then at 42°C, in a water bath, for 45 seconds to heat shock the competent cells into taking up the DNA. The vial was removed from the 42°C water bath and immediately placed into ice. To the vial, 250 µL of pre-warmed SOC medium was added before incubation at 37°C for 1 hour at 225 rpm. Two volumes of the transformation reaction were plated, 100 µL and 200 µL, onto LB amp plates. The plates were inverted and incubated at 37°C for 18 hours.
General procedure for overnight cultures of successful transformation colonies

A transformant was picked from the LB agar plates containing the appropriate antibiotic and inoculated into 5 mL aliquots of LB medium containing the same antibiotic to select for your expression plasmid. The overnight cultures were incubated at 37°C for 18 hours at 225 rpm.

General procedure for making glycerol stocks

Glycerol stocks were prepared by combining 800 µL of the overnight culture with 200 µL of glycerol. The samples were stored at –80°C.

General procedure for extracting and purifying plasmid DNA

The remaining overnight culture samples were harvested by centrifugation at 4000 rpm for 10 minutes. The supernatant was discarded and the bacterial cell pellet was dried by inversion of the tubes. The bacterial cell pellet was then purified using a Qiagen Plasmid Prep Kit (27106) as according to the manual.

Restriction digest analysis of pPIC9 Cal B construct

The restriction digest reactions contained 14 µL of dH2O, 3 µL of Buffer 2 (provided with restriction enzyme from NEB), 1 µL of BSA (10 mg/mL, provided with XbaI restriction enzymes), 10 µL of pPIC9 Cal B plasmid DNA, 1 µL of SphI restriction enzyme (20 U/µL) and 1 µL of XbaI restriction enzyme (20 U/µL). The reaction mixture was incubated at 37 °C for 1.75 hours and was analysed on a 0.8% (w/v) agarose gel containing SYBR Safe DNA gel stain 10,000x concentrate in DMSO.

General procedure for ethanol precipitation for sequencing

To 1 µg of plasmid DNA, 10 µL of 10 M ammonium acetate and 125 µL of 100% pre-chilled ethanol were added. The sample was submitted for centrifugation for 15 minutes at 13,000 rpm. The supernatant was carefully pipetted off and 150 µL of 70% ethanol was
added before it was centrifuged again for 15 minutes at 13,000 rpm. The supernatant was carefully removed again and the sample was air dried for 1 hour.

**PCR of the Cal B gene using designer primers for sub-cloning into pET 16b and Champion™ pET SUMO vectors**

To a mini eppendorf tube 5 µL of 10x Taq buffer, 5 µL of DMSO, 34 µL of dH₂O, 1 µL of dNTP mix (10 mM), 1 µL DNA template (50 ng/µL), 3 µL of a mix of the forward and backward primers (100 µM) and 1 µL of Taq polymerase (20 U) were added (Table 5.1). Five samples of each of the two vectors in the reaction mixture were prepared and they were placed in a thermocycler PCR machine with the conditions set out below (Table 5.2). The PCR reaction was analysed on a 0.8% agarose gel containing SYBR Safe DNA gel stain 10,000x concentrate in DMSO. The PCR product was then purified using a QIAquick PCR purification kit (Qiagen PCR purification kit) as according to the manual.

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**Table 5.1:** Primers for cloning into the vectors Champion™ pET SUMO and pET 16b.

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</table>

**Table 5.2:** PCR reaction conditions.
Double digestion of the amplified Cal B gene and pET 16b vector

To 1 µg of each of the purified insert and vector, 5 µL of 10x Buffer 4 (provided by NEB with the restriction enzymes), 1 µL of NdeI (20 U/µL), 1 µL of XhoI (20 U/µL), 0.5 µL of BSA (10 mg/mL) and 26.5 µL of dH2O were added. The reaction mixtures were incubated at 37 °C for 3 hours. An analysis gel (0.8% agarose gel) was run to monitor the reaction which was judged to be complete after 3 hours. The reaction mixtures were then run on a 0.8% agarose gel and extracted under a blue light transilluminator and purified using a QIAquick Gel Extraction Kit according to the manual.

Sub-cloning the Cal B gene into pET 16b vector

Two ligation reactions were set up with a 1:1 ratio of insert to vector and 3:1 ratio of insert to vector. The 1:1 insert to vector reaction mixture contained 1 µL of insert (4 µg/mL), 8.3 µL of pET 16b vector (3 µg/mL), 2 µL of T4 buffer, 5.7 µL of ultrapure water and 1 µL of T4 ligase (20 U/µL). The 3:1 insert to vector reaction mixture contained 3 µL of insert (4 µg/mL), 8.3 µL of pET 16b vector (3 µg/mL), 2 µL of T4 buffer, 5.7 µL of ultrapure water and 1 µL of T4 ligase (20 U/µL). Both reaction mixtures were incubated at 4 °C for 4 days.

Transformation of One Shot® BL21 competent cells with pET 16b Cal B

Two vials of One Shot® BL21 competent cells (50 µL) were thawed on ice prior to addition of 1 µL of each of the ligation reactions (1:1 and 3:1 ratio of insert to vector). The vials
were incubated on ice for 30 minutes and then at 42°C, in a water bath, for 45 seconds to heat shock the competent cells into taking up the DNA. The vials were removed from the 42°C water bath and immediately placed into ice. To the vials, 250 µL of pre-warmed SOC medium was added before they were incubated at 37°C for 1 hour at 225 rpm. Two volumes of the transformation reaction were plated, 50 µL and 100 µL, onto LB amp plates. The plates were inverted and incubated at 37°C for 18 hours.

**Sub-cloning of the Cal B gene into Champion™ pET SUMO**

Two ligation reactions were set up with a 1:1 ratio of insert to vector and 3:1 ratio of insert to vector. The 1:1 insert to vector reaction mixture contained 1 µL of insert (11 µg/mL), 2 µL of Champion™ pET SUMO vector (25 µg/mL), 1 µL of T4 buffer, 5 µL of ultrapure water and 1 µL of T4 ligase (20 U/µL). The 3:1 insert to vector reaction mixture contained 3 µL of insert (11 µg/mL), 2 µL of Champion™ pET SUMO vector (25 µg/mL), 1 µL of T4 buffer, 5 µL of ultrapure water and 1 µL of T4 ligase (20 U/µL). Both reaction mixtures were incubated at 4°C for 18 hours.
Transformation of the pET SUMO Cal B construct into Mach1 T1-R competent cells for propagation

Two vials of One Shot® Mach1 T1-R competent cells (50 µL) were thawed on ice prior to addition of 2 µL of each of the ligation reactions (1:1 and 3:1 ratio of insert to vector). The vials were incubated on ice for 30 minutes and then at 42°C, in a water bath, for 45 seconds to heat shock the competent cells into taking up the DNA. The vials were removed from the 42°C water bath and immediately placed into ice. To the vials, 250 µL of pre-warmed SOC medium was added before they were incubated at 37°C for 1 hour at 225 rpm. Two volumes of the transformation reaction were plated, 50 µL and 100 µL, onto LB kan plates. The plates were inverted and incubated at 37°C for 18 hours.

Restriction digest analysis to confirm the orientation of the gene in pET 16b Cal B and pET SUMO Cal B plasmid DNA

Two restriction digest analysis reactions were set up. One eppendorf tube contained 14 µL of dH₂O, 3 µL of BamHI buffer (provided with restriction enzyme from NEB), 1 µL of BSA (10 mg/mL, provided with appropriate restriction enzymes), 10 µL of pET SUMO Cal B plasmid DNA, 1 µL of SphI restriction enzyme (20 U/µL) and 1 µL of BamHI (20 U/µL) restriction enzyme. The second eppendorf tube consisted of 15 µL of dH₂O, 3 µL of Buffer 2 (provided with restriction enzyme from NEB), 10 µL of pET 16b Cal B plasmid DNA, 1 µL of MlnI restriction enzyme (20 U/µL) and 1 µL of SacII restriction enzyme (20 U/µL). The reaction mixtures were incubated at 37°C for 3 hours. The reactions were analysed on a 0.8% (w/v) agarose gel containing SYBR Safe DNA gel stain 10,000x concentrate in DMSO.

Transformation of One Shot® BL21 competent cells with pET SUMO Cal B

A vial of One Shot® BL21 competent cells (50 µL) was thawed on ice prior to addition of 1 µL pET SUMO Cal B plasmid DNA (42 µg/mL). The vial was incubated on ice for 30 minutes and then at 42°C, in a water bath, for 45 seconds to heat shock the competent cells into taking up the DNA. The vial was removed from the 42°C water bath and immediately placed into ice. To the vial, 250 µL of pre-warmed SOC medium was added before it was incubated at 37°C for 1 hour at 225 rpm. Two volumes of the transformation reaction were
plated, 75 µL and 150 µL, onto LB kan plates. The plates were inverted and incubated at 37°C for 18 hours.

### 5.3.3 Protein production

**Transformation of Origami 2 competent cells with pET 16b Cal B**

A vial of Origami 2 competent cells (200 µL) was thawed on ice prior to sub-dividing into aliquots (20 µL). To a thawed aliquot of Origami 2 competent cells 1 µL of pET 16b Cal B was added. The vials were incubated on ice for 5 minutes and then at 42°C, in a water bath, for 30 seconds to heat shock the competent cells into taking up the DNA. The vials were removed from the 42°C water bath and immediately placed into ice for 3 minutes. To the vials, 80 µL of pre-warmed SOC medium was added before they were incubated at 37°C for 1 h at 250 rpm. Two volumes of the transformation reaction were plated, 20 µL and 50 µL, onto LB amp plates with volumes less than 25 µL being added to a pool of SOC medium (50 µL) before spreading. The plates were incubated at rt for 15 minutes before inverting and incubating at 37°C for 24 h.

**General procedure for overnight cultures of successful transformation colonies**

A transformant was picked from the LB agar plates containing the appropriate antibiotic and inoculated into 5 mL aliquots of LB containing the same antibiotic to select for your expression plasmid. The overnight cultures were incubated at 37°C for 18 h for BL21(DE3) *E. coli* and for 24 h for Origami 2(DE3) *E. coli* at 250 rpm.

**Expression of pET 16b Cal B in Origami 2 *E. coli***

LB amp (500 mL) was inoculated with the overnight culture (5 mL). The culture was allowed to grow to an OD₆₀₀ of 0.6 in a 37 °C shaking (250 rpm) incubator. Once the cells had grown to the correct optical density, IPTG (1 M, 500 µL) was added and the cells were incubated at 16 °C for 24 h. The cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min.
Expression of pET 16b Cal B in BL21 E. coli

LB amp (500 mL) was inoculated with the overnight culture (5 mL). The culture was allowed to grow to an OD$_{600}$ of 0.6 in a 37 °C shaking (250 rpm) incubator. Once the cells had grown to the correct optical density, the cells were incubated at 30 °C or 16 °C for 18 h or 24 h. The cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min.

General procedure for the chemical lysis of bacterial cell pellets

The bacterial cell pellet (1 g) was resuspended in BugBuster protein extraction reagent (5 mL). To the solution, protease inhibitor cocktail (P8849, 50 µL), purchased from Sigma-Aldrich Company Ltd., and lysonase (71230-4, 10 µL), purchased from Merck Biosciences Ltd., were added and the reaction mixture was allowed to mix at rt for 20 minutes before centrifuging at 20,000 rpm and 4 °C for 20 minutes. The cell free extract was decanted from the cell pellet.

General procedure for the mechanical lysis of bacterial cell pellets

The bacterial cell pellet (1 g) was resuspended in potassium phosphate buffer (pH 7.4, 0.1 M, 5 mL) before addition of protein inhibitor cocktail (P8849, 50 µL), purchased from Sigma-Aldrich Company Ltd., and lysozyme (L6876, 10 µL of 10 mg in 1mL of H$_2$O). The reaction was incubated at 37 °C for 20 minutes before incubating in ice and sonicating for 15 seconds on, 15 seconds off for 3 cycles.

General procedure for SDS-PAGE and Western blot

To 2x Laemmli sample buffer (S3401, 20 µL), sample (20 µL) was added. The reaction was heated at 95 °C for 5 minutes before applying onto a Criterion Tris-HCl precast gel (Bio-Rad Laboratories UK). The gel was run at 150 mA for 80 minutes.

For a Western blot, the SDS-gel and 2 sheets of blotting paper were incubated in cold Towbin buffer for 5 minutes. The PVDF membrane was first incubated in methanol for 5 minutes before incubating in Towbin buffer for 5 minutes. The materials were arranged in the blotter as follows: blotting paper, PVDF membrane, SDS-gel and blotting paper. The
air bubbles were rolled out of the stack and excess liquid removed before blotting at 150 mA for 70 minutes. After blotting, the SDS-gel was stained for 15 minutes at rt. The PVDF membrane was incubated in blocking buffer overnight at 4 °C. The membrane was then washed 3 times with PBS with Tween 20 and then once with PBS. The membrane was then incubated in blocking buffer with monoclonal Anti-polyHistidine–Peroxidase antibody for 1.5 h before washing again with PBS with Tween 20 3 times and once with PBS. The membrane was then incubated in SigmaFast 3,3'-diaminobenzidine solution for 5 minutes. The membrane was dried before handling.

**General procedure for protein purification on the ÄKTA Explorer 100 FPLC**

A HiTrap Chelating HP affinity column was washed with filtered dH2O (5 column volumes) at a “drop to drop” rate then the metal ion solution (0.1 M, NiSO4 or CuSO4, 0.5 mL) was applied. The column was equilibrated with water (5 column volumes) and then the syringe filtered cell free extract was applied. The column was then attached to the equilibrated ÄKTA Explorer 100 FPLC and the method run. The fractions (1 mL) were collected in a 96 well plate and stored at 4 °C.

To concentrate and to desalt a sample for use in the pNPA assay, the fractions containing Cal B were combined before concentrating to 3 mL in a Vivaspin column with a 30,000 molecular weight cut-off. The concentrated sample was then applied to a prepared PD10 column. The protein was eluted off the PD10 column with potassium phosphate buffer (pH 7.4, 0.05 M, 3 mL) and used in the pNPA assay and BCA assay.

**5.3.4 Testing the activity of recombinant Cal B**

**General procedure for the esterification of 2-bromopropionic acid and 2-fluoropropionic acid using recombinant Cal B**

A stock solution was prepared containing iso-hexane (2 mL per reaction), substrate (5g per L) and n-butanol (5 equiv.). The stock solution was distributed equally into 7 mL glass vials with screw on caps containing a single molecular sieve (4 Å, 10 – 18 mesh) per reaction. The biotransformations were started by addition of recombinant Cal B (10 or 100
μL of cell free extract) and the reactions were incubated at 30°C and shaken at 225 rpm for the length of the biotransformation.

**General procedure for the pNPA assay**

To each well enzyme preparation (5 µL) was added into potassium phosphate buffer (pH 7.4, 0.05 M, 185 µL). The pNPA substrate (0-80 mM, 10 µL) was added prior to monitoring at 405 nm for 200 cycles.

**5.3.5 High throughput enantioselective screen development**

**Synthesis of 4-nitrophenyl 2-bromopionate 118**

![Chemical structure of 4-nitrophenyl 2-bromopionate](attachment:structure.png)

2-Bromopropionyl chloride (5 mL, 49.6 mmol) was added dropwise to a vigorously stirred solution of sodium 4-nitrophenoxide (7.26 g, 45.1 mmol) in 1 N sodium hydroxide (54.2 mL, 54.2 mmol) and water (300 mL) cooled in an ice bath. The mixture was allowed to stir in the ice bath for 5 minutes and the precipitate was collected under vacuum filtration and washed with water. The title compound 118 (1.87 g, 14%) was obtained by crystallisation from cyclohexane as a white solid (m.p. 41-43 °C). $^1$H NMR (400 MHz, 298 k, CDCl$_3$): $\delta$ = 1.41 (3H, d, J = 6.8), 4.1 (1H, q, J = 6.8), 7.6 (2H, C3H, dd), 8.2 (2H, C4H, dd).

**5.3.6 Characterisation of the different preparations of Cal B**

**General procedure for the pNPB assay**

To each well enzyme preparation (5 µL) was added into potassium phosphate buffer (pH 7.4, 0.05 M, 185 µL). The pNPB substrate (0-80 mM, 10 µL) was added prior to monitoring at 405 nm for 200 cycles.
5.3.7 Molecular modelling

All modelling studies were prepared using Accelrys Discovery Studio 2.0. Substrates, 2-bromopropionic acid, 2-fluoropropionic acid and ethyl-2-bromopropionate, were docked into the active site of wild-type Cal B using Accelrys Discovery Studio 2.0 software. The docking studies were performed with the help of Dr. K. Bailey and Dr. S. Willies.

5.3.8 Genetic modification

Protein engineering of Cal B

The gene encoding Cal B, within a pET 16b expression plasmid, was subjected to mutagenesis of targeted amino acids (Thr40, Asp134, Thr138, Gln157, Ile189, Val190, Leu278 and Ile 285). The saturation mutagenesis was prepared using the Stratagene QuikChange® Site-directed Mutagenesis Kit, according to the manufacturer’s instructions and the forward and backward primers were designed using the QuikChange® Primer Design Program and adhering to the rules specified in the Stratagene QuikChange® Site-directed Mutagenesis Kit manual.

To a mini eppendorf tube 5 µL of 10x Taq buffer, 1 µL forward primer (125 ng/µL), 1 µL backward primer (125 ng/µL), 40 µL of dH2O, 1 µL of dNTP mix (10 mM), 1 µL DNA template (50 ng/µL), and 1 µL of PfuTurbo DNA polymerase (2.5 U/µL) were added. Each of the PCR reactions was prepared and they were placed in a thermocycler PCR machine with the conditions set out below (Table 5.23).

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature °C</th>
<th>Time/ mins</th>
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<tr>
<td>1</td>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
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<td>3</td>
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<td>1</td>
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<tr>
<td>5</td>
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<td>10</td>
</tr>
<tr>
<td>6</td>
<td>4</td>
<td>hold</td>
</tr>
</tbody>
</table>

Table 5.3: PCR reaction conditions.
After PCR amplification, the DpnI restriction enzyme (20 U/μL) was added to digest the parental supercoiled dsDNA at 37 °C for 1 h, before inactivation of the restriction enzyme at 80 °C for 20 mins. The DNA containing the mutations were then used to transform *E. coli* XL1-Blue supercompetent cells. Transformants were then plated out on LB amp plates and individual colonies were picked and used to inoculate LB amp (5 mL of LB amp). The cultures were grown overnight at 37 °C before harvesting the cells by centrifugation. The bacterial cell pellet was then purified using a Qiagen Plasmid Prep Kit as according to the manual. The DNA was then sent for sequencing (Appendix 8 – Sequencing primers).

DNA samples containing the correct mutation were used to transform *E. coli* Origami 2 competent cells. Transformants were then plated out on LB amp plates and individual colonies were picked and used to inoculate LB amp (5 mL of LB amp). The cultures were grown overnight at 37 °C before being used to inoculate fresh media for production of protein. The cultures were grown at 37 °C to an OD$_{600}$ of ~ 0.6 before addition of IPTG (1 M, 500 µL) and incubation of the cells at 16 °C for 24 h. The cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 minutes before mechanical lysing. The cell free extract was used directly for biotransformation reactions.

The substrate, ethyl-2-bromopropionate **123** (5 g/L), was added to potassium phosphate buffer (0.1 M, pH 7.0, 4 mL) containing ethanol (0.36 v/v%). The enzyme was added to start the reaction; biocatalysis was carried out at 30 °C and 250 rpm in a thermoshaker. Samples (100 µL) were taken at various time points and subjected to denaturisation at 95 °C for 5 mins and then centrifugation. The supernatant was analysed by reverse phase chiral HPLC.
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Appendices

Appendix 1  Rate of conversion for 2-bromopropionic acid

Equation 1  Conversion = \( \frac{A_{\text{prod}}/\epsilon}{(A_{\text{sub}}/\epsilon) + (A_{\text{prod}}/\epsilon)} \)

where:
- \( \epsilon \) is the molar extinction coefficient of 2-bromopropionic acid
- \( A_{\text{prod}} \) is the total area under product peaks
- \( A_{\text{sub}} \) is the total area under substrate peaks

Appendix 2  Enantiomeric excess

Equation 2

\[
\text{ee} = \frac{\text{conc. of the (S)-enantiomer} - \text{conc. of the (R)-enantiomer}}{\text{conc. of the (S)-enantiomer} + \text{conc. of the (R)-enantiomer}}
\]

Appendix 3  Selectivity E for kinetic resolution calculated from the enantiomeric excess of the substrate

Equation 3  \( E = \ln[(1-c)(1-\text{ee}(S))) \div \ln[(1-c)(1+\text{ee}(S))] \)

where:
- \( c \) is the conversion
- \( \text{ee}(S) \) is the enantiomeric excess of the substrate
Appendix 4  Rate of conversion for 2-fluoropropionic acid

Equation 4  Conversion = (([blank]/ε)-(substrate)/ε)) ÷ ([blank]/ε)

where:
ε is the extinction coefficient of 2-fluoropropionic acid
(substrate) is the concentration of 2-fluoropropionic acid in mg/mL
(blank) is the concentration of 2-fluoropropionic acid in mg/mL in the biotransformation reaction with no enzyme

Appendix 5  ng of insert to vector to use

Equation 5

Ng of insert = ng of vector × (kb of insert / kb of vector) × ratio of insert to vector

Appendix 6  Amino acids

Smaller amino acids

H2N CO2H
H2N
H

Glycine (Gly, G) Alanine (Ala, A)

OH
H2N CO2H
H2N
OH

Serine (Ser, S) Threonine (Thr, T)

SH
H2N CO2H
H2N
SH

Cysteine (Cys, C)

Hydrophobic amino acids

H2N CO2H
H2N

Valine (Val, V) Leucine (Leu, L) Isoleucine (Ile, I)

CO2H
H2N

Methionine (Met, M) Proline (Pro, P)

Aromatic amino acids

H2N CO2H
H2N

Phenylalanine (Phe, F) Tyrosine (Tyr, Y) Tryptophan (Trp, W)
Appendix 7  Selectivity E for kinetic resolution calculated from the enantiomeric excess of the product

Equation 6  \[ E_R = \frac{\ln[(1-c)(1+\text{ee}(P))] \div \ln[(1-c)(1-\text{ee}(P))]} \]

where:
c is the conversion
\text{ee}(P) is the enantiomeric excess of the product

Appendix 8  Primers for sequencing mutants of Cal B

<table>
<thead>
<tr>
<th>Position</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thr40</td>
<td>T7 forward standard primers</td>
</tr>
<tr>
<td>Asp134, Thr138 and Gln157</td>
<td>GCGCAGCTGGGTACACACC</td>
</tr>
<tr>
<td>Ile 189 and Val190</td>
<td>GTTGCACAGTGGGTCTGACC</td>
</tr>
<tr>
<td>Leu278 and Ile 285</td>
<td>T7 reverse standard primers</td>
</tr>
</tbody>
</table>