BIOTRANSFORMATIONS USING NITRILE HYDROLYSING ENZYMES FOR STEREOSELECTIVE ORGANIC SYNTHESIS

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By

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DECLARATION

I hereby certify that this material, is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text. It is based on research carried out within the department of Chemical and Life Science at Waterford Institute of Technology.

Signed: _____

Date: _____

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ABSTRACT

Nitrile hydrolysing enzymes have found wide use in the pharmaceutical industry for the production of fine chemicals. This work presents a strategy that facilitates the rapid identification of bacterial isolates demonstrating nitrile hydrolysing activity. The strategy incorporates toxicity, starvation and induction studies along with subsequent colorimetric screening for activity, further focusing the assessment towards the substrates of interest. This high-throughput strategy uses a 96 well plate system, and has enabled the rapid biocatalytic screening of 256 novel bacterial isolates towards *β*-hydroxynitriles. Results demonstrate the strategy's potential to rapidly assess a variety of *β*-hydroxynitriles including aliphatic, aromatic and dinitriles. A whole cell catalyst *Rhodococcus erythropolis* SET1 was identified and found to catalyse the hydrolysis of 3-hydroxybutyronitrile with remarkably high enantioselectivity under mild conditions, to afford (*S*)-3-hydroxybutyric acid in 42% yield and >99.9% ee. The biocatalytic capability of this strain including the variation of parameters such as temperature and time were further investigated and all results indicate the presence of a highly enantioselective if not enantiospecific nitrilase enzyme within the microbial whole cell.

We present substrate evaluation with 34 chiral nitriles of *Rhodococcus erythropolis* SET1. These substrates consist primarily of *β*-hydroxy nitriles with varying alkyl and aryl groups at the *β*-position containing in some cases, various substituents at the *α*-position. In the case of *β*-hydroxy nitriles unsubstituted at the *α*-position, acids were the major products as a result of suspected nitrilase activity of the isolate. Unexpectedly, amides were found to be the major products when *β*-hydroxynitriles were substituted at the α-position with a vinyl group. Therefore this novel isolate has demonstrated additional NHase behaviour which is dependent on the functionality at the *α*-position. In order to probe this mechanism further related substrates were evaluated and amide was observed where other electron withdrawing groups were present at the *α*-position. Additionally various parameters which may influence the biocatalytic hydrolysis by SET1 were studied and are presented herein.

ABBREVIATIONS

Ar	Aryl
t-Bu	*tert*-Butyl
Bz	Benzyl
COSY	Correlated Spectroscopy
DABCO	1,4-diazobicyclo[2.2.2.]octane
DEPT	Distortionless Enhancement by Polarization Transfer
DMAP	4-Dimethylaminopyridine
DMF	*N*,*N*-Dimethylformamide
DMF-DEA	*N*,*N*-Dimethylformamide diethylacetal
DMF-DMA	*N*,*N*-Dimethylformamide dimethylacetal
EDCI	1-Ethyl-3-(3-dimethylaminopropyl) carbodimide
ee	Enantiomeric excess
ESI	electrospray ionization
Et	Ethyl
HPLC	High performance liquid chromatography
LC-MS	Liquid chromatography- mass spectrometry
HMQC	Heteronuclear Multiple-Quantum Coherence
Me	Methyl
MTBE	Methyl *tert*-butyl ether
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
Ph	Phenyl
rt	Room temeprature
TBDMS	*tert*-butyldimethylsilyl
TFA	Trifluoroacteic acid
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TMS	Trimethylsilyl

CONTENTS

CHAPTER 1

NITRILE HYDROLYSING ENZYMES FOR STEREOSELECTIVE ORGANIC SYNTHESIS

NITRILE HYDROLYSING ENZYMES FOR STEREOSELECTIVE ORGANIC SYNTHESIS

1.1 INTRODUCTION

Pharmaceutical research and manufacturing is increasingly turning to biotechnology for more efficient synthesis of complex compounds. Biocatalysis is rapidly evolving in organic synthesis, the historic backbone of the pharmaceutical industry, offering advantages in green-technology and stereoselective control [1]. The use of enzymes in organic reactions can negate the need for expensive reagents and offers a cost-effective alternative to the pharmaceutical industry. Biocatalysts often use milder reaction conditions than chemical synthetic methods reducing the need for hazardous reagents and the production of hazardous waste by-products [2]. Reactions can be carried out in aqueous media minimising the use of organic solvents and may be carried out at lower temperatures, under ambient atmosphere, generating safer processes [3]. In addition, in a large synthetic sequence less chemical steps (protection/deprotection) are often required. Therefore the 'atom economy'; a measure of the incorporation of the reactants used in the final product of a chemical reaction, can be increased [4].

As many drug molecules are chiral, stereoselectivity is increasingly important in the pharmaceutical sector due to regulatory requirements by authorities such as the FDA. Single enantiomer chiral drugs are typically difficult and expensive to manufacture and there is a need for new efficient synthetic methods. One of the major advantages of biocatalysts is that they often possess excellent regioselective and stereoselective properties generating enantiopure products difficult to achieve using traditional chemical methods, resulting in products of increased value [5].

The focus of Chapter 2 of this thesis lies in the development of a high-throughput screening strategy for the rapid identification of active nitrile hydrolysing enzymes towards of a range of β-hydroxynitriles, including aliphatic, aromatic and dinitriles.

The factors that influence enzyme activity, including bioconversion conditions such as; temperature and pH, the effect of metal ions on activity and the potential use of organic solvents are discussed in Chapter 3. This Chapter also includes an overview of the various chromatographic techniques used to attempt to purify and characterize the desired enzyme from the bacterial whole cells.

Chapter 4 examines the substrate scope of the isolate *R. erythropolis* SET1 identified using the high-throughput screening strategy. A variety of structurally diverse nitrile analogues were prepared and examined to investigate the effect of various substituents on the reaction activity and enantioselectivity.

This review chapter will present a brief history of biotransformations including successful industrial examples. The drawbacks of traditional asymmetric methods for the production of chiral molecules will also be discussed. Some key findings in the area of nitrile hydrolysing enzymes will be presented including a review of various substrates and strategies for enhancing enantioselectivity, to place context to the work presented in later Chapters.

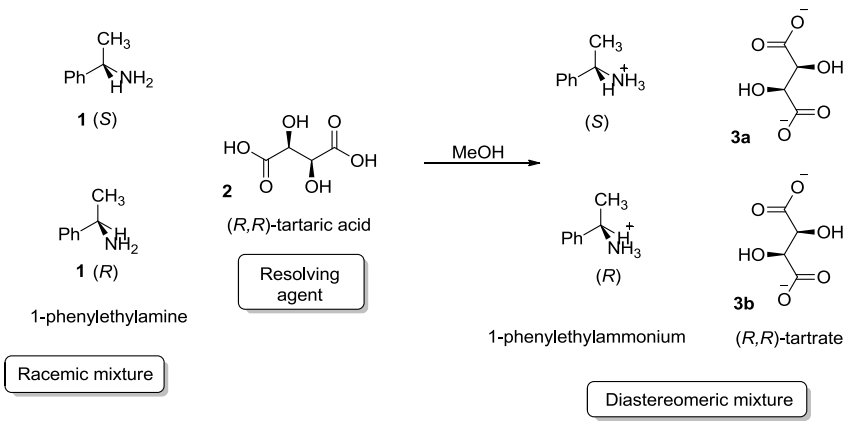
1.2 ASYMMETRIC SYNTHESIS FOR THE PRODUCTION OF ENANTIOPURE COMPOUNDS

A major challenge for the synthesis of chiral pharmaceutical compounds is the generation of products as single enantiomers [3]. The presence of an undesirable enantiomer can lead to an increase in the dose required and can often also led to adverse side effects, such as with thalidomide [6].

This section will provide a brief overview of traditional methods for the production of enantiomerically pure pharmaceutical actives and intermediates. It will focus on classical resolution of racemic mixtures and various methods for the production of pure enantiomers including the use of chiral reagents, chiral auxillaries and chiral catalysts.

1.2.1 RESOLUTION OF RACEMIC MIXTURES TO GENERATE SINGLE ENANTIOMERS

The resolution of racemic mixtures necessitates the presence of a chiral environment. The classical method to resolve a racemate is to react the mixture of enantiomers with an enantiomerically pure compound as shown in Scheme 1.1 [6]. The resulting diastereomeric products may be separated based on their physical properties by crystallization or chromatography. The separated diastereomers may then be converted back to the enantiomers of the original compound by cleaving the resolving agent [6].

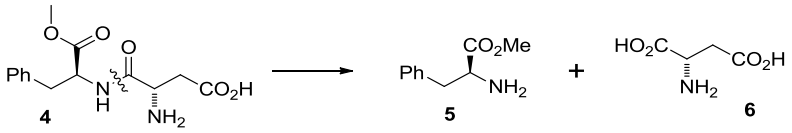


Scheme 1.1: Resolution of 1-phenylethylamine using (*R,R*)-tartaric acid as a resolving agent.

For example, the racemic amine **1** may be separated using (L)-(+)-tartaric acid **2** (also known as the (*R,R*)-form) as the resolving agent. The two salts formed *(S)*-(1)-phenylethyl ammonium-(*R,R*)-tartrate **3a** and *(R)*-(1)-phenylethyl ammonium-(*R,R*)-tartrate **3b** may be separated by crystallisation. The free amine can be recovered by reaction of the recovered ammonium salt with a strong base i.e., sodium hydroxide or sodium carbonate and extraction into organic solvent. One disadvantage of chiral resolution of racemates compared to direct asymmetric synthesis of one of the enantiomers is that only 50 % of the desired enantiomer is obtained. While some naturally occurring resolving agents such as L-tartaric acid are readily available, the opposite enantiomer may be expensive and difficult to obtain.

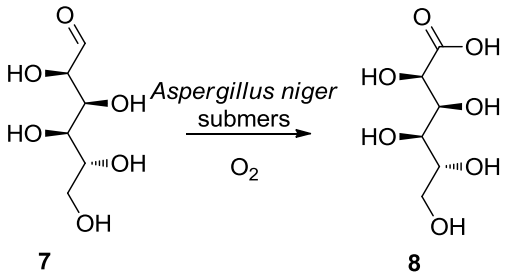
1.2.2 ASYMMETRIC SYNTHESIS USING THE CHIRAL POOL

This strategy relies on finding a suitable enantiomerically pure natural product – a member of the "chiral pool" that can easily be transformed into the target molecule. The "chiral pool" is a collection of cheap, readily available pure natural products usually amino acids or sugars, containing the desired chiral centres which can be taken and incorporated into the product [7]. An example of such a method is the synthesis of aspartame **4**, a dipeptide which starts with two members of the chiral pool, the constituent (*S*)-amino acids, phenylalanine **5** and aspartic acid **6** as shown in Scheme 1.2.



Scheme 1.2: Production of aspartame from *(S)*-phenylalanine and *(S)*-aspartic acid.

Natural chiral building blocks have also been used for the production of enantiomerically pure sugar acids. In industrial synthesis sacharides can be oxidized selectively. Oxidations with hypochlorite [8] and electrochemical oxidisations [9] have been developed for the production of D-gluconic acid **8** from D-glucose **7**. These have been replaced by biotechnological processes in which the monosaccharide is selectively oxidised with microorganisms (mainly *Aspergillus niger* and *Gluconobacter*) in a submersed aerobic process as shown in Scheme 1.3 [10].

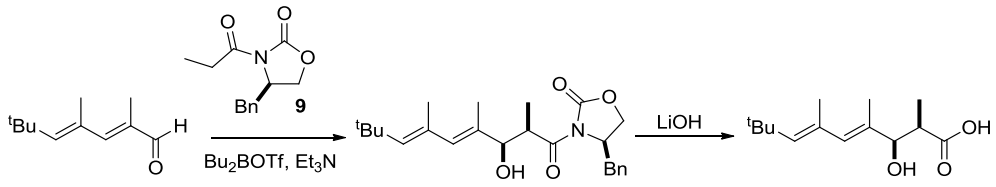


Scheme 1.3: Selective enzymatic oxidation of D-glucose to D-gluconic acid.

However in the cases of the use of the chiral pool, the number of possible reactions the molecule can undergo is restricted and difficult synthetic routes may be required. This approach also requires a stochiometric amount of the enantiopure starting material which may be expensive, limiting its synthetic applications.

1.2.3 CHIRAL AUXILIARIES TO INFLUENCE REACTION SELECTIVITY

Another common approach to selectively synthesise a single enantiomer compound is to employ a chiral auxiliary. A chiral portion is temporarily incorporated into a reaction to control the stereochemical outcome. In the first step, the auxiliary is covalently coupled to the substrate; the resulting compound undergoes a diastereoselective transformation and the auxiliary is then removed without racemisation of the product [11]. The Evans oxazolidone chiral auxiliary **9** has been used to control the face-selectivity in enolate reactions as shown in Scheme 1.4.



Scheme 1.4: Evans chiral auxiliary aldol condensation used in the synthesis of an antillatoxin isomer.

The strategy does suffer from the disadvantage that two extra steps are required to add and remove the chiral auxiliary. In addition a full equivalent of the chiral auxiliary is required.

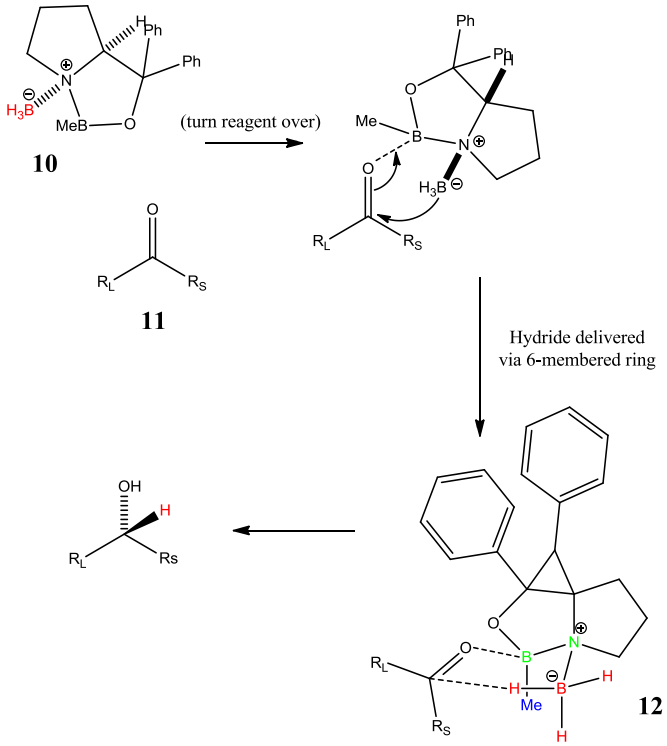
1.2.4 CHIRAL REAGENTS IN ASYMMETRIC SYNTHESIS

Another chiral influence which can be employed in the generation of a chiral centre is the use of a chiral reagent or catalyst [7]. In the case of carbonyl reduction, chiral reducing agents are often used. Here the reagent exerts the chiral effect.

Chiral borohydride analogues developed by Corey, Bakshi and Shibita [12], are effective reagents for the enantioselective reduction of ketones. The reagent is based upon a stable boron heterocycle **10** made from an amino alcohol derived from proline (Scheme 1.5), and is known as the CBS reagent after its developers. Such CBS reductions are most efficient when the ketones two substituents are well-differentiated sterically.

The mechanism involves complexation of the ketone **11** with the electrophilic boron atom, which activates BH_3 as the hydride donor. The boron of the catalyst coordinates to the ketone at the electron lone pair of the smaller substituent. This preferential binding minimizes steric interactions and aligns the carbonyl for a favorable, face selective hydride transfer through the six membered transition state **12** (Scheme 1.5) [7].

The disadvantage of utilising chiral reagents for asymmetric synthesis is that the reaction requires a stochiometric amount of the chiral material, in addition to the various parameters which need to be controlled in order to obtain the desired enantioselectivity. The CBS reagent must be used under anhydrous conditions as water has a significant effect on enantiomeric excess.



Scheme 1.5: Reduction of a ketone by CBS reagent developed by Corey, Bakshi and Shibita.

1.2.5 CHIRAL CATALYSTS IN ASYMMETRIC SYNTHESIS

Chiral inorganic catalysts have been investigated in the synthesis of enantiomerically pure compounds. The main disadvantage to this strategy is that the catalysts often involve a difficult synthesis and have toxicity issues especially when considering the use of heavy metals in the manufacture of pharmaceuticals. In addition, high enantioselectivity is often difficult to achieve [13].

An example of the use of a chiral catalyst for the synthesis of single enantiomer drugs is the catalytic asymmetric hydrogenation of alkenes [14]. Here the catalyst selects a single enantiotopic face of a double bond and selectively adds hydrogen across it. Noyori demonstrated that well defined, mononuclear catalysts such as Ru-BINAP ((2,2'-bis(diphenylphosphino)-1,1'-binaphthyl)) had an incredible range of efficiency in this reaction [14]. The ligand BINAP **13** as shown in Figure 1.1, is a chelating diphosphine: the metal sits between the two phosphorous atoms. BINAP **13** itself does not contain a chiral centre; instead it possesses axial chirality by virtue of restricted rotation [7]. BINAP ruthenium is particularly efficient at catalysing the hydrogenation of allylic alcohols and α, β unsaturated carboxylic acids.

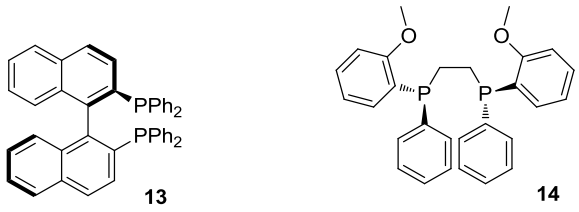
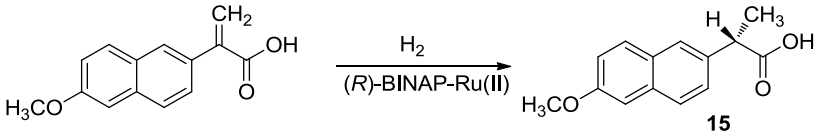


Figure 1.1: Structure of BINAP and DIPAMP chiral auxillary.

Additionally BINAP **13** has been used to synthesise (*S*)-naproxen **15**, the active ingredient in several over the-counter nonsteroidal anti-inflammatory drugs, in greater than 98 % enantiomeric excess (Scheme 1.6) [7].



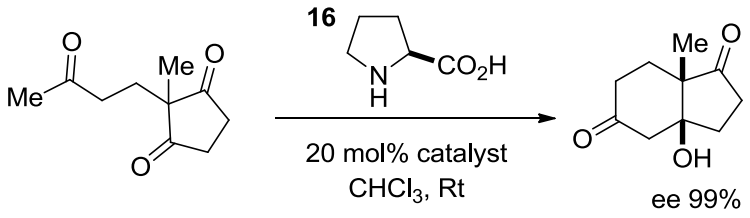
Scheme 1.6: *(R)*-BINAP catalysed synthesis of *(S)*-naproxen

If the double bond to be reduced also bears an amino group, a catalyst based on rhodium DIPAMP **14** (Figure 1.1) may also be used. The successful development of Rh-DIPAMP as a catalyst for L-DOPA with enantiomeric excess of up to 96 %, enabled Monsanto to be the primary supplier of the main drug used in stabilising the effects of Parkinson's disease for many years [13].

Several disadvantages associated with chiral catalysts include product inhibition due to binding of the reaction product with the catalyst. Metal catalysts may react with oxygen making the exclusion of air and moisture imperative, and they possess limited functional group and solvent tolerance. Due to the heavy metal content of the catalyst, issues may also arise during disposal. Additionally, the high cost involved makes catalyst recycling essential which is not always possible due to degradation.

1.2.6 CHIRAL ORGANOCATALYSTS IN ASYMMETRIC SYNTHESIS

Organocatalysis, or the use of small organic molecules to catalyse organic transformations, has received much attention over recent years [15]. The proline **16**-catalysed intramolecular aldol reaction as shown in Scheme 1.7 is an example of such an approach. In this case attachment of the catalyst **16** at the nucleophile with formation of an iminium ion occurs to influence the forming carbon-carbon bond. Central to the success of this aldol transformation is a proposed hydrogen bond between the carboxylic acid of proline and the carbonyl electrophile [15].



Scheme 1.7: Proline catalysed intramolecular aldol reaction as adapted from [15].

Organocatalysts have several advantages over metal based chiral catalysts. Generally organic molecules are insensitive to oxygen and moisture in the atmosphere. A wide variety of organic reagents- such as amino acids, carbohydrates and hydroxy acids- are available from biological sources as single enantiomers to prepare organocatalysts. Finally organic molecules are typically non-toxic and environmentally friendly [11].

In summary the advantages and disadvantages of the various chemical methods for producing optically active compounds as discussed above are outlined in Table 1.1.

Table 1.1: Summary of the advantages and disadvantages of traditional chemical based methods of asymmetric synthesis [16].

Method	Advantages	Disadvanteages	Examples
Resolution	Both enantiomers available	Maximum 50 % yield	Synthesis of BINAP
Chiral pool	100 % ee	Often only one enantiomer available	Amino acid- and sugar – derived synthesis
Chiral auxiliary	Often excellent ees; can recrystallise to purify to high ee	Extra steps to introduce and remove auxiliary	Oxazolidinones
Chiral reagent	Often excellent ees; can recrystallise to purify to high ee	Only a few reagents are successful and often for few substances	CBS reducing agent
Chiral catalyst (metal)	Economical: only small amounts of recyclable material used	Only a few reactions are successful: recrystallisation can improve high ees	Asymmetric hydrogenation, epoxidation, dihydroxylation.
Chiral organocatalyst	Low cost and toxicity, tolerant of moisture and oxygen, often excellent ee.	Limited substrate scope for a particular organocatalyst.	Proline, phenylalanine, binol, thiourea

1.3 ENZYMATIC REACTIONS IN ORGANIC SYNTHESIS

Enzymes can catalyse a broad range of transformations relevant to organic chemistry, including for example, redox reactions, carbon-carbon bond forming reactions, and hydrolytic reactions [1]. Enzymes are typically classified according to the types of reaction they influence. In enzyme nomenclature classification, they are subdivided and categorised into six main enzyme groups as outlined in Table 1.2 [17].

Table 1.2: Classification of enzymes according to the general types of reaction they catalyse as adapted from [18].

Enzyme class	EC number	Selected reactions
Oxidoreductases	1	Reduction of C=O and C=C; reductive amination of C=O; oxidation of C-H, C=C, C-N, and C-O; cofactor reduction/oxidation
Transferases	2	Transfer of functional groups such as amino, acyl, phosphoryl, methyl, glycosy, nitro, and sulphur containing groups
Hydrolases	3	Hydrolysis of esters, amides, lactones, lactams, epoxides, nitriles, and so on
Lysase	4	Addition of small molecules to double bonds such as C=C, C=N and C=O
Isomerase	5	Transformation of isomers (isomerisations) such as racemisations, epimerisations, and rearrangement reactions
Ligase	6	Formation of complex compounds (in analogy to lyases) but enzymatically active only when combined to ATP

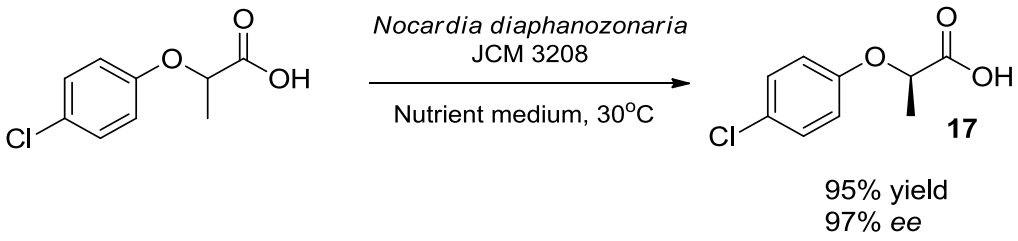
1.3.1 ENZYMATIC RESOLUTION OF RACEMIC MIXTURES

The unique functions of enzymes as catalytically active proteins are a result of their three-dimensional structures and active site [18]. This enables highly specific recognition of substrates, leading to excellent selectivity. Besides chemoselectivity they can also exhibit regioselectivity, diastereoselectivity as well as enantioselectivity during these reactions [17].

Enzymatic resolution is based on the ability of enzymes to distinguish between (*R*)- and (*S*)-enantiomers. The criterion for a successful enzymatic resolution is that one enantiomer is a preferred substrate for the enzyme, which leads to one enantiomer transforming preferentially. Generally the enantioselectivity is quite high as enzyme-catalysed reactions involve a specific fit of the substrate into the catalytic site [19]. The same necessity for a substrate fit however is the primary limitation on enzymatic resolution.

In principle a Dynamic Kinetic Resolution (DKR) can result in quantitative yields of a single enantiomer. DKR combines the resolution step of kinetic resolution with an *in situ* equilibration or racemisation of the chirally labile substrate. The racemisation step can be either enzyme (e.g. racemase) or non-enzyme (e.g. transition metal) catalysed [20].

Ohta *et al.* reported the microbial resolution of 2-aryl and 2-aryloxypropanoic acids using *Nocardia diaphanozonaria* JCM 3208 as demonstrated in Scheme 1.8. In both cases the (*R*)-enantiomer was preferentially obtained from the racemate. In optimal cases the acid **17** was isolated in yields of up to 95 % with an associated ee of 97 % [21].



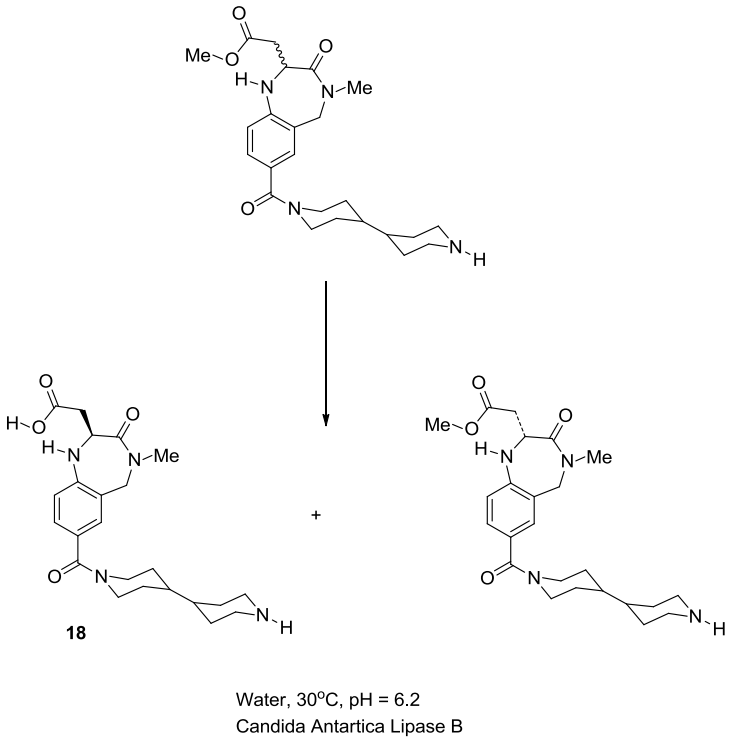
Scheme 1.8: Preparation of 2-(-4-chlorophenoxy)propanoic acid) by *Nocardia diaphanozonaria* JCM 3208. [21]

About two thirds of resolutions employ hydrolytic transformations involving ester and amides bonds using proteases, esterases or lipases [18]. Other hydrolases can cleave nitriles to form amides and carboxylic acids [1].

In nature, hydrolases display a digestive catalytic activity [1]. However, their catalytic behaviour is more heterogenous and they can be employed as biocatalysts for many organic transformations. Hydrolases, more particularly lipases, present advantages over other biocatalysts as they do not require co-factors for their catalytic behaviour; they are active not only in aqueous medium but also in organic solvents, allowing the transformation of non-water soluble compounds [22]. Furthermore many of them are commercially available and easy to handle biocatalysts; they commonly display low substrate specificity with high regio-, chemo- and enantioselectivity [23].

Lotrafiban **18**, is an orally active fibrinogen receptor antagonist designed for the prevention of thrombotic events and is produced by GlaxoSmithKline [24]. *Candida antartica* lipase B quickly hydrolysed the (*S*)-enantiomer in aqueous solution and was found to be extremely stereoselective (Scheme 1.9) [24]. Once all of the (*S*)-isomer of

the starting ester had been converted resulting in 50 % yield and 100 % ee, the reaction ceased and no hydrolysis of the (*R*)-enantiomer could be detected [24].



Scheme 1.9: Bioresolution to produce Lotrafiban 18 employing CALB [24].

Lipases have been successfully employed in resolutions to hydrolyse esters and amides, or for enantioselective transesterifications of secondary alcohols and amines [25]. Lipase from *C. Antartica* (CALB) as discussed above, shows great versatility in the field of biotransformations [25]. CALB shows very low substrate specificity and has been employed by Schering- Plough in the desymmetrisation of the prochiral diethyl 3-[3,4-dichlorophenyl]glutarate to the corresponding *(S)*-monoester **19** (Figure 1.2). This is a chiral intermediate for the synthesis of NK1/NK2 antagonists; compounds with potential activity in the treatment of asthma, arthritis and migraine. The enzymatic process has been scaled up to produce 200 kg on 80 % isolated yield and enantiomeric excess greater than 99 % [23, 26].

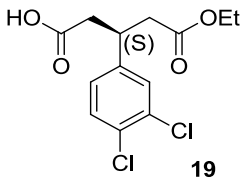
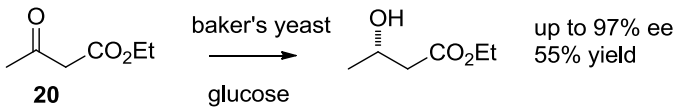


Figure 1.2: Chiral building block of NK1/NK2 dual antagonists produced by Schering-Plough using *Candida antartica* lipase B [23].

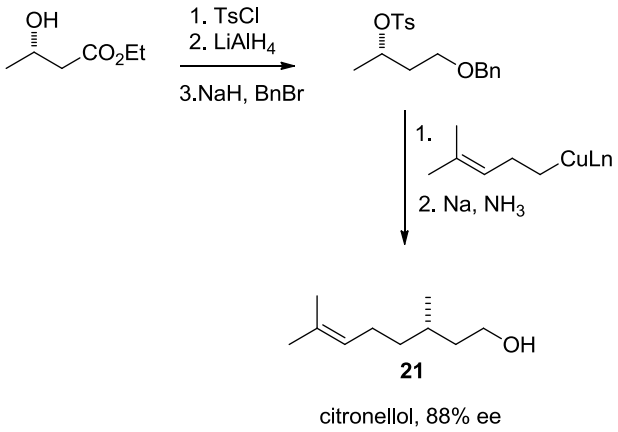
Chirality can also be introduced into a prochiral molecule in a single asymmetrisation step in some enzymatic reactions and may result in both 100 % yield and ee, if a selective enzyme is used. The use of redox enzymes for the production of chiral building blocks offers an attractive alternative from an environmental point of view compared with the use of chemical redox catalysts, usually containing transition metals [27]. Asymmetric reduction of prochiral ketones by enzymes is one of the most investigated methods to produce chiral compounds [28].

Reduction reactions performed with baker's yeast are often efficient reactions [28]. However if different oxidoreductases of the yeast compete for the same substrate the stereodifferentiation becomes moderate and difficult to predict. The best enantioselectivities are obtained when the ketone carries a *β*-ester group as shown in substrate **20** in Scheme 1.10 [29].



Scheme 1.10: Baker's yeast reduction of a *β*-keto ester.

An important application of this baker's yeast reduction is in the synthesis of citronellol **21** [30]. After reduction and protection of the ester formed in Scheme 1.10, S_N2 substitution of the secondary tosylate group could be achieved with inversion using a copper nucleophile. Citronellol **21** may be obtained using this method in 88 % ee, as shown in Scheme 1.11.



Scheme 1.11: Subsequent steps after reduction in the synthesis of citronellol 21 [30].

1.4 BIOTRANSFORMATIONS OF NITRILE SUBSTRATES

Nitriles are an important synthetic intermediate because of their easy preparation and versatile transformations. Chemical hydrolysis of nitriles is frequently applied in both academia and industry to produce carboxamides and carboxylic acids [31]. Nitrile groups can be introduced into molecules by reacting potassium cyanide with alkyl halides, and enzymatic conversion can lead to many synthetically useful products as seen in Figure 1.3

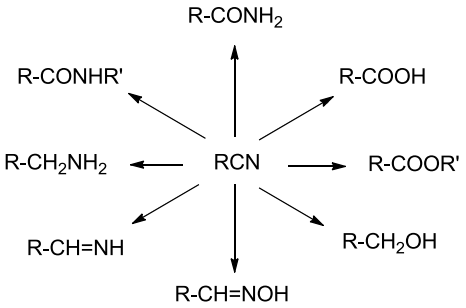


Figure 1.3: Structural types accessible through possible conversion of nitriles, R and R' represent alkyl or aryl groups.

1.4.1 TYPES OF NITRILE DEGRADING ENZYMATIC PATHWAYS

There are five possible enzymatic mechanisms for the degradation of cyanide and nitriles, as shown in Figure 1.4 [32].

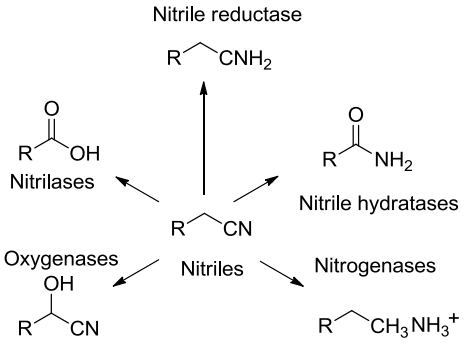
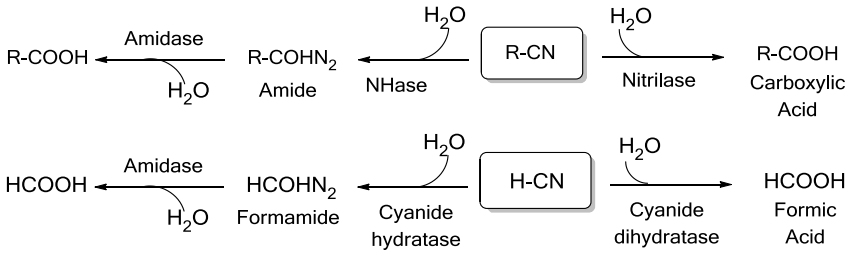


Figure 1.4: Enzymatic nitrile degrading pathways.

Nitriles may be degraded by the use of nitrilases, oxygenases, nitrile hydratases, nitrile reductase and nitrogenases. The oxidation of cyanide has been demonstrated by *Pseudomonas flourescans* [33], while nitriles have been hydrogenated by nitrogenase enzymes found in nitrogen fixing bacteria [34]. More recently nitrile reduction was reported with the nitrile reductase (QueF) found to be highly substrate specific [35]. Both nitrilases and nitrile hydratases are capable of hydrolysing nitriles to the

corresponding amide and acid. This pathway is most common for the microbial metabolism of nitriles and is well studied. As nitrile hydrolysis is the only mechanism of concern to this study, this mechanism will be discussed in further detail in the following sections.

Hydrolysis of nitriles by microbial isolates may proceed *via* two different enzymatic pathways as shown in Scheme 1.12. In the first route, nitrilase and cyanide dihydratase catalyse the direct hydrolysis of nitriles into the corresponding carboxylic acid and ammonia. In an alternative pathway nitrile hydratase may convert nitriles to the corresponding amide, which is then hydrolysed to the corresponding carboxylic acids and ammonia by an amidase enzyme [36]. While nitrilases hydrolyse larger nitriles (RCN), cyanide dihydratases and cyanide hydratases hydrolyse hydrogen cyanide (HCN) only.



Scheme 1.12: Enzymatic pathways for nitrile hydrolysis.

Microbial degradation *via* hydrolysis is considered as an effective way of removing highly toxic nitriles from the environment. A number of bacteria (*Acinetobacter, Corynebacterium, Arthrobacter, Norcadia* and *Rhodococcus* for example) are known to metabolise nitriles as a sole source of carbon and nitrogen [31]. Usually, the microbes contain either a nitrilase or a NHase-amidase system [37]. However, some microorganisms such as *R. rhodochrous* J1 [38] and *R. rhodochrous* PA–34 [39] contain both nitrilase and NHase-amidase systems. The three enzymes responsible for nitrile hydrolysis can be induced selectively by substitution of one of the nitriles or amides within the enzyme expression media [40]. In the following section NHase/ amidase will be briefly discussed before attention will be focused on nitrilase enzymes.

1.4.2 NITRILE HYDRATASE ENZYMES

Nitrile hydratase was first isolated from the bacterium *Arthrobacter* sp. J-1 by Asano *et al.* in 1982 [41, 42]. A number of microorganisms with NHase activity have since been isolated, purified and characterised [37]. These enzymes have shown a wide range of

physiochemical properties and substrate specificities. NHases are divided in two classes: ferric NHases and cobalt NHases, based on the type of metal ion incorporated into the active site. Metal ions may be incorporated into the active site as they are good catalysts for –CN hydratation, and they may also be required for protein folding [31]. Generally Fe-type NHases catalyse the hydration of aliphatic nitriles and Co-type catalyse the hydration of aromatic nitriles [43]. The genetic organisation of NHases consists of two usually separated NHase subunits fused in one protein [40]. In general only one type of NHase is produced by one organism. However, *Rhodococcus rhodochrous* J1 produces two types of NHases and their expression is regulated by the supplementation of a specific inducer for each in the culture media [40].

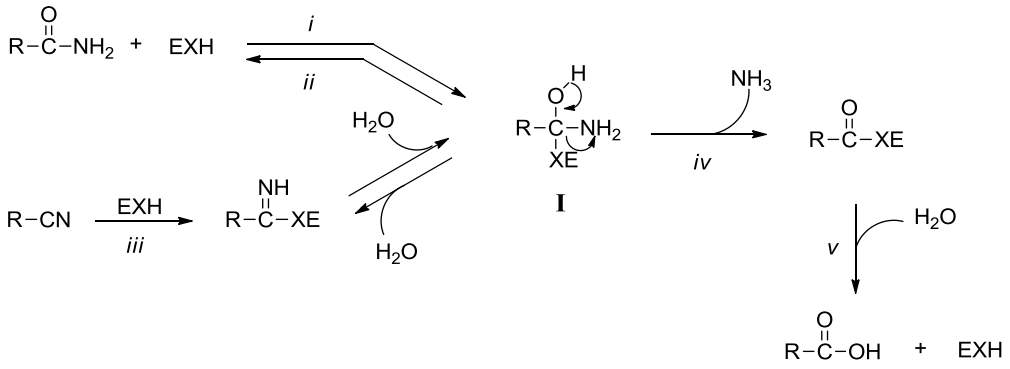
Most of the reported NHase producing organisms are Gram-positive bacteria, which have rigid and strong cell walls [40]. Therefore, the maximum release of NHase from the cells depends on the enzymes ability to bypass the cell wall. Purified active NHases generally have molecular masses ranging from 54 kDa to 530 kDa and are usually composed of two distinct α- and β- subunits having individual molecular masses in the range of 22 kDa to 29 kDa. A distinct NHase has been discovered in *Rhodococcus* sp. RHA1 which contains one cobalt, two copper and one zinc atom per functional unit of the enzyme [44]. The physiological pH optima of NHases vary between 6.5 and 8.5 and a rapid loss of activity was reported by decreasing the pH [40]. Most of the NHases are thermo-labile and their optimum temperature ranges are between 20-35 °C. The reaction involving the hydration of nitrile to amide is generally carried out at a low temperature (< 25 °C) as amidases exhibit maximum activity at 40-60 °C, therefore carrying out the reaction at 25 °C drastically lowers the activity of amidase to negligible levels and significantly improves the NHase activity [40]. The catalytic site of NHase (Fe-/Co-ion centre) is deeply buried in the protein scaffold, the nitrile molecule therefore must reach the interior of NHase for catalysis [40].

There are three significant disadvantages to using NHases in a biotransformation. Firstly, they can be unstable and are therefore tedious to work with. Secondly, NHases are sensitive to steric factors thus restricting the range of potential substrates. Lastly, using aqueous solvent in the process can restrict their applicability in the hydration of nitriles that are hydrophobic (4).

1.4.3 AMIDASE ENZYMES

As mentioned previously, amidases catalyse the hydrolysis of amides to free carboxylic acids and ammonia. Theses enzymes are involved in nitrile metabolism in both prokaryotic and eukaryotic cells [31]. They comprise branch 2 of the nitrilase superfamily and examples include amidases from *Pseudomonas aeruginosa, Rhodococcus erythropolis, Helicobacter pylori* and *Bacillus stearothermophilus* [45]. In industry they are employed in combination with nitrile hydratase for the production of commercially important organic acids. Amidase classifications exist based on catalytic activity, amino acid sequence and phylogenetic relationships. The cluster analysis of amino acid sequences of enantioselective amidases showed distinct amino acids of glycine 17, aspartic acid 19 and serine 23 [46] [47]. In aliphatic amidases, a cysteine residue was reported to act as the nucleophile in the catalytic mechanism and the putative catalytic triad Cys-Glu-Lys was shown to be conserved in all the members of the nitrilase superfamily [48]. Unlike NHases, the association of amidases with metals such as cobalt or iron is reported only in the case of *K. pneumonia* [31].

Kobayashi *et al.* proposed a mechanism for the hydrolysis by amidase as shown in Scheme 1.13, which is analogous to that of nitrilase. The carbonyl group of the amide is attacked by the nucleophilic amino acid of the amidase (step *i*), resulting in the formation of a tetrahedral intermediate **I**. The intermediate is converted into the acyl-enzyme complex by removal of ammonia (step *iv*). This is then hydrolysed to the acid in step *v* [49] [50].



Scheme 1.13: Proposed mechanism of hydrolysis catalysed by amidase. The tetrahedral intermediate is indicated by I [51].

Some amidase enzymes are specific for aliphatic amides, others cleave amides of aromatic acids. However, signature amidases have wide substrate specificity (aliphatic, aromatic amides and amides of α-substituted carboxylic acids) and they also exhibit

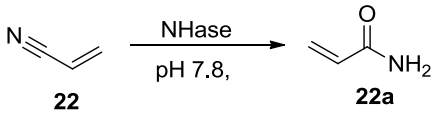
stereoselectivity [47]. Aliphatic amidases are also known as nitrilase related amidases containing a conserved nucleophilic cysteine residue at the active site [52]. This will be discussed in more detail in the nitrilase superfamily section later in the Chapter.

1.4.4 BIOTECHNOLOGICAL POTENTIAL OF NHASE AND AMIDASE ENZYMES

THE PRODUCTION OF ACRYLAMIDE USING NHASE

The application of nitrile biotransformations was first demonstrated in 1985 when the enzymatic production of acrylamide **22a**, a typical commodity chemical, was performed on an industrial scale [38]. The production of **22a** using nitrile hydratase enzymes was first established by Nagasawa *et al.* using an enzyme isolated from *Rhodococcus* sp. N-774 [53]. Now approximately 10,000 tons of acrylamide is produced per year using this process. Whole cells of *Pseudomonas chlorophis* B23 [54], and *Rhodococcus rhodochrous* J1 [53] were also selected as efficient catalysts for the production of the amide **22a** (Table 1.3) [54].

Table 1.3: Acrylamide production comparing NHase enzymes from different sources.



22 **22a**

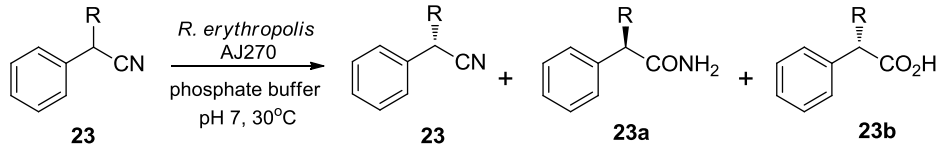
	Microorganism used		
	Rhodococcus sp. N-774	*Pseudomonas chlororaphis* B23	*Rhodococcus rhodochrous* J1
Yield of acrylamide produced	20 %	27 %	40 %
First year of production scale	1985	1988	1991
Total annual production (tons)	4000	6000	>30000

In contrast to the conventional chemical process for the production of **22a**, the recovery of unreacted nitrile **22** is not necessary in the biochemical process because of the high conversion rate. Overall, the enzymatic process is simpler and more economical. It is carried out below 10 °C under mild reaction conditions [55], [38]. *R. rhodochrous* J1 has a more powerful ability to produce acrylamide than *P. chlororaphis* B23. The nitrile hydratase of *P. chlororaphis* B23 contains ferric ions as a cofactor in comparison to *R. rhodochrous* J1 which contains cobalt ions [38]. *R. rhodochrous* J1 nitrile hydratase is much more heat stable and more tolerant to high concentrations of **22** [56].

1.4.5 NHASE/AMIDASE CATALYSED HYDROLYSIS OF CHIRAL SUBSTRATES

The following section will provide a brief overview of chiral substrates which have been hydrolysed by a NHase/amidase bienzymatic system. One well documented example is that of *Rhodococcus erythropolis* AJ270, which has been studied extensively by Wang *et al.* The isolate *R. erythropolis* AJ270 was identified among a large number of nitrile metabolizing strains taken from a disused industrial site on the banks of the River Tyne in the north east of England. This strain has been shown to selectively hydrolyse a wide range of aliphatic, aromatic and heterocyclic nitriles and amides [57-61]. In this bi-enzymatic pathway the stereoselectivity is generally associated with the amidase coexpressed with NHase [47].

Wang *et al.* [62] demonstrated that *Rhodococcus sp.* AJ270 was able to catalyse the stereoselective conversions of α-substituted phenylacetonitriles **23-29** under very mild conditions into amides **23a- 29a** and carboxylic acids **23b-29b**, as shown in Scheme 1.14.



24: R = Me
25: R = Et
26: R = *n*-Pr
27: R = *i*-Pr
28: R = *n*-Bu
29: R = MeO

Scheme 1.14: Biotransformation of α-alkylated arylacetonitriles using *Rhodococcus* sp. AJ270 [62]

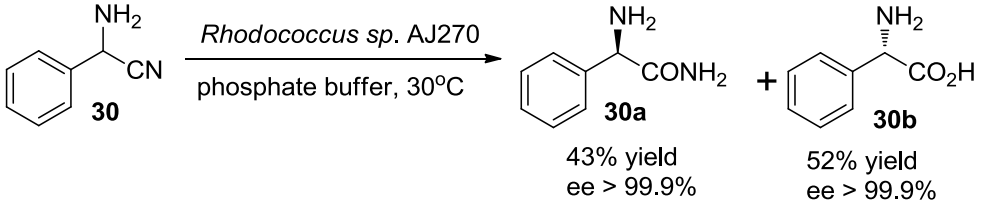
The results as shown in Table 1.4, demonstrate that *Rhodococcus* sp. AJ270 can hydrolyse phenylacetonitriles bearing a variety of substituents at the α-position. The reaction outcome was strongly influenced by the nature of the α-substituent with both electronic and steric factors dramatically affecting the reactivity and, more importantly the enantioselectivity [62].

Table 1.4: Biotransformation results of α-alkylated phenylacetonitrile by *R. erythropolis* AJ270

Entry	Substrate	R	Nitrile Yield(%), ee (%)	Amide (a) Yield(%), ee (%)	Acid (b) Yield(%), ee (%)
1	**24**	Me	-	42, *R* > 99	48, *S* 90
2	**25**	Et	-	34, *R* 96	40, *S* >99
3	**26**	*n*-Pr	55, *S*, 24	27, *S* 41	8, *S*, >99
4	**27**	*i*-Pr	-	47, *R* > 99	46, *S* >99
5	**28**	*n*-Bu	36, *S*, 36	34, *S* 20	23, *S* 98

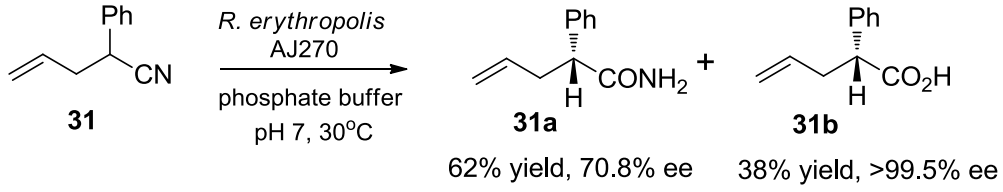
6	**29**	MeO	-	78, 0	-

When nitrile **27** (Table 1.4, entry 4) underwent hydrolysis, the acid **27b** was prepared in 46 % yield and 99 % ee. However, when the isopropyl group was replaced by n-propyl **26** or n-butyl (nitrile **28)** the hydrolysis proceeded very slowly and a low yield was obtained. Wang *et al.* also discovered that introduction of a polar moiety such as methoxy **29** (Table 1.4, entry 6) instead of an alkyl at the α-position, gave no effect on the rate of hydration [62]. The amidase demonstrated high (*S*)-enantiocontrol against amides, while the nitrile hydratase exhibited low (*R*)-selectivity against nitriles. The results suggest that the amidase in *Rhodococcus sp*. AJ270 seems to have the ability to recognize substituents of different sizes and of marginally different polarities [62]. This is also true in the biotransformation of phenylglycine nitriles **30** which efficiently produced optically active D-phenylglycine amides **30a** and acid **30b** in excellent yields with >99 % ee, as seen in Scheme 1.15 [57].



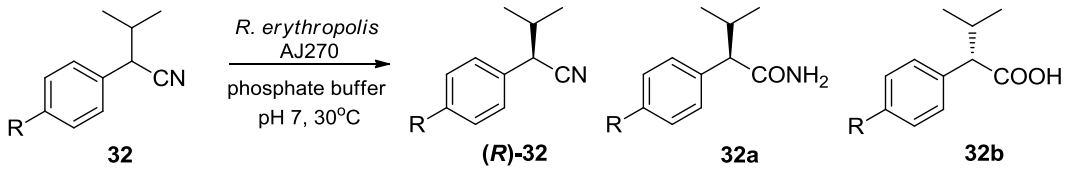
Scheme 1.15: Hydrolysis of racemic phenylglycine nitrile by *Rhodococcus erythropolis* AJ270

Wang *et al.* also studied the biotransformations of a range of racemic 2-aryl-4-pentenenitriles, an example of which is shown in Scheme 1.16 [63]. Highly optically pure (*R*)- amide **31a** and (*S*)- acid **31b** were obtained in almost quantitative yields after about 3 days incubation [64], with >99.5 % enantiomeric excess and 38 % yield of the acid.



Scheme 1.16: Biotransformations of racemic 2-aryl-4-pentenenitriles using AJ270 [64].

R. erytrhropolis AJ270 was also examined in the hydrolysis of 2-aryl-3-methylbutyronitriles **32-37** bearing either an electron-withdrawing or electron donating group on the aromatic ring, as demonstrated in Scheme 1.17.



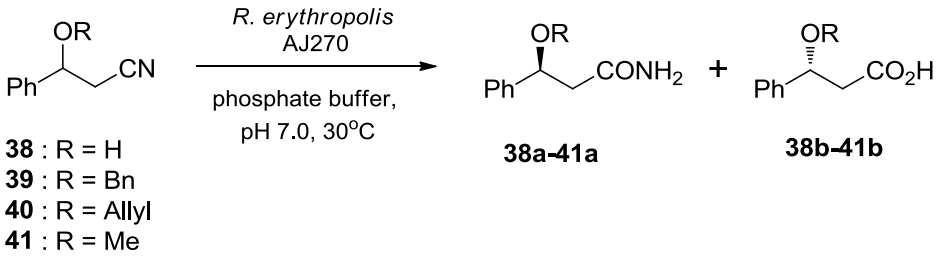
Scheme 1.17: Biotransformations of 2-aryl-3-methylbutyronitriles [65]

It was found that the reaction rate was strongly dependent upon the steric nature of the *para*–substituent on the aromatic ring, and bromo- and methoxy substituted analogs proceeded slowly with the recovery of more than half the starting material [65]. The addition of smaller substituents such as fluoro (substrate **34** Table 1.5, entry 2) and chloro (substrate **35,** Table 1.5, entry 3) at the *para*–position of the phenyl ring, proved more efficient as seen in Table 1.5. The biotransformation of all substrates tested produced *(S)*-(+)-carboxylic acids **33b-37b** in excellent optical yields (ee > 99 %) [65].

Table 1.5: Biotransformations of 2-aryl-3-methylbutyronitriles, adapted from [65].

Entry	Substrate	R	Nitrile Yield(%), ee (%)	Amide (*R*)-a Yield(%), ee (%)	Acid (*S*)- b Yield(%), ee (%)
1	**33**	H	-	45, > 99	48, > 99
2	**34**	F	-	45, > 99	43, > 99
3	**35**	Cl	45, 29	20, 18	29, > 99
4	**36**	Br	59, *5*	8, > 99	16, > 99
5	**37**	OMe	58, > 41	16, 0	15, > 99

Wang *et al.* studied the microbial hydrolysis of racemic *β*-hydroxy and *β*-alkoxy nitriles **38-41** and observed a dramatic *O*-substituent effect on the enantioselectivity of the biocatalytic reactions [66]. Racemic **38**, a *β*-hydroxy carbonitrile substrate was found to produce amide **38a** and acid **38b** in quantitative yields in the presence of AJ270 (Scheme 1.18). However, the enantiomeric excess values of both the amide and acid products were found to be extremely low (Table 1.6, entry 1) [67].



Scheme 1.18: Biotransformations of racemic *β*-hydroxy and *β*-alkoxy nitriles [67].

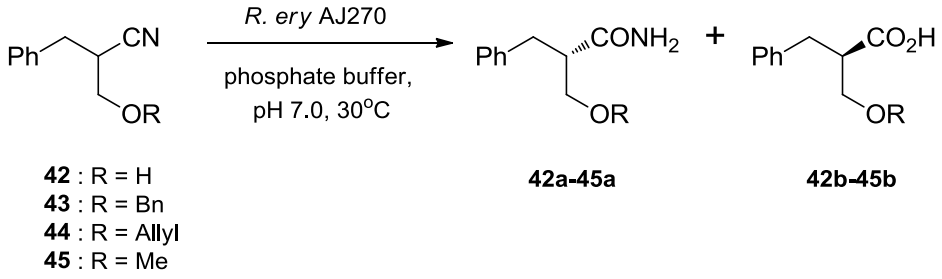
They found that by protecting the free β-hydroxy of **38** with a methyl group to generate **41** (Table 1.6, entry 4), the enantioselectivity was increased dramatically to 77 % for the amide **41a** and 59 % for the acid **41b** (Table 1.6, entry 4) [67].

Table 1.6: Biotransformations of racemic β-hydroxy and β-alkoxy nitriles [67].

Entry	Substrate	R	Amide (*S*)-a (yield %), (ee%)	Acid (*R*)-b (yield %), (ee%)
1	**38**	H	(50), (1.6)	(49), (4.0)
2	**39**	Bn	(56), (2.4)	(0)
3	**40**	Allyl	(47), (40)	(40, 29.4)
4	**41**	Me	(49), (77.6)	(51), (59.2)

Wang *et al.* found that the biotransformation of *O*-benzylated form of the substrate **39** proceeded very slowly without the formation of the acid **39b**. This suggests that the presence of a phenyl group rather than an alkyl group in the substrates poses steric hindrance to both the nitrile hydratase and amidase present [67].

Wang *et al.* then studied the biotransformations of racemic 2-hydroxymethyl-3-phenylpropionitrile **42**, a β^2-hydroxy carbonitrile species (Scheme 1.19). The substrate **42** underwent rapid and efficient biotransformation to produce optically active (*S*)-amide **42a** and (*R*)-acid **42b** in excellent yields with moderate enantioselectivity, as shown below in Table 1.7.



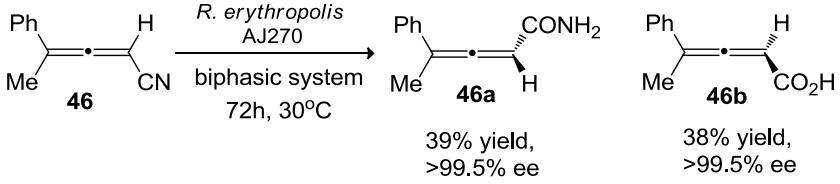
Scheme 1.19: Biotransformations of racemic β^2-hydroxy and β^2-alkoxy nitriles [67].

Table 1.7: Biotransformations of racemic β^2-hydroxy and β^2-alkoxy nitriles [67].

Entry	Substrate	R	Amide a (yield %), (ee%)	Acid b (yield %), (ee%)
1	**42**	H	(43), (71)	(47), (81.1)
2	**43**	Bn	(21), (68.4)	(0)
3	**44**	Allyl	(47), (57.6)	(51), (54.8)
4	**45**	Me	(50), (96.2)	(50), (96.2)

In this case better stereodiscrimination in the unprotected substrate **42** when compared to **38**, is in agreement with the notion that the closer the stereogenic center is to the reaction site, the higher the chiral induction [67]. The hydroxyl group was then protected by several different substituents **43-45**, and the effect of the *O*-substituent was examined. The enantioselectivity of the *O*-allylated substrate **44** (Table 1.7, entry 3) decreased compared to the reaction of hydroxy containing substrates **42**. Protection *via* a methyl group (substrate **45**) resulted in an increase in ee to 96.2 % (Table 1.7, entry 4) [67].

Recently the hydrolysis of racemic axially chiral 2,3-allenenitrile **46** in an aqueous phosphate buffer-n-hexane biphasic system by whole cells of *R. erythropolis* AJ270 was reported [68]. The nitrile **46** underwent hydrolysis to form (a, R)-amide **46a** and (aS)-acids **46b** with ee's of up to 99.5 % as shown in Scheme 1.20. The nitrile hydratase enzyme catalysed the non-selective hydration of the nitrile, followed by the amide hydrolysis catalysed by the substrate dependant enantioselective amidase [68].



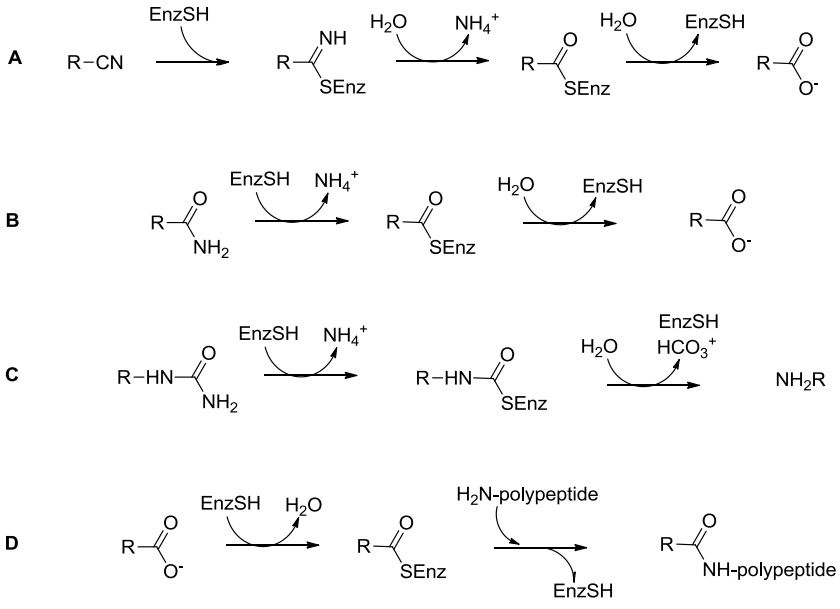
Scheme 1.20: Hydrolysis of 2,3-allenenitriles by *R. erythropolis* AJ270 in an aqeous organic biphasic system.

1.5 NITRILASE AS A MEMBER OF THE NITRILASE SUPERFAMILY

Over the past five decades various nitrilase-producing organisms including bacteria, filamentous fungi, yeasts and plants have been described [69-71]. Nitrilases, cyanide dihydratases and cyanide hydratases have been grouped into a single enzyme family known as the nitrilase/cyanide hydratase family, which also includes the less closely related aliphatic amidases [52]. The nitrilase superfamily consists of thiol containing enzymes involved in natural product biosynthesis. On the basis of sequence similarity, the superfamily can be classified into 13 branches of which have known or deduced specificity for specific nitrile- or amide- hydrolysis or amide condensation reactions [45]. The nitrilase superfamily also consists of carbamylases which are specific for the decarbamylation of D-amino acids [72]. Nitrilases and α/β hydroxylases belonging to the nitrilase super-family, have been widely acknowledged as valuable alternatives to chemical catalysts as they have proven to transform an immense variety of organic

nitriles under mild conditions, often in a stereoselective or regioselective manner [73]. The members in the superfamily share a common fold with a conserved catalytic Cys-Glu-Lys site, which is surrounded by signature sequences conserved within each branch with differences between branches [45].

The four types of reactions performed by the nitrilase superfamily are shown schematically in Scheme 1.21. The nitrilase branch contains enzymes responsible for nitrile hydrolysis directly from a nitrile to a carboxylic acid. Nitrilases are perhaps the best characterised of all members of the superfamily with numerous examples identified across kingdoms [74]. Eight of the remaining branches can be classified as amidases of various specificities. Branch 2, 3, 4 and 5 consist of aliphatic amidases, amino-terminal amidases, biotinidase and β–ureidopropionase. Nitrile hydratases which convert a nitrile to the corresponding amide are not members of the nitrilase superfamily [45]. Only branch 9 enzymes, namely the alipoprotein N-acyltransferases, catalyse amide condensation reaction, represented as D in Scheme 1.21. The function of enzymes in branches 11, 12 and 13 are unknown.



Scheme 1.21: Four types of reaction carried out by the nitrilase superfamily. (A) Nitrile hydrolysis catalysed by branch 1 nitrilase enzymes. (B) Amide hydrolysis catalysed by the amidase enzymes (branch 2-4), glutamine specific amidases are classified as branch (7-8). (C) The carbamylase reaction is a special case of the amidase reaction (branch 5-6). (D) N-acyltransferases perform the amidase reaction in reverse, transferring a fatty acid from a phospholipid to a polypeptide amino terminus. This figure is adapted from [45].

Cyanide dihydratases are classified under branch 1 enzymes of the nitrilase superfamily, which hydrolyse cyanide to formate and ammonia. These enzymes have been identified

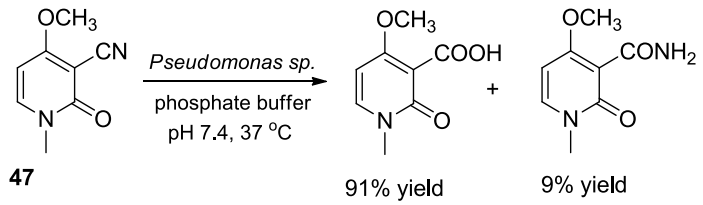
in *Alcaligenes xylosoxidans* subsp. *denitrificans* [75], *Bacillus pumillus* C1 [76], *Pseudomonas fluorescens* NCIMB 11764 [77] and *Pseudomonas stutzeria* AK61 [78]. None of these enzymes were reported to form formamide in the reactions they catalyse.

Another group of branch 1 nitrilases, namely the cyanide hydratases, hydrolyse cyanide to formamide [45]. The enzyme cyanide hydratase was first identified in the fungus *Stemphylium loti*, a pathogen of cyanogenic plants [79]. This enzyme is not related to the metal containing nitrile hydratases. Cyanide hydratases which have been studied so far include *Gleocercospora sorghi* [80], *Fusarium lateritium* [81] and *Leptospheria maculans*

Since the nitrilase enzyme is the focus of this body of work, specific attention will be given to this enzyme. The next section of this review will concentrate on nitrilase occurrence, catalytic reaction mechanism, reaction properties, substrate scope and high-throughput screening.

1.5.1 NITRILASE ENZYMES IN BIOCATALYSIS

Nitrilase activities exist extensively in nature, especially in bacteria, filamentous fungi and plants. The first nitrilase enzyme was discovered in barley leaves in 1948, and this nitrilase is known to convert indole-3-acetonitrile to indole-3-acetic acid in plants [31]. Substrate analysis on purified enzyme with 26 nitriles indicated that the enzyme has a broad substrate range [37]. The first bacterial nitrilase enzyme was isolated from soil in 1964 and was found to hydrolyse the ricinine nitrile group of **47** (Scheme 1.22), this isolate was identified as belonging to the *Pseudomonas* genus and formed acid predominantly [82].



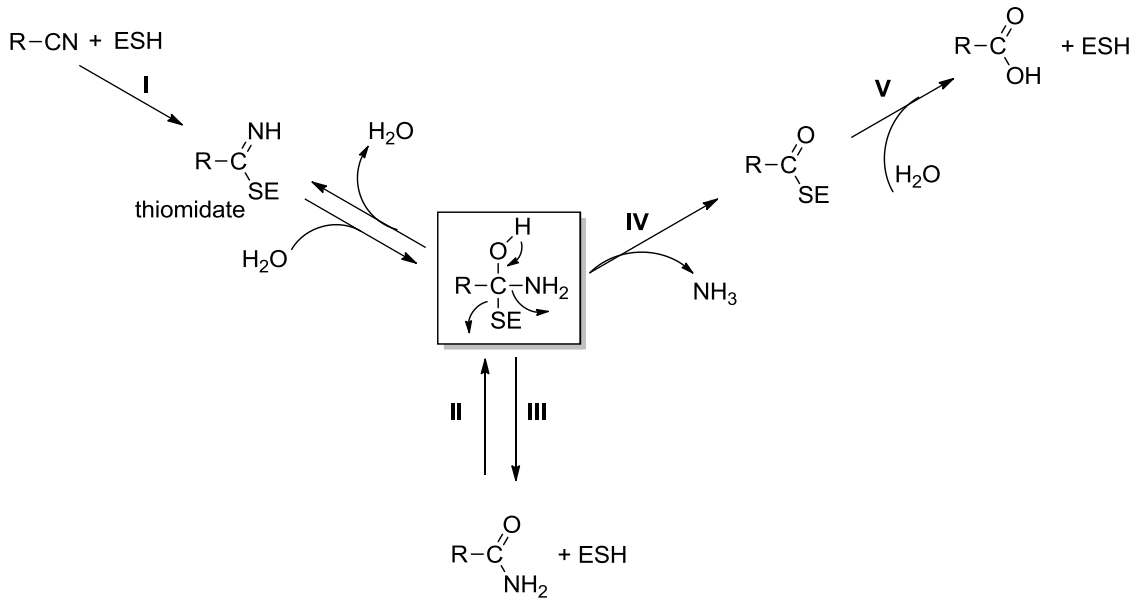
Scheme 1.22: Hydrolysis of ricinine nitrile by the nitrilase of *Pseudomonas* sp. [82].

The biotechnological potential of nitrile hydrolyzing enzymes has led to the isolation of a range of bacteria and fungi capable of hydrolyzing nitriles. Nitrilases have been isolated from bacteria such as that of *Rhodococcus* [83], *Nocardia* [84], *Acinetobacter* [85], *Pseudomonas* [86] and *Alcaligenes* [87]. These nitrilases exhibit a broad substrate

range and diverse biochemical characteristics [43]. Nitrilase activity was also reported in several fungi strains including *Fusarium* [88], *Gibberella*, *Aspergillus* [89], and *Penicillium* [70]. Most of these were isolated using a particular nitrile as a carbon and/ or nitrogen source [37].

Nitrilases are generally inducible enzymes composed of one or two types of subunits of different size and number [31]. Most nitrilases consist of a single polypeptide with a molecular mass of approximately 40 kDa, which aggregates to form the active enzyme. The preferred form of nitrilase enzymes seems to be a large aggregate of 6-26 subunits [74]. Nitrilases unlike NHases, do not show the presence of any metal co-factor or prosthetic group [31]

The major disadvantage of nitrilases in industrial applications is their relatively poor stability. The presence of a critical thiol (-SH) group at the active site of the enzyme may contribute to this instability [90]. A possible mechanism for the nitrilase-catalysed hydrolysis as demonstrated in Scheme 1.23, includes a nucleophilic attack by a thiol group on the carbon atom of the nitrile (Scheme 1.23, step I), with concomitant protonation of the nitrogen to form a tetrahedral thiomidate intermediate. Subsequent steps (Scheme 1.23, steps IV and V) involve attack by two water molecules and protonation of the nitrogen atom which is lost as ammonia [74].



Scheme 1.23: Proposed mechanism of nitrilase activity adapted from O'Reilly *et al.* [74].

1.5.2 NITRILASE SUBSTRATE SPECIFICITY AND ACTIVITY

Nitrilase enzymes can be divided into three subgroups according to their physical properties and substrate specificities and the best substrate can be different for each enzyme [37]. Substrate analysis on a wide variety of purified nitrilases indicates that while most nitrilases show their highest activity with aromatic nitriles, some nitrilases have a preference for arylacetonitriles and others for aliphatic nitriles [74]. Various other nitrilase subgroups based on substrate specificity also exist such as, bromoxynil specific nitrilases, cyanide dihydratase and cyanide hydratase

A number of nitrilase enzymes demonstrate activity towards both aliphatic and aromatic substrates. These nitrilase enzymes will be addressed in more detail in the following sections. However, below is an outline of the various nitrilase producing bacteria according to the preferred substrate type.

AROMATIC NITRILASE ENZYMES

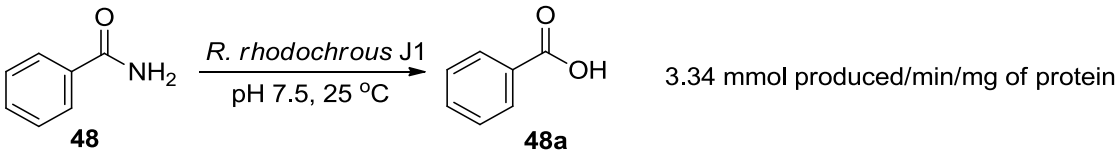
Those enzymes which have been classified as aromatic nitrilases preferentially hydrolyse benzonitrile and related aromatic substrates. A variety of these enzymes have been purified from the bacteria *Pseudomonas* sp [82], *Nocardia* sp. NCIMB 11215 and NCIMB 11216 [91], *Arthobacter* sp. Strain J1 [92], *R. rhodochrous* [93], *Klebsiella* [94], and the fungus *Fusarium solani* [88, 95] as outlined in Table 1.8.

Table 1.8: Characteristics of aromatic nitrilases from various microorganism in the literature.

Organism	Subunit (SDS/PAGE) (kDa)	pH	Temp opt °C	Amide by-product	Ref
Arthobacter sp. Strain J1	30	8.5	40	-	[92]
Bacillus pallidus DAC 521	41	7.6	65	-	[96]
Fusarium solani IMI196840	76	7.8-9.1		-	[88]
Fusarium oxysporum f. Sp. Melonis	37	6-11	40	detected	[97]
Fusarium solani 01	40	8	40-45	-	[95]
Nocardia (Rhodococcus) NCIMB 11215	45	7-9.5	30	-	
Nocardia (Rhodococcus) NCIMB 11216	45.8	8	30		[91]
Rhodococcus rhodochrous ATCC39484	40	7.5	45	detected	[98]
Rhodococcus rhodochrous J1	41.5	7.6	45	detected	[93]
Aspergillus niger K10	38.5	8.0	45		[99]
Rhodococcus rhdochrous P*A*–34	45	7.5	35		[100]

One of the most extensively studied aromatic nitrilases is that of *R. rhodochrous* J1, which was purified and found to be specific for nitrile groups attached to an aromatic or a heterocyclic ring. Nagasawa *et al.* had reported that *R. rhodochrous* J1 formed a large amount of nitrilase by the addition of isovaleronitrile as an inducer [101]. It was also found that *ε*-caprolactam acts as a more powerful inducer than isovaleronitrile [102]. Both physiochemical properties and identical N-terminal amino acid sequences suggested that both inducing nitriles resulted in the formation of identical nitrilase enzymes [91]

Nitrilases were previously reported not to catalyse the hydrolysis of amides [103]. However, Kobayashi *et al.* reported in 1998 that *Rhodococcus rhodochrous* J1 contained a nitrilase that surprisingly catalysed the hydrolysis of amide to acid and ammonium stochiometrically (Scheme 1.24) [49]. This was found to occur at the same catalytic site as the nitrile hydrolysis reaction. This nitrilase was highly purified from an *Escherichia coli* transformant which suggests that the nitrilase did not contain any other contaminant proteins displaying amidase activity. Koboyashi *et al.* found that the disappearance of benzamide **48** corresponded to the appearance of benzoic acid **48a**. The relative activity of the hydrolysis of amide **48** was 0.00022 % of the activity towards benzonitrile which was taken as 100 %. With the nitrilase, 1 mol of benzoic acid and ammonia were formed per mole of benzamide degraded. Although the catalytic activity towards benzonitrile was 6 orders of magnitude greater than that towards benzamide, the amidase activity was ascribed to be a property of the nitrilase.

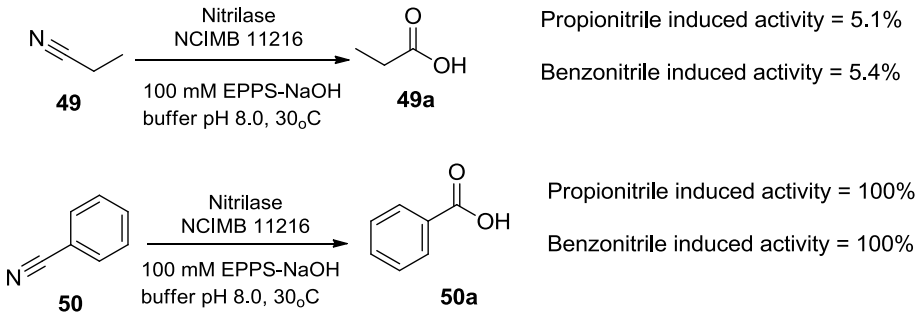


Scheme 1.24: Benzamide hydrolysis by the nitrilase of *Rhodococcus rhdochrous* J1.

A kinetic study of this nitrilase also demonstrated competitive inhibition, suggesting competition for the same binding region by benzonitrile and benzamide at the enzyme active site cysteine residue [49].

The nitrilase of *R. rhodochrous* NCIMB 11216 was found to be capable of biotransforming a wide variety of nitriles. Hoyle *et al.* found that using either propionitrile or benzonitrile as the sole source of carbon and nitrogen during the

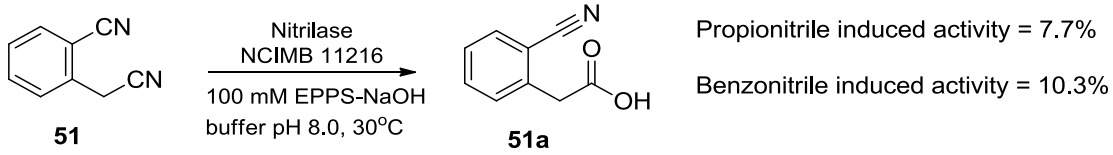
induction stage resulted in different enzyme activity [91]. NCIMB 11216 was capable of hydrolysing short-chain aliphatic nitriles when the isolate was induced with both propionitrile and benzonitrile [91]. However very low activity was found for the hydrolysis of aliphatic mononitrile such as **49** when compared with that of benzonitrile **50** (Scheme 1.25).



Scheme 1.25: Nitrilase catalysed hydrolysis of aromatic and aliphatic nitriles by *R. rhodochrous* NCIMB 11216. Where 100 % activity = mmol of ammonia produced from the biotransformation of 20 mM benzonitrile min^{-1}/ mg^{-1} dry cell weight [91].

It was demonstrated that this nitrilase exhibits the greatest activity towards aromatic mononitriles such as **50** and unsaturated aliphatic dinitriles. This was proposed to be due to the greater structural rigidity of these compounds caused by the presence of a double bond on the aromatic ring. It was also suggested that a more compact substrate structure can result in increased activity [91].

The substrate α–cyano-o-tolunitrile **51** was also investigated in order to provide an insight into the regioselective capabilities of NCIMB 11216 (Scheme 1.26). This compound contains two nitrile groups, one attached to the aromatic ring and the other to the aliphatic side chain [91]. Hoyle *et al.* found that the mononitrile-monoacid **51a**, was produced; therefore this enzyme also displayed regioselectivity as nitrile hydrolysis occurred only at the cyano group of the aliphatic side chain.



Scheme 1.26: Nitrilase catalysed hydrolysis of α–cyano-o-tolunitrile by the nitrilase enzyme of *R. rhodochorous* NCIMB 11216.

ALIPHATIC NITRILASE ENZYMES

Aliphatic nitrilases are so classified because they demonstrate preferential activity towards aliphatic substrates as opposed to those containing an aromatic ring. A number of these enzymes were isolated from various microorganisms and are outlined in Table 1.9. A variety of these enzymes have been purified from the bacteria *Acinetobacter* sp [82, 85], *Bradyrhizobium* [104], *Pseudomonas* [105], and *Rhodococcus* [93]. This table also contains an example of a bromoxynil specific nitrilase enzyme isolate from *Klebsiella ozaenae* [106], and an adiponitrile selective nitrilase found in *Comomonas testeroni* [107].

Table 1.9: Characteristics of aliphatic nitrilases from various microorganisms reported in the literature.

Organism	Substrate specificity	Subunit (SDS/PAGE) (kDa)	pH	Temp opt °C	Ref
Acinteobacter AK226	Aliphatic	41/43	8.0	50	[85]
Synechocystis sp PCC6803	Aliphatic	40	7.0	40-45	[108]
Bradyrhizobium japonicum USDA110	Aliphatic	34.5	7.0-8.0	45	[104]
Pseudomonas fluorescens Pf-5	Aliphatic	33	7.0	45	[105]
Acidovorax facillis 72W	Aliphatic	40	8-9	65	[109]
R. rhodochrous K22	Aliphatic	41	5.5	50	[83]
R. rhodochrous J1	Aliphatic and aromatic	41.5	7.5	45	[93]
Acinetobacter AK226	Aliphatic and aromatic	-	8.0	50	[110]
Comomonas testeroni	Adiponitrile	38	7.0	25	[107]
Klebsiella ozaenae	Bromoxynil	37	9.2	35	[111]
Fusarium oxysporum	Aliphatic and aromatic	27	-	4	[97]

A novel nitrilase identified as *Rhodococcus rhodochrous* K22 originally isolated from soil was found to catalyse the hydrolysis of aliphatic nitriles to their corresponding carboxylic acids [83]. The *R. rhodochrous* K22 enzyme is characteristic in its broad substrate specificity for aliphatic nitriles (Figure 1.5), which were previously thought to be unaccepted by nitrilase enzymes. Aliphatic olefinic nitriles with two to five carbon atoms were remarkably active as substrates for the enzyme.

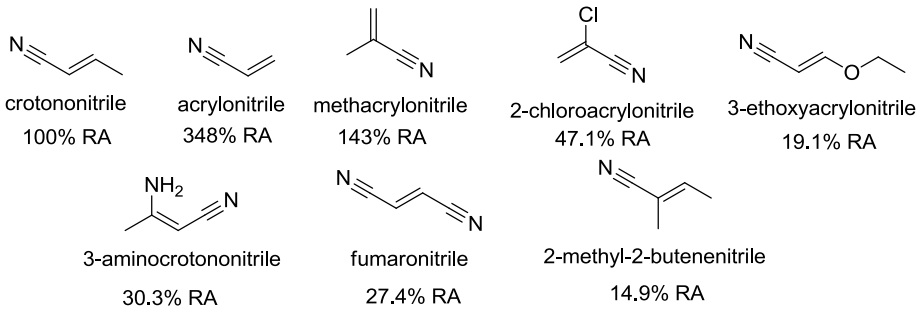
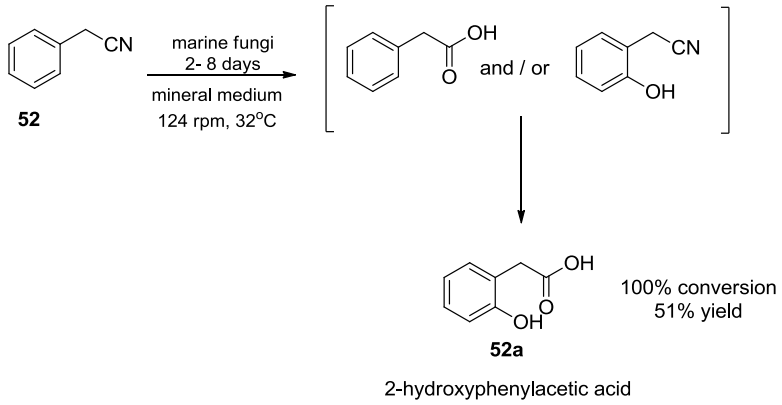


Figure 1.5: Aliphatic olefinic nitriles hydrolysed by the nitrilase of *R. rhodochrous* K22, RA = Activity relative to crotononitrile.

ARYLACETONITRILASE ENZYMES

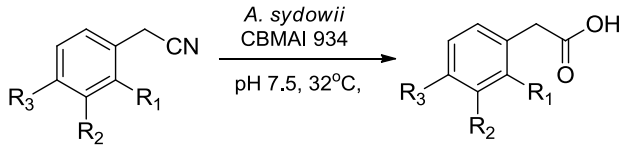
These nitrilase enzymes demonstrate a preference for the hydrolysis of arylacetonitrile analogues. The production of 2-hydroxyphenylacetic acid **52a** from phenylacetonitrile **52** was described by Oliviera *et al.* using marine fungi belonging to the genera *Aspergillus, Penicillium, Cladosporium, and Bionectria* [112]. The phenylacetonitrile was first hydrolysed to the carboxylic acid, and then, an enzymatic hydroxylation at the *ortho* position resulted in the formation of the acid **52a** with 51 % isolated yield (Scheme 1.27). In an alternative route, the hydroxylation could occur first and subsequently the hydrolysis of the nitrile group. However intermediate compounds such as phenylacetic acid or 2-hydroxylphenylacetonitrile, were not observed.



Scheme 1.27: Proposed pathway for the biotransformation of phenylacetonitrile to 2-hydroxyphenylacetic acid by induced marine fungi in liquid medium containing mineral salts, glucose, and phenylacetonitrile [112].

They also examined the hydrolysis of 4-fluorophenylacetonitrile to determine the sequence of enzymatic reactions. In this case only the corresponding non-hydroxylated acid was detected with 100 % conversion and 51 % isolated yield [112].

In a subsequent study Oliveira *et al.* examined the biotransformations of methylphenylacetonitriles **53-55** by Brazilian marine fungus *Aspergillus sydowi* CBMAI 934. The substrates 2-methylphenylacetonitrile **53**, 3- methylphenylacetonitrile **54** and 4- methylphenylacetonitrile **55** were hydrolysed to the corresponding carboxylic acids **53a-55a** (Scheme 1.28) [113]. It was found that when the substrate contained substituents at the *ortho, meta* or *para* position in the aryl ring, the hydroxylation previously observed did not occur.



53: $R_1 = CH_3$, $R_2 = R_3 = H$ **(2)**
54: $R_1 = R_3 = H$, $R_2 = CH_3$ **(3)**
55: $R_1 = R_2 = H$, $R_3 = CH_3$ **(4)**

53a = (76%)
54a= (52%)
55a= (46%)

Scheme 1.28: Biotransformation of phenylacetonitrile derivatives by marine fungus *A. sydowii* CBAMI 934 [113].

It was unusual to note that substrate **53** containing a methyl group at the *ortho* position of the aromatic ring was fully transformed to the carboxylic acid. This is in contrast to other reports where a lack of activity due to steric hindrance due to proximity is usually observed [74]. Hence, *A. swodowii* CBMAI 934 is a promising biocatalyst for the preparation of arylaliphatic and aliphatic carboxylic acids.

1.6 NITRILASE CATALYSED HYDROLYSIS OF SUBSTRATES CONTAINING A CHIRAL CENTRE.

Several nitrilase enzymes have been examined in the hydrolysis of chiral nitrile analogues bearing various substituents at the chiral centre. In this section emphasis will be placed on the hydrolysis of nitriles with a chiral centre α- or β- to the nitrile functional group containing hydroxyl, alkyl and amino groups.

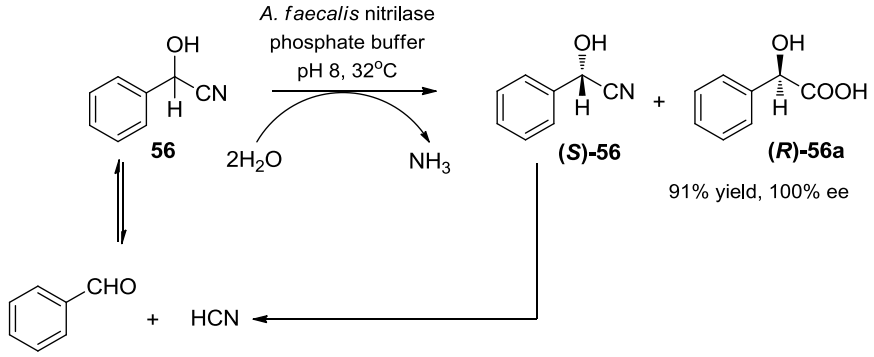
1.6.1 NITRILASE CATALYSED HYDROLYSIS OF SUBSTRATES BEARING AN α-STEREOCENTRE

HYDROLYSIS OF α-HYDROXY SUBSTITUTED NITRILES

The compound (*R*)-(-)-Mandelic acid **(*R*)-56a** is an optical resolving reagent and the source of pharmaceuticals such as semisynthetic cephlosporins [114]. *A. Faecalis* ATCC 8750 contained an enantioselective nitrilase for mandelonitrile **56** and amidase for mandelamide based on the partial purification of these enzymes [115]. Similar to

other nitrilase enzymes such as *Alcaligenes faecalis* JM3, an inducer is essential for the nitrilase expression [87].

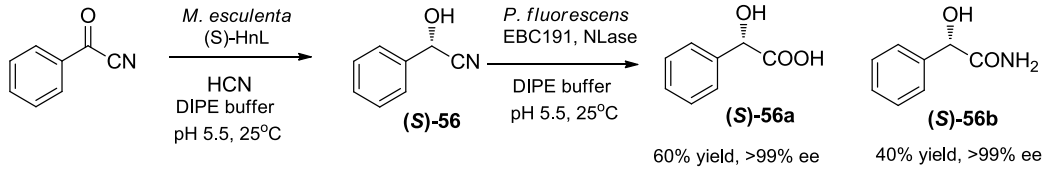
A DKR of (*R*)-(-)-Mandelic **(*R*)-56a** acid may be produced from racemic mandelonitrile **56** by *Alcaligenes faecalis* ATCC 8750 in up to 91 % yield and 100 % ee (Scheme 1.29) [114]. In this case, the remaining (*S*)- nitrile of **56** was spontaneously racemised due to the chemical equilibrium and then subsequently used as the substrate for the nitrilase, thereby being consumed and converted to (*R*)- acid **56a** [114].



Scheme 1.29: Synthesis of *(R)*-mandelic acid through the enantioselective nitrilase catalysed hydrolysis of mandelonitrile by *A. faecalis* ATCC 8750.

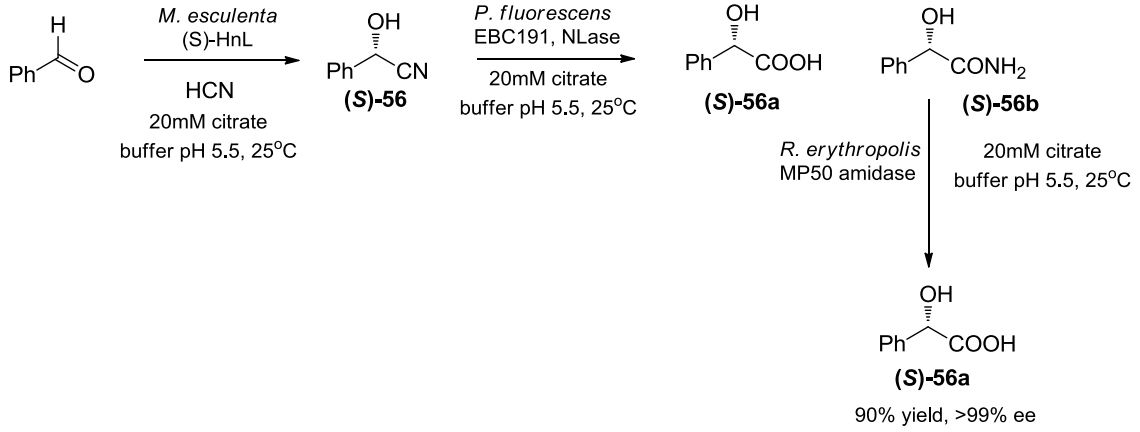
This organism was also capable of hydrolysing aliphatic nitriles such as chloroacetonitrile, but had difficulty hydrolysing aromatic nitriles such as benzonitrile. As such, Yammamoto suggested that it be classified as an aliphatic nitrilase [115].

Mateo *et al.* demonstrated that benzaldehyde could be converted into an enantiomerically pure (*S*)-mandelic acid **(*S*)-56a** by sequential HCN addition and hydrolysis in the presence of a cross-linked enzyme aggregate composed of the (*S*)-selective oxynitrilase from *Manihot exculenta*, and a non-selective recombinant nitrilase from *Pseudomonas fluorescens* EBC 191 (Scheme 1.30) [116]. The enzymatic hydrocyanations were performed at pH < 5 to suppress the degradation of nitrile **(*R*)-56** to benzaldehyde and cyanide. Few nitrilases were reported to display activity under acidic pH conditions, and most of these were strongly biased towards the (*R*)-enantiomer of **56** and only converted **(*S*)-56** sluggishly. However, the nitrilase from *P. fluorescens* EBC 191 hydrolysed both (*S*)- and (*R*)- **56** at pH 5.5. The formation of mandelamide **(*S*)-56b** as a by-product suggested that this nitilase displayed NHase activity towards **56**. This is discussed in more detail in section 1.6.4. The hydrolysis of **56** resulted in the formation of acid **(*S*)-56a** in 99 % ee [116].



Scheme 1.30: Bienzymatic pathway for the production of mandelic acid using a HnL and a NLase in tandem.

Chumra *et al.* produced enantiomerically pure **(*S*)-56a** acid from benzaldehyde by sequential hydrocyanation and hydrolysis using the enzymatic oxynitrilase/ nitrilase cross linked enzyme aggregate (CLEA), as previously described by Mateo *et al.* [116]. However, by including an amidase from *Rhodococcus erythropolis* they were able to suppress amide formation and **(*S*)-56a acid** was isolated as the sole product in 90 % yield and >99 % enantiomeric purity (Scheme 1.31) [117]. Preliminary tests revealed that the amide **(*S*)-56b** was completely hydrolysed in the presence of *penicillin acylase* as well as the amidase from *Rhodococcus erythropolis* MP50. Both enzymes when added to the cascade reaction mixture, mediated the *in situ* hydrolysis of amide into acid [117].



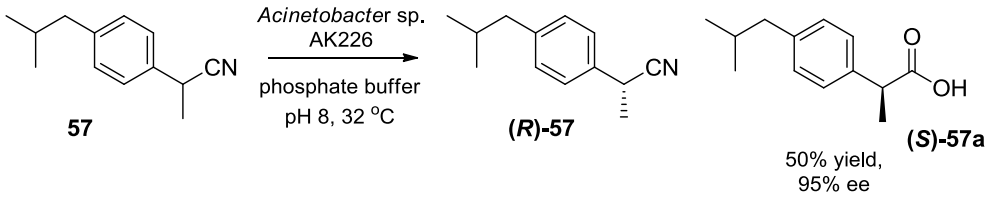
Scheme 1.31: enzymatic pathway for the production of mandelic acid using a HnL, NLase and an amidase in the form of a CLEA.

The enantioselective hydrolysis of nitrile **(*S*)-56** with whole cells of a recombinant *E. coli* expressing the nitrilase from *Alcaligenes* sp. ECU0401 was examined by Zhang *et al.* [118]. Using the recombinant *E. coli* cells as a biocatalyst, a high concentration (200mM) of **(*S*)-56** could be completely transformed within 4 h, forming the acid product **(*S*)-56a** in 98.5 % yield and 99.0 % ee. Substrate inhibition was observed when the biotransformation was attempted with concentrations as high as 300mM, and the yield dropped abruptly to below 10 %. To circumvent this problem Zhang *et al.*

investigated the use of biphasic organic solvents. They envisaged that a water-organic biphasic system would cause a hydrophobic substrate such as mandelonitrile to be retained in the organic phase, which acts as a reservoir for the toxic substrate, thus regulating substrate concentration around the enzyme and minimizing substrate inhibition. In the case of toluene, the substrate partitioned mainly in the organic phase while the product, was mainly found in the aqueous phase. The inhibitory effect of the mandelic acid **56a** on the enzyme was negligible. Toluene was therefore found to be an ideal solvent for nitrile **56** but a poor one for acid **56a**, which permits easy recovery of the product.

NITRILASE CATALYSED HYDROLYSIS OF α–ALKYL SUBSTITUTED NITRILE

Yamamoto *et al.* found that the nitrilase from *Acinetobacter* sp. Strain AK226 was found to hydrolyse Ibu-CN **57** to acid **(*S*)-57a** with 95 % ee [110] (Scheme 1.32). The time course of the reaction was investigated and it was found that concentration of nitrile **57,** was reduced rapidly until it reached 50 % conversion of the initial concentration. The corresponding amide was not detected in the reaction mixture throughout the reaction. There was a slight increase in the production of (*R*)-ibuprofen **(*R*)-57a** when the reaction was left for prolonged periods. This appeared to be caused by a slow attack of the nitrilase towards the remaining nitrile **(*R*)-57**, as the cells demonstrated no racemising activity for the (*S*)-nitrile starting material [110].



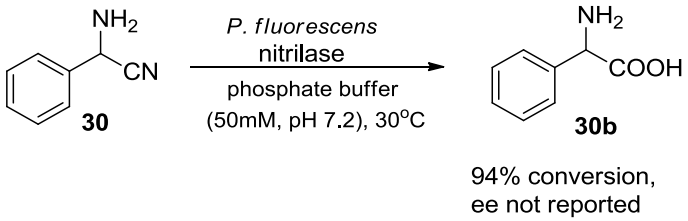
Scheme 1.32: Hydrolysis of ibuprofen nitrile to (S)-ibuprofen by the nitrilase of Acinetobacter AK226.

NITRILASE CATALYSED HYDROLYSIS OF α-AMINO NITRILES

α-amino nitriles are important substrates as upon hydrolysis they generate both natural and unnatural, α-amino acids. The amino acid D-phenylglycine is an important chiral building block in the synthesis of antibiotics such as ampicillin and cefalexin [119] [120]. The nitrilases of *Rhodococcus rhodochrous* PA-34 [100] and of *Acinetobacter* sp [85], for example, were reported to convert several aliphatic DL -α-amino nitriles (including phenylglycinonitrile) into L-amino acids in moderate to high enantiomeric

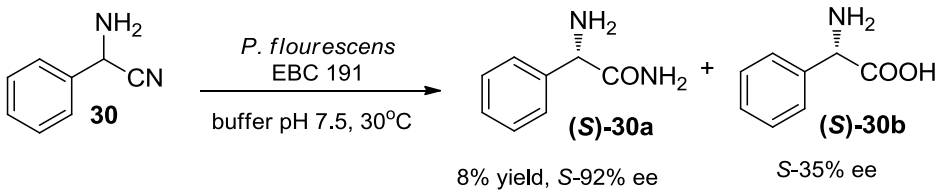
excesses. The nitrilase of *Aspergillus furmigatus* catalysed the hydrolysis of DL-phenylglycinonitrile giving D-acid in 80 % enantiomeric excess [2].

Brady *et al.* examined the hydrolysis of 2-phenylglycinonitrile **30** by a number of nitrilase enzymes and only the nitrilase of *P. fluorescens* exhibited activity for this α-amino substituted nitrile (Scheme 1.33) [121]. The yield of 2-phenylglycine **30b** from nitrile **30** was reduced by several percent due to the limited spontaneous decomposition of the substrate to yield benzaldehyde.



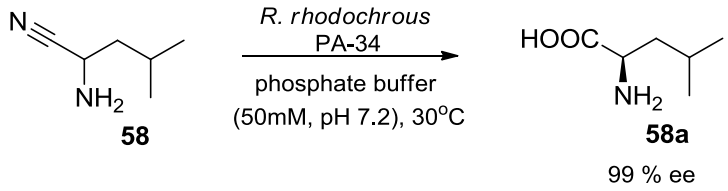
Scheme 1.33: Nitrilase catalysed conversion of 2-phenylglycinonitrile demonstrated by Brady *et al.* [121].

Kiziak *et al.* also reported the hydrolysis of 2-phenylglycinonitrile **30** by *Pseudomonas flourseceńs* EBC191, and produced acid **(*S*)-30b** with an enantiomeric excess of 35 % [86] (Scheme 1.34). The conversion of nitrile **30** also resulted in the formation of relatively low amounts of amide **30a** (8 %) and this proved to be enantioselective, giving the (*S*)-isomer in 92 % ee [86].



Scheme 1.34: *Pseudomonas flourseceńs* EBC191, catalysed conversion of 2-phenylglycinonitrile.

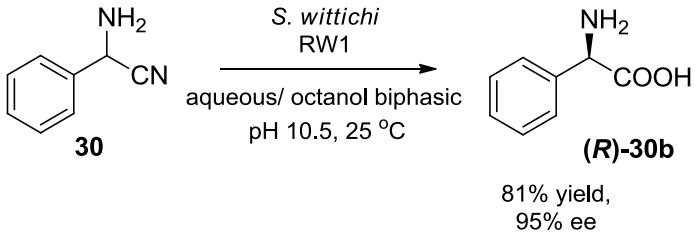
Rhodococcus rhodochrous PA–34 isolated from soil as a propionitrile-utilising microorganism, was found to hydrolyse several α–aminonitriles to optically active amino acids [100]. This isolate was chosen after 65 strains were screened for their ability to produce valine **58a** from α–aminoisovaleronitrile **58** (Scheme 1.35). The optical purity of the **58a** produced exceeded 99 % ee.



Scheme 1.35 Nitrilase catalysed hydrolysis of α–amino-isocapronitrile by *R. rhodochrous* PA–34.

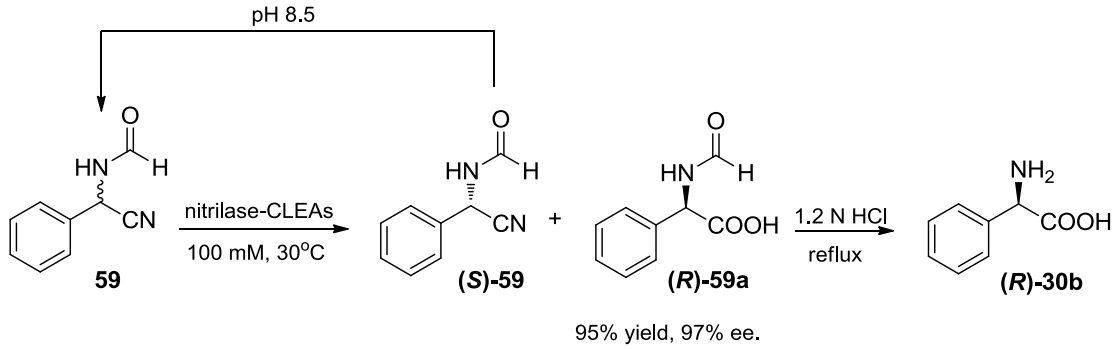
Additionaly L-amino acids were produced where substrates had long or branched alkyl substituents, whereas D-amino acids were produced from α–aminopropionitrile. Although this isolate demonstrated a high substrate specificity for benzonitrile (695.2 % relative activity to α–amino-isocapronitrile), it also showed high activity for alkane nitriles and α–aminoalkane nitriles [100].

More recently, Qiu *et al.* prepared **(*R*)-30b**, from racemic nitrile **30** using the nitrilase of *S. Wittichi* RW1 in an aqueous-1-octanol biphasic system (Scheme 1.36). Due to the efficient suppression of the decomposition of **30** at pH 10.5, a maximum yield of 81 % and 95 % ee was obtained [119].



Scheme 1.36: Production of D-phenylglycine by the nitrilase of *S. wittichi* in an aqueous/ octanol biphasic system at pH 10.5.

Qiu *et al.* also reported the preparation of D-N-formyl-phenylglycine **59** by a nitrilase-CLEA mediated dynamic kinetic resolution. The CLEA was prepared with the nitrilase from *S. Wittichi* RW1 as discussed above, and D-acid **(R)-59a** was produced with a yield of 95 % and 97 % ee at pH 8.5, as shown in Scheme 1.37 [122]. This product could be hydrolysed to give free amide **(*R*)-30b** without racemisation of the chiral centre.



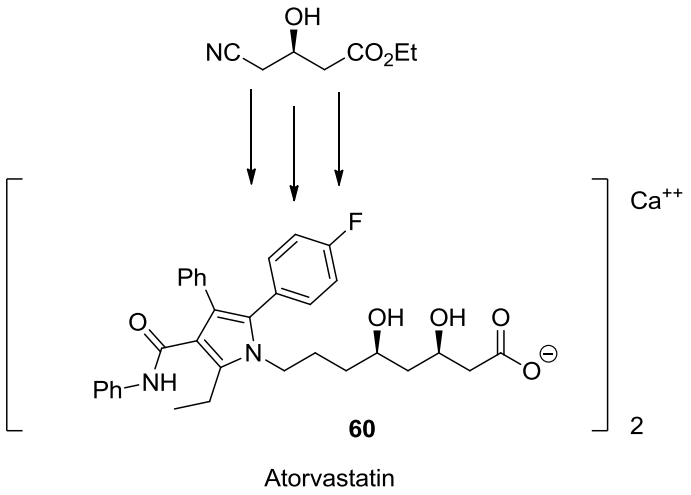
Scheme 1.37: Preparation of D-N-formyl-phenylglycine using CLEAs containing the nitrilase from *S. Wittichi* RW1.

1.6.2 NITRILASE CATALYSED HYDROLYSIS OF CHIRAL SUBSTRATES BEARING A β-STEREOCENTRE

Biotransformations of nitriles having a chiral centre remote from the cyano or amido functional group have been reported to proceed with, in most cases, disappointingly low enantioselectivity and chemical yield [123-127]. It is generally believed that the movement of a stereocentre from the reactive site (α position to the functional group) to a remote region gives rise to the decrease of enantioselecivity. However, this notion may not be true for enzymatic reactions since the chiral recognition site of an enzyme might be located some distance from the catalytic centre [128].

NITRILASE CATALYSED HYDROLYSIS OF β-HYDROXYNITRILES

An example of industrial biotransformations incorporating the use of a nitrile catalysed hydrolysis of a β-hydroxynitrile, is the desymmetrisation of 3-hydroxyglutaronitrile for the preparation of a statin side chain intermediate. Statins are HMG-CoA reductase inhibitors that are used for the treatment of hypocholesterolemia. Atorvastatin (Lipitor) **60**, [129, 130] a statin launched in 1997, is now one of the world's most profitable drugs (Scheme 1.38). The key step in its synthesis employs a highly volume-efficient nitrilase-catalysed desymmetrisation of the meso-dinitrile species 3-hydroxyglutaronitrile, forming the chiral building block *(R)*-ethyl-4-cyano-3-hydroxybutyric acid in 100 % yield and 99 % ee, developed by DeSantis *et al.* [131] [132].

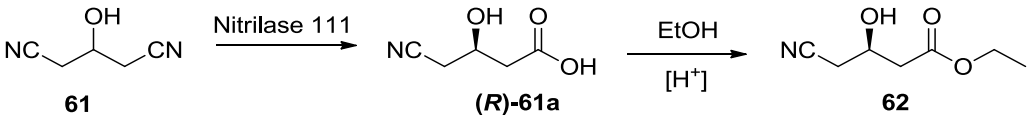


60

Atorvastatin

Scheme 1.38: Preparation of Atorvostatin (Lipitor) [132].

In their development of the route, DeSantis *et al.* believed the few known nitrilases from cultured sources were not efficient catalysts for the hydrolysis of related *β*–hydroxynitrile substrates. They examined their isolate library for it's ability to desymmetrize the readily available prochiral substrate 3-hydroxyglutaronitrile **61** to afford 4-cyano-3-hydroxybutyric acid **(*R*)-61a** (Scheme 1.39) which once esterified, may be used as an intermediate for the production of Lipitor **60** [131].



61 **(*R*)-61a** **62**

Scheme 1.39: Nitrilase catalysed desymmetrisation of prochiral 3-hydroxyglutaronitrile, for the formation of a chiral intermediate for the production of Lipitor.

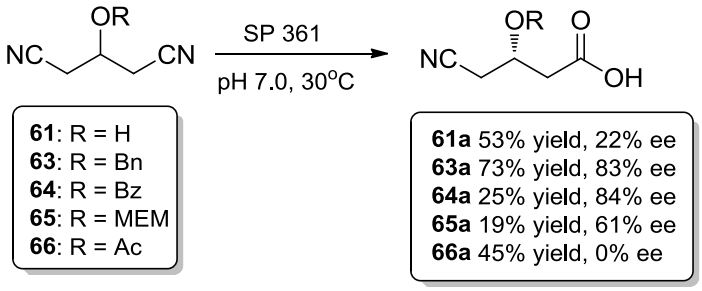
Four unique enzymes that provided the required product **(*R*)-61a** with >95 % conversion and >90 % ee were identified. This process was operated on a 1.0 g scale (240 mM) and after incubation for 22 hours at 22 °C the required acid **(*R*)-61a** was obtained in 98 % yield and 95 % ee. The same screening program also identified 22 nitrilases that afford the opposite enantiomer **(*S*)-61a** with 90-98 % ee [131].

DeSantis *et al.* later reported the application of a novel directed evolution technique, the gene site saturation mutagenesis (GSSM) to help improve the enantioselectivity and substrate tolerance of the above nitrilase enzyme towards nitrile **61**. The GSSM technology involved replacing amino acids with each of the other 19 naturally occurring amino acids, and created a comprehensive library from a wild type nitrilase. There were 10,528 genetic variants and 31,584 clones to be screened. To do this, DeSantis *et al.*

developed a novel high-throughput screening strategy which will be discussed in detail in the next section [133]. It was found that amino acid changes at 17 different residues lead to enhanced enantioselectivity over the wild type strain. The mutants were also examined at 2.25M substrate concentration and the serine, histidine and threonine 190 variant resulted in significant improvement. The Ala190His mutant was found to be the most selective with complete conversion to **(*R*)-61a** in 98 % ee within 15 hours [133].

The nitrilase enzyme (BD9570) previously discovered by DeSantis *et al*. [131] was examined by Bergeron *et al.* [132]. Under optimized conditions the biotransformation using rehydrated nitrilase was shown to form the 3-hydroxy-monoacid **(*R*)-61a** in 99 % conversion within 16 hours at pH 7.5. Enantioselectivity data was not reported.

In their work, Crosby and Turner *et al*. prepared a series of protected 3-hydroxyglutaronitrile derivatives via protection of 3-hydroxyglutaronitrile at the hydroxyl position (Scheme 1.40) [134].

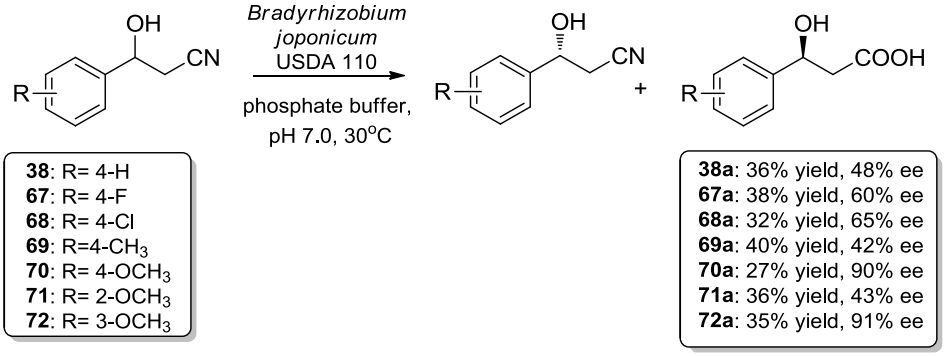


Scheme 1.40: Hydrolysis of *β*–hydroxy substituted dinitriles analogues with SP 361.

The analogues were converted to the corresponding nitrile carboxylic acids with no evidence for the formation of nitrile amides. Without protection 3-hydroxyglutaronitrile **61** was hydrolysed to form the monoacid in 52 % yield and 22 % enantiomeric excess. The efficiency of the hydrolysis was dramatically increased by incorporating a benzyl docking group **63** and both the yield and the enantioselectivity increased to 73 % and 83 % respectively. It was thought that the aromatic ring may serve to enhance the enantioselectivity [134]. The configuration of acid formed was found to be (*S)* in each case.

Kamila *et al.* found that the nitrilase bll6402 from *Bradyrhizobium japonicum* USDA 110, was found to enantioselectively hydrolyse aromatic *β*–hydroxy nitriles **38** and **67-72** to their corresponding enantioenriched *β*–hydroxy carboxylic acids (Scheme 1.41).

As discussed previously, this nitrilase was an efficient catalyst for the hydrolysis of mandelonitrile and its derivatives [104].



Scheme 1.41: Enantioselective hydrolysis of b-hydroxynitriles batalysed by nitrilase bll6402.

The hydrolysis of 3-hydroxy-3-phenylpropionitrile **38** formed the corresponding (*S*)-acid **38a** in 36 % yield and 48 % ee. Analysis of the aryl substituted biotransformation products **67-72** suggested that the substituent on the benzene ring did not dramatically alter enzyme activity but exerted some effect on enantioselectivity [135]. The *para*–methoxy substituted analogue **70** resulted in the highest enantioselectivity 90 % ee, whereas the *ortho*-methoxy analogue **71** resulted on the formation of the corresponding acid **(*S*)-71a** in 43 % ee.

Kamila *et al.* suggested that the enantioselectivity of the nitrilase was influenced by both the steric and electronic factors of the substituents on the aryl ring [135]. It was surprising that the nitrilase was capable of hydrolyzing β-hydroxynitriles as the biotransformation of mandelonitrile, an α-hydroxynitrile was not found to be enantioselective (Figure 1.6). This was in contrast to the observation that a chiral carbon atom at the β-position would not be recognized as readily as those possessing a chiral centre at the α-position.

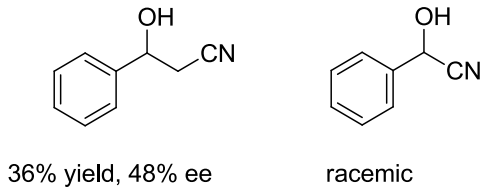


Figure 1.6: Comparison of the nitrilase catalysed hydrolysis of β–methyl, β-hydroxy and α-hydroxy nitriles.

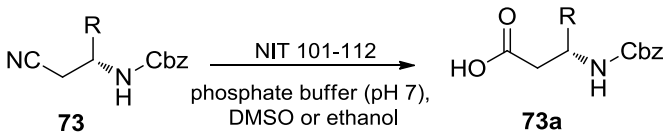
Brady *et al.* examined 6 different enzymes towards 33 structural nitrile analogues including *β*-hydroxynitriles. The nitrilase enzymes included that of *P. fluorescens* DSM 7155, *Arabdopsis thaliana* and four other sources obtained from BioCatalytics, USA [121]. Each enzyme demonstrated a characteristic substrate profile on a wide range of substrates. Only two nitrilases, *P. fluorescens* and commercial nitrilase 1006 were active against unsubstituted phenylacetonitriles, *α*-substituted arylaliphatic nitriles including 2-phenylpropionitrile, mandelonitrile and 2-phenylbutyronitrile. Even the enzymes that accepted *α*-substituents were slow to convert 2-phenylbutyronitrile. Having investigated 3-phenylpropionitrile and 3-hydroxy-3-phenylpropionitrile, Brady *et al.* found that the activity decreased with the addition of a *β*–hydroxy substituent for all nitrilase enzymes. This decrease in activity may be contributed to the steric bulk and *β*-hydroxy substituent [121].

NITRILASE CATALYSED HYDROLYSIS OF β-AMINO NITRILES

β-amino acids are constituents of compounds such as the antitumor drug Taxol, the antifungal antibiotic Cispentacin, and the antidiabetic drug Sitagliptin [136], [137]. Single enantiomer *β*-amino amides and acids can be obtained through the enzymatic hydrolysis of the related nitriles [137]. However, the biotransformation of unprotected amino nitriles is severely hindered due to difficulties arising with reaction monitoring and product isolation of these strongly polar compounds.

The effect of protecting groups has been examined by Preiml *et al*. who evaluated the use of N-protecting groups with respect to their applicability in biotransformation of *β*-amino nitriles using a NHase/amidase system [138]. However, there is less literature available dealing with the nitrilase catalysed hydrolysis of *β*-amino nitriles.

Several commercially available nitrilases were investigated with regard to their potential to hydrolyse protected β^3-amino nitrile **73** into their corresponding *N*-protected β^3-amino acid **73a**. The best hydrolysis results were achieved for the *N*-Cbz-β^3-amino nitrile derived from L-alanine using the NIT-107 enzyme (Scheme 1.42). However, no biotransformation into the corresponding acids was observed for *N*-sulfonylamide β^3-amino nitriles (139).

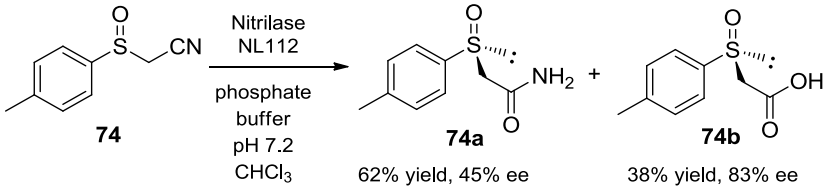


Entry	Enzyme	Nitrile %	Acid %
1	NIT-104	54	46
2	NIT-107	0	100
3	NIT-108	80	20
4	NIT-109	54	46
5	NIT-110	55	45
6	NIT-112	55	45

Scheme 1.42: Biotransformation of β^3-amino nitriles into the corresponding carboxylic acid using nitrilase [139].

NITRILASE CATALYSED HYDROLYSIS OF β-SULFINYL NITRILES

The enantioselective hydrolysis of cyanomethyl *p*-tolylsulfoxide **74** into the corresponding amide **74a** and acid **74b** as shown in Scheme 1.43, was examined using several nitrilases by Kiełbasinski *et al.* [140].



Scheme 1.43: Nitrilase catalysed hydrolysis of cyanomethyl p-tolylsulfoxide, adapted from [140]

In the case of most nitrilases examined, the reactions were not selective and resulted in the formation of both amide **74a** and acid **74b**. Examining the stereochemical outcome of the experiments provided an insight into the catalytic mechanism, as both the amide and acid products exhibited the same stereochemical configuration. Kiełbasinski *et al.* suggested that this was proof of a bidirectional mechanism in which both products are formed concurrently by the nitrilase. To further probe the catalytic mechanism, they examined the hydrolysis of amide **74a** by the various nitrilases. However, acid was not observed. This may also suggest that externally added amide cannot enter the active site, whereas if the amide was generated within the cells by hydrolysis of the nitrile it could still react. Examining the hydrolysis of the enantiopure nitrile substrate, they found that the preferential formation of amide was from the (*R*)-nitrile whereas acid was formed from the (*S*)-nitrile. This demonstrates that the absolute configuration of the substrate had a substantial effect on the reaction outcome [140].

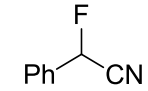
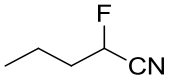
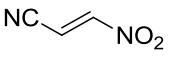
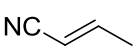
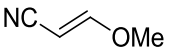
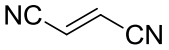
1.6.3 NITRILASES DISPLAYING NHASE ACTIVITY

Nitrilases usually catalyse the addition of two equivalents of water to a nitrile, to directly produce the corresponding carboxylic acid and ammonia. However in some instances, products other than carboxylic acids such as amides have been generated [141]. This was noted when reviewing the literature above. The mechanisms proposed in the literature for the formation of amide will also be outlined.

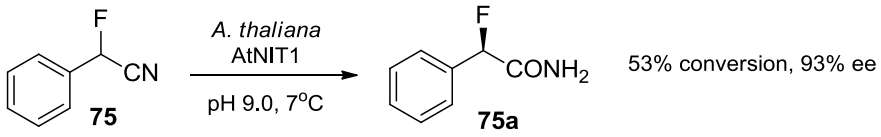
The substrate scope of a nitrilase AtNIT1 from *Arabdopsis thaliana* was investigated using structurally varied aromatic and aliphatic nitriles (Table 1.10) by Osswald *et al.* [141]. The biotransformation was found to be strongly dependent on the nature of the substituent at the α- position in aromatic systems (Table 1.10 entry 1 *vs* Table 1.10 entry 2) [141].

As amide formation was thought to occur when the substrate contained electron withdrawing substituents at the α–position, Osswald *et al.* examined three substituted acrylonitrile analogues. The biotransformation of 3-nitroacrylonitrile (Table 1.10, entry 3) resulted in the formation of the corresponding amide as the major product. Whereas when substrates contained electron donating substituents (Table 1.10, entry 5), acids were found to be the major products.

Table 1.10: **Product distribution of amide to acid in the AtNIT1 catalysed nitrile hydrolysis. Adapted from [141]**

Entry	Substrate	Relative activity (%)	Product distribution (amide : acid)
1		26	85 : 15
2		127	5 : 95
3		12	95 : 5
4		27	1 : 99
5		24	5 : 95
6		47	93 : 7

The resolution of (*R*,*S*)-2-fluoroarylacetonitriles **75** by AtNIT1 produced the corresponding (*R*)-amides **47a**, and not the expected carboxylic acids as the major products in 88 to 93 % ee at 53 % conversion (Scheme 1.44) [141].



Scheme 1.44: Conversion of 2-fluorobenzyl cyanide to *(R)*-2-fluoro-2-phenylacetamide by AtNIT1.

The substrate specificity of a nitrilase ZmNIT2 isolated from maize was examined with a structurally diverse collection of nitriles by Mukherjee *et al.* [142]. The nitrilase of ZmNIT2 was found to be active towards aliphatic nitriles, phenylacetonitriles and aromatic nitriles. In these cases acids were the major products with concomitant formation of amides. During the hydrolysis of aliphatic nitriles the acid to amide ratio varied depending on the structure of the aliphatic nitriles [142]. Amide formation increased with increasing chain length of the substrate as can be seen in Figure 1.7.

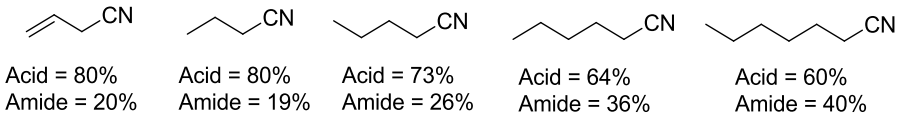
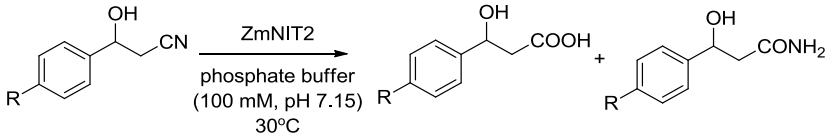


Figure 1.7: Increasing amide formation with increasing chain length of aliphatic nitriles.

Mukherjee *et al.* performed a kinetic study of 3-(4-fluoropheny)-3-hydroxypropionitrile and 3-(4-methylphenyl)-3-hydroxypropionitrile to gain insight into the mechanism of amide formation. By examining the acid to amide ratio at various time points, no significant change for either substrate over the time course of the reaction was observed. The concurrent formation of amide and acid clearly suggested that the acid was not produced from amide and enzyme inactivation was proposed to be responsible for the constant acid to amide ratio [142].

When *β*–hydroxynitriles were examined with the nitrilase ZmNIT2, the major products were amides with the conversion of 63-88 %, along with acids as minor products (Table 1.11). However, this nitrilase showed little enantioselectivity (less than 40 %) in the hydrolysis reaction [142]. The enantioselectivity data for the substrates outlined in Table 1.11 (entry 1-6) was not reported in this study.

Table 1.11: Hydrolysis of *β*-hydroxynitriles catalysed by nitrilase ZmNIT2 [142].



Entry	Substrate	R	Conversion (%)	
			Acid	Amide
2	**67**	4-F	17 %	73 %
3	**68**	4-Cl	13 %	85 %
1	**69**	4-CH_3	14 %	85 %
5	**70**	4-OCH_3	37 %	63 %
4	**76**	4-Br	12 %	88 %
6	**77**	4-CH_3CO	27 %	68 %

Mukherjee *et al.* assessed the importance of intra–molecular bonding (and the formation of a proposed 6 membered ring) during the formation of amide. They examined the biotransformaton of 4-phenylbutyronitrile and 4-hydroxy-4-phenylbutyronitrile (Figure 1.8).

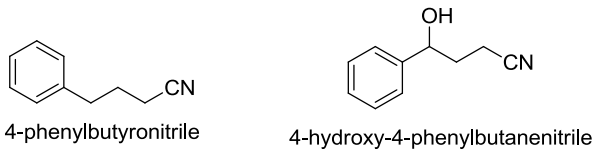
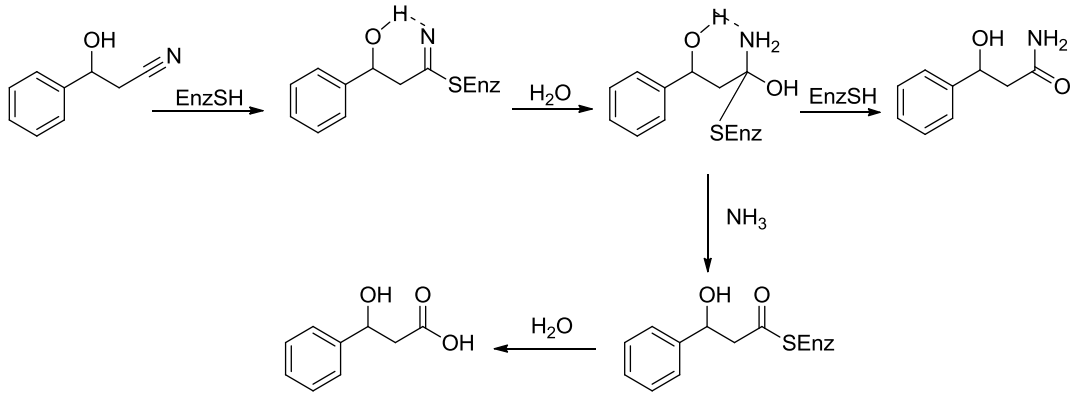


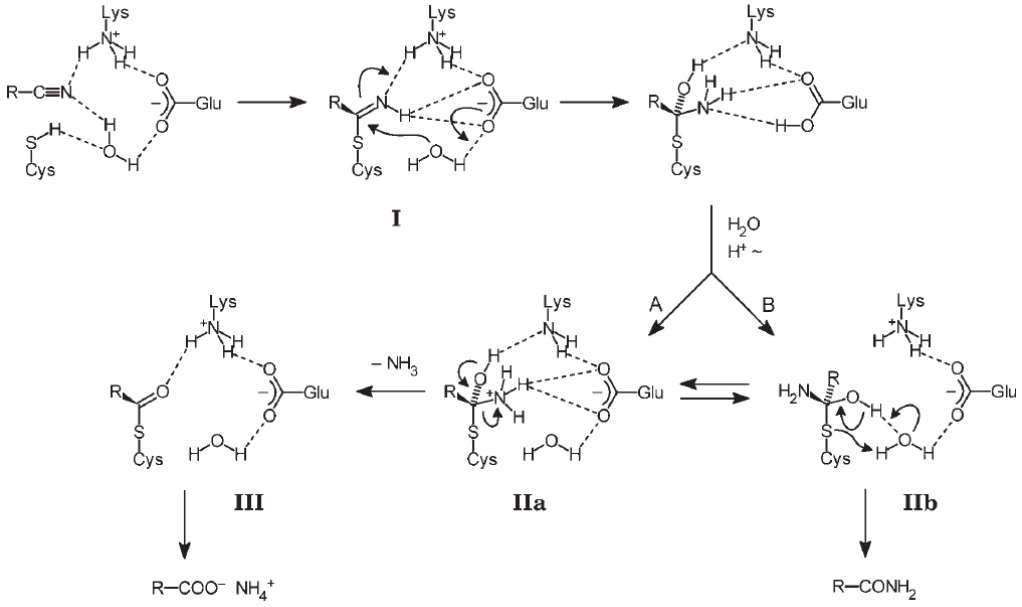
Figure 1.8 Nitriles chosen to assess the importance of intra-molecular bonding in amide formation

In each case carboxylic acids were formed as the major products [142]. As the formation of a seven membered ring would occur in the case of the above substrates, it was proposed that *ψ*-hydroxyl groups did not promote amide formation. This indicated that the *β*–hydroxy group played a critical role in formation of large amounts of amide (Scheme 1.45).



Scheme 1.45: Proposed nitrilase mechanism by Mukerjhee *et al.* [142]

A nitrilase mechanism to account for amide formation in mandelonitrile **56** hydrolysis in the presence of *P. fluroescens*, was also proposed by Fernandes *et al.* In this case amides would result from cleavage of the C-S bond in the tetrahedral intermediate (IIb) (Scheme 1.46 below). They suggest this elimination of ammonia from the tetrahedral intermediate *via* pathway B, occurs when an electron demanding R-group destabilizes the positive charge on the reactant N (in IIa) [143].



Scheme 1.46: Proposed mechanism for the formation of acid (A) and amide (B), adapted from [143]

Using this mechanism, Fernandes *et al.* proposed that amides were major products of the biotransformation of substrates containing electron withdrawing substituents at the α–position and that of π conjugated nitriles [143].

Mateo *et al.* found that during the hydrolysis of mandelonitrile **56** with *P. fluorsecens* EBC191, *(S)*-mandelamide **(*S*)-56b** (40 %) accompanied the formation of mandelic acid **(*R*)-56a**. The hydrolysis of enantiomerically pure *(R)*- and *(S)*- mandelonitrile **56** was investigated and a minor amount of the amide was formed from the *(R)*- enantiomer (acid/amide = 8), whereas the amide was a major product from the *(S)*-enantiomer. The acid to amide ratio was found to stay constant under the reaction conditions suggesting that the formation of amide was not an intermediate during the formation of the acid. This was also supported by the fact that no acid was produced when the enzyme was incubated with the amide product. Mateo *et al.* concluded that amides are products of the nitrilase and that the stereochemical configuration of the nitrile exerts a major

influence on their formation, in addition to the electronic density at the α–carbon atom [116].

1.7 SCREENING STRATEGIES FOR NITRILE HYDROLYZING ENZYMES

Among the approaches utilised in the search for new catalytic activities, high throughput microbial screening and the enrichment technique are considered to be the most reliable [144]. The initial goal is to identify microorganisms capable of performing the desired transformation. Cultures may be isolated from soil, seaweed, or they may be obtained in pure form from standard culture collection centres [71].

1.7.1 SELECTION AND ENRICHMENT TECHNIQUES

Selection methods are based on using a selective medium which allows only the colonies with the desired enzyme to grow. Selection is a method of choice for isolation of nitrile hydrolysing microorganisms as nitrilase and amidase convert their substrates into ammonia which are utilised as the N source. Therefore, microorganisms producing the enzymes of interest are often able to grow in the presence of nitriles or amides as sole N sources [145]. Most nitrile-converting micro-organisms were obtained by using a selection technique designed as an enrichment culture, which consists of repeated subcultivations of mixed microbial populations with the target compound [145]. Therefore, they are more difficult to develop and provide only qualitative results but have very high throughput [128].

Different microbial strains are selected when using different nitrile or amide compounds. The strains usually show high-relative activities for substrates which were used during the selection procedure. Selection criteria such as temperature or pH can also be varied according to the desired properties of the enzyme. However, the enrichment technique has some disadvantages as some nitriles and amides inhibit growth. In this case structurally similar substrates can be used. For instance microorganisms catalysing the hydration of acrylonitrile were isolated by enrichment with acetonitrile or isobutyronitrile [145].

Enrichment strategies may provide a relationship between the nitrogen source and enzyme systems that are produced by the isolates and the enzyme specificities for the utilization of the nitriles [128]. Thus an enrichment strategy may be performed to obtain a wide range of nitrile degrading isolates whose properties may be analysed with respect

to their selectivity and activity towards differently substituted nitriles [146]. In general, nitrile-hydrolysing bacteria become readily available in enrichments with various nitriles. The isolates usually show high relative activities against their selective nitrile [128]. This technique has proven to be a very powerful tool to obtain highly active bacteria for nearly every possible target substrate [146]. Kaul *et al.* demonstrated that selection of a substrate analogue as a sole source of carbon or nitrogen during enrichment culture, gives access to bacterial enzyme systems that are highly adapted to the target substrate [147]. Kaul *et al.* identified three new bacterial isolates, *Pseudomonas putida*, *Microbacterium paraoxydans,* and *Microbacterium liquefaciens* with activity towards mandelonitrile. These isolates were obtained when phenylacetonitrile was used as the sole source of carbon and nitrogen during the enrichment stage [90].

Layh *et al.* performed enrichments with 2-phenylpropionitrile, 2-(2-methoxyphenyl)propionitrile, ibuprofen nitrile, 2-phenylbutyronitrile, naproxen nitrile and benzonitrile as the sole source of nitrogen (0.5 mmol). A selection of the isolates reported using this enrichment technique, including the enzymes induced and the activity of these enzymes, are outlined in Table 1.12. The isolated strains were all Gram-negative bacteria [148].

Table 1.12: Nitrile hydrolysis and enantioselectivity of new isolates towards their respective enrichment substrates, adapted from Layh *et al.* [149].

Strain	**Nitrile source**	**Enzyme system**	**ee. (%) of acid or amide after hydrolysis of (% conversion of racemate)**	
			Nitrile	Amide
Pseudomonas sp. A3	PPN	Nitrilase	63 % (*R*) acid (28 %)	
Rhodococcus sp. C311	NN	NHase	99 % (*S*) acid (40 %)	
		Amidase		99 % (*S*) acid (43 %)
Rhodococcus sp. OP8E	PBN	NHase	99 % (*S*) acid (25 %)	
		Amidase		99 % (*S*) acid (29 %)
Rhodococcus sp. OP5M	MPPN	NHase	41 % (*S*) acid (23 %)	
		Amidase		99 % (*S*) acid (41 %)
Alcaligenes B1b	KA	Amidase		21 % (*S*) acid (17 %)

PPN 2-phenylpropionitrile, MPPN 2-(2-methoxyphenyl)propionitrile, PBN 2-phenylbutyronitrile, NN naproxen nitrile, KA ketoprofen amide. Enantioselectivity of the enzymes was usually determined with the nitrile used as a substrate during the enrichment.

By examining the ability of the isolates to catalyse the hydrolysis of various nitriles and amides, Layh *et al.* demonstrated that the isolates displayed a broad substrate range. A number of isolates exhibited both nitrilase and NHase/amidase enzymes. The nitrilases were *(R)*- or *(S)*- selective and exhibited only low enantioselectivity in contrast to the amidase which were *(S)*- selective and had high enantioselectivity. Layh *et al.* also

determined that the structure of the nitrile is of crucial importance in the isolation of nitrile-hydrolysing bacteria and in determining the nature of the nitrile hydrolysing system, which is again demonstrated by the data reported in Table 1.12.

Screening methods are largely readily available and quantitative, but every single colony must be analysed for enzyme activity. Reaction products have been most often separated by chromatographic methods prior to spectrophotometric quantitation or determined in coupled reactions [145]. Liquid or gas chromatography is useful for accurate analysis of the reaction products and especially for enantioselectivity assays, but unsustainable for high throughput screens. Reverse phase high performance liquid chromatography coupled with UV detection is used whenever possible for most of the aromatic and arylaliphatic compounds [145]. Because of the low absorbance of cyano, amido and carboxy groups, this method is not suited for saturated aliphatic nitriles, which are mainly analysed by gas chromatography coupled with flame ionisation detection. GC or HPLC with mass spectrometric detection is invaluable for identification of unknown compounds [145].

1.7.2 NITRILE HYDROLYSING ACTIVITY ASSAYS

Most of the methods currently used to measure activity of microbes containing nitrilase/ amidase enzyme systems, rely on the measurement of ammonia formed from nitrile hydrolysis [145]. Colorimetric or fluorimetric determination of ammonia as a common product of nitrilases and amidases, offers a promising fundament for the development of faster screening methods which is discussed below [145].

The parameters (detection and quantitation limits, selectivity, safety, costs etc) of several analytical methods are available for the detection of ammonia i.e colorimetric Nessler's, indophenols and indothymol methods, fluorimetric modified Roths method, chemiluminiscent methods and ion selective electrodes were reviewed by Molins-Legua *et al.* in 2006 [150]. Several of the above methods are briefly discussed below.

It must be noted that ammonia determination may not always provide a true estimation of the enzyme activity for several reasons. Ammonia may be formed or utilised by side reactions in cell lysates or whole cells and the enzyme activity leading to the formation of amide, which makes up a considerable part of the nitrilase product, will not be reflected [145].

NESSLERISATION AS A METHOD FOR AMMONIA DETECTION

Nesslerisation refers to the determination of ammonia using the Nesslers method. Addition of Nesslers reagent to a solution containing ammonia produces an orange brown colour after development for a certain amount of time [151].

In the Nesslerisation reaction NH_3 and NH_4^+ react with an excess of Nessler's reagent to form a yellow brown complex as shown in Figure 1.9. The intensity of the colour is proportional to the ammonia concentration and is read at 425 nm. The sensitivity of the assay is from 20 μg/L up to 5 mg/L. Concentrations above this range can be diluted to bring them into the linear range of the assay.

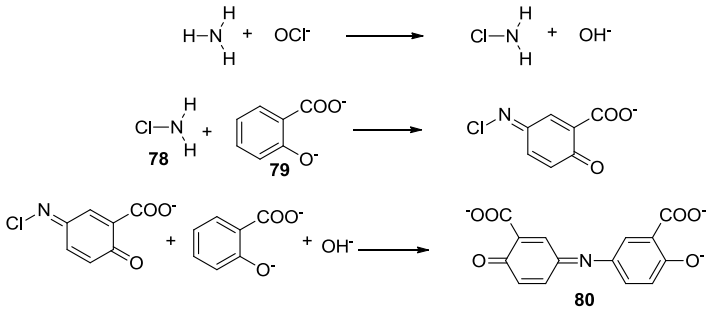
$$2(HgI_4)^{2-} + NH_3 + 3OH^- \longrightarrow Hg_2ONH_2I + 7I^- + 2H_2O$$

Figure 1.9: Chemical reaction which occurs in the Nesslers assay for the detection of ammonia.

Unfortunately, the assay can suffer from interference with several inorganic ions and some organic solvents. Calcium and magnesium ions for example, can cause cloudiness of the reagent [128]. Nesslerisation is a common method for determination of nitrile hydrolysing activity and may be used to measure quantitatively, the concentration of ammonia produced by the reaction [145].

THE BERTHELOT METHOD FOR AMMONIA DETECTION

The Berthelot method for measuring nitrile-hydrolysing activity is referred to as the phenol/hypochlorite method. In this method, ammonia reacts with hypochlorous acid to produce chloramine **78**. The reaction of **78** with an alkaline phenol **79** results in the formation of indophenol blue **80** [152]. The corresponding chemical reactions are depicted in Scheme 1.47 [153]. Ammonia liberated from nitrile hydrolysis is quantified colorimetrically at 640 nm.



Scheme 1.47: Generalised method of the Berthelot reaction for ammonia detection, adapted from [153]

The procedure requires heating of the test solution at 90 °C for 30 minutes or 100 °C for 5 minutes [154]. Heating is disadvantageous as vaporisation of toxic phenols can occur. Also, insoluble MnO_2 (used as a catalyst) is formed, which interferes with the spectrophotometric determination [155]. There are several disadvantages to this method other than the use of corrosive reagents, including the requirement for large samples [156].

pH SENSITIVE INDICATORS AS A METHOD FOR AMMONIA DETECTION

Bromothymol blue (BTB) **81** as shown in Figure 1.10, is a pH indicator for weak acids and bases. BTB appears yellow when protonated and blue when deprotonated. It is a blue green colour in a neutral solution. The deprotonated form produces **82** on reaction with ammonia and results in a highly conjugated structure **83**, accounting for the difference in colour as depicted in Scheme 1.48.

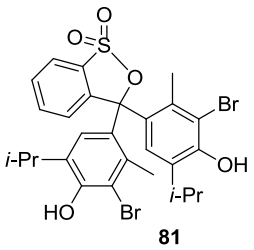
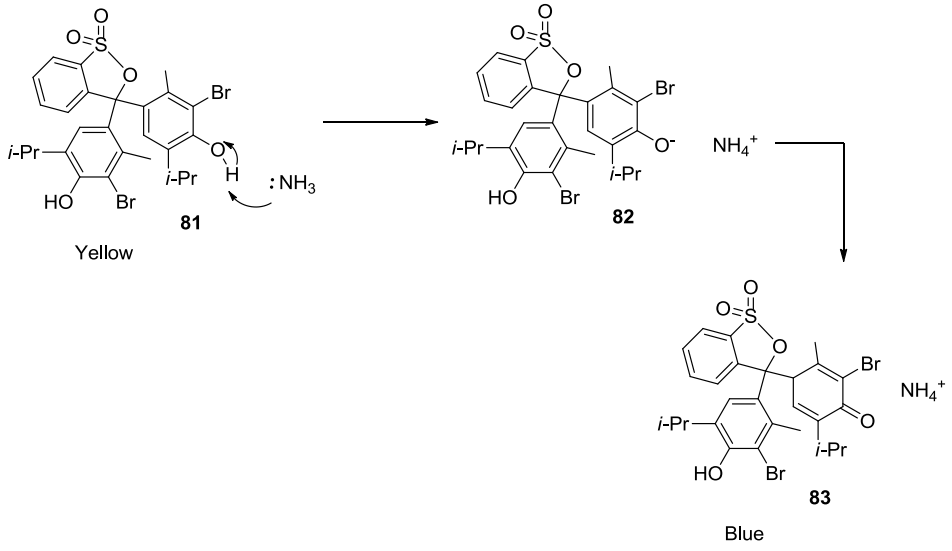


Figure 1.10: Chemical structure of Bromothymol blue.



Scheme 1.48: Mechanism of phenolic proton extraction from BTB by ammonia molecule adapted from [157].

Banerjee *et al.* [90] described the measurement of nitrilase activity in several microorganisms using a pH-sensitive indicator-based colorimetric assay based on the

reaction concepts shown in Figure 1.11. This method is based on a pH drop that occurs as the rection proceeds due to formation of the acid. The pH drop is reflected by the colour change of the indicator, provided the colour profile of the indicator falls into the pH range of the enzyme activity [158].

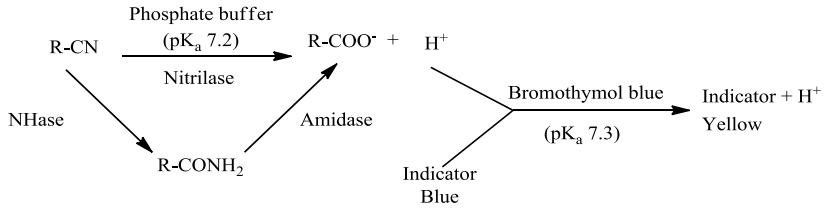


Figure 1.11: Screening of nitrile hydrolysing microorganisms with pH indicators [158].

The reaction mixtures consisted of (BTB) **81** as indicator, mandelonitrile as substrate, and whole cell culture as the source of enzyme. The colour change (green to yellow) could be monitored over two hours as shown in Figure 1.12.

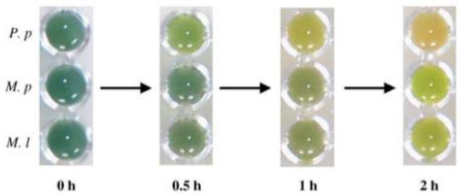
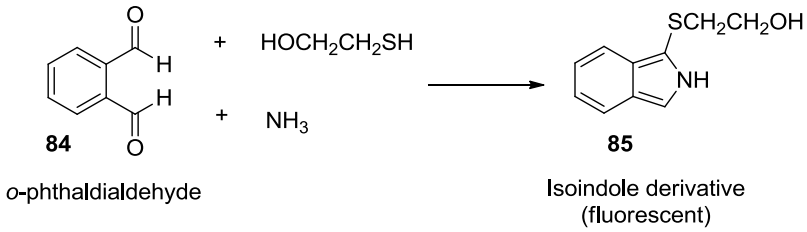


Figure 1.12: **Visualisation of nitrile hydrolysis using the pH sensitive indicator based assay by Kaul *et al.* [90]**

This method suffers from limited applicability due to poor sensitivity and the possibility of false reactions due to generation of metabolic acids by the microorganisms [158].

FLOURIMETRIC METHODS FOR THE MEASUREMENT OF AMMONIA

Banerjee *et al.* described a fluorimetric assay method for the measurement of nitrilase activity in which 3-cyanopyridine was hydrolysed to nicotinic acid using *Rhodococcus rhodochrous* [159]. The ammonia liberated in the reaction is reacted with a buffered OPA (*O*-phthaldialdehyde) **84** solution containing 2-mercaptoethanol and allowed to form a fluorescent isoindole **85** derivative as shown in Scheme 1.49.



Scheme 1.49: formation of fluorescent isoindole derivative.

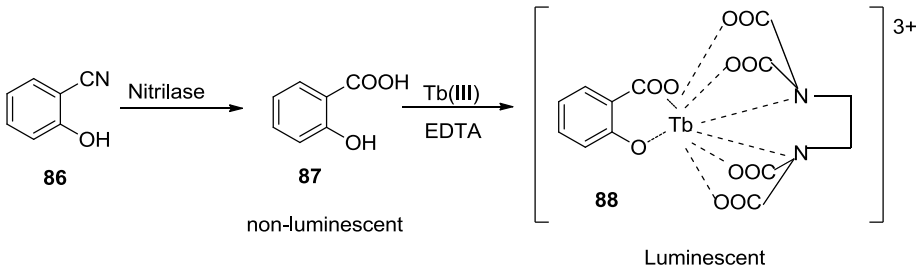
The fluorescence intensity of the resultant fluorochrome is measured using excitation and emission wavelengths of 412 and 467 nm, respectively [51]. Unknown concentrations of ammonia were determined from a standard curve constructed using known concentrations of NH_4Cl. One unit of nitrile hydrolysing activity was determined as the amount capable of generating 1 μmol of NH_3/ min per mg of cells.

1.7.3 HIGH THROUGHPUT SCREENING STRATEGIES

The major hurdle in the quest to find novel nitrilase activities from nature as well as *via* directed evolution is the lack of a robust high-throughput assay for nitrilases. At present apart from the conventional methods, few high-throughput methods for efficient screening of nitrilase activity are documented [160, 161].

A review of screening methods has been published by Martinkova [128] and Gong [71]. Many strategies facilitate the screening of novel nitrilase producing microorganisms through detection of ammonia released during the hydrolysis reaction. In some cases the colorimetric activity screening assays as described previously have been applied to 96 and 384 well plate systems for the selection of nitrile metabolising isolates [158].

Outside of ammonia detection, Zhu *et al.* reported a high-throughput screening strategy for detecting nitrilase activity. In this case o-hydroxy-benzonitrile **86** derivatives can be hydrolysed to the corresponding salicylic acid derivatives **87**, which upon binding to Tb_3^+, serve as a photon antenna and sensitise Tb_3^+ luminescence **88** (Scheme 1.50).

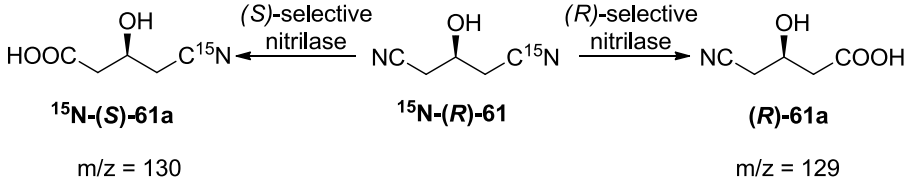


Scheme 1.50: Design principle of the time-resolved luminescent nitrilase probes [160].

Because the detection was performed on a 96- or 384- well plate, the activity of the nitrilases from microorganisms could be determined quickly [160].

DeSantis *et al.* developed a novel high-throughput screen based on spectrometric detection of isotopically differentiated products from the nitrilase catalysed hydroysis of

3-hydroxyglutaronitrile. In this assay the screening substrate and the target substrate differ only by an isotope label ensuring that the identified nitrilases will perform equally well on the native unlabeled substrate [133]. The use of chiral ^{15}N-(*R*)-3-hydroxyglutaronitrile **61** was important since the labelled atom would be removed from the product upon hydrolysis with a (*R*)-selective nitrilase, whereas the use of a (*S*)-selective nitrilase results in the detection of labelled N atom as depicted in Scheme 1.51. The hydrolysis products differ by one mass unit and are distinguished and quantified by mass spectrometry [133]. Using directed evolution there were 10,528 genetic variants and 31,584 clones to be screened using this method. [133]. It was found that amino acid changes at 17 different residues led to enhanced enantioselectivity over the wild type strain [133].



Scheme 1.51: High-throughput screen of isotopically labeled 3-hydroxyglutaronitrile based on mass spectrometry [133].

CONCLUSIONS

In summary, this Chapter has detailed the advantages of the use of biocatalysts, in particular the use of nitrilase enzymes in the production of chiral carboxylic acids and amides. Particular attention was given to nitrilase substrate specificity and the effect that analogues bearing α- and β-stereocentre have on the reaction outcome. Various techniques to screen for nitrile hydrolysing enzymes based on the detection of ammonia have also been outlined. Chapter 2 will describe the experimental work conducted to screen an isolate library for nitrile hydrolysing enzymes towards the chosen substrates (β-hydroxy nitriles).

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CHAPTER 2

DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING STRATEGY TO IDENTIFY NOVEL NITRILE HYDROLYSING ENZYMES

DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING STRATEGY TO IDENTIFY NOVEL NITRILE HYDROLYSING ENZYMES

2.1 INTRODUCTION

In recent years, the use of enzymes or whole cell bacterial isolates to afford enantiomerically pure pharmaceutical actives and intermediates has become a significant and well studied area in biotechnology and organic chemistry. This is due to the many advantages offered by biotransformations, which have been reviewed in detail in Chapter 1.

A major consideration in the development of a specific biotransformation is the identification of an appropriate biocatalyst or strain from culture collections by using selection methods [1, 2]. Selective enrichment for the isolation of nitrile hydrolysing micro-organisms, where nitrile substrates are used as a nitrogen source requires efficient methods to screen libraries of isolated strains to identify those possessing the desired reactivity and selectivity [1]. Several high-throughput screening methods have been developed for nitrile hydrolysing enzymes, and have been reviewed by Martinkova [2] and Gong [3] and are discussed in chapter 1.

Colorimetric determination of ammonia using reagents such as Nesslers provides a promising method for rapid screening [4] and in particular offers the potential for the evaluation of nitrile hydrolysing enzymes in 96 well plate systems.

2.2 PROJECT AIM

The objective of this study was to screen a novel bacterial isolate bank (previously collected from soil samples in the laboratory) and identify those with activity towards three pharmaceutically relevant β-hydroxynitriles [5] [6]. The main research questions to be addressed by this work are as follows:

- Can a high-throughput screening strategy using a 96 well plate format be developed for the rapid identification of nitrile hydrolysing isolates?
- Do any of the available novel bacterial isolates possess interesting biocatalytic activity?
- Can a suitable bacterial isolate for the production of enantiomerically pure pharmaceutical actives and intermediates be identified?

2.3 ISOLATE SOURCE

Molecular biotechnology research carried out by O'Reilly *et al*. involving environmental sampling and sequence analysis resulted in the discovery of over 250 nitrile-metabolising bacterial isolates [7, 8]. Soil samples had previously been collected from a disused mining site in the Silvermines district in Co. Tipperary, the banks of a stream in Silvermines village and the banks of the River Suir, Kings Channel, Waterford. These samples had been chosen due to suspected elevated metal content and a number of cyanide and nitrile degrading bacteria had previously been isolated from these soils, even after storage at 4 °C for several years [9]. Uncontaminated suburban soil samples were also collected, from two suburban sites in Melbourne, Victoria, Australia, and from a forest in Poland. Soils were stored at 4 °C before enrichment.

Samples of six seaweed species; *Polysiphonia lanosa*, *Ascophyllum nodesum*, *Ulva intestinalus*, *Ulva lacutuca*, *Palmari palmate* and *Ceranium codiola* were collected from Baginbun Beach, Hook Peninsula, Co. Wexford and Annestown beach, Co. Waterford [6]. Soil and seaweed samples were isolated using the enrichment technique with different nitriles as the N source (acetonitrile, benzonitrile, adiponitrile, mandelonitrile, acrylonitrile or phenylacetonitrile) which were not the nitriles to be evaluated by this study [6]. The isolate bank includes 187 isolates from the soil enrichment, and 67 isolates purified from the seaweed enrichments.

A sample of the nature and the origin of the novel bacterial isolates, along with the enrichment source used during the isolation stage are presented in Table 2.1 below (suburban soil isolates). Tables for the foreign soil and seaweed isolates may be found in the appendix section. The tables also include genes detected in previous studies as reported by Coffey *et al*. [7, 8].

Table 2.1: Suburban soil isolates enriched on various nitriles and genes previously detected.

Isolate	Source	Geographic origin	Enrichment	Gene detected
SS1 1	9	Ireland	Acetonitrile	
Rhodococcus sp. SS1-2	9	Ireland	Acetonitrile	NHase
SS1 3	9	Ireland	Acetonitrile	
SS1 4, *R. erythropolis* sp.	10	Ireland	Acetonitrile	
SS1 5	10	Ireland	Acetonitrile	
SS1 6, *Enterobacter* sp.	8	Ireland	Acetonitrile	
Rahnella sp. SS1-7	8	Ireland	Acetonitrile	NHase
SS1 8	8	Ireland	Acetonitrile	
SS1 9	8	Ireland	Acetonitrile	

2.4 SUBSTRATES OF INTEREST

The substrates chosen for this screen were *β*-hydroxy nitriles **38**, **61** and **89** (Figure 2.1), which can act as sources of *β*-hydroxy carboxylic acids *via* hydrolysis reactions. However, they can be prone to elimination reactions under classical acid/base conditions [10]. Alternatively, nitrile biocatalysis can often selectively facilitate this hydrolysis without affecting other acid- or alkali- labile functional groups present [11, 12].

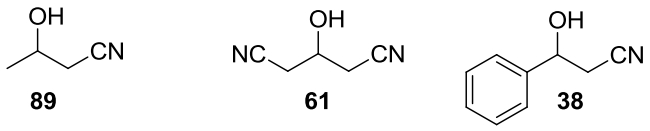


Figure 2.1: Structure of *β*-hydroxy nitriles of interest.

Of particular commercial interest is the nitrilase catalysed hydrolysis of 3-hydroxyglutaronitrile **61**, the ethyl-ester **62** of which is an intermediate to the cholesterol lowering drug Lipitor (atorvastatin) **60** as shown in Figure 2.2 [13], [14]. Also 3-hydroxy-3-phenylpropionic acid and its derivatives have been used as precursors to chiral drugs such as nisoxetine [15], fluoxetine [16] and tomoxetine (Figure 2.3) [15].

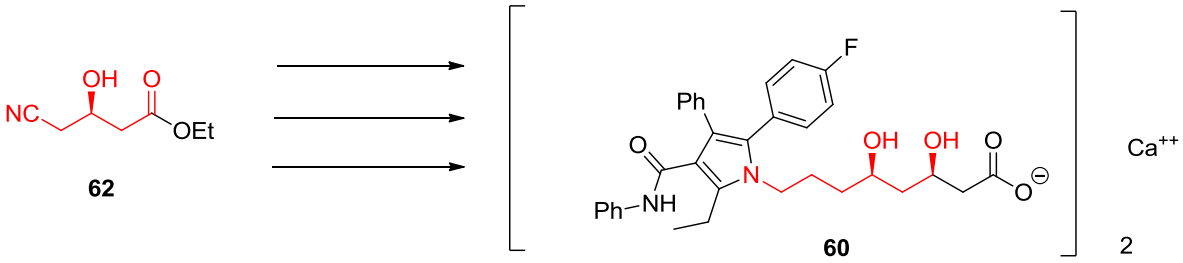


Figure 2.2 : Ethyl-(*R*)-4-cyano-3-hydroxybutyric acid as a chiral building block for the production of atorvastatin (Lipitor) [13].

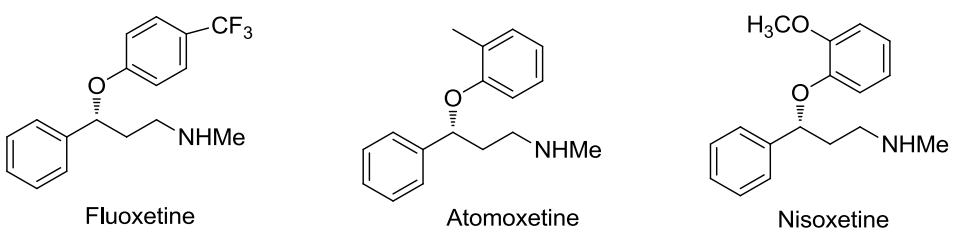


Figure 2.3 : Enantiomerically pure 3-hydroxy-3-phenylpropionic acid used as a chiral building block for the production of pharmaceuticals [16].

A challenge of working with these substrates is that having the chiral centre remote from the cyano functional group often results in disappointingly low enantioselectivity and chemical yield following biotransformation [17] [18-21].

Initial work in the laboratory ensured that fresh working cultures and duplicate archive stocks of all isolates were generated and maintained to serve the duration of the project. The isolates were grown in rich medium liquid broth (LB broth) and stored in duplicate, using microtitre-based high throughput growth techniques.

2.5 IDENTIFICATION OF NITRILE HYDROLYSING ISOLATES

2.5.1 TOXICITY SCREENING

The bacterial isolates were subjected to toxicity studies with the 3 nitriles (**89**, **61** and **38**) of interest. By attempting to grow the different isolates in rich medium in the presence of β-hydroxynitriles, the isolates that are sensitive to the nitrile and cannot grow in its presence were determined and excluded from further screening. This data was useful in later studies when analysing activity towards the key nitriles, where a lack of growth due to toxicity may have been mistaken for a lack of activity [2].

Cells were grown in LB broth containing 10 mM concentrations of the nitriles of interest and checked daily for growth by measuring the optical density @ 600 nm using a NanoDrop spectrophotometer. Cells were then subcultured in nutrient rich agar, containing the key nitrile, using a 96 well stamp format and incubated for up to 10 days. Plates were examined visually for the appearance of colonies.

A representative sample of data from the growth studies (suburban soil) is presented in Table 2.2. Tables of the foreign soil and seaweed isolates may be found in the appendix section of this body of work.

Table 2.2: Suburban soil isolates that could grow in the presence of β–hydroxynitriles.

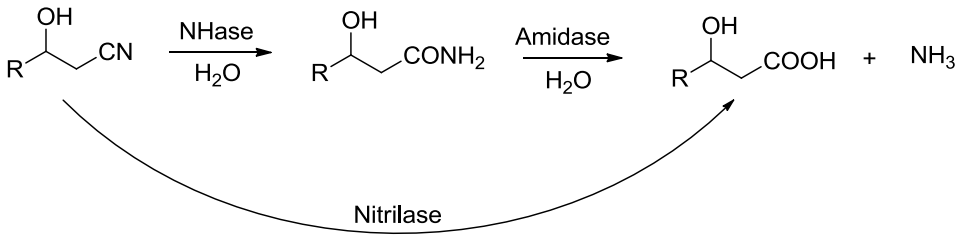
	3-HGN 61	**3-HBN 89**	**3-HPPN 38**
SS1 1		✓	
Rhodococcus sp. SS1-2	✓	✓	
SS1 3		✓	
SS1 4, *R. erythropolis* sp.	✓	✓	
SS1 5		✓	✓
SS1 6, *Enterobacter* sp.	✓	✓	✓
Rahnella sp. SS1-7		✓	
SS1 12	✓	✓	✓
SS1 14	✓	✓	✓
Microbacterium sp. SS1-15	✓	✓	✓

Of the initial 256 isolates, 145 could grow in the presence of 3-hydroxybutyronitrile **89**, with 77 demonstrating growth after a 48 hour period and the remaining 68 after 144 hours. A total of 65 of the isolates grew in the presence of 3-hydroxy-3-phenylpropionitrile **38** and 107 in the presence of 3-hydroxyglutaronitrile **61**. The toxicity study demonstrated that some isolates show no growth in the presence of the nitriles, whereas 16 isolates, such as isolate SS1-12 showed growth against two or more nitriles.

2.5.2 ISOLATE INDUCTION AND ACTIVITY SCREENING

The tolerant isolates then underwent a starvation process in order to ensure that the *β*-hydroxynitrile would be utilised as the sole nitrogen source during the induction stage. The subsequent induction stage involved attempting to grow the isolates in M9-minimal media using the *β*-hydroxynitrile as the sole nitrogen source (10 mmol/L).

In order to screen the isolates to determine activity, a suitable analytical screening method was required. As bacterial isolates utilizing *β*-hydroxynitriles as a nitrogen source result in the release of ammonia as seen in Scheme 2.1 below, enzyme activity may be monitored using the technique of Nesslerisation [22].



Scheme 2.1:Ammonia is a by-product of the microbial hydrolysis of nitriles

In the Nesslerisation reaction NH_3 and NH_4^+ react with an excess of Nesslers reagent to form a yellow-brown complex. The intensity of the colour is directly proportional to the ammonia concentration present and is read at 425 nm [22]. A high-throughput microscale colorimetric assay was developed to detect the ammonia and adapted to a 96 well format to enable rapid screening

Appropriate standards were prepared containing 1 – 5 mmol/L of ammonium chloride. This concentration was based on the predicted quantity of ammonia produced from a biotransformation containing 10 mmol/L of substrate. Aliquots of the standards were reacted with Nessler's master-mix and the absorbance measured at 425 nm. A standard

curve (Figure 2.4) was prepared by plotting absorbance versus concentration resulting in a linear line with a correlation coefficient $R^2 = 0.999$.

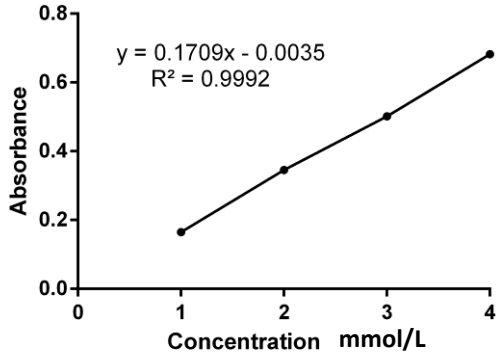


Figure 2.4: Standard curve for the detection of ammonia using the Nesslers Colorimetric assay.

To assess each isolate after the biotransformation, HCl was added to quench the hydrolysis and the cell debris was pelleted by centrifugation. The supernatant was transferred to a 96 well plate and the Nesslers mastermix was added. The plates were then allowed to stand for 10 minutes before the absorbance was read at 425 nm. A representative plate after Nesslerisation for 3-hydroxybutyronitrile with isolates is shown in Figure 2.5.

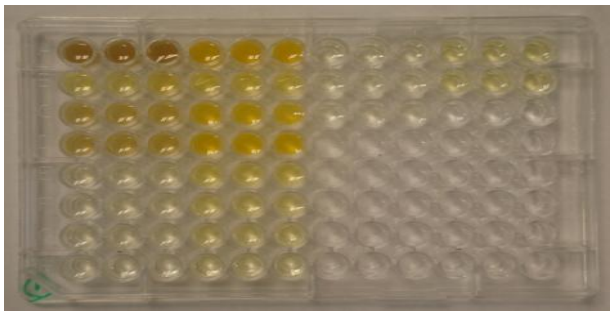


Figure 2.5: Nesslers colorimetric activity assay towards 3-hydroxybutyronitrile, an example of library screening for nitrile-hydrolyzing enzyme activity in a 96-well plate format.

Nitrile hydrolyzing activity was calculated by relating the sample absorbance to the standard curve shown earlier. Each sample was performed in triplicate and the average absorbance used to calculate activity.

Based on ammonia production, the isolates were divided into three subcategories (0.08-0.6 mmol/L = low activity, 0.6-5.8 mmol/L = good activity and >5.8 mmol/L = high activity). Preliminary results revealed that 47 isolates demonstrated activity towards 3-

hydroxybutyronitrile **89**, 34 isolates towards 3-hydroxyglutaronitrile **61**, while only 4 of the initial 256 isolates catalysed the hydrolysis of 3-hydroxy-3-phenylpropionitrile **38.**

A total of 7 isolates demonstrated "low" activity while 23 isolates were classified as having "good" activity towards 3-hydroxyglutaronitrile **61**. Both SS1-4 and SET1 demonstrated high activity towards this nitrile, producing 6.08 and 8.1 mmol of NH_3 respectively (Table 2.3). In contrast, these isolates demonstrated low to good activity towards 3-hydroxybutyronitrile. Neither SET1 nor SS1-4 demonstrated activity towards 3-hydroxy-3-phenylpropionitrile **38**.

Table 2.3: Isolates demonstrating activity towards 3-hydroxyglutaronitrile.

Low activity	**NH_3 (mmol/L) produced**	**Good activity**	**NH_3 (mmol/L) produced**	**High activity**	**NH_3 (mmol/L) produced**
F66	0.11	F32	2.47	SS1-4	6.08
F70	0.4	F41	2.86	SET1	8.1
F62	0.117	F57	2.17		
NN12	0.22	F37	3.86		
F71	0.33	F45	2.62		
NN36	0.59	F59	1.66		
F34	0.11	F68	1.45		
		F71	0.84		
		F30	3.28		
		F46	0.69		
		F69	0.88		
		SS1-12	4.18		
		SS1-16	2.12		
		SS1-22	5.8		
		SS1-24	1.97		
		SS1-18	2.94		
		SW1-15	0.81		
		Nit (-) 9	1.66		
		NN30	4.56		
		F73	0.86		
		F25	1.38		
		F19	1.21		
		F72	1.74		

A total of 25 isolates demonstrated low activity towards 3-hydroxybutyronitrile **89**, 22 isolates were found to have good activity, whereas none of the isolates could be classified as having high activity. Isolate F36 demonstrated the highest activity toward **89**, producing 2.53 mmol/L of NH_3 (Table 2.4).

Table 2.4 : Isolates demonstrating activity towards 3-hydroxybutyronitrile

Low activity	NH_3 (mmol/L) produced	Good activity	NH_3 (mmol/L) Produced
F62	0.21	F31	0.77
F45	0.29	F65	0.85
F74	0.19	F20	1.43
SS1-3	0.25	F36	2.53
SS1-1	0.29	F61	0.94
F66	0.28	F64	0.71
SS1-4	0.13	F71	1.6
F73	0.22	F69	1.83
SS1-14	0.36	F71	1.52
SS1-22	0.27	SS1-12	1.63
NN20	0.34	SET1	1.51
SS1-17	0.35	SW1-7	0.63
SS1-24	0.52	Nit (-) 9	0.78
SW1-3	0.27	NN32	1.94
Nit(-)36	0.2	LC3c	2.14
SS1-27	0.21	SW2-33	1.89
SW1-4	0.57	NN12	0.78
NN5a	0.19	NN30	1.44
NN31	0.33	Nit(-)17	0.74
F5	0.5	SS1-31	2.12
F17	0.18	F13	1.22
F35	0.17	F38	0.7
F2	0.55		
F15	0.155		
SS1-29	0.36		

However, the number of isolates with activity towards 3-hydroxy-3-phenylpropionitrile **38** was poor (Table 2.5), with only 4 isolates demonstrating activity after 24 hours. Two of these isolates fell within the high and one within the good activity range. This may be due to steric hindrance by the aromatic ring present on 3-hydroxy-3-phenylpropionitrile affecting enzyme function. This is also supported by isolate SS1-24 demonstrating good activity against 3-hydroxglutaronitrile **61** (1.97 mmol/L) as opposed to low with nitrile **38** (0.21 mmol/L).

Table 2.5 :Isolates demonstrating activity towards 3-hydroxy-3-phenylpropionitrile

Low activity	NH_3 (mmol/L) produced	Good activity	NH_3 (mmol/L) produced	High activity	NH_3 (mmol/L) produced
SS1-24	0.21	SS1-12	1.61	F62	7.64
				SS1-14	8.07

Following the preliminary studies as outlined above, Table 2.6 summarises the isolates chosen with potential activity against each substrate. Ten isolates with good to high activity were chosen for 3-hydroxyglutaronitrile **61** and 3-hydroxybutyronitrile **89**, and

all 4 with activity 3-hydroxy-3-phenylpropionitrile **38** were chosen for further screening,

Table 2.6: Isolates demonstrating the highest activity towards β-hydroxynitriles.

3-HGN	3-HBN	3-HPPN
F32	F36	SS1-24
F41	F69	SS1-12
F37	F71	F62
F30	SS1-12	SS1-14
SS1-12	SET1	
SS1-22	NN32	
SS1-18	LC3c	
NN30	SW2-33	
SS1-4	NN30	
SET-1	SS1-31	

A combination of this assay with toxicity, starvation and induction studies, allowed for the rapid identification of 10 isolates potentially containing nitrile hydrolysing enzymes from a bank of 256 isolates. A key advantage of this assay is the incorporation of the 96 well plate which enables rapid detection of activity.

2.5.3 TEMPERATURE GROWTH STUDIES AS AN INDUCTION GUIDELINE

Reaction temperature is a key parameter in biotransformations and can significantly influence the activity, enantioselectivity and stability of a biocatalyst [23]. As the bacterial strains were isolated from various locations such as soil and seaweed, these isolates may have exhibited different growth patterns at various temperatures.

In order to determine an optimal temperature for future induction studies, the growth of the chosen isolates in the presence of the key nitriles was monitored across a range of temperatures between 15 – 30 °C. A total of 6 isolates demonstrated the highest optical density at 15 °C when grown in the presence of 3-hydroxyglutaronitrile **61** (Table 2.7). Isolate SET1 demonstrated optimum cell growth on both 3-hydroxyglutaronitrile **61** and 3-hydroxybutyronitrile **89** at the lower temperature of 15 °C. Tables of the optical density of the isolates at various temperatures are outlined below.

Table 2.7: Growth studies of isolates with activity towards 3-hydroxyglutaronitrile.

Isolate	Optical density 600nm				Optimum growth temperature
	15 °C	20 °C	25 °C	30 °C	
F32	1.63	0.63	0.43	0.11	15 °C
F41	1.06	0.74	0.37	0.2	15 °C
F37	2.5	0.73	0.47	0.13	15 °C
F30	1.87	0.52	0.32	0.13	15 °C
SS1-12	2.37	0.55	0.35	0.06	15 °C
SS1-22	0.51	0.33	1	0.14	25 °C
SS1-18	0.16	0.72	1.48	0.15	25 °C
NN30	0.23	0.88	0.48	0.14	20 °C
SS1-4	0.42	0.66	0.36	0.45	20 °C
SET1	2.08	0.47	0.3	0.13	15 °C

The optimum temperature of isolates grown in the presence of nitrile **89** is shown in Table 2.8. In the case of those isolates utilising 3-hydroxybutyronitrile **89** as the nitrogen source, a total of 5 demonstrated an optimal OD_{600nm} when grown at 15 °C, while only LC3c and SW2-33 were found to have an optimum growth temperature of 30 °C with an OD_{600nm} of 1.00 and 0.76 respectively.

Table 2.8: Growth studies of isolates with activity towards 3-hydroxybutyronitrile.

Isolate	Optical density 600nm				Optimum growth temperature
	15 °C	20 °C	25 °C	30 °C	
F36	1.63	2.13	0.89	1.25	20 °C
F69	1.06	1.79	0.97	1.07	20 °C
F71	2.5	2.27	1.02	1.03	15 °C
SS1-12	1.87	1.1	0.5	0.61	15 °C
SS1-TE	2.37	1.31	0.66	0.67	15 °C
NN32	0.51	0.29	0.29	0.07	15 °C
LC3c	0.16	0.84	0.44	1.00	30 °C
SW2-33	0.23	0.37	0.39	0.76	30 °C
NN30	0.42	0.23	0.19	0.37	15 °C
SS1-31	2.08	2.44	1.23	1.87	20 °C

The four isolates with activity towards 3-hydroxy-3-phenylpropionitrile **38** demonstrated the highest optical density when induced at lower temperatures, with the highest optical density being observed at 15 °C, with little or no growth at 30 °C (Table 2.9).

Table 2.9: Growth studies of Isolates with activity towards 3-hydroxy-3-phenylpropionitrile

Isolate	Optical density 600nm				Optimum growth temperature
	15 °C	20 °C	25 °C	30 °C	
SS1-24	2.19	0.09	0.08	0	15 °C
SS1-12	2.85	0.02	0.03	0.04	15 °C
F62	0.05	0.04	0	0.01	15 °C
SS1-14	3.65	0.03	0	0	15 °C

2.5.4 INVESTIGATION INTO THE EFFECT OF TEMPERATURE OF GROWTH ON ACTIVITY

The initial findings outlined above demonstrated that optimum growth for the majority of isolates took place at 15 °C. In order to determine the optimum temperature for biotransformation reactions, growth temperature of isolates and the resultant activity towards the β-hydroxynitriles of interest was investigated.

Following growth at 15 °C, 20 °C, 25 °C and 30 °C, whole cells were then incubated with the relevant nitrile at 25 °C for 24 hours. The activity was determined by quantifying the ammonia produced using the Nesslers micro-scale colorimetric assay as reported previously.

Results presented in Table 2.10 show that in the majority of cases the isolates demonstrated optimum activity towards 3-hydroxyglutaronitrile **61** when grown and induced at 25 °C. In fact, the hydrolysis of 3-hydroxyglutaronitrile **61** did not proceed when isolates were grown at 15 °C and assayed at 25 °C, and only four of the isolates demonstrated activity when grown at 30 °C and assayed at 25 °C.

Table 2.10: Investigation into the temperature of induction on the activity of isolates towards 3-hydroxyglutaronitrile.

Isolate	Concentration of ammonia produced at 25 °C (mmol)				Optimum activity	Optimum growth temperature
	15 °C	20 °C	25 °C	30 °C		
	Growth temperature					
F32	ND	0.752	1.726	ND	25°C	20 °C
F41	ND	ND	1.629	0.667	25°C	20 °C
F37	ND	0.397	1.140	0.301	25°C	15 °C
F30	ND	1.866	2.150	ND	25°C	15 °C
SS1-12	ND	0.018	0.772	ND	25°C	15 °C
SS1-22	ND	1.433	1.330	ND	25°C	15 °C
SS1-18	ND	ND	0.910	ND	25°C	30 °C
NN30	ND	0.637	1.790	0.603	25°C	30 °C
SS1-4	ND	1.126	1.468	0.111	25°C	15 °C
SET1	ND	0.430	1.144	ND	25°C	20 °C

Isolates which did not demonstrate any activity at 25 °C, were re-screened by carrying out the assay at the same temperature as cell growth and induction (e.g. those grown at 15 °C were assayed at 15 °C). Reaction temperature significantly influenced the activity of isolates when the biotransformation was performed at a range of temperatures between 15 °C and 30 °C. The majority of isolates demonstrated their highest activity at 25 °C however, optimum cell growth was observed at lower temperatures.

This was also observed during the biotransformation of 3-hydroxybutyronitrile **61** as can be seen in Table 2.11. Three isolates F71, SS1-12 and SET1 displayed optimum activity when grown at 25 °C and four isolates; F69, NN32, NN30 and SS1-31 when grown at 20 °C, while only isolate F36 displayed maximum activity when grown at 15 °C and one at 30 °C.

Table 2.11: Investigation into the temperature of induction on the activity of isolates towards 3-hydroxybutyronitrile.

Isolate	Concentration of ammonia produced at 25 °C (mmol/L)				Optimum activity	Optimum growth temperature
	15 °C	20 °C	25 °C	30 °C		
	Growth temperature					
F36	2.630	0.012	0.515	ND	15 °C	20 °C
F69	ND	1.206	0.424	ND	20 °C	20 °C
F71	0.603	0.820	4.389	ND	25 °C	15 °C
SS1-12	0.222	0.582	1.430	0.420	25 °C	15 °C
SET1	ND	0.983	1.021	0.699	25 °C	15 °C
NN32	0.073	0.377	2.91	3.090	20 °C	15 °C
LC3c	ND	1.521	0.640	1.650	30 °C	30 °C
Sw2-33	ND	1.000	0.778	ND	20 °C	30 °C
NN30	ND	3.102	0.494	0.178	20 °C	15 °C
SS1-31	1.951	2.126	ND	2.42	20 °C	20 °C

This indicates that cell growth/induction temperatures need careful consideration in order to achieve a suitable degree of activity during the biotransformation of the nitrile to the corresponding carboxylic acid. This is evident in the contrast between the optimum conditions required for cell growth and the optimum activity associated with the isolates. This indicates that the temperature of growth does not always result in the best activity for the biotransformations.

2.5.5 ALTERNATIVE INDUCTION METHODS

It was observed that when induced with the chosen β-hydroxynitriles, the growth rate of isolates was relatively slow with long periods of time required to reach an optical density of greater than one. Isolate SS1-14 for example, required two weeks incubation

in M9 minimal media when aromatic nitrile **38** was used as the sole nitrogen source. In order to overcome this shortcoming, the possibility of inducing the desired enzymes using alternative nitriles was explored. In addition nitrile **89** is a relatively expensive substrate and as such it is not economical to use this nitrile as a nitrogen source during the induction stage. Several factors were taken into account when deciding which nitriles could be used to replace the three key nitriles in the growth and induction stage. These include the cost and availability of the alternative nitriles, the structural similarity of the new inducing nitriles to the key nitriles, and the ability to determine if the alternative nitriles induced the same specific enzyme which showed activity against the original β-hydroxynitriles.

Various nitrile inductions were performed to obtain a wide range of nitrile degrading isolates so that the properties of the isolates could be analysed with respect to their selectivity and activity towards the three key nitriles.

ALTERNATIVE INDUCTION OF ISOLATES WITH ACTIVITY FOR 3-HYDROXYGLUTARONITRILE

The substrate 3-hydroxyglutaronitrile **61**, which is a dinitrile containing a hydroxyl functionality at the β-position is structurally similar to adiponitrile and succinonitrile with the exception of the hydroxyl functional group (Figure 2.6). Both adiponitrile and succinonitrile are dinitriles containing 6 and 4 carbon atoms respectively.

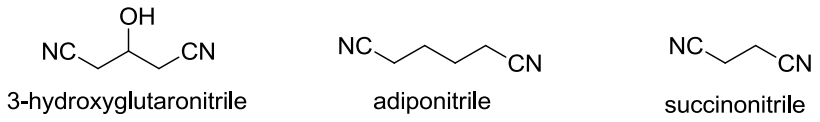


Figure 2.6: 3-hydroxyglutaronitrile alternative inducing nitriles.

The isolates showed good growth when induced with either adiponitrile or succinonitrile (Table 2.12). Isolate F41 for example gave an OD_{600nm} = 1.37 when grown in M9 minimal media containing succinonitrile for 3 days. In contrast to growth obtained, the isolates demonstrated poor activity when assayed towards substrate **61** at 25 °C (Table 2.12). These isolates were then examined in the biotransformation of succinonitrile and were found to be active towards this inducing substrate.

Table 2.12: Results of activity assay towards succinonitrile and 3-hydroxyglutaronitrile when induced using succinonitrile.

Isolate	Optical density (600 nm)	Activity towards 3-hydroxyglutaronitrile (NH_3 mmol/L)	Activity towards succinonitrile (NH_3 mmol/L)
F37	0.61	0	0.248
SS1-22	0.73	0	0.222
SS1-4	0.35	0	0.906
F32	0.76	0	0
F30	0.89	0	0
SS1-18	0.53	0	0
SS1-15	0.43	0	0.132
F41	1.37	0	0.073
SS1-12	0.9	0	0.078
NN30	0.83	0	0.708

ALTERNATIVE INDUCTION OF ISOLATES WITH ACTIVITY TOWARDS 3-HYDROXYBUTYRONITRILE

The substrate 3-hydroxybutyronitrile **89** contains a hydroxyl functionality at the β-position and is structurally similar to butyronitrile and isovaleronitrile (Figure 2.7) with the exception of the hydroxyl functional group. Butyronitrile contains 4 carbon atoms, however isovaleronitrile differs from 3-hydroxybutyronitrile by the methyl substituent at the β-position.

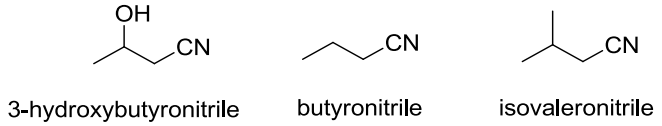


Figure 2.7: 3-hydroxybutyronitrile alternative inducing nitriles.

Isolates grown in M9-minimal media in the presence of 10 mmol butyronitrile showed good growth characteristics (OD_{600nm} > 1). For example, isolate SS1-12 reached an OD_{600nm} = 1.37 after 3 days incubation at 25 °C. The isolates induced with butyronitrile were examined in the biotransformation of 3-hydroxybutyronitrile **89**, however as with 3-hydroxyglutaronitrile **61**, the hydrolysis did not proceed to any extent at 25 °C. The isolates were then examined for their ability to hydrolyse butyronitrile, results of this study are outlined in Table 2.13.

Table 2.13: Results of Nesslers activity assay towards butyronitrile and 3-hydroxybutyronitrile when induced using butyronitrile.

Isolate	Optical density (600 nm)	Activity towards 3-hydroxybutyronitrile (NH_3 mmol)	Activity towards butyronitrile (NH_3 mmol)
F36	0.76	0	0.078
F69	0.89	0	0.708
F71	0.53	0	0.906
SS1-12	1.37	0	0
SET1	0.9	0	0
NN32	0.83	0	0
LC3c	0.43	0	0
SW2-33	0.98	0	0.12
NN30	0.89	0	0.07
SS1-31	0.62	0	0.34

Of the 10 isolates, 6 demonstrated activity towards butyronitrile. F71 for example, did not demonstrate any activity towards 3-hydroxybutyronitrile however produced 0.906 mM of ammonia during the hydrolysis of butyronitrile. Isolate SS1-12 which showed good growth characteristics when butyronitrile was used as the sole nitrogen source, did not exhibit activity towards 3-hydroxybutyronitrile **89** or butyronitrile. This was also demonstrated in the case of those isolates grown in the presence of isovaleronitrile. These isolates demonstrated activity towards isovaleronitrile rather than their target 3-hydroxybutyronitrile **89**.

ALTERNATIVE INDUCTION OF ISOLATES WITH ACTIVITY TOWARDS 3-HYDROXY-3-PHENYLPROPIONITRILE

The substrate 3-hydroxy-3-phenylpropionitrile **38** contains a hydroxyl functionality at the β-position and is structurally similar to mandelonitrile with the exception of a methylene group as shown in Figure 2.8. Good growth (OD_{600nm} > 1) was observed with isolates in the presence of mandelonitrile however no activity against nitrile **38** was observed as shown in Table 2.14.

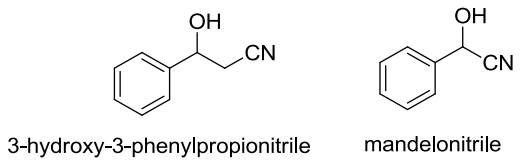


Figure 2.8: 3-hydroxy-3-phenylpropionitrile alternative inducing nitrile with mandelonitrile.

It was found that all isolates were capable of hydrolysing mandelonitrile, however, low activity was observed based on the concentration of ammonia produced. Isolate SS1-12 demonstrated the highest activity of the 4 isolates, producing 0.65 mmol of ammonia,

whereas isolate F62 resulted in 0.13 mmol of ammonia when assayed with mandelonitrile. These results (Table 2.14) suggested that the optimum isolates require the key nitriles for growth in order to induce the required enzymes capable of transforming the β-hydroxy substrate to the desired products.

Table 2.14: Results of Nesslers activity assay towards 3-hydroxy-3-phenylpropionitrile and mandelonitrile when induced using mandelonitrile.

Isolate	Optical density (600 nm)	Activity towards 3-hydroxy-3-phenylpropionitrile (NH_3 mmol)	Activity towards mandelonitrile (NH_3 mmol)
SS1-24	0.58	0	0.54
SS1-12	0.46	0	0.65
F62	0.29	0	0.13
SS1-14	0.37	0	0.27

Weak binding forces are thought to stabilise the three dimensional structure of an enzyme. These forces can be van Der Waals interactions of aliphatic chains, $\pi-\pi$ stacking of aromatic units and salt bridges between charged parts of molecules [24], and are thought to be essential to maintaining the three dimensional structure of the enzyme and thus its catalytic activity [25]. These binding forces may be influenced during growth and induction. Hence, the inducing nitrile and its structure can influence the activity and enantioselectivity of the isolate.

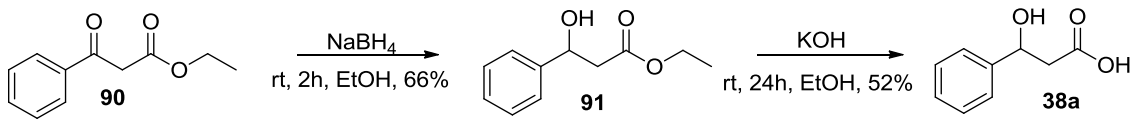
In the case of the nitrilase enzyme, it has been observed that the subunits of nitrilase self-associate to convert the enzyme to its active form. This association is thought to be accelerated by temperature and enzyme concentration as described by Harper *et al* [26]. Stevenson *et al*. [27] suggested that *R. rhodochrous* cells are converted to the active form by subunit association when incubated with the substrate [27]. Nagasawa *et al*. [28] suggested that various substrates can expose hydrophobic sites on the enzyme and alter selectivity; a so-called hydrophobic effect. The use of alternative inducing nitriles may result in the induction and generation of different nitrilase enzymes as previously observed in the case of *R. rhodochrous* NCIMB 11216 [28]. It is also possible that NHase/ amidase enzymes may be produced depending on the nitrogen source during induction and growth.

2.6 SCREENING FOR ENANTIOSELECTIVE ISOLATES ACTIVE TOWARDS β-HYDROXYNITRILES.

Having narrowed down the bacterial isolates from 256 to 24 having good to high activity towards β-hydroxynitriles, it was decided to focus attention on determining the enantioselectivity of the isolates. As the purity of the enzymes and the specific enzyme content of the cells were unknown, the isolates were screened for activity to produce both amide and the corresponding carboxylic acid as these are the expected products of nitrile hydratase/amidase and/ or nitrilase systems. The corresponding β-hydroxy carboxylic acid and amide standards were synthesized according to the relevant literature as outlined in the next section. This was performed in order to develop a chromatographic method capable of resolving the enantiomers of these racemic compounds. Following chromatographic method development each isolate was screened for enantioselectivity.

2.6.1 SYNTHESIS OF STANDARDS FOR CHROMATOGRAPHIC METHOD DEVELOPMENT

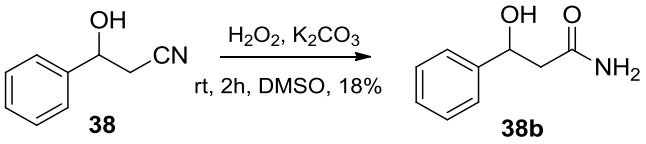
The standard 3-hydroxy-3-phenylpropionic acid **38a** was prepared by reduction followed by hydrolysis of ethylbenzoylacetate **90** as demonstrated below in Scheme 2.2.



Scheme 2.2: Synthesis of 3-hydroxy-3-phenylpropionic acid from commercially available ethyl-benzoylacetate.

The initial reduction of **90**, with $NaBH_4$ was carried out at room temperature over 2 hours. The product **91** was purified using flash chromatography with a mobile phase of (50: 50) hexane: ethyl acetate to yield **91** (66 %) as colourless oil. Key ^{1}H spectroscopic resonances included the triplet integrating for 1 proton at 4.7 ppm corresponding to C<u>H</u>OH. GC-MS analysis resulted in a molecular ion m/z 194 corresponding to the M^+ ion. Subsequent ester hydrolysis of **91** using KOH in methanol over 24 hours gave the acid **38a** in 56 % yield as a white solid following extractive work up and chromatographic purification. The ^{1}HNMR spectrum showed the loss of the ethoxy <u>CH_3</u>CH_2 signals which previously appeared as a triplet at 1.24 ppm for CH_3 integrating for 3 protons and quartet for <u>CH_2</u>CH_3 integrating for 2 protons at 2.7 ppm in the ester starting material.

Along with the acid standard; 3-hydroxy-3-phenylpropionamide **38b** [29, 30] was also produced synthetically (Scheme 1.4). The product was prepared by reacting nitrile **38** with 30 % H_2O_2 and K_2CO_3 in DMSO over 2 hours.



Scheme 2.3: Synthesis of 3-hydroxy-3-phenylpropionamide from 3-hydroxy-3-phenylpropionitrile [29].

The reaction was quenched with H_2O as outlined in literature. The amide **38b** however, did not crash out of solution as expected and alternatively was extracted into diethylether and dried over anhydrous $MgSO_4$. The crude product was purified using preparative TLC with a mobile phase of (2: 1) petroleum ether: acetone to yield amide **109** (18 %) as a white solid. Key ^{1}H NMR spectroscopic resonances included the methylene protons which appear as two doublets of doublets integrating for 1 proton each at 2.41 and 2.29 ppm. The ^{13}C NMR signal corresponding $CONH_2$ at 174.3 ppm also verifies the formation of the amide.

In order to determine the conversion of 3-hydroxy-3-phenylpropionitrile **38**, standard solutions of the acid **38a**, amide **38b** and nitrile **38** were prepared with concentrations ranging from 0.5 – 2 mg/mL. The concentrations of the standards were based on the predicted quantity from a biotransformation containing 10 mmol of substrate. The mixed standards were then injected on a C18 HPLC column and the absorbance measured at 215 nm. A standard curve (Figure 2.9) was prepared by plotting absorbance versus concentration resulting in a linear line for nitrile, amide and acid with correlation coefficients R^2 = 0.998, 0.976 and 0.993 respectively.

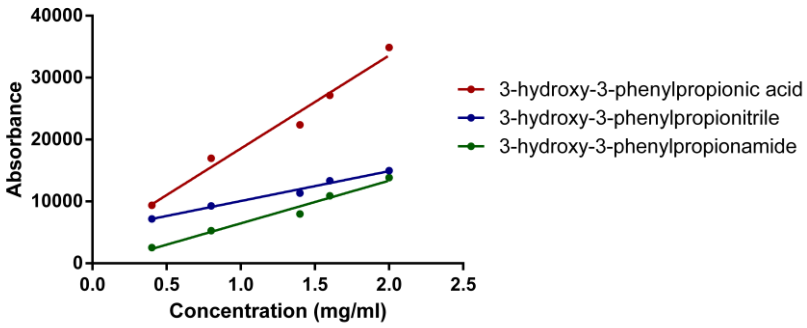


Figure 2.9: Standard curve of the hydrolysis products of 3-hydroxy-3-phenylpropionitrile.

Chromatographic methods were also developed to determine the enantioselectivity of isolates towards nitrile **38**. Chiral HPLC methods were developed using the synthesised and characterised racemic standards. The enantiomers of 3-hydroxy-3-phenylpropionic **38a** were separated using a Daicel AD-H chiral column employing a mobile phase of hexane: IPA (90:10) with 0.1 % TFA as an additive, at a flow rate of 0.8 mL/min at λ=215 nm. The chromatography showed sufficient base line resolution with the enantiomers eluting at 12.3 and 13.8 minutes. The response factor which is the ratio between a signal produced by an analyte and the quantity of analyte which produces the signal, and was determined to be 1.023.All spectroscopic and chromatographic data correlated to that previously reported within the literature [30].

Chromatographic methods were developed to determine the enantiomeric excess of nitrile **38** and the potential amide product **38b**. Both the nitrile and amide were resolved using a Daicel OJ-H column, employing a mobile phase of hexane: IPA (90:10) with a flow rate of 0.8 mL/min and at λ=215 nm. The enantiomers of nitrile **38** eluted at 32.9 and 41.4 minutes with a response factor of 1.012. In addition amide **38b** eluted at 19.4 and 22.27 minutes with a response factor of 1.01.

The biotransformation of racemic aromatic nitrile **38** was initially investigated, with the formation of acid **38a** and amide **38b** expected as products. Isolates were induced using nitrile **38** for optimum activity and selectivity. The biotransformation was performed in triplicate by incubating the isolates (OD_{600nm} = 1) in potassium phosphate buffer (100 mM, pH 7) containing the 3-hydroxy-3-phenylpropionitrile **38** (10 mM) for 24 hours at 25 °C. The reaction was quenched by the addition of 2M HCl and the biomass removed by centrifugation. Following extractive work up with ethyl acetate and removal of the solvent under vacuum, the products were dissolved in IPA and analysed by HPLC on an AD-H chiral column. The effect of time and temperature on the enantioselectivity of the hydrolysis reaction was also investigated.

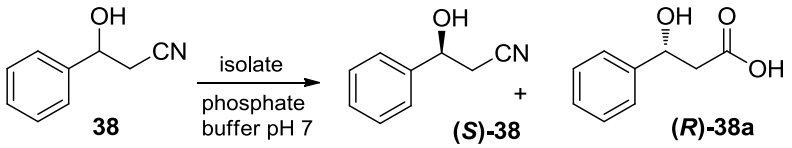
Due to lack of growth of F62 at the predetermined growth conditions, only 3 of the 4 isolates chosen could be screened for conversion and enantioselectivity. The hydrolysis reaction of SS1-14 also gave no chemical yields of acid **38a** or amide **38b** (Table 2.15, entry 7, 8 and 9) after 6 or 24 hours at 25 °C or over 24 hours at 15 °C, in each case nitriles were recovered in quantitative yields. In the case of SS1-24 and SS1-12 only low enantiomeric excess values were obtained for the (*R*)-acid product **38a**, with no

amide detected. The configuration was determined to be (*R*)- acid **38a** in all cases, by comparison with a commercial standard of (*R*)-acid **38a**, which eluted at 13.8 minutes. Comparison of the optical rotation to the authentic sample also enabled the configuration of the acid to be determined [30]. The best ee value (18.1 %) was obtained with isolate SS1-12 (Table 2.15, entry 4) after 24 hours at 25 °C. When the temperature was reduced to 15° C over 24 hours (Table 2.15, entry 5), the enantioselectivity decreased to 5.5 % for this isolate.

In all cases the absence of the amide product **38b** suggested that the isolates may contain a nitrilase enzyme, as at both time points and temperatures no amide was detected, either by reverse phase or chiral HPLC.

The low ee values of the recovered nitrile and acid produced in both cases, were not enhanced by performing the biotransformation at lower temperatures. A decrease in temperature from 25 °C to 15 °C also resulted in a lower yield of the corresponding acid as can be seen with isolate SS1-24 (Table 2.15, entry 1, 2) which decreased from 81 % to 47 % respectively.

Table 2.15: Biotransformation of 3-hydroxy-3-phenylpropionitrile.



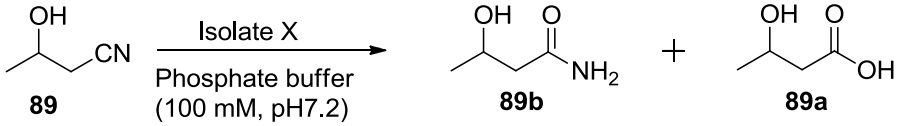
38 **(*S*)-38** **(*R*)-38a**

Entry	Isolate[a]	Time (Hours)	Temp (°C)	Nitrile[b]		Acid[b]	
				Yield %	ee %	Yield %	ee %
1	SS1-24	24	25	18.0	5.2	81.0	0.4
2	SS1-24	24	15	42.3	1.2	47.0	0.0
3	SS1-24	6	25	18.5	2.4	80.2	4.9
4	SS1-12	24	25	83.4	3.6	13.1	18.1
5	SS1-12	24	15	87.2	3.4	11.3	5.5
6	SS1-12	6	25	89.0	6.0	9.5	8.0
7	SS1-14	24	25	98.1	2.8	ND	ND
8	SS1-14	24	15	97.5	1.9	ND	ND
9	SS1-14	6	25	98.6	0.2	ND	ND

[a]Biotransformation was carried out by incubating 3-hydroxy3-phenylpropionitrile (10 mmol) in a suspension of the named isolate (OD=1) in phosphate buffer (pH 7.0). [b]Determined by HPLC analysis using a chiral column.

2.6.2 ENANTIOSELECTIVITY SCREENING OF ISOLATES TOWARDS 3-HYDROXYBUTYRONITRILE

Isolates were next screened towards 3-hydroxybutyronitrile **89** for enantioselectivity (Scheme 2.4).



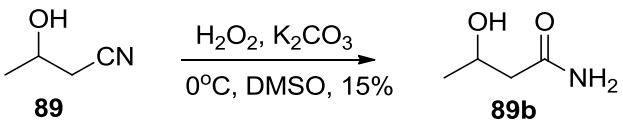
Scheme 2.4: Enantioselectivity screening of isolates towards 3-hydroxybutyronitrile.

For HPLC analysis racemic 3-hydroxybutyric acid **89a** was commercially available and obtained from Sigma Aldrich. The corresponding *β*-hydroxy amide standard **89b** was synthesized in order to facilitate chromatographic method development.

A number of synthetic approaches were investigated for the production of amide **89b**. The 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) coupling of acid **89a** and NH_3 catalysed by 4-dimethylaminopyridine (DMAP) in dichloromethane (DCM) proved difficult (Scheme 2.5), so an alternative synthesis was evaluated.

Scheme 2.5: DMAP catalysed coupling of 3-hydroxybutyric acid with NH_3 to produce 3-hydroxybutyramide.

Preparation of amide **89b** from nitrile **89** proved to be challenging. During initial attempts to hydrolyse nitrile **89** using H_2O_2 and K_2CO_3 in dimethyl sulfoxide (DMSO) (Scheme 2.6), the reaction was quenched with water after 15 minutes and extracted with DCM as per literature procedure. The amide product **89b** was not present however after this time.

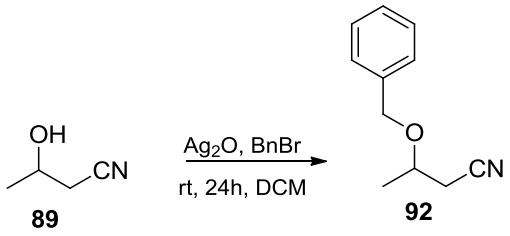


Scheme 2.6: Preparation of 3-hydroxybutyramide from 3-hydroxybutyronitrile using H_2O_2.

The reaction was repeated and a kinetic study performed using LC-MS to monitor the progress of the reaction. The production of the amide **89b** was monitored at various time points by detecting the amide MH^+ adduct at m/z 104. After approximately 4

hours, the reaction was quenched with H_2O and following extractive work up and chromatographic purification, the expected product was obtained in 15 % yield. LC-MS analysis produced the MNa^+ adduct at m/z 126. The structure of this compound was also verified by ^1H and ^{13}C NMR spectroscopy. The key NMR spectroscopic resonances which confirmed the successful reaction and verified the product, were the ^1H NMR amide NH_2 peaks at 6.75 ppm and ^{13}C NMR $CONH_2$ peak at 173 ppm.

Chromatographic method development for the detection of 3-hydroxybutyronitrile **89** and the corresponding products **89a** and **89b**, proved to be a complex task. 3-hydroxybutyronitrile and the corresponding amide and acid products are not intrinsically UV active. A derivitisation method was therefore explored (Scheme 2.7), to add a UV active-benzyl group for more facile analysis and monitoring of the reaction.

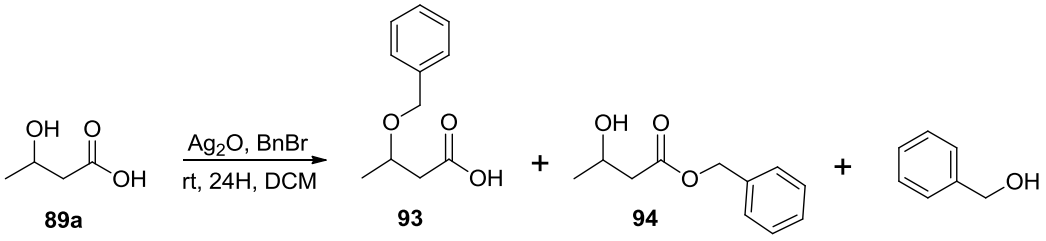


Scheme 2.7: Benzylation of 3-hydroxybutyronitrile.

The introduction of a benzyl group onto the hydroxy of nitrile **89**, was achieved by the reaction of **89** with benzyl bromide in the presence of Ag_2O in acetone, as previously described by Wang *et al*. [31]. The reaction product **92** was purified using preparative TLC and characterised using NMR. Key ^1H NMR resonances included the benzyl CH_2 peaks at 4.63 ppm and aromatic protons at 7.30-7.48 ppm. A chiral HPLC method was developed using an OJ-H chiral column to separate the enantiomers of **92**, and adequate resolution was achieved with enantiomers eluting at 19.2 and 23.1 minutes, which corresponded to data reported in the literature [32] [31].

The benzylation conditions were investigated in order to develop an *in situ* derivatisation procedure at the biotransformation scale of 10 mmol (Scheme 2.8). The quantity of DCM required was determined by investigating the reaction at 5 mg/mL and 5 mg/25 mL concentrations. Other parameters investigated included variation of the duration of the derivitisation procedure. Final conditions employed were the addition of 2 mL of solvent to biotransformation products leaving the reaction for 12 hours in the dark. This correlates with the expected concentration of the remaining nitrile if the

biotransformation was to proceed to 50 % conversion. Racemic acid **89a** was also subjected to the derivitisation parameters as outlined above. Although Ag_2O should selectively benzylate the hydroxy functional group, both possible derivatives were obtained [31].



Scheme 2.8: Benzylation of 3-hydroxybutyric acid for the production of 3-benzyloxy butyric acid and benzyl-3-hydroxybutanoate.

Chiral HPLC analysis of the crude reaction product indicated the presence of both 3-benzyloxybutyric acid **93** and benzyl-3-hydroxybutanoate **94** by-products, along with benzyl bromide. The reaction was repeated on a larger scale so that each benzylated product could be purified and characterised. GC-MS analysis of the crude reaction mixture again suggested that benzylation occurs at both functional groups, and also revealed the identity of one of the by-products as benzyl alcohol. The products were separated by preparative TLC and characterised by NMR spectroscopy. ^{1}H NMR analysis indicated that the major product was **93** as opposed to **94**. This is evident in the chemical shift value of the benzyl CH_2 ether peak at 4.72 ppm. This is distinctly different from the benzyl CH_2 ester peak which occurs as a singlet integrating for 2 protons at 5.2ppm. LC-MS analysis showed a MH^+ ion with m/z 195 in both cases. Chiral HPLC method development was carried out using an AD-H chiral column. Adequate baseline resolution was achieved and the ee was calculated based on separation of the benzyl ether enantiomers. The (*R*)-enantiomer was found to elute at 11.94 min and the(*S*)-enantiomer at 12.34 min, which correlated with literature values previously reported by Wang *et al* [31].

Having developed chromatographic techniques to screen for enantioselectivity, the isolates were induced using 3-hydroxybutyronitrile **89** for optimum activity and selectivity. The biotransformation was carried out in phosphate buffer containing 10 mM of nitrile **89** on a 6 mL scale. The isolates were assayed over 6 and 24 hours at both 15 and 25 °C. The reaction was quenched by removal of the biomass, acidified using 1M HCl and extracted with ethyl acetate. The crude reaction mixture was subjected to

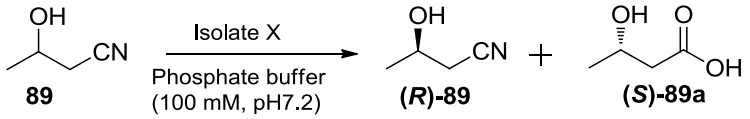
the benzylation process by the addition of BnBr (4 equiv) and Ag_2O (1 equiv) in 2mL of DCM with stirring in the dark over night. The sample was filtered, evaporated and then diluted with mobile phase prior to injection on an AD-H chiral column. The amide was also subjected to the biotransformation conditions outlined above. The corresponding benzyl ether was injected onto the AD-H chiral column and the enantiomers were found to elute at 18.4 and 19.6 minutes.

The biotransformations of the 10 isolates were carried out as per the standard procedure, at 15 and 25 °C over 6 and 24 hours. In all cases, only the corresponding acid was detected. Also the nitrile was partially consumed during the incubation period which is in agreement with the results obtained using the high-throughput screening strategy. The following Table 2.16 reports the enantioselectivity values for both remaining nitrile and the corresponding acid for the 10 chosen isolates.

The chiral screening study identified a single isolate SET1 (Table 2.16, entry 5) which demonstrated exceptional enantioselectivity towards nitrile **89**. In this case only the corresponding acid was detected, with >99.9 % ee after 24 hours. Isolate NN30 (Table 1.16, entry 9) demonstrated the second highest enantioselectivity with 58.5 % ee after 24 hours at 15 °C, followed by F36 and F69 (Table 2.16, entry 1 and 2) with 31.6 % and 27.6 % respectively.

It should be noted that several of the isolates demonstrated higher enantioselectivity at lower temperatures, for example an increase in enantioselectivity was observed in the case of F36 from 21.3 % to 31.6 % ee when the temperature was reduced from 25 °C to 15 °C (Table 2.16, entry 1). In the case of isolate SET1, the corresponding acid was produced in >99.9 % ee at both temperatures. A slight decrease in activity from 1.02mmol/L to 0.98mmol/L was observed when the temperature was reduced from 25 °C to 15 °C respectively. Also, a slight decrease in activity was observed from 1.02 mmol/L produced after 24 hours, to 0.86 mmol/L when the time was reduced to 6 hours, at 25 °C.

Table 2.16: Enantioselective hydrolysis of 3-hydroxybutyronitrile catalysed by novel bacterial isolates.



Entry	Isolate	Optimum temperature[b]	Nitrile ee [c] (%)	Acid ee [c] (%)	Activity[d]	ref
1	F36	15 °C	13.9	31.6	Low	
2	F69	15 °C	1.4	27.6	Good	
3	F71	15 °C	12.5	2.0	Good	
4	SS1-12	25 °C	26.2	14.0	Good	
5	SET1	25 °C	ND	99.9	High	
6	*Paenibacillus* sp. NN32	15 °C	1.3	25.5	High	[7]
7	*Burkholderia* sp. LC3c	15 °C	5.2	13.1	High	[7]
8	SW2-33, *Serratia* sp.	15 °C	2.3	ND	Low	
9	*Bacillus* sp. NN30	15 °C	22.3	58.5	Good	[7]
10	SS1-31	25 °C	16.2	18.7	Good	

[a] Biotransformation was carried out by incubating 3-hydroxybtyronitrile (10 mmol/L) in a suspension of the named isolate (OD600nm = 1) in phosphate buffer (pH 7.0) for 24 hours. [b] Temperature for optimum enantioselectivity. [c] Determined by HPLC analysis using a chiral column. [d] Activity determined using Nessler's colorimetric activity assay (at optimum temperature).

To compare isolate activity and enantioselectivity, isolate SET1 (Table 2.16, entry 5) fell within the 'high' activity range of the screening scale. However it did not have the highest activity as determined using the Nesslers colorimetric assay. In comparison, isolate NN32 (Table 2.16, entry 6) showed the highest activity but demonstrated much lower enantiocontrol.

Work performed by Erica Owens (unpublished) involved screening strain SET1 for the presence of nitrilase, nitrile hydratase (α and β subunits) and amidase genes using conventional PCR. While no nitrilase genes were detected by this screening, the aforementioned results suggest that there is an undetected active nitrilase present in isolate SET1. Sequencing of the 16s rRNA gene (by Erica Owens) indicates that SET1 is a strain of *Rhodococcus erythropolis.* It is 100 % identical over the sequenced region to *Rhodococcus* sp. TMS1-19 (GenBank acession JX949804)

During this work it was identified that isolate SET1 also contained an amidase enzyme. Most nitrile hydrolysing micro-organisms have been shown to contain either a nitrilase or N-Hase/amidase system. However, some micro-organisms such as *Rhodococcus*

rhodochrous J1 contain both nitrilase and N-Hase/ amidase biotransformation pathways [33].

It is also surprising to note that in the case of SET1, the corresponding unreacted (*R*)-nitrile **89** could not be detected in the reaction mixture after the 24 hour reaction time. The general extraction procedure was examined in order to ensure that any remaining unreacted nitrile substrate could be efficiently extracted and recovered. Both centrifugation and celite pad filtration were evaluated as methods for biomass removal. Ethyl acetate was substituted with DCM and the pH varied, however in all cases the nitrile product was not recovered. The various parameters for the extraction procedure were also carried out on the nitrile substrate in the absence of the isolate. No substantial difference in nitrile recovery was observed, indicating that any unreacted nitrile present should be recoverable from the biotransformation and may be metabolised by other enzymes during the biotransformation.

To determine the isolated yield and configuration of the acid product formed using isolate SET1 during the hydrolysis of racemic nitrile **89**, a large scale biotransformation was carried out. The isolate was incubated with 3-hydroxybutyronitrile (10 mM) in potassium phosphate buffer (100 mM, pH 7) for 24 hours at 25 °C. The biomass was removed by centrifugation, following an acid base work up, the acid products were purified using preparative TLC in 41 % yield and >99.9 % ee. The ee value of acid **89a** was measured by chiral HPLC analysis after derivitisation, and the absolute configuration was determined as (*S*) by comparison of optical rotation with an authentic sample [34]. The unreacted nitrile **(*R*)-89** could not be recovered from the reaction mixture.

An enantioselectivity versus time study was carried out where the biotransformation was monitored after 3, 6, 9, 24 and 36 hours (Figure 2.10). Results indicate that isolate SET1 is highly enantioselective and possibly enantiospecific with >99.9 % ee of acid **(*S*)-89a** at each sample point. It is believed the hydrolysis of the (*S*)-nitrile proceeds rapidly, as only the remaining nitrile **(*R*)-89** is detected after 3 hours. This was further demonstrated by the formation of only *(S)*-3-hydroxybutyric acid after a 10 day incubation period. In addition, the reduction in intensity of the remaining nitrile **(*R*)-89** is also evident after various time points, indicating the potential metabolism of the

unreacted nitrile **(*R*)-89** along with the desired biotransformation. Attempts to recover the remaining nitrile **(*R*)-89** after 6 hours, also failed to yield the nitrile.

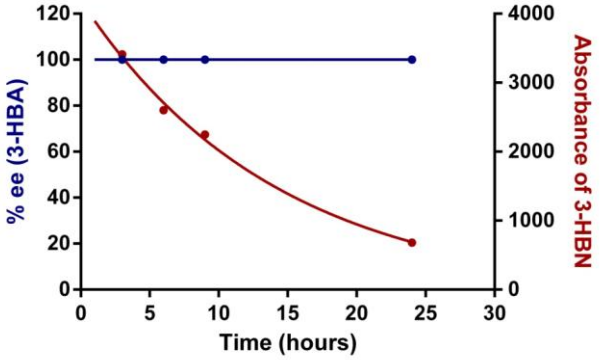


Figure 2.10: Enantioselectivity study over time demonstrating the exponential decay of (*R*)- 3-hydroxybutyronitrile.

The exponential decay of the unreacted nitrile **(*R*)-89** may be due to other enzyme systems present within the microbial whole cells. It was thought a possible loss of yield may be due to hydrolysis of the nitrile to the corresponding acid **89a** followed by isomerisation, as this would lead to an increase in yield of the (*S*)-isomer **89a** at the expense of the (*R*)-isomer. An investigation into this racemisation was carried out by incubating racemic 3-hydroxybutyric acid **89a** with the isolate over a 24 hour period; however unchanged racemic acid (96 % yield, 0.3 % ee) was recovered. Other options include the possibility of an isomerase converting the remaining nitrile **(*R*)-89** to (*S*)-nitrile **89** which may then be converted to the corresponding acid **(*S*)-89a**. However the isolated yield never exceeded 50 %. Other enzymes such as an aldoxime or alcohol dehydrogenase may also account for the loss of mass balance but were not investigated further.

An alternative possibility is that nitrile **89** enters the bacterial whole cells by passive diffusion and may be retained in the cell; therefore removal of the biomass by centrifugation may be responsible for the difficulty encountered when attempting to extract the remaining nitrile. Brammar *et al*. suggested that acetamide and other aliphatic amides were taken up by *P. aeruginosa* via a constitutive transport system [35]. Subsequently Betz & Clarke, found that *P. aeruginosa* was freely permeable to a range of amides [36].

It has been reported that the enzyme catalysed kinetic resolution of *β*-substituted nitriles can demonstrate low enantioselectivity in some cases [37], [18], [19], [20], [38], [21]. For example in the biotransformation of 3-hydroxybutyronitrile **89** using whole cells of *Rhodococcus erythropolis* AJ270 containing a nitrile hydratase/amidase system, low enantiocontrol was observed with the formation of (*S*)-3-hydroxybutyric acid in 17.8 % ee [31]. The enantioselectivity of the reaction was dramatically improved using a benzyl protection/ docking strategy which resulted in the formation of the acid with 86.2 % ee. In the case of SET1 >99.9 % ee may be achieved using 3-hydroxybutyronitrile **89** with the free unprotected hydroxy group. Such high enantioselectivity demonstrated by isolate SET1 may be as a result of the position of the chiral recognition site in the enzyme located remotely to the catalytic centre [34]. This has also been observed by previous authors [17, 31]. While the biocatalytic hydrolysis of *β*-hydroxynitriles to corresponding *β*-hydroxy acids and amides using microorganisms possessing nitrile hydratase/amidase have been well reported [39], [21] and [37], in contrast, microorganisms with nitrilase activity often demonstrate low or extremely low activity towards the hydrolysis of *β*-hydroxynitriles [10, 12, 21, 37]. To the best of our knowledge, this is the first isolate potentially demonstrating enantiospecificity towards 3-hydroxybutyronitrile **89**.

Due to the success of this isolate, Chapter 3 will describe experimental work involving the optimisation *R. erythropolis* SET1. In addition, several techniques employed to try and purify and characterise this enzyme will be discussed.

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CHAPTER 3

ISOLATE EVALUATION AND ATTEMPTED PROTEIN PURIFICATION

ISOLATE EVALUATION AND ATTEMPTED PROTEIN PURIFICATION

3.1 INTRODUCTION

The advantages of utilising biocatalysts for producing optically active, higher-value molecules have been previously discussed. However in reality, the range of chemical reactions open to biocatalysis is limited. This is due to the reaction conditions necessary for enzyme function (an aqueous environment at near physiological conditions) not being conducive to reactivity with their non-natural substrates, such as larger more complex chemical compounds. In order for an enzyme to be industrially useful it must possess characteristics such as tolerance for higher substrate concentration, broadened pH and thermostability, and broad substrate specificity [1]. Several such parameters influencing the biocatalytic reaction of SET1 towards the hydrolysis of 3-hydroxybutyronitrile its optimum substrate, were examined and are discussed herein.

3.2 INFLUENCE OF TEMPERATURE DURING THE BIOTRANSFORMATION OF 3-HYDROXYBUTYRONITRILE BY SET1

Temperature is one of the most crucial factors for enzymatic reaction rate and can significantly influence the activity, enantioselectivity, and stability of a biocatalyst as well as the equilibrium of a reaction (Table 3.1) [2]. The structure and the nature of the enzyme may also be influenced by temperature due to protein folding interactions [3-5].

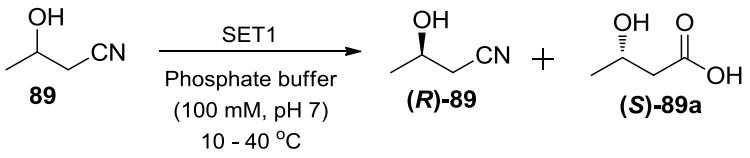
Table 3.1:Reported bacteria with nitrilase activity and their optimum temperature as reported in previous literature adapted from Gong *et al.* [2]

Entry	Bacteria	MW (kDa)	Optimum temp (^{O}C)	Stability temp (^{O}C)	Ref
1	*Pseudomonas sp.*	-	-	-	[6]
2	*P. putida*	412	40	below 50	[7]
3	*Pysococcus abyssi* GE5	60	80	60-90	[8]
4	*R. rhodochrous* NCIMB 11216	45.8	30	-	[9]
5	*P. fluorescens* DSM 7155	130	55	-	[10]
6	*R. rhodochrous* K22	650	50	55	[11]
7	*R. rhodochrous* PA-34	45	35	35	[12]
8	*A faecalis* ATCC 8750	460	45	20-50	[13]
9	*Acinetobacter sp.* AK226	580	50	below 50	[14]
10	*Comamonas testosterone sp.*	-	-	-	[15]
11	*R. rhodochrous J1*	78	45	20-50	[16]
12	*Nocardia* sp. NCIB 11216	560	-	-	[9]
13	*Brevibacterium* strain R 312	-	35	below 30	[17]

The effect of temperature on SET1 activity towards 3-hydroxybutyronitrile **89** was investigated. Enzyme activity and enantioselectivity were determined after 24 h incubation with nitrile **89** (10 mM) at temperatures ranging from 10 °C to 40 °C

(Scheme 3.1). The ammonia produced during the hydrolysis was quantified using the Nesslers colorimetric activity assay, and the enantioselectivity determined after derivitisation to the corresponding benzyl ether and HPLC analysis on an AD-H chiral column.

The effect of temperature on nitrilase activity and enantioselectivity is shown in Figure 3.1. Relative activity was calculated based on the activity observed and concentration of ammonia produced during the hydrolysis of nitrile **89** (10 mM) in potassium phosphate buffer at pH 7 at 25 °C. A minor decrease in activity was observed when the temperature was reduced to 10 °C with 82 % relative activity, whereas 104 % relative activity occurred when the temperature was increased from 25 °C to 30 °C. A further increase in temperature from 30 °C to 40 °C significantly increased the relative activity to 132 %.



Scheme 3.1: An investigation into the effect of temperature on the SET1 nitrilase catalysed hydrolysis of 3-hydroxybutyronitrile.

Although a lower temperature (10 °C) was successful in maintaining the enantioselectivity, the reaction activity decreased (Figure 3.1). In contrast, above 30 °C the nitrilase enantioselectivity was rapidly lost. The biotransformation at 30 °C caused an approximate 63 % loss of the initial enantioselectivity with a further decrease to 84 % at 40 °C.

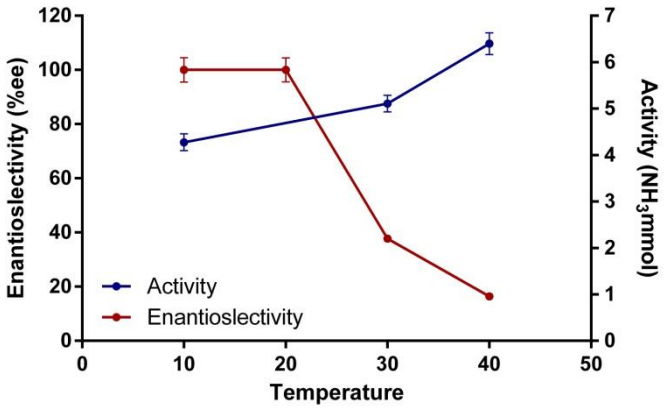


Figure 3.1: **Effects of temperature on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) at 10, 20, 30 and 40 °C. Activity was determined using the Nesslers microscale colorimetric assay. Enantioselectivity was determined by HPLC analysis using a chiral column.**

As compared to literature values, SET1 appears to follow the trend that biotransformations at higher temperatures (30 - 40 °C) result in higher activity. For example, *R. rhodochrous* NCIMB 11216 (Table 3.1, entry 4) was found to have much higher activity at 40 °C and 50 °C during the hydrolysis of benzonitrile [9]. The optimal temperature for *R. rhodochrous* K22 (Table 3.1, entry 6) towards crotononitrile was found to be 50 °C however above 55 °C enzyme activity was rapidly lost [14].

In the case of SET1 the optimal temperature for the biotransformation of nitrile **89** was determined to be 25 °C. Although higher temperatures resulted in higher activity, enantioselectivity was lost.

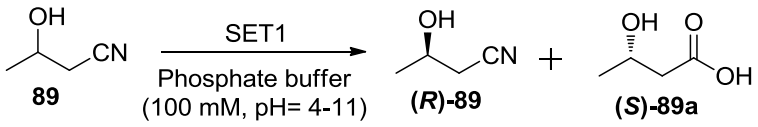
3.3 INFLUENCE OF pH ON THE NITRILASE ACTIVITY OF SET1

In addition to temperature, pH may affect the structure and the nature of an enzyme thereby influencing the enzyme activity. The optimal pH for the biotransformation of a variety of nitrilase enzymes which originate from bacteria are shown in Table 3.2. This indicates that most nitrilases exhibit their best activity in the pH range of 7.0-8.0 [2].

Table 3.2:The reported bacteria with nitrilase activity and their optimum pH as reported in previous literature adapted from Gong *et al.* [2]

Entry	Bacteria	MW (kDa)	Optimum pH	Stability pH	Ref
1	*Pseudomonas sp.*	-	-	7.4-8.8	[6]
2	*P. putida*	412	7	6.5-8	[7]
3	*Pysococcus abyssi* GE5	60	7.4	4.5-8.	[8]
4	*R. rhodochrous* NCIMB 11216	45.8	8	-	[9]
5	*P. fluorescens* DSM 7155	130	9	-	[10]
6	*R. rhodochrous* K22	650	5.5	-	[11]
7	*R. rhodochrous* PA-34	45	7.5	-	[12]
8	*A faecalis* ATCC 8750	460	7.5	7-8	[13]
9	*Acinetobacter sp.* AK226	580	8	5.8-8	[14]
10	*Comamonas testosterone sp.*	-	-	-	[15]
11	*R. rhodochrous J1*	78	7.6	-	[16]
12	*Nocardia* sp. NCIB 11216	560	8	-	[9]
13	*Brevibacterium* strain R 312	-	7	-	[17]

To evaluate the alkaline and acid tolerance of *R. erythropolis* SET1, resting cells expressing nitrilase activity were incubated with 3-hydroxybutyronitrile **89** overnight at 25 °C at various pH values between 4 and 11 in phosphate buffer (100 mM KH_2PO_4) (Scheme 3.2). Following extractive work-up and derivitisation using previously reported methods in Chapter 2, both activity and enantioselectivity were determined.



Scheme 3.2: An investigation into the effect of pH on the SET1 nitrilase catalysed hydrolysis of 3-hydroxybutyronitrile.

The results in Figure 3.2 show the effect of pH on nitrilase activity and enantioselectivity. Activity was maximal at pH 7.0, falling sharply at both higher and lower values. Isolate SET1 was found to have a pH optimum of 7, with maximum activity and enantioselectivity both retained. When the pH was reduced to 6, 86 % of the maximum activity relative to that observed at pH 7 (25 °C) was retained. A further decrease of pH resulted in a further loss of activity with 67 % relative activity observed at pH 4.

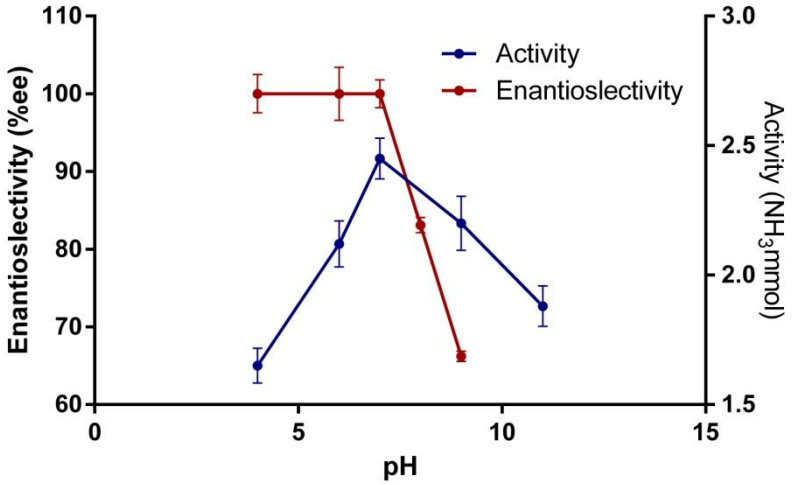


Figure 3.2: Effect of pH on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours at 25 °C in potassium phosphate (100 mM) buffered at pH 4, 6, 7, 9 and 11. Activity was determined using the Nessler's microscale colorimetric assay. Enantioselectivity was determined by HPLC analysis using a chiral column.

A similar trend is observed with biotransformations carried out under alkaline conditions where activity is lost at higher pH values. However the drop in activity is not as sharp under basic conditions, with 89 % relative activity at pH 9 and 76 % at pH 11. The enantioselectivity of the biotransformation however was less dramatically influenced by the acid pH of the biotransformation; >99.9 % ee was retained under acidic conditions even at pH 4. However a significant decrease in ee was found under alkaline conditions, with approximately 83 % and 66 % of the initial enantioselectivity remaining at pH 8 and pH 9 respectively.

To compare isolate SET1 with other nitrilases, Yamamoto *et al.* [18] examined the pH optimum of *Actinobacter sp* AK226 (Table 3.2, entry 9) towards various nitriles with activity being reported relative to ibuprofen-nitrile. They determined that this nitrilase displayed optimal activity at pH 8, which reduced rapidly or gradually at lower or higher pH respectively. *R. rhodochrous* sp. NCIMB 11216 (Table 3.2, entry 4) had a higher pH optimum of 8.5 towards benzonitrile [9], and *R. rhodochrous* K22 (Table 3.2, entry 6) demonstrated a maximum activity in the more acidic region of pH 5.5 towards crotononitrile. This is in contrast to the pH optimum of other nitrilases previously reported and discussed above which have a pH range of 7.5 to 9 [11, 14, 19].

Therefore the relatively high activity observed under acidic pH conditions with SET1, which enabled the biotransformation to be performed without any substantial loss in enantioselectivity, is promising for biotransformations of nitrile analogues which may decompose at a higher pH. This is discussed later in more detail for the hydrolysis of mandelonitrile, which as a substrate undergoes rapid decomposition to produce benzaldehyde and cyanide which may act as enzyme inhibitors.

3.4 INFLUENCE OF METAL IONS AND OTHER REAGENTS ON THE ACTIVITY OF SET1

Numerous enzyme modifiers including inhibitors, stabilisers and metal ions, can affect enzyme activity [2]. The majority of metals including mercury, cadmium, arsenic, lead, chromium, copper, nickel, cobalt and zinc, at elevated levels are classified as highly toxic to biocatalysts. The toxicities of heavy metal ions arise from their action on enzymes where they may act by; (1) binding to the sulfhydryl –SH groups of the enzyme, (2) displacing an essential metal cofactor of the enzyme or (3) inhibiting the synthesis of the enzyme [20].

The inhibition of nitrilase by heavy metal ions has been ascribed to the reaction of these ions with the thiol –SH group of the catalytically conserved cysteine residues, resulting in the formation of thiolates [21]. With metal ions, thiolates behave as ligands to form transition metal thiolate complexes [21]. According to hard/soft acid/base theory, sulphur is a relatively soft, polarisable atom [22]. This explains the tendency of thiols to bind to softer elements or ions such as mercury, lead or cadmium. Additives that include Ag^{+} and Hg^{2+} ions, prefer soft donors such as sulphur, and exhibit the following preferential binding site sequence: S> N> O. In contrast, Cu^{2+} although having a higher

affinity for nitrogen- and oxygen- containing ligands, are also capable of forming stable complexes with various ligands such as sulphur [22].

Importantly, heavy metal ions can also bind to functional groups other than thiols. These mainly include nitrogen- (histidine) and oxygen-(aspartic and glutamic acids) containing functional groups. In fact, the relative frequency of sites reported as utilised by metals in metaloproteins follows the order, His, Cys, Asp and Glu [23].

The effects of various inorganic compounds ($CuSO_4.5H_2O$, $FeCl_3.6H_2O$, $Pb(CH_3COO)_2.3H_2O$, $FeSO_4.7H_2O$, $AgNO_3$, $HgCl_2$, $Cu_2SO_4.5H_20$, $MnSO_4.4H_2O$, EDTA, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$, $NiSO_4.6H_2O$ and $ZnSO_4.7H_2O$) were examined in the biotransformation of nitrile **89** to acid **89a** using whole cells of *Rhodococcus erythropolis* SET1. High sensitivity to these reagents may indicate the importance of the thiol group for catalytic activity. The influence of the metal ions was examined at 1 and 5 mM concentrations of additive. Samples containing the whole cells were incubated with nitrile **89** (10 mM) at 25 °C for 24 hours. The standard activity was determined using the Nesslers colorimetric assay, along with cell blanks and metal ion blanks. The activity of the isolate was calculated relative to the activity of SET1 in the biotransformation of 3-hydroxybutyronitrile **89** in 100 % phosphate buffer (100 mM at pH 7) and the results are outlined in Table 3.3.

Table 3.3: Influence of various reagents, and chelating agents on the nitrilase of *R. erythropolis* SET1.

Compounds	Relative nitrilase activity %	
	1mM	5mM
None	100	
Fe^{3+}	119	108
Pb^{2+}	101	83
Ag^{+}	6	0
Fe^{2+}	75	63
Hg^{2+}	0	0
Cu^{2+}	109	107
Mn^{2+}	15	0
EDTA	43	40
Mg^{2+}	113	121
Co^{2+}	106	100
Ni^{2+}	12	10
Zn^{+}	38	8

Samples containing the whole cells (10 mM) and a reagent (1-5 mM) were incubated with 3-hydroxybutyronitrile at 25 °C for 24 hours. The standard activity was determined using the Nesslers colorimetric assay, along with cell blanks and metal ion blanks.

Several of the reagents at a concentration of 1 and 5 mM, completely inhibit the activity of *R. erythropolis* SET1 as already demonstrated for other nitrilases [8, 16]. The isolate displayed high sensitivity towards thiol binding metal ions, in particular Zn^{2+}, Ag^{+} and Hg^{2+}. These metal ions can bind to a variety of sites, although they may be predominantly coordinated with proteins via the sulphur thiol moieties of Cys residues [21] as shown in Figure 3.3.

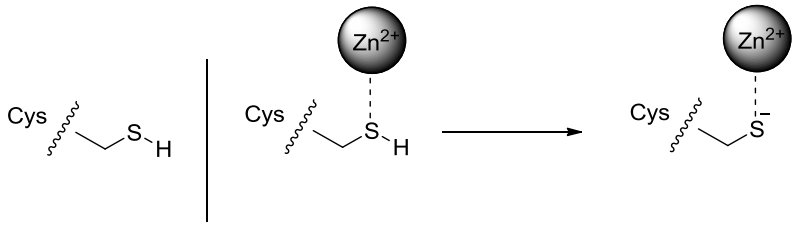
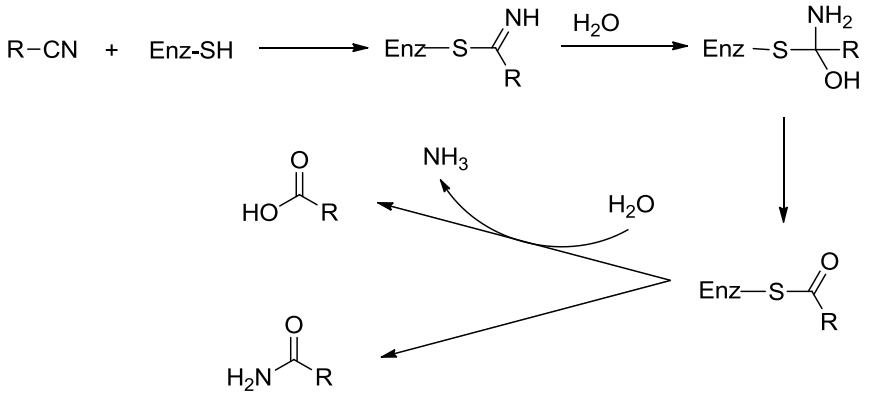


Figure 3.3: Coordination of zinc ions to thiol functionality of a cysteine residue.

These results as shown in Table 3.3 may support the presence of a nitrilase in SET1, following the proposed mechanism for nitrilases as shown in Scheme 3.3. This includes a nucleophilic attack on the nitrile carbon atom by a thiol group at the active of the enzyme [24], [1]. The resulting enzyme-iminothiol ester could be hydrolysed via the tetrahedral amino- hydroxy-thioester intermediate to the thiol ester, which is further hydrolysed to the acid.



Scheme 3.3: Proposed mechanism for nitrilase catalysed hydrolysis indicating the importance of the thiol functionality of the cysteine residue.

Other metals such as Ni^{2+} also caused a marked decrease in nitrilase activity with only 12 % relative activity observed at 1mM concentrations of $NiSO_4.6H_2O$. This was also observed in the case of *R. rhodochrous* PA-34, where it was proposed that this nitrilase contains sulfhydryl groups at the active site of the enzyme [12]. This could also possibly be attributed to the ability of Ni^{2+} to form co-ordination complexes near the active site involving other catalytically active thiol groups, thereby inhibiting activity. This

mechanism was also proposed by Goldlust *et al.* for the interaction of *Fusarium oxysporum* f. sp. melonis with Ni^{2+} salt [7].

The enzyme was partially affected by Fe^{2+} with only 75 % and 63 % relative activity retained at $FeSO_4$ concentrations of 1mM and 5mM respectively. It was found that 5 mM concentration of Mn^{2+} completely inhibited enzyme activity and this was also the case observed with the nitrilase of *Nocardia* sp. NCIB 11216 [25]. Heavy metal ions may also be involved in redox processes and may participate in redox cycling in which reactive oxygen species are generated, and these are thought to be responsible for oxidation of protein functional groups such as thiols [26]. The influence of the iron Fe^{2+} ions could be due to such a redox effect or due to the formation of an iron complex with the thiol group of the enzyme.

To compare Fe^{2+} and Fe^{3+} ions and their effect, there is a large decrease in activity from Fe^{3+} (119 %) to Fe^{2+} (75 %). This may be due to sulphur's high polarisability which decreases the ionic character of its bonds and hence, sulphur containing ligands do not easily displace oxygen ligands from the more electropositive metal ions of Mg^{2+} and Fe^{3+} [26]. This can be seen from the activity maintained in each of these cases.

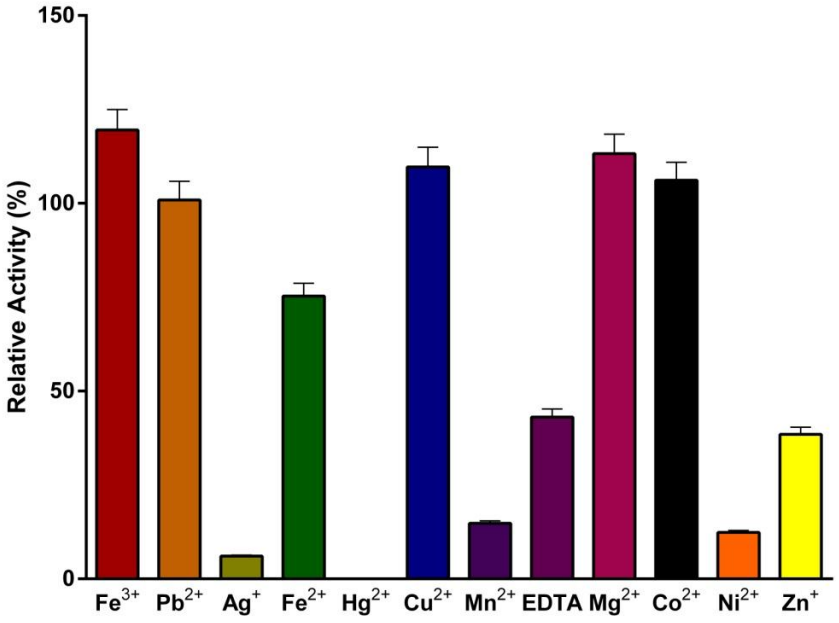


Figure 3.4: The effect of metal ions on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) containing 1 mM of inorganic metal ions. Activity was determined in triplicate using the Nesslers microscale colorimetric assay and is reported relative to that observed in the absence of metal ions.

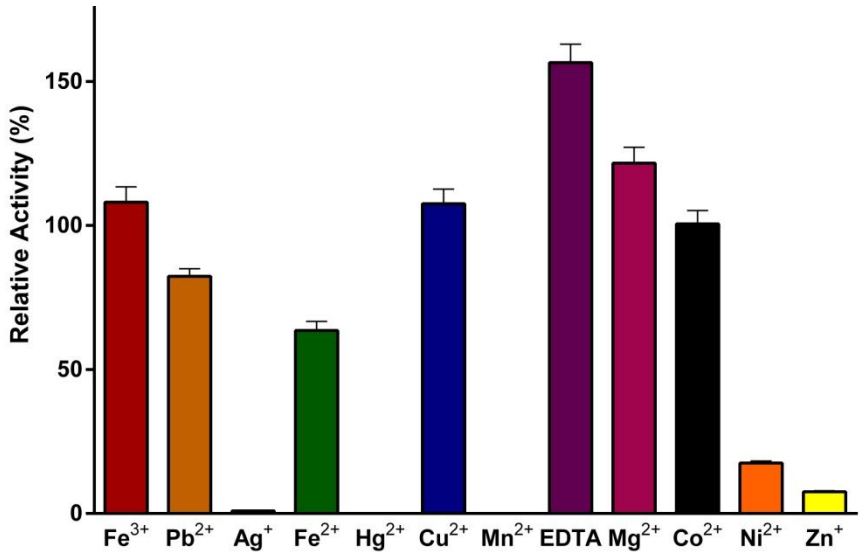


Figure 3.5: The effect of metal ions on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) containing 5 mM of inorganic metal ions. Activity was determined in triplicate using the Nesslers microscale colorimetric assay and is reported relative to that observed in the absence of metal ions.

In summary these results agree with the mechanism for the nitrilase proposed by Thimann and Mahadevan, as all the results outlined in this section point towards the sensitivity of a thiol functionality of the catalytically reserved cysteine residue at the active site [2, 27].

3.5 EFFECT OF ORGANIC SOLVENTS ON NITRILASE ACTIVITY OF SET1

Aqueous media can limit the use of nitrile hydrolysing enzymes in the hydration of inherently hydrophobic nitriles, though added quantities of organic solvents can be used to aid substrate availability to the enzyme in some cases [28].

Several approaches have been reported in the literature including monophasic systems with solvents such as DMSO [29] and biphasic systems such as toluene/ H_2O and hexane/ H_2O [30]. The activity of a recombinant nitrilase from *Synechocystis* sp. PCC6803 demonstrated enhanced activities in buffer-organic solvent monophasic or biphasic mixtures (40 % dimethylsulfoxide, 20 % methanol or 40 % *n*-heptane) [29]. A biphasic system containing toluene was chosen as the optimal solvent for the biotransformation of mandelonitrile with *Alcaligenes* sp. ECU0401 and the optimal ratio of water phase to organic phase was considered to be 9:1 (v/v), at which a high product yield (>99.0 %) and a satisfactory ee (98.0 %) could be achieved easily [30].

In order to assess the solvent tolerance of SET1 and determine an optimum solvent system, a series of solvent studies were carried out. An important criterion for the

selection of a potential organic solvent is its biocompatibility towards the enzyme. Eight organic solvents were examined in this work. These included toluene, hexane, ethyl acetate, butanol, IPA, ethanol, DMSO and THF. Initial work examined water miscible organic solvents, such as IPA, DMSO, ethanol and THF for the effect on the activity retention of the whole cells and results are presented in Figure 3.6. Whole cells of *R. erythropolis* SET1 were incubated with 3-hydroxybutyronitrile **89** dissolved in aqueous buffer with various additional co-solvents at quantities between 5 and 50 % (v/v). The reaction mixture was incubated at 25 °C for 24 hours and enzyme activity was monitored using the technique of Nesslerisation which has previously been described in this body of work. The appropriate cell blanks in the various solvent ratios allowed for careful determination of the nitrilase activity. Each biotransformation and subsequent activity assay was performed in triplicate. Relative activity was calculated based on the activity observed from the standard reaction conditions without added solvent to account for variability arising from different cell cultures., using the concentration of ammonia produced during the hydrolysis of nitrile **89** (10 mM) in potassium phosphate buffer at pH 7.

The addition of 35 % (v/v) IPA resulted in 100 % relative activity to that in aqueous solution. As the ratio of IPA increased (50 % v/v) so too did the activity (144 %). This is in contrast to DMSO, ethanol and THF, where the activity decreases when the percentage of organic solvent is increased; this is also shown in Figure 3.6. The addition of 5 % ethanol and 5 % THF resulted in a decrease in relative activity of 54 % and 47 % respectively. The relative activity decreased as the percentage of organic solvent increased and the enzyme appeared to be inactive (0 % relative activity) at concentrations of 50 % v/v ethanol and THF. This significant drop in enzyme activity may indicate that the proteins become denatured in the presence of higher concentrations of these organic solvents.

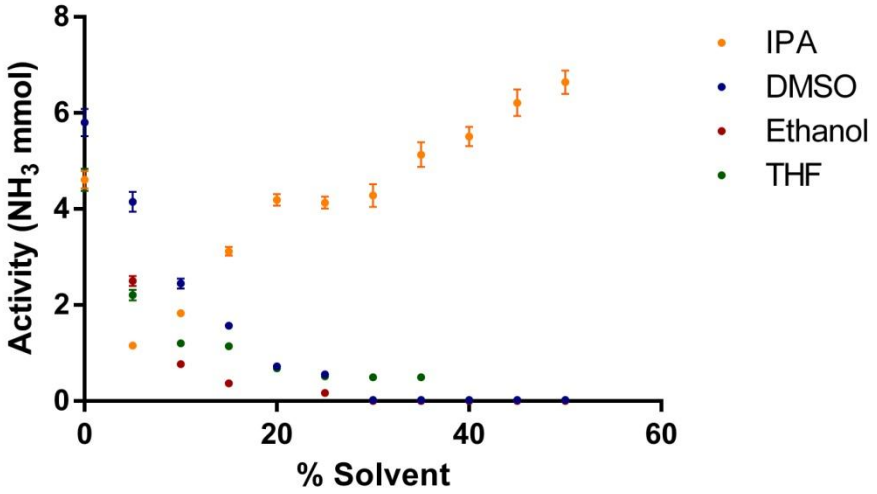


Figure 3.6: Effects of monophasic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) solvent mixture at 25 °C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay

Low concentrations of DMSO (5 % v/v) resulted in retention of 89 % relative activity, while higher concentrations of DMSO (50 % v/v) further reduced the activity (4 %). In summary, although the presence of IPA (35 % v/v) maintained the initial activity, higher concentration of the solvent (50 % v/v) increased the activity to 144 %. Other co solvents examined decreased the relative activity of the reaction in all cases.

In order to relieve the biocatalyst from substrate inhibition in the aqueous monophasic reaction system, a water organic biphasic system may provide an attractive alternative. In this case the hydrophobic substrate will be mainly retained in the organic phase which can act as a reservoir for the toxic or insoluble substrate, thus regulating the substrate concentration around the biocatalyst and minimizing the substrate inhibition [31]. Taking into account the poor water solubility of some nitriles, this allows higher substrate concentrations and also facilitates the product recovery. However, it is unknown whether the biotransformation product 3-hydroxybutyric acid **89a** will be retained only in the aqueous phase due to its high water solubility, and if this will have any inhibitory effect on the enzyme activity.

Four water immiscible organic solvents; ethyl acetate, toluene, butan-1-ol and hexane between 5-50 % v/v were examined for the effect on the activity retention of the whole cells and the results are presented in Figure 3.7

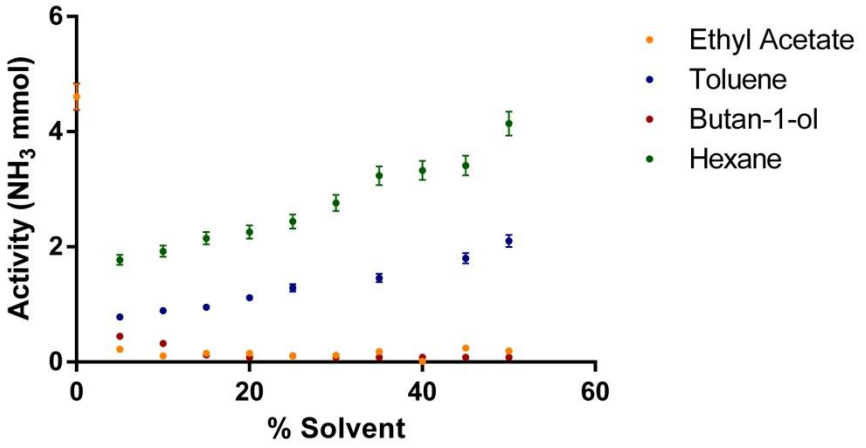


Figure 3.7: Effects of biphasic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours with mechanical shaking in potassium phosphate (100 mM, pH 7.0) and up to 50 % volume of organic solvent added to the mixture at 25 ^{O}C. Activity was determined in triplicate using the Nesslers microscale colorimetric assay.

The highest relative activities were obtained in hexane and toluene which had log P values of 3.5 and 2.5 respectively (Table 3.4). The maximum activity retention was obtained in 50/50 hexane/ aqueous (log P = 3.5) which was 89 % of that in neat aqueous buffer. This may be due to the more rigid and stable conformational structure of the enzyme in this solvent [32]. Although toluene has a similar log P value of 2.5, the activity in 50/50 solvent/ aqueous decreased to 48 %. It was observed that the incorporation of low concentrations of ethyl acetate (5 %) and butan-1-ol (5 %) decreased the relative activity of the reaction to 4.8 % and 9.7 % respectively.

Table 3.4: Shows the logP values of the solvents examined and the solvent ratio at which maximum activity was observed.

Solvent	log P	Solvent/ aqueous ratio
Hexane	3.5	50/50
Ethyl acetate	0.68	5/95
Toluene	2.5	50/50
Butan-1-ol	0.88	5/95
DMSO	-1.49	5/95
IPA	0.38	50/50
Ethanol	0.07	5/95
THF	0.40	5/95

This result indicates that in a biphasic system log P ≥ 2.5 is preferable and that water immiscible solvents with low log P values such as ethyl acetate and butan-1-ol (0.68 and 0.88 respectively) may inhibit the activity of SET1. The optimum percentage of relative activity retained during the biotransformation of nitrile **89** in the presence of

various organic solvents (water miscible and immiscible) is shown in Figure 3.8. The results of the biotransformation of nitrile **89,** catalysed by the nitrilase from SET1 suggested that this enzyme maybe suitable for use in selected organo-aqueous media at selected ratios only.

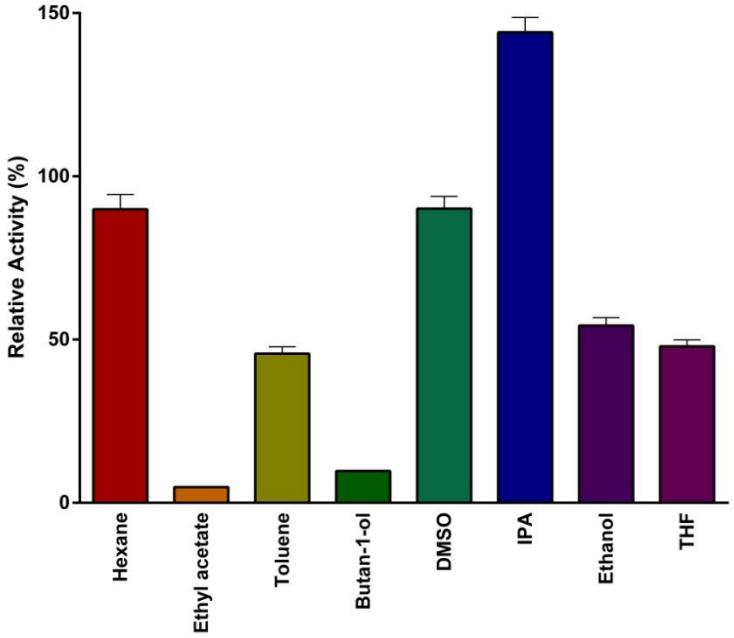


Figure 3.8: Effects of organic solvents on the nitrilase activity of *R. erythropolis* SET1. Reactions were run for 24 hours in potassium phosphate (100 mM, pH 7.0) solvent mixture at 25 °C. Activity was determined using the Nesslers microscale colorimetric assay.

A preliminary investigation into the effect of solvent on the enantioselectivity of the hydrolysis was also carried out. Whole cells of SET1 were incubated in the optimum ratio of each solvent for 24 hours at 25 °C and the biomass was removed through celite pad filtration rather than centrifugation. Extraction of the acid product was attempted with ethyl acetate; however no acid was obtained after work up. The polarity of the extraction solvent/ reaction solvent mix may have affected the product recovery. Therefore work is still required to determine if the solvent and solvent ratio have a detrimental effect on the enantioselectivity of the biotransformation. Additional experiments performed in which the organic solvent in the reaction media is first removed followed by subsequent extraction of the aqueous phase with ethyl acetate did not enable product recovery.

3.6 SUBSTRATE CONCENTRATION AND ITS EFFECT ON THE ACTIVITY AND ENANTIOSELECTIVITY OF THE BIOTRANSFORMATION

Substrate concentration is an important issue associated with nitrile biotransformations. In general, nitrile compounds are known to be detrimental to nitrilases, making the

substrate concentrations used in their biotransformations usually very low (typically 5-40 mM) [2].

The substrate concentration dependence of SET1 was examined in the range of 0.01 to 0.1 M of 3-hydroxybutyronitrile **89**. The reactions were performed by incubating SET1 in a solution of nitrile **89** for 24 hours at 25 °C. Following biomass removal and acid base extractive work up, the crude products were purified by preparative TLC with a 50/50 mixture of hexane and ethyl acetate. Products were verified by their spectroscopic data including ^{1}H and ^{13}C NMR. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase and correlated with literature data [33]. Table 3.5 summarises the quantities of amide and acid formed from the microbial hydrolysis of nitrile **89** by SET1, and also demonstrates the effect of substrate concentration on the enantioselectivity of the reaction.

Table 3.5: Effect of substrate concentration on the enantioselective hydrolysis of 3-hydroxybutyronitrile.

Substrate	Concentration	Time	Amide (yield%, ee%)	Acid (yield%, ee%)
3-HBN	10 mM	24 hours	ND	42.0, > 99.9.2
3-HBN	50 mM	24 hours	18.7, 27.0	21.6, 66 .1
3-HBN	100 mM	24 hours	18.4, 19.1	19.6, 57 .2

In contrast to the lower concentration of 10 mM 3-hydroxybutyronitrile **89**, at 50 mM and 100 mM quantities of the substrate, both the corresponding amide **89b** and acid **89a** were produced. At 50 mM of nitrile **89**, the corresponding amide **89b** and acid **89a** were obtained in 18.7 % and 21.6 % yield respectively with a total product yield of 40.3 %. At 100 mM concentration the amide **89b** product was formed in 18.4 % yield and acid **89a** in 19.6 %. The total product yield was determined to be 38 %. In both cases the unreacted nitrile **89** was not recovered from the biotransformation and the total yields of amide and acid never exceeded 50 %. This fits with previous postulations relating to the recovery of the unreacted (*R*)-nitrile **89**. It is disappointing to note that the ee of acid **(*S*)-89a** produced decreased as the substrate concentration increased and amide **89b** was produced. Enantioselectivity remained greater than 99.9 % ee at 10 mM substrate concentration with no amide detected, while at 50 mM substrate concentration the enantioselectivity of the corresponding acid **(*S*)-89a** decreased to 66 % ee and a further decrease to 57 % ee was observed at 100 mM.

The production of amide **89b** may be a result of high substrate concentration, indicating that the nitrilase enzyme may reach saturation and release the amide intermediate. This is also supported by the loss of mass balance and the absence of unreacted nitrile, which again suggests that the transformation is an intracellular process. Allowing longer reaction times may give an additional insight into the amide formation at higher substrate concentrations. With little evidence of enzyme inactivation or inhibition, biotransformations may be carried out at a higher substrate concentration to determine the maximum substrate concentration tolerated by the enzyme before loss of activity.

3.7 PROTEIN PURIFICATION OF THE NITRILASE IN *R. ERYTHROPOLIS* SET1

It was envisaged that identification and characterisation of the enzyme structure would allow for more detailed insights into the catalytic mechanism. Purification and characterisation of the enzyme would enable a recombinant strain to be produced expressing only the identified nitrilase enzyme. This would ensure that no additional nitrile hydrolysing capabilities that may be present within the whole cells, account for the production of amide or the subsequent hydrolysis to that of the acid product. Hence, expression of the nitrilase enzyme in a recombinant host would ensure that the NHase activity of the nitrilase observed is in fact due to the electronic and steric effects of the α-subtituents and not due to additional NHase and amidases enzymes present as outlined in subsequent chapters.

Nitrilases are typically intracellular enzymes that are fragile and susceptible to degradation in the extracellular environment [2]. Several nitrilase enzymes have been purified and characterised [11, 13, 34-38], which have been recently reviewed by Gong et al. [2]. Some other examples are outlined below in Table 3.6.

Table 3.6: Nitrilase purification methods as adapted from Gong *et al.* [39]

Organism	Purification process	Purification fold	Specific activity	Yield (%)	Ref
Pseudomonas sp.	DEAE-Cellulose; Ammonium sulfate fractionation; Starch electrophoresis	400	12	14	[6]
Nocardia sp. NCIB 11215	DEAE-Cellulose; Ammonium sulfate fractionation; Sephadex G-200	15.82	1.74	9.1	[34]
F. oxysporum f sp. *melonis*	DEAE-Sephacel; TSK-phenyl 5 PW	39.71	143	50	[37]
R. rhodochrous K22	DEAE-Sephacel; Ammonium sulfate fractionation; Phenyl-Sepharose CL-4B; Cellulofine GCL-200	8.3	0.737	9.08	[11]
Acinetobacter sp. AK 226	1st DEAE-cellulose; 1st hydroxyapatite,2nd DEAE-cellulose; 2nd hydroxyapatite; Sephacryl S-400	4.41	0.156	20.1	[18]
A faecalis ATCC 8750	Ammonium sulfate fractionation; Phenyl-Sepharose CL-4B; DEAE-Cellulose	29.0	3.10	17.9	[13]
C testosteroni sp.	Q-sepharose; Superdex 200; Hydroxyapatite.	60	68	79	[15]
R. rhodochrous PA 34	Ammonium sulfate fractionation; 1st Sephacryl S-300 HR; 2nd Sephacryl S-300 HR; DEAE-cellulose toyopearl 650 S	14.1	3.52	34.8	[12]
P. fluorescens DSM 7155	Phenyl-sepharose FF; Mono Q; Superose 12	259	90	10	[10]
P. putida MTCC5110	Ammonium sulfate fractionation; Superdex 200; Q-sepharose; Phenyl-Sepharose	35.01	3.26	10.45	[7]
F. solani O1	Phenyl sepharose; Sephacryl S-200; Q-Sepharose	9.9	156	25.9	[40]
F. solani IMI 196840	Phenyl sepharose; Sephacryl S-200; Q-Sepharose	20.3	144.0	26.9	[41]

Purification process lists the adopted column chromatography techniques after preparation of a cell free extract

A variety of these techniques; preparation of a cell free extract, ammonium sulphate fractionation, hydrophobic interaction chromatography and gel filtration chromatpgoraphy (outlined in Table 3.6) were examined and are discussed below.

3.7.1 PREPARATION OF A CELL FREE EXTRACT

The first step of intracellular protein purification requires methods of disrupting the cells and releasing the enzyme into an aqueous extract. Bacteria range from fairly fragile organisms that can be broken up by digestive enzymes or osmotic shock, to more resilient species with thick cell walls, needing vigorous mechanical treatment for cell disruption [42, 43].

AN INVESTIGATION INTO ENZYMATIC LYSIS CONDITIONS

Cell disruption of *Rhodococcus erythropolis* SET1 was performed by incubating the whole cells in BugBuster® Protein Extraction Reagent (Novagen), which contains non-ionic detergents that are capable of cell wall perforation without denaturing soluble proteins [44]. Dialysis was carried out over 24 hours in phosphate buffer (100mM pH=7) to remove the BugBuster solution using a Float-A-Lyzer (Sigma-Aldrich) with a 20 kDa cut off. Following dialysis, enzyme activity was examined towards 3-hydroxybutyronitrile using the Nessler's colorimetric activity assay. In order to ensure the Nessler's colorimetric assay was compatible with bug buster solution a series of ammonium chloride standards were prepared in BugBuster. Figure 3.9 demonstrates a linear correlation (r^2= 0.9972) between absorbance of the standards and the concentration of ammonia.

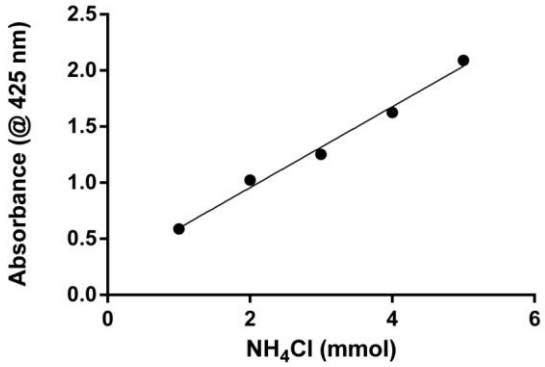


Figure 3.9: Standard curve for ammonium chloride in BugBuster.

Examination of the cell lysate using the Nessler's colorimetric activity assay demonstrated that no acid was produced; this indicated loss of activity as the nitrile was recovered unchanged after 24 hours of interaction. The activity of the soluble cell extract was also examined towards 3-hydroxybutyronitrile over time, in order to determine if the enzyme was still active after centrifugation i.e was the enzyme soluble and present in the supernatant? Nesslers colorimetric assay was again used to examine activity. However no activity was observed indicating that the enzyme was sensitive and may have lost activity or alternatively that the enzyme may be present in the cell debris. This was investigated by streaking the crude lysate, supernatant and cell pellet onto nutrient agar. Following three days of incubation there was substantial growth on all plates, indicating that the lysis conditions were not sufficient.

THE USE OF SONICATION FOR PREPARATION OF A CELL FREE EXTRACT

The effect of sonication on the lysis of microbial whole cells was then examined. Whole cells of *R. erythropolis* SET1 grown in M9 minimal media containing 3-hydroxybutyronitrile as the sole N source were subjected to sonication using an SNE soniprep 150 using a 1 cm probe over various times. Measures were taken to cool the system during cell disruption; the sonication procedure was performed on ice using short 5 second bursts of treatment followed by a 5 second cooling period. Due to the robust nature of *R. erythropolis*, the lysis conditions were examined over 6 and 8 minutes. The "extract" was prepared and the cell debris was pelleted at 20,000 g for 20 minutes.

Both lysis times resulted in almost equal activity when examined using the Nesslers colorimetric assay. It was decided to carry out all future procedures over 6 minutes, as extended times may eventually result in less activity because the treatment denatures proteins either by heating, or by the vigorous shearing nature of the disintegration method.

3.7.2 SDS-PAGE ANALYSIS OF CELL FREE EXTRACTS

Having optimised the cell lysis conditions, it was then necessary to use SDS-PAGE to separate and visualise the proteins present in the cell-free extract. Protein separation using SDS-PAGE may give an indication of the relative molecular mass of the protein to be purified; running molecular weight markers of standard proteins of known size allows for approximation of the molecular weight determination of the proteins present in the sample.

In order to determine which protein is responsible for nitrilase activity, isolate *R. erythropolis* was grown in M9 minimal media containing 3-hydroxybtyronitrile (10 mM) as the sole nitrogen source; which is known to induce the desired enzyme. For comparative purposes the isolate was also grown in M9-minimal media using ammonium chloride (10 mM) as the nitrogen source. Both bacterial cultures were harvested by centrifugation at 4,000 g for 20 minutes and resuspended in 5 mL of water. The cells were subjected to sonication to prepare a cell-free extract as described above. SDS- PAGE (Figure 3.10) was performed in order to determine if induction with the nitrile produced an obvious protein band which may correspond to the enzyme.

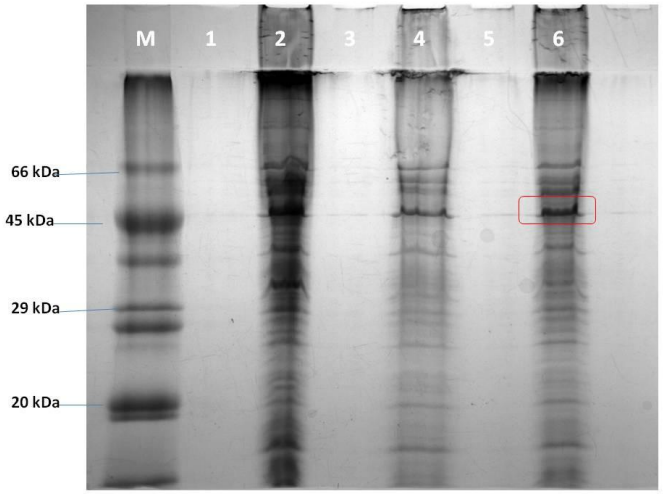


Figure 3.10: M- Low range molecular weight marker (Sigma), 1.blank, 2. ammonium chloride induced cell free extract, 3. blank, 4. 3-hydroxybutyronitrile induced cell free extract, 5. blank 6. 3-hydroxybutyronitrile induced cell free extract.

There is a stronger band observed in the gel when the isolate was grown utilising 3-hydroxybutyronitrile as the sole N source. This band was the most intense of the proteins present; this may be due to the fact that the enzyme is expressed in a higher concentration during the induction stage.

The relative molecular mass of the resolved protein can be determined by comparison of the band to a Sigma low weight molecular weight marker (6,500-66,000 Da) run on the same gel. The gel was then analysed to obtain the R_f values for each band. The R_f is defined as the migration distance of the protein through the gel divided by the migration distance of the dye front. A plot of log MW versus R_f (Figure 3.11) was generated from the bands in the gel shown in Figure 3.10 to determine the MW of the unknown protein.

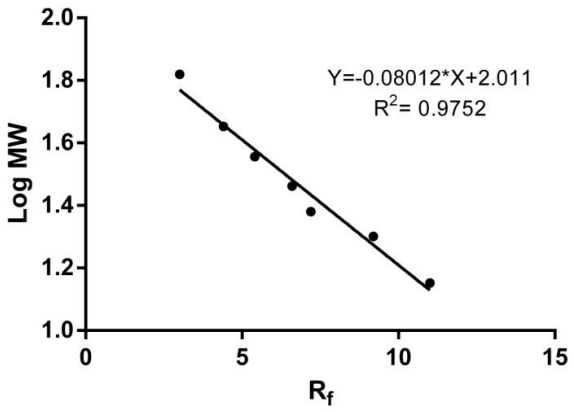


Figure 3.11: Determining the MW of an unknown protein by SDS-PAGE.

The strong linear relationship ($r^2 > 0.97$) between the molecular weight (MW) of the proteins and the migration distance demonstrates the reliability in predicting MW. Based on the formula Y=MX+C from the linear standard curve, the protein outlined in red (Figure 3.10) was found to be 47.2 kDa.

3.7.3 PROTEIN PRECIPITATION

To initiate protein purification, precipitation of this enzyme was investigated. Proteins precipitate at different salt concentrations depending upon size. Protein precipitation is a crude method and very much a starting point for protein purification. In general, proteins of higher molecular weight precipitate in lower concentrations of ammonium sulphate [45]. The solubility of ammonium sulphate varies very little in the range of 0-30 °C; a saturated solution of ammonium sulphate in pure water is approximately 4M [45]. Having decided to examine the use of ammonium sulphate precipitation, the next step was to decide what percentage saturation results in precipitation of the desired enzyme. The ammonium sulphate concentration was therefore examined at 20 %, 30 %, 40 %, 50 % and 60 % saturation.

Ammonium sulphate precipitation was carried out on the cell-free extract prepared by sonication as described above. The total volume of the protein solution was 5 mL. In this process, an initial concentration of 20 % ammonium sulphate is added with subsequent increases in concentration of the salt created by addition of ammonium sulphate (each time adjusting for volume).The 20 % solution was stirred on ice for 20 minutes before centrifugation at 4,000 g for 10 minutes. The supernatant was then subjected to a further increase in concentration and the precipitated protein stored on ice.

In order to examine the activity of the precipitated protein fractions, it was necessary to remove any ammonium sulphate from the cell pellet. This was carried out by resuspending the pellets in distilled water and concentrating the protein using microcentrifuge filters with a nominal molecular weight limit (NMWL) of 10,000 Da. This was repeated 3 times and the final sample resuspended in water. SDS-PAGE was carried out on each fraction in order to determine those with the desired enzyme (data not shown). Due to the lack of discernable purification and little difference in demonstrated activity between ammonium sulphate concentrations, an alternative method was sought as a means of purifying the cell-free extracts.

3.7.4 HYDROPHOBIC INTERACTION CHROMATOGRAPHY, PHENYL SEPHAROSE CL-4B

Hydrophobic Interaction Chromatography is a separation technique that uses the properties of hydrophobicity to separate proteins from one another. This technique was examined as a means of purifying the nitrilase enzyme from the cell-free extract. The protein solution was concentrated using a microcentrifuge tube with a 10 kDa cut off and resuspended in 5 mL of 2 M NaCl in 10 mM HEPES. The resulting suspension was injected onto a phenyl sepharose stationary phase and eluted with a decreasing salt gradient of 2 M NaCl in 10 mM HEPES over 40 minutes. Proteins were detected at 280 nm and collected in 4 mL fractions. However, an initial broad peak was observed after the sample was loaded. The chromatogram appeared clean. In order to determine where the target protein eluted, the fractions were examined using SDS-PAGE (Figure 3.12).

SDS-PAGE revealed that the proteins did not interact with the hydrophobic stationary phase and eluted immediately after the sample was loaded. This indicates that either hydrophobic interaction chromatography is not suitable for this means of protein purification or alternatively a higher concentration of NaCl must be used in order to enhance the protein- stationary phase interaction.

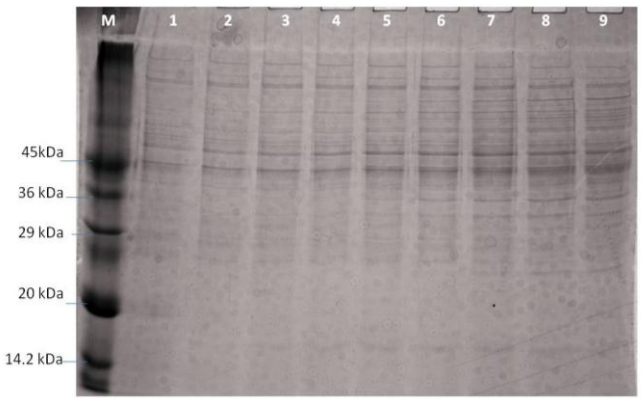


Figure 3.12: M= Low range molecular weight marker (Sigma), 1. Fraction 16 2. Fraction 17, 3. Fraction 18, 4. Fraction 19, 5. Fraction 20, 6**. Fraction 21, 7. Fraction 22, 8. Fraction 23, 9. Fraction 24.**

In order to enhance hydrophobic interactions, it was decided to increase the ionic strength of the starting buffer from 2 M NaCl in 10 mM HEPES to 4 M NaCl in 10 mM HEPES at pH 7. The sample eluted immediately and no other peaks were observed during the run. The fractions collected were again examined using SDS-PAGE, the protein was identified in the initial fractions. As such, this form of chromatography as a

procedure for purifying the nitrilase protein was deemed inappropriate and the use of gel filtration chromatography was investigated.

3.7.5 GEL FILTRATION SEPHACRYL S-200

This technique is based on the ability of the gel filtration media to separate the proteins according to size. Following the preparation of a cell free extract and removal of the debris by centriguation, the supernatant was concentrated using a concentrating centrifuge tube with a 10 kDa cut off from approximately 10 mL down to 0.6 mL in 200 mM NaCl in 10 mM HEPES at pH 7. The protein mixture appeared as a viscous yellow/ orange solution and was injected directly onto the Sephacryl S-200 column, using a mobile phase of 200 mM NaCl in 10 mM HEPES, at a flow rate of 1 mL/minute for 2 hours. The peak obtained from the chromatogram appeared very broad with the absorbance increasing between test-tubes 17-33. In order to determine where the target protein eluted, these fractions were examined using SDS-PAGE (Figure 3.13 and Figure 3.14).

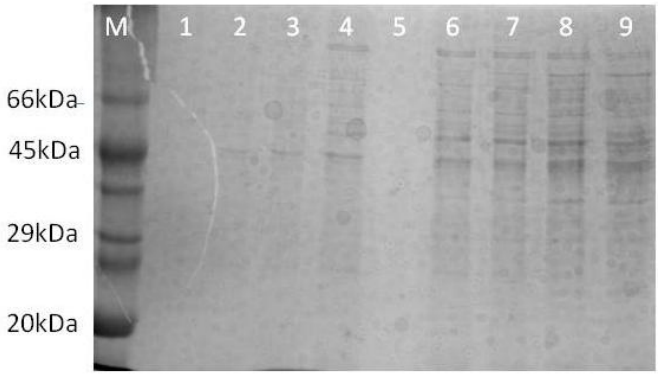


Figure 3.13: M- Low range molecular weight marker (Sigma), 1. Blank, 2. Fraction 17, 3. Fraction 18, 4. Fraction 19, 5. Fraction 20, 6. Fraction 21, 7. Fraction 22, 8. Fraction 23, 9. Fraction 24.

No obvious purification resulted from gel filtration column chromatography as observed from SDS-PAGE analysis. It was expected that larger molecules would elute first as they cannot permeate the pores and therefore take the shortest path length through the column. The Nessler's colorimetric activity assay does not indicate any substantial activity in any one fraction, although the later fractions appear to have more activity than the earlier eluting fractions. SDS-PAGE was repeated for fractions up to 49, again no one single fraction appeared to be pure.

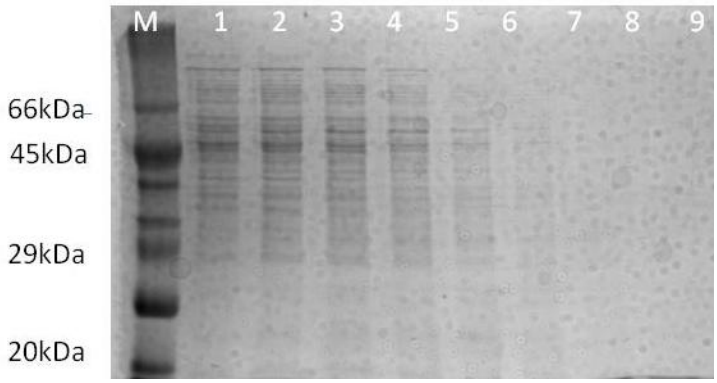


Figure 3.14: Low range molecular weight marker (Sigma), 1. Fraction 24, 2. Fraction 25, 3. Fraction 26, 4. Fraction 27, 5. Fraction 28, 6. Fraction 29, 7. Fraction 30, 8. Fraction 31, 9. Fraction 32.

3.7.6 PROTEIN IDENTIFICATION USING TRYPSIN

None of the chromatographic techniques utilised resulted in a purified fraction containing the nitrilase of interest. Therefore, a different approach was adopted. The protein could potentially be identified by obtaining primary sequence information by mass spectrometry. Usually sequence information is obtained on the peptides that result from internal proteolytic cleavage of the protein with trypsin [46]. A single SDS-PAGE separation is often adequate to isolate the proteins of interest. Cell lysates could be separated by SDS-PAGE as a first step in peptide mapping. A single protein band in a single lane of an SDS-PAGE gel is sufficient material for dozens of peptide maps [46].

There are many different methods for the cleavage of proteins. Because differences in primary structure are to be accentuated, a cleavage reagent or a combination of cleavage reagents which generate many fragments should be used [46]. It was decided to use trypsin; an enzymatic cleavage reagent with cleaves at the carboxy side of Arg and Lys residues.

Trypsin has a pocket with a negative charge due to an asparagine residue at the lining of the pocket (Asp189), which facilitates the binding of positively charged arginine (NH_2^+) and lysine (NH_3^+) residues. Trypsin cleaves peptides on the carbonyl side of the basic amino acids, arginine or lysine (Figure 3.15); it forms salt bridges with negatively charged aspartic acid and the positive substrates.

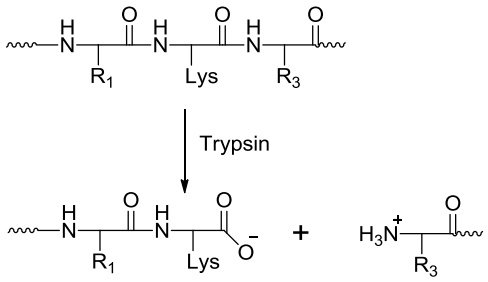


Figure 3.15: Trypsin digestion of peptides at the carbonyl side of the basic amino acids, arganine or lysine.

Matrix assisted laser desorption ionisation (MALDI) time of flight (TOF) can be used to obtain a peptide "mass map" which contains the masses of all the peptides derived from the digestion of the protein. This map provides a unique "fingerprint" of that protein, which can be computed against the predicted mass map of all known proteins to aid in identifying the protein [46]. Alternatively, liquid chromatography tandem MS (LC-MS/MS) can be used to obtain primary sequence information on peptides within the digest. This sequence information can also be used to identify a protein.

It was envisaged that the mass spec analysis, by obtaining a partial sequence, would enable the characterisation of the nitrilase enzyme. This would enable a primer or (degenerate primers) to be designed for the original gene sequence encoding for the unknown nitrilase. This could potentially allow the sequencing of the entire gene and subsequent recombinant production of a purified nitrilase by expression of the gene in a recombinant host such as *E. coli.*

The proteins from the cell-free extracts were separated as one-dimensional (1D) bands on SDS gel electrophoresis as described before (Figure 3.10). It was decided to segment the gel into 8 sections of equal size and a rough indication of molecular weight was recorded as a comparative to the molecular weight markers. The protein segments were then excised into fractions of equal size with a scalpel.

The colloidal Coomassie blue stain was removed using the de-staining procedure as outlined in the experimental section. The protein gel was then dehydrated and the gel slices were dried in a centrifugal evaporator, followed by the addition of a trypsin solution on ice for 30 minutes. Additional NH_4HCO_3 was added and the resulting solution and incubated overnight at 37 °C. The reaction was quenched by the addition of acetonitrile and trifluoro acetic acid. The liquid portion was retained as it contains the

digested peptides, however further digested peptides were recovered from the gel slices by the addition 83 % (v/v) ACN/ 0.2 % (v/v) TFA. Both extracts were combined and frozen at -80 °C before freeze-drying overnight. The lyophilised portion was then solubilised in 30 µL of buffer containing 5 % ACN, 95 % ACN/ 0.1 % formic acid.

The resulting peptides were separated by reversed-phase HPLC, and analysed with ion trap MS/MS, this was performed by Andrew Porter in Nurthumbria University. Peptide ions were selected automatically for MS/MS analysis. The peptide sequences obtained were subject to a search (peptide) query using the Matrix Science Mascot search tool. This is a commonly utilised bioinformatics tool used to compare a peptide query with a large online database of known peptide sequences.

Of all of the peptide fragments obtained only a single 11-residue fragment matched any known nitrilase enzymes. The mass spectrum obtained for this nitrilase peptide fragment is shown in Figure 3.17. The top aligned sequence was that of a nitrilase/cyanide hydratase from *Bradyrhizobium* (sp. ORS 278); a partial sequence for which is shown in Figure 3.18 with the 11 residue peptide identified from the sequencing highlighted in red.

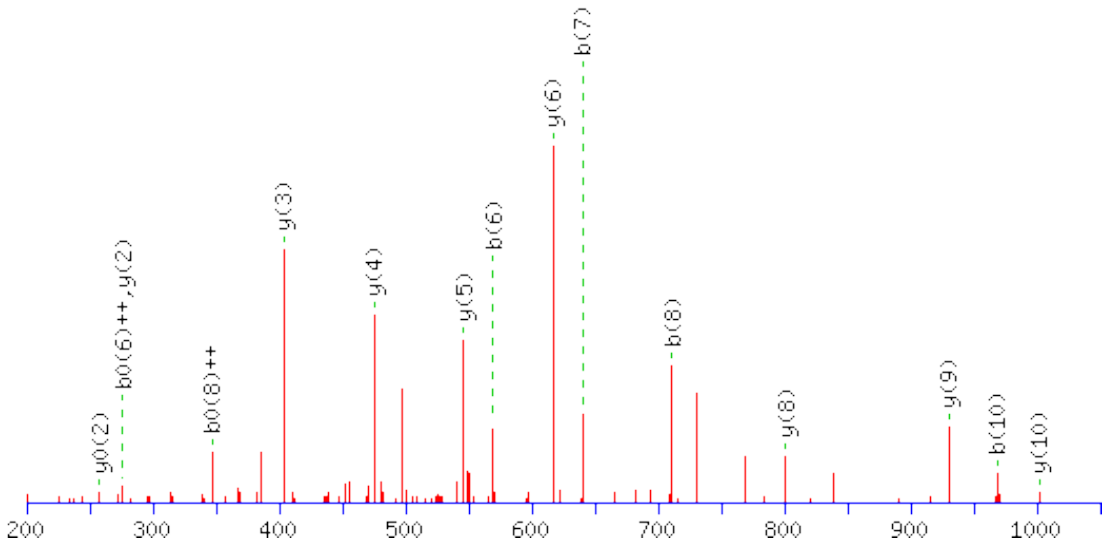


Figure 3.16: The mass spectrum obtained for the nitrilase peptide fragment corresponding to *Bradyrhizobium* (sp. ORS 278)

```
  1 MPMTYKAATV QFEPVLAGKE RNIEGLLALC EQAAASGAKL IVTPEMGTTG
 51 YCWYDRAEVA PYVEKVPGST THRFASLARR HDCYIVIGMP EVDDDDIYYN
101 SAVLIGPDGV IGRHRKTHPY ISEPKWAAAG DLHNQVFETP IGRIALLICM
151 DIHFVETARL MALGGADIIC HISNWLAERT PAPYWISRAF ENSCYVIESN
201 RWGLERTVQF SGGSCVIAPD GSLPAVIDKG DGVAFAEIDL AWARARKVLG
251 EPVFAQRRPE LYPELLTNTY SWNPRDFFGL YGHEPWPVGK RSRVSVAQFT
301 PSPDVASNLA RIAELAAAAK EKGAELVVFP ELAATGLTHS AETAEPIPGR
351 ITAALTELAA ERGLTLVCGL AERDGNTIYN SAVLVTPDGK ISTYRKTHLT
401 TAERSWATAG DEWTVVDTPM GRIGILIGHD AVFPEAGRVL ALRGCDLIVC
451 PSAVKGIFSA AHAGTKVMQP SPIPTGADPY HWHHFRVRGG ENNAYFAFAN
501 VCDAADEDCG LSGVFGPDTF AFPRREAIVD RGEGIATLEI DTGNLDSVYP

551 TNVVRRKDLV CMRMPHSYRA LVQSKASNF
```

Figure 3.17: Partial sequence with the 11 residue peptide identified in the sequence (*Bradyrhizobium* sp. ORS 278) highlighted in red

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance. It decreases exponentially as the Score (S) increases. The ions score for an MS/MS match is based on the calculated probability, P, that the observed match between the experimental data and the database sequence is a random event. The lower the E value, or the closer it is to zero, the more "significant" the match is. Bearing in mind that shorter sequence searches have higher E values as there is a higher probability of occurring in the database by chance. Therefore, the E value of 0.39 predicted for the 11 amino acid peptide corresponding to the nitrilase, is indicative that the match is not due to chance.

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CHAPTER 4

AN INVESTIGATION INTO THE SUBSTRATE SCOPE OF *R. ERYTHROPOLIS* SET1

SUBSTRATE EVALUATION OF *R. ERYTHROPOLIS* SET1

4.1 INTRODUCTION

Chapter 1 detailed the potential of biocatalysts for the production of enantiomerically pure pharmaceutical actives and intermediates, and Chapter 2 described the development of a high throughput bacterial isolate screening strategy which resulted in the identification of a novel nitrile metabolising enzyme within *Rhodococcus erythropolis* SET1. This strain subsequently demonstrated enantiospecificity towards 3-hydroxybutyronitrile. The work reported in this chapter details an investigation into the substrate range of this novel bacterial isolate, with β-hydroxynitriles; including aliphatic, aromatic and dinitriles as well as Baylis Hillman β-hydroxy-α-methylene nitriles and various alternative secondary nitriles (as shown in Figure 4.1).

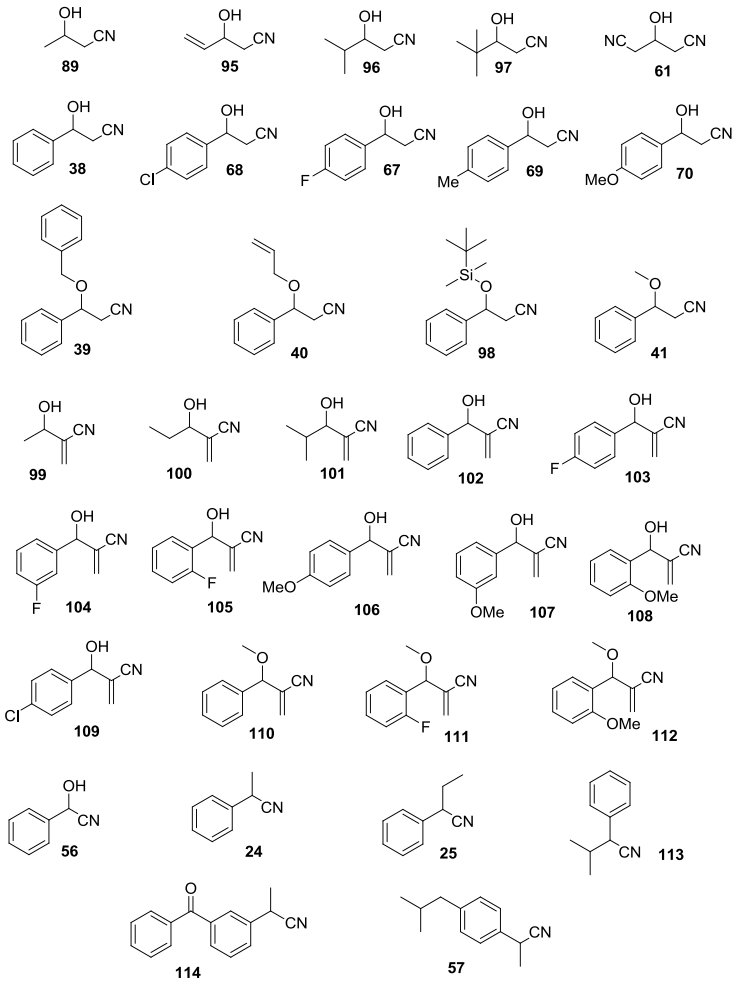


Figure 4.1: The 34 nitrile substrates evaluated in this study.

As discussed previously, enantiomerically pure β-hydroxy and β-amino acids and their derivatives are important intermediates in the synthesis of natural products and biologically important compounds [1]. Optically pure β-hydroxy carboxylic acids for example, contain both a hydroxy and a carboxyl functional group, which allow for easy modification and the introduction of a second chiral centre [2]. Biotransformations of β-hydroxy-nitriles provide an alternative method for the synthesis of β-hydroxy acids and amides.

It was envisaged that the evaluation of substrate promiscuity of *R. erythropolis* SET1 with a wide variety of structurally diverse nitriles (with features summarised in Figure 4.2) would provide an insight into the chiral recognition mechanism between the enzyme and the substrate. Several features, such as substituents at the β–position, proximity of the chiral centre to the cyano-group and free/protected hydroxyl at the β-position were evaluated.

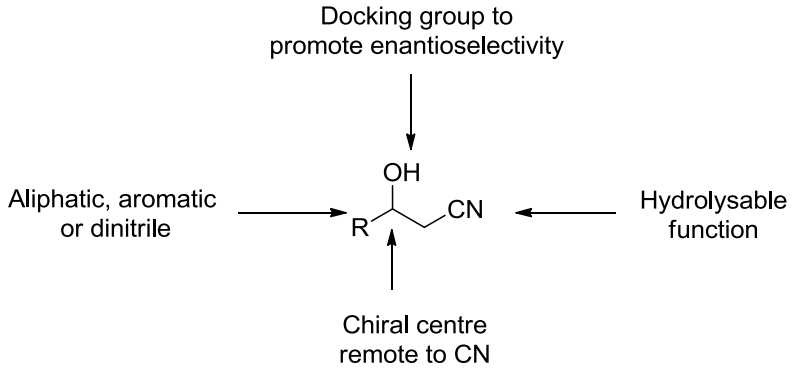


Figure 4.2: Features associated with β-hydroxynitrile substrates.

Due to the lack of enantioselectivity data reported in Section 3.4 when examining the use of organic solvents, it was decided that biotransformation reactions should be carried out in aqueous media. In the case of 3-hydroxybutyronitrile **89** >99.9 % ee was achieved in 100 % phosphate buffer (100 mM, pH 7). For consistency purposes, and also to enable direct comparison of catalytic performance, the substrate range was initially examined when isolate SET1 was grown in M9-minimal media containing 3-hydroxybutyronitrile **89** as the sole nitrogen source.

4.2 ALIPHATIC β- HYDROXYNITRILES AS A SUBSTRATE

Due to the initial success of isolate SET1 in the enantioselective biotransformation of 3-hydroxybutyronitrile, it was decided to explore the isolates catalytic activity towards a variety of aliphatic β-hydroxynitriles of potential pharmaceutical relevance. The nitriles shown in Figure 4.3 were chosen as they contain alkyl groups of various sizes at the β-position and hence may indicate the importance of steric interactions at this site during the biotransformations.

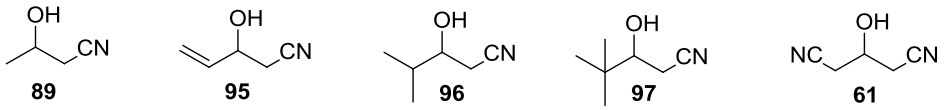
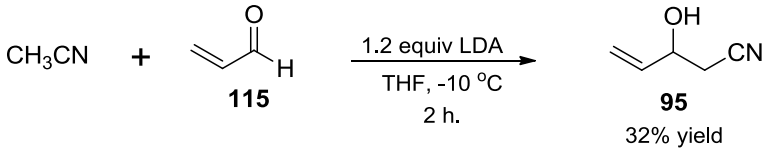


Figure 4.3: β-hydroxy aliphatic nitriles proposed for enantioselectivity screening of *R. erythropolis* SET1.

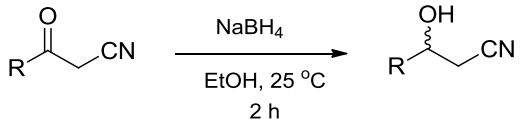
These β–hydroxy alkanenitriles can be prepared from condensation of acetonitrile with various aldehydes in the presence of LDA [3]. The racemic nitrile **95** was synthesised according to procedures outlined by Ma *et al*. [4] and Itoh *et al* [5] utilising the route as shown in Scheme 4.1.



Scheme 4.1: Condensation reaction of acrolein with acetonitrile in the presence of LDA.

The synthesis involved the addition of acetonitrile to a solution of lithium diisopropyl amide (LDA) in dry THF, under an inert N_2 atmosphere at -10 °C. Acroelin **115** was subsequently added and stirred for 2 h at -10 °C. The reaction was quenched with saturated ammonium chloride and following extractive work and purification by flash chromatography, nitrile **95** was obtained in 32 % yield. The product identity was confirmed by LC-MS showing an ion at m/z 112. Key ^{1}H NMR spectroscopic resonances included the β-proton which appeared as a multiplet integrating for 1 proton at 4.45 ppm, and the introduced vinyl protons at 5.8, 5.1 and 5.01 ppm. This data correlated to that reported previously in the literature [4].

The *β*-hydroxynitriles **96** and **97** were prepared by an alternative route which involved the reduction of corresponding *β*-ketonitriles with $NaBH_4$ at 0 °C. In each case $NaBH_4$ was added to a solution of the ketone in ethanol and the reaction was allowed to reach room temperature (Scheme 4.2). When the reaction had proceeded to completion, the mixture was quenched with 5 % HCl and extracted with ethyl acetate. Solvent was removed *in vacuo* and the residues were subjected to silica gel column chromatography using a 50:50 mixture of hexane and ethyl acetate.



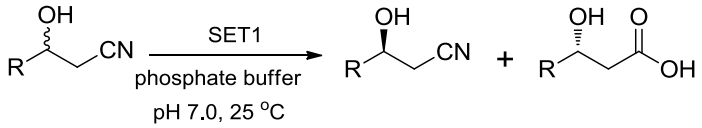
R	Yield	Key ^{1}H NMR
R= $CH(CH_3)_2$	59 %	δ = 3.70ppm (m, 1H, *β*-H)
R= $C(CH_3)_3$	62 %	δ = 3.63ppm (m, 1H, *β*-H)

Scheme 4.2: Synthesis of 3-hydroxy-4-methylpentanenitrile and 3-hydroxy-4,4-dimethylpentanenitrile.

The products were characterised by ^{1}H and ^{13}C NMR spectroscopy and compared with spectral data reported in the literature [4]. Racemic nitrile **96** was isolated as colourless oil in a 59 % yield. The appearance of the multiplet at 3.68 ppm integrating for 1 proton in ^{1}H NMR spectroscopy indicated that the ketone had been reduced. The alkyl nitrile **97** was obtained as a yellow oil in 62 % yield, and again the product was characterised using ^{1}H NMR with the presence of a multiplet at 3.63 ppm integrating for one proton confirming the reduction procedure had taken place. Also in both cases, the absence of the carbonyl ketone was evident in the ^{13}C NMR spectrum.

The biotransformations of racemic 3-hydroxy-alkanenitriles **89, 95, 96, 97** and **61** were subsequently investigated**.** Both 3-hydroxybutyronitrile **89** and 3-hydroxyglutaronitrile **61** were commercially available. In each case, alkane nitriles **89, 95, 96, 97** and **61** (10 mM) were suspended in phosphate buffer (100 mM, pH 7) with whole cells of SET1 (OD_{600nm} = 1) at 25 °C. The reaction products were monitored by TLC over 72 hours. The biomass was removed by centrifugation and following an acid/base work up, the products were purified using preparative TLC. Table 4.1 outlines the results obtained during the biotransformation.

Table 4.1: Biotransformations of racemic *β*-hydroxyalkanenitriles



Entry	Substrate	R	Time (hours)	Nitrile Yield %	Nitrile ee%	Acid Yield %	Acid ee%
1	**89**	Me	24	ND	ND	42	(*S*) > 99.9
2	**95**	CH_2CHCH_2	48	50	1.4	49	1.20
3	**96**	$CH(CH_3)_2$	72	66	3.0	ND	ND
4	**97**	$C(CH_3)_3$	72	72	1.8	ND	ND
5	**61**	CH_2CN	48	22	ND	67	(*S*)-32.0

[a]Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET1 (OD=1) in phosphate buffer (pH 7.0) at 25 °C. [b] Isolated yield after flash chromatography. [c] Determined by HPLC analysis using a chiral column. ND = Not detected.

The hydrolysis of nitrile **89** (Table 4.1, entry 1) proceeded with excellent enantioselectivity to afford (*S*)-3-hydroxybutyric acid **89a** in 42 % yield and >99.9 % ee as discussed in Chapter 2 [6]. The hydrolysis of nitrile **95** (Table 4.1, entry 2) proceeded over the course of 48 hours resulting in 50 % yield of nitrile and 50 % acid after work up and purification. The product was characterised by LC-MS and ^{1}H & ^{13}C NMR spectroscopy and results were compared to those reported in the literature [4]. In particular, evidence of the acid COOH peak at 175 ppm in the ^{13}C NMR spectrum confirmed hydrolysis. LCMS analysis verified the formation of the acid with the MNa^{+} adduct found at m/z 139. The enantiomers were separated using a chiralpak AD-H column with isocratic elution consisting of 90:10 hexane: *iso*-propanol with UV detection at 215 nm and retention times correspond to literature values. Unfortunately however, low ee (1.2 %) of acid were obtained with no amide detected. Configuration was tentatively assigned based on the order of elution in previously reported chiral HPLC analysis.

Unfortunately the biotransformation of racemic nitrile **96** (Table 4.1, entry 3) and nitrile **97** (Table 4.1, entry 4) did not proceed to acid or amide, and only the starting nitriles were recovered with reduced yield after 72 hours. This experimental data suggests that the presence of a bulky group close to the chiral centre in the substrate causes steric hindrance to the enzyme systems involved within the isolate.

The biotransformation of the prochiral substrate **61** (Table 4.1, entry 5) was also examined, the ethyl ester of which (as discussed in the literature review) is an intermediate used in the manufacture of the cholesterol lowering drug Lipitor **60** [1],[7, 8].

In this case, in order to develop chromatographic methods to determine the enantioselectivity of the biotransformation (Figure 4.4) with the available chiral LC and GC columns, racemic 4-cyano-3-hydroxybutyric acid standards were required. As this standard is not commercially available, a number of synthetic routes to prepare the acid and ester derivatives were examined.

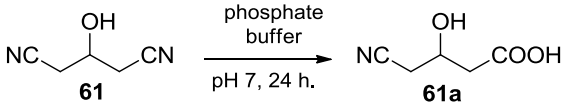
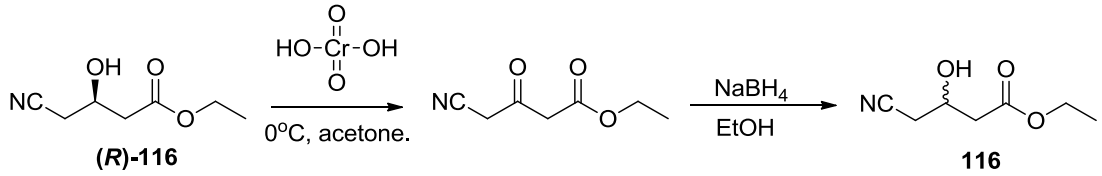


Figure 4.4: Biotransformation of prochiral 3-hydroxyglutaronitrile.

Initial work focused on the racemisation of commercially available ethyl-ester **(*R*)-116** by Jones oxidation and subsequent reduction with $NaBH_4$ (Scheme 4.3).



Scheme 4.3: Proposed Jones oxidation of (*R*)-ethyl-4-cyano-3-hydroxybutyrate followed by $NaBH_4$ reduction to yield racemic 4-cyano-3-hydroxybutyrate.

Jones reagent was prepared and added to a solution of ester **(*R*)-116** at 0 °C in acetone. However, the reaction did not proceed to any appreciable extent when monitored by TLC. The crude reaction mixture was examined using LC-MS. The major peak was identified with m/z 159. This corresponded to starting material and indicated that the reaction had not occurred.

An alternative procedure explored the Mitsinobu inversion of **(*R*)-116**. The Mitsunobu reaction is the standard method for inversion of configuration of secondary alcohols [9],[10]. Recently Yang *et al*. [10] reported the inversion of **(*S*)-117** (Figure 4.5) a structurally similar alcohol to **(*R*)-116**. Lipshutz *et al*. [9] also reported the use of DEAD for the inversion of **(*S*)-118**.

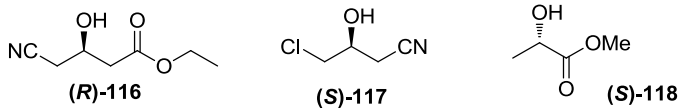
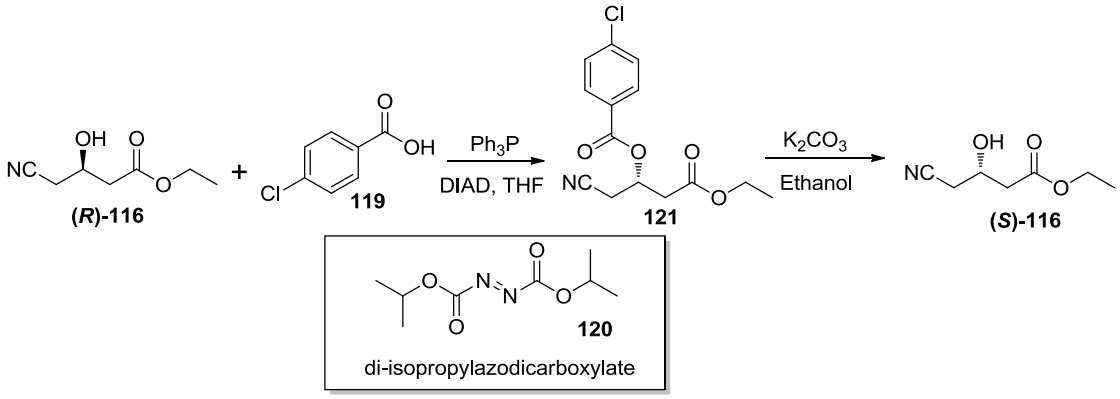


Figure 4.5: Structurally similar secondary alcohols reported in the Mitsunobu inversion, (*S*)-4-chloro-3-hydroxybutyronitrile [10] and (*R*)-ethyl-4-cyano-3-hydroxybutyrate [9].

The Mitsunobu reaction was carried out according to the method reported by Yang *et al*. [10]. Initially ester **(*R*)-116** was added to a solution of 4-chlorobenzoic acid **119** in the presence of PPh_3 in anhydrous THF under a N_2 atmosphere at 0 °C (Scheme 4.4). The resulting solution was treated with di-isopropylazo-di-carboxylate (DIAD) **120** and the reaction mixture was monitored by TLC.



Scheme 4.4: Proposed Mitsunobu inversion of (*R*)-ethyl-4-cyano-3-hydroxybutyrate followed by the ester hydrolysis to produce the desired (*S*)-product.

Analysis of the reaction mixture by both TLC and LCMS indicated the reaction did not proceed to any appreciable extent. A large amount of by-products were also formed and it was difficult to isolate the desired product using flash chromatography.

Due to the difficulty encountered trying to synthesise a racemic acid standard, it was decided to proceed with GC method development, using the ester **(*R*)-116** standard available initially.

Previous literature reports commented on the difficulty encountered when using chromatographic techniques to detect the acid product [1, 8]. Derivatisation to an ester appeared to be favoured, with formation of the corresponding methyl ester reported using diazomethane [8]. However, as diazomethane is explosive and highly toxic, alternative GC derivatising agents were investigated. The use of dimethylformamide-dimethylacetal DMF-

DMA **122** and dimethylformamide-diethylacetal DMF-DEA **123** (Figure 4.6) seemed appropriate to form the corresponding methyl and ethyl ester respectively as an alternative to diazomethane [11] .

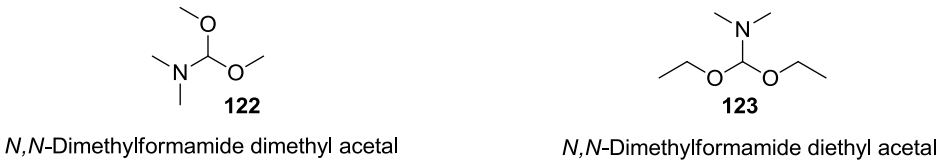
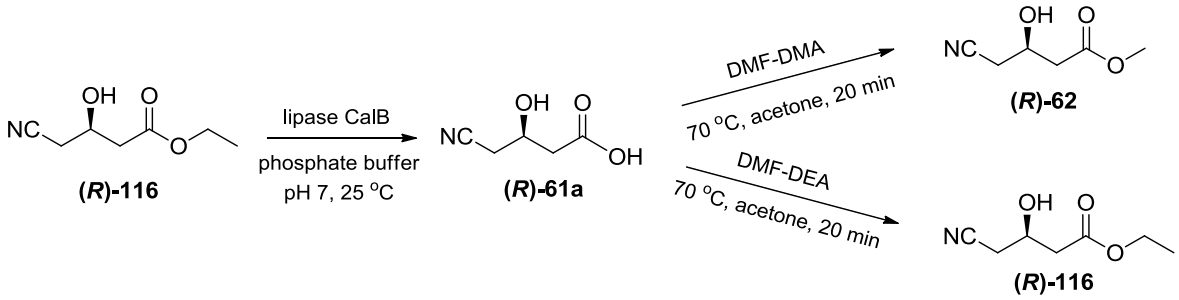


Figure 4.6: Commercially available GC derivatising agents.

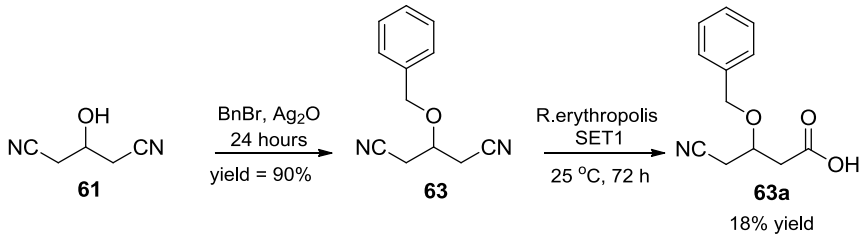
The lipase from *Candida antartica* CALB was used to hydrolyse a commercial sample of **(*R*)-116** (Scheme 4.5). The hydrolysis was performed by incubating the ester (10 mM) in phosphate buffer (100 mM, pH 7) with the enzyme at 25 °C over 24 hours. Following extractive work up and purification by preparative TLC, the acid product **(*R*)-61a** was obtained in 13 % yield. Confirmation of reaction progress by ^{1}H NMR included the absence of the (CH_2CH_3) ethyl protons. ^{13}C NMR spectra also verified the formation of the acid COOH peak at 176 ppm. This data correlated to that previously reported [12].



Scheme 4.5: Lipase catalysed hydrolysis of (*R*)-ethyl-4-cyano-3-hydroxybutanoate followed by derivitisation to the corresponding methyl and ethyl ester.

Derivatisation using DMF-DMA **122** and DMF-DEA **123** were examined by reacting the acid **(*R*)-61a** with the reagent in acetone at 70 °C for 20 minutes. Following dilution of the reaction mixture, the samples were injected onto both a β- and γ- cyclodextrin chiral column using instrument methods previously reported [1, 12]. However neither resulted in the detection of the corresponding ester derivatives.

A final approach using chiral HPLC and an available AD-H chiral column was employed. As nitrile **61** and the corresponding acid **61a** and amide products are not UV active a docking strategy was used to aid in the detection (Scheme 4.6).



Scheme 4.6: Benzylation 3-hydroxyglutaronitrile for chiral HPLC method development.

Crosby *et al.* [7] investigated the hydrolysis of prochiral dinitriles, and reported chiral HPLC conditions and optical rotation data for the benzylated nitrile **63** and acid **63a** products using an IA chiral column, which is similar to the AD-H column. The biotransformation of nitrile **63** was attempted to obtain potential benzylated acid **63a;** products which could be compared with these literature values and retention times obtained. The docking strategy could be used post biotransformation of free nitrile **61** as in the case of 3-hydroxybutyronitrile **89** described previously.

The substrate **63** was prepared in high yields (90 %) by the addition of benzyl bromide and Ag_2O to a solution of 3-hydroxyglutaronitrile **61** in dichloromethane [7]. The reaction mixture was filtered and the solvent removed *in vacuo*. The residue was purified using silica gel column chromatography to yield yellow oil. Characterisation of the product using LC-MS and ^{1}H & ^{13}C NMR spectroscopy correlated to that reported in the literature [7, 13]. The ^{1}H NMR data showed signals consistent with the addition of 5 aromatic protons at 7.3-7.4 ppm, and the addition of a signal for CH_2 as a singlet at 4.8 ppm, supporting benzylation.

The substrate **63** was subjected to whole cells of isolate SET1 over the course of one week. The biomass was removed by centrifugation. Following extractive work up and purification by preparative TLC the corresponding benzylated acid **63a** was isolated in 18 % yield. Key spectroscopic ^{1}H NMR peaks indicated the formation of the acid with the $\underline{CH_2}COOH$ peak as a doublet at 2.25 ppm, integrating for 2 protons. This was also supported by the detection of the carboxylic acid peak in the ^{13}C NMR spectrum at 176 ppm. Detection of the MH^+ ion

at m/z 220 using LCMS also identified the product as benzylated acid, and this correlated with the literature data [7, 13].

Comparison of the sign of optical rotation with that reported in the literature [7] suggested that the product contained a mixture of enantiomers, allowing a chiral HPLC method to be developed. Separation of the enantiomers was achieved using a chiralpak AD-H column, with isocratic elution with mobile phase (90: 10, hexane: IPA, 0.1 % TFA) with UV detection at 215 nm. The enantiomers eluted at t_{major}= 14.80 min, t_{minor}= 16.31min.

The biotransformation of unprotected nitrile **61** was carried out in a suspension of phosphate buffer (0.1M, pH 7.0, 200 mL) with whole cells of *R .erythropolis* SET1 @ OD_{600nm} = 1. The reaction was monitored by TLC and LC-MS, and the resulting aqueous solution was basified to pH 12 with NaOH (2M). Extraction with ethyl acetate, removal of the solvent under vacuum, and purification using preparative TLC, yielded the remaining nitrile (22 %). The aqueous solution was then acidified using aqueous HCl (2M) to pH 2 and extracted with MEK. The acid product **61a** was obtained in 67 % yield after work up and purification by preparative TLC. Analysis of the purified product using LCMS identified the MNa^+ peak with m/z 152.2, corresponding to the acid. Key ^{1}H NMR spectroscopic peaks included the C$\underline{H_2}$COOH peak which appeared as a multiplet integrating for 2 protons. This was also supported by the detection of the carbonyl peak in ^{13}C NMR at 176.2 ppm. The data matched that previously reported by DeSantis *et al*. [8].

The acid **61a** was transformed to the benzyl ether **63a** using benzyl bromide in the presence of silver oxide in DCM as previously reported. Chiral HPLC analysis of the product after work up was performed on an AD-H chiral column to determine enantiomeric excess and the peaks obtained correlated to that of the benzyloxy standard described earlier (t_{major}= 14.77min, t_{minor}= 16.23min). Further characterisation using both GC-MS and LC-MS m/z 224, indicated that acid **61a** had been derivatised to the corresponding benzyl ether **63a**. Also evident in the HPLC chromatogram, were additional peaks due to benzyl alcohol and benzyl bromide. The product 4-cyano-3-hydroxybutyric acid **61a** was formed in 32 % ee.

In summary, the complete lack of amide produced in all cases (Table 4.1, entry 1-5) lead us to believe that a nitrilase enzyme was present in the isolate. However, low ee values were

obtained for the acid product of aliphatic substrates other than 3-hydroxybutyronitrile, along with moderate chemical yields and degradation of the substrate in some cases.

The low enantioselectivity results also demonstrated by isolate SET1 with aliphatic substrates are not surprising. Ma *et al*. previously reported the observation of low enantioselectivity when investigating these nitriles with *R. erythropolis* AJ270, obtaining amide in 13-14 % yield with ee =2-12 %, and acid 12-32 % yield with the ee never exceeding 17.8 % [14].

4.3 ENANTIOSELECTIVITY SCREENING TOWARDS AROMATIC *β*–HYDROXYNITRILES

A series of related aromatic substrates were next examined in the biotransformation using SET1. The nitriles shown in Figure 4.7 were chosen as they contain aryl groups with various substituents on the benzene ring *β*- to the nitrile.

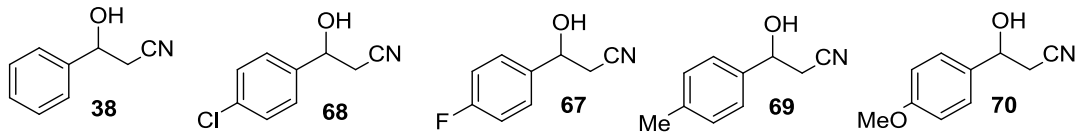
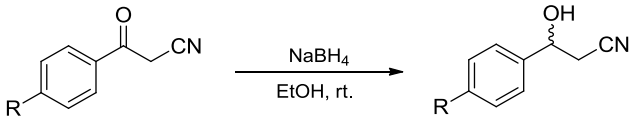


Figure 4.7: *β*-hydroxy aromatic nitriles proposed for enantioselectivity screening of R erythropolis SET1.

These substituted aromatic nitriles were prepared by reduction of the corresponding ketones using $NaBH_4$ in ethanol (Scheme 4.7) [15]. After the reaction had proceeded to completion, the mixture was quenched with 5 % HCl and extracted with ethyl acetate. After removal of the solvent *in vacuo*, the residues were subjected to silica gel column chromatography.



Scheme 4.7: Sodium borohydride reduction of *para* substituted benzoylacetonitrile for the preparation of *β*-hydroxy aromatic ring substituted nitriles.

The hydroxy nitriles **67-70** were prepared in yields ranging from 52-80 % as sumarised in Table 4.2. In each case the appearance of a signal at approximately 5 ppm in ^{1}H NMR, and the disappearance of the carbonyl in the ^{13}C NMR spectrum confirmed the reaction had

occurred, also ^{1}H NMR and ^{13}C NMR spectra correlated to that published in the literature [15].

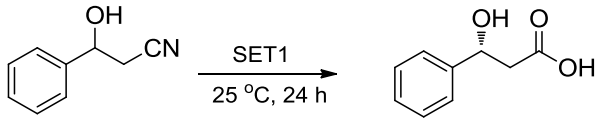
Table 4.2: Preparation of aromatic *β*-hydroxynitriles from the corresponding ketone.

Entry	Substrate	R	Yield	Key ^{1}H NMR	LCMS
1	**68**	4-Cl	52 %	δ=5.04 ppm (t, *J*= 6.4 Hz, 1H)	MH^+ m/z = 182
2	**67**	4-F	60 %	δ=5.02 ppm (t, *J*= 5.8 Hz 1H),	MH^+ m/z = 165
3	**69**	4-CH_3	80 %	δ=5.00 ppm (m, *J*= 2.75 Hz, 1H)	MH^+ m/z = 162.1
4	**70**	4-OCH_3	62 %	δ=5.06 ppm (m, *J*= 2.9 Hz, 1H)	MH^+ m/z = 190

In order to develop chiral HPLC conditions for the *para*-substituted *β*-hydroxy nitriles, the racemic nitriles were injected on a Chiralpak AD-H column with isocratic elution consisting of 90:10 hexane: *iso*-propanol with UV detection at 215 nm. All standards demonstrated adequate base line resolution, and retention times correlated with literature values [15].

In initial studies the biotransformation of 3-hydroxy-3-phenylpropionitrile **38** using isolate SET1 induced separately with 3-hydroxybutyronitrile **89** and the target substrate nitrile **38** was evaluated. It has been shown that the selection of a nitrile substrate as a sole source of nitrogen during induction, can give rise to bacterial enzyme systems highly adapted to the target substrate [16].

The substrate 3-hydroxy-3-phenylpropionitrile was treated in both cases with whole cells of *R. erythropolis* SET1 in potassium phosphate buffer (100 mM, pH 7) and the reaction mixture incubated at 25 °C (Scheme 4.8). The reaction was monitored in each case by TLC and LC-MS. The biomass was removed by centrifugation and the aqueous phase was then extracted using an acid/ base work up procedure as discussed previously.



Inducer	Acid	
	Yield %	ee %
3-HBN	20.0%	(*R*)- 7.8%
3-HPPN	19.1%	(*R*)- 19.0%

Scheme 4.8: *R. Erythropolis* SET1 catalysed hydrolysis of 3-hydroxy-3-phenylpropionitrile.

In each case the identities of the products were confirmed as the starting nitrile and the corresponding acid using ^{1}H and ^{13}C NMR spectroscopy. The ^{13}C NMR peak

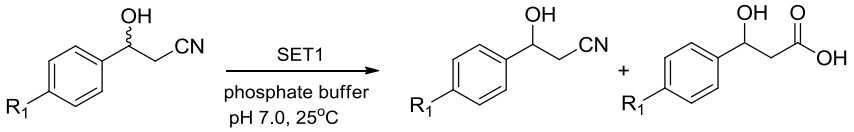
corresponding to COOH at 177.3 ppm, in particular verified the formation of the acid. All spectroscopic data correlated to that reported in the literature [17]. The acid product was injected onto a chiralpak AD-H column with isocratic elution consisting of 90:10 hexane: *iso*-propanol with UV detection at 215 nm, the enantiomers eluted at t_{major}= 12.2 min, t_{minor} = 13.6 min.

However, only low enantiomeric excesses were obtained for the (*R*)-acid product, along with low chemical yields in both cases. It was also noted that a longer reaction time was required for the hydrolysis of aromatic substrates (5-7 days) in comparison with that of 3-hydroxybutyronitrile (1 day). The configuration of acid produced was determined to be (*R*), by comparison with a commercial standard of (*R*)-3-hydroxy-3-phenylpropionic acid, which eluted from the AD-H chiral column at 13.6 minutes. Comparison of the optical rotation to the authentic sample also enabled the configuration of the acid to be determined [18]. Although 3-hydroxy-3-phenylpropionitrile induced cells demonstrated a higher ee (19 %) in comparison to 3-hydroxybutyronitrile ee (7.8 %), the latter was more feasible in that a higher OD_{600nm} (>5) at the growth stage was reached in a shorter amount of time (3 days *versus* 7 days). It was decided to continue using SET1 grown in the presence of 3-hydroxybutyronitrile for future studies.

SET1 was then examined for the catalysed hydrolysis of the other aryl substituted 3-hydroxy propionitrile analogues bearing electron donating and electron withdrawing *para* substituents **67-70** (Table 4.3, entry 1-5), to determine if this would have an effect on ee values [15]. Nitrilase enzymes have been shown to give higher yields and ee with *meta*- and *para*- substituted aromatic rings, with poor or no activity seen with *ortho*-substituted substrates. This may be due to steric hindrance of the *ortho*-substituted compounds close to the nitrile group [19].

The substituted aromatic *β*-hydroxy nitriles were treated with whole cells of *R. erythropolis* SET1 (induced on 3-hydroxybutyronitrile), in potassium phosphate buffer and the reaction mixture incubated at 25 °C. The reaction was monitored by TLC and LC-MS and worked up as outlined previously by acid/base extraction. The crude *β*-hydroxy carboxylic acids and unreacted *β*-hydroxy nitriles were purified by preparative thin layer chromatography.

Table 4.3: Biotransformation of *para*-substituted *β*-hydroxynitriles.



Entry	Substrate	R_1	Time	Nitrile		Acid	
				yield% [b]	ee% [c]	yield% [b]	ee% [c]
1	**38**	H	5 days	80.0	*S* 5.0	19.3	*R* 7.0
2	**68**	Cl	5 days	74.1	*R* 8.7	20.1	*S* 15.8
3	**67**	F	5 days	35.2	*R* 23.2	42.0	*S* 19.8
4	**69**	Me	5 days	90.0	*S* 7.4	ND	ND
5	**70**	OMe	5 days	55.1	*S* 21.5	28.2	*R* 7.4

[a] Biotransformation carried out by incubating nitrile (10 mmol) in a suspension of *Rhodococcus erythropolis* SET1 (OD_{600nm} = 1) in phosphate buffer (50 mL, pH 7.0) at 25 °C. [b] Isolated yield. [c] Determined by HPLC analysis using a chiral column (see section). Configuration determined by comparison to literature values [15].

The biotransformation products **38a, 67a, 68a** and **70a** were verified by LC-MS and also by NMR spectroscopy with their ^{1}H and ^{13}C NMR signals correponding to previous reports as acids and nitrile starting material (Table 4.4) [15]. The ee values of both the product acids and the recovered nitriles were measured by chiral HPLC analysis, and their absolute configurations were determined by comparing the sign of optical rotation with the literature data [15].

Table 4.4: Characterisation data for *para*-substituted *β*-hydroxy acids.

Entry	Product	R	Isolated yield	Key ^{13}C NMR peaks	LCMS
1	**38a**	H	19.3 %	δ = 177.3 ppm (COOH)	MH^+ m/z = 167
2	**68a**	Cl	20.1 %	δ = 177.3 ppm (COOH)	MH^+ m/z = 201
3	**67a**	F	42.0 %	δ = 177.4 ppm (COOH)	MH^+ m/z = 185
5	**70a**	OMe	28.2 %	δ = 177.3 ppm (COOH)	MH^+ m/z = 197

The substituent on the benzene ring of *β*-hydroxynitriles exerted an effect on enantioselectivity and activity in some cases. Among the *para*-substituted aryl *β*-hydroxy nitriles, the hydrolysis of the substrate **67** with an electron withdrawing *para*-fluoro group on the aromatic ring (Table 4.3, entry 3), showed the highest enantioselectivity (19.8 %) and also the highest isolated yield (42 %).

The increase in ee also held for *p*-chloro substituted nitrile **68** (Table 4.3, entry 2) but in this case no yield improvement was observed when compared to results with unsubstituted aryl nitrile **38** (Table 4.3, entry 1). While ee improved for aromatic nitriles substituted with electron-withdrawing groups, it remained low for electron-donating groups present on the aryl ring as observed with *para*- substituted methyl group **69** (Table 4.3, entry 4), which did not demonstrate any activity with the starting nitrile recovered in 90 % yield and 7.4 % ee. It is also worth noting that the configuration of the product changed from (*R*) to (*S*) when electron-withdrawing groups were present in comparison to electron-donating groups. This has previously been observed by Robertson *et al.* [20] who suggested that while enzymes predominantly show selectivity towards one enantiomer, changes in the way the active site binds to the substrate may result in reversal of stereochemistry.

Results for such ring substituted aromatic *β*-hydroxynitriles as published by Kamila *et al*., demonstrated that the various substituents on the benzene ring did not generally effect the enzyme activity of nitrilase b116402, but exerted some effect on enantioselectivity [15]. The results obtained in our study are in contrast to these results. Kamila *et al*. found that the biotransformation of the *para*-methoxy substituted analogue showed the highest enantioselectivity generating the corresponding acid with 90 % ee [15]. In contrast, electron withdrawing substituents at the *para* position resulted in the corresponding acid having a reduced ee of 60-65 % [15].

In all cases the isolate was induced using 3-hydroxybutyronitrile **89**. It may be that varying the inducing nitrile to 3-hydroxy-3-phenylpropionitrile may enhance the activity and enantioselectivity of the isolate towards the larger substrates. However, there was difficulty in producing a sufficient quantity of biomass when 3-hydroxy-3-phenylpropionitrile was used as the sole N source during growth of isolates for biotransformations. It is also of note that no amide was formed in each case. This again indicates that the isolate may contain a nitrilase enzyme.

4.4 ENHANCEMENT OF ENANTIOSELECTIVITY USING A DOCKING/ PROTECTION STRATEGY

Molecular recognition of an enzyme towards a substrate is a crucial attribute in enantiospecific or highly enantioselective biotransformations. Substrate engineering has

been shown to be useful in improving the efficiency and selectivity of biocatalysis [21]. Such substrate modifications can tailor the structure of small organic molecules to suit the active site of the enzyme (Figure 4.8).

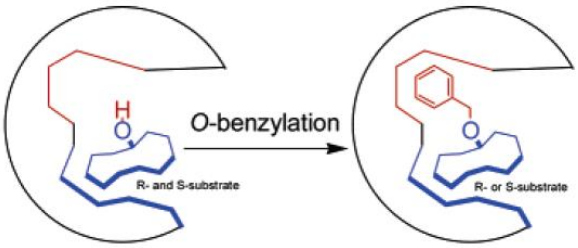


Figure 4.8: Enhancement of enantioselectivity using a benzyl docking/ protection strategy reprinted from [3].

Ma *et al*. found that the addition of a removable docking group at the *β*-hydroxy position improved ee values and recognition in the biotransformation of *β*-hydroxynitriles [14]. It was envisioned that the introduction of such a removable protecting group on the free hydroxyl moiety, might result in the enhanced chiral recognition of SET1 with aromatic substrates. This could potentially lead to improved enantioselectivity in the biotransformation of 3-hydroxy-3-phenylpropionitrile **38** which was low (19 % yield, 7.8 % ee).

In choosing a protection or docking group, several criteria need to be considered. Ma *et al*. found that adding a benzyl group dramatically improved enantioselectivity [14]. The introduction and removal of a protection group should be readily carried out under mild conditions and not cause contamination of the product. Care should be taken when choosing the protecting group to avoid racemisation under the conditions required for deprotection. When using whole cell biocatalysts, the protecting group should be stable to other enzymes such as esterases. A UV-active protection group is also beneficial because it facilitates the detection of both substrates and products and therefore, simplifies monitoring of the biotransformation [21]. An extra advantage of utilising a large protecting group is the prevention of further metabolism of the corresponding products and the increase in hydrophobicity, both leading to the isolation of the products in potentially higher yields [21].

In order to improve the ee of 3-hydroxy-3-phenylpropionitrile, the protecting groups –OMe, -OBn, -OAllyl and -OSi were chosen (Figure 4.9). In each case well established procedures exist for the removal of the docking group [22], [23].

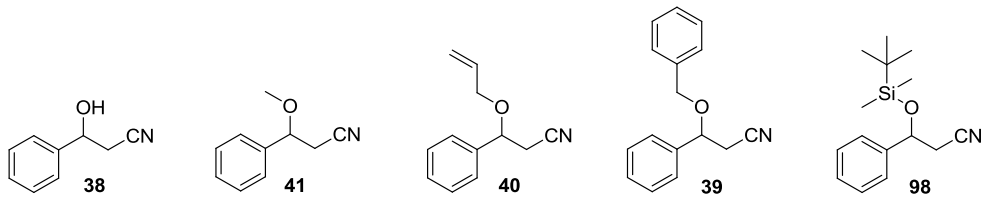
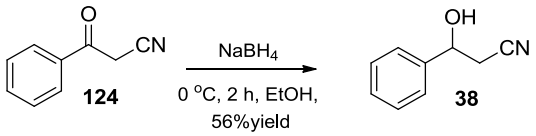


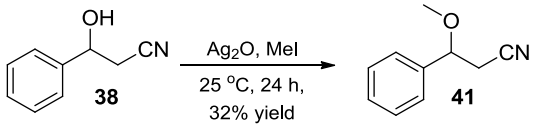
Figure 4.9: Racemic *β*-hydroxy and *β*-alkoxy nitriles synthesised for enantioselectivity screening of *R. erythropolis* SET1.

While in previous studies a commercial sample of 3-hydroxy-3-phenylpropionitrile **38** was used, for this work the substrate was prepared from the reduction of benzoylacetonitrile **124**, in moderate yields using $NaBH_4$ as the reducing agent (Scheme 4.9). The nitrile was obtained in 56 % yield as yellow oil. Characterisation of the product by ^{1}H and ^{13}C NMR spectroscopy gave data that correlated with literature reported and an available commercial sample.



Scheme 4.9: Preparation of 3-hydroxy-3-phenylpropionitrile from benzoylacetonitrile.

The various hydroxy protected analogues reported in this study were synthesized according to procedures previously reported by Ma *et al.* [14]. Racemic 3-methoxy-3-phenylpropionitrile **41** was prepared by the reaction of nitrile **38** with methyl iodide in the presence of Ag_2O (Scheme 4.10), over 24 hours in the dark.

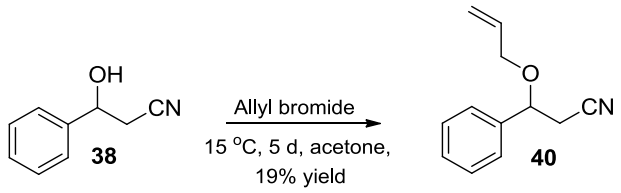


Scheme 4.10: Preparation of 3-methoxy-3-phenylpropionnitrile [18].

After work up, the residue was subjected to silica gel column chromatography to give the title product **41** in 32 % yield as a colourless oil. The ^{1}H NMR spectrum showed the

introduction of the methoxy signal at 3.28 ppm as a singlet integrating for 3 protons when compared to the free hydroxy nitrile. All ^{1}H NMR spectroscopic resonances were assigned and correlated to that reported in the literature [14]. LC-MS analysis detected a molecular ion at m/z 162 (MH^{+}).

Racemic 3-allyloxy-3-phenylpropionitrile **40** was synthesized from nitrile **38** by reaction with allyl bromide in the presence of potassium carbonate (Scheme 4.11) over 5 days. After filtration and removal of the solvent *in vacuo,* the residue was subjected to silica gel column chromatography to give **40** in 19 % yield as colourless oil.



Scheme 4.11: Preparation of racemic 3-allyloxy-3-phenylpropionnitrile [18].

The purity and structure were confirmed using ^{1}H and ^{13}CNMR and correlated to the literature values [18]. Key ^{1}H NMR signals included the vinyl protons (CH_2=C<u>H</u>) at 5.87 ppm integrating for 1 proton and (C<u>H$_2$</u>=CH) at 5.28 ppm which appeared as a doublet integrating for 2 protons. LC-MS analysis was used to identify the MNa^{+} adduct m/z 188.

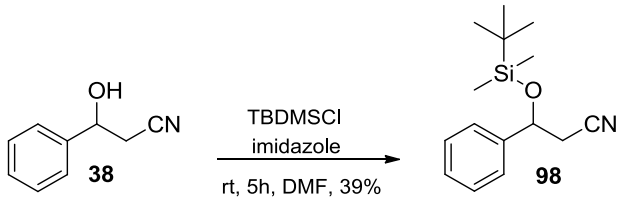
To prepare the benzyl analogue, nitrile **38** was converted into the corresponding benzyloxy ether **39** by the reaction of the nitrile with benzyl bromide in the presence of Ag_2O (Scheme 4.12) as previously described. The product, a colourless liquid, was isolated in 70 % yield after flash chromatography.

Scheme 4.12: Preparation of racemic 3-benzyloxy-3-phenylpropionnitrile [18].

Benzylation was confirmed by the ^{1}H NMR spectroscopic resonances which showed a multiplet at δ 7.33 for the 10 aromatic protons, and O<u>CH$_2$</u> peak at 4.52 ppm integrating for

2 protons and LCMS which gave MH^+ ion at m/z 238. This correlated with previous reports by Wang *et al.* [4].

Finally the silyl derivative **98** was prepared by reaction of nitrile **38** in dimethylformamide (DMF) with *tert*-butyldimethylsilyl chloride for 5 hours in the presence of imidazole. The reaction mixture was quenched by the addition of dihydrogen carbonate (Scheme 4.13).

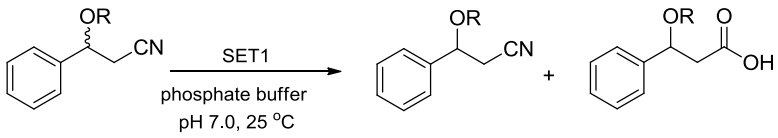


Scheme 4.13: Preparation of racemic 3-((tert-butydimethylsilyl)oxy)3-phenylpropionitrile.

The silyl protected analogue **98** was obtained in 39 % yield as colourless liquid after extractive work up and silica gel column chromatography. The *tert*-butyl signal integrating for 9 protons at approximately 0.9 ppm confirmed the presence of the TBDMS protecting group in ^{1}H NMR spectra [24].

In each case, the biotransformations were carried out by subjecting the *β*-hydroxy substituted nitriles to *R. erythropolis* SET1 in phosphate buffer at pH 7.0 and monitored over time. The reaction was worked up as previously discussed. The biotransformation results are shown in Table 4.5.

Table 4.5: Biotransformation of *β*-hydroxy protected nitriles.



Entry	Substrate	R	Time	Nitrile		Acid	
				Yield %[b]	ee % [c]	Yield %[b]	ee % [c]
1	**38**	H	5 days	80	(*S*) 5.0	19.3	(*R*) 7.0
2	**39**	Bn	7 days	91	2.1	ND	ND
3	**40**	Allyl	5 days	97	3.4	ND	ND
4	**98**	TBDMS	7 days	96	1.2	ND	ND
5	**41**	Me	5 days	86	(*S*) 4.2	14.0	(*R*) >99.9

[a]Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET1 (OD=1) in phosphate buffer (pH 7.0) at 25 ^{O}C. [b] Isolated yield after flash chromatography.[c] Determined by HPLC analysis using a chiral column. ND = Not detected.

The biotransformation of nitrile **39** (Table 4.5, entry 2), **40** (Table 4.5, entry 3) and **98** (Table 4.5, entry 4) did not proceed to any large extent, only the starting nitriles were recovered after 7 days. No acid product was observed. This experimental data suggests that the presence of a phenyl group or a sterically bulky group in the substrate close to hydroxyl may interfere with the enzyme systems involved within the isolate.

The hydrolysis of 3-methoxy substituted nitrile **41** (Table 4.5, entry 5) was carried out over 5 days. The acid reaction product was isolated after work up and purification in 14 % yield. The product was verified by LC-MS and also by NMR spectroscopy with its ^{1}H & ^{13}C NMR signals corresponding to previous reports [14]. The ee was determined on the crude reaction mixture using chiral HPLC with a Chiralpak AD-H column and isocratic elution consisting of 90:10 hexane : *iso*-propanol with 0.1 % TFA and UV detection at 215 nm with the major enantiomer eluting at 6.67 min [18]. Evidence of the formation of the single enantiomer correlated to results obtained from the specific optical rotation in chloroform [14] and in both cases results indicated the formation of the (*R*)-enantiomer only.

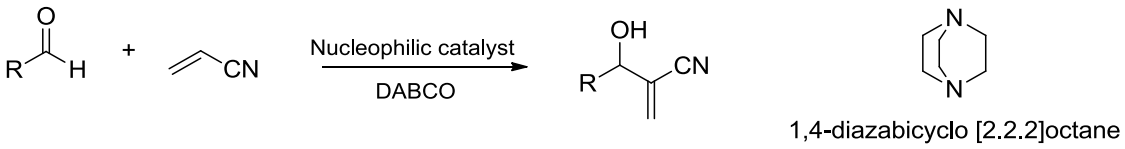
In this case, introduction of a methyl group onto the free hydroxy of 3-hydroxy-3-phenylpropionitrile, led to a dramatic increase of enantioselectivity (100 %) albeit with a slightly lower yield (19 % vs 14 %), over a longer reaction period (5 days). The low isolated yield of acid may be due to the toxicity of the nitrile or corresponding acid product which may cause enzyme inhibition [14]. It appears that size of the protecting group at this position also has an impact on ee values. In contrast, where a more sterically demanding group was present in the case of **39, 40** and **98**, no acid was detected. Again, in all cases no amide was detected after biotransformation indicating a possible nitrilase enzyme present in the isolate.

It is also worth noting that the biotransformation of racemic-3-benzyloxy substituted nitrile **39** was also investigated when *Rhodococcus erythropolis* SET1 was induced and grown with 3-hydroxy-3-phenylpropionitrile **38** rather than 3-hydroxybutyronitrile **89**. Induction in the presence of the aromatic nitrile may result in different protein folding arrangements and hence alter the shape of the active site. However, nitrile **39** was recovered in quantitative yields and no acid was produced.

The reaction outcome was therefore proposed to be strongly influenced by the nature of the *β*-hydroxy protecting group, with both steric and possibly electronic factors affecting the reactivity and more importantly, the enantioselectivity [25].

4.5 BIOTRANSFORMATION OF α-METHYLENE-β-HYDROXY ALKYL AND ARYL NITRILES

The Baylis-Hillman reaction is a three-component reaction involving the coupling of the *α*-position of activated alkenes e.g., *α,β,*-unsaturated esters, amides, nitriles and ketones with carbon electrophiles such as aldehydes, ketones or imines under the catalytic influence of a bicyclic amine such as 1,4-diazabicyclo [2,2,2]octane (DABCO), to give multifunctional products (Scheme 4.14) [26]. A new carbon-carbon bond is formed between the *α*-carbon of the activated olefin component and carbon electrophiles. This reaction combines the aldol and Michael reactions in a single pot to convert two relatively simple molecules into a highly functionalised product [27].



Scheme 4.14: DABCO promoted Morita Baylis Hillman reaction [28].

This reaction has important features such as complete atom economy, the possibility of being performed in an aqueous medium or in the absence of solvents under the influence of organocatalysis, to generate single enantiomer products[28].

Baylis Hillman derivatives find wide applications in the production of pharmaceutical compounds, and often demonstrate antibacterial, anti-tumor and antifungal activity. For a recent review of the literature and to outline the applications shown in Figure 4.10, see the following review [29].

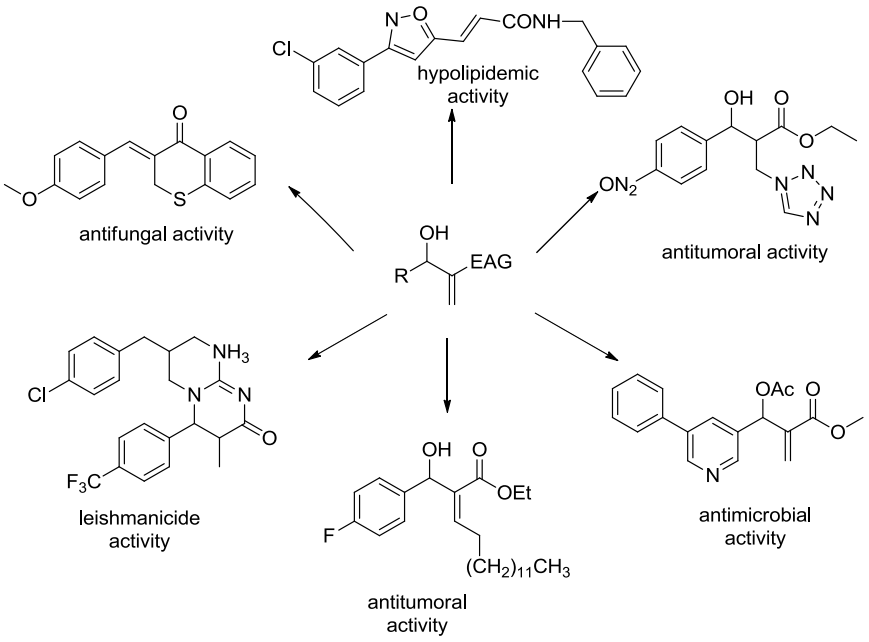
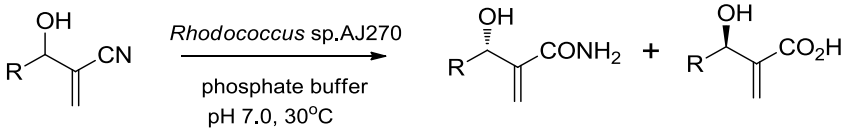


Figure 4.10: Some example of compounds prepared from MBH products and its biological activities adapted from [29].

Wang *et al*. previously investigated the biocatalytic activity of *Rhodococcus sp* AJ270 for the production of enantiomerically pure α-methylene-β-hydroxy carboxylic acids and amides from precursor nitriles (Table 4.6) [30].

Table 4.6: Biotransforamtions of α-methylene-β-hydroxy-β-phenlpropiononitrile using [31].



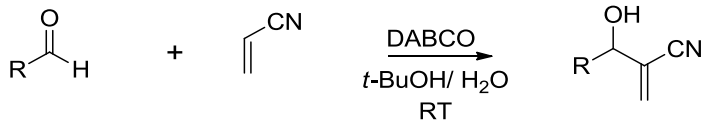
Entry	Substrate	Ar	Amide		Acid	
			yield %	ee %	yield %	ee %
1	**102**	C_6H_5	90	0	-	-
2	**106**	4-MeO-C_6H_4	43	41	52	27
3	**107**	3-MeO-C_6H_4	48	81	45	75
4	**108**	2-MeO- C_6H_4	50	79	44	70
5	**109**	4-Cl- C_6H_4	45	49	49	39
6	**100**	Et	44	0	51	56
7	**101**	Me_2CH	43	61	51	56

Under the influence of AJ270 the reaction generated optically active (*S*)-(+)-amide and (*R*)-(-)-acid. Wang suggested that although the electronic nature of the substituent affected the

enantiomeric excess of the products, it was the substitution pattern that mainly governed the reaction enantioselectivity [31].

In order to evaluate the effect of introducing an α-methylene functionality into β-hydroxy nitriles, several Baylis Hillman nitriles were synthesised using standard conditions reported by DeSouza *et al.* [32]. These again included both aliphatic and ring substituted aromatic analogues in order to determine the effect on activity and enantioselectivity demonstrated by isolate SET1.

De Souza *et al*. reported the reaction between *p*-nitrobenzaldehyde, acrylonitrile and DABCO in various polar protic solvents [32]. The optimum results were obtained using *tert*-butanol and water as solvents [32]. This approach was used in the synthesis of α–methylene-β-hydroxy aryl and alkyl nitriles, employing acrylonitrile, DABCO and the appropriate aliphatic and aromatic aldehydes (Scheme 4.15).



Scheme 4.15: DABCO promoted Baylis Hillman reaction of various aldehydes with acrylonitrile for the production of α-methylene-β-hydroxy nitriles [27].

Each reaction was stirred at room temperature overnight and monitored by TLC. Following extractive work up and purification by flash chromatography the products **99-101** were isolated (Figure 4.11).

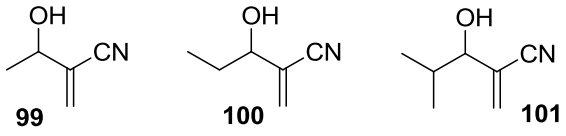
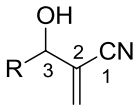


Figure 4.11: Aliphatic α-methylene-β-hydroxynitriles to be examined by SET1.

The DABCO mediated coupling of aliphatic aldehydes with acrylonitrile resulted in the formation of the α-methylene-β-hydroxynitriles **99-101** in yields ranging from 63-69 %. The products were confirmed using ^{1}H & ^{13}C NMR spectroscopy. The key features which identified the substrates were the presence of the ^{1}H NMR spectroscopic peaks corresponding to the α-methylene functionality, which appeared as two non-equivalent

signals as apparent singlets for **99** (Table 4.7, entry 1) and **101** (Table 4.7, entry 3), or in the case of **100** (Table 4.7, entry 2) a singlet at 6.02 ppm integrating for 2 protons. The presence of β-C<u>H</u>OH peak in all three substrates integrating for 1 proton, also verified that the coupling reaction had occurred. LC-MS analysis in all cases indicated the formation of the sodium adduct MNa^+.

Table 4.7: Key spectroscopic data used to verify the preparation of aliphatic Baylis Hillman nitriles.



Entry	Substrate	R	Isolated yield	Key ^{1}H NMR peaks	LCMS
1	**99**	CH_3	69 %	δ = 5.99 (s, 1H, C=C<u>H</u>$_2$) δ = 5.93 (s, 1H, C=C<u>H</u>$_2$) δ = 4.42 (q, 1H,C<u>H</u>OH)	MNa^+ m/z = 121
2	**100**	C_2H_5	72 %	δ = 6.02 (s, 2H, C=C<u>H</u>$_2$) δ = 4.16 (dd, 1H, C<u>H</u>OH)	MNa^+ m/z = 134
3	**101**	$CH(CH_3)_2$	63 %	δ = 6.01 (s, 1H, C=C<u>H</u>$_2$) δ = 5.96 (s, 1H, C=C<u>H</u>$_2$) δ = 3.98 (d, 1H, C<u>H</u>OH)	MNa^+ m/z = 149

A series of aromatic analogues **102-109** bearing electron donating and withdrawing functional groups on the aryl ring were synthesized using the same procedure (Figure 4.12). Products were obtained in yields 58-79 %.

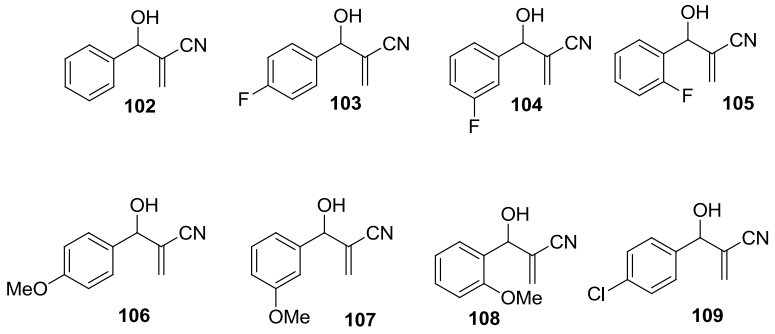


Figure 4.12: aryl-substituted α-methyl-β-hydroxy nitriles to be examined.

The identity of the products was confirmed again by LCMS and ^{1}H and ^{13}C NMR spectroscopic techniques. The identifying ^{1}H NMR peaks, as noted also for aliphatic analogues, are the vinyl methylene peaks which appear in most cases as 2 non-equivalent

singlets, and the newly introduced β- CHOH peak. These are reported in Table 4.8 along with LCMS data.

Table 4.8: Aromatic and aryl substituted α-methylene-β-hydroxynitriles for substrate screening.

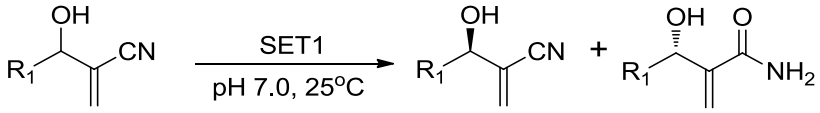
Entry	Substrate	R	Isolated yield	Key ^{1}H NMR peaks	LCMS
1	**102**	C_6H_5	79 %	δ = 6.12 (s, 1H, C=C$\underline{H}_2$) δ = 6.02 (s, 1H, C=C$\underline{H}_2$) δ = 5.27 (br, 1H,C$\underline{H}$OH)	MH^+ m/z = 160
2	**103**	4-F-C_6H_4	58 %	δ = 6.09 (s, 1H, C=C$\underline{H}_2$) δ = 6.01 (s, 1H, C=C$\underline{H}_2$) δ = 5.26 (s, 1H, C$\underline{H}$OH)	MH^+ m/z = 178
3	**104**	3-F-C_6H_4	58 %	δ = 6.06 (s, 1H, C=C$\underline{H}_2$) δ = 5.99 (s, 1H, C=C$\underline{H}_2$) δ = 3.98 (s, 1H, C$\underline{H}$OH)	MH^+ m/z = 178
4	**105**	2-F-C_6H_4	62 %	δ = 6.05 (s, 1H, C=C$\underline{H}_2$) δ = 6.00 (s, 1H, C=C$\underline{H}_2$) δ = 5.58 (s, 1H,C$\underline{H}$OH)	MH^+ m/z = 178
5	**106**	4-OMe-C_6H_4	66 %	δ = 6.31 (s, 1H, C=C$\underline{H}_2$) δ = 6.01 (s, 1H, C=C$\underline{H}_2$) δ = 5.33 (s, 1H, C$\underline{H}$OH)	MH^+ m/z = 190
6	**107**	3-OMe-C_6H_4	70 %	δ = 6.09 (s, 1H, C=C$\underline{H}_2$) δ = 6.01 (s, 1H, C=C$\underline{H}_2$) δ = 5.25 (s, 1H, C$\underline{H}$OH)	MH^+ m/z = 190
7	**108**	2-OMe-C_6H_4	68 %	δ = 5.91 (s, 2H, C=C$\underline{H}_2$) δ = 5.41 (s, 1H, C$\underline{H}$OH)	MH^+ m/z = 190
8	**109**	4-Cl-C_6H_4	65 %	δ = 6.11 (s, 1H, C=C$\underline{H}_2$) δ = 6.01 (s, 1H, C=C$\underline{H}_2$) δ = 5.34 (br, 1H, C$\underline{H}$OH)	MH^+ m/z = 194

Separation of the nitrile enantiomers in each case was achieved using a Chiralpak AD-H or OJ-H column, with isocratic elution with mobile phase (90: 10, hexane: IPA, 0.1 % TFA) with UV detection at 215 nm.

4.5.1 BIOTRANSFORMATIONS OF ALIPHATIC β-HYDROXY-α-METHYLENE NITRILES

Each nitrile **99-101** was subjected to the biotransformation with SET1 as reported previously and after a length of time determined by TLC or LCMS, worked up and purified by preparative TLC (Table 4.9).

Table 4.9: Biotransformations of aliphatic α-methylene-β-hydroxy nitriles.



Entry	Substrate	R	Time	Nitrile		Amide	
				Yield %[b]	ee % [c]	Yield %[b]	ee % [c]
1	**99**	Me	48 h	42.0	(*R*) 76.5	43.2	(*S*) 87.8
2	**100**	Et	72h	69.3	4.2	22.0	(*S*) 10.8
3	**101**	$CH(CH_3)_2$	72h	42.2	(*R*) 12.0	44.1	(*S*) 16.1

[a] Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET1 (OD=1) in phosphate buffer (pH 7.0) at 25 °C. [b] Isolated yield using flash chromatography. [c] Determined by HPLC analysis using a chiral column. ND = Not detected.

Interestingly, in each case the product was identified as amide, with evidence of amide C=O in ^{13}C NMR and also by LCMS adducts correlating to amide (Table 4.10). In all cases the enantioselectivity was determined using AD-H chiral column and data correlated to that reported in the literature [30].

Table 4.10: Characterisation data of aliphatic α-methylene-β-hydroxy amides from the biotransformation of nitriles.

Entry	Product	R	Isolated yield	Key ^{13}C NMR peaks δ = ppm	LCMS
1	**99a**	Me	43.2 %	δ = 171ppm, ($\underline{C}ONH_2$)	MNa^+ m/z = 138
2	**100a**	Et	22.0 %	δ = 171.1 ppm, ($\underline{C}ONH_2$)	MNa^+ m/z = 152
3	**101a**	$CH(CH_3)_2$	44.1 %	δ = 172.0 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 143

R. erythropolis SET1 influenced the conversion of nitrile **99** into the corresponding (*S*)-amide **99a** in 87.8 % ee in 43 % yield (Table 4.9, entry 1) and this is a slight decrease from >99.9 % ee and 42 % yield observed for 3-hydroxybutyronitrile **89** without a substituent at the α-position. The amide product however is in sharp contrast to behaviour observed previously while examining SET1, where the whole cell biocatalyst efficiently catalysed the hydrolysis of non α-substituted-β-hydroxy nitriles to give acids with (*S*) configuration as the major products. In all previous substrate studies towards a variety of β-hydroxy nitriles including aromatic, aliphatic and dinitriles, with both the free hydroxyl and protected hydroxyl groups, amide was not detected.

It also appears from this data that size at the β- position has an impact on ee values for aliphatic α-methylene nitriles. For example, where a more sterically demanding group was

present in the substrate the ee values decreased from 87 % ee in 43.2 % yield for substrate **99** (Table 4.9, entry 1), to 10.8 % ee in 22.0 % yield for nitrile **100** (Table 4.9, entry 2). The reaction outcome therefore, also appeared to be strongly influenced by the nature and presence of the alkyl group at the chiral centre. This is in contrast to previous reports by Wang *et al*. who found that during the hydrolysis of aliphatic Baylis Hillman nitriles by *R. erythropolis* AJ270 (a NHase/ amidase containing isolate) that the enantioselectivity increased with increasing size of the alkyl substituent [31].

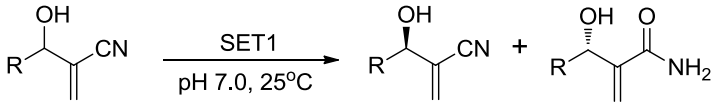
The results initially indicated that there may be an active nitrile hydratase in the microbial cells which demonstrates enantioselectivity towards the Baylis Hillman nitrile specifically. Although all previous studies suggested the presence of a highly enantioselective if not enantiospecific nitrilase enzyme within SET1, other nitrile hydrolyzing enzymes such as nitrile hydratase/ amidase may also be present within the microbial whole cell. Many strains have been shown to contain either a nitrilase or nitrile hydratase/ amidase [33]. However some micro-organisms such as *Rhodococcus rhodochrous* LL 100-21 (formerly *Nocardia rhodochrous* LL 100-21) [34] and *Rhodococcus rhodochrous* J1 [35] contain both nitrilase-catalysed and nitrile hydratase/ amidase catalysed biotransformation pathways. However, nitrile hydratase does not generally exhibit enantioselectivity [36],[14]. In most cases the enantioselectivity is demonstrated by the corresponding amidase enzyme, to preferentially form one enantiomer of acid. Another plausible explanation is that the enzyme system involved within *R. erythropolis* SET1 contains an enantioselective nitrilase which is demonstrating NHase activity [37]. This will be further discussed later in this Chapter.

4.5.2 BIOTRANSFORMATIONS OF ARYL SUBSTITUTED α-METHYLENE-β-HYDROXY NITRILES

Having previously detected the formation of amide during the biotransformation of aliphatic *α*-methylene-*β*-hydroxy nitriles, the hydrolysis of aryl substituted *α*-methylene-*β*-hydroxy nitriles was next evaluated. This study also examined the positional effect that the substituent on the aryl ring had on the reaction outcome. To this end, the biotransformation of nitriles **102-109** were examined using the same conditions as outlined before. Results are outlined in Table 4.11.

As expected *Rhodococcus* sp. SET1 converted nitriles, **102-109** into the corresponding amides in 12.8-30.2 % yield after purification using preparative TLC (Table 4.11, entry 1-8). In all cases of aryl substituted β-methylene nitriles derived, the reaction gave optically active (*S*)-amide. The configuration was assigned by comparison of the sign of optical rotation with previous literature reports [30]. Consideration was also taken for the order of elution on the AD-H and OJ-H chiral column.

Table 4.11: Biotransformations of aromatic α- methylene-β- hydroxy nitriles.



102-109 **(R)-102-109** **102a-109a**

Entry	Substrate	R	Time	Nitrile		Amide a	
				Yield %[b]	ee % [c]	Yield %[b]	ee % [c]
1	**102**	C_6H_5	120h	72.4	(*R*) 18.7	22.2	(*S*) 42.3
2	**103**	4-F-C_6H_5	120h	61.3	(*R*) 22.5	30.2	(*S*) 50.4
3	**104**	3-F-C_6H_5	120h	43.2	(*R**) 46.0	30.1	(*S**) 4.9
4	**105**	2-F-C_6H_5	120h	69.5	(*R**) 14.3	26.4	(*S**) 38.4
5	**106**	4-OMe-C_6H_5	120h	64.6	(*R*) 10.8	28.5	(*S*) 32.1
6	**107**	3-OMe-C_6H_5	120h	45.2	(*R*) 38.9	27.3	(*S*) 11.6
7	**108**	2-OMe-C_6H_5	120h	82.3	3.8	12.8	(*S*) 60.5
8	**109**	4-Cl-C_6H_5	120h	97.0	2.8	ND	ND

[a] Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET-1 (OD=1) in phosphate buffer (pH 7.0) at 25 °C. [b] Isolated yield using flash chromatpgraphy. [c] Determined by HPLC analysis using a chiral column. ND = Not detected. [*]Configuration was tentatively assigned.

The isolated amide produced from each biotransformation was analysed by ^{1}H and ^{13}C NMR spectroscopy. In each case the appearance of the amide carbonyl $\underline{C}ONH_2$ peak at approximately 170 ppm in the ^{13}C NMR spectrum identified the product. This data correlated to that previously reported in the literature by Wang *et al.* [30]. LC-MS analysis served as further evidence of amide formation with the MH^+ adduct. A summary of the results obtained during characterisation of the products is outlined in Table 4.12, entry 1-7

Table 4.12: Characterisation data for isolated α-methylene-β-hydroxy amides produced from the corresponding nitrile using SET1.

Entry	Product	R	Isolated yield	Key ^{13}C NMR peaks	LCMS
1	**102a**	C_6H_5	22.2 %	δ = 171.3 ppm, ($\underline{C}ONH_2$)	MH^+ m/z = 178
2	**103a**	4-F-C_6H_4	30.2 %	δ = 169.5 ppm, ($\underline{C}ONH_2$)	MH^+ m/z = 196
3	**104a**	3-F-C_6H_4	30.1 %	δ = 169.4 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 196
4	**105a**	2-F-C_6H_4	26.4 %	δ = 171.5 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 196
5	**106a**	4-OMe-C_6H_4	28.5 %	δ = 169.1 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 208
6	**107a**	3-OMe-C_6H_4	27.3 %	δ = 171.0 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 208
7	**108a**	2-OMe-C_6H_4	12.8 %	δ = 172.0 ppm ($\underline{C}ONH_2$)	MH^+ m/z = 208

In comparison to the biotransformation of 3-hydroxy-3-phenylpropionitrile **38** which resulted in the formation of acid **38a** (19.3 % yield, 7.0 % ee) (Table 4.5, entry 1), its corresponding α-methylene substituted analogue **102** gave amide (22.2 % yield, 42.3 % ee) as product (Table 4.11, entry 1). Both an increase in yield and ee were found for this Baylis Hillman analogue.

Several trends were observed during this work. In the case of substituted aryl β-hydroxy methylene nitriles **103-105**, bearing electron withdrawing groups such as F on the aromatic ring, the optimum ee (50.4 %) was obtained when it was present at the *para* position **103** (Table 4.11, entry 2). While electron-donating groups such as OMe (Table 4.11, entry 7) are present, the optimum enantioselectivity (60.5 %) was observed when the group was present at the *ortho*-position **108**. This is supported by results published by Wang *et al*. who found that the *ortho*-methoxy analogue **108** resulted in the highest enantioselectivity with the amide formed in 79 % ee and the acid in 70 % ee [31]. In the case of SET1, when electron donating or electron withdrawing groups were present at the *meta*-position **104** (Table 4.11, entry 3) and **107** (Table 4.11, entry 6) poor enanantioselectivity was observed, with the amide produced in 4.9 % and 11.6 % ee respectively. The preferential enantiopreference for the *ortho*-substituted aromatic substrates in this study is intriguing, as for other α-substituted nitriles and amides it is generally the *para*-substitution on the aromatic ring that leads to the increase in enantioselectivity values [31].

It is worth noting that Martinkova *et al*. observed amide formation (Figure 4.13) in the case of two α-methylene-β-hydroxy nitriles when subjected to an isolate containing a nitrilase from *Aspergillus niger* K10 [38]. No enantioselectivity data was reported.

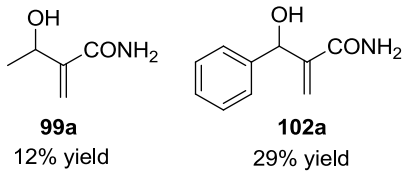


Figure 4.13: Amide formation observed by Martinkova *et al*. during the biotransformation of α-methylene-β-hydroxy nitriles.

They propose that the nitrile converting enzyme is either a nitrilase affording extremely large amounts of some specific amides or a nitrile hydratase. However, NHase possessing broad substrate specificity is not usually observed in the case of filamentous fungus [38].

To understand the role the hydroxy group played in the biotransformation, it was protected by methylation as previously discussed in Section 4.3, in the case of nitrile **38**. The –OMe group **41** was chosen as it gave the best increase in enantioselectivity (Table 4.5, entry 5).

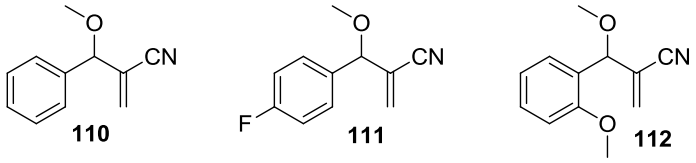
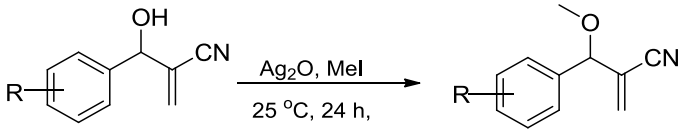


Figure 4.14 O-methylated Baylis Hillman nitrile analogues to determine the role the hydroxyl group plays in the biotransformation.

Three methoxy protected analogues (Figure 4.14) were prepared in yields ranging from 32-41 %, by the addition of methyl iodide to a solution of the nitrile in dichloromethane in the presence of silver oxide (Scheme 4.16). Following extractive work up as previously described and chromatographic purification, the O-alkylated nitriles were characterised (Table 4.13).



Scheme 4.16: Synthesis of methyl protected α-methylene-β-hydroxy aromatic nitriles.

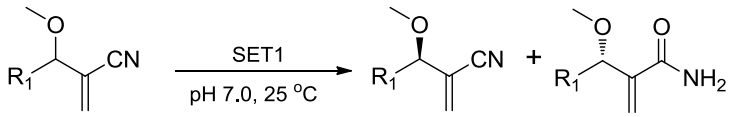
The key ^{1}H NMR spectroscopic resonances which confirmed the successful reaction and verified the protection strategy, were the newly introduced methoxy peaks at 3.35 ppm which appeared as a singlet integrating for 3 protons [30]. LCMS also verified the methylation and characterisation results are outlined in Table 4.13.

Table 4.13: Key characterization data for methyl protected α-methylene-β-hydroxy aromatic nitriles.

Entry	Substrate	R	Isolated yield	Key ^{1}H NMR peaks δ = ppm	LCMS
1	**110**	C_6H_5	32.0 %	δ = 3.35, (s, 3H, COC$\underline{H}_3$)	MH^+ m/z = 174
2	**111**	4-F-C_6H_4	41.1 %	δ = 3.42, (s, 3H, COC$\underline{H}_3$)	MH^+ m/z = 192
3	**112**	2-OMe-C_6H_4	39.2 %	δ = 3.37, (s, 3H, COC$\underline{H}_3$)	MH^+ m/z = 204

The resulting substrates (10 mM) were then hydrolysed by whole cells of SET1 (OD_{600nm} = 1) in phosphate buffer (100 mM, pH 7) at 25 °C. The reaction products were monitored by TLC over 72 hours, and worked up as reported previously. In each case amide was again formed ranging from 12.2-27.1 % yield (Table 4.14). Separation of the amide enantiomers was achieved using a Chiralpak OJ-H or AD-H columns, with isocratic elution with mobile phase (90: 10, hexane: IPA, 0.1 % TFA) with UV detection at 215 nm. Configuration was tentatively assigned based on the order of elution with data previously reported by Wang *et al.* [30].

Table 4.14: Biotransformations of aryl β-methoxy-α-methylene nitriles.



110-112 **(*R*)-110-112** **110a-112a**

Entry	Substrate	R	Time	Nitrile		Amide a	
				Yield % [b]	ee % [c]	Yield % [b]	ee % [c]
1	**110**	C_6H_5	120h	58.6	8.2	27.1 %	(*S*) 33.0
2	**111**	4-F-C_6H_5	120h	60.8	(*R**) 10.3	22.0 %	(*S**) 26.4
3	**112**	2-OMe-C_6H_5	120h	76.3	1.5	12.2 %	(*S*) 31.3

[a]Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET-1 (OD=1) in phosphate buffer (pH 7.0) at 25^{O}C. [b] Isolated yield using flash chromatpgraphy. [c] Determined by HPLC analysis using a chiral column. ND = Not detected. [*]Configuration was tentatively assigned.

Amide structure was confirmed by ^{1}H and ^{13}C NMR spectroscopy and data correlated to that previously reported in the literature [31]. The key ^{13}C NMR peaks included the amide

$\underline{C}ONH_2$ peak at approximately 170 ppm. Generation of the corresponding MH^+ ion during LCMS analysis also verified the formation of amide (Table 4.15).

Table 4.15: Characterisation data of the amides resulting from the biotransformaton of aryl β-methoxy-α-methylene nitriles by SET1.

Entry	Product	R	Isolated yield	Key ^{13}C NMR peaks δ = ppm	LCMS
1	**110a**	C_6H_5	32.0 %	δ = 168.3 ppm, ($\underline{C}ONH_2$)	MH^+ m/z = 194
2	**111a**	4-F-C_6H_4	41.1 %	δ = 171.0 ppm, ($\underline{C}ONH_2$)	MH^+ m/z = 209
3	**112a**	2-OMe-C_6H_4	39.2 %	δ = 171.3 ppm, ($\underline{C}ONH_2$)	MH^+ m/z = 222

It was observed that enantioselectivity decreased upon protection for these substrates. For example the enantioselectivity obtained for the free hydroxy substrate **102** decreased from 42.3 % (Table 4.11, entry 1) to 33 % ee for the methoxy analogue **110** (Table 4.14, entry 1). This is in contrast to reports by Wang *et al*. who found that the enantioselectivity increased for such methyl protected substrates [31]. This indicated that during the hydrolysis by SET1, the β-hydroxyl group may play a role in the formation of β-hydroxy amides and may exert some effect on enantioselectivity (this is discussed in more detail later).

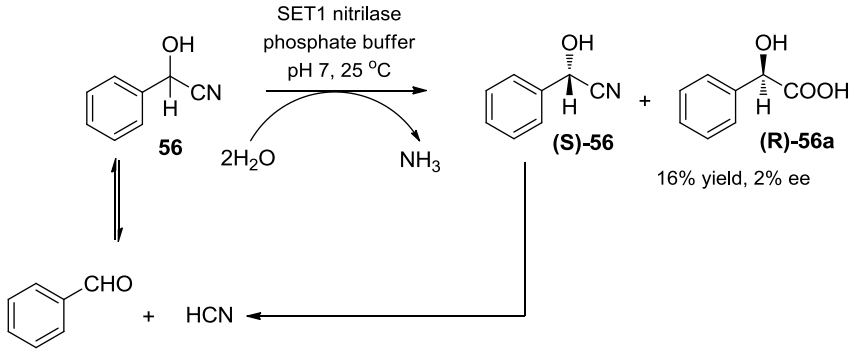
4.6 BIOTRANSFORMATIONS OF α-SUBSTITUTED NITRILES

4.6.1 BIOTRANSFORMATION OF α-HYDROXY SUBSTITUTED NITRILES

As noted previously, (*R*)-Mandelic acid **(*R*)-56a** is an important chiral intermediate for the preparation of pharmaceuticals such as antitumor agents and anti-obesity agents [39]. Industrial application of nitrilase catalysed hydrolysis of mandelonitrile **56** is attractive because of the potential to carry out a dynamic kinetic enzymatic resolution which can theoretically provide 100 % yield of product.

As mandelonitrile **56** differs from 3-hydroxybutyronitrile **89** and 3-hydroxy-3-phenylpropionitrile **38** in the position of the OH group, α- rather than β-, it was initially decided to evaluate mandelonitrile as a substrate to investigate the role that the position of the hydroxy group plays in the enantioselective hydrolysis by *R. erythropolis* SET1. During the hydrolysis of mandelonitrile **56** by the resting cells of *R. erythropolis* SET1 at pH 7.0, racemic mandelic acid **56a** was obtained in low yields (16 %) after 24 hours, without any detectable amount of mandelamide as a possible by-product (Scheme 4.17). The resulting

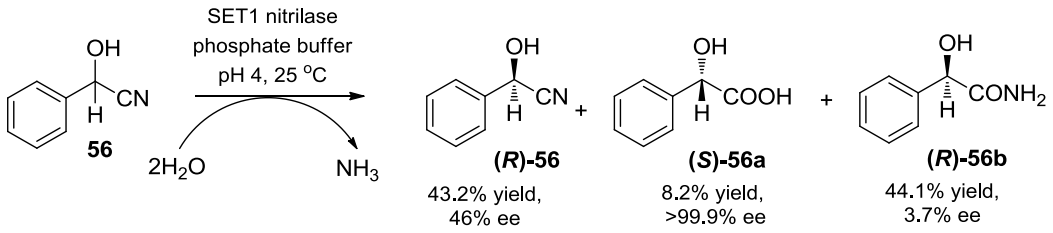
mandelic acid enantiomers were separated on an AD-H chiral column. A by-product of the degradation of mandelonitrile, benzaldehyde, was detected in the crude product mixture using GC-MS.



Scheme 4.17: Biotransformation of racemic mandelonitrile using *R. erythropolis* SET1 at pH 7.

The stability of mandelonitrile in aqueous solution is an obstacle that requires careful consideration during the biotransformation. Mandelonitrile can spontaneously decompose into benzaldehyde and hydrogen cyanide; the process is reversible and is the major racemisation pathway of enantiomerically pure mandelonitrile as shown in Scheme 4.17. A potential factor that must be carefully assessed is the pH of the biotransformation media. Sheldon *et al*. observed that in studies using *P. fluroescens* EBC191, mandelonitrile was stable at pH < 4-5 and lower temperature. However these conditions also had a negative effect on nitrilase activity [37].This was previously discussed in Chapter 1.

The activity of *R. erythropolis* SET1 towards 3-hydroxybutyronitrile **89** as reported in Chapter 3, was found to reduce when the biotransformation was undertaken at lower pH. The enantioselectivity was however retained at pH 4-6. The hydrolysis of mandelonitrile at pH 4 by SET1 was examined. The products were found to be mandelamide **(*R*)-56b** (44 % yield) and also mandelic acid **(*S*)-56a** (8.2 % yield) after 48 hours. Although the yield of the acid **(*S*)-56a** was low the product was of extremely high optical purity (> 99.9 %). In both cases all spectroscopic and LC data correlated with known standards. In addition unreacted nitrile was recovered in 43.2 % yield and 46 % ee (Scheme 4.18).



Scheme 4.18: Hydrolysis of mandelonitrile by *R. erythropolis* SET1 at pH 4.

It was decided to monitor the time course of the reaction to determine if amide and acid were formed concurrently, or if the acid produced was a result of amide hydrolysis. For this work a series of standards were prepared for nitrile **56**, acid **56a** and amide **56b**. Injection of these mixed standards onto reverse phase C18 HPLC column using ACN: water as the mobile phase generated a linear response for the standard curve as can be seen in Figure 4.15. The amide, acid and nitrile standards resulted in R^2 values of 0.992, 0.994, and 0.986 respectively.

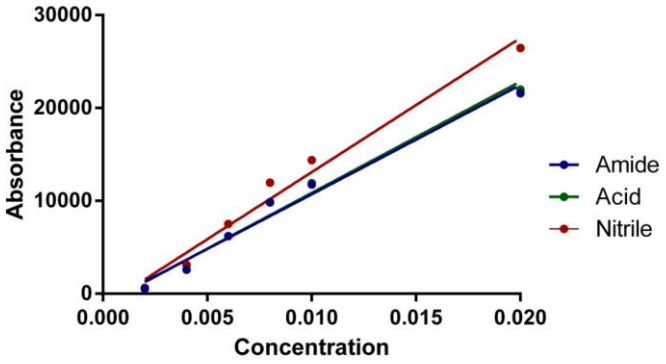


Figure 4.15: Standard curve for the determination of mandelonitrile, mandelamide and mandelic acid concentrations using reverse phase HPLC.

The reaction was monitored at various time points over a 48 hour period using HPLC to determine conversion. From the time course of the reaction it is apparent that mandelamide and mandelic acid are formed concurrently as shown in Figure 4.16. The reaction appears to be complete after 4 hours with no additional amide produced up to 96 hours. It can be seen that formation of the acid also ceases after 4 hours and that as the time increases, so too does the production of benzaldehyde due to spontaneous degradation with a decrease in concentration of mandelonitrile over 48 hours.

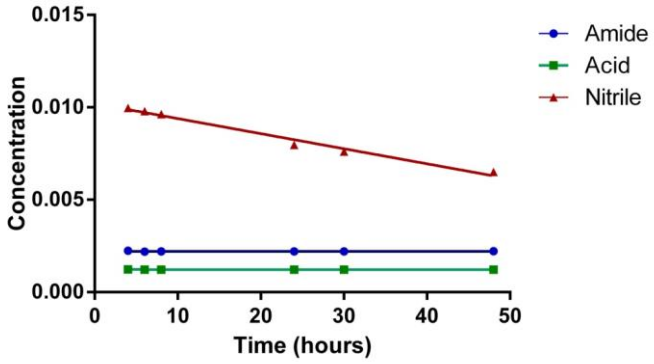


Figure 4.16: SET1 catalysed hydrolysis of mandelonitrile at pH 4 over 48 hours.

It was necessary to exclude any possibility that the production of acid **(*S*)-56a** was due to amide **(*R*)-56b** hydrolysis by an amidase within the bacterial whole cell. Accordingly *R. erythropolis* SET1 was incubated with racemic mandelamide at pH 4. However, no reaction was observed with amide recovered in quantitative yields (96 %) after 48 hours. This finding suggests that mandelamide is not an intermediate in the production of acid during the hydrolysis of mandelonitrile by SET1.

It appears from this data that the position of the stereocentre α- to the nitrile group has an impact on the reaction outcome. The biotransformation of nitrile **56** tended towards the formation of amide **(*R*)-56b** (44 % yield) with the acid **(*S*)-56a** only produced in 8.7 % yield. This is in contrast to 3-hydroxy-3-phenylpropionitrile **38**, where the hydroxyl stereocentre is located at the β-position to the nitrile. During the hydrolysis of nitrile **38** by SET1, only the corresponding acid was produced in 19.3 % yield and 7 % ee (Table 4.3, entry 1) without amide.

The reaction outcome therefore, appeared to be strongly influenced by the position of the hydroxyl group or stereocentre relative to the nitrile. This fits with previous nitrilase catalysed biotransformations with mandelonitrile where both the acid and amide were produced. It appears that the nature of the electron withdrawing hydroxyl group at the α-position may have influenced the relative quantity of acid and amide produced. For example, Wang *et al.* had previously observed in the case of *Pseudomonas fluorescens* EBC191 which contains a nitrilase, that mandelonitrile **56** was converted to mandelic acid **56a** and mandelamide **56b** with a product ratio of 80:20 [40]

4.6.2 BIOTRANSFORMATIONS OF RACEMIC α-ALKYL SUBSTITUTED ARYL NITRILES

Optically active 2-arylpropionic acids are useful as pharmaceutically active substances, especially the non-steroidal anti-inflammatory drugs referred to as profens (Figure 4.17). The activity of profens resides almost almost exclusively in their (*S*)- enantiomers [41],[42].

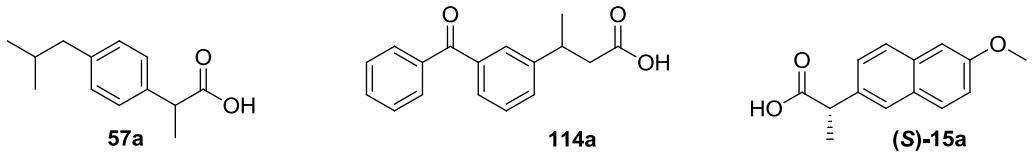


Figure 4.17: Pharmaceutically active 2-arylpropionic acids.

The unexpected quantity of amides formed from α-methylene-β-hydroxy nitriles, along with amide formation when the hydrolysis of mandelonitrile was carried out at pH 4, was surprising. This appeared to be due to substituent groups at the α- position, in particular when electron withdrawing groups were present, and was thought to warrant further investigation. A final set of α-aryl, secondary nitrile substrates were evaluated. In this case alkyl groups replaced the hydroxyl functionality in mandelonitrile to again assess the possible effect of the electron withdrawing hydroxyl group at the α-position. The size at the α-position was also varied as shown in Figure 4.18.

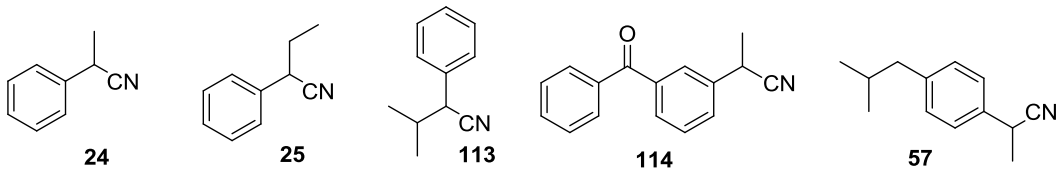
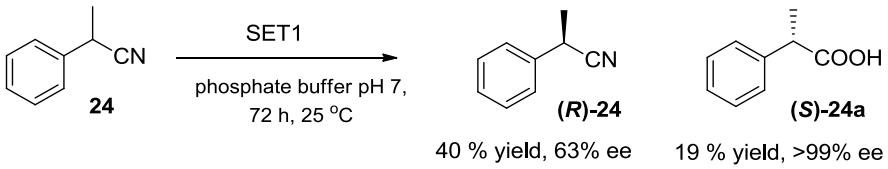


Figure 4.18: α-alkyl substituted nitrile to be investigated.

As ibuprofen **57a**, is one of the most well-known non-steroidal anti-inflammatory agents and falls within this class, the nitrile **57** was also evaluated along with ketoprofen nitrile **114**; a related analogue. The α-alkyl substituted aryl nitriles were purchased from commercial sources and used without additional purification.

The hydrolysis of 2-phenylpropionitrile **24** was performed by incubating the substrate with the isolate in potassium phosphate buffer at 25 °C for 72 hours (Scheme 4.19). Following

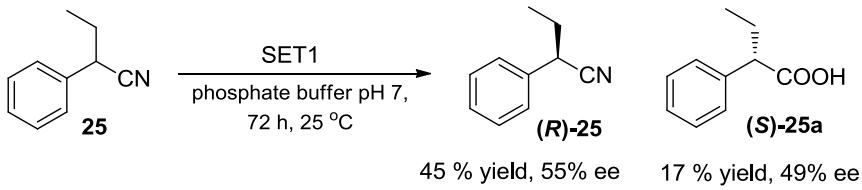
extractive work up and purification using preparative TLC the corresponding acid **(*S*)-24a** was isolated in low yields (19.3 %) with >99.9 % ee.



Scheme 4.19: Nitrilase catalysed hydrolysis of 2-phenylpropionitrile by SET1.

The enantiomers were separated using an AD-H column and data correlated with spectra in the literature [33, 43-45]. Again all spectroscopic data including ^{1}H and ^{13}C NMR provided evidence of acid formation. The variation in ^{1}H NMR chemical shift of the C<u>H</u>CH_3 proton which appears as a quartet integrating for 1 proton in the nitrile to a more downfield position for the corresponding acid at 3.66 ppm, along with an acid COOH peak at 196 ppm in ^{13}C NMR was observed. LC-MS analysis in positive mode also demonstrated the corresponding MH^+ adduct at m/z 254. The configuration of the acid was determined to be (*S*)- by comparison of optical rotation data to that reported in the literature [46], and > 99 % ee was obtained.

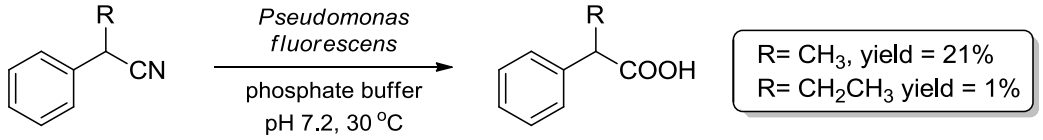
The biotransformation of 2-phenylbutyronitrile **25** resulted in the formation of acid **(*S*)-25a** in 17 % yield and 49.5 % ee (Scheme 4.20), with the same trends in ^{1}H NMR and ^{13}C NMR observed along with LCMS confirmation obtained [33, 43-45]. In this case there was a decrease in the enantioselectivity from >99.9 % ee to 49.5 % ee for the α-ethyl substituted analogue **25**.



Scheme 4.20: Nitrilase catalysed hydrolysis of 2-phenylbutyronitrile by SET1.

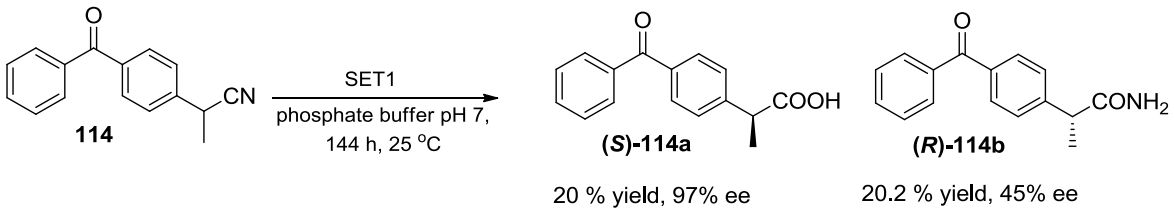
In the case of the biotransformation of 2-methyl-2-phenylbutyronitrile **113**, the reaction did not proceed, with the starting nitrile being recovered in 73 % yield after 72 hours interaction with whole cells of SET1.

In both successful cases in contrast to mandelonitrile, the nitrilase generated the corresponding carboxylic acid, with no quantities of the amide produced. Although the enzyme accepted α- alkyl substituents, it was slow to convert the ethyl and even slower to convert the bulkier isopropyl substituted analogue (where the biotransformation did not proceed at all). The decreasing conversion of nitrile **24** and **25** may be ascribed to the increasing bulk of the α–substituent as previously observed by Brady *et al.* [47]. A range of α–substituted aryl nitriles (Scheme 4.21) were examined using *Pseudomonas fluorescens* nitrilase. Brady *et al.* found that this nitrilase could convert 2-phenylpropionitrile to the corresponding acid in 21 % yield, where as 2-phenylbutyric acid was formed in only 1 % yield (enantioselectivity data was not reported in this study).



Scheme 4.21: Conversion of α-substituted phenylacetonitriles by the nitrilase of *Pseudomonas fluorescens*.

The hydrolysis of ketoprofen nitrile **114** was carried out over 5 days in the presence of SET1. In this case both amide and acid were obtained in 20 % yield as products after work up and purification by preparative TLC (Scheme 4.22).



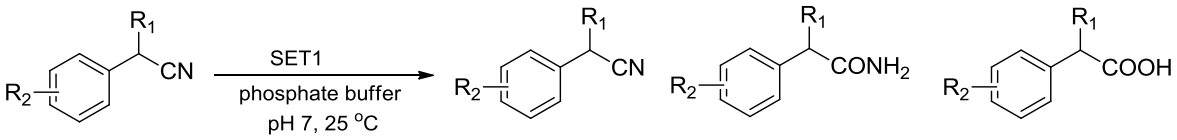
Scheme 4.22: Nitrilase catalysed hydrolysis of 2-(3-benzoylphenyl)propanenitrile by SET1.

Both products were verified by LC-MS and also by NMR spectroscopy with ^{1}H & ^{13}C NMR signals corresponding to previous reports [14]. The C<u>H</u>-CH_3 proton appeared as a quartet integrating for 1 proton at 3.8 ppm for the acid product **(*S*)-114a** in the ^{1}H NMR spectrum, with the same signal appearing at 3.97 ppm for the amide product **(*R*)-114b**. ^{13}C NMR spectroscopic analysis also verified the production of both products with the acid COOH peak at 178.1 ppm and the amide $CONH_2$ peak at 176.7 ppm. After HPLC analysis

with a chiral column, it was determined that the amide was formed in 45 % ee and the acid 97 % ee. Both comparison of the order of elution and the specific optical rotation data indicated the *(S)*- configuration of the acid.

The hydrolysis of ibuprofen nitrile **57** (Table 4.16, entry 5) was also performed under the standard conditions. However, after 120 hours the reaction had stalled with the nitrile recovered in 89 % yield. There was no evidence of the formation of the corresponding acid or amide when the crude reaction product was monitored by LCMS.

Table 4.16: Biotransformations of α-alkyl substituted aryl nitriles



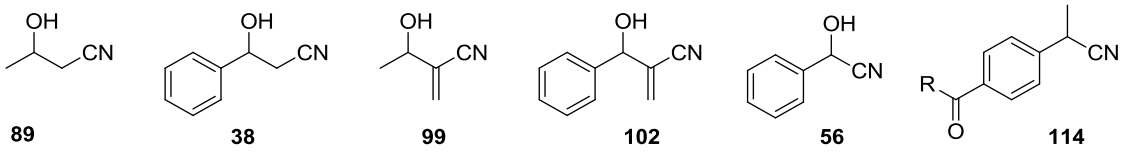
Entry	Substrate	R_1	R_2	Nitrile (yield%)[b], (ee%)[c]	Amide c (yield%)[b], (ee%)[c]	Acid b (yield%)[b], (ee%)[c]
1	**24**	Me	H	(40.1), (63.0)	ND	(19.3), (*S* >99.9)
2	**25**	Et	H	(45.0), (55.5)	ND	(17.0), (49.5)
3	**113**	*i*-Pr	H	(73.1), (1.2)	ND	ND
4	**114**	Me	3-COPh	(54.1), (41.7)	(20.2), (*R* 45.7)	(20.0), (*S* 96.7)
5	**57**	Me	*4-i*-Pr	(89.2), (1.2)	ND	ND

[a]Biotransformation was carried out by incubating substrate (10 mmol/L) in a suspension of the *Rhodococcus erythropolis* SET-1 (OD=1) in phosphate buffer (pH 7.0) at 25^{O}C. [b] Isolated yield using flash chromatography. [c] Determined by HPLC analysis using a chiral column. ND = Not detected.

In summary, the hydrolysis of α- alkyl arylacetonitriles resulted in the formation of acids as the sole product. In contrast mandelonitrile and ketoprofen nitrile **114** gave amides **114b** varying in yield up to 20.2 %. This again supplements previous work demonstrating that amide may be formed where electron withdrawing groups are present at the α-position. In the case of ketoprofen nitrile **114**, the electron withdrawing effect may be *via* a carbonyl *meta*- to the ring inductively withdrawing from the α-carbon.

A complete comparison of the effects of alkyl and aryl substitution at the β-position along with variation in the substituent present at the α-position of evaluated nitriles on the reaction outcome, including the acid/amide ratio is summarised in Table 4.17.

Table 4.17: Electronic and steric effects on the acid to amide ratio.



Substrate	89	38	99	102	56	114
Acid (%)	42.2	19.3	ND	ND	8.2	20.0
ee (%)	>99.9 *(S)*	7.0 *(R)*	ND	ND	>99.9 *(S)*	98.7 *(S)*
Amide (%)	ND	ND	43.2	22.2	44.1	20.2
ee (%)	ND	ND	87.8 *(S)*	42.3 *(S)*	3.7 *(R)*	45.7 *(R)*

Isolate SET1 demonstrated a correlation between the structure of the substrates and the relative proportions of the amide formed as seen in Table 4.17. The largest amounts of amide were formed from α-methylene-β-hydroxy analogues, and lower amounts of amide were formed in the case of mandelonitrile and ketoprofen nitrile (Table 4.16, entry 4). No amide formation was observed for β-hydroxy aliphatic or β-hydroxy aryl unsubstituted at the α-position analogues (Tables 4.1, 4.3 and 4.5). This correlates with Kiziak *et al* [46] who suggested that the synthesis of amide intermediates increased with the electron withdrawing properties at the 2-position, although other properties might influence the degree of amide formation.

4.7 MECHANISTIC INSIGHTS INTO NITRILASE/ NHASE BEHAVIOR

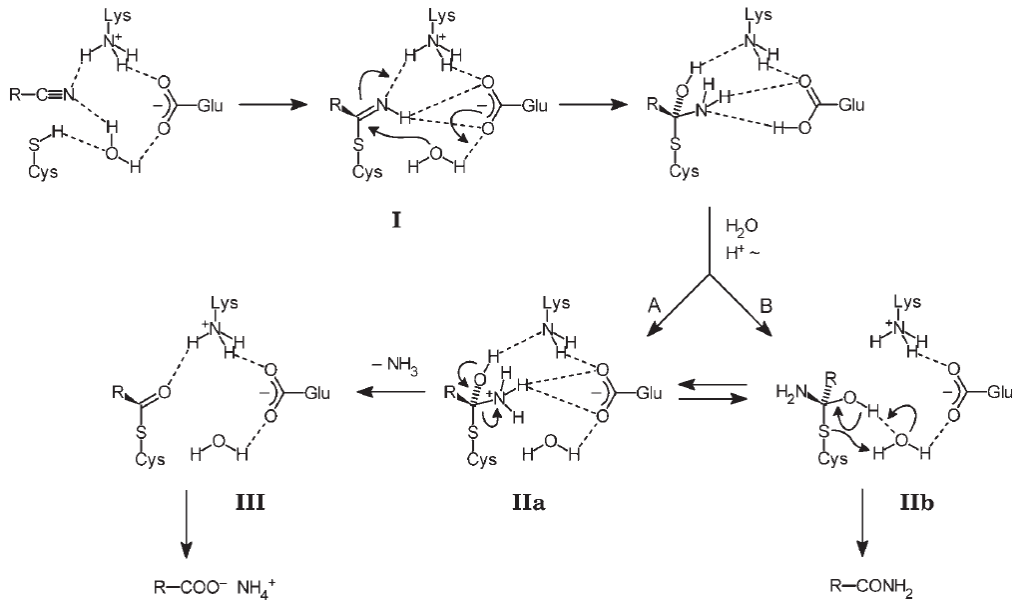
There are several reports on the formation of amides as minor products in the nitrilase catalysed hydrolysis of nitrile substrates, along with various rationales for the production of amides [48-50]. Of relevance to this work are the results obtained with AtNit1 and AtNit4 from *Arabdopsis thaliana* [49, 51, 52], which gave amide formation, the quantity of which was found to be strongly dependent on the nature of the substituent at the α-position [49]. Osswald *et al*. found that amide was formed preferentially when nitriles contained a α-fluoro substituent along with an α- aromatic ring, or when acceptor groups such as CN or NO_2 were present in π conjugated nitriles.

Osswald's work fits with results obtained with SET1 where mandelonitrile and ketoprofen nitrile, both containing electron withdrawing groups along with aromatic rings at the α-

position, gave varying amounts of acid and amide. In addition the α-methylene-β-hydroxy nitriles containing π conjugated nitriles with additional possible inductive withdrawal at the α-position *via* the β-hydroxy group and the aromatic ring, exclusively formed amide.

Osswald *et al*. proposed that stabilization of the tetrahedral intermediate formed after the addition of H_2O to the thioimidate intermediate, led to this amide formation. How this stabilisation assists the elimination of cysteine was unknown [49]. Work by Mateo *et al*. in particular in the case of mandelonitrile, again points to the electron density at the α-position affecting the quantity of acid and amide formed. In their case differing acid/amide ratios were obtained when enantiomerically pure (*R*)- and (*S*)- mandelonitrile were subjected to hydrolysis by PfNLase. They suggest in addition to electron density, stereochemical configuration of the nitrile can lead to amide formation [50].

Bruno *et al*. proposed a detailed mechanism (Scheme 4.23) to explain the formation of amide when electron deficient α- substituted nitriles such as mandelonitrile were used as substrates with *Pseudomonas fluorescens* EBC191 [37].

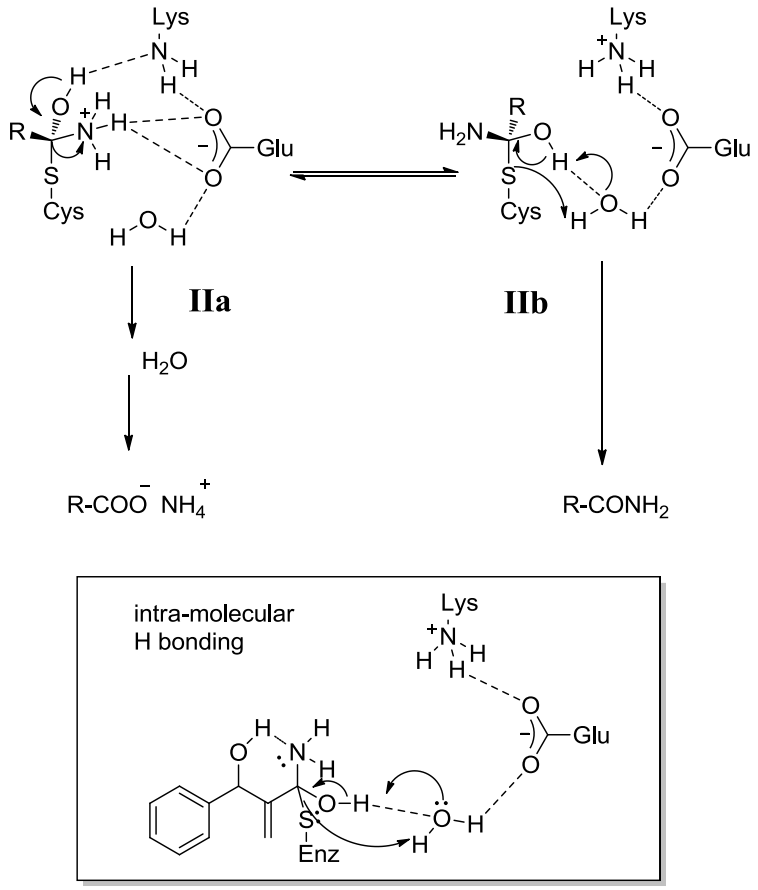


Scheme 4.23: Proposed mechanism for the formation of acid (A) and amide (B), adapted from [37].

They explained amide formation where the Glu and Lys residues along with cysteine are involved in stabilizing intermediates and facilitating proton transfer [37]. They suggest

stabilisation of the positively charged aryl intermediate IIa is key for acid formation. They postulate that if IIa is sufficiently stabilized by the Glu residue, acid will form preferentially. In contrast, if the positive charge lies on the amino Lys residue as in intermediate IIb, thiol elimination may occur to form amide [37].

They suggest that if an electron withdrawing group is present at the α-position, it will destabilize the acid forming Glu interacting intermediate IIa, ultimately producing amide *via* IIb. In the case of the work with SET1 and results obtained with mandelonitrile, ketoprofen nitrile and the α-methylene-β-hydroxy nitriles the relative quantity of amide could be linked to destabilization via conjugation or inductive effects at the α-position, resulting in preferential formation of IIb rather than IIa (Scheme 4.24).



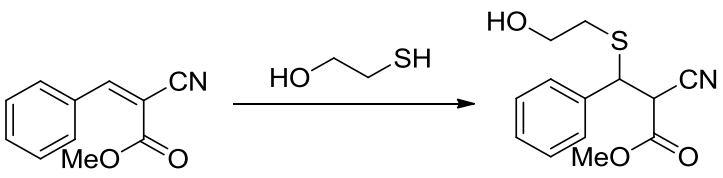
Scheme 4.24: Proposed nitrilase mechanism of amide formation in SET1 adapted from [37].

In the case of α-methylene-β-hydroxy nitriles, in addition intra-molecular hydrogen bonding via a 6 membered ring of the β-hydroxyl with the amino moiety may stabilise the

thiol eliminating intermediate IIb, further leading to increased amide formation (Scheme 4.24). This is demonstrated by loss of ee when this hydroxyl functionality is protected as methoxy. Stabilisation of a similar intermediate *via* H-bonding was proposed by Mukherjee *et al*. to be responsible for amide formation in the nitrilase catalysed hydrolysis of *β*-hydroxy nitriles by ZmNit2 [48]. Finally along with such electronic effects, steric interaction could play a role in destabilising IIa by weakening the interactions between the charged N atom and Glu residue.

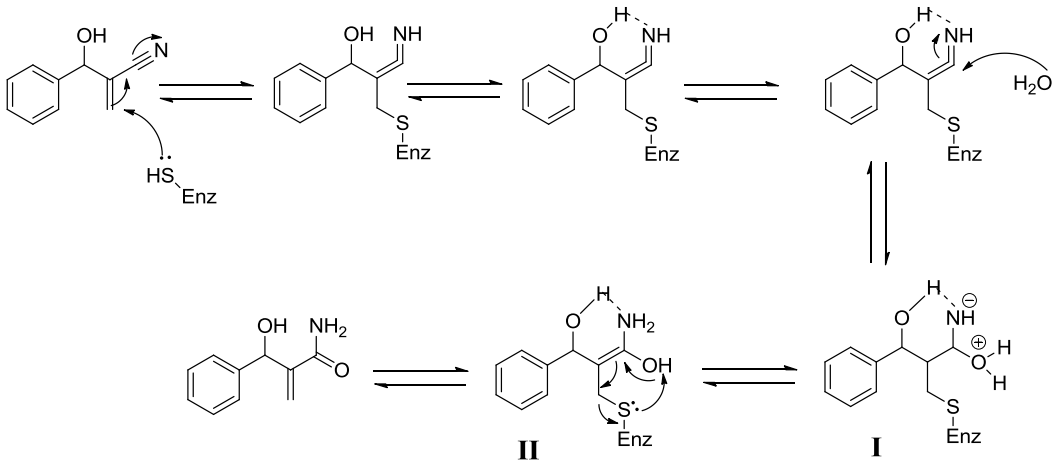
It should also be noted that the other amino acids present near the active site could also affect the amide to acid ratio. Sosedov *et al.* [53], discovered while examining *Pseudomonas fluorescens* EBC191 a Cys_{163} residue was present in the direct neighbourhood of the catalytically active Cys residue. They then changed this Cys residue with various amino acid residues which are present in other nitrilases at this position. The mutants were analysed towards 2-phenylpropionitrile, and changes were observed in their amide forming capacities. Especially for the exchange of Cys_{163} with glutamine or asparginine which resulted in significantly increased amounts of amides formed, this points to active participation of Glu as in Scheme 4.23. Whereas analine or serine residues at this position decreased the amounts of amide formed [53]. This indicates the importance of obtaining the crystal structure of the nitrilase of SET1, as amino acids in the vicinity of the catalytic cysteine residue may also promote amide formation of the substrate analogues.

Reversible thiol conjugate addition has been shown to be possible in activated acrylonitriles [54]. Iana *et al.* examined experiments showing that the addition of a nitrile group on the acidity of *α*C-H bond facilitates rapid elimination of thiol adducts at physiological pH and at the same time accelerates the rate of thiol addition [54]. The resulting electrophiles react with cysteine thiols as shown in Scheme 4.25.



Scheme 4.25: Conjugate addition reaction of *β*-mercaptoethanol with cinnamonitrile.

In the case of the hydrolysis of α-methylene-β-hydroxy nitriles in particular, it could be postulated that enzyme linkage to the unsaturated nitrile *via* a Michael type conjugate addition may be an alternative. This may be followed by addition of water and subsequent elimination of the thiol containing cysteine residue from the tetrahedral intermediate restoring unsaturation (Scheme 4.26). Alternative amino acid residues may facilitate the reversible conjugate addition process.



Scheme 4.26: Postulated mechanism for the formation of amide from α-methylene-β-hydroxy nitriles.

In addition, intra-molecular H bonding via 6 membered rings may facilitate this mechanism by stabilising such intermediates as I and II shown above.

In summary, by examining the hydrolysis of a range of α and β-substituted alkyl and aryl nitrile analogues, a mechanism for the formation of amide by this nitrilase has been postulated. This postulation is supported by the results observed when the nitrile analogues contained electron withdrawing functional groups at the α-position to the nitrile. The data within this study further supports previous postulations made by Mukerjhee *et al*. [48], Bruno *et al*. [37] and Osswald *et al*. [50].

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CHAPTER 5

CONCLUSIONS AND FUTURE WORK

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In summary, the work reported in this thesis described the identification of isolates with nitrile hydrolysing activity towards β-hydroxy nitriles. This was achieved through the development of a high-throughput screening strategy. The combination of techniques enabled isolates to be screened for their ability to transform aliphatic and aromatic nitriles along with dinitriles. The addition of a toxicity screen towards the various nitriles further focuses this approach. By attempting to grow the different isolates in rich medium in the presence of β-hydroxynitriles, the isolates that are sensitive to the nitrile and cannot grow in its presence were determined and excluded from further screening. The toxicity study identified 145 isolates tolerant towards 3-hydroxybutyronitrile, 107 towards 3-hydroxybutyronitrile and 65 capable of growing in the presence of 3-hydroxy-3phenylpropionitrile. Following a starvation process the isolates were then induced on the β-hydroxynitrile of interest as the sole N source. A high-throughput microscale colorimetric assay was developed to detect ammonia and this was adapted to a 96 well format to enable rapid screening. While investigating the use of alternative nitriles during the growth and induction stage it was found that the inducing nitrile can dramatically alter activity. Temperature for growth and also biotransformation is another parameter that also requires careful consideration in order to achieve a suitable degree of activity during the biotransformation. This is evident in the contrast between the optimum conditions required for cell growth and the optimum activity associated with the isolates.

In subsequent enantioselectivity screening of isolates demonstrating the desired activity towards 3–hydroxybutronitrile, an isolate demonstrating a highly enantioselective if not enantiospecific activity towards 3-hydroxybutyronitrile in particular, was identified. Consequently an efficient method for the production of (*S*)-3-hydroxybutyric acid from racemic 3-hydroxybutyronitrile was established using this novel *Rhodococcus erythropolis* strain SET1. It is of note also that, isolates with higher enantioselectivity generally did not display the highest activity, for example; SET1 demonstrated moderate activity towards 3-hydroxybutyronitrile, however it had the highest enantioselectivity. This highlights the importance of carefully selecting the activity specifications of isolates chosen for enantioselectivity screening.

The factors influencing the nitrilase activity and bioconversion of *R. erythropolis* SET1 were also examined. The temperature optimum for the biotransformation of 3-hydroxybutyronitrile was determined to be 25 °C, although higher temperatures resulted in higher activity, enantioselectivity was lost. This enzyme was also found to be stable over a broad pH range with enantioselectivity retained under acidic pH conditions, although a slight decrease in activity was observed. It was also found that this isolate is suitable for use in selected organo-aqueous media at selected ratios. The sensitivity of this isolate to thiol binding reagents agrees with proposed catalytic mechanisms for a nitrilase previously proposed by Thimann and Mahadevan *et al.* [1].

Further studies have shown that *R. erythropolis* SET1 catalysed the hydrolysis of a variety of structural analogues of 3-hydroxybutyronitrile to afford both acid and amide in high ee and yield in several cases, as discussed in Chapter 4. Key structural attributes which may influence the activity of the isolate have been determined. In the case of β-aryl analogues of 3-hydroxybutyronitrile, ee may be improved dramatically by the use of a docking strategy at the hydroxy position, in particular with a methyl group present. Consequently, it appears that protecting group size at this position also has an impact on ee values. In contrast, where a more sterically demanding group was present no acid was detected. It was also noted that the hydrolysis of β-aryl substituted nitriles is much slower than the corresponding β-alkyl analogues.

It is also consistently observed that where no electron withdrawing group is present at the α-position, acid is exclusively formed by suspected nitrilase activity. Although the enzyme accepted α- alkyl substituted analogues, the decreasing conversion of 2-phenylpropionitrile and 2-phenylbutyronitrile may be ascribed to the increasing bulk of the group present at the α–position. In contrast, in the case of α-methylene-β-hydroxy nitriles containing a conjugating group at the α-position, amides were produced with (*S*) configuration as the sole product. Ammonia release from the tetrahedral intermediate may be disfavoured with these substrates which may be due to hydrogen bonding [2]. To the best of our knowledge this is the first report of a nitrilase enzyme exhibiting enantioselective NHase activity towards β-hydroxy nitrile substrates bearing an α-methylene functionality. Similar trends for SET1 have also been observed as for other isolates discussed in the literature, which have demonstrated NHase activity. Our work adds to this knowledge, highlighting the potential of dual nitrilase/ NHase activity

which is highly structure dependant. Further studies of *R. erythropolis* SET1 towards the understanding of its enantioselectivity and NHase activity are required.

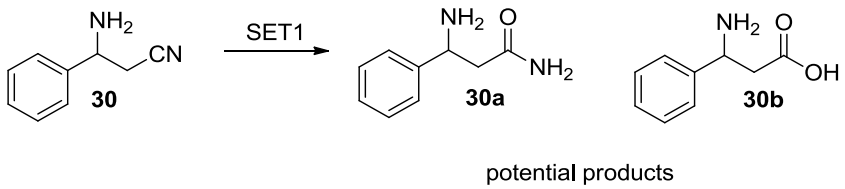
Ongoing work within the PMBRC involves attempts to isolate the enzyme of SET1. As eluded to in Chapter 3, when attempting to purify the desired enzyme from SET1 the development of degenerate primers for the 11 amino acid sequence obtained by peptide sequencing, did not prove successful. Therefore, in order to identify and subsequently characterise the enzyme fully, it is necessary to isolate the gene responsible for its production. Work is currently underway to obtain a whole genomic library representing the entire gene content of SET1.

To date a library of 2,000 *E. coli* clones each harbouring a fragment of the SET1 genome has been constructed for screening purposes. At present, there are two clones capable of utilising 3-hydroxyglutaronitrile as the sole nitrogen source. Following the identification of an *E. coli* clone containing a fosmid vector with the nitrilase gene inserted, the sequence of this gene will be determined. The gene will then be cloned into an *E. coli* expression vector for high level expression.

At present, the structure of the active enzymatic site of nitrilases has been defined, but a number of questions need to be addressed in order to understand this enzyme better and to facilitate its industrial applications. The relationship between substrate structure and enzymatic activity of the nitrilase of *R. erythropolis* SET1 could be investigated. Various techniques for determining protein structure include circular dichroism, which refers to the differential absorption of left and right handed circularly polarised light. In particular, UV circular dichroism is used to investigate the secondary structure of proteins. This technique has been reported in the characterisation of a nitrilase from *Fusarium solani* IMI96840 by Vejvoda *et al.* [3]. X-ray diffraction may be used to determine the crystal structure which would provide additional insight into the catalytic mechanism and determine key residues surrounding the binding site. A homology model could then be built based on the crystal structure of the enzyme and a docking simulation of the natural substrate from the spacial comparison mutations which may be performed by gene site saturation mutagenesis, as previously described by Brenner *et al*. [4]. These differences in structure may lead to significant diversity in catalysis of the hydration of the nitrile group.

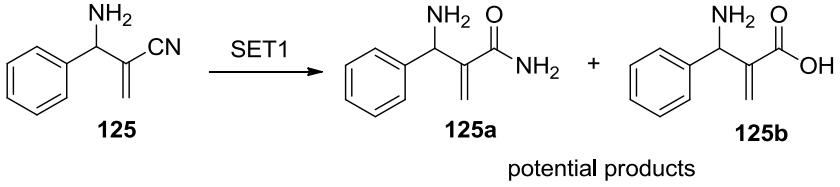
Possible future work arising from the results described in Chapter 4 would include assessing the functional group tolerance and mechanistic action of SET1 towards *β*-aminonitrile and cyanomethyl sulfoxide substrates. As mentioned in Chapter 1, *β*-amino acids and amides can be used as building blocks for the synthesis of pharmaceutical intermediates [5]. Trends observed for these substrates could be similar to those observed for *β*-hydroxy nitriles studied in the present work

This could be achieved by synthesising various aryl substituted, protected and unprotected 3-amino-3-phenylpropionitriles using methods previously reported by Chhiba *et al.* [5]. As discussed in Chapter 1, the hydrolysis of unprotected *β*-amino nitriles by nitrilase enzymes is quite rare (Scheme 5.1). Hence, the hydrolysis of *β*-amino substituted nitriles could be examined. N-protected *β*-substituted derivatives could potentially include *N*-methyl, *N*-acetyl, *N*-tosyl and *N*-boc protected 3-amino-3-phenylpropionitrile, to determine the role the protection group plays during the hyrolysis of *β*-amino nitriles. As observed for aromatic *β*-hydroxy nitriles, the size of the protecting group was important and O-methyl proved most efficient.



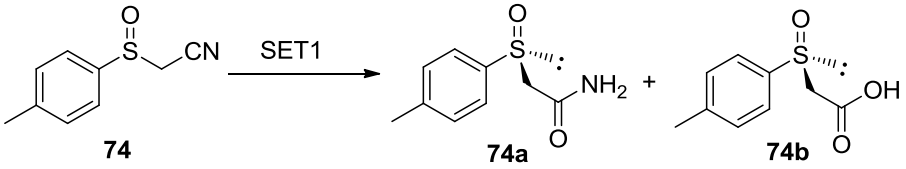
Scheme: 5.1: Biocatalytic conversion of *β*-aminonitriles to *β*-amino amides and acids.

The *α*-substituted, *β*–amino-*α*-methylene nitriles may also be prepared by the *Aza* Baylis Hillman reaction to investigate if amide is formed also when the *β*-amino group is present, to provide additional insight into the NHase behaviour of the suspected nitrilase within *R. erythropolis* SET1 (Scheme 5.2). Similar *β*-amino nitriles have been examined by Winkler *et al*. in the presence of NHase/ amidase containing isolates [6].



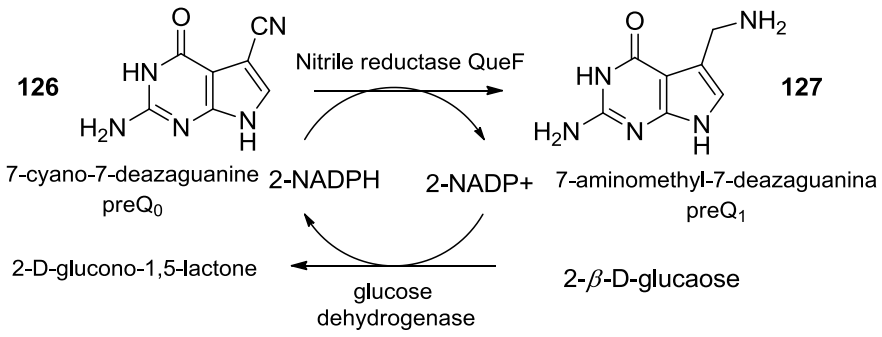
Scheme: 5.2: Biotransformation of *β*-amino-*α*–methyene nitriles to examine *β*-substituent effect on reaction outcome.

The nitrilase catalysed kinetic resolution of racemic cyanomethyl sulfoxide (Scheme 5.3) could also be examined. Kielbasinski *et al*. previously reported the synthesis and biotransformation of this nitrile and generated amide as a product along with acid [7]. This would allow a comparison to be made between *β*-hydroxy substituted and *β*-sulfinyl substituted analogues, again to evaluate potential amide generation.



Scheme: 5.3: Biocatalytic conversion of cyanomethyl sulfoxides.

In further work the novel isolate library may be screened to detect isolates containing enzymes capable of catalysing other types of nitrile metabolism; such as nitrile reductases and amidases capable of acyl transfer. Nitrile reduction can provide a useful method of access to amines that are ubiquitous in pharmaceutical compounds. Nitrile reductases have been shown to reduce nitrile groups to amines in the presence of NADPH (Scheme 5.4) [8-10]. The discovery of this enzyme class offers a new sustainable eco-friendly method for the synthesis of amines.

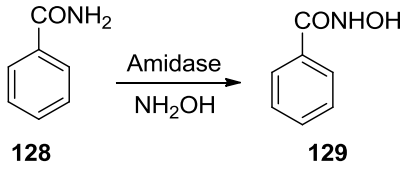


Scheme: 5.4:Enzymatic reduction of 2-amino-5-cyanopyrrolo[2,3-*d*]primidin-4-one (preQ$_0$) by the nitrile reductasse QueF, adapted from [8].

It may be possible to obtain / prepare a range of these enzymes by screening and identifying isolates within the library using a high through-put method operating in a 96-well plate format. Initial work would develop a colorimetric assay to detect amines, based on the use of TNBS 2,4,6-trinitrobenzene sulfonic acid, which upon reaction with primary amines forms a highly chromogenic product that can be readily measured by UV at 335nm [11, 12]. Screening of the isolates with both the natural substrate preQ$_0$ and various structural analogues; could potentially identify isolates capable of resolving

racemic mixtures of nitriles *via* the selective reduction of one enantiomer. To date, the wild type nitrile reductases have displayed limited activity towards nitriles other than the natural substrate (examples nitrile reductase from *G. kaustophilus* and EcoNR from *Escherichia coli* K-12) [13]. However in the case of a homology model prepared of EcoNR, polar amino acid residues in the active site of EcoNR, may help to accommodate polar compounds in the active site and aid the alignment of $preQ_0$ correctly for reduction [13].

Beside amide hydrolysis, amidases have also been used for acyl-transfer from amides onto nucleophiles other than water, which makes use of the same type of enzymes but a differential acyl acceptor (hydroxylamine) (Scheme 5.5).



Scheme: 5.5: Enzymatic synthesis of hydroxyamic acids by isolates with amidase activity.

The acyl transfer from amides onto hydroxylamine can be detected by colorimetric enzyme assays, as hydroxyamic acids can be quantified after complexation with ferric ions when they form a UV active compound [14]. During the screening studies reported in this thesis, the Nesslers assay identified a series of nitrile hydrolysing isolates and some of these may contain NHase/amidase systems. Further screening of these isolates may identify amidases capable of such acyl transfer.

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CHAPTER 6

EXPERIMENTAL

6.1 GENERAL EXPERIMENTAL CONDITIONS

All commercial chemicals were obtained from Sigma-Aldrich and were used as received unless otherwise stated. Nuclear magnetic resonance spectroscopy (NMR) spectra were recorded on a Joel ECX-400 spectrometer operating at 400 MHz, using deuterochloroform as the solvent with tetramethylsilane as an internal reference (δ TMS = 0.0) unless otherwise stated. The following abbreviations (and combinations thereof) have been used to describe the signal multiplicity: b-broad, s- singlet, d- doublet, q-quartet, and m- multiplet.

All infra-red spectra were recorded on a Shimadzu FTIR-8400s, melting points were determined using a Stuart Scientific melting point apparatus smp3, compounds were weighed out on an Explorer OHAUS analytical balance, GC-MS analysis was carried out Varian Saturn 2000 GC/MS/MS. Optical rotations were measured on an AA series polar 20 automatic polarimeter.

Thin layer chromatography was performed using silica coated plastic plates (60 F_{254}) supplied by Merck. They were visualized using ultra violet radiation or developed in potassium permanganate solution or iodine. Flash column chromatography was performed using flash silica (230-400 mesh) 9385 supplied by Merck.

Preparative TLC was carried out on glass plates pre-coated with silica 60 PF254 (1.00748) supplied by Merck. Chiral HPLC was conducted on a HP 1050 HPLC using a chiral column (Daicel Chiralpak AD-H, OJ-H or IA columns). Chromatographic protein purification procedures were performed using an AKTA Explorer 100 (GE Healthcare) fast-performance liquid chromatography (FPLC) system controlled by the Unicorn software.

The organic layers were routinely dried over anhydrous magnesium sulphate and they were concentrated by rotary evaporation. The last traces of solvent were removed on a high vacuum pump. Solvents were purchased pre-dried.

6.2 DEVELOPMENT OF A HIGH-THROUGHPUT SCREENING STRATEGY TO IDENTIFY NOVEL NITRILE HYDROLYSING ENZYMES

6.2.1 IDENTIFICATION OF NITRILE HYDROLYSING ISOLATES

a. Bacterial isolates

The 254 bacterial isolates used in this study are those described in Coffey *et al* [1]. All cultures were maintained in glycerol stocks stored at -80 °C.

b. Nitrile Toxicity studies

96 well Megablock® plates (Sarstedt Ltd) containing 250 µl LB broth (Merck) and 10 mM *β*-hydroxy nitrile, were inoculated from glycerol stocks of the isolate library and incubated at 25 °C with constant agitation (250 rpm) for 72 hours. 5 µL of these cultures were then transferred in a 96 well format to M9 agar [2], [3] (Merck agar-agar ultrapure (15 % w/v) prepared in M9-media), containing *β*-hydroxy nitrile (10 mM) and incubated at 25 °C for 72 hours.

c. Preparation of M9-minimal media

Preparation 5X M-9 basis*:* To a solution of sodium dihydrogen ortho-phosphate dodecahydrate (85.33 g, 240 mmol) in deionised water (1000 mL), was added potassium dihydrogen ortho-phosphate (15g, 110 mmol), and sodium chloride (2.5 g, 62.5 mmol), the white suspension was stirred at room temperature until the solution was fully dissolved, and autoclaved at 121 °C for 15 minutes.

Preparation of Trace elements: Zinc sulphate heptahydrate (29 mg, 0.1 mmol), manganese chloride tetrahydrate (198 mg, 1 mmol), cobalt chloride hexahydrate (254 mg, 1 mmol), copper chloride dihydrate (17 mg, 0.1 mmol) and calcium chloride dihydrate(147 mg, 1 mmol) were dissolved to 100 mL with deionised water and autoclaved at 121 °C for 15 minutes.

Preparation of 20 % (w/v) glucose: 20 g of glucose was dissolved in 100 mL of deioinsed water and autoclaved at 121 °C for 15 minutes.

Preparation 0f 0.1M calcium chloride dihydrate***:*** calcium chloride dihydrate (1.43 g, 100 mmol) was dissolved in 100 mL of deionised water and autoclaved at 121°C for 15 minutes.

Preparation of magnesium sulphate heptahydrate: magnesium sulphate heptahydrate (24.65 g, 100 mmol) was dissolved in 100 mL of deionised water, prepared freshly and filtered through 0.45 μm filter.

Preparation of ferrous sulphate heptahydrate: ferrous sulphate heptahydrate (140 mg, 50 mmol) was dissolved in 100 mL of deionised water and autoclaved at 121 °C for 15 minutes.

Preparation of M-9 minimal media: to M-9 basis (20 mL) was added trace elements (100 μL), ferrous sulphate heptahydrate (100 μL), glucose (2 mL), calcium choride dehydrate (100 μL) and magnesium sulphate heptahydrate (100 μL) the total volume was made up to 100 mL using deionised water. Media was prepared under aseptic conditions.

d. Preparation of potassium phosphate buffer

Potassium phosphate buffer was prepared by dissolving monobasic potassium phosphate (5.3 g) and dibasic potassium phosphate (10.6 g) in deionised water (1000 mL). The pH was adjusted to 7 using 2 M NaOH. This solution was sterilised by autoclaving at 121 °C for 15 minutes.

e. Optical density of isolates

The optical density of all cell cultures was measured using the NanoDrop® spectrophotometer ND1000. The volume required to produce a suspension of each isolate with an optical density of 1 (OD_{600nm} = 1) was calculated. The required volume was transferred to an Eppendorf tube and centrifuged at 13,000 *g* for 10 minutes. The supernatant was removed and the remaining cells resuspended in the desired volume of phosphate buffer.

f. Nitrogen Starvation

Successful isolates from 6.2.1 b were inoculated into 250 µl M9 minimal media broth [2], [3] containing 10 mM *β*-hydroxynitrile as the sole source of N. Each well was then inoculated into M9-agar containing *β*–hydroxynitrile (10 mM) before incubation at 25 °C with orbital shaking at 250 rpm for 24 hours.

g. Induction of Nitrile Metabolising Enzyme Activity

96-well Megablocks® containing 500 µL of M9 minimal media broth [2], [3] [4] and *β*-hydroxy nitrile (10 mM) were inoculated with nitrogen starved isolates (20 µL) from 6.1.1 f, and incubated with shaking for 72 hours at 25 °C and 250 rpm. 500 µL of 50 % glycerol solution was then added to each culture before storage at -80 °C. These cultures of induced isolates served as inoculation and activity screening stocks for subsequent analyses.

h. An investigation of temperature on the growth of various isolates

96-well Megablocks® containing 500 µL of M9 broth and *β*-hydroxy nitrile (10 mM) were inoculated with nitrile induced isolates (20 µL) from 6.1.1 g and incubated with shaking for 72 hours at 15 °C, 20 °C, 25 °C and 30 °C at 250 rpm. The OD_{600nm} was measured and recorded for future studies.

i. Standard curve for Nesslers colorimetric assay

Ammonium chloride (100 mM, 5.35 g) was dissolved in deionised water (1000 mL). This solution was used as a stock to prepare standards with the following concentrations; 1 mM, 2 mM, 3 mM, 4 mM and 5 mM. The absorbance was then measured at 425 nm. At NH_3 concentrations of up to 10 mmol/L, the $A_{425\ nm}$ was directly proportional to NH_3 concentration (R^2= 0.9992).

j. Nesslers Microscale Ammonia Assay

Induced isolates from 6.1.1g were initially screened for activity towards *β*-hydroxynitriles using the Nesslers colorimetric assay [5], in 96-well microtitre plates (Sarstedt Ltd). Fresh cultures of each isolate were grown in M9-minimal media containing 10 mM nitrile (before washing 3 times with 500 µL of phosphate buffer). Each 150 µl reaction contained 10 mM nitrile and cells (OD_{600nm}=0.1) in potassium phosphate buffer (100 mM, pH 7.0). Microtitre plates were sealed using adhesive film (Sarstedt) and incubated at 25 °C at 250 rpm for 24 hours. The reaction was then quenched by adding 37.5 µL of 250 mM HCl. Plates were centrifuged at 500 *g* for 10 minutes to pellet the cell debris. 20 µl of the supernatant was transferred to a microtitre plate, 181 µL of assay mastermix was added (155 µL deionised water, 1 µL 10N NaOH, 25 µL Nesslers reagent (Merck)). The reaction was allowed to stand for 10 minutes and

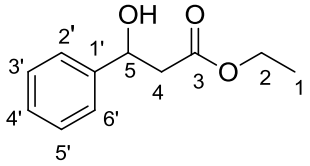
then the absorbance read at 425 nm. Cell blanks contained cells @ OD_{600nm}=0.1 in phosphate buffer. Nitrile blanks contained 150 μL of 10 mM nitrile in phosphate buffer.

k. Alternative induction of nitrile metabolising isolates

The use of alternative nitriles during the induction stage was investigated by attempting to grow the isolates in M9-minimal media containing 10 mM of the alternative inducing nitrile. The isolates activity was then examined using Nesslers colourimetric assay as described in 6.1.1 j.

6.2.2 SYNTHESIS OF STANDARDS FOR CHIRAL HPLC METHOD DEVELOPMENT

(*R,S*)-Ethyl-3-hydroxy-3-phenylpropanoate 91



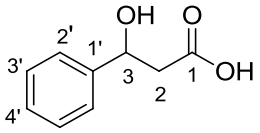
Ethyl benzoylacetate (10.4 mmol, 2 g) was dissolved in ethanol (20 mL) and to this stirred solution was added dropwise a solution of sodium borohydride (12mmol, 0.454 g) dissolved in ice water (6 mL). The progress of the reaction was monitored by TLC. The mixture was slowly quenched after 40 minutes by the addition of 40 mL of water containing 4 mL of conc hydrochloric acid. The reaction mixture was extracted with ethyl acetate (3 × 40 mL), the combined ethyl acetate organics were washed with saturated sodium chloride, dried with $MgSO_4$, filtered and solvent removed *in vacuo*. Purification by silica gel column chromatography using a mobile phase of 8 % ethyl acetate in dichloromethane, yielded the title compound as clear oil (66 %).

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.24-7.37(5H, m, Ar), 5.10-5.13 (1H, m, C-5), 4.14-4.19 (q, J= 9.7, 2H, C-2), 3.25-3.26 (d, J= 8.6,1H, C-4), = 2.70-2.74 (2H, m, C-4), 1.23 -1.27 (t, J= 8.6, 3H, C-3).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 170.5 (C-3), 143.6 (C-1’), 128.9 (C-3’ and C-5’), 127.6 (C-4’), 127.1 (C-2’ and C-6’), 71.2 (C-5), 62.3 (C-2), 51.3 (C-4), 14.1 (C-1)

GCMS- (EI), retention time = 8.643 min, m/z = 194, 177, bp = 135, 105, 77.

(*R*,*S*)- 3-hydroxy-3-phenylpropionic acid 38a [6]



To a solution of ethyl-3-hydroxy-3-phenylpropionate (0.5 g , 2.5 mmol) in ethanol (6.25 mL) was added aqueous potassium hydroxide (0.7 g in 1.25 mL of water). The reaction mixture was stirred overnight at room temperature and monitored by TLC. After this time the yellow coloured reaction mixture was added to water (6.25 mL) and acidified by dropwise addition of concentrated hydrochloric acid. The aqueous reaction media was extracted with ethyl acetate (3 × 25 mL), the organic layer washed with saturated sodium chloride, dried over anhydrous magnesium sulphate and the solvent removed *in vacuo*. The crude product was purified by dissolving the solid in ethyl acetate and washed with 2M sodium hydroxide (2 × 30 mL) the aqueous layer was washed with ethyl acetate (3 × 30 mL). The aqueous layer was then acidified to pH 2 with 2M HCl and extracted with ethyl acetate (3 × 30 mL), washed with saturated sodium chloride, dried over anhydrous magnesium sulphate and the solvent removed *in vacuo* to yield the title product (58 %) as a white solid. mp= 95-96 °C.

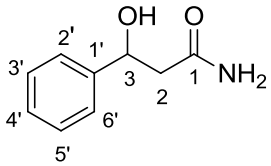
^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.31 (5H, m, Ar), 5.13 (dd, *J*=9.2 Hz, *J*=3.6 Hz, 1H, H-3), 2.84 (dd, *J*=16.7 Hz, *J*= 9.1 Hz, 1H, H-2), 2.76 (dd, *J*=16.7 Hz, *J*= 3.6 Hz, 1H, H-2).

^{13}C NMR (100 MHz, CDCl3), δ = 177.3 (C-1), 143.8 (C-1’), 128.9 (C-4’), 127.6 (C-3’), 127.1 (C-2’), 70.9 (C-3), 45.3 (C-2).

MS Electrospray low res: $M+H^+$ = 167.1, $M+Na^+$ = 189

GCMS (EI) retention time = 8.828 minutes, m/z = 166, 149 (100 %), 131, 107, 77

(*R,S*)- 3-hydroxy-3-phenylpropionamide 38b



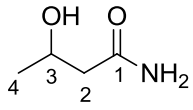
To a stirred solution of 3-hydroxy-3-phenylpropionitrile (1 mmol, 0.147g, 132 μL) in dimethylsulfoxide (300 μL) cooled in an ice bath, was added 30 % hydrogen peroxide (120 μL) and anhydrous potassium carbonate (0.02g). The reaction was stirred for 15 minutes at room temperature and quenched with water (5 mL). The aqueous portion was extracted with dichloromethane (3×5 mL) dried over anhydrous magnesium sulphate and the solvent removed *in vacuo* to yield the crude product. The residue was purified by preparative thin layer chromatography (2:1 petroleum ether: acetone), and the amide extracted from the silica using a mixture of dichloromethane : methanol (95:5) and solvent removed *in vacuo* to yield the pure product.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.15-7.35 (m, 5H, Ar), δ = 6.82 (br, 1H, NH_2), δ = 5.38 (1H, s, br, OH), δ = 4.91-4.96 (m, 1H, H-3), δ = 2.41 (dd, J= 14.3, 1H, H-2), δ = 2.29 (dd, J= 14.3, 1H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 174.3 (C-1), 143.8 (C-1’), 129.4 (C-2’), 128.9 (C-6’), 127.6 (C-3’), 127.1 (C-5’), 71.0 (C-3), 51.6 (C-2).

MS Electrospray low res: $M+Na^+$ = 188.2

(*R,S*)- 3-hydroxybutyramide 89b



To a stirred solution of 3-hydroxybutyronitrile (2 mmol, 0.170 g, 178 μL) in dimethyl-sulfoxide (600 μL) cooled in an ice bath, was added 30 % hydrogen peroxide (240 μL) and anhydrous potassium carbonate (40 mg). The reaction was monitored by TLC and quenched after 15 minutes by the addition of 5 mL of water. The aqueous portion was extracted with dichloromethane (3× 5 mL) dried over anhydrous magnesium sulphate, solvent removed *in vacuo*.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 4.60 (s, 1H), 3.91 (m, 1H, H-3), 2.13- 2.21 (dd, 1H, H-2), 2.03-2.10 (dd, 1H, H-2), 1.04- 1.10 (d, 3H, H-4),

^{13}C NMR (100 MHz, $CDCl_3$), δ = 174.3, 143.8, 129.4, 128.9, 127.6, 127.1, 71.0, 51.6.

MS Electrospray low res: $M+Na^+$ = 134.7

6.2.3 ENANTIOSELECTIVITY SCREENING

Chiralcel AD-H and OJ-H columns (all from Daicel Chemical Industries) were used for chiral analysis. Chiralcel AD-H was used for the resolution of *β*-hydroxyacids. Analytical conditions applied: 90 % hexane, 10 % IPA and 0.1 % TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm. Chiralcel OJ-H was used for the resolution of *β*-hydroxyamides and nitriles using the same mobile phase conditions with the exception of TFA. The biotransformation products of 3-hydroxybutyronitrile were first derivatised to their corresponding *β*–benzyloxyethers before analysis.

General procedure for enantioselectivity screening towards 3-hydroxy3-phenylpropionitrile

The biotransformation was carried out in a suspension of phosphate buffer (0.1M, pH 7.0) containing induced cells (OD_{600nm} = 1). The cells were activated by incubation at 25 ^{O}C for 30 minutes with mechanical shaking. Racemic nitrile (10 mM) was added in one portion to the flask and the mixture incubated at 25 °C with mechanical shaking (250 rpm) for 24 hours. The reaction was quenched by removing the biomass through centrifugation. The resulting aqueous solution was acidified to pH 2 with aqueous HCl (2M). Extraction with ethyl acetate gave, after drying over anhydrous $MgSO_4$, removal of the solvent under vacuum, the crude products and the remaining nitrile. All experiments were performed in triplicate. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase. % enantiomeric excess is calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

General procedure for enantioselectivity screening towards 3-hydroxybutyronitrile

Racemic nitrile (5.1 mg, 5.9μL, 10 mM) was added in one portion to a solution of potassium phosphate buffer (0.1M, pH= 7.2, 6 mL) containing induced cells (OD_{600nm}

=1), and activated at 25 ° C for 30 minutes with orbital shaking (250 rpm). The reaction was quenched after 24 hours by removal of the biomass by centrifugation at 3,000 g. The resulting aqueous solution was acidified by the addition of 1M HCl (200 μL). The aqueous portion was then extracted with ethyl acetate, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. Silver oxide (1 equiv, 0.06 mmol, 13.6 mg), benzylbromide (4 equiv, 0.24 mmol, 28 μL) and dichloromethane (2 mL) were added and the mixture stirred in the dark for 24 hours. The reaction mixture was diluted with acetone and filtered through a 0.45 μm filter and solvent was removed under vacuum. 1 mL of mobile phase (90 % hexane : 10 % IPA) was added before the solution was injected on the Chiral HPLC system. All experiments were performed in triplicate. % enantiomeric excess is calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

General procedure for large scale biotransformation towards 3-hydroxybutyronitrile

The procedure for large scale biotransformation of racemic 3-hydroxybutyronitrile was similar to the general procedure, with the exception of the use of potassium phosphate buffer (0.1M, pH 7.0, 100 mL) containing 3-hydroxybutyronitrile (85.1 mg, 10 mM). The resulting aqueous solution was basified to pH 12 with aqueous NaOH (2M) and extracted with ethyl acetate (3×100 mL). The aqueous solution was acidified using aqueous HCl (2M) to pH 2 and extracted with ethyl acetate (3×100 mL), dried over $MgSO_4$ and the solvent removed under vacuum. The crude product was subjected to silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (1:1) to give 3-hydroxybutyric acid in 42 % yield (44 mg, 4.23 mmol) as clear oil. The configuration of the corresponding acid was determined by comparing the direction of specific rotation with that of an authentic sample. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase and correlated with literature. (*R*)-enantiomer elutes at 11.94 min, (*S*)-enantiomer elutes at 12.34 min [7]. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.19 – 4.27 (1H, m), δ = 2.45 – 2.58 (2H, m), δ = 1.23 (3H, d, *J*=6.3Hz). ^{13}C NMR (400 MHz, $CDCl_3$) δ = 117, 76, 64, 42, 22 $[\alpha]^{d}_{25}$ = + 4.0 (c = 2.5, MeOH), and compared with that in the literature $[\alpha]^{d}_{25}$= + 4.1 (c = 2.7, MeOH) [8]. This experiment was performed in triplicate.

6.3 ISOLATE EVALUATION AND ATTEMPTED PROTEIN PURIFICATION

6.3.1 DETERMINATION OF OPTIMUM BIOCATALYTIC REACTION PARAMETERS

a. Enzyme assay

Enzyme activity at the various reaction conditions was monitored using the Nesslers microscale colorimetric assay as previously described [3].

b. Temperature studies

Nitrilase activities towards 3-hydroxybutyronitrile (10 mM) was assayed as described above using whole cells (SET1, OD_{600nm} = 1) in KH_2PO_4 (100 mM, pH 7.0). The hydrolysis was performed at 0 °C, 10 °C, 20 °C, 30 °C, and 40 °C. Chiral HPLC analysis was used to measure enantiomeric excess.

c. Influence of pH

Nitrilase activities towards 3-hydroxybutyronitrile (10 mM) was assayed as described above using whole cells (SET1, OD_{600nm} = 1) in KH_2PO_4 (100 mM,). The hydrolysis was performed pH 4, 5, 7, 9 and 10 at 25 °C. Chiral HPLC analysis was used to measure enantiomeric excess.

d. Inhibitors, metal ions and other reagents

The effects of various inorganic compounds ($CuSO_4.5H_2O$, $FeCl_3.\ 6H_2O$, $Pb(CH_3COO)_2.3H_2O$, $FeSO_4.7H_2O$, $AgNO_3$, $HgCl_2$, $Cu_2SO_4.5H_20$, $MnSO_4.4H_2O$, EDTA, $MgSO_4.7H_2O$, $CaCl_2.6H_2O$, $NiSO_4.6H_2O$ and $ZnSO_4.7H_2O$) were examined. Samples containing the whole cells of *Rhodococcus erythropolis* SET1 and 3-hydroxybutyronitrile (10 mM) were incubated with the above reagents (1-5 mM) at 25 °C for 24 hours. The standard activity was determined using the Nesslers colorimetric assay, along with cell blanks and metal ion blanks.

e. Solvent tolerance

Whole cells of *R. erythropolis* SET1 were incubated with 3-hydroxybutyronitrile dissolved in aqueous buffer with various additional co-solvents at quantities between 5 and 50 % (v/v). The reaction mixture was incubated at 25 °C for 24 hours and enzyme activity was monitored using the technique of Nesslerisation, along with cell blanks and solvent blanks.

f. Effect of substrate concentration

The substrate concentration dependence of SET1 was examined in the range of 0.01 to 0.1 M of 3-hydroxybutyronitrile. The reactions were performed by incubating SET1 in a solution of 3-hydroxybutyronitrile for 24 hours at 25 °C. Following biomass removal and acid base extractive work up, the crude products were purified by preparative TLC with a 50.50 mixture of hexane and ethyl acetate. Products were verified by their spectroscopic data including ^{1}H and ^{13}C NMR. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase and correlated with literature data [3].

6.3.2 NITRILASE PURIFICATION OF R. ERYTHROPOLIS SET1

a. *Microorganism and culture conditions.*

R. erythropolis SET1 which was previously isolated from soil was used as the source of the enzyme for the purification. SET 1 was inoculated into 500 mL of nitrogen-free M9 media (in a 1 L Erlenmeyer flask) containing 3-hydroxybutyronitrile (10 mM) as the sole nitrogen source. The culture was incubated at 25 °C with reciprocal shaking at 250 rpm for 72 hours, after which the cells were collected by centrifugation (4,000g, at 4 °C for 30 minutes).

b. *Purification of nitrilase*

All purification procedures were performed at 0 to 5 °C. The buffer solution used through-out was 100 mM potassium phosphate buffer, pH 7.0.

c. *Preparation of a cell free extract*

Enzymatic lysis

The harvested cells were re-suspended in sterile distilled water and transferred in 1 mL aliquots to microfuge tubes for centrifugation at 12,000 *g*. The wet weight of the cell pellet was determined. BugBuster® Master Mix (Novagen) (10 mL) was added to approximately 1.2 g wet weight of *Rhodococcus erythropolis* SET1 [9]. Gentle vortexing was carried out to resuspend the cell pellet. The cell suspension was incubated on a shaking platform for 1 hour. The insoluble cell debris was removed by centrifugation at 16,000 *g* for 20 minutes at 4 °C.

In order to remove the BugBuster from the cell lysate, dialysis was performed in potassium phosphate buffer (100mM pH=7), using a Float-A-Lyzer G2 with a 20kDa cutoff. The Float-A-Lyzer G2 was first activated by submerging the device in deionised water for 30 minutes, followed by soaking in potassium phosphate buffer. The Float-A-Lyzer was placed vertically in the dialysate reservoir (potassium phosphate buffer 100mM pH=7), the stirring rate was adjusted to form a gentle rotating current. The sample was dialysed at 4 °C over the course of 24 hours with 5 buffer changes at 1, 3, 5, 8 and 20 hours. The dialysed cell suspension was then subjected to incubation with 3-hydroxybutyronitrile for 24 hours. Following extractive work up and purification, 3-hydroxybutyronitrile was recovered unchanged.

Sonication

Cells were harvested from the liquid culture 6.2.2 c by centrifugation at 4,000 *g* for 20 minutes. The cell pellet was washed with phosphate buffer (2 x 10 ml) and then re-suspended in sterile distilled water (5 mL). The cell suspension was then subjected to sonication using a SNE Soniprep 150 with a 1 cm probe at 14,000 Hz for 5 second blasts for a total of 6 minutes while being stored on ice. Following sonication the crude cell lysate was centrifuged at 20,000 *g* for 20 minutes at 4 °C.

d. *General Procedure for Sodium- dodecylsulphate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Preparation of 12 % SDS-PAGE resolving gel.

Acrylonitrile 40 % - 3 mL

Molecular grade water – 4.5 mL

Buffer B – 2.5 mL (150 mL Tris HCl pH 8.8, 8 mL 10 % SDS, H_2O 42 mL)

Ammonium per sulphate (10 %) – 50 μL

Tetremethyl-ethylenediamine (TEMED) - 10 μL

Preparation of 5 % SDS-PAGE stacking gel.

Acrylonitrile 40 % - 0.5 mL

Molecular grade water – 2.5 mL

Buffer C – 1 mL (100 mL Tris HCl pH 6.8, 8 mL 10 % SDS, H_2O 92 mL)

Ammonium per sulphate (10 %) – 30 μL

Tetremethyl-ethylenediamine (TEMED) - 10 μL

Running buffer

1 in 10 dilution of (30.3 g Tris, 142 g glycine, 10 g SDS)

Instrument settings

170 volts for 70 minutes

Staining buffer

0.1 % w/v Coomassie Brilliant Blue R-250, 45 % v/v methanol, 10 % v/v acetic acid

Destaining buffer

Methanol (100 mL), glacial acetic acid (100 mL) and H_2O (800 mL).

e. SDS-PAGE

Rhodococcus erythropolis SET1 cells were harvested and lysed using the methods as described above. Samples were loaded in SDS-loading buffer. Analytes were then run on 12 % w/v acrylamide gels with a 5 % w/v stacking gel (37:1 acrylamide to bis-acrylamide) at a constant 170 volts for 70 minutes. Staining was performed with a standard Coomassie stain (0.1 % w/v Coomassie Brilliant Blue R-250, 20 % v/v methanol, 10 % v/v acetic acid) for 2 hours and de-stained for approximately 4 hours in de-staining solution (10 % methanol, 10 % acetic acid, 80 % H_2O) with constant agitation. Gels were photographed on a standard desktop scanner.

f. Ammonium sulphate precipitation

Rhodococcus erythropolis SET1 cells were harvested and lysed using the methods as described above. The total volume of the protein solution was 5 mL. Solid ammonium sulphate was added to the resulting supernatant solution to give 20 % saturation. After

being stirred on ice for 2 hours, the precipitate was removed by centrifugation, and ammonium sulphate was added to the supernatant solution to give 30 % saturation, and the prior steps repeated for up to 60 % saturation. Removal of ammonium sulphate was carried out by resuspending the pellets in 400 μL of distilled water and concentrating the protein using microcentrifuge filters nominal molecular weight limit (NMWL), 10,000, PLGC. This was repeated 3 times and the final sample resuspended in 100 μL of water.

Chromatography

Chromatography experiments were performed using an AKTA Explorer 100 (GE Healthcare) fast-performance liquid chromatography (FPLC) system controlled by the Unicorn software.

g. Hydrophobic interaction chromatography Phenyl sepharose CL-4B

R. erythropolis SET1 cells were harvested and lysed using the methods as described previously. The protein solution was concentrated using a microcentrifuge tube with a 10 kDa cut off and resuspended in 5 mL of 2 M NaCl in 10 mM HEPES. The column chosen contained a phenyl sepharose stationary phase. The column was equilibrated with 2 M NaCl in 10 mM HEPES buffered at pH 7. The system was run using a gradient elution in which the first 25 minutes involved pumping 2 M NaCl in 10 mM HEPES, the concentration was then decreased to 10 mM HEPES over 40 minutes followed by a further 25 minutes isocratic elution using 2 M NaCl in 10 mM HEPES. Proteins were detected at 280 nm and collected in 4 mL fractions. In order to determine where the protein eluted, both SDS-PAGE and the Nesslers colorimetric activity assay were used to examine each fraction.

h. Gel filtration chromatography Sephacryl S-200

R. erythropolis SET1 cells were harvested and lysed using the methods as described previously. The protein solution was concentrated using a microcentrifuge tube with a 10 kDa cut off and resuspended in 5 mL of 2 M NaCl in 10 mM HEPES (pH 7). The superdex 200 prep grade column was equilibrated with 2 M NaCl in 10 mM HEPES buffered at pH 7. The crude cell extract was injected onto the superdex 200 prep grade column and eluted with 2 M NaCl in 10 mM HEPES at a flow rate of 1 mL/minute for 2 hours. Proteins were detected at 280 nm and collected in 4 mL fractions. In order to

determine where the protein eluted, both SDS-PAGE and the Nesslers colorimetric activity assay were used to examine each fraction as previously described.

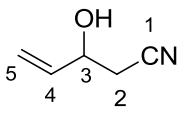
l. Protein identification using trypsin

Rhodococcus erythropolis SET1 was grown and prepared as a cell free extract as described above. SDS-PAGE was performed on the crude cell lysate as previously reported. The protein band of the crude enzyme was manually excised from SDS polyacrylamide gel. Protein spots were cut into 1 mm × 1 mm gel slices, and transferred to a clean 1 mL silanised micro-centrifuge tube. Colloidal Coomassie blue stain was removed by the addition of 100 μL of 100 mM NH_4HCO_3 and 60 μL of acetonitrile. The tube was shaken for 30 minutes and the liquid was removed by aspiration. This wash step was repeated three times. The protein gel was then dehydrated by the addition of 50 μL of acetonitrile, and incubated at room temperature for 5 minutes. The liquid was removed and the dehydration step repeated. The gel slices were dried in a centrifugal evaporator for 15 minutes and were preincubated in 25 μL of 20 μg/ml trypsin solution, on ice for 30 minutes. NH_4HCO_3 (30 μL, 50 mM) was then added to cover the gel. The digestion mixture was then incubated at 37 °C overnight. The treated gel was lyophilised and the tryptic peptide fragments analysed using a MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) and identified based on peptide LIFT spectra using MS/MS ion search of Mascot programme (Matrix Science)

6.4 AN INVESTIGATION INTO THE SUBSTRATE SCOPE OF *R. ERYTHROPOLIS* SET1

6.4.1 PREPARATION OF STARTING NITRILES

(R/S)-3-hydroxy-pent-4-enenitrile 95 [10]



A solution of anhydrous acetonitrile (1.2 equiv, 42 mmol, 1.02 g) in anhydrous THF (20 mL) was added *via* cannulation, under a nitrogen atmosphere to a solution of lithium diisopropylamide (1.2 equiv, 42 mmol, 23 mL) prepared from a 2M stock solution in THF. The resulting solution was stirred at -10 °C for 30 minutes and allowed to reach room temperature and stirred for a further 30 minutes. A solution of acroelin (1 equiv, 35.6 mmol, 2 g) in anhydrous THF (10 mL) was added *via* cannulation. The reaction

mixture was allowed to stir for 2 hours before being quenched by the addition of saturated NH_4Cl (2 mL) and adjusted to pH 3 using 2M HCl. The mixture was extracted using diethyl ether (3 × 50 mL). After drying over anhydrous $MgSO_4$ and removal of the solvent *in vacuo*, column chromatography using silica gel (eluted with a mixture of hexane and ethyl acetate (60:40) gave the desired product as a brown oil (1.10 g, 32 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 9.03 min, t_2 = 9.40 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 5.90(dt, *J*=2.8 Hz, 1H, H-4), 5.40 (dd, *J*= 2.6 Hz, 1H, H-5'), 5.31(dd, J=2.5 Hz, 1H, H-5), 4.45-4.38 (m, 1H, H-3), 2.64-2.63 (m, 2H, H-2).

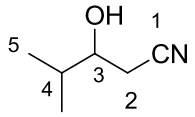
^{13}C NMR (100 MHz, $CDCl_3$), δ = 137.24 (C-4), 117.7 (C-1), 116.2 (C-5), 68.64 (C-3), 25.9 (C-2).

MS Electrospray low res: $M+Na^+$ = 120.4

General procedure for the reduction of *β*-ketonitriles

The *β*-hydroxynitriles **38, 67, 68, 69, 70, 96** and **97** were prepared by the following reduction procedure. Sodium borohydride (3.3 equiv) was added at 0 °C to a solution of *β*-ketonitrile (1 equiv) in ethanol (10 mL). The reaction mixture was allowed to reach room temperature and monitored by TLC. The mixture was quenched using 5 % HCl and extracted with ethyl acetate (3 × 30 mL). The organic layer was dried over anhydrous $MgSO_4$ and the solvent removed under vacuum to afford the crude product. The residues were subjected to silica gel column chromatography using a mobile phase of hexane: ethyl acetate (50/50). All products were characterized by ^{1}H and ^{13}C NMR, and comparison of the spectral data and melting points with that reported in the literature [11]

(*R*,*S*)- 3-hydroxy-4-methylpentanenitrile 96 [10]



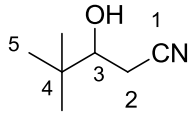
The crude product was purified to give the title compound as colourless oil (0.76 g, 59 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 10.8 min, t_2 = 12.7 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 3.70-3.66 (m, 1H, H-3), 2.58-2.43 (m, 2H, H-2, H-2'), 2.23 (s, 1H, Br, OH), 1.85-1.75 (m, 1H, H-4), 0.96 (d, *J*= 6.8 Hz, 3H, H-5), 0.93 (d, *J*= 6.8 Hz, 3H, H-5').

^{13}C NMR (100 MHz, $CDCl_3$), δ = 117.1 (C-1), 73.9 (C-3), 33.5 (C-4), 22.4 (C-2), 18.1 (C-5), 17.2 (C-5')

GCMS (EI), retention time = 6.83 min, m/z = 113.1 (11.1 %), 96.2 (59.6 %), 73.0 (60.8 %), 55 (55.6 %), 43 (100 %).

(*R*,*S*)- 3-hydroxy-4,4-dimethylpentanenitrile 97 [10]



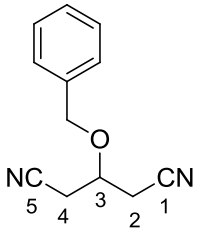
The title compound was obtained after purification as yellow oil (0.49g, 62 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 13.6 min, t_2 = 14.2 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 3.64-3.62 (m, 1H, H-3), δ = 2.52 (dd, *J*= 16.72, *J*= 3.21 Hz, 1H, H-2), δ = 2.41 (dd, *J*= 16.72 Hz, *J*= 9.62 1H, H-2'), δ = 0.91 (s, 9H, H-5).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 119.0 (C-1), 75.4 (C-3), 34.9 (C-4), 25.21 (C-5), 21.5 (C-2).

GCMS (EI), retention time = 7.37 min, m/z = 128.3 (26.3 %), 110 (16.1 %), 83.2 (46.1 %), 69.3 (12.4 %), 57.3 (100 %).

***(R,S)*- 3-benzyloxyglutaronitrile 63** [12]



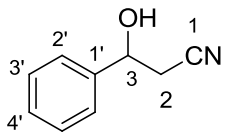
To a solution of 3-hydroxygluaronitrile **61** (1g, 9 mmol, 1 equiv) in DCM (20 mL) was added Ag_2O (2.097 g, 1 equiv, 9 mmol) and benzyl bromide (4 equiv, 36 mmol, 4.34 mL). The solution was stirred overnight in the dark at room temperature. The reaction mixture was quenched with acetone, filtered and the solvent removed *in vacuo.* The crude product was subjected to silica gel column chromatography (50 % hexane: 50 % ethyl acetate) to give the title product as a yellow oil (1.62 g, 90 %). chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and 2-propanol (90:10) with 0.1 % TFA, at a flow rate of 0.8 mL/min as the mobile phase, t_1= 15.4, t_2= 17.4 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.35 – 7.32 (m, 5H, Ar-H), 4.67 (s, 2H), 3.98-3.97 (m, 1H, H-3), 2.69-2.67 (d, J=2.5, 4H, H-2 and H-4).

^{13}C NMR (100 MHz, $CDCl_3$) = δ = 137.5 (C-Ar), 128.6 (C-Ar), 127.8 (C-Ar), 127.4 (C-Ar), 127.1 (C-Ar), 117.1 (C-1 and C-5), 71.1 ($\underline{C}H_2$-O) , 69.1 (C-3), 22.0 (C-2 and C-4).

MS Electrospray low res: $M+H^+$ = 201.2

(*R,S*)- 3-hydroxy-3-phenylpropionitrile 38 [11]



The title compound was obtained after purification as yellow oil (1.82 g, 56 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column

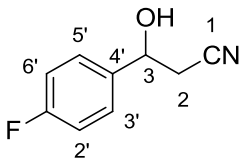
with a mixture of hexane and 2-propanol (90:10) at a flow rate 0.8 mL/min as the mobile phase, t_1= 23.7 min, t_2= 30.1 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.35 – 7.38 (m, 5H, Ar-H), δ = 4.98 (t, *J*= 5.9 Hz, 1H, H-3), δ = 3.09 (br, 1H, OH), δ = 2.72 (d, *J*= 6.41 Hz, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ =138.7 (C-1'), 129.0 (C-2'), 126.4 (C-3'), 125.0 (C-4'), 117.3 (C-1), 78.9 (C-3), 27.0 (C-2).

MS Electrospray low res: $M+H^+$ = 148.2

(*R,S*)-3-(4-fluorophenyl)-3-hydroxypropionitrile 67 [11]



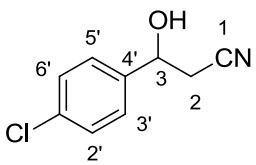
The title compound was obtained after purification as yellow oil (0.48 g, 60 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min, t_1= 12.4 min, t_2= 13.0 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = (m, *J*= 5.5 Hz, 2H, H-5', H-3'), 7.08 (m, *J*= 7.3 Hz, 2H, H-2', H-6'), 5.02 (s, 1H), 2.70 (d, *J*= 6.41 Hz, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 163.9 (C-1'), 136.7 (C-4'), 127.4 (C-3'), 127.3 (C-5'), 117.1 (C-1), 115.7 (C-2' and C-6'), 69.4 (C-3), 28.0 (C-2).

MS Electrospray low res: $M+H^+$ = 165.1

(*R,S*)-3-(4-chlorophenyl)-3-hydroxypropionitrile 68 [11]



The title compound was obtained after purification as colourless oil (0.35 g, 52 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H

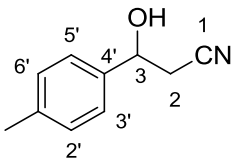
column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 13.7 min, t_2= 14.6 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.35 (m, 4H, Ar-H), 5.04 (t, *J*= 6.4 Hz, 1H, H-3), 2.74 (d, *J*= 6.4 Hz, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 160.2 (C-1'), 133.6 (C-5' and C-3'), 126.9 (C-2' and C-6'), 114.4 (C-1), 69.9 (C-3), 28.01 (C-2),

MS Electrospray low res: $M+H^+$ = 182.3

(*R,S*)-3-hydroxy-3-(p-toluoyl)-propionitrile 69 [11]



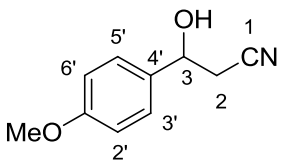
The title compound was obtained after purification as colourless liquid (0.82 g, 80 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 11.8 min, t_2= 12.6 min.

^{1}H NMR (400MHz, $CDCl_3$) δ (ppm) = 7.22 (d, *J*= 8.3 Hz, 2H, Ar-H), 7.19 (d, *J*= 8.3 Hz, 2H, Ar-H), 5.01-4.98 (m, 1H, H-3), 2.73 (dd, *J*= 6.4 Hz, 2.3 Hz, 2H, H-2), 2.50 (d, *J*= 3.2 Hz, 1H), 2.35 (s, 3H, CH_3).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 139.4 (C-4'), 138.5 (C-CH_3), 130.0 (C-5' and C-3'), 126.1 (C-6' and C-2'), 118.2 (C-1), 70.9 (C-3), 28.1 (C-2).

MS Electrospray low res: $M+H^+$ = 162.1

(*R,S*)-3-hydroxy-3-(4-methoxyphenyl)-propionitrile 70 [11]



The title compound was obtained after purification as colourless liquid (0.60 g, 62 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H

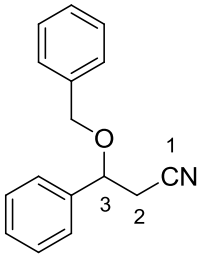
column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 17.1 min, t_2= 18.0 min.

^{1}H NMR (400MHz, $CDCl_3$) δ (ppm) = 7.87 (d, *J*= 8.7 Hz, 2H, H-5' and H-3'). 6.95 (d, *J*= 8.7 Hz 2H, H-6' and H-2'), 5.01-4.98 (t, J= 5.97, 2H, H-3), 3.97 (s, 3H, C<u>H</u>$_3$-O), 2.76 (m, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 160.2 (C-1'), 133.6 (C-4), 126.9 (C-5' and C-3'), 117.2 (C-1), 114.4 (C-6' and C-2'), 69.9 (C-3), 55.4 (<u>C</u>H$_3$-O), 28.0 (C-2).

MS Electrospray low res: $M+H^+$ = 190.0.

(*R,S*)- 3-benzyloxy-3-phenylpropionitrile 39 [13]



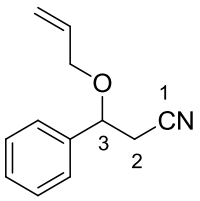
To a solution of 3-hydroxy-3-phenylpropionitrile (6.8 mmol, 1 g, 1 equiv) in dichloromethane (5 mL) were added Ag_2O (10.2 mmol, 2.37 g, 1.5 equiv), and benzyl bromide (7.5 mmol, 1.1 equiv), and the resulting reaction mixture was stirred in the dark for 4 hours at room temperature. The reaction mixture was filtered and the solvent removed under vacuum. The product was subjected to silica gel column chromatography eluted with a mixture of hexane and ethylacetate (7:1) to give the title compound as a colourless oil (1.12 g, 70 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 24.0 min, t_2= 25.8 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.33-7.41 (m, 10H, Ar-H), 4.64 (dd, *J*= 7.5 Hz, *J*= 5.5 Hz, 1H, H-3), 4.52 (d, *J*= 11.4 Hz, 1H, OCH_2), 4.33 (d, *J*= 11.4 Hz, 1H, OCH_2'), 2.80 (dd, *J*= 16.7 Hz, *J*= 7.7 Hz, 1H, H-2), 2.70 (dd, *J*= 16.7 Hz, *J*= 5.5 Hz, 1H, H-2').

^{13}C NMR (100 MHz, $CDCl_3$), δ = 138.7, 137.3, 129.0 (2C), 128.6, 128.0 (2C), 127.8 (2C), 126.6, 117.2 (C-1), 72.2 (C-3), 70.8 (O<u>C</u>H$_2$), 27.3 (C-2).

MS Electrospray low res; $M+H^+$ = 238.2.

***(R,S)*- 3-allyloxy-3-phenylpropionitrile 40** [13]



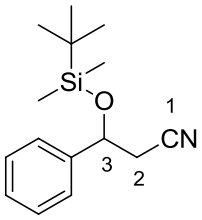
Allyl bromide (20.4 mmol, 2.42 g, 3 equiv) and potassium carbonate (13.6 mmol, 1.87 g, 2 equiv) were added to a solution of 3-hydroxy-3-phenylpropionitrile (6.8 mmol, 1 g, 1 equiv) in acetone (10 mL) and the resulting mixture was stirred at room temperature for 5 days monitored by TLC. The solution was filtered and solvent removed under vacuum before being subjected to silica gel column chromatography using a mobile phase 7:1 hexane : ethylacetate. To give the title compound as a colourless oil (0.22 g, 19 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 21.2 min, t_2= 22.3 min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.25-7.41 (m, 5H, Ar-H), 5.85-5.89 (m, 1H, CH_2-C<u>H</u>), 5.28 (d, *J*= 16.0 Hz, 1H, C<u>H</u>$_2$-CH), 5.20 (d, *J*= 10.5 Hz, 1H, C<u>H</u>$_2$-CH), 4.60 – 4.64 (m, 1H, OCH_2), 3.83 (dd, *J*= 12.6 Hz, *J*= 6.4 Hz, 1H, H-3), 2.78 (dd, *J*= 16.8 Hz, *J*= 6.9 Hz, 1H, H-2), 2.68 (dd, *J*= 16.5 Hz, *J*= 5.5 Hz, 1H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ =142.1 (C-Ar), 138.9 (C-Ar), 128.6 (2C, C-Ar), 128.4 (C-Ar), 125.6 (2C, C-Ar), 117.6 (C-1), 71.6 (C-3), 25.7 (C-2).

MS Electrospray low res: $M+Na^+$ = 188.1

_(R,S)_- 3-((tert-butyldimethylsilyl)oxy)-3-phenylpropionitrile 98



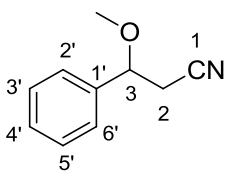
tert-butyldimethylsilyl chloride (8.84 mmol, 1.33 g, 1.3 equiv) was added to a solution of 3-hydroxy-3-phenylpropionitrile (6.8 mmol, 1g, 1 equiv) in DMF (4.7 mL). This was followed by the addition of imidazole (10.2 mmol, 0.693 g, 1.5 equiv) and the reaction mixture was stirred for 5 hours. The solution was quenched by the addition of sodium dihydrogen carbonate and extracted with diethyl-ether (2 × 25 mL). The ether extract was then washed with sodium dihydrogen carbonate, H_2O and saturated NaCl. The ether layer was then dried over anhydrous $MgSO_4$ and concentrated *in vacuo*. The product was purified using silica gel column chromatography eluted with a mixture of hexane and ethylacetate (7:1). The title compound was obtained as colourless oil (0.69 g, 39 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 32.1 min, t_2= 34.5min.

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.54 – 7.35 (m, 5H, Ar-H), 4.95 (dd, *J*= 7.1 Hz, *J*= 5.0 Hz, 1H, H-3), 2.68 (dd, *J*= 16.4 Hz, *J*= 7.3 Hz 1H, H-2), 2.68 (dd, *J*= 16.4 Hz, *J*= 4.5 Hz 1H, H-2), 0.90 (9H, s, $C(C\underline{H}_3)_3$),

^{13}C NMR (100 MHz, $CDCl_3$), δ = 142.1 (C-Ar), 128.6 (C-Ar), 128.4 (C-Ar), 125.6 (C-Ar), 117.6 (C-1), 71.6 (C-3), 29.8 (C-($\underline{C}H_3)_3$), 25.7 (C-2).

MS Electrospray low res: $M+H^+$ = 262.

_(R,S)_- 3-methoxy-3-phenylpropionitrile 41 [13]



Solid silver oxide (1.62 g, 6.8 mmol, 1 equiv) and iodomethane (3.84 mL, 27.2 mmol, 4 equiv) were added to 3-hydroxy-3-phenylpropionitrile (1 g, 6.8 mmol, 1 equiv) in DCM

and the resulting mixture was stirred in the dark overnight. The suspension was diluted with acetone (5 mL) and filtered by gravity. The solvent was removed *in vacuo* to afford the crude product. This was subjected to silica gel column chromatography eluted with a mixture of hexane : ethyl acetate (10 : 1). The title compound was obtained as colourless oil (0.52 g, 32 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 19.1 min, t_2= 19.9min.

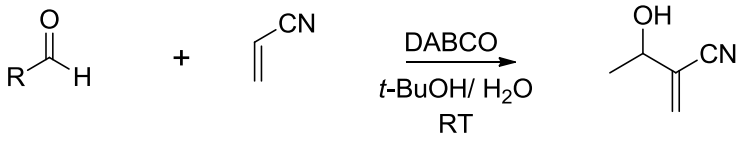
The title compound was isolated as colourless oil (32 %).

^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.37 – 7.32 (m, 5H, Ar-H), 4.45 (apparent t, *J*= 5.5 Hz, 1H, H-3), 3.28 (3H, s, OCH_3), 2.73 (dd, *J*= 16.7 Hz, *J*= 6.9 Hz, 2H, H-2), 2.65 (dd, *J*= 16.3 Hz, *J*= 5.5 Hz, 2H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ =137.2 (C-1'), 129.0 (C-4'), 128.1 (2C, C-3'and C-5'), 127.4 (2C, C-2' and C-6'), 117.7 (C-1), 79.7 (C-3), 55.9 (CH3-O), 25.3 (C-2).

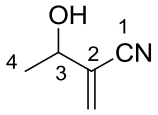
MS Electrospray low res: $M+H^+$ = 162.2

General method for Baylis-Hillman reaction [14]



Acrylonitrile (10 mmol, 1 equiv), and DABCO (1 equiv) were added to a solution of aldehyde (10 mmol, 1 equiv) in *tert*-butanol / water (60/40) (20 mL). The reactions were stirred over night at room temperature. TLC was used to determine reaction completion. The reaction solution was then extracted with ethyl acetate (3 × 50 mL). The organic phase was dried with anhydrous $MgSO_4$ and concentrated under reduced pressure. The residues were subjected to silica gel column chromatography using a mobile phase of hexane: ethyl acetate (60/40) to yield the title compound in each case.

***(R,S)*-3-hydroxy-2-methylenebutanenitrile 99** [15] [16]



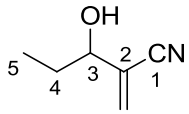
The crude product was purified to give the title compound as colourless oil (1.01 g, 69 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 8.0 min, t_2= 8.6min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 5.99 (s, 1H, C=C$\underline{H}_2$), 5.93 (s, 1H, C=C$\underline{H}_2$), 4.42 (q, *J*= 6.4 Hz, 1H, H-3), 1.42 (d, *J*= 6.4 Hz, 3H, H-4).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 129.1 ($\underline{C}H_2$-C), 127.7 (C-2), 116.9 (C-1), 68.3 (C-3), 22.2 (C-4).

MS Electrospray low res: $M+Na^+$ = 148.0.

(*R,S*)-3-hydroxy-2-methylenepentanenitrile 100 [14]



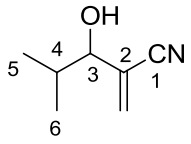
The crude product was purified to give the title compound as colourless oil (1.59 g, 72 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 8.1 min, t_2= 8.9min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 6.02 (s, 2H, C=C$\underline{H}_2$), 4.16 (dd, *J*= 11.9 Hz, *J*= 5.9 Hz, 1H, H-3), 1.75- 1.69 (m, 2H, H-4), 0.97 (t, *J*= 7.6 Hz, 3H, H-5).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 130.7 (CH_2=C), 120.9 (C-2), 117.3 (C-1), 74.9 (C-3), 26.6 (C-4), 9.2 (C-5).

MS Electrospray low res: $M+Na^+$ = 134.13.

***(R,S)*-3-hydroxy-4-methyl-2-methylenepentanenitrile 101** [14]



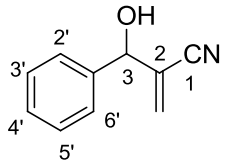
The crude product was purified to give the title compound as colourless oil (0.79 g, 63 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 7.5 min, t_2= 7.9min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 6.01 (s, 1H, C=C$\underline{H}_2$), 5.96 (s, 1H, C=C$\underline{H}_2$), 3.98 (d, *J*= 5.5 Hz, 1 H, H-3), 1.98 (m, 1H, H-4), 0.96 (d, *J*= 4.5 Hz, 3H, H-5), 0.94 (d, *J*= 4.5 Hz, 3H, H-6)

^{13}C NMR (100 MHz; $CDCl_3$) δ= 130.1 ($\underline{C}H_2$=C), 126.2 (C-2), 118.1 (C-1), 72.5 (C-3), 18.5 (C-4), 17.2 (C-5 and C-6).

MS Electrospray low res: M+Na^+ = 148.09.

***(R,S)*-2-(hydroxyl(phenyl)methyl)acrylonitrile 102** [14]



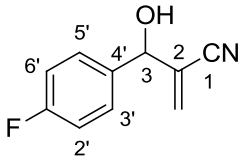
The crude product was purified to give the title compound as colourless oil (1.26 g, 79 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 9.25 min, t_2= 10.51min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.35- 7.37 (m, 5 H, Ar-H), 6.12 (s, 1H, C=C$\underline{H}_2$), 6.02 (s, 1H, C=C$\underline{H}_2$), 5.28-5.26 (br, 1H, H-3), 2.48 (d, *J*= 4.12 Hz, 1H, OH).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 141.2 (C-1'), 131.6 ($\underline{C}H_2$=C), 130.1 (C-5' and C-3'), 128.9 (C-4'), 127.9 (C-6' and C-2'), 127.1 (C-2), 117.8 (C-1), 71.4 (C-3).

MS Electrospray low res: M+H^+ = 160.2.

***(R,S)*-2-((4-fluorophenyl)(hydroxyl)methyl)-acrylonitrile 103** [17] [14]



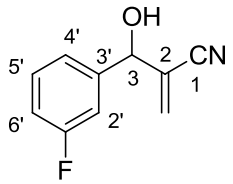
The crude product was purified to give the title compound as colourless oil (0.65 g, 58 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 9.4 min, t_2= 10.3min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.34 (dd, *J*= 8.7 Hz, *J*= 5.4 Hz, 2 H, H-6'& H-2'), 7.06 (t, *J*= 8.5 Hz, 2H, H-3' & H-5'), 6.09 (s, 1H, C=C$\underline{H}_2$), 6.01 (s, 1H, C=C$\underline{H}_2$), 5.26 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 160.8 ($\underline{C}$-F), 130.9 (C-4'), 126.0 ($\underline{C}H_2$=C), 124.4 (C-5' and C-3'), 124.3 (C-2), 122.0 (C-6' and C-2'), 111.7 (C-1), 69.4 (C-3).

MS Electrospray low res: $M+H^+$ = 178.06

***(R,S)*-2-((3-fluorophenyl)(hydroxyl)methyl)-acrylonitrile 104** [18]



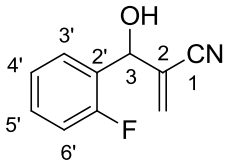
The crude product was purified to give the title compound as colourless oil (0.54 g, 58 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 9.00min, t_2= 10.36min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.31- 7.19 (m, 1H, H-2'), 7.04- 6.99 (m, 3H, Ar-H, H-4', H-5', H-6'), 6.06 (s, 1H, C=C$\underline{H}_2$), 5.99 (s, 1H, C=C$\underline{H}_2$), 5.24 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 130.6 (Ar), 130.5 (C-F), 130.2 (CH_2=C), 125.7 (C-5'), 124.1 (C-2), 116.6 (C-4'), 116.0 (C-6'), 115.8 (C-2'), 113.3 (C-1), 73.5 (C-3).

MS Electrospray low res: $M+H^+$ = 178.0

***(R,S)*-2-((2-fluorophenyl)(hydroxyl)methyl)-acrylonitrile 105** [19]



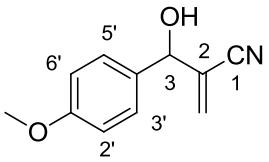
The crude product was purified to give the title compound as colourless oil (0.58 g, 62 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 9.28 min, t_2= 10.15 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.47 apparent triplet (t, *J*= 5.5 Hz, 1H, H-5'), 7.31 (m, 1H, H-3'), 7.19 (m, 1H, H-6'), 7.06 apparent triplet (t, *J*= 9.1 Hz, 1H, H-4'), 6.05 (s, 1H, C=CH_2), 6.00 (s, 1H, C=CH_2), 5.58 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 160.0 (C-F), 141.1 (C-2'), 130.6 (CH_2=C), 128.5 (C-5') 127.6 (C-3'), 126.2 (C-4'), 124.9 (C-2), 116.5 (C-6'), 115.4 (C-1), 67.7 (C-3).

MS Electrospray low res: $M+H^+$ = 178.02

***(R,S)*-2-(hydroxy(4-methoxyphenyl)methyl)-acrylonitrile 106** [14]



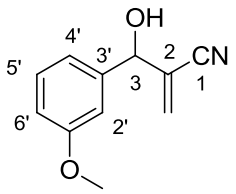
The crude product was purified to give the title compound as colourless oil (0.65 g, 66 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 13.3 min, t_2= 17.3 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.30 (d, *J*= 8.7 Hz, 2 H, H-3' & H-5'), 6.9 (d, *J*= 8.7 Hz, 2H, H-2' & H-6'), 6.31 (s, 1H, C=C$\underline{H}_2$), 6.01 (s, 1H, C=C$\underline{H}_2$), 5.33 (s, 1H, H-3), 3.84-3.81 (s, 3H, OC$\underline{H}_3$).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 137.3 (C-1'), 130.8 ($\underline{C}H_2$=C), 128.8 (C-5' and C-3'), 126.9 (C-2), 125.0 (C-4'), 116.8 (C-6' and C-2'), 83.0 (C-2), 57.9 ($\underline{C}H_3$-O).

MS Electrospray low res: M+H$^+$ = 190.2.

***(R,S)*-2-(hydroxy(3-methoxyphenyl)methyl)-acrylonitrile 107** [14]



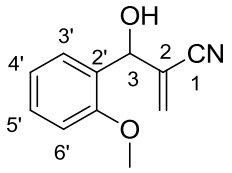
The crude product was purified to give the title compound as colourless oil (0.68 g, 70 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 14.5 min, t_2= 18.6 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.29 (t, *J*= 7.8 Hz, 1 H, H-5'), 6.91-6.88 (m, 3H, Ar-H), 6.09 (s, 1H, C=C$\underline{H}_2$), 6.01 (s, 1H, C=C$\underline{H}_2$), 5.25 (s, 1H, H-3), 3.79 (s, 3H, OC$\underline{H}_3$).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 159.9 ($\underline{C}$-1'), 140.7 (C-3'), 130.0 ($\underline{C}H_2$=C), 129.8 (C-5'), 125.9 (C-2), 118.7 (C-4'), 116.8 (C-1), 114.5 (C-6'), 111.9 (C-2'), 74.1 (C-2), 55.3 ($\underline{C}H_3$-O).

MS Electrospray low res: M+H$^+$ = 190.2.

***(R,S)*-2-(hydroxy(2-methoxyphenyl)methyl)-acrylonitrile 108** [14]



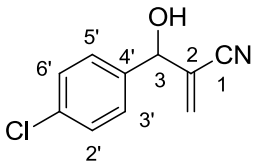
The crude product was purified to give the title compound as colourless oil (0.65 g, 68 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 14.3 min, t_2= 15.2 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.25-7.22(m, 2 H, H-5' & H-3'), 6.93 (t, *J*= 6.4 Hz, 1H, H-4'), 6.84 (d, *J*= 8.7 Hz, 1H, H-6'), 5.91 (s, 2H, C=C$\underline{H}_2$), 5.41 (s, 1H, H-3), 3.78 (s, 3H, OC$\underline{H}_3$).

^{13}C NMR (100 MHz; $CDCl_3$) 156.6 (C-1'), 130.0 ($\underline{C}H_2$=C), 129.7 (C-5'), 127.9 (C-3'), 126.9 (C-2'), 125.8 (C-2), 121.1 (C-1), 117.2 (C-6'), 115.9 (C-4'), 70.8 (C-3), 56.1 ($\underline{C}H_3$-O).

MS Electrospray low res: $M+H^+$ = 190.2.

***(R,S)*-2-((4-chlorophenyl)(hydroxyl)methyl)-acrylonitrile 109** [14]



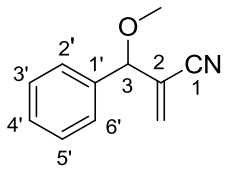
The crude product was purified to give the title compound as colourless oil (0.53 g, 58 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 10.4 min, t_2= 11.3 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.35-7.28 (m, 4H, Ar-H), 6.11 (s, 1H, C=C$\underline{H}_2$), 6.01 (s, 1H, C=C$\underline{H}_2$), 5.34 (br, 1H, H-3), 2.47 (br, 1H, OH).

^{13}C NMR (100 MHz; $CDCl_3$) δ= 169.6 (C-4'), 148.3 (C-Cl), 140.9 (CH_2=C), 136.8 (C-6' and C-2'), 129.3 (C-5' and C-3'), 127.5 (C-2), 118.0 (C-1), 71.7 (C-3).

MS Electrospray low res: $M+H^+$ = 194.6.

***(R,S)*-2-(methoxy(phenyl)methyl)acrylonitrile 110** [20]



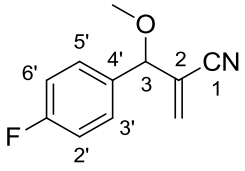
Solid Ag_2O (0.78 g, 3.4 mmol, 1 equiv) and iodomethane (1.92 g, 13.6 mmol, 4 equiv) were added to 2-(hydroxy(phenyl)methyl)acrylonitrile **102** (0.5 g, 3.4 mmol, 1 equiv) successively and the resulting mixture was stirred in the dark overnight. The reaction mixture was diluted with acetone (5 mL) and gravity filtered. The solvent was removed *in vacuo* to afford the crude product. The crude product was subjected to silica gel column chromatography eluted with a mixture of hexane: ethyl acetate (80: 20). The title product was obtained as colourless oil (0.19 g, 32 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 23.0 min, t_2= 24.6 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.37- 7.33 (m, 5 H, Ar-H), 6.01 (s, 1H, CH_2=C), 5.98 (s, 1H, CH_2=C), 4.72 (s, 1H, H-3), 3.35 (s, 3H, CH_3-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 137.0 (C-1'), 131.1 ($\underline{C}H_2$=C), 129.5 (2C) (C-3' and C-5'), 127.6 (2C) (C-2' and C-6'), 125.1 (C-2), 117.3 (C-1), 83.1 (C-3), 57.2 ($\underline{C}H_3$-O).

MS Electrospray low res: $M+H^+$ = 174.09.

***(R,S)*-2-((4-fluorophenyl)(methoxy)methyl)acrylonitrile 111** [21]



Solid Ag_2O (0.646g, 2.82 mmol, 1 equiv) and iodomethane (1.59 g, 11.3 mmol, 4 equiv) were added to 2-((4-fluorophenyl)(hydroxy)methyl)acrylonitrile **103** (0.5 g, 2.82 mmol, 1 equiv) successively and the resulting mixture was stirred in the dark overnight.

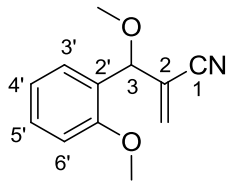
The reaction mixture was diluted with acetone (5 mL) and gravity filtered. The solvent was removed *in vacuo* to afford the crude product. The crude product was subjected to silica gel column chromatography eluted with a mixture of hexane: ethyl acetate (80:20). The title product was obtained as colourless oil (0.22 g, 41 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 11.0 min, t_2= 11.5 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.51 (td, *J*= 7.3 Hz, *J*= 1.8 Hz, 1 H, Ar-H), 7.35-7.31 (m, 1 H, Ar-H), 7.22-7.20 (m, *J*= 1.4 Hz, 1H, Ar-H), 7.09-7.05 (m, 1 H, Ar-H), 6.05 (s, 1 H, C$\underline{H}_2$=C), 5.99 (s, 1 H, C$\underline{H}_2$=C), 5.13 (s, 1 H, H-3), 3.42 (s, 3H, C$\underline{H}_3$-O),

^{13}C NMR (100 MHz; CDCl3) δ = 164.0 (C-1'), 134.1 (C-4'), 128.1 (C$\underline{H}_2$=C), 128.0 (C-2' and C-6'), 116.9 (C-2), 116.0 (C-1), 115.1 (C-3' and C-5'), 78.1 (C-3), 57.0 (C$\underline{H}_3$-O),

MS Electrospray low res: $M+H^+$ = 192.2

***(R,S)*-2-(methoxy(2-methoxyphenyl)methyl)acrylonitrile 112** [22]



Solid Ag_2O (0.619g, 2.6 mmol, 1 equiv) and iodomethane (1.45 mL, 10.4 mmol, 4 equiv) were added to 2-(hydroxy-2-(methoxyphenyl)methyl)acrylonitrile **108** (0.5 g, 2.6 mmol, 1 equiv) successively and the resulting mixture was stirred in the dark overnight. The reaction mixture was diluted with acetone (5 mL) and filtered by gravity. The solvent was removed *in vacuo* to afford the crude product. The product was subjected to silica gel column chromatography eluted with a mixture of hexane: ethyl acetate (60:40). The title product was obtained as colourless oil (0.20 g, 39 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_1= 11.2 min, t_2= 12.2 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.45 (d, *J*= 7.5 Hz, 1 H, Ar-H), 7.29 (t, *J*= 7.3 Hz, 1 H, Ar-H), 7.01 (t, *J*= 6.4 Hz, 1 H, Ar-H), 6.88 (d, *J*= 8.2 Hz, 1 H, Ar-H), 5.96 (d, *J*= 6.8 Hz, 2 H, CH_2=C)), 5.20 (s, 1 H, H-3), 3.82 (s, 3H, O-CH_3), 3.37 (s, 3H, CH_3-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 169.1 (C-1'), 147.8($\underline{C}H_2$=C), 145.5 (C-5'), 143.6 (C-3'), 128.3 (C-2'), 127.4 (C-2), 127.1 (C-4'), 117.8 (C-1), 71.4(C-3), 57.6, ($\underline{C}H_3$-O), 56.1 ($\underline{C}H_3$-O).

MS Electrospray low res: $M+H^+$ = 204.1

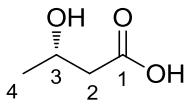
6.4.2 GENERAL PROCEDURE FOR THE BIOTRANSFORMATION OF NITRILES

Cells of *R. erythropolis* SET1 cells (OD_{600nm} = 1) were added to a 100 mL erlenmeyer flask along with potassium phosphate buffer (0.1 M, pH 7.0, 80 mL). These resting cells were activated at 25 °C for 0.5 h with orbital shaking. Racemic nitrile was added in one portion to the flask and the mixture was incubated at 25 °C using an orbital shaker (250 rpm). The reaction, monitored by TLC and LC-MS, was quenched after a specified period of time by removal of the biomass using centrifugation. The resulting aqueous solution was basified to pH 12 with aqueous NaOH (2 M). Extraction with ethyl acetate (3 x 60 mL) gave, after drying over $MgSO_4$, concentration, and silica gel column chromatography eluted with a mixture of petroleum ether and acetone (2:1), the remaining nitrile. The retained aqueous solution was then acidified using aqueous HCl (2 M) to pH 2 and extracted with ethyl acetate (3 x 60 mL). The acid product was obtained after drying over with $MgSO_4$, removal of the solvent under vacuum and preparative TLC. All products were characterized by their spectral data and comparison of the melting points and specific rotation values with those of the known compounds. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase.

The characterisation data below represents the acids and amides produced during the biocatalytic hydrolysis of the corresponding nitriles. The remaining nitriles were characterised by ^{1}H and ^{13}C NMR and did not vary from the data previously reported in the substrate synthesis section.

6.4.3 BIOTRANSFORMATION OF RACEMIC β-HYDROXY ALKANE NITRILES

***(S)*-3-hydroxybutyric acid 89a** [3, 8]



The crude product was purified to give the title compound as a white solid (0.44 mg, 42 % yield). The enantiomers were separated by HPLC analysis after derivitisation to the corresponding benzyl ether using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave (*R*)-enantiomer t_{minor}= 11.2 min, (*S*)-enantiomer t_{major}= 12.3 min. $[\alpha]^{d}_{25}$ = + 4.0 (c = 2.5, MeOH), and compared with that in the literature $[\alpha]^{d}_{25}$= + 4.1 (c = 2.7, MeOH).

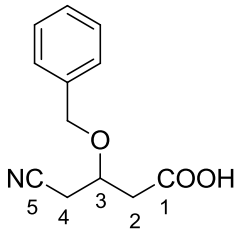
^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.19 – 4.27 (m, *J*=2.7Hz, 1H, H-3), δ = 2.55 (dd, *J*=16.6 Hz, *J*=2.4Hz, 1H, H-2), δ = 2.48 (dd, *J*=16.7 Hz, *J*=8.2Hz, 1H, H-2), δ = 1.23 (d, *J*=6.3Hz, 3H, H-4).

^{13}C NMR (400 MHz, $CDCl_3$) δ = 176.2 (C-1), 64.1 (C-3), 42.3 (C-2), 22.4 (C-4).

MS Electrospray low res: $M+Na^{+}$ = 127.1

The remaining nitrile **89** was purified and all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis after derivitisation to the corresponding benzyl ether **93** using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 19.2 min, t_{major}= 23.1 min.

3-(benzyloxy)-4-cyanobutyric acid 63a [12]



The crude product was purified to give the title compound as a white solid (0.13 mg, 18 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H

column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 14.7 min, t_{major}= 16.2 min.

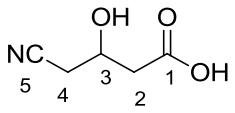
^{1}H NMR (400 MHz, $CDCl_3$) δ = 7.3-7.2 (m, 5H, Ar-H), 2.71 (d, *J*=2.5 Hz, 2H, H-4), 2.48 (m, 1H, H-3), 2.25 (d, *J*=2.6 Hz, 2H, H-2).

^{13}C NMR (400 MHz, $CDCl_3$) δ = 176.2 (C-1), 128.6 (2C, Ar), 127.8 (C-Ar), 127.4 (C-Ar), 117.1 (C-5), 64.1 (C-3), 42.3 (C-2), 22.4 (C-4).

MS Electrospray low res: $M+H^+$ = 220.

The remaining nitrile **63** was purified and all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 15.4 min, t_{major}= 17.4 min.

4-cyano-3-hydroxybutyric acid 61a [23]



The crude product was purified to give the title compound as a white solid (0.52 mg, 67 % yield). The enantiomers were separated by HPLC analysis after derivitisation to the corresponding benzyl ether using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 16.3 min, t_{major}= 14.8 min.

^{1}H NMR (400MHz, D_2O) δ = 4.25-4.31 (m, 1H, H-3), δ = 2.51-2.79– (m, 4H, H-2 and H-4).

^{13}C NMR (100MHz, D_2O) δ = 171.9 (C-1), 118.7 (C-5), 63.4 (C-3), 41.2 (C-4), 25.2 (C-2).

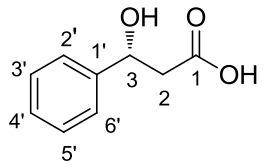
MS Electrospray low res: $M+H^+$ = 130.2

The remaining nitrile **61** was purified and all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis after derivitisation to the corresponding benzyl ether **63** using a Chiralcel AD-H column with

a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 15.4 min, t_{major}= 17.4 min.

6.4.4 ENANTIOSELECTIVITY SCREENING TOWARDS AROMATIC β–HYDROXYNITRILES

(*R*)-3-hydroxy-3-phenylpropionic acid 38a [11]



The title compound was obtained as a white solid (65 mg, 80 % yield). Mp 117-120 °C (lit 118-120 °C). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{major}= 12.2 min, t_{minor} = 13.6 min. $[\propto]_D^{25}$ = +30.3 (c 1.2, CH_3OH) (*R*) compared with literature values $[\propto]_D^{25}$ = +22.4 (c 4.1, CH_3OH).

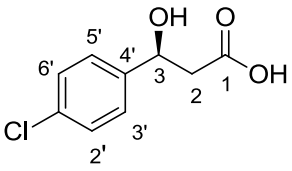
^{1}H NMR (400 MHz, $CDCl_3$), δ = 7.31 (5H, m, Ar), 5.13 (dd, *J*=9.2 Hz, *J*=3.6 Hz, 1H, H-3), 2.84 (dd, *J*=16.7 Hz, *J*= 9.1 Hz, 1H, H-2), 2.76 (dd, *J*=16.7 Hz, *J*= 3.6 Hz, 1H, H-2).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 177.3 (C-1), 143.8 (C-1’), 128.9 (C-4’), 127.6 (C-3’ and C-5’), 127.1 (C-2’ and C-6’), 70.9 (C-3), 45.3 (C-2).

MS Electrospray low res: $M+H^+$ = 167.1

The remaining nitrile **38** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 23.7 min, t_{major}= 30.1 min.

(*S*)-3-(4-chlorophenyl)-3-hydroxypropionic acid 68a [11]



The title compound was obtained as a white solid (25 mg, 20 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column, with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA , with a flow rate of 0.8 mL/min as the mobile phase, t_{major}= 13.70 min, t_{minor} = 14.6min. $[\propto]_D^{25}$= -11.6 (*c* 0.32, $CHCl_3$) (*S*) compared with literature values $[\propto]_D^{25}$= -15.8 (*c* 0.56, CH_3Cl_3).

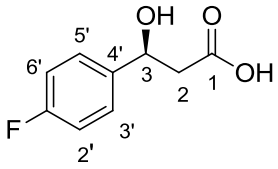
^{1}H NMR (400MHz, $CDCl_3$) δ (ppm) = 7.36-7.28 (m, 4H, Ar-H), 5.04 (m, 1H, H-3), 2.75 (d, *J*=5.8, 2H, H-2), 2.49 (s, 1H, OH).

^{13}C NMR (100 MHz, $CDCl_3$), δ = 177.3 (C-1), 139.4 (C-4'), 129.2 (C-1'), 127.0 (C-3' and C-5'), 116.9 (C-6' and C-2'), 69.6 (C-3), 28.1 (C-2)**.**

MS Electrospray low res: $M+H^+$ = 201.

The remaining nitrile **68** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 13.7 min, t_{major}= 14.6 min.

(*S*)-3-(4-fluorophenyl)-3-hydroxypropionic acid 67a [11]



The title compound was obtained as a white solid (41 mg, 42 % yield). mp= 75-76 °C, (lit mp = 71-72 °C) The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column, with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA , with a flow rate of 0.8 mL/min as the mobile phase, t_{major}= 12.42 min, t_{minor} = 13.03 min**.** $[\propto]_D^{25}$= -4.8 (*c* 0.32, CH_3OH) (*S*) compared with literature values $[\propto]_D^{25}$= -12.5 (*c* 0.55, CH_3OH).

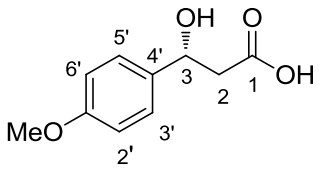
^{1}H NMR (400MHz, $CDCl_3$) δ (ppm) = 7.34 (dd, *J*=8.7 Hz, *J*=5.5 Hz, 2H, Ar-H). 7.05 (t, *J*=8.7 Hz, 2H, Ar-H), 5.12 (dd, *J*=9.2 Hz, *J*=3.6 Hz, 1H, H-3), 2.80 (dd *J*=16.5 Hz, *J*=9.2 Hz, 1H, H-2), 2.72 (dd *J*=16.5 Hz, *J*=3.6 Hz, 1H, H-2).

^{13}C NMR (100MHz, $CDCl_3$) δ (ppm) = 177.4 (C-1), 161.5 (C-1'), 138.1 (C-4'), 127.9 (C-5'), 127.8 (C-3'), 116.0 (C-6'), 115.6 (C-2'), 70.0 (C-3), 43.5 (C-2).

MS Electrospray low res: $M+H^+$ = 185.2.

The remaining nitrile **67** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 13.0 min, t_{major}= 12.4 min.

(*R*)-3-hydroxy-3-(4-methoxyphenyl)-propionic acid 70a [11]



The title product was obtained as colourless oil (30 mg, 28 %yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column, with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA , with a flow rate of 0.8 mL/min as the mobile phase, t_{major}= 12.61 min, t_{minor} = 11.86 min. $[\propto]_D^{25}$= +16.7 (*c* 0.4, CH_3OH) (*R*) compared with literature values, $[\propto]_D^{25}$= +24.5 (*c* 0.96, CH_3OH).

^{1}H NMR (400MHz, $CDCl_3$) δ (ppm) = 7.34-7.32 (m, 2H, H-2' and H-6'), 6.91-6.92 (m, 2H, H-5' and H-3'), 5.08-5.09 (m, 1H, H-3), 3.77 (s, 3H, CH_3-O), 2.64 -2.62 (m, 2H, H-2).

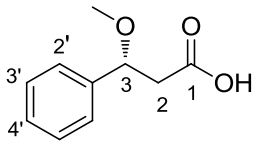
^{13}C NMR (100 MHz, $CDCl_3$), δ = 177.3 (C-1), 159.9 (C-1'), 134.2 (C-4'), 126.9 (C-3' and C-5'), 115.1 (C-2' and C-6'), 70.4 (C-3), 55.8 ($\underline{C}H_3$-O), 43.4 (C-2).

MS Electrospray low res: $M+H^+$ = 197.2

The remaining nitrile **70** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 17.1 min, t_{major}= 18.0 min.

6.4.5 ENHANCEMENT OF ENANTIOSELECTIVITY USING A DOCKING/ PROTECTION STRATEGY

(*R*)-3-methoxy-3-phenylpropionic acid 41a [6]



The title product was obtained as a white solid (30 mg, 14 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column, with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA , with a flow rate of 0.8 mL/min as the mobile phase, t_{major}= 6.67 min). $[\propto]_D^{25}$= +18.6 (*c* 0.1, $CHCl_3$) (*R*) compared with literature values $[\propto]_D^{25}$= -49.3 (*c* 0.3, $CHCl_3$).

^{1}H NMR (400MHz, $CDCl_3$) δ = 7.35-7.37 (m, *J*=7.3, 5H, Ar-H), 4.62 (dd, *J*=9.6, *J*=4.1, 1H, H-3), 3.25 (s, 1H, C$\underline{H}_3$-O), 2.83 (dd, *J*=15.6, *J*=9.6, 1H, H-2), 2.63 (dd, *J*=15.6, *J*=4.1, 1H, H-2).

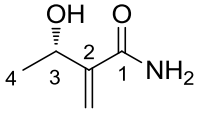
^{13}C NMR (100 MHz, $CDCl_3$) δ =176.2 (C-1), 140.1 (C-1'), 128.7 (C-3' and C-5'), 128.2 (C-2' and C-6'), 126.6 (C-4'), 79.8 (C-3), 56.8 ($\underline{C}H_3$-O), 43.2 (C-2).

MS Electrospray low res: $M+H^+$ = 181.2.

The remaining nitrile **41** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 19.1 min, t_{major}= 19.9min.

6.4.6 BIOTRANSFORMATION OF α-METHYLENE-β-HYDROXY ALKYL AND ARYL NITRILES

(*S*)-3-hydroxy-2-methylenebutanamide 99a [15]



The title product was obtained as a as a white solid (28 mg, 43.2 % yield). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol

(90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 8.4 min, t_{major} = 10.8 min.

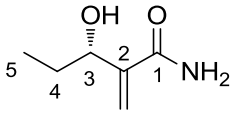
^{1}H NMR (400 MHz; $CDCl_3$) δ = 6.01 (s, 1H, (C<u>H</u>$_2$=C)), 5.95 (s, 1H, C<u>H</u>$_2$=C), 4.43 (q, *J*=6.4 Hz, 1H, H-3), 1.44 (d, *J*=6.4 Hz, 3H, H-4).

^{13}C NMR (100 MHz; $CDCl_3$) δ =171.0 (C-1), 147.9 (C-2), 117.4 (<u>C</u>H$_2$=C), 66.6 (C-3), 21.4 (C-4).

MS Electrospray low res: M+Na^+ = 138.0.

The remaining nitrile **99** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 8.6 min, t_{major}= 8.0 min.

(*S*)-3-hydroxy-2-methylenepentanamide 100a [20]



The title product was obtained as a white solid (28 mg, 22.0 % yield). The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 9.03 min, t_{major} = 9.40 min. $[\propto]_D^{25}$= +2.8 (*c*= 0.1, CH_3OH) compared with literatre values $[\propto]_D^{25}$= 0 (*c*= 0.4, CH_3OH).

^{1}H NMR (400 MHz; MeOD) δ = 5.81 (s, 1H, C<u>H</u>$_2$=C), 5.58 (s, 1H, C<u>H</u>$_2$=C), 4.36 (t, *J*= 6.4 Hz, 1H, H-3), 1.66 (m, 1H, H-4), 1.54 (m, 1H, H-4), 0.92 (t, *J*= 7.3 Hz, 3H, H-5).

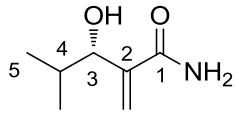
^{13}C NMR (100 MHz; MeOD) δ =171.1 (C-1), 130.0 (C-2), 126.7 (<u>C</u>H$_2$=C), 73.6 (C-3), 28.7 (C-4), 9.3 (C-5).

MS Electrospray low res: M+Na^+ = 152.0

The remaining nitrile **100** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H

column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 8.9 min, t_{major}= 8.1 min.

(*S*)-3-hydroxy-4-methyl-2-methylenepentanamide 101a [20]



The title product was obtained as a white solid (34 mg, 44.1 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 9.4 min, t_{major} = 8.6 min. $[\propto]_D^{25}$= +2.7 (*c*= 0.1, CH_3OH) compared with literature $[\propto]_D^{25}$= -10.17 (*c*= 3.0, CH_3OH).

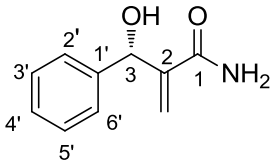
^{1}H NMR (400 MHz; MeOD) δ = 5.81 (s, 1H, C$\underline{H}_2$=C), 5.58 (s, 1H, C$\underline{H}_2$=C), 4.16 (d, *J*= 6.4 Hz, 1H, H-3), 1.83 (m, *J*= 6.4 Hz, 1H, H-4), 0.93 (d, *J*= 6.8 Hz, 3H, H-5), 0.89 (d, *J*= 6.8 Hz, 3H, H-5).

^{13}C NMR (100 MHz; MeOD) δ = 172.1 (C-1), 147.9 (C-2), 117.4 ($\underline{C}H_2$=C), 66.6 (C-3), 32.2 (C-4), 21.4 (C-5).

GCMS- general 240 isocratic (EI), retention time = 9.32 min, m/z = 143 (11.7 %), 126 (1641 %), 100 (100 %), 83 (73.1 %), 55 (58.6 %).

The remaining nitrile **101** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 7.9 min, t_{major}= 7.5 min.

(*S*)-2-(hydroxyl(phenyl)methyl)acrylamide 102a [20]



This title product was obtained as a white solid (26 mg, 22 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol

(90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 17.95 min, t_{major} = 19.03 min.

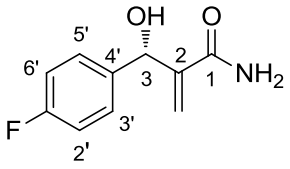
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.38-7.19 (m, 5 H, Ar), 6.12 (s, 1H, C$\underline{H}_2$=C), 6.12 (s, 1H, C$\underline{H}_2$=C), 6.0 (s, 1H, C$\underline{H}_2$=C), 5.81 (s, 1H, H-3)

^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.3 (C-1), 143.9 (C-2), 137.7 (C-1'), 130.5 (C-4'), 128.9 (2C) (C-2' and C-6'), 127.6 (C$\underline{H}_2$=C), 127.1 (2C) (C-3' and C-5'), 69.8 (C-3).

MS Electrospray low res: M+H$^+$ = 178.

The remaining nitrile **102** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 10.51 min, t_{major}= 9.25 min.

(*S*)-2-((4-fluorophenyl)(hydroxy)methyl)acrylamide 103a [24]



The title product was obtained as a white solid (26 mg, 30.2 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 16.09 min, t_{major} = 17.20 min.

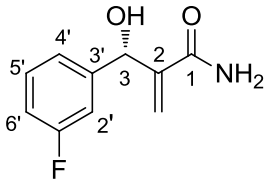
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.31-7.27 (m, 1 H, Ar-H), 7.10 (t, *J*= 7.8 Hz, 2H, Ar-H), 6.95 (m, 1H, Ar-H), 5.92 (s, 1H, C$\underline{H}_2$=C), 5.86 (s, 1H, C$\underline{H}_2$=C) 5.51 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 169.5 (C-1), 161.7 (C-1'), 143.9 (C-2), 143.4 (C-4') 130.1 (CH_2=C), 122.6 (C-5'), 121.7 (C-3'), 114.7 (C-6'), 113.2 (C-2'), 73.9 (C-3).

MS Electrospray low res: M+H$^+$=196.1

The remaining nitrile **103** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 10.3 min, t_{major}= 9.4 min.

(*S)-(2-((3-fluorophenyl)(hydroxy)methyl)acrylamide 104a**



The product 2-((3-fluorophenyl)(hydroxy)methyl)acrylamide was obtained as a white solid (30.1 % yield), (*S* 4.9 % ee). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 17.27 min, t_{major} = 18.10 min.

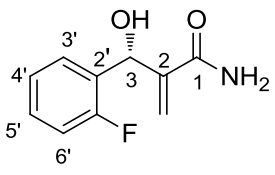
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.31-7.27 (m, *J*= 5.9 Hz, 1 H. Ar), 7.10 (t, *J*= 7.8 Hz, 2H, Ar), 6.95 (td, *J*= 5.0 Hz, *J*= 3.2 Hz, 1H, Ar), 5.92 (s, 1H, C$\underline{H}_2$=C), 5.51 (s, 1H, C$\underline{H}_2$=C), 5.50 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 169.45 (C-1), 161.67 (C-1'), 143.9 (C-2), 143.4 (C-3'), 130.1 (C-5'), 122.63 (CH_2=C), 121.7 (C-4'), 114.7 (C-2'), 113.2 (C-6'), 73.92 (C-3).

MS Electrospray low res: M+H^+=196.1

The remaining nitrile **104** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 10.36 min, t_{major}= 9.00 min

(*S)-(2-((2-fluorophenyl)(hydroxy)methyl)acrylamide 105a**



The title product was obtained as a white solid (29 mg, 26.4 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 18.7 min, t_{major} = 20.10 min.

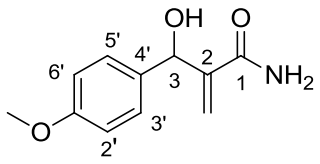
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.53 (td, *J*= 7.6 Hz, *J*= 1.8 Hz, *J*= 1.4 Hz,1 H, Ar), 7.24-7.16 (m, 1H, Ar), 7.16 (t, *J*= 7.6 Hz, 1H, Ar), 5.86 (s, 1H, C$\underline{H}_2$=C), 5.79 (s, 1H, C$\underline{H}_2$=C), 5.46 (s, 1H, H-3).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.5 (C-1), 142.9 (C-F), 139.2 (C-2) 129.5 (C-2'), 127.9 (C-3'), 124.3 (CH_2=C), 122.0 (C-5'), 115.3 (C-4'), 115.1 (C-6'), 68.9 (C-3).

MS Electrospray low res: $M+H^+$ = 196.

The remaining nitrile **105** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 10.15 min, t_{major}= 9.28 min.

(*S*)-2-(hydroxy(4-methoxyphenyl)methyl)acrylamide 106a [20]



The title product was obtained as a white solid (26 mg, 28.5 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 27.8 min, t_{major} = 32.5 min. $[\propto]_D^{25}$= +3.6 (*c*= 0.2, CH_3OH).

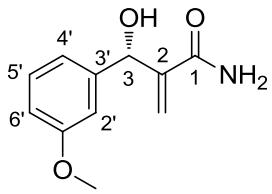
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.26-7.22 (m, 2 H, H-3'and H-5'), 6.94- 6.83 (m, 2H, H-2' and H-6'), 6.06 (s, 1H, C$\underline{H}_2$=C), 5.71 (s, 1H, C$\underline{H}_2$=C), 5.32 (s, 1H, H-3), 3.79 (s, 3H, C$\underline{H}_3$-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.1 (C-1), 159.2 (C-1'), 145.1 (C-2), 136.4 (C-4'), 125.1 (C-5' and C-3'), 123.0 (C$\underline{H}_2$=C), 115.1 (C-6' and C-2'), 70.4 (C-3), 55.8 (C$\underline{H}_3$-O)

MS Electrospray low res: $M+H^+ = 208.1$

The remaining nitrile **106** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 17.3 min, t_{major}= 13.3 min.

(*S*)-2-(hydroxy(3-methoxyphenyl)methyl)acrylamide 107a [20]



The title product was obtained as a white solid (14 mg, 11.6 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 25.0 min, t_{major} = 29.7 min

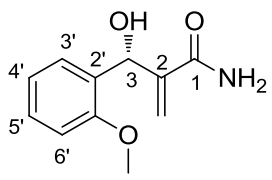
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.23 (m, 2 H, Ar-H), 6.89 (m, 2H, Ar-H), 6.06 (s, 1H, C$\underline{H}_2$=C), 5.71 (s, 1H, C$\underline{H}_2$=C), 5.32 (s, 1H, H-3), 3.79 (s, 3H, C$\underline{H}_3$-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.0 (C-1), 160.8 (C-1), 145.2 (C-2), 129.1 (C-5'), 124.3 (C$\underline{H}_2$=C), 119.5 (C-4'), 116.3 (C-6'), 114.5 (C-2'), 73.5 (C-3), 55.1 (C$\underline{H}_3$-O)

MS Electrospray low res: $M+H^+ = 208.1$.

The remaining nitrile **107** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 18.6 min, t_{major}= 14.5 min.

(*S*)-2-(hydroxy(2-methoxyphenyl)methyl)acrylamide 108a [20]



The title product was obtained as a white solid (21 mg, 12.8 % yield). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 26.4 min, t_{major} = 28.3 min. $[\propto]_D^{25}$= +5.6 (*c*= 0.1, CH_3OH) compared with literature $[\propto]_D^{25}$= +54.24 (*c*= 0.88, CH_3OH).

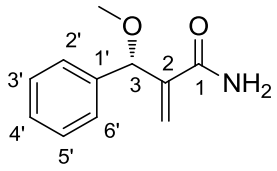
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.26-7.22 (m, 2 H, Ar), 6.94- 6.83 (m, 2H, Ar), 6.06 (s, 1H, C$\underline{H}_2$=C), 5.71 (s, 1H, C$\underline{H}_2$=C), 5.32 (s, 1H, H-3), 3.79 (s, 3H, C$\underline{H}_3$-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 172.1 (C-1), 156.2 (C-1), 143.1 (C-2), 128.6 (C-5'), 128.1 (C-2'), 127.9 (C-3'), 124.2 ($\underline{C}H_2$=C), 121.4 (C-4'), 118.2 (C-6'), 65.9 (C-3), 56.1 ($\underline{C}H_3$-O)

MS Electrospray low res: $M+H^+$ = 208.0.

The remaining nitrile **108** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 15.2 min, t_{major}= 14.3 min.

(*S*)-2-(methoxy(phenyl)methyl)acrylamide 110a [20]



The title product was obtained as a white solid (32 mg, 27.1 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 13.01 min, t_{major} = 12.55 min. $[\propto]_D^{25}$= +8.9 (*c*= 0.15, CH_3OH) compared with literature $[\propto]_D^{25}$= +26.7 (*c*= 0.6, CH_3OH).

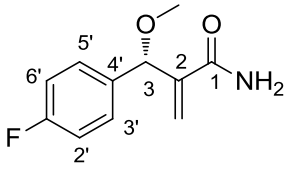
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.33-7.24 (m, 5 H, Ar-H), 6.26 (s, 1H, C$\underline{H}_2$=C), 5.57- (s, 1H, C$\underline{H}_2$=C), 5.06 (s, 1H, H-3), 3.41 (s, 3H, CH_3-O).

^{13}C NMR (100 MHz; $CDCl_3$) δ =168.2 (C-1), 141.6 (C-2), 138.5, (C-1') 128.4 (2C) (C-3' and C-5'), 127.9 ($\underline{C}H_2$=C), 126.3 (2C) (C-2' and C-6'), 125.6 (C-4'), 83.2 (C-3), 56.9 ($\underline{C}H_3$-O).

MS Electrospray low res: $M+H^+$ = 192.1.

The remaining nitrile **110** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 24.6 min, t_{major}= 23.0 min.

(*S)-(2-((fluorophenyl)(methoxy)methyl)acrylamide 111a**



The title product was obtained as a white solid (19 mg, 22.0 % yield). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 11.60 min, t_{major} = 11.11 min.

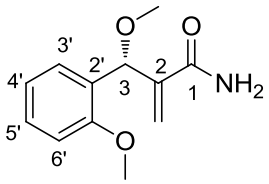
^{1}H NMR (400 MHz; MeOD) δ = 7.38-7.29 (m, *J*= 7.3 Hz, 2 H, Ar), 7.16 (t, *J*= 7.7 Hz, 1H, Ar), 7.06 (t, *J*= 10.1 Hz, 1H, Ar), 5.92 (s, 1H, H-3), 5.51 (d, *J*= 10.1 Hz, 2H, $C\underline{H}_2$=C), 3.28 (s, 3H, $C\underline{H}_3$O-CH).

^{13}C NMR (100 MHz; MeOD), δ =171.0 (C-1), 143.6 (C-1'), 129.7 (C-2), 129.6 ($\underline{C}H_2$=C), 128.4 (C-4'), 124.0 (C-5' and C-3'), 119.6 (C-6' and C-2'), 75.1 (C-3), 56.2 ($\underline{C}H_3$-O).

GCMS- (EI), retention time = 11.48 min, m/z = 209 (26.3 %), 194 (16.1 %), 178 (46.1 %), 161 (12.4 %), 139 (100 %), 123, 44.

The remaining nitrile **111** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 11.5 min, t_{major}= 11.0 min.

(*S*)-2-(methoxy(2-methoxyphenyl)methyl)acrylamide 112a



The title product was obtained as a clear oil (15 mg, 12.2 % yield). Chiral HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 17.04 min, t_{major} = 20.4 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.36 (dd, *J*= 7.7 Hz, *J*= 1.8 Hz, 1 H, H-3'), 7.28 (dd, *J*= 7.3 Hz, *J*= 1.8 Hz, 1H, H-5'), 6.97 (t, *J*= 7.3 Hz, 1H, H-4'), 6.91-6.86 (d, *J*= 8.2 Hz, 1H, H-6'), 6.18 (s, 1H, H-3), 5.51(s, 1H, C$\underline{H}_2$=C), 5.43 (s, 1H, C$\underline{H}_2$=C), 3.82 (s, 3H, C$\underline{H}_3$-O-Ar), 3.38 (s, 3H, C$\underline{H}_3$-O).

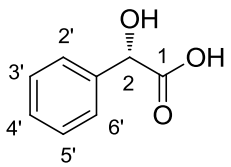
^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.3 (C-1), 157.2 (C-1'), 141.3 (C-2), 130.3 (C-3'), 130.1 (C-5'), 129.3 (C-2'), 127.2 (C$\underline{H}_2$=C), 124.8 (C-4'), 120.8 (C-6'), 57.2 (Ar-O$\underline{C}H_3$), 55.5 ($\underline{C}H_3$O-C).

MS Electrospray low res: $M+H^+$ = 222.1.

The remaining nitrile **112** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 12.2 min, t_{major}= 11.2 min.

6.4.7 BIOTRANSFORMATIONS OF RACEMIC α-SUBSTITUTED NITRILES

(*S*)-2-hydroxy-2-phenylacetic acid 56a [25]



The title product was obtained as a white solid (15 mg, 8.2 % yield), (99.9 % ee). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-

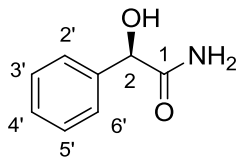
ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 18.3 min, t_{major} = 16.4 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.38-7.26 (m, 5 H, Ar), 5.28 (s, 1H, H-2).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 178.1 (C-1), 132.8 (C-1'), 129.8 (C-3 and C-5''), 127.2 (C-4'), 126.8 (C-2' and C-6'), 73.4 (C-2).

MS Electrospray low res: $M+H^+$ = 153.0

(*R*)-2-hydroxy-2-phenylacetamide 56b [25]



The title product was obtained as a white solid (52 mg, 44.1 % yield). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 14.0 min, t_{major} = 17.8 min.

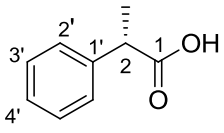
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.79-7.44 (m, 5 H, Ar), 5.45 (s, 1H, H-2).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 175.5 (C-1), 134.1 (C-1'), 129.8 (C-2' and C-6'), 129.2 (C-3' and C-5'), 127.6 (C-4'), 76.6 (C-2)

MS Electrospray low res: $M+H^+$ = 152.0

The remaining nitrile **56** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 9.6 min, t_{major}= 8.8 min.

(*S*)-2-phenylpropionic acid 24a [26] [27]



The title product was obtained as a white solid (22 mg, 19.3 % yield), (98.5 % ee). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 7.5 min, t_{major} = 8.6 min. $[\propto]_D^{25}$= +18.6 (*c*= 0.15, $CHCl_3$) compared with literature (*R*)- $[\propto]_D^{25}$= -57.3 (*c*= 0.48, $CHCl_3$).

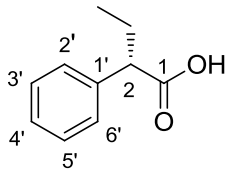
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.79-7.44 (m, 5 H, Ar), 3.76 (q, *J*= 6.9 Hz, 1H, H-2), 1.51 (d, *J*= 7.3 Hz, 3H, C$\underline{H}_3$-CH).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 176.7 (C-1), 141.8 (C-1'), 128.9 (C-2' and C-6'), 128.7 (C-3' and C-5'), 128.5 (C-4'), 46.5 (C-2), 18.6 ($\underline{C}H_3$-CH).

MS Electrospray low res: $M+H^+$ = 151.2.

The remaining nitrile **24** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 14.3 min, t_{major}= 13.1 min.

(*S*)-2-phenylbutanoic acid 25a [26]



The title product was obtained as a white solid (22 mg, 17.0 % yield), ee = 45.7 %. Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 7.4 min, t_{major} = 8.2 min. $[\propto]_D^{25}$= +18.6 (*c*= 0.15, $CHCl_3$), (*S*)- compared with literature $[\propto]_D^{25}$= +104 (*c*= 01.2, toluene)

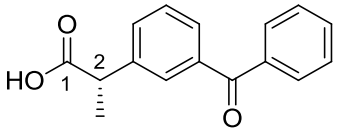
^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.38-7.28 (m, 5 H, Ar-H), 3.41 (t, *J*= 7.28 Hz, 1H, H-2), 2.07 (m, 1H, C$\underline{H}_2$-CH_3), 1.79 (m, 1H, C$\underline{H}_2$-CH_3), 0.89 (t, *J*= 7.33, 3H, C$\underline{H}_3$-CH_2).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 171.1(C-1), 132.0 (C-1'), 128.9 (2C) (C-3'and C-5'), 126.1(2C) (C-2' and C-6'), 125.5 (C-4'), 53.2 (C-2), 26.2 ($\underline{C}H_2$-CH_3), 12.1 ($\underline{C}H_3$-CH_2).

MS Electrospray low res: $M+H^+$ = 165.

The remaining nitrile **25** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel OJ-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 9.7 min, t_{major}= 8.9 min

(*S*)-2-(3-benzoylphenyl)propanoic acid 113a [28] [29]



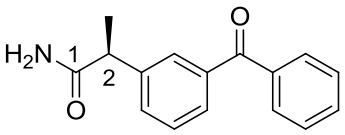
The title product was obtained as a white solid (19 mg, 20 % yield), (96.7 % ee). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 18.5 min, t_{major} = 22.4 min. $[\propto]_D^{25}$= +18.9 (*c*= 0.25, CH_2Cl_2) (S)- compared with literature $[\propto]_D^{25}$= +54.4 (*c*= 2.7, CH_2Cl_2).

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.78-7.76 (m, 3 H, Ar-H), 7.67-7.65 (m, 1 H, Ar-H), 7.58=7.53 (m, 2 H, Ar-H), 7.47-7.40 (m, 3 H, Ar-H), 3.80 (q, *J*= 7.3 Hz, 1H, H-2), 1.53 (d, *J*= 7.3 Hz, 3H, $C\underline{H}_3$-CH).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 179.8 (C=O), 178.1 (C-1), 140.0 (C-Ar), 137.3 (C-Ar), 132.5 (C-Ar), 131.6 (C-Ar), 130.0 (C-Ar), 129.3 (C-Ar), 129.2 (C-Ar), 128.5 (C-Ar), 128.3 (C-Ar), 45.1 (C-2), 18.1 ($CH\underline{C}H_3$).

MS Electrospray low res: $M+H^+$ = 255.

(*R*)-2-(3-benzoylphenyl)propanamide 114b [28]



The title product was obtained as a white solid (19 mg, 20 % yield), (45.7 % ee). Chiral HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase with a flow rate of 0.8 mL/min gave t_{minor}= 25.80 min, t_{major} = 24.06 min.

^{1}H NMR (400 MHz; $CDCl_3$) δ = 7.78-7.76 (m, 3 H, Ar-H), 7.67-7.65 (m, 1 H, Ar-H), 7.58=7.53 (m, 2 H, Ar-H), 7.47-7.40 (m, 3 H, Ar-H), 3.97 (q, *J*= 7.3 Hz, 1H, H-2), 1.65 (d, *J*= 7.3 Hz, 3H, CH_3-CH).

^{13}C NMR (100 MHz; $CDCl_3$) δ = 196.6 (C=O), 176.7 (C-1), 141.8 (C-Ar), 138.1 (C-Ar), 137.4 (C-Ar), 132.7 (C-Ar), 131.6 (C-Ar), 130.2 (C-Ar), 129.4 (C-Ar), 129.2 (C-Ar), 128.9 (C-Ar), 128.7 (C-Ar), 128.5 (C-Ar), 46.5 (C-2), 18.6 (CH<u>C</u>H_3).

MS Electrospray low res: $M+H^+$ = 254.

The remaining nitrile **114** was purified, all spectroscopic data correlated to that reported previously. The enantiomers were separated by HPLC analysis using a Chiralcel AD-H column with a mixture of hexane and propan-2-ol (90:10) 0.1 % TFA as the mobile phase, t_{minor}= 15.3 min, t_{major}= 17.2 min.

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APPENDIX 1

TABLES ACCOMPANYING CHAPTER 2

APPENDIX 1

Table a: Irish soil isolates enriched on various nitriles and genes previously detected.

Isolate	Source	Geographic origin	Enrichment	Gene detected
Bacillus sp. NN1	Soil 1	Ireland	Acetonitrile	NHase
Rhodococcus sp. NN2	Soil 1	Ireland	Acetonitrile	NHase
Nit (-)3, Bacillus sp.	Soil 2	Ireland	Acetonitrile	
Nit(-)4, R. erythropolis	Soil 2	Ireland	Acetonitrile	
Rhodococcus sp. NN5a	Soil 1	Ireland	Acetonitrile	NHase
Rhodococcus sp. NN5b	Soil 1	Ireland	Acetonitrile	NHase
Rhodococcus sp. NN6	Soil 7	Ireland	Acetonitrile	NHase
Nit(-)7, Burkholderia sp.	Soil 7	Ireland	Acetonitrile	
Burkholderia sp. NN8	Soil 2	Ireland	Acetonitrile	NHase
Nit(-)9, A. aurescens	Soil 3	Ireland	Benzonitrile	
Rhodococcus sp. NN10	Soil 3	Ireland	Benzonitrile	NHase
Nit(-)11, Rhodococcus sp.	Soil 7	Ireland	Benzonitrile	
Rhodococcus sp. NN12	Soil 1	Ireland	Benzonitrile	NHase
Rhodococcus sp. NN13	Soil 5	Ireland	Benzonitrile	NHase
Nit(-)14, Microbacterium sp.	Soil 5	Ireland	Benzonitrile	
Nit(-)15, Burkholderia sp.	Soil 7	Ireland	Acrylonitrile	
Nit(-)16, R. erythropolis	Soil 1	Ireland	Adiponitrile	
Nit(-)17	Soil 1	Ireland	Adiponitrile	
Nit(-)18, R. erythreus	Soil 1	Ireland	Adiponitrile	
Nit(-)19, Burkholderia sp.	Soil 1	Ireland	Adiponitrile	
Burkholderia sp. NN20	Soil 3	Ireland	Adiponitrile	NHase
Nit(-)21, mixed	Soil 3	Ireland	Adiponitrile	
Nit(-)22, Burkholderia sp.	Soil 4	Ireland	Adiponitrile	
Nit(-)23	Soil 6	Ireland	Adiponitrile	
Nit(-)24	Soil 6	Ireland	Adiponitrile	
Nit(-)25	Soil 6	Ireland	Adiponitrile	
Nit(-)26, mixed	Soil 4	Ireland	Acryonitrile	
Arthrobacter sp. NN27	Soil 3	Ireland	Acrylonitrile	NHase
Nit(-)28, Burkholderia	Soil 3	Ireland	Acrylonitrile	
Burkholderia sp. NN29	Soil 6	Ireland	Acrylonitrile	NHase
Bacillus sp. NN30	Soil 4	Ireland	Mandelonitrile	NHase
Bacillus sp. NN31	Soil 5	Ireland	Mandelonitrile	NHase
Paenibacillus sp. NN32	Soil 1	Ireland	Phenylacetonitrile	NHase
Nit(-)33, Paenibacillus sp.	Soil 1	Ireland	Phenylacetonitrile	
Paenibacillus sp. NN34	Soil 1	Ireland	Phenylacetonitrile	NHase
Nit(-)35, Actinobacterium	Soil 4	Ireland	Phenylacetonitrile	
Nit(-)36, Arthrobacter sp.	Soil 6	Ireland	Phenylacetonitrile	
Nit(-)37, Arthrobacter sp.	Soil 6	Ireland	Phenylacetonitrile	

Table b : Foreign soil isolates enriched on various nitriles and genes previously detected.

Isolate	Source	Geographic origin	Enrichment	Gene detected
F1	11	Australia	Acetonitrile	
F2	11	Australia	Acetonitrile	
F3	11	Australia	Acetonitrile	
F4	11	Australia	Acetonitrile	
F5	11	Australia	Acetonitrile	
F6	11	Australia	Acetonitrile	
F7, *Arthrobacter* sp.	11	Australia	Acetonitrile	
F8	11	Australia	Benzonitrile	
F9	11	Australia	Adiponitrile	
Bacillus sp. LCF10	12	Australia	Acetonitrile	NHase
F11	12	Australia	Acetonitrile	
F12	12	Australia	Acetonitrile	
F13	12	Australia	Acetonitrile	
F14	12	Australia	Acetonitrile	
F15	13	Poland	Acetonitrile	
F16	13	Poland	Acetonitrile	
Rhodococcus sp. LCF17	13	Poland	Acetonitrile	NHase
F18, *Rhodococcus* sp.	13	Poland	Acetonitrile	
Rhodococcus sp. LCF19	13	Poland	Acetonitrile	NHase
F19	13	Poland	Acetonitrile	
F20	11	Australia	Benzonitrile	
F21	11	Australia	Benzonitrile	
F22	11	Australia	Benzonitrile	
F23	11	Australia	Benzonitrile	
F24	12	Australia	Acetonitrile	
F25	12	Australia	Acetonitrile	
F26, *Bacillus pumilus*	12	Australia	Benzonitrile	
F27	12	Australia	Benzonitrile	
F28	12	Australia	Benzonitrile	
F29	12	Australia	Benzonitrile	
Rhodococcus sp. LCF30	12	Australia	Benzonitrile	NHase
F31, *Rhodococcus* sp.	13	Poland	Benzonitrile	
F32	13	Poland	Benzonitrile	
F33	13	Poland	Benzonitrile	
F34	13	Poland	Benzonitrile	
F35	13	Poland	Benzonitrile	
F36	11	Australia	Adiponitrile	
Rhodococcus sp. LCF37	11	Australia	Adiponitrile	NHase
F38	11	Australia	Adiponitrile	
F39	12	Australia	Adiponitrile	
F40	12	Australia	Adiponitrile	
F41	12	Australia	Adiponitrile	
F42	12	Australia	Adiponitrile	
F43	12	Australia	Adiponitrile	
F44	13	Poland	Adiponitrile	
F45	13	Poland	Adiponitrile	
F46	13	Poland	Adiponitrile	
F47	13	Poland	Adiponitrile	
F48	13	Poland	Adiponitrile	
F49	13	Poland	Adiponitrile	
Curtobacterium sp. LCF50	11	Australia	Mandelonitrile	NHase
F51	11	Australia	Mandelonitrile	
Rhodococcus sp. LCF52	11	Australia	Mandelonitrile	NHase

F53	11	Australia	Mandelonitrile	
F54	12	Australia	Mandelonitrile	
Microbacterium sp. LCF55	12	Australia	Mandelonitrile	NHase
F56	12	Australia	Mandelonitrile	
F57	12	Australia	Mandelonitrile	
F58	12	Australia	Mandelonitrile	
F59	13	Poland	Mandelonitrile	
F60	13	Poland	Mandelonitrile	
Streptomyces sp. LCF61	13	Poland	Mandelonitrile	NHase
F62, *Bacillus* sp.	13	Poland	Mandelonitrile	
F63	11	Australia	Acrylonitrile	
F64	11	Australia	Acrylonitrile	
F65	11	Australia	Acrylonitrile	
F66	11	Australia	Acrylonitrile	
F67	11	Australia	Acrylonitrile	
F68	12	Australia	Acrylonitrile	
F69	12	Australia	Acrylonitrile	
F70	13	Poland	Acrylonitrile	
F71	13	Poland	Acrylonitrile	
F72	13	Poland	Acrylonitrile	
Burkholderia sp. LCF73	13	Poland	Acrylonitrile	NHase
F74, *Microbacterium* sp.	11	Australia	Phenylacetonitrile	
F75	11	Australia	Phenylacetonitrile	
F76	12	Australia	Phenylacetonitrile	
F77	12	Australia	Phenylacetonitrile	
F78	13	Poland	Phenylacetonitrile	

Table c: Suburban soil isolates enriched on various nitriles and genes previously detected.

Isolate	**Source**	**Geographic origin**	**Enrichment**	**Gene detected**
SS1 1	9	Ireland	Acetonitrile	
Rhodococcus sp. SS1-2	9	Ireland	Acetonitrile	NHase
SS1 3	9	Ireland	Acetonitrile	
SS1 4, *R. erythropolis* sp.	10	Ireland	Acetonitrile	
SS1 5	10	Ireland	Acetonitrile	
SS1 6, *Enterobacter* sp.	8	Ireland	Acetonitrile	
Rahnella sp. SS1-7	8	Ireland	Acetonitrile	NHase
SS1 8	8	Ireland	Acetonitrile	
SS1 9	8	Ireland	Acetonitrile	
SS1 10	9	Ireland	Adiponitrile	
SS1 11	9	Ireland	Adiponitrile	
SS1 12	9	Ireland	Adiponitrile	
SS1 13	9	Ireland	Adiponitrile	
SS1 14	9	Ireland	Adiponitrile	
Microbacterium sp. SET1	9	Ireland	Adiponitrile	NHase
SS1 16	9	Ireland	Adiponitrile	
SS1 17	9	Ireland	Benzonitrile	
SS1 18	10	Ireland	Benzonitrile	
SS1 19	10	Ireland	Benzonitrile	
SS1 20	10	Ireland	Benzonitrile	
SS1 21	10	Ireland	Benzonitrile	
SS1 22	8	Ireland	Benzonitrile	
SS1 23	8	Ireland	Benzonitrile	
SS1 24,	8	Ireland	Benzonitrile	
Rahnella sp. SS1-25	9	Ireland	Benzonitrile	NHase

SS1 26	10	Ireland	Benzonitrile	
SS1 27, *Rahnella* sp.	8	Ireland	Benzonitrile	
SS1 28	8	Ireland	Acrylonitrile	
Rhodococcus sp. SS1-29	8	Ireland	Acrylonitrile	NHase
SS1 30, *Arthrobacter* sp.	9	Ireland	Acrylonitrile	
SS1 31	9	Ireland	Acrylonitrile	
SS1 32	10	Ireland	Acrylonitrile	
SS1 33	10	Ireland	Acrylonitrile	
SS1 34	10	Ireland	Acrylonitrile	
SS1 35	10	Ireland	Acrylonitrile	
SS1 36	8	Ireland	Acrylonitrile	
SS1 37	8	Ireland	Acrylonitrile	
SS1 38	8	Ireland	Acrylonitrile	

Table d :Seaweed isolates enriched on various nitriles and genes previously detected.

Isolate	**Source**	**Geographic origin**	**Enrichment**	**Gene detected**
SW1	Ulva	Ireland	Acetonitrile	
SW2	Ulva	Ireland	Acetonitrile	
SW3	Asco	Ireland	Acetonitrile	
SW4	Asco	Ireland	Acetonitrile	
SW5	Ulva	Ireland	Acetonitrile	
SW6	Poly	Ireland	Acetonitrile	
SW7	Asco	Ireland	Acetonitrile	
SW8	Ulva	Ireland	Benzonitrile	
SW9	Asco	Ireland	Benzonitrile	
SW10	Poly	Ireland	Benzonitrile	
SW11	Poly	Ireland	Benzonitrile	
SW12	Asco	Ireland	Benzonitrile	
SW13	Asco	Ireland	Benzonitrile	
SW14	Asco	Ireland	Benzonitrile	
SW15	Ulva	Ireland	Adiponitrile	
SW16	Ulva	Ireland	Adiponitrile	
SW17	Ulva	Ireland	Adiponitrile	
SW18	Ulva	Ireland	Adiponitrile	
SW19	Poly	Ireland	Adiponitrile	
SW20	Asco	Ireland	Adiponitrile	
SW21	Ulva	Ireland	Adiponitrile	
SW22	Poly	Ireland	Adiponitrile	
SW23	Asco	Ireland	Adiponitrile	
SW24	Ulva	Ireland	Acrylonitrile	
Bacillus sp. SW1-25	Poly	Ireland	Acrylonitrile	NHase
SW26	Asco	Ireland	Acrylonitrile	
SW27	Ulva	Ireland	Acrylonitrile	
SW28	Ulva	Ireland	Acrylonitrile	
Rhodococcus sp. SW1-29	Poly	Ireland	Acrylonitrile	NHase
Arthrobacter sp. SW1-30	Asco	Ireland	Acrylonitrile	NHase

Table e:Seaweed isolates enriched on various nitriles and genes previously detected.

Isolate	Source	Geographic origin	Enrichment	Gene detected
SW2 1	Fucus	Ireland	Acetonitrile	
SW2 2	Fucus	Ireland	Acetonitrile	
Rhodococcus sp. SW2-3	Ulva I	Ireland	Acetonitrile	NHase
SW2 4, mixed	Ceranium	Ireland	Acetonitrile	
SW2 5	Ceranium	Ireland	Acetonitrile	
SW2 6	Ceranium	Ireland	Acetonitrile	
SW2 7	Ceranium	Ireland	Acetonitrile	
SW2 8	Ulva I	Ireland	Acetonitrile	
SW2 9	Ceranium	Ireland	Acetonitrile	
SW2 10	Ulva I	Ireland	Adiponitrile	
SW2 11	Ulva L	Ireland	Adiponitrile	
SW2 12, *Agrobacter* sp.	Ulva L	Ireland	Adiponitrile	
SW2 13	Ulva L	Ireland	Adiponitrile	
SW2 14	Palmari	Ireland	Adiponitrile	
SW2 15	Ceranium	Ireland	Adiponitrile	
SW2 16	Ceranium	Ireland	Adiponitrile	
SW2 17	Ulva I	Ireland	Benzonitrile	
Serratia sp. SW2-18	Ulva L	Ireland	Benzonitrile	NHase
SW2 19	Ulva L	Ireland	Benzonitrile	
Bacillus sp. SW2-20	Palmari	Ireland	Benzonitrile	NHase
SW2 21, *Bacillus subtilis*	Palmari	Ireland	Benzonitrile	
SW2 22, *Rahnella* sp.	Ceranium	Ireland	Benzonitrile	
SW2 23	Ceranium	Ireland	Benzonitrile	
SW2 24, *Serratia* sp.	Ceranium	Ireland	Benzonitrile	
Microbacterium sp. SW2-25	Ceranium	Ireland	Benzonitrile	NHase
SW2 26, mixed	Ceranium	Ireland	Benzonitrile	
SW2 27, *Citrobacter* sp.	Ceranium	Ireland	Benzonitrile	
Buttiauxella SW2-28	Ulva L	Ireland	Acrylonitrile	NHase
SW2 29, *Halomonas* sp.	Fucus	Ireland	Acrylonitrile	
SW2 30, *Exiquobacterium* sp.	Fucus	Ireland	Acrylonitrile	
SW2 31	Palmari	Ireland	Acrylonitrile	
SW2 32	Palmari	Ireland	Acrylonitrile	
SW2 33, *Serratia* sp.	Palmari	Ireland	Acrylonitrile	
SW2 34	Ceranium	Ireland	Acrylonitrile	
SW2 35	Ceranium	Ireland	Acrylonitrile	
SW2 36, *Klebsiella* sp.	Ceranium	Ireland	Acrylonitrile	
SW2 37	Ceranium	Ireland	Acrylonitrile	

Table f:Foreign soil isolates that could grow in the presence of β-hydroxynitriles of interest.

	3-HGN	3-HBN	3-HPPN
F3		✓	
F9			✓
Rhodococcus sp. LCF19		✓	✓
F19		✓	
F20	✓	✓	
Rhodococcus sp. LCF30	✓		✓
F31, *Rhodococcus* sp.		✓	✓
F32	✓	✓	
F33		✓	
F36	✓	✓	✓
Rhodococcus sp. LCF37	✓		
F41	✓		✓
F42		✓	✓
F43		✓	
F44		✓	
F45	✓	✓	
F46	✓	✓	
F47	✓	✓	✓
F48		✓	
F49			✓
Curtobacterium sp. LCF50		✓	
F53	✓	✓	
F54	✓	✓	✓
Microbacterium sp. LCF55		✓	
F56	✓	✓	
F57	✓	✓	
F58		✓	
F59	✓	✓	
F60		✓	
Streptomyces sp. LCF61		✓	
F62, *Bacillus* sp.	✓	✓	✓
F63	✓	✓	
F64	✓	✓	
F65	✓	✓	✓
F66	✓	✓	
F67		✓	
F68	✓	✓	
F69	✓	✓	
F70	✓	✓	
F71	✓	✓	✓
F72		✓	✓
Burkholderia sp. LCF73		✓	
F74, *Microbacterium* sp.		✓	
F75		✓	
F76		✓	
F78		✓	

Table g:Suburban soil isolates that could grow in the presence of β–hydroxynitriles of interest.

	3-HGN	3-HBN	3-HPPN
SS1 1		✓	
Rhodococcus sp. SS1-2	✓	✓	
SS1 3		✓	
SS1 4, *R. erythropolis* sp.	✓	✓	
SS1 5		✓	✓
SS1 6, *Enterobacter* sp.	✓	✓	✓
Rahnella sp. SS1-7		✓	
SS1 12	✓	✓	✓
SS1 14	✓	✓	✓
Microbacterium sp. SET1	✓	✓	✓
SS1 16	✓		
SS1 17	✓	✓	✓
SS1 18	✓	✓	✓
SS1 19			✓
SS1 22	✓	✓	✓
SS1 24,	✓	✓	
Rahnella sp. SS1-25		✓	✓
SS1 27, *Rahnella* sp.		✓	
SS1 34	✓	✓	
SS1 36			✓
SS1 37	✓		

Table h: Seaweed soil isolates that could grow in the presence of β-hydroxynitriles of interest.

	3-HGN	3-HBN	3-HPPN
SW1			
SW2	✓		
SW3	✓	✓	
SW4		✓	
SW5	✓	✓	
SW6			
SW7		✓	
SW8	✓		
SW28	✓		
Arthrobacter sp. SW1-30	✓		

Table i: Seaweed soil isolates that could grow in the presence of β–hydroxynitriles of interest.

	3-HGN	3-HBN	3-HPPN
SW2 2	✓		
SW2 13			✓
SW2 17		✓	
SW2 21, *Bacillus subtilis*			✓
SW2 22, *Rahnella* sp.			✓
SW2 27, *Citrobacter* sp.	✓		
SW2 30, *Exiquobacterium*			✓
SW2 32			✓
SW2 33, *Serratia* sp.		✓	✓
SW2 34	✓		✓
SW2 35	✓		✓
SW2 36, *Klebsiella* sp.	✓		

Table j:Isolates demonstrating activity towards two or more β-hydroxynitriles.

Isolate	3-HBN	3-HGN	3-HPPN
F66	low	low	
F62	low	low	high
NN12	good	low	
F71	good	low	
NN36	low	low	
F45	low	good	
F71	good	good	
F69	good	good	
SS1-12	good	good	good
SS1-22	good	low	
SS1-24	good	low	low
Nit (-)9	good	good	
NN30	good	good	
F73	good	low	
SS1-4	high	low	
SET1	high	low	

Table k: Isolates demonstrating the highest activity towards β-hydroxynitriles.

3-HGN	3-HBN	3-HPPN
F32	F36	SS1-24
F41	F69	SS1-12
F37	F71	F62
F30	SS1-12	SS1-14
SS1-12	SS1-5	
SS1-22	NN32	
SS1-18	LC3c	
NN30	SW2-33	
SS1-4	NN30	
SET1	SS1-31	

Growth studies of Isolates with activity towards β-hydroxynitriles

Table l : Growth studies of Isolates with activity towards 3-hydroxyglutaronitrile.

Isolate	Optical density 600nm				Optimum
	15 °C	20 °C	25 °C	30 °C	temperature
F32	1.63	0.63	0.43	0.11	15 °C
F41	1.06	0.74	0.37	0.2	15 °C
F37	2.5	0.73	0.47	0.13	15 °C
F30	1.87	0.52	0.32	0.13	15 °C
SS1-12	2.37	0.55	0.35	0.06	15 °C
SS1-22	0.51	0.33	1	0.14	25 °C
SS1-18	0.16	0.72	1.48	0.15	25 °C
NN30	0.23	0.88	0.48	0.14	20 °C
SS1-4	0.42	0.66	0.36	0.45	20 °C
SS1-TE	2.08	0.47	0.3	0.13	15 °C

Table m: Growth studies of Isolates with activity towards 3-hydroxybutyronitrile.

Isolate	Optical density 600nm				Optimum
	15 °C	20 °C	25 °C	30 °C	temperature
F36	1.63	2.13	0.89	1.25	20 °C
F69	1.06	1.79	0.97	1.07	20 °C
F71	2.5	2.27	1.02	1.03	15 °C
SS1-12	1.87	1.1	0.5	0.61	15 °C
SS1-TE	2.37	1.31	0.66	0.67	15 °C
NN32	0.51	0.29	0.29	0.07	15 °C
LC3c	0.16	0.84	0.44	1.00	30 °C
SW2-33	0.23	0.37	0.39	0.76	30 °C
NN30	0.42	0.23	0.19	0.37	15 °C
SS1-31	2.08	2.44	1.23	1.87	20 °C

Table n:Growth studies of Isolates with activity towards 3-hydroxy-3-phenylpropionitrile

Isolate	Optical density 600nm				Optimum
	15 °C	20 °C	25 °C	30 °C	temperature
SS1-24	2.19	0.09	0.08	0	15 °C
SS1-12	2.85	0.02	0.03	0.04	15 °C
F62	0.05	0.04	0	0.01	15 °C
SS1-14	3.65	0.03	0	0	15 °C

Investigation into the temperature of induction on the activity

Table o: Investigation into the temperature of induction on the activity of isolates towards 3-hydroxyglutaronitrile.

Isolate	Concentration of ammonia produced at 25 °C (mmol)				Optimum	Optimum
	15 °C	20 °C	25 °C	30 °C	activity	Growth
F32	0	0.752	1.726	0	25 °C	20 °C
F41	0	0	1.629	0.667	25 °C	20 °C
F37	0	0.397	1.140	0.301	25 °C	15 °C
F30	0	1.866	2.150	0	25 °C	15 °C
SS1-12	0	0.018	0.772	0	25 °C	15 °C
SS1-22	0	1.433	1.330	0	25 °C	15 °C
SS1-18	0		0.910	0	25 °C	30 °C
NN30	0	0.637	1.790	0.603	25 °C	30 °C
SS1-4	0	1.126	1.468	0.111	25 °C	15 °C
SET1	0	0.430	1.144	0	25 °C	20 °C

Table p: Investigation into the temperature of induction on the activity of isolates towards 3-hydroxybutyronitrile.

Isolate	Concentration of ammonia produced at 25 °C (mmol)				Optimum	Optimum
	15 °C	20 °C	25 °C	30 °C	activity	Growth
F36	2.630	0	0.515	0	15 °C	20 °C
F69	0	1.206	0.424	0	20 °C	20 °C
F71	0.603	0.820	4.389	0	25 °C	15 °C
SS1-12	0.222	0.582	1.430	0.420	25 °C	15 °C
SET1	0	0.983	1.021	0.699	25 °C	15 °C
NN32	0.073	0.377	2.91	3.090	20 °C	15 °C
LC3c	0	1.521	0.640	1.650	30 °C	30 °C
Sw2-33	0	1.000	0.778	0	20 °C	30 °C
NN30	0	3.102	0.494	0.178	20 °C	15 °C
SS1-31	1.951	2.126	0	2.42	20 °C	20 °C

Alternative Induction

Table q: Results of Nesslers activity assay towards succinonitrile and 3-hydroxyglutarinitrile when induced using succinonitrile.

Isolate	Optical density (600 nm)	Activity towards 3-hydroxyglutaroitrile	Activity towards succinonitrile
F37	0.61	0	0.248
SS1-22	0.73	0	0.222
SS1-4	0.35	0	0.906
F32	0.76	0	0
F30	0.89	0	0
SS1-18	0.53	0	0
SET1	0.43	0	0.132
F41	1.37	0	0.073
SS1-12	0.9	0	0.078
NN30	0.83	0	0.708

Table r :Results of Nesslers activity assay towards butyronitrile and 3-hydroxybutyronitrile when induced using butyronitrile.

Isolate	Optical density (600 nm)	Activity towards 3-hydroxybutyroitrile	Activity towards butyronitrile
F36	0.76	0	0.078
F69	0.89	0	0.708
F71	0.53	0	0.906
SS1-12	1.37	0	0
SS1-TE	0.9	0	0
NN32	0.83	0	0
LC3c	0.43	0	0
SW2-33	0.98	0	0.12
NN30	0.89	0	0.07
SS1-31	0.62	0	0.34

APPENDIX 2

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A high throughput screening strategy for the assessment of nitrile-hydrolyzing activity towards the production of enantiopure β-hydroxy acids

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ABSTRACT

Nitrile hydrolysing enzymes have found wide use in the pharmaceutical industry for the production of fine chemicals. This work presents a strategy that facilitates the rapid identification of bacterial isolates demonstrating nitrile hydrolysing activity. The strategy incorporates toxicity, starvation and induction studies along with subsequent colorimetric screening for activity, further focusing the assessment towards the substrates of interest. This high-throughput strategy uses a 96 well plate system, and has enabled the rapid biocatalytic screening of 256 novel bacterial isolates towards β-hydroxynitriles. Results demonstrate the strategy's potential to rapidly assess a variety of β-hydroxynitriles including aliphatic, aromatic and dinitriles. A whole cell catalyst *Rhodococcus erythropolis* SET1 was identified and found to catalyse the hydrolysis of 3-hydroxybutyronitrile with remarkably high enantioselectivity under mild conditions, to afford (*S*)-3-hydroxybutyric acid in 42% yield and >99.9% ee. The biocatalytic capability of this strain including the variation of parameters such as temperature and time were further investigated and all results indicate the presence of a highly enantioselective if not enantiospecific nitrilase enzyme within the microbial whole cell.

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1. Introduction

The awareness of the importance of chirality in conjunction with biological activity has led to an increasing demand for efficient methods for the industrial synthesis of enantiomerically pure compounds [1]. Many chiral drugs now employ chiral synthons in their synthesis typically utilising the chiral pool, kinetic resolution techniques, or asymmetric synthesis [2] to afford single enantiomer compounds. Drawbacks associated with these approaches can be the use of expensive substrates and/or chiral metal complex catalysts/reagents which also in the latter case may require specialised conditions, can be toxic and not easily recovered/reused [3]. The combination of chemical procedures with biocatalytic methods can offer an excellent alternative strategy for the production of such compounds [4,5].

The distinct features of enzymatic transformations of nitriles in particular are the formation of enantiopure carboxylic acids and the generation of enantiopure amides, which are valuable compounds in synthetic chemistry [6]. β-Hydroxy nitriles such as 3-hydroxybutyronitrile, 3-hydroxyglutaronitrile and 3-hydroxy-3-phenylpropionitriles can act as sources of β-hydroxy carboxylic acids *via* hydrolysis reactions however can be prone to elimination reactions under classical acid/base conditions [7]. Alternatively, nitrile biocatalysis can selectively facilitate this hydrolysis without affecting other acid- or alkali-labile functional groups present [8,9]. This potential has resulted in significant work to identify bacteria and fungi capable of hydrolysing such nitriles [10,11], whose products can be widely used as chiral precursors for pharmaceutical compounds. For example, 3-hydroxy-3-phenylpropionic acid and its derivatives, have been used as precursors to chiral drugs such as nisoxetine [12], fluoxetine [13] and tomoxetine [12]. Additionally, of particular commercial interest, is the nitrilase catalysed hydrolysis of 3-hydroxyglutaronitrile, the ethyl-ester of which is an intermediate to the cholesterol lowering drug (atorvastatin) Lipitor [14,10]. However the biotransformations of substrates having such a chiral centre remote from the cyano functional group have been reported to proceed with, in many cases, disappointingly low enantioselectivity and chemical yield [6,15–18].

A major issue in the development of a specific biotransformation is to find the appropriate biocatalyst. If there are no commercially available enzyme preparations the desired activities must be found either by screening for enzymatic activity in strains from culture collections or using microbial selection methods [19,20].

Selective enrichment is a method of choice for the isolation of nitrile hydrolysing micro-organisms, where nitrile substrates are used as a nitrogen source [19]. It is subsequently important

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to efficiently screen libraries of isolated strains to identify those possessing the desired reactivity and selectivity. Developing conventional methods such as high performance liquid chromatography (HPLC) or gas chromatography can be tedious and time consuming for assaying a large number of isolates.

Various high-throughput screening methods have been developed for nitrile hydrolysing enzymes, which have been reviewed by Martinkova [20] and Gong [21] recently. For example He et al. reported a simple, rapid and high throughput screening method for assaying nitrile hydrolysing enzymes based on ferric hydroxymate spectrophotometry [22]. Lin et al. found that a combination of ferrous and ferric ions could be used to distinguish α-amino nitriles, α-amino amides and α-amino acids in aqueous solution [23]. Zhang et al. recently developed a solid screen selective culture medium plate with bromcresol purple to screen isolates capable of converting iminodiacetonitrile to iminodiacetic acid [24]. However, colorimetric determination of ammonia as a common product of nitrilases and NHases/amidase activity provides a promising method for rapid screening [25]. For example the Berthelot method which quantifies ammonia formation colorimetrically at 640 nm after reaction with a phenol hypochlorite reagent [26]. Alternatively addition of Nesslers reagent to a solution containing ammonia produces an orange brown colour after development for a certain amount of time [27].

As mentioned previously highly effective and enantioselective biotransformations of β-hydroxynitriles would provide a convenient approach to producing optically active β-hydroxy carboxylic acids [28]. The objective of this study was to screen and identify bacteria with activity towards the β-hydroxynitriles from a novel bacterial isolate bank (previously collected from soil and seaweed) in our laboratory [29,30]. From the soil enrichment cultures a total of 187 isolates were purified, and 67 isolates were obtained from the seaweed enrichments [30]. We now report a high throughput screening strategy for the rapid identification of bacterial isolates possessing nitrile hydrolysing activity towards a variety β-hydroxynitriles including 3-hydroxybutyronitrile, 3-hydroxy-3-phenylpropionitrile and 3-hydroxyglutaronitrile, which represent an aliphatic, aromatic nitrile and dinitrile respectively. We also demonstrate the application of this strategy to a successful enantioselectivity screening study, resulting in the identification of an isolate demonstrating >99.9% ee for 3-hydroxybutyronitrile specifically.

2. Materials and methods

2.1. Materials

Racemic 3-hydroxybutyronitrile and 3-hydroxy-3-phenylpropionitrile were purchased from Sigma–Aldrich. All other chemicals were of analytical grade and obtained from various commercial sources.

2.2. Bacterial isolates

The 254 bacterial isolates used in this study are those described in Coffey et al. [30].

2.3. Nitrile toxicity studies

96 well Megablock® plates (Sarstedt Ltd.) containing 250 μl LB broth (Merck) and 10 mM β-hydroxy nitrile were inoculated from glycerol stocks of the isolate library and incubated at 25 °C with shaking at 250 rpm for 72 h. 5 μL of these cultures then transferred in a 96 well format to M9 [31] agar with modifications [32], (Merck agar-agar ultrapure (15% w/v) prepared in M9-media), containing β-hydroxy nitrile (10 mM) and incubated at 25 °C for 72 h.

2.4. Nitrogen starvation and induction of isolates

2.4.1. Nitrogen starvation

Successful isolates from Section 2.3 were inoculated to 250 μl M9 minimal media broth [31] with modifications [32], containing 10 mM β-hydroxynitrile as the sole source of N. Each well was then inoculated from M9-agar containing β-hydroxynitrile (10 mM) from Section 2.3 before incubation at 25 °C with orbital shaking at 250 rpm for 24 h.

2.4.2. Induction of nitrile metabolising enzyme activity

96-well Megablocks® containing 500 μL of M9 minimal media broth [31] with modifications [32] and β-hydroxy nitrile (10 mM) were inoculated with nitrogen starved isolates (20 μL) from Section 2.4.1 and incubated with shaking for 72 h at 25 °C and 250 rpm. 500 μL of 50% glycerol solution was then added to each culture before storage at −80 °C. These cultures of induced isolates served as inoculation and activity screening stocks for subsequent analyses.

2.5. An investigation of temperature on the growth of various isolates

96-well Megablocks® containing 500 μL of M9 broth and β-hydroxy nitrile (10 mM) were inoculated with nitrile induced isolates (20 μL) from Section 2.4.2 and incubated with shaking for 72 h at 15 °C, 20 °C, 25 °C and 30 °C at 250 rpm. The OD_{600nm} measured and recorded for future studies.

2.6. Standard curve for Nesslers colorimetric assay

Ammonium chloride (100 mM, 5.35 g) was dissolved in deionised water (1000 mL), this solution was used as a stock solution to prepare standards with the following concentration 1 mM, 2 mM, 3 mM, 4 mM and 5 mM. The absorbance was then measured at 425 nm. At NH_3 concentrations up to 10 mmol/L, the $A_{425\,nm}$ was directly proportional to NH_3 concentration $R^2 = 0.9992$.

2.7. Nesslers microscale ammonia assay

Induced isolates from Section 2.4.2 were initially screened for activity towards β-hydroxynitriles using the Nesslers colorimetric assay [27] modified and outlined in [33], in 96-well microtitre plates (Sarstedt Ltd.). Fresh cultures of each isolate were grown in M9-minimal media containing 10 mM nitrile as in Section 2.4.2 before washing 3 times with 500 μL of phosphate buffer). Each 150 μl reaction contained 10 mM nitrile and cells ($OD_{600\,nm} = 0.1$) in potassium phosphate buffer (100 mM, pH 7.0), Microtitre plates were sealed using adhesive film (Sarstedt) and incubated at 25 °C at 250 rpm for 24 h. The reaction was then quenched by adding 37.5 μl of 250 mM HCl. Plates were centrifuged at 500 g for 10 min to pellet the cells/debris. 20 μl of the supernatant was transferred to a microtitre plate, 181 μL of assay mastermix was added (155 μL deionised water, 1 μL 10 N NaOH, 25 μL Nesslers reagent (Merck). The reaction was allowed to stand for 10 min and then the absorbance read at 425 nm. Cell blanks contained cells @ $OD_{600nm} = 0.1$ in phosphate buffer. Nitrile blanks contained 150 μL of 10 mM nitrile in phosphate buffer.

2.8. General procedure for enantioselectivity screening towards 3-hydroxybutyronitrile

Racemic nitrile (5.1 mg, 5.9 μL, 10 mM) was added in one portion to a solution of potassium phosphate buffer (0.1 M, pH = 7.2, 6 mL) containing induced cells ($OD_{600\,nm}$ = 1), and activated at 25° C for 30 min with orbital shaking (250 rpm). The reaction was quenched after 24 h by removal of the biomass by centrifugation at 3000 × *g*. The resulting aqueous solution was acidified by the addition of 1 M HCl (200 μL). The aqueous portion was then extracted with ethyl acetate, the extracts were dried over $MgSO_4$ and the solvent removed under vacuum. Silver oxide (1 equiv, 0.06 mmol, 13.6 mg), benzylbromide (4 equiv, 0.24 mmol, 28 μL) and dichloromethane (2 mL) were added and the mixture stirred in the dark for 24 h. The reaction mixture was diluted with acetone and filtered through a 0.45 μm filter and solvent was removed under vacuum. 1 mL of mobile phase (90% hexane: 10% IPA) was added before the solution was injected on the Chiral HPLC system. All experiments were performed in triplicate. % enantiomeric excess is calculated from the ratio of the enantiomer by the difference in peak area divided by the sum of the peak areas for the major and minor enantiomers.

2.9. General procedure for large scale biotransformation towards 3-hydroxybutyronitrile

The procedure for large scale biotransformation of racemic 3-hydroxybutyronitrile was similar to the general procedure, with the exception of the use of potassium phosphate buffer (0.1 M, pH 7.0, 100 mL) containing 3-hydroxybutyronitrile (85.1 mg, 10 mM). The resulting aqueous solution was basified to pH 12 with aqueous NaOH (2 M) and extracted with ethyl acetate (3 × 100 mL). The aqueous solution was acidified using aqueous HCl (2 M) to pH 2 and extracted with ethyl acetate (3 × 100 mL), dried over $MgSO_4$ and the solvent removed under vacuum. The crude product was subjected to silica gel column chromatography eluted with a mixture of hexane and ethyl acetate (1:1) to give 3-hydroxybutyric acid in 42% yield (44 mg, 4.23 mmol) as clear oil. The configuration of the corresponding acid was determined by comparing the direction of specific rotation with that of an authentic sample. Enantiomeric excess values were obtained from HPLC analysis using a column of chiral stationary phase and correlated with literature. (*R*)-enantiomer elutes at 11.94 min, (*S*)-enantiomer elutes at 12.34 min [34]. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 4.19–4.27 (1H, m), δ = 2.45–2.58 (2H, m), δ = 1.23 (3H, d, J = 6.3 Hz). ^{13}C NMR (400 MHz, $CDCl_3$) δ = 117, 76, 64, 42, 22 $[\alpha]^d_{25}$ = +4.0 (c = 2.5, MeOH), and compared with that in the literature $[\alpha]^d_{25}$ = +4.1 (c = 2.7, MeOH) [35]. This experiment was performed in triplicate.

2.10. Chiral HPLC separations

Chiralcel AD-H and OJ-H columns (all from Daicel Chemical Industries) were used for chiral analysis. Chiralcel AD-H was used for the resolution of β-hydroxyacids. Analytical conditions applied: 90% hexane, 10% IPA and 0.1% TFA, with a flow rate of 0.8 mL/min and a detection wavelength of 215 nm. Chiralcel OJ-H was used for the resolution of β-hydroxyamides and nitriles using the same mobile phase conditions with the exception of TFA. The biotransformation products of 3-hydroxybutyronitrile were first derivatised to their corresponding β-benzyloxyethers before analysis.

2.11. Conventional PCR screening and DNA Sequencing

Bacterial genomic DNA was purified using the DNeasy Tissue Kit (Qiagen, Germany) as per the manufacturer's instructions. The 16S ribosomal DNA was amplified using primers 63f and 1387r [36]. The 15 μl reaction mix contained 7.5 μl GoTaq® Green Master Mix (Promega UK), 15 pmoles each primer and 30 ng DNA. The PCR conditions used for amplification were; 1 cycle of 95 °C for 5 min, 30 cycles of 95 °C for 1 min, 56 °C for 1 min, 72 °C for 1 min, followed by 1 cycle of 72 °C for 5 min. PCR products were purified using the DNA Clean and Concentrator – 5 kit (Zymo Research, CA, USA), as per the manufacturer's instructions. DNA sequencing of PCR products was performed using the BigDye 3.1 kit (Applied Biosystems) as per the manufacturer's instructions and analysed using an ABI Prism 310 Genetic Analyser (Applied Biosystems, CA, USA).

Nucleotide sequences were analysed using the BLASTn or BLASTx software [37] (http://www.ncbi.nlm.nih.gov/BLAST/) from the GenBank (NCBI) database The sequence of isolate SET1 was deposited in GenBank with accession number KF156942.

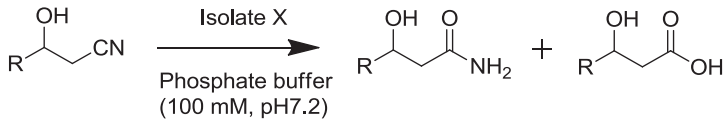
Fig. 1. Nesslers colorimetric activity assay towards 3-hydroxybutyronitrile, an example of library screening for nitrile-hydrolyzing enzyme activity in a 96-well plate format. The biotransformation was carried out at 25 °C with 10 mM nitrile. The reaction was then quenched by the addition of HCl. The biomass was removed by centrifugation. 20 μl of the supernatant was transferred to a microtitre plate, 181 μL of assay mastermix was added. The reaction was allowed to stand for 10 min and then the absorbance read at 425 nm.

3. Results and discussion

3.1. High throughput activity screening

The bacterial isolates were first subjected to toxicity studies with the nitriles of interest. By attempting to grow the different isolates in rich medium in the presence of β-hydroxynitriles, the isolates that were sensitive to the nitrile were identified and excluded from further screening. 145 of the initial 256 isolates could grow in the presence of 3-hydroxybutyronitrile, with 77 demonstrating growth after a 48 h period and the remaining 68 after 144 h. A total of 65 of the isolates grew in the presence of 3-hydroxy-3-phenylpropionitrile and 107 in the presence of 3-hydroxyglutaronitrile. The toxicity study demonstrated that some isolates show no growth in the presence of the nitriles, where as 16 isolates showed growth against two or more nitriles such as isolate SS1-12. The tolerant isolates then underwent a starvation process in order to ensure that the β-hydroxynitrile would be utilised as the nitrogen source during the induction stage, which involved attempting to grow the isolates in minimal media using the β-hydroxynitrile as the sole nitrogen source. As bacterial isolates utilising β-hydroxynitriles as a sole nitrogen source result in the release of ammonia, enzyme activity was monitored using the technique of Nesslerisation. A high-throughput technique was developed using the Nesslers microscale colorimetric assay to screen for activity in the hydrolysis of β-hydroxynitriles to β-hydroxycarboxylic acids using the Nesslers colorimetric assay [27] modified and outlined in [33], in 96 well microtitre plates (Sarstedt Ltd.) as shown in Fig. 1. Nitrile hydrolyzing activity was calculated by measuring the absorbance of the sample at 425 nm, and relating the absorbance to the standard curve in order to determine the concentration of NH_3 in the sample.

Table 1
Activity screening towards β-hydroxynitriles.



Isolate	R	Temperature for optimum growth (°C)	Optimum activity temperature (°C)	Activity[a] (mmol/L)
3-Hydroxyglutaronitrile				
F41	CH_2CN	15	25	6.29
F30	CH_2CN	15	25	2.150
NN30	CH_2CN	20	25	1.790
F32	CH_2CN	15	25	1.726
SS1-4	CH_2CN	15	25	1.468
SS1-24	CH_2CN	20	20	1.433
SET-1	CH_2CN	15	25	1.144
F37	CH_2CN	15	25	1.140
SS1-18	CH_2CN	25	25	0.910
SS1-12	CH_2CN	15	25	0.772
3-Hydroxybutyronitrile				
NN32	CH_3	15	25	9.91
F71	CH_3	15	25	4.389
NN30	CH_3	15	20	3.120
SS1-31	CH_3	20	30	2.42
LC3c	CH_3	30	20	1.521
SS1-12	CH_3	15	25	1.430
F69	CH_3	20	20	1.206
SET-1	CH_3	15	25	1.021
Sw2-33	CH_3	30	20	1.00
F36	CH_3	20	25	0.515
3-Hydroxy-3-phenylpropionitrile				
SS1-14	Ph	15	25	8.07
F62	Ph	15	25	7.64
SS1-12	Ph	15	25	1.61
SS1-24	Ph	15	25	0.21

[a] Activity determined using Nessler's colorimetric activity (24 h) assay expressed in terms of concentration (mmol/L) of NH_3 produced @ reported optimum temperature (column 4).

Reaction temperature is a key parameter in biotransformations which can significantly influence the activity, enantioselectivity and stability of a biocatalyst [38]. The isolate library was first screened for the hydrolysis of β-hydroxynitriles to β-hydroxycarboxylic acids at 25 °C and subsequently at various temperatures (Table 1). Based on the activity assay results the isolates were then divided into three subcategories based on the concentration of NH_3 produced (0.08–0.6 mmol/L = low activity, 0.6–5.8 mmol/L = good activity and >5.8 mmol/L = high activity). Preliminary results revealed that 47 isolates demonstrated activity towards 3-hydroxybutyronitrile, 34 isolates towards 3-hydroxyglutaronitrile while only 4 of the initial 256 isolates catalysed the hydrolysis of 3-hydroxy-3-phenylpropionitrile. This indicated sensitivity towards the phenyl ring at the b-position on the enzymatic reaction, and this is demonstrated in the ability of isolate to SS1-24 to show good activity towards 3-hydroxyglutaronitrile (1.43 mmol/L) where the same isolate demonstrates low activity (0.21 mmol/L) towards 3-hydroxy-3-phenylpropionitrile. This enabled the 10 isolates demonstrating the highest activity towards the target substrates to be selected for subsequent screening and optimisation.

As the bacterial strains have been isolated from various locations such as soil and sea weed, these isolates may show different growth patterns at various temperatures. It was decided also to monitor the growth of the 24 chosen isolates in the presence of the key nitriles at various temperatures ranging from 15 to 30 °C, and in each case an optimum temperature for induction was also determined for all future studies.

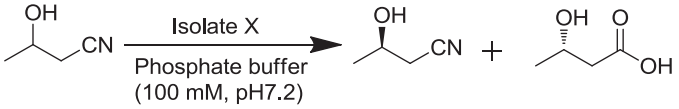
No significant influence of reaction temperatures on the activity of the chosen isolates was observed when the isolates were assayed at various temperatures ranging from 15 °C to 30 °C. The majority of isolates demonstrated their highest activity at 25 °C however in contrast optimum growth was observed at lower temperatures in many cases. Table 1 indicates the temperature for optimum growth and also the temperature which resulted in the optimum activity of the isolate.

This high throughput screening strategy allowed for the rapid identification of isolates demonstrating activity towards β-hydroxynitriles. The toxicity testing, nitrogen starvation and activity screening serve as a preliminary study where active isolates can be identified. The ultimate aim of this work was to identify novel enantioselective strains. In previous studies strains were isolated by enrichment with nitriles such as acetonitrile, benzonitrile, adiponitrile and acrylonitrile [29]. Our work attempted to induce strains directly with the target substrates to determine if enantioselective enzyme systems could be produced. Following the preliminary studies reported above the 10 isolates demonstrating the highest activity towards 3-hydroxybutyronitrile were chosen for further enantioselectivity screening towards 3-hydroxybutyronitrile (Table 1) to identify a link between our activity screening and enantioselectivity, results are presented in Table 2. This work serves as a demonstration of the application of this high-throughput screening strategy for the identification of both active and enantioselective nitrile hydrolysing isolates.

3.2. *Screening for enantioselective biotransformations of 3-hydroxybutyronitrile*

Having reduced the bacterial isolates from 256 to 24 having good to high activity towards β-hydroxynitriles, attention was focused on determining the enantioselectivity of the isolates. Initial enantioselectivity screening involved the biotransformation of racemic

Table 2
Enantioselective hydrolysis of 3-hydroxybutyronitrile catalysed by novel bacterial isolates.



Entry	Isolate	Optimum temperature[b] (°C)	Nitrile ee[c] (%)	Acid ee[c] (%)	Activity[d]	Refs.
1[a]	F36	15	13.9	31.6	Low	
2[a]	F69	15	1.4	27.6	Good	
3[a]	F71	15	12.5	2.0	Good	
4[a]	SS1-12	25	26.2	14.0	Good	
5[a]	SET1	15	ND	99.9	High	
6[a]	Paenibacillus sp. NN32	15	1.3	25.5	High	[1]
7[a]	*Burkholderia sp. LC3c*	15	5.2	13.1	High	[2]
8[a]	SW2 33, Serratia sp.	15	2.3	ND	Low	
9[a]	*Bacillus sp. NN30*	15	22.3	58.5	Good	[1]
10[a]	*SS1-31*	25	16.2	18.7	Good	

[a] Biotransformation was carried out by incubating 3-hydroxybutyronitrile (10 mmol/L) in a suspension of the named isolate (OD = 1) in phosphate buffer (pH 7.0) for 24 h.
[b] Temperature for optimumenantioselectivity.
[c] Determined by HPLC analysis using a chiral column (see Section 2.8).
[d] Activity determined using Nessler's colorimetric activity assay (at optimum temperature).

3-hydroxybutyronitrile. The reaction was carried out conveniently using whole cells of the isolates at 25° C in aqueous phosphate buffer (pH 7.0). The hydrolysis products were transformed to the corresponding benzyloxy derivatives *in situ* for ease of detection using HPLC. As the specific nitrile metabolising enzyme content of the cells was unknown the isolates were assayed for activity to produce both amide and the corresponding carboxylic acid. It was assumed that the cells possessed a nitrilase system as during the hydrolysis of nitriles with resting cells, no amide could be detected and, furthermore the amide was not used as a substrate. In all cases the nitrile was partially consumed during the incubation period, which is in agreement with the results obtained using the high throughput screening strategy.

This chiral screening study identified a single isolate SET1 which demonstrated exceptional enantioselectivity towards 3-hydroxybutyronitrile (>99.9% ee), (entry 5, Table 2). The effect of temperature on the enantioselectivity of the biotransformation was examined at 15 °C and 25 °C. Several isolates demonstrated higher enantioselectivity at lower temperatures, for example an increase in enantioselectivity was observed in the case of F36 from 21.3% to 31.6% ee, when the temperature was varied from 25 °C to 15 °C (entry 1, Table 2). In the case of isolate SET1 at both temperatures the corresponding acid was produced in >99.9% ee. In this case only a slight decrease in activity from 1.02 mmol/L to 0.98 mmol/L was observed when the temperature was reduced from 25 °C to 15 °C respectively.

Isolate SET1 (entry 5, Table 2), fell within the 'high' activity range of the screening scale however it did not have the highest activity as determined using the Nesslers colorimetric assay. In comparison, isolate NN32 (entry 6, Table 2) showed the highest activity (Table 1) but demonstrated much lower enantiocontrol. Isolate SET1 also only generated the corresponding (*S*)-carboxylic acid, and no amide was detected; thus it is believed that this isolate possesses a highly enantioselective nitrilase. It is also surprising to note that in the case of SET-1 the corresponding unreacted (*R*)-nitrile could not be detected in the reaction mixture after the 24 h reaction time.

To determine yield and configuration of the acid product formed using isolate SET1in the hydrolysis of racemic 3-hydroxybutyronitrile, a large scale biotransformation was carried out over 24 h. The *ee* value of 3-hydroxybutyric acid was measured by chiral HPLC analysis, and the absolute configuration was determined as (*S*) by comparison of optical rotation with an authentic sample [35]. The product 3-hydroxybutyric acid was isolated from the reaction mixture using column chromatography in 41% yield and >99.9% *ee*. The unreacted (*R*)-nitrile could not be recovered from the reaction mixture.

Having identified this very promising isolate SET1 we decided to further focus our efforts on this isolate and explore the high enantioselectivity isolate SET1 demonstrated towards 3-hydroxybutyronitrile. An enantioselectivity *vs.* time study was carried out where the biotransformation was monitored after 3, 6, 9, 24 and 36 h; results indicate that isolate SET1 is highly enantioselective and possibly enantiospecific with >99.9% ee of (*S*)-acid at each sample point. It is believed the hydrolysis of the (*S*)-nitrile proceeds rapidly, as only the remaining (*R*)-nitrile is detected after 3 h. This was further demonstrated by the formation of only *(S)*-3-hydroxybutyric acid after a 10 day incubation period. While the reaction afforded the corresponding (*S*)-3-hydroxybutyric acid with excellent enantioselectivity, neither amide nor the starting nitrile could be recovered after this time. In addition the reduction in intensity of the remaining (*R*)-nitrile is also evident after various timepoints, with complete consumption after 24 h, indicating the potential metabolism of the unreacted (*R*)-nitrile along with the desired biotransformation.

The exponential decay of the unreacted nitrile may be due to other enzyme systems present within the microbial whole cells. A possibility may be hydrolysis of the nitrile to the corresponding acid followed by isomerisation. An investigation into the deracemisation of the racemic 3-hydroxybutyric acid was carried out by incubation with the isolate over a 24 h period; however unchanged racemic acid was recovered. Other options include the possibility of an isomerase converting the remaining (*R*)-nitrile to (*S*)-nitrile which may then be converted to the corresponding (*S*)-acid, however the isolated yield never exceeds 50%. Other enzymes such as an aldoxime or alcohol dehydrogenase may also account for the loss of mass balance.

It has been reported that the enzyme catalysed kinetic resolution of β-substituted nitriles can demonstrate low enantioselectivity in some cases [39,15,16,17,40,18]. For example in the biotransformation of 3-hydroxybutyronitrile using whole cells of *Rhodococcus erythropolis* AJ270 containing a nitrile hydratase/amidase system, low enantiocontrol was observed with the formation of *(S)*-3-hydroxybutyric acid (17.8% ee) [34]. The enantioselectivity of the reaction was dramatically improved using a benzyl protection/docking strategy which resulted in the formation of the acid with 86.2% enantiomeric excess. In the case

of SET1 >99.9% ee may be achieved using 3-hydroxybutyronitrile with the free unprotected hydroxy group. The high enantioselectivity demonstrated by isolate SET1 may be as a result of the position of the chiral recognition site in the enzyme which is possibly located remote to the catalytic centre [35], this has also been observed by previous authors [6,34]. While the biocatalytic hydrolysis of β-hydroxynitriles to corresponding β-hydroxy acids and amides using microorganisms possessing nitrile hydratase/amidase have been well reported [41,18,39], in contrast microorganisms with nitrilase activity often demonstrate low or extremely low activity towards the hydrolysis of β-hydroxynitriles [7,9,18,39]. To the best of our knowledge this is the first isolate potentially demonstrating enantiospecificity towards 3-hydroxybutyronitrile.

DNA sequencing of a 432 bp region of the 16s rDNA of isolate SET1 indicates that it is a strain of *Rhodococcus erythropolis*. It is 100% identical over the sequenced region to *Rhodococcus* sp. TMS1-19 (GenBank acession JX949804) a strain isolated from a glacier in China.

4. Conclusion

In summary, this work describes the development of a high-throughput screening strategy for the identification of isolates with nitrile hydrolysing activity towards β-hydroxynitriles. This high-throughput method was used to identify a range of isolates demonstrating activity towards several β-hydroxynitriles. In a subsequent enantioselectiveity screening study of isolates demonstrating the desired activity towards 3-hydroxybutronitrile, an isolate demonstrating a highly enantioselective if not enantiospecific activity towards 3-hydroxybutyronitrile was identified. Hence a biosynthetic pathway for the production of (*S*)-3-hydroxybutyric acid from racemic 3-hydroxybutyronitrile was established using this novel *Rhodococcus erythropolis* strain SET1. This cost effective screening strategy developed in this study can be used to screen a large number of microorganisms for their ability to transform aliphatic, aromatic nitriles and dinitriles within a short time period, in particular the addition of a toxicity screen towards the various nitriles, further focuses the strategy. Further screening techniques can be applied to candidates to determine their enantioselectivity; of note in this particular study isolates with higher enantioselectivity generally did not display the highest activity, for example SET1 demonstrated moderate activity towards 3-hydroxybutyronitrile, however it had the highest enantioselectivity. This highlights the importance of carefully selecting the activity specifications of isolates chosen for enantioselectivity screening. Further studies of *R. erythropolis* SET1 towards the understanding of its remarkable enantioselectivity and the substrate scope towards various β-hydroxynitriles, including the effect of various substituents on the enantioselectivity of the biotransformations are actively being investigated in our laboratory.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molcatb.2013.08.001.

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