Biocatalytic Routes to the Synthesis of Chiral Pharmaceutical Intermediates in Ionic Liquids.

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Dedicated to

My Family

Abstract

Ionic liquids have been shown to offer significant advantages when used as alternative reaction media, such as enhanced yields, when compared to traditional organic solvent systems. At present, however, there is limited data on their application to industrial processes. The main objective of this thesis is to identify a generic approach for the application of ionic liquids to bioconversions. Key factors for the operation of bioconversions in ionic liquids have been identified and product recovery options investigated.

Two bioconversions were examined. The first was the hydrolytic resolution of racemic 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349) to (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid (SB-240101) by immobilised *Candida antarctica* lipase B, CALB (Novozyme 435), performed industrially in t-butanol. Initial studies showed this reaction occurred in several ionic liquids with different physico-chemical properties. Simply replacing the organic solvent with an ionic liquid under otherwise identical conditions reduced the rate of conversion and overall yield. The key factors influencing the rate and yield of this bioconversion in ionic liquids were the type of ionic liquid and the substrate solubility, the reaction temperature and the water content. The final optimised reaction in ionic liquids shows an eighteen-fold enhancement in product formation compared to the optimised t-butanol system.

In order for ionic liquids to be applied commercially there are still many issues which still need to be resolved these include: the extraction of substrates and products from the ionic liquid media for down stream processing, and the recycle of the media for subsequent reactions. The next step having optimised the CALB bioconversion of SB-235349 in ionic liquid media was to extract the SB-240101 product and the un-reacted SB-235349 substrate in order to recycle the ionic liquid. The SB-240101 produced by the reaction was removed by liquid-liquid extraction with 50mM bicarbonate buffer (pH10); overall 93% of the SB-240101 produced was removed from the ionic liquid into the aqueous buffer phase. The un-reacted 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-240098) was removed by liquid-liquid extraction with isopropyl alcohol, and 91% was removed from the ionic liquid.

The ionic liquid was then regenerated with sodium hydroxide for recycle. The results from the bioconversion with fresh and recycled ionic liquid were almost identical, in both cases around $1.8 g.L^{-1}$ of product was produced in 6 hours. The two-phase extractions were subsequently studied in the Lewis cell, and the mass transfer rate (K_o) examined for the extraction of both the substrate and the product from the ionic liquid. Values of K_0 determined in the Lewis cell over the Reynolds number range for which a flat, non-perturbed, interface could be maintained were in the range 1.0 - 3.5 x 10^{-6} $m.s^{-1}$ for both product and substrate extraction. In both extraction experiments there was a linear increase in K_0 with Reynolds number.

The second bioconversion the thymidine phosphorylase catalysed synthesis of thymidine from thymine, which is traditionally performed in aqueous media, was then examined in ionic liquids. Initial investigations of this conversion step showed that replacing the aqueous media with an ionic liquid like [BMIM][PF_6] under otherwise identical reaction conditions reduced the overall yield, which is attributed to the low solubility of the reagents in this ionic liquid. As the first constraint of the aqueous system was the solubility of the substrates and products; work then focused on those ionic liquids that showed a high solubility for thymine and especially thymidine. A study of the conversion in these high solubility ionic liquids showed conversion to the same degree as that demonstrated in aqueous media. An examination of product recovery from [EMIM][tosylate] following the thymidine transformation showed that the most likely method of product recovery was adsorption to an anion exchange resin 1-X8.

In summary the results presented in this thesis show that ionic liquids offer significant advantages as alternative reaction media in industrial bioconversions. These are related to the excellent solvation properties of ionic liquids, and the tunable physico-chemical properties of ionic liquids such as miscibility (or immiscibility) with water by changes in the anion or cation. Overall this thesis has identified generic procedures for the design of bioconversions and product recovery options in ionic liquids that have been exemplified using two different bioconversion systems.

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Contents

List of Figures

List of Tables

Nomenclature

A	interfacial area (m^2) (Equation 2.3)
C_{aq}	concentration of the substrate or the product in the aqueous phase ($kg.m^{-3}$) (Equation 2.3)
C_{IL}	concentration of the substrate or the product in the ionic liquid phase ($kg.m^{-3}$) (Equation 2.3)
C_{org}	concentration of the substrate or the product in the organic solvent phase ($kg.m^{-3}$) (Equation 2.3)
Di	impeller diameter (m^2) (Equation 2.2)
D	diffusion coefficient (Equation 4.1)
j_p	extraction rate of the product (Equation 2.3)
j_s	extraction rate of the substrate (Equation 2.4)
K	partition coefficient (Equation 2.7)
K_o	overall mass transfer coefficient ($m.s^{-1}$) (Equation 2.3)
$LogP$	octanol/water partition coefficient
m	equilibrium partition coefficient
m_{eq}	molar equivalent
M	solute molecular weight
N	impeller speed ($m.s^{-1}$) (Equation 2.2)
Re	Reynolds number (Equation 2.2)
t	time (seconds)
T	temperature (°C)
U	units of enzyme activity ($\mu molmin^{-1}$)
V	volume of the ionic liquid phase (m^3) (Equation 2.3)
V_R	volume ratio
V_m	molecular volume of solute (Equation 4.1)
x_a	association factor of solvent (Equation 4.1)
ρ	medium density ($kg.m^{-3}$) (Equation 2.2)
μ	medium viscosity ($kg.s^{-1}$) (Equation 2.2)
μ_l	liquid viscosity (Equation 4.1)

Abbreviations

atm	atmospheres
CALB	*candida antarctica* lipase B
DNA	deoxyribonucleic acid
HPLC	high pressure liquid chromatography
g	grams
IPA	isopropyl alcohol
TFA	trifluoroacetic acid
L	litres
LD_{50}	lethal dose 50 %
MW	molecular weight
NADH	nicotinamide adenine dinucleotide (reduced)
NMR	nuclear magnetic resonance
RNA	ribonucleic acid
RO	reverse osmosis
rpm	revolutions per minute
Tris	tris-(hydroxymethyl) aminomethane hydrochloride
TP	thymidine phosphorylase
UV	ultraviolet
v/v	volume per unit volume
w/w	weight per unit weight
[BMIM]Cl	1-Butyl-3-Methylimidazolium Chloride [BMIM]Cl
[BMIM][PF_6]	1-Butyl-3-Methylimidazolium Hexafluorophosphate
[BMIM][BF_4]	1-Butyl-3-Methylimidazolium Tetrafluoroborate
[BMIM] [$N(SO_2CF_3)_2$]	1-Butyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)amide
[BMIM][CF_3SO_3]	1-Butyl-3-Methylimidazolium Trifluoromethane sulfonate
[Oc_3MeN] [$N(SO_2CF_3)_2$]	Methyl-trioctyl-imidazolium Bis(trifluoromethylsulfonyl)amide
[BMIM] [$MDEGSO_4$]	1-Butyl-3-Methylimidazolium Diethyleneglycol Monomethylether

[EMIM][tosylate]	1-Ethyl-3-Methylimidazolium Tosylate
[BMIM][$OcSO_4$]	1-Butyl-3-Methylimidazolium Octylsulphate
[CABHEM] [$MeSO_4$]	ECOENG™ 500 (PEG-5 Cocomonium Methosulfate)
[HMIM] [$N(SO_2CF_3)_2$]	1-Hexyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)amide
[MOEMIM][OMs]	1-Methoxyethyl-3-Methylimidazolium Methanesulfonate
[MMIM][$MeSO_4$]	1-Methyl-3-Methylimidazolium Methanesulfonate
[OMIM][PF_6]	1-Octyl-3-Methylimidazolium Hexafluorophosphate
[EMIM] [$N(SO_2CF_3)_2$]	1-Ethyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)amide
[EMIM][BF_4]	1-Ethyl-3-Methylimidazolium Tetrafluoroborate
[MTOA] [$N(SO_2CF_3)_2$]	Trioctylmethyl-Ammonium Bis(trifluoromethylsulfonyl)amide
[4-MBP][BF_4]	4-Methyl-N-Butyl-Pyridinium Tetrafluoroborate
[EtPy][CF_3COO]	N-Ethyl Pyridinium Trifluoroacetate
[BMIM][SbF_6]	1-Butyl-3-Methylimidazolium Hexafluoroantimonate

1.0 Introduction

1.1 Biocatalysts and Bioconversions

Biological catalysts are increasingly being exploited in the pharmaceutical, agrochemical and fine chemical industries because of the significant advantages they possess over certain chemical catalysts, as shown in Table 1.1.

Table 1.1- Advantages of biological catalysts over chemical catalysts (Faber et al., 1997).

Advantages of biological catalysts over chemical catalysts
The ability to operate under mild conditions, typically 37 °C, pH 7 and 1 atm
Their high stereo-, regio- and positional specificity
Their ability to perform complex reactions with no equivalent in traditional chemistry
Low environmental impact due to the biodegradable nature of enzymes
Potential for fewer side reactions due to the mild operating conditions and high selectivity of the biocatalyst
High atom efficiency due to the avoidance of protection and deprotection steps

A bioconversion is the use of a biocatalyst, either as an isolated enzyme or a whole cell, to catalyse a specific chemical reaction in which defined substrate(s) are converted to defined product(s) (Lilly, 1994). Biocatalysts are used in a wide range of chemical transformations in synthetic organic chemistry such as redox reactions and carbon-carbon bond formation in addition to well established hydrolysis and esterification reactions (Faber et al., 1997, Schulze and wubbolts, 1999). Improvements in recombinant DNA technology have meant that a wider range of biocatalysts are available and can be produced in the amounts that are required for large scale processes (Lilly, 1994). As a result, the cost of biocatalysts and chemical catalysts is comparable (Rozzell, 1999) and it is estimated that over two hundred biocatalytic processes are operated commercially (Liese et al., 2000). Moreover, global trends such as 'greener' more environmentally friendly processes and the increasing structural complexity of new drug compounds in recent years have

encouraged companies to investigate alternatives to traditional synthetic chemistry routes.

Biocatalysts have naturally evolved for use in aqueous environments, at neutral pH and temperatures below 40 °C. For industrial bioconversion processes, however, many of the substrates and/or products of interest have low aqueous solubilities, or water is the product of the transformation. This has lead to the development of bioconversions operated in 'non-conventional' media, usually an organic solvent or a water-solvent biphasic mixture, in order to effect bioconversions at higher overall concentrations (Lilly and Woodley, 1985).

1.1.1 Biocatalysis in 'Non-conventional' Media

The first record of a biocatalyst functioning in the presence of organic media appeared in the 1930's (Halling and Kvittingen, 1999). It was over 40 years later, however, that the industrial potential of the technology was realised with the publication of cholesterol modification in aqueous-organic biphasic media by whole Nocardia cells (Buckland et al., 1975), and the recognition that enzymes can function in anhydrous, or nearly anhydrous, organic solvents (Zaks and Klibanov, 1984). The primary benefits of using organic solvents as reaction media are the solubilisation of poorly water-soluble substrate and/or products at higher concentrations, control of substrate and product partitioning (to overcome inhibitory or toxic effects), and the ability to shift reaction equilibrium towards product formation (Lilly and Woodley, 1985; Brink et al., 1988). Other potential benefits offered by this type of reaction medium are the suppression of undesirable hydrolyses and easier product recovery. Based on the quantity of the aqueous phase present it is possible to distinguish three main reaction systems when considering biocatalysis in 'non-conventional' media (Kragl et al., 2003);

1. as a pure solvent
2. as a co-solvent in aqueous systems (for organic solvents miscible with water)
3. in a biphasic system (for organic solvents immiscible with water)

In each case the biocatalyst may be free or immobilised, which in the case of isolated enzymes, converts them into an insoluble form. The pure solvent system may contain

small amounts of added water (typically <5 % v/v) or be totally anhydrous. In many cases the application of organic solvents compared to transformations conducted in entirely aqueous media show significant improvements in space-time yields (Lilly and Woodley, 1985; Brink et al., 1988). Most organic media however, have a number of generic disadvantages that are shown in Table 1.2.

Table 1.2- Disadvantages offered by the use of organic solvents

Disadvantages associated with the use of organic solvents
Toxicity to both process operators and the environment
Potential explosion hazard due to their volatile and flammable nature
Damage to the membranes and cell walls of bacterial biocatalysts reducing their long-term operational stability
Polar solvents can act as denaturants of enzyme biocatalysts
Exposure to aqueous-organic interfaces can lead to denaturation of enzyme biocatalysts

One potential alternative to the use of organic solvents is the use of room temperature ionic liquids in biocatalytic processes (described later in Section 1.2), which could potentially overcome many of the issues associated with organic solvents.

1.1.2 Biocatalyst Stability in 'Non-conventional' Media

The use of bioconversions in industrial organic synthesis is a rapidly expanding area of industrial biocatalysis (Margolin, 1993; Schulze and Wubbolts, 1999; Liese and Villela, 1999). Biocompatibility of the solvent is an important issue for biocatalysis in 'non-conventional' media. A positive correlation is found between the hydrophobicity of the solvent and a decrease in toxicity to the biocatalyst. Hydrophilic solvents tend to affect biocatalyst cell membranes making them more fluid and reducing enzyme activity (Osborne et al., 1990). Rules have been developed to enable the rational selection of solvents for use with particular biocatalysts and transformations (Laane et al., 1987; Bruce and Dauglis, 1991). A considerable amount of work has been performed to understand solvent effects on the structure and function of both whole cells and isolated enzymes in order to select more stable

biocatalysts (De Bont et al., 1998). One of the general rules for the optimisation of biocatalytic systems in organic solvents is the logarithm of the partition coefficient (LogP) scale of a solvent in a water-octanol two-phase system, as a quantitative measure of solvents polarity, i.e. the more hydrophobic the solvent the greater proportion will be in the octanol phase. Biocatalysis has been shown to be low at log P values < 2, moderate in solvents with log P values between 2 and 4, and high in apolar solvents with log P values > 4 (Laane et al., 1986). Solvent tolerance has been shown to be slightly higher in Gram negative cells than in Gram positive ones (Vermue et al., 1993), and some bacteria have been shown to be especially tolerant, possessing membrane pumps for the active removal of solvent, or solvent impermeable membranes (De Bont et al., 1998).

With isolated enzymes there are several factors that contribute to their diminished catalytic activity in non-aqueous media; including the loss of critical water residues from the enzyme surface, a drastic decrease in the polarity of the enzymes microenvironment, the decrease conformational mobility of the protein structure, and the loss of activity in the preparation of enzymes for use in non-aqueous media (e.g. lyophilization). Although several techniques have been developed to improve this loss in catalytic activity, including lyophilization in the presence of lyoprotectants (Dabulis and Kilbanov, 1993), or the use of protein engineering to 'design' more functional mutant enzymes (Arnold and Volkov, 1999). However, such techniques have only yielded modest improvements in enzyme activity and, in most cases, activation levels beyond 10-100-fold have never been achieved (Lee and Dordick, 2002).

1.2 Room Temperature Ionic Liquids

Ionic liquids are solutions composed entirely of ions, also known as molten salts with relatively low melting points, many below ambient temperature. A wide range of ionic liquids are available which are stable under ambient conditions and in the presence of air (Osteryoung et al., 1998; Seddon, 1997). In recent years there has been considerable interest in ionic liquids for clean organic synthesis (Seddon, 1997). A variety of chemical transformations have been successfully performed in ionic liquids,

such as alkylation reactions, Diels-Alder cyclisations and Heck coupling reactions. In many cases significant yield improvements compared to reactions in conventional organic solvents have been observed (Welton, 1999; Sheldon, 2002). The key physico-chemical properties of ionic liquids are summarized in Table 1.3.

Table 1.3- The interest in ionic liquids for application in biocatalysis in 'Non-conventional' media (Seddon, 1997 and Cull et al., 2000).

Properties of Room Temperature Ionic Liquids
Non-volatile, non-flammable, low toxicity
Liquid and stable over a wide temperature range (typically –80 °C to 200 °C)
Relatively low viscosity with Newtonian rheology
Non-corrosive and compatible with common materials of construction
Good solvents for many organic, inorganic and polymeric materials
Immiscible with a wide range of organic solvents
Immiscible (or miscible) with water
Able to suppress solvation and solvolysis phenomena
Tunable physico-chemical properties ("designer solvents")

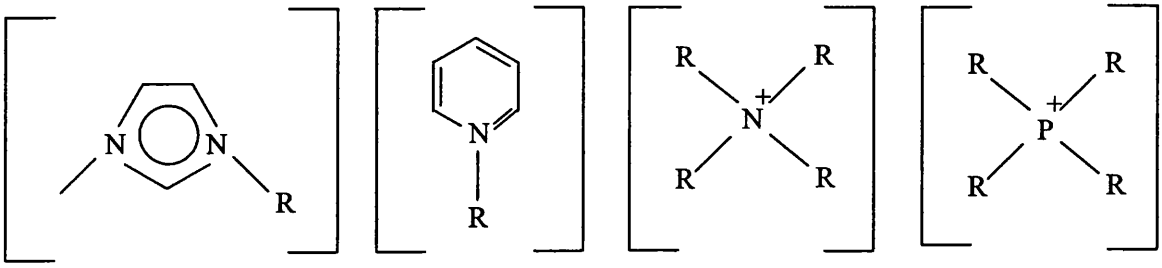
A wide range of ionic liquids exist and there are a number of different approaches to their synthesis (Gordon, 2003; Ren, 2003; Varma, 2003). Two of the most frequently used ionic liquids to date are 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] and 1-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4] as shown in Figure 1.1.

Figure 1.1- Structure of 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] (left) and 1-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4] (right).

In both cases the cation, [BMIM], is large compared to simple inorganic cations, which accounts for their low melting point and relatively low viscosity at ambient temperature (Visser et al., 2003). They are un-reactive with water and are frequently

used in research. Also of interest is the ability to readily alter the physico-chemical properties of these solvents by simple structural modifications to the cation or changes in the anion (Seddon, 1997). This has lead to ionic liquids being termed "designer solvents" (Freemantle, 1998) with the potential to design more biocompatible ionic liquids having now been demonstrated (Walker and Bruce, 2004).

Commonly used cations for ionic liquids



1-alkyl-3-methyl-imidazolium | N-alkyl-pyridinium | Tetraalkyl-ammonium | Tetraalkyl-phosphonium

Commonly used anions for ionic liquids

Water insoluble	⟶	Water soluble
$[PF_6]^-$	$[BF_4]^-$	$[CH_3CO_2]^-$
$[N(SO_2CF_3)_2]^-$	$[CF_3SO_3]^-$	$[CF_3CO_2]^-$, $[NO_3]^-$
$[BR_1R_2R_3R_4]^-$		Br^-, Cl^-, I^-

Figure 1.2- Structure of some commonly used cations and anions for the production of ionic liquids.

The increasing water solubility of ionic liquids such as [BMIM][PF_6], [BMIM][BF_4] and [BMIM]Cl is attributed to the large differences in the dipolarities, although they do have similar polarity (Anderson et al., 2002).

1.2.1 Physico-chemical Properties of Ionic Liquids

Ionic liquids are considered to be relatively polar solvents, but can be non-coordinating (mainly dependent on the anion present). Ionic liquids are similar to dipolar, aprotic solvents and short-chain alcohols in their solvent characteristics and

promote the dissolution of a vast array of pharmaceutical intermediates and final drug substance molecules (Aki et al., 2001). Ionic liquids are much more viscous than typical organic solvents, and high viscosity is probably inherent to ionic liquids. Strong intermolecular forces between solvent molecules cause high viscosity, while ionic liquids with shorter alkyl chains are less viscous than those with longer alkyl chains. Addition of co-solvents lowers the viscosity of ionic liquids, but cancels many of the advantages of using ionic liquids (Park et al., 2003). An important aspect when considering the application of ionic liquids to reactions is their phase behaviour, with other compounds. As described previously substitutents on the cation and anion can be selected to enhance or suppress solubility of ionic liquids in other compounds, or the solubility of other compounds in ionic liquids. The solubility of various gases in ionic liquids is also important when considering ionic liquids as reaction media. The ionic liquid [BMIM][PF_6] has been shown to have very large solubility differences between the relatively high-solubility gases (CO_2, C_2H_4, C_2H_6 and CH_4) and the low-solubility gases (CO, H_2, O_2 Ar and N_2). Unfortunately, the gases of interest for reactions (H_2, O_2 and CO) are only sparingly soluble.

1.2.2 Biocatalysis in Ionic Liquids

A number of recent publications have shown the potential to carry out enzymatic and whole cell bioconversions in ionic liquids and there are already a number of reviews (Park et al., 2003; Van-Rantwijk et al., 2003; Roberts and Lye, 2002; Kragl et al., 2002; Sheldon et al., 2002). Results from the use of ionic liquids as pure solvent, as co-solvent and for biphasic systems have been reported and are briefly summarized in the following sections. Precise details of the reaction systems, including the type of biocatalyst and ionic liquid used are given below in Tables 1.4, 1.5 and 1.6. Table 1.4 examines whole cell biocatalytic systems in ionic liquids, Table 1.5 examines isolated lipase enzyme systems, and Table 1.6 examines all other isolated enzyme systems.

1.2.3 Whole cell Systems in Ionic Liquids

To date there have been limited numbers of publications on whole-cell systems in ionic liquids. The first publication on the use of a whole cell biocatalyst in a biphasic aqueous-ionic liquid system was the hydration of poorly water-soluble aromatic

dinitriles (Cull et al., 2000). This conversion involves the successive action of both nitrile hydratase and an amidase enzyme respectively (Bunch, 1998), and is therefore best performed using a whole cell system. Experiments showed the bioconversion kinetics with the ionic liquid-aqueous system were similar to those in an organic solvent-aqueous system, phase ratio 0.2 v/v. This system was also examined in a single ionic liquid phase and showed the rate of conversion was dependent on the amount of water present up to 9 % v/v (Roberts and Lye, 2002). Results show that in a two phase aqueous-[BMIM][PF_6] system the Rodococcus R312 cells showed enhanced stability (Cull et al., 2000). In the single [BMIM][PF_6] phase results suggest the ionic liquid had no adverse effect on the structure and function of bacterial cell membranes, and that the cells showed no evidence of lysis and remained viable (Roberts and Lye, 2002).

The reduction of ketones with bakers' yeast and the recovery of n-butanol from fermentation broth was also examined in a two-phase system with [BMIM][PF_6] (Howarth et al., 2001; Fadeev and Meagher, 2001). In all cases where the ionic liquid

Table 1.4- Whole-cell systems in ionic liquids.

Biocatalyst	Reaction System	Ionic Liquid	Reference
Whole cells of *Rhodococcus* R312	Biotransformation of 1,3-dicyanobenzene	[BMIM][PF_6]/buffer (two-phase) [BMIM][PF_6]	Cull et al., 2000. Roberts and Lye, 2002.
Whole cells of bakers' yeast	Reduction of ketones	[BMIM][PF_6]/buffer (two-phase)	Howarth et al., 2001.
Whole cells of yeast	Recovery of n-butanol from fermentation broth	[BMIM][PF_6]/buffer (two-phase)	Fadeev and Meagher, 2001.

was used in a two-phase system with whole cells the ionic liquid was used as a substrate reservoir and/or for *in situ* removal of the inhibitory product formed.

1.2.4 Isolated Enzymes Lipases in Ionic Liquids

Lipases have been described as the 'work horses' of biocatalysis (Bornscheuer and Kazlauskas, 1999). Several groups have investigated lipases in ionic liquids, due to the widespread use of these enzymes in industry and the considerable literature on their use in organic solvents (Liese and Villela, 1999, Goderis et al., 1986). Sheldon and co-workers first demonstrated lipases to be active in anhydrous ionic liquids (Lau et al., 2000). They examined the activity of both free and immobilised *Candida antarctica* lipase B (CALB) in ionic liquids for alcoholysis, ammoniolysis and perhydrolysis, discovering similar reaction rates compared to reactions performed in organic solvents such as propan-2-ol and butan-1-ol.

The kinetic resolution of 1-phenylethanol was investigated with eight different lipases and two-esterases in ten ionic liquids (Schofer et al., 2001). Vinyl acetate was used for the transesterification. No activity was observed for the esterases, with the lipases however, improved enantioselectivity was observed in [BMIM][$N(CF_3SO_2)_2$], compared to the organic solvent system. In particular *Candida antarctica* lipase B showed the best results in [BMIM][CF_3SO_3], [BMIM][$N(CF_3SO_2)_2$] and [OMIM][PF_6]. No activity was observed in either [BMIM][PF_6] or [BMIM][BF_4], which conflicts with other groups, who obtained good activities for transesterification or ammoniolysis in the same ionic liquid (Lau et al., 2000, Lozano et al., 2001, Kim et al., 2001, Itoh et al., 2001). Impurities may cause these differences. Halide ions are a possible impurity as many ionic liquids are synthesised from 1-alkyl-3-methylimidazolium chloride or bromide and the halide is exchanged with the desired anion, but an incomplete exchange leaves halide anions behind. Not all ionic liquids are suitable for biocatalysis. Lipases have been shown to be inactive in ionic liquids with anions such as Cl, Br and CF_3SO_3 and active in ionic liquids with PF_6 BF_4 and $N(SO_2CF_3)_2$ cations (Karr et al., 2003). This is thought to be due to the lower hydrogen bond basicity of the enzyme-compatible anions (Anderson et al., 2002).

Parks and Kazlauskac investigated the regioselective acylation of glucose in ionic liquids and the acylation of ascorbic acid with oleic acid in ionic liquids (Park et al., 2003, Park and Kazlauskas, 2001). The results obtained for both conversions showed much higher yield and selectivity than in commonly used organic solvents. This was due to the high solvation properties of ionic liquids for both hydrophobic and

hydrophilic reactants. Furthermore, this group also demonstrated the influence of an additional washing regime, following ionic liquid synthesis, to remove impurities and enhance enzyme activity. This is of particular significance when silver salts (e.g. of BF_4 and PF_6) are used to prepare ionic liquids, silver being a well-known enzyme inhibitor (Park and Kazlauskas, 2001). Other groups have demonstrated the importance of controlling the water content in ionic liquids in order to achieve higher conversions (Eckstein et al., 2002, Berberich et al., 2003).

Table 1.5- Examples of lipase systems in ionic liquids.

Biocatalyst		**Reaction system**	**Ionic liquid**	**Reference**
Lipase	*Candida antarctica* (CALB)	Alcoholysis, amminolysis, perhydrolysis	[BMIM][PF_6] [BMIM][BF_4]	Lau et al., (2000).
Eight lipases and two esterases		Kinetic resolution of (R,S)-1-phenylethanol	10 different ionic liquids	Schofer et al., (2001).
Lipase	*Candida antarctica*	Synthesis of butyl buterate by transesterification	[EMIM][BF_4] [BMIM] [$(CF_3SO_2)_2N$] [BMIM][PF_6]	Lozano et al., (2001).
Lipases	*Candida antarctica* *Pseudomonas cepacia*	Kinetic resolution of secondary alcohols	[EMIM][PF_6] [BMIM][PF_6]	Kim et al., (2001).
Lipases	*Candida antarctica* *Pseudomonas cepacia* *Candida rugosa* Porcine liver	Kinetic resolution of allylic alcohols	[BMIM][PF_6] [BMIM] [CF_3SO_3] [BMIM][BF_4] [BMIM] [$(CF_3SO_2)_2N$] [BMIM][SbF_6]	Itoh et al., (2001).

Lipases	*Candida antarctica* (CALB)	Transesterification	Several ionic liquids with different properties	Kaar et al., (2003).
Lipases	*Candida antarctica* *Pseudomonas cepacia*	Kinetic resolution of (R,S)-1-phenylethanol; acylation of β–glucose	Several ionic liquids; washing with aqueous sodium carbonate	Park and Kazlauskas, (2001).
Lipase	*Candida antarctica* (CALB)	Acylation of ascorbic acid with oleic acid	Several ionic liquids with different properties	Park et al., (2003).
Lipase	*Pseudomonas sp.*	Kinetic resolution of (R,S)-1-phenylethanol; influence of water activity and temperature	[BMIM] $[(CF_3SO_2)_2N]$	Eckstein et al., (2002).
Lipase	*Pseudomonas cepacia*	Transesterification and polyester synthesis	[BMIM][PF_6]	Nara et al., 2002. Nara et al., 2003
Lipase	*Candida antarctica* (CALB) *Mucor Miehei* *Humicola lanuginosa*	Synthesis of simple esters	[BMIM][PF_6] [BMIM][BF4]	Husum et al., (2001).

1.2.5 Isolated Enzymes other than Lipases in Ionic Liquids

Isolated enzyme biocatalysis is the most developed area of research to date. The first publication on the use of enzyme catalysis in ionic liquids concerned the synthesis of

Z-aspartame by the protease thermolysin (Erbeldinger et al., 2000). Aspartame is currently commercially produced using the soluble enzyme in an aqueous system (Liese et al., 2000). Working with [BMIM][PF_6] containing 5% v/v water, Russell and co-workers found comparable rates and yields as with the use of relatively polar solvents such as ethanol and *tert*-amyl alcohol. Additionally, increased enzyme stability was observed and the ionic liquid was recycled several times after removal of non-converted substrates by extraction with water and product precipitation. This work was followed by publications concerning the protease α–chymotrypsin used for the transesterification of N-acetyl-l-phenylalanine ethyl ester (Laszlo and Compton, 2001) and N-acetyl-l-tyrosine ethyl ester (Lozano et al., 2001; Lozano et al., 2003) into the corresponding propyl esters. Laszlo and Compton compared the use of ionic liquids to organic solvents and found that rates were comparable, and that a certain amount of water was required for reaction as with polar organic solvents. Lozano et al., (2001) studied the stabilising effect of ionic liquids compared to 1-propanol for the transesterification of N-acetyl-l-tyrosine ethyl ester. Results showed that although the enzyme activity was decreased in the ionic liquid the increased stability resulted in higher final product concentrations.

Other groups have investigated galactosylation reactions using ionic liquids as co-solvents rather than discreet phases. The hydrolytic activity of β-galactosidase from *E.coli* and the protease subtilisin were examined in a 50 % v/v aqueous solution of the water miscible [BMIM][BF_4], results were comparable to those with an organic solvent as co-solvent (Husum et al., 2001). The galactosylation with β–galactosidase from *Bacillus circulans* for the synthesis of N-acetyl-lactosamine, starting from lactose and N-acetyl-glucosamine has also been investigated (Kaftzik et al., 2002). Results showed that the addition of the water miscible ionic liquid [MMIM][$MeSO_4$] as a co-solvent suppressed the undesirable secondary hydrolysis of the product observed in the aqueous system. This group also showed the repeated use of the enzyme after reaction and filtration. Another enzyme that has been investigated in ionic liquids is formate dehydrogenase from *Candida boidinii* which was found to be active and stable in mixtures of [MMIM][$MeSO_4$] with buffer (Kaftzik et al., 2002). The enzyme esterase from *Bacillus stearothermophilus* was also found to have increased stability in ionic liquids (Persson and Bornscheuer, 2003). The enzymatic resolution of amino acid esters has also been examined with the addition of an ionic

liquid, results showed that the concentration of the ionic liquid in the mixed solvent system effected both the enantioselectivity and the yield (Zhao and Malhotra, 2002).

Table 1.6- Isolated enzymes other than lipases in ionic liquids.

Biocatalyst	Reaction System	Ionic Liquid	Reference
Thermolysin	Synthesis of Z-aspartame	[BMIM][PF_6]	Erbeldinger et al., 2000.
α-Chymotrypsin	Transesterification of N-acetyl-$_L$-phenylalanine ethyl ester with 1-propanol	[OMIM][PF_6] [BMIM][PF_6] [EMIM][$N(SO_2CF_3)_2$] [BMIM][$N(SO_2CF_3)_2$]	Laszlo and Compton, 2001. Eckstein et al., 2002.
α-Chymotrypsin	Transesterification of N-acetyl-$_L$-tyrosine ethyl ester with 1-propanol	[EMIM][BF_4] [EMIM][$N(SO_2CF_3)_2$] [BMIM][PF_6] [BMIM][BF_4] [MTOA][$N(SO_2CF_3)_2$]	Lozano et al., 2001. Lozano et al., 2003.
β-Galactosidase subtilisin	Hydrolytic activity	[BMIM][BF_4]/buffer (one-phase)	Husum et al., 2001.
β-Galactosidase *Bacillus circulans*	Synthesis of N-acetyl-lactosamine	[MMIM][$MeSO_4$]/buffer (one-phase)	Kaftzik et al., 2002.
Formate dehydrogenase	Regeneration of NADH	[MMIM][$MeSO_4$] [4-MBP][BF_4]	Kaftzik et al., 2002.
Peroxidases	Oxidation of guaiacol	Imidazolium-based ionic liquids either anhydrous ionic liquid or ionic liquid/buffer (one-phase)	Hinckley et al., 2001. Laszlo and Compton, 2002.
Esterase from *Bacillus Stearothermo-philus*	Transesterification	Several ionic liquids with different properties	Persson and Bornscheuer, 2003.

Protease Novo alcalase	Enantioselective resolution of N-acetyl amino acids	[EtPy][CF_3COO]/buffer (one-phase)	Zhao and Malhotra, 2002.

Apart from lipases only a few other enzyme systems have been studied none of these in detail in ionic liquids.

1.2.6 Biocompatibility of Ionic Liquids

As discussed previously, enzymes and whole cells have been shown to be active in ionic liquids. Not all ionic liquids are suitable for biocatalysis, however, enzymes are usually active in ionic liquids containing the anions BF_4, PF_6 and $N[SO_2CF_3)_2]$, and not in the anions Cl, NO_3, trifluoroacetate and acetate anions (Karr et al., 2003). This has been attributed to the lower hydrogen bond basicity of the enzyme-compatible anions, which interferes with the internal hydrogen bonds of an enzyme. Several groups have reported increased stability of enzymes in ionic liquids compared to organic solvents. For example α-chymotrypsin has been shown to be seventeen times more stable (Lozano et al., 2001), CALB three times (Lozano et al., 2001), and *Bacillus Stearothermophilus* esterase thirty times (Persson and Bornscheuer, 2003); the reasons for this are still unclear. Research has also revealed the importance of controlling the water activity in ionic liquids; the driest conditions have been shown to give the highest enzyme activity (Eckstein et al., 2002, Berberich et al., 2003). The polar nature of ionic liquids can increase the ionization of acids, which can increase their acidity (Thomazeau et al., 2003). This increased acidity of acids in ionic liquids, generated from the hydrolysis of acyl donors, may inactivate enzymes. At present little is known about the toxicity of ionic liquids, although the acute toxicity of 1-hexyloxymethyl-3-methylimidazolium tetrafluoroborate has been assessed. The values were found to be LD_{50} = 1400 $mg.kg^{-1}$ for female Wister rats, and LD_{50} = 1370 for males. The authors concluded that the tetrafluoroborate salt could be used safely (Pernak et al., 2001). Studies on ionic liquid toxicity to mammalian cells have shown that imidazolium ionic liquids are generally less toxic than conventional solvents, and that increasing the alkyl chain length increases the toxicity (Ranke et al., 2004). The purified enzyme acetylcholinesterase has been shown to be inhibited by ionic liquids, which indicates they have a toxic potential for the environment. The inhibitory

potential depends on the type of cationic structure and the alkyl side chains. Ionic liquids with large bulky cations such as imidazolium and phosphonium ionic liquids are less inhibitory than pyridinium ionic liquids and results indicate the influence of the anion does not dominate the toxicity of the ionic liquid (Stock et al., 2004).

1.2.7 Product Recovery from Ionic Liquids

Issues such as the efficient recovery, product isolation and re-use of an ionic liquid are key to their industrial application due to the current lower cost of organic solvents. These issues are important to the 'greenness' of ionic liquids. To date investigation of methods such as extraction with benign solvents like water or supercritical carbon dioxide, pervaporation or distillation have been investigated, all are methods suitable on an industrial scale and several groups have investigated extraction with organic solvents. Typically hexane or ethane have been found to be unsuitable for the extraction of polar substrates (Itoh et al., 2001; Visser et al., 2000). Distillation has been shown to be a suitable method of recovery for volatile substrates (Schofer et al., 2001). Supercritical carbondioxide has been shown to remove nonpolar substrates (Reetz et al., 2002), and column chromatography a method widely used in organic chemistry has also been investigated (Kim et al., 2001; Kaftzik et al., 2002). Further consideration of various product recovery techniques and their application to specific bioconversions will be studied in this thesis and described later in Section 4.2.

1.3 Lipase Catalysed Bioconversion Studied in this Thesis

The *Candida antarctica* lipase B (Novozyme 435) catalysed resolution of 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349), a key Lotrafiban intermediate, will be investigated in this research. Lotrafiban, as shown in Figure 1.3, is a potent non-peptidic glycoprotein antagonist that inhibits platelet aggregation (Samanen et al., 1996). Phase III clinical trials of Lotrafiban have been undertaken with a view to determine whether the repeat of secondary thrombotic events such as heart attack or stroke could be prevented (Scarborough and Gretler, 2000). The molecule has the (*S*)- stereochemistry and only this enantiomer is active. CALB was identified as an enzyme which was highly

selective for hydrolysis of the (*S*)-enantiomer of SB-235349 to give the (*S*) acid, a key Lotrafiban intermediate, with greater than 99.5% chiral purity. The anhydrous nature of the reagents led to Novozyme 435 (a CALB preparation immobilised on acrylic resin) being selected as the preferred catalyst (Anderson et al., 1998).

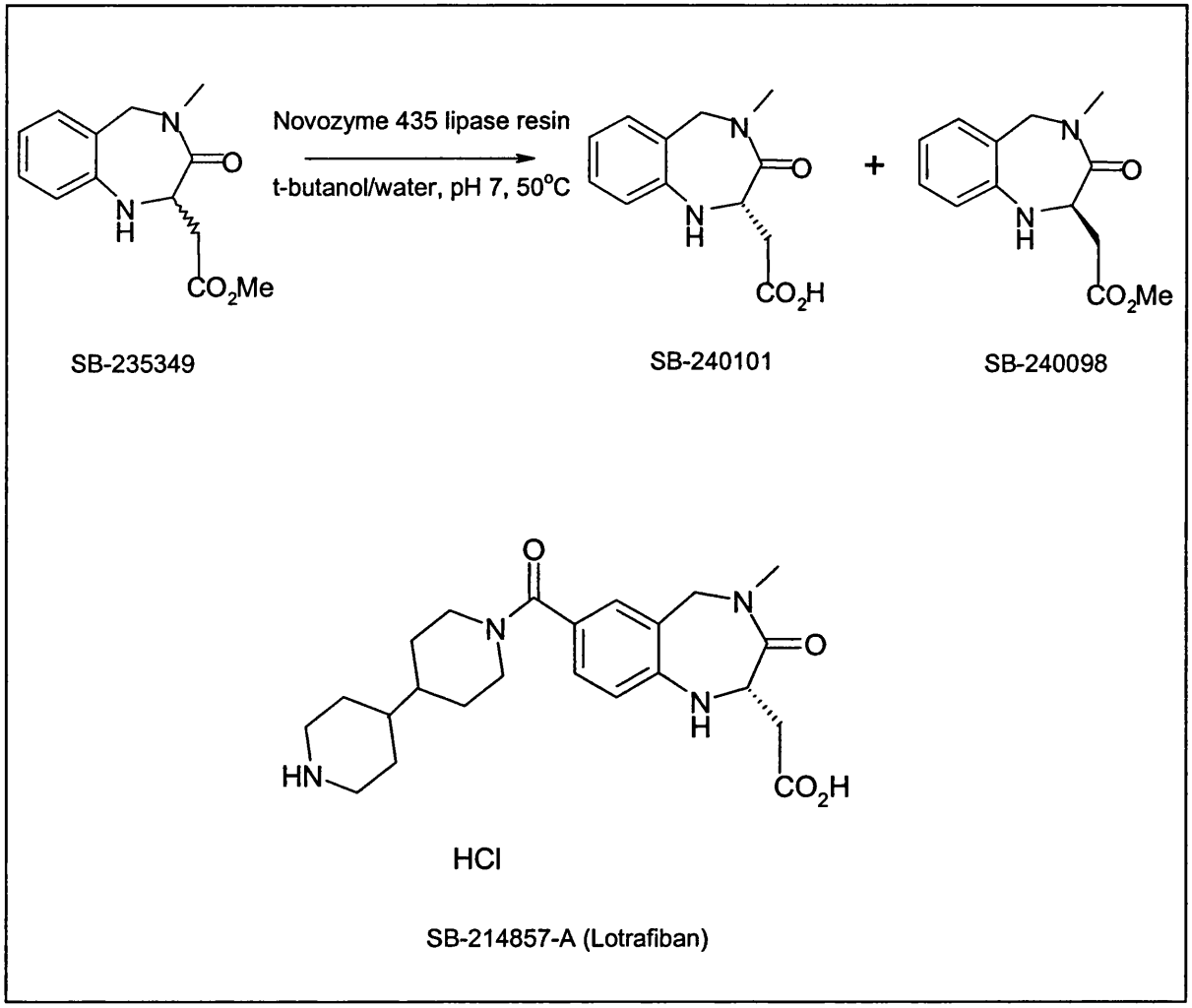


Figure 1.3- Details of the industrial process for the lipase catalysed resolution of (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349) to (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid (SB-240101) leaving the un-reacted (2R)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-240098). SB-240101 is a key intermediate in the synthesis of the glycoprotein antagonist Lotrafiban (SB-214857-A (Figures re-drawn from Atkins et al., 2003).

Industrial process research and development for Lotrafiban focused on finding the optimum conditions for the enzymatic resolution of SB-235349 with respect to: (i)

chiral purity of the product, (ii) reaction rate and yield, and (iii) re-use of the resin. Novozyme 435, at a catalogue price of £500 kg^{-1}, would contribute significantly to the cost of goods and maximum re-use would be required. Commercially available t-butanol (88% v/v) was identified as the best reaction solvent, having the advantage of being liquid at temperatures above –10 °C. This contrasts to the use of anhydrous t-butanol, which has a melting point of 25 °C and is therefore impractical for industrial use. Increasing the reaction temperature was found to cause a decrease in the re-usability of the Novozyme 435, but was necessary to dissolve the substrate at satisfactory levels. Therefore 50 °C was finally selected as the most appropriate reaction temperature (Atkins et al., 2003). This enzymatic resolution has been operated under these conditions at scales of up to 3000L.

1.4 Nucleoside Chemistry in Ionic Liquids

Nucleotides are synthesised from simple precursors. They function as energy carriers, as regulatory molecules, and as precursors of coenzymes and nucleic acids (Zubay, 1998). The biosynthesis of nucleotides is a vital process, since these compounds are indispensable precursors for the synthesis of both RNA and DNA. Without RNA synthesis, protein synthesis will not occur, and without DNA cells cannot divide. It follows, therefore, that inhibitors of nucleotide biosynthesis are very toxic to cells, and this has been utilised in the treatment of cancer (MacCross and Robins, 1990) as well as in the treatment of certain diseases resulting from infections from viruses and bacteria (Robins and Revankar, 1998). Protected nucleosides also serve as building blocks for the synthetic oligonucleotides extensively used as probes for diagnostic purposes (Lerman, 1986), and in antisense therapeutics (Crooke and Lebleu, 1993).

As a result, nucleoside chemistry is an important area of research for both industry and academia. The term "nucleoside phosphorylase" is a generic name for enzymes involved in the phosphorolysis of an N-glycoside bond in a nucleoside in the presence of phosphoric acid. The enzymes, which may be generally classified into purine nucleoside phosphorylases and pyrimidine nucleoside phosphorylases, are widely distributed and present in the tissue of mammals, birds and fish, yeasts and bacteria. Purine and pyrimidine nucleoside phosphorylases have been isolated from a number of bacterial cells (Utagawa et al., 1985).

One of the major obstacles in nucleoside development, however, is the limited solubility of many nucleosides in aqueous and organic solvents. To date there has been limited investigation of nucleoside chemistry in ionic liquids. The ionic liquid [MOEMIM][OMs] has been shown to be a 'green' alternative to conventional organic solvents used as reaction medium in nucleoside chemistry (Uzagare et al., 2003). This group investigated the acylation and peracylation of 2'deoxyribonucleosides in [MOEMIM][OMs] by chemical synthesis. The solubility of thymidine and 2-deoxyribonucleosides was examined in a range of ionic liquids and the range of suitable ionic liquids available was determined as limited, due to the high viscosity of most ionic liquids with suitable solubility.

1.4.1 Nucleoside Bioconversion Studied in this Thesis

The natural 2′-deoxynucleoside, thymidine (as shown in Figure 1.4), is a precursor for a number of important antiviral nucleosides such as 3′-deoxy-3′-azidothymidine (AZT) and dideoxydidehydrothymidine (d4T) and other potential antiviral compounds referred to as antisense oligonucleotides (Pal and Nair, 1997). The preparation of the nucleoside compound thymidine occurs from reacting 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt and thymine in the presence of thymidine phosphorylase. Thymidine phosphorylase (EC 2.4.2.4) is an example of a nucleoside phosphorylase as described in Section 1.~~42.~~3. Reaction conditions in aqueous media were determined from the European patent (EP 1179598A1, 2002), which described the preparation of a nucleoside compound by reacting a pentose-1-phosphate and a nucleic acid base, or it's analogue in the presence of enzyme activity of nucleoside phosphorylase. This is an equilibrium reaction and the patent describes a method in order to shift the equilibrium toward the direction of nucleoside synthesis, by the addition of a metal salt capable of forming a water-insoluble salt with phosphoric acid, a by-product, to eliminate it from the reaction system, leading to an improved yield of the desired nucleoside compound. The metal salts identified as capable of forming a water-insoluble salt with phosphoric acid are those with the calcium, barium and aluminium ions, such as calcium nitrate a water-soluble salt. The results

indicate formation of 2.28mM thymidine with a yield of 91.2 % (EP 1179598A1, 2002).

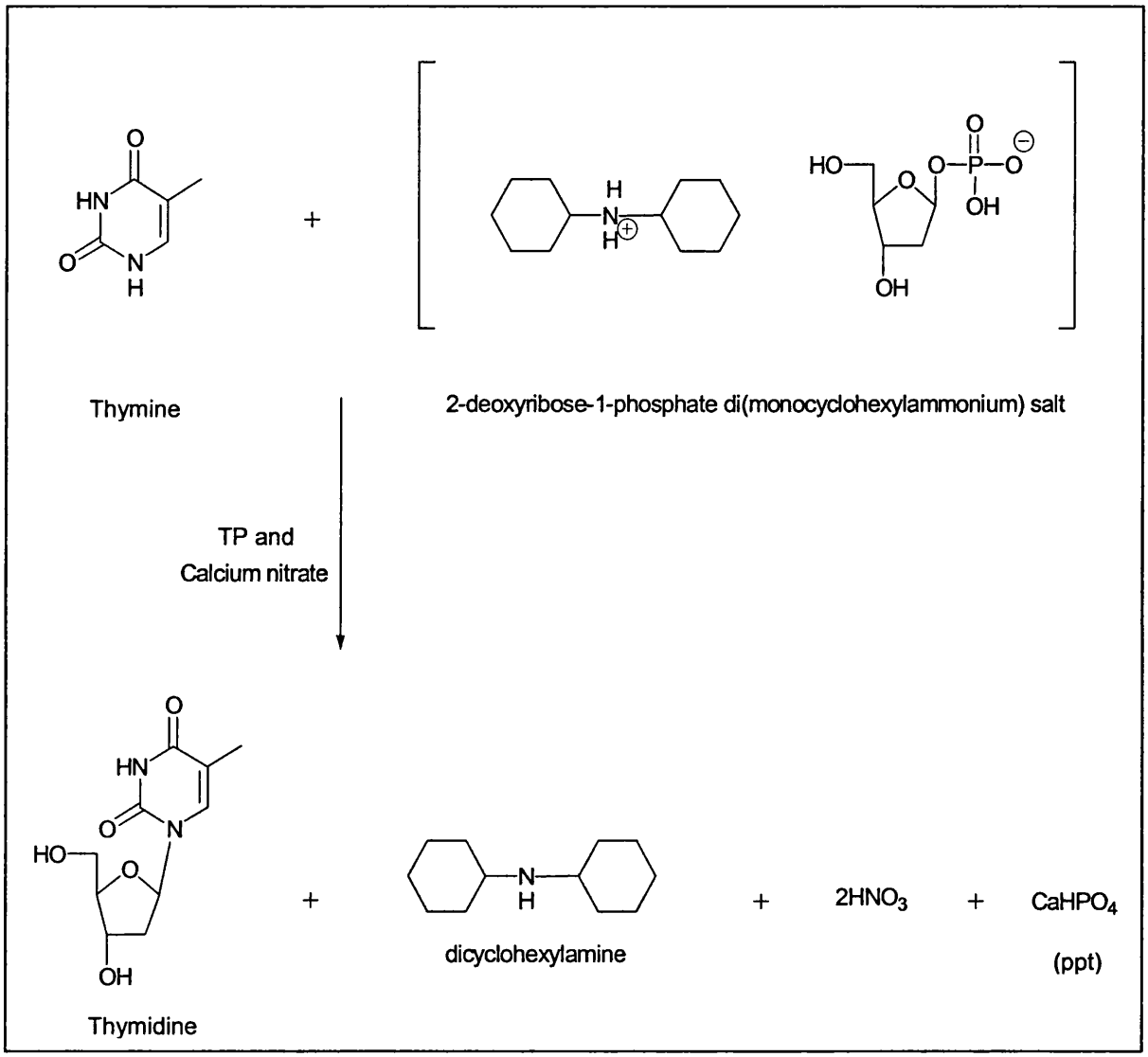


Figure 1.4- Details of the thymidine phosphorylase (TP) catalysed synthesis of thymidine from thymine and 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt (European patent no. EP 1179598A1, 2002). Thymidine is a key precursor for a number of important antiviral nucleosides such as 3′-deoxy-3′-azidothymidine (AZT) and dideoxydidehydrothymidine (d4T) and other potential antiviral compounds referred to as antisense oligonucleotides (Pal and Nair, 1997). (ppt water insoluble precipitate).

1.5 Aims of the Project

The primary aim of this research was to establish a generic design procedure for the study and optimisation of bioconversion processes in ionic liquids, together with methods for product recovery and re-use of the ionic liquid reaction media. In this work the *Candida Antarctica* lipase B (Novozyme 435) catalysed resolution of 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349), a key Lotrafiban intermediate, has been chosen for investigation (Figure 1.3). The reaction has previously been investigated and optimised in the organic solvent t-butanol as described in Section 1.3. This industrially optimised conversion will be extensively applied to ionic liquid media, and product recovery routes investigated in order to determine a generic method for the application of ionic liquids to make the future design of such a process more rational. Finally, an example of the application of the generic features identified will be applied to a second bioconversion which is the thymidine phosphorylase catalysed conversion of thymine and 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt to thymidine, dicyclohexylamine, nitric acid and calcium phosphate (Figure 1.4) compared to conventional aqueous solvents as described in Section 1.4.1 The specific objectives of the investigation are summarised below:

- Ionic liquids have been shown to offer substantial benefits in selectivity and yield when used as reaction media, as discussed in Section 1.2. The initial objective of this work was to characterise the lipase-catalysed bioconversion in ionic liquids as discussed in Section 1.3. The rate, yield and chiral purity of the reaction will be investigated as a function of reaction temperature, substrate concentration and water content of the ionic liquids. The re-use of the enzyme will also be explored. The results obtained will be compared to those for the previously optimised industrial t-butanol process (Atkins et al., 2003) in order to determine any advantages the application of ionic liquids offers, and to identify key factors influencing the bioconversion process in ionic liquids. The results of this work are presented and discussed in Chapter 3.

- A generic approach to assess product recovery and ionic liquid recycling options will also be examined for the lipase-catalysed bioconversion. Data will then be determined to establish a route for product recovery, un-reacted substrate enantiomer recovery, and re-use of the ionic liquid. As part of the investigation the solute mass transfer kinetics controlling mass transfer process will be ascertained, in order to enable selection of the most appropriate operating conditions. The results of this work are presented and discussed in Chapter 4.

- Ultimately the generic features identified for the application of ionic liquids to bioconversions will be used as the basis for the application of the thymidine phosphorylase bioconversion process as described in Section 1.4.1 The operating parameters for this conversion will also be determined and compared to those for the previously optimised industrial process. The results of this work are presented and discussed in Chapter 5.

Finally, in Chapters 6 and 7 the main findings of this work and generic features regarding the use of ionic liquids in industrial bioconversions will be discussed along with ideas for future work.

During the course of this research funds were obtained from the Centre for Scientific Enterprise (a collaboration between UCL and the London Business School) to carry out an evaluation of the use of ionic liquid technology as a replacement for commonly used organic solvents in the manufacture of pharmaceuticals as a new technology venture. A copy of the evaluation is included in Appendix VI of this thesis, and is included as one of the requirements for the Centre of Scientific Enterprise funding.

2.0 Materials and Methods

2.1 Reagents and Suppliers

Reverse osmosis (RO) purified water was used in all experimental work. The substrate and product standards of the lipase resolution (Figure 1.3) were generously donated by GlaxoSmithKline PLC (Tunbridge, UK). Ionic liquids used were either synthesised in-house or were obtained from Acros Organics (Geel, Belgium) or Solvent Innovation GmbH (Koln, Germany). All other chemicals and biochemicals were purchased from Sigma Chemical Co. (Dorset, UK) and were of the highest purity available. The enzyme CALB pre-immobilised on a polystyrene resin and marketed as Novozyme 435 or prepared as a lyophilized powder was purchased from Sigma. The enzyme thymidine phosphorylase was also purchased from Sigma. All the commercial adsorbents evaluated were purchased either from: Biorad (California, USA) AG® 1-X8 and AG® 50W-X8 resins; (Rohn and Hass Co, France) Amberlite XAD-7, XAD-16 and IRA 488 resins, and the activated charcoal was purchased from Sigma.

2.2 Synthesis and Preparation of Ionic Liquids

2.2.1 1-Butyl-3-Methylimidazolium Chloride [BMIM]Cl

[BMIM]Cl was prepared in the laboratory: N-methylimidazolium (500 mL, 6.3 moles) was mixed together with chlorobutane (730 mL, 6.9 moles) in a round-bottomed flask in an oil bath heated to 80 °C and stirred for 24 hours under reflux. After this the mixture was dissolved in an excess of acetonitrile to make it less viscous. To remove the excess reactants the mixture was washed (~4 times) with an equal volume of ethyl acetate. The ethyl acetate is immiscible with the acetonitrile/[BMIM]Cl and was decanted off. With the final wash of ethyl acetate still present, the mixture was placed in a fridge until the [BMIM]Cl solidified. The solid was then broken up and washed with ethyl acetate again before being recovered by filtration through an S4 (porosity 4) filter. The [BMIM]Cl solid was then stored in a

clean dry sealed vessel. To remove the remaining acetonitrile the [BMIM]Cl was placed on a rotary evaporator with heating (~50 °C) until no further solvent condensed. Finally the [BMIM]Cl was immersed in an oil bath, and attached to a high-vacuum line, incorporating a trap system each loop cooled in liquid nitrogen. The oil bath was heated to ~50 °C and the [BMIM]Cl was exposed to these conditions for 6 hours to evaporate any remaining solvent. After this time whilst still hot the [BMIM]Cl was poured out on a flat tray under a nitrogen atmosphere, and when it had cooled to a solid it was placed in an airtight container. To determine the purity of the [BMIM]Cl a sample was taken for NMR analysis in d-chloroform as described in Section 2.5.4.1.

2.2.2 1-Butyl-3-Methylimidazolium Hexafluorophosphate [BMIM][PF_6]

[BMIM][PF_6] was prepared in the laboratory: [BMIM]Cl (174.67 g) was dissolved in ~250 mL of distilled H_2O in a round bottomed flask and when the salt had dissolved the flask was immersed in an ice bath and stirred. Using a separating funnel HPF_6 (60 % w/w aqueous solution 114 mL, 0.7744 moles) was added drop wise to the aqueous solution of the halide salt, and was then left stirring for 24 hours at room temperature. After this time a two-phase mixture had formed. The upper aqueous phase was decanted off and the residual ionic liquid dissolved in dichloromethane (DCM). The DCM-ionic liquid mixture was then washed with ~200 mL distilled H_2O (~ 4 times) in a separating funnel. After the final wash, $MgSO_4$ was added, to remove any excess water, and shaken with the DCM-ionic liquid mixture. The $MgSO_4$ was then removed by filtration through an S4 filter. To remove the remaining DCM the mixture was placed on a rotary evaporator with heating (~50 °C) until no further solvent condensed. The retained crude ionic liquid was immersed in an oil bath, and attached to a high-vacuum line, incorporating a trap system each loop being cooled in liquid nitrogen. The oil bath was heated to ~50 °C and the ionic liquid exposed to these conditions for 6 hours. The remaining liquid was then removed from the oil bath and left to cool. The purity of the ionic liquid was determined by NMR analysis in d-chloroform as described in Section 2.5.4.1.

[BMIM][PF_6] was also obtained from Solvent Innovation GmbH, at a quoted purity of 97 %. To ensure the purchased ionic liquid was saturated with water before use, and to remove any remaining extractable acidic components, the ionic liquid supplied was washed 10 times with an equal volume of reverse osmosis (RO) water. The pH of the water phase after the final wash was measured to ensure a pH of 6.5. Before the ionic liquid was used, pH measurements taken after each wash stage indicated an increase in pH from low initial values, ~ pH 2.5, to a steady value of ~ pH 6.5. After washing the saturation water content of the ionic liquid was measured as described in Section 2.5.4.2. For reactions at higher water contents, RO water was added to give overall concentrations of between 3 – 15 % H_2O w/w.

2.2.3 1-Butyl-3-Methylimidazolium Tetrafluoroborate [BMIM][BF_4]

[BMIM][BF_4] was prepared in the laboratory: [BMIM]Cl (172.5 g) was dissolved in distilled H_2O (~200 mL). Na[BF_4] (119.28 g) was then added and the reaction mixture left stirring for 24 hours at room temperature. The solution was then mixed with 2 L of DCM and formed a two-phase mixture. The remaining [BMIM]Cl and the [BMIM][BF_4] dissolved in the lower DCM phase while the NaCl and $NaBF_4$ were decanted off in the upper aqueous phase. To remove the remaining [BMIM]Cl small amounts of distilled H_2O were used. Although both the ionic liquid and the [BMIM]Cl both dissolve in H_2O, [BMIM]Cl preferentially dissolves in small amounts of H_2O (10 x 50 mL washes). Then two spatulas of $MgSO_4$ were added, to remove the remaining water, and the DCM-ionic liquid mixture shaken. The $MgSO_4$ was then removed by filtration through an S4 filter. To remove the remaining DCM the mixture was placed on a rotary evaporator with heating (~50 °C) until no further solvent condensed. Finally the ionic liquid was immersed in an oil bath, and attached to a high-vacuum line, incorporating a trap system each loop being cooled in liquid nitrogen. The oil bath was heated to ~70 °C and the ionic liquid exposed to these conditions for 8 hours. The ionic liquid was then removed from the oil bath and left to cool. To determine the purity of the ionic liquid a sample was taken for NMR analysis as described in Section 2.5.4.1. The residual chloride content was measured as described in Section 2.5.4.3.

[BMIM][BF_4] was also obtained from Solvent Innovation GmbH at a given purity of 99 %. Since this ionic liquid is miscible with water it was used without additional purification. The water content as supplied was measured as described in Section 2.5.4.2 and the ionic liquid either used at this water content (Table 2.1) or RO water was added to give an overall water content of 12 % H_2O w/w before use (Section 2.3.1).

2.2.4 1-Butyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)amide [BMIM][$N(SO_2CF_3)_2$]

[BMIM][$N(SO_2CF_3)_2$] was prepared in the laboratory: [BMIM]Cl (200 g) was mixed with $LiN(CF_3SO_2)_2$ (237.12 g) in distilled water in a round bottomed flask and stirred for 24 hours at room temperature. After this time a two-phase mixture had formed. The upper aqueous phase was decanted off and the residual ionic liquid dissolved in DCM. The DCM-ionic liquid mixture was then washed with ~200 mL of distilled water (~ 4 times) in a separating funnel. After the final wash, $MgSO_4$ was added, to remove any excess water, and shaken with the DCM-ionic liquid mixture. The $MgSO_4$ was then removed by filtering through an S4 filter. To remove the remaining DCM the mixture was placed on a rotary evaporator with heating (~50 °C) until no further solvent condensed. The retained crude ionic liquid was then immersed in an oil bath, and attached to a high-vacuum line, incorporating a trap system each loop being cooled in liquid nitrogen. The oil bath was heated to ~50 °C and the ionic liquid exposed to these conditions for 6 hours. The remaining mixture was then removed from the oil bath and left to cool. The purity of the ionic liquid was determined by NMR analysis in d-chloroform as described in Section 2.5.4.1. The water content of the ionic liquids was measured as described in Section 2.5.4.2 and the ionic liquid used at this water content (Table 2.1) or RO water was added to give an overall water content of 12 % H_2O w/w before use (Section 2.3.1).

2.2.5 1-Butyl-3-Methylimidazolium Trifluoromethane sulfonate [BMIM][CF_3SO_3]

[BMIM][CF_3SO_3] was obtained from Acros Organics at a stated purity of 99 %. Since this ionic liquid is miscible with water it was used without further purification. The water content of the ionic liquid was measured as described in Section 2.5.4.2 and the ionic liquid used at this water content (Table 2.1), or with RO water added to give an overall water content of 12 % H_2O w/w before use (Section 2.3.1).

2.2.6 Methyl-trioctyl-imidazolium Bis(trifluoromethylsulfonyl)amide [Oc_3MeN][$N(SO_2CF_3)_2$]

[Oc_3MeN][$N(SO_2CF_3)_2$] was obtained from Solvent Innovation GmbH at a purity of 98 %. Although this ionic liquid is immiscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied (Section 2.3.2), or with RO water added to give an overall water content of 12 % H_2O w/w before use (Section 2.3.1).

2.2.7 1-Butyl-3-Methylimidazolium Diethyleneglycol Monomethylether [BMIM][$MDEGSO_4$]

[BMIM][$MDEGSO_4$] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Since this ionic liquid is miscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied, or RO water was added to give an overall water content of 12 % H_2O w/w before use (Section 2.3.1), or 10-20 % H_2O w/w depending on the reaction (Section 2.3.2).

2.2.8 1-Ethyl-3-Methylimidazolium Tosylate [EMIM][tosylate]

[EMIM][tosylate] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Since this ionic liquid is miscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied or with RO water was adjusted to give an overall water content of between 10-50 % H_2O w/w depending on the reaction (Section 2.3.2).

2.2.9 1-Butyl-3-Methylimidazolium Octylsulphate [BMIM][$OcSO_4$]

[BMIM][$OcSO_4$] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Since this ionic liquid is miscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied (Section 2.3.2).

2.2.10 ECOENG™ 500 (PEG-5 Cocomonium Methosulfate) [CABHEM][$MeSO_4$]

[CABHEM][$MeSO_4$] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Since this ionic liquid is miscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied (Section 2.3.2).

2.2.11 1-Hexyl-3-Methylimidazolium Bis(trifluoromethylsulfonyl)amide [HMIM][$N(SO_2CF_3)_2$]

[HMIM][$N(SO_2CF_3)_2$] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Although this ionic liquid is immiscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied (Section 2.3.2).

2.2.12 1-Methoxyethyl-3-Methylimidazolium Methanesulfonate [MOEMIM][OMs]

[MOEMIM][OMs] was obtained from Solvent Innovation GmbH at a stated purity of 98 %. Since this ionic liquid is miscible with water it was used without further purification. The ionic liquid was used with a minimal water content as supplied (Section 2.3.2).

2.3 Bioconversion Experiments

2.3.1 Bioconversion of SB-235349 to SB-240101

The reaction scheme was shown in Figure 1.3 All reactions were performed in a small, jacketed cone-shaped stirred reactor (h = 8 cm, d_{top} = 3.5 cm, d_{base} = 1 cm). This was fitted with a single flat-bladed impeller having a diameter of 0.4 cm, which was situated 1 cm from the base of the reactor and coupled to an overhead stirrer operated between 1000 - 3000 rpm. The reactor was charged with fresh Novozyme 435 resin (1.4 $g.L^{-1}$, 1 unit corresponds to the amount of enzyme which liberates 1 μmol butyric acid per minute pH 8 at 40 °C) or the free enzyme as a lyophilized powder (1.4 $g.L^{-1}$ 1 unit will hydrolyse 1 μmol equivalent of fatty acid from a triglyceride in 1 hour pH 7.7 at 37 °C), and 5 – 40 $g.L^{-1}$ SB-235349 depending on the particular experiment dissolved in either t-butanol (88 % v/v) or various ionic liquids (each with 12 % H_2O w/w unless otherwise stated). The reaction vessel jacket was pre-heated via a circulating water bath, which controlled the temperature to 25 – 75 ± 0.1 °C depending on the particular experiment. A 2 μL sample of the reaction mixture was taken every hour and analysed as described in Section 2.5.1. When the reaction was deemed to be complete (conversion > 47% based on HPLC peak area ratio of SB-235349 to SB-240101) the Novozyme 435 preparation could be recovered by filtration. In certain experiments a radiometer autotitrator was used to maintain a constant pH of 7.0 in the reaction mixture (this will be discussed later in Section 3.4.2), by automated addition of a 1.5M ammonia solution in either t-butanol (88% v/v) or various ionic liquids (each with 12 % H_2O w/w) (Atkins et al., 2003).

2.3.1.1 Examination of Enzyme Re-use

The re-use of the Novozyme 435 preparation was investigated over 10 reaction cycles at 50 °C. Initially 1.4 $g.L^{-1}$ of the immobilised enzyme, was added to the reaction vessel containing SB-235349 (5 $g.L^{-1}$) in [BMIM][PF_6] (containing 12 % H_2O w/w). The reaction was then repeated over 10 cycles as described in Section 2.3.1. After each resolution was deemed complete the reaction mixture was filtered through an S4

filter. The immobilised enzyme was then washed with water, re-weighed, and added to a fresh batch of substrate solution.

2.3.1.2 Determination of Solubility Limit for SB-235349

The solubility limit of the substrate in [BMIM][PF_6] was estimated by adding increasing quantities to a known volume of ionic liquid. The suspensions were then placed in a heated water bath set at 50 °C and mixed with a magnetic stirrer after each addition for up to 24 hours until the suspension became clear. The point at which no more solute would dissolve was taken as the solubility limit. The integrity of the dissolved solutes during the course of the experiment was confirmed by HPLC as described in Section 2.5.1.

2.3.1.3 Determination of Stability of SB-235349 and SB-240101

The concentration of the SB-235349 substrate and the SB-240101 product was measured over a six-hour period at both 50 °C and 75 °C. The integrity of the dissolved solutes at both temperatures did not change over the course of the experiment and was confirmed by HPLC as described in Section 2.5.1.

2.3.2 Bioconversion of thymine to thymidine

The reaction scheme is shown in Figure 1.4. All reactions were performed in triplicate, in a small cylindrical glass vessel (h = 2 cm, d = 1 cm). The reactor was charged with thymine (2.5mM), 2-deoxyribose-1-phosphate di(monocyclohexylammonium)salt (2.5mM) dissolved in either 10mM Tris hydrochloride buffer (pH7.4) or various ionic liquids (with varying water contents of 0 – 50 % H_2O w/w). The reaction vessel was immersed in a heated water bath at 30 ± 0.1 °C and stirred with a magnetic stirrer. When the substrates had dissolved the reaction was started by the addition of thymidine phosphorylase (12 units/mL 1 unit will convert 1 μmol each of thymidine and phosphate to thymine and 2-deoxyribose-1-phosphate per minute at pH 7.4 at 25 °C). Calcium nitrate (10mM) was added to the reaction mixture either before reaction or at equilibrium depending on the particular reaction. A 10 μL sample was taken every hour over the course of the reaction and

analysed as described in Section 2.5.3. The reaction was deemed complete when the reaction reached equilibrium (50 % conversion of thymine to thymidine) and a white precipitate had formed.

2.3.2.1 Determination of Solubility limit for thymine and thymidine

Thymine and thymidine are crystalline solids at room temperature. The solubility limit of thymine and thymidine was determined in a range of ten different ionic liquids (Table 5.2), obtained as discussed in Section 2.2. The solubility limit was determined for both thymine and thymidine by the addition of a measured concentration to a known volume of ionic liquid, which was then mixed at 1800rpm on a bench shaker (IKA labortechnik) for 24 hours at room temperature. If the substrate or product had dissolved the procedure was repeated, until a solid was still visible in the ionic liquid. The integrity of the dissolved solutes during the course of the experiment was confirmed by HPLC as described in Section 2.5.3.

The solubility limit for both thymine and thymidine was repeated in the ionic liquids [EMIM][tosylate] and [BMIM][$MDEGSO_4$] with different added water concentrations present depending on a particular experiment (water contents of 0 – 50 % H_2O w/w).

2.4 Product Recovery Experiments

2.4.1 Recovery of SB-235349 and SB-240101 following Bioconversion

2.4.1.1 Solvent Screening for Extraction of SB-235349 and SB-240101 from Ionic Liquids

A range of 35 polar, non-polar, aqueous and organic solvents (as described later in Table 4.2) were mixed with an equal volume of [BMIM][PF_6] containing 2.5 $g.L^{-1}$ of either SB-235349 or SB-240101. Both phases were mixed for 1 hour and then left for 24 hours to equilibrate. If the two phases were largely immiscible a sample was then

taken from both the [BMIM][PF_6] phase and the solvent phase, and analysed as described in Section 2.5.1.

2.4.1.2 Adsorbent Screening

An evaluation of an anionic and cationic resin and hydrophobic activated charcoal was undertaken, for the adsorption of SB-235349 and SB-240101 from [BMIM][PF_6] as shown in Table 4.3. [BMIM][PF_6] containing either 2.5 $g.L^{-1}$ SB-235349 or 2.5 $g.L^{-1}$ SB-240101 was prepared. A measured quantity of adsorbent (5 g per 100 mL) was then added (used as supplied) and mixed for 1 hour. After mixing the ionic liquid was removed from the adsorbent by filtration through a 1 mL syringe filter and analysed as described in Section 2.5.1.

2.4.1.3 Determination of the Distribution Ratio for the Extraction of SB-240101

[BMIM][PF_6] solutions containing 2.5 $g.L^{-1}$ of SB-235349, or 2.5 $g.L^{-1}$ of SB-240101, were prepared. 2 ml of each was contacted with an equal volume of 50mM aqueous buffer at a range of pH values (2-12). pH 2-3, 6-8 and 11-12: phosphate buffer, pH 4-5: acetate buffer, and pH 9-10 bicarbonate buffer. The ionic liquid-aqueous mixtures were subsequently agitated at 200 rpm and room temperature for 1 hour in sealed glass vials. The two-phases were then left to separate for 24 hours and a sample of an equal volume from both layers of each concentration analysed by HPLC, as described in Section 2.5.1. The concentration of the solutes were determined from the calibration curve from each phase, and the partitioning of the solute was calculated as a distribution ratio as in equation 2.1

$$D = \frac{Concentration\ in\ lower\ ionic\ liquid\ phase}{Concentration\ in\ upper\ aqueous\ phase} \qquad (2.1)$$

2.4.1.4 Extraction of SB-240101 from Bioconversion Reaction Mixture

Initially 1.4 $g.L^{-1}$ of the immobilised enzyme, was added to the reaction vessel containing SB-235349 (5 $g.L^{-1}$) in [BMIM][PF_6] (containing 8 % H_2O w/w), the

conversion of SB-235349 was then carried out at 75 °C as described in Section 2.3.1. When the reaction was deemed complete and the enzyme was removed, the [BMIM][PF_6] was mixed with an equal volume of 50mM bicarbonate buffer (pH10) for 1 hour and left for 24 hours to equilibrate. The upper aqueous buffer layer was then decanted off and a sample from both phases analysed as described in Section 2.5.1. The procedure was then repeated with fresh buffer each time, until 90 % w/w of the SB-235349 had been removed into the aqueous phase.

2.4.1.5 Extraction of SB-240098 from Bioconversion Reaction Mixture

The unconverted enantiomer of SB-240098 left after the bioconversion reaction had reached completion (Section 2.3.1) and extraction of SB-240101 (Section 2.4.1.4), was subsequently recovered by liquid-liquid extraction with isopropyl alcohol. An equal volume of isopropyl alcohol and ionic liquid reaction mixture were mixed together for 1 hour and left for 24 hours to equilibrate. The top layer of isopropyl alcohol was decanted off and a sample from both phases analysed as described in Section 2.5.1. The procedure was then repeated with fresh isopropyl alcohol each time, until 90 % w/w of the unreacted SB-240098 enantiomer had been removed into the alcohol phase.

2.4.1.6 Regeneration of [BMIM][PF_6] for Reuse

The ionic liquid phase from a bioconversion experiment (Section 2.3.1) following extraction with aqueous buffer (Section 2.4.1.4), then isopropyl alcohol (Section 2.4.1.5), was subsequently washed with 50mM sodium hydroxide to remove any residual SB-235349 or SB-240101. An equal volume of the sodium hydroxide was mixed with the ionic liquid for 1 hour and then left for 24 hours to equilibrate. The upper aqueous layer was then decanted off and the ionic liquid analysed as described in Section 2.5.1. Finally the reaction mixture was washed with an equal volume of 50mM potassium phosphate buffer (pH7) for 1 hour and left for 24 hours to equilibrate. The upper aqueous buffer layer was then decanted off and the pH of the ionic liquid measured using a pH meter.

2.4.2 Recovery of Thymine and Thymidine Following Bioconversion

2.4.2.1 Solvent Screening for Extraction of Thymine and Thymidine

A range of polar and non-polar organic solvents (as described later in Table 5.1) were mixed with an equal volume of [EMIM][tosylate] containing 5mM of either thymine or thymidine. Both phases were mixed for 1 hour and then left for 24 hours to equilibrate. If the two phases were immiscible a sample was taken from both the ionic liquid phase and the solvent phase and analysed as described in Section 2.5.3.

2.4.2.2 Adsorbent Screening

A range of anionic and cationic resins and hydrophilic activated charcoal were evaluated, for the adsorption of thymine and thymidine from ionic liquids as described later in Section 5.5.2. [EMIM][tosylate] containing either thymine (5mM) or thymidine (5mM) was prepared. A measured quantity of adsorbent (5 g per 100 mL) was then added (used as supplied) and mixed for 1 hour. After mixing the ionic liquid was removed from the adsorbent by filtration through a 1 mL syringe filter and analysed as described in Section 2.5.3.

2.4.2.3 Adsorption kinetics

The adsorption kinetics of both thymine and thymidine to activated charcoal and the anion exchange resin 1-X8 were examined as described later in Section 5.5.3. [EMIM][tosylate] (10 % H_2O w/w) containing either thymine (5mM) or thymidine (5mM) was prepared. A measured quantity of adsorbent either activated charcoal or the anion exchange resin 1-X8 (5 g per 100 mL) was then added (used as supplied) and mixed for six hours. Samples were removed from the adsorbent by filtration every hour through a 1 mL syringe filter and analysed as described in Section 2.5.3.

2.4.2.4 Adsorption and elution of thymine and thymidine

Both activated charcoal and the anion exchange resin 1-X8 were examined for adsorption and elution of thymine and thymidine following the synthesis of thymidine in both Tris HCl buffer and [EMIM][tosylate] (10 % H_2O w/w) as described in Section 5.5.4. Following conversion as described in Section 2.3.2, the reaction mixture was filtered to remove the calcium phosphate precipitate and then mixed with either activated charcoal or the anion exchange resin 1-X8 (5 g per 100 mL) for 6 hours. The adsorbent was then filtered off and the thymine and thymidine removed from the adsorbent by elution with an equal volume of either ethanol for the activated charcoal or 50mM sodium chloride for the anion exchange resin 1-X8.

2.4.3 Lewis cell extraction

2.4.3.1 Lewis cell operation

As described in Section 2.4.1.4 and Section 2.4.1.5 the extraction of SB-240101 and SB-240098 respectively, were examined from the ionic liquid reaction medium following conversion of SB-235349. The extraction of SB-240101 from the ionic liquid [BMIM][PF_6] was studied with an aqueous phase (pH 10) and the remaining unreacted SB-240098 was extracted from [BMIM][PF_6] into an organic phase (IPA). In order to study the mass transfer kinetics of these extractions both were examined within the Lewis cell. The initial solute concentration used in both cases was 5 $g.L^{-1}$ in [BMIM][PF_6].

The Lewis cell was designed as described by Lye and Stuckey (2001). Experiments were performed with equal Reynolds numbers (Re) in each phase to ensure comparable hydrodynamics either side of the interface. Re is defined as follows:

$$\mathrm{Re} = \frac{NDi^2\rho}{\mu} \qquad (2.2)$$

where N is the impeller speed ($m.s^{-1}$) determined using Lutron tachometer Model DT-2236, Di is the impeller diameter (m), ρ is the density ($kg.m^{-3}$) and μ is the viscosity ($kg.s^{-1}$). Measurements of ρ and μ were carried out as described in Section 2.5.5. The Lewis cell was operated at 25 $\pm$ 0.1 °C in all experiments and this was maintained by a temperature controlled water bath circulating through the vessel jacket. The lower ionic liquid phase was funnelled to the bottom of the vessel first to avoid wetting the upper half of the cell. The upper aqueous phase was then carefully added on top of the ionic liquid in order to not disturb the ionic liquid layer. Both phases were then brought to a temperature of 25 °C and samples taken of each phase before the impellers were switched on at their pre-set speeds. Equal volumes of both phases had previously been equilibrated, in the absence of product or substrate, to ensure that these were the only transferring species. The water content of the ionic liquid phase was measured following equilibration with the aqueous phase as described in Section 2.5.4.2. Each experiment lasted for 6 hours during which time 200 µL samples from both phases were extracted through the sampling ports for analysis as described in Section 2.5.1.

2.4.3.2 Lewis cell extraction

For the extractions studied in the Lewis cell, the two-film theory of mass transfer is applicable, the extraction rate of SB-235349 (j_s) or SB-240101 (j_p) out of the ionic liquid phase, is given by:

$$j_P = \left(\frac{V}{A}\right)\frac{-dC_{IL}}{dt} = K_0\{C_{IL} - (1/m)C_{aq}\} \tag{2.3}$$

$$j_S = \left(\frac{V}{A}\right)\frac{-dC_{IL}}{dt} = K_0\{C_{IL} - (1/m)C_{org}\} \tag{2.4}$$

Where K_0 ($m.s^{-1}$) is the overall mass transfer coefficient, C ($kg.m^{-3}$) is the concentration of the substrate or the product in the phase under consideration, m is the

equilibrium partition coefficient, V (m^3) is the volume of the ionic liquid phase, A (m^2) is the interfacial area of the cell, and t (s) is the extraction time (Lye and Stuckey, 2001).

Integrating the extraction rate expression yields the following equation that allows determination of K_0 by linear regression. For the product:

$$\ln[\{C_{IL}(0)-(1+1/m)C_{aq}\}/C_{IL}(0)] = -\left(\frac{A}{V}\right)(1+1/m)K_0 t \qquad (2.5)$$

For the substrate:

$$\ln[\{C_{IL}(0)-(1+1/m)C_{org}\}/C_{IL}(0)] = -\left(\frac{A}{V}\right)(1+1/m)K_0 t \qquad (2.6)$$

In all the cases studied, plots of Equation (2.5) and (2.6) produced a straight line and confirm the application of this model to the experimental data. When m is large (m>5) the calculated value of K_0 did not change significantly therefore the above equation reduces to

$$\ln[C_{IL}/C_{IL}(0)] = -\left(\frac{A}{V}\right)K_0 t \qquad (2.7)$$

Hence equation (2.7) was used to calculate K_0 during all extraction experiments since in all cases m>5 (see Table 4.4 for the experimentally determined values of m).

2.5 Analytical Techniques

2.5.1 Quantification of SB-235349 and SB-240101

Bioconversion samples in either t-butanol or ionic liquids (injection volume 100 µl) were analysed by HPLC (Dionex PeakNet system) using a Phenomenex 5 µm C18 column (150 x 4.6 mm) as described by Atkins et al. (2003). Eluents used were A: 0.1% Trifluoroacetic acid (TFA) in water and B: 0.1% TFA in acetonitrile. The mobile phase flow rate was 1 mL.min^{-1} with a gradient of 25%-75% B over 10 minutes with UV detection at 254 nm.

The UV response for a particular solute concentration in a sample was determined as an integrated peak area on a HPLC chromatogram. External calibration curves were used to quantify these responses for the pure SB-235349 substrate and pure SB-240101 products. Typical calibration curves of concentration against UV response (Figs: I.3 and I.4) and sample chromatograms (Figs: I.1 and I.2) are shown in Appendix I for all the types of sample analysed. The maximum coefficient of variance for the quantification of SB-235349 and SB-240101 concentrations was 2.5 % and 2 % respectively.

2.5.1.1 Determination of solute equilibrium partition coefficients in ionic liquid-aqueous biphasic systems

In order to quantify the concentration of SB-235349 and SB-240101 present in samples prepared after extraction into aqueous buffer it was necessary to first determine the equilibrium partition coefficient of the solutes in ionic liquid-aqueous buffer biphasic systems. [BMIM][PF_6] with five different concentrations of SB-235349 and SB-240101 were made up at concentrations between 0.0-2.5 gL^{-1}. 2 ml of each concentration was contacted with an equal volume of 50mM aqueous buffer. The ionic liquid-aqueous buffer mixture was subsequently agitated at 200 rpm at room temperature for 1 hour in sealed glass vials. The two-phases were then left to separate for 24 hours and the upper aqueous layer from each concentration transferred to glass vials for HPLC analysis, as described in Section 2.5.1. By determining the aqueous concentration of SB-235349 and SB-240101 in each phase at equilibrium, the aqueous-ionic liquid partition coefficient, K, can be determined as the ratio between the equilibrium concentration of solute in the aqueous buffer, C_{aq}, and the equilibrium concentration of solute remaining in the ionic liquid, C_{IL}, using Equation 2.8. These results are shown in Appendix II (Figure II.2) together with the HPLC calibration curves (Figure II.1).

$$K = \frac{C_{aq}}{C_{IL}} \tag{2.8}$$

2.5.1.2 Determination of solute equilibrium partition coefficient in an ionic liquid-IPA biphasic system

In order to quantify the concentration of SB-235349 and SB-240101 present in ionic liquid samples prepared by extraction into IPA, it was necessary to first determine the ionic liquid-organic solvent partition coefficient for the compound. [BMIM][PF_6] with five different concentrations of the substrate, SB-235349, and product SB-240101 were made up at concentrations between 0.0-2.5 gL^{-1}. 2 ml of each concentration was contacted with an equal volume of the IPA. The ionic liquid-organic solvent mixture was subsequently agitated at 200 rpm at room temperature for 1 hour in sealed glass vials. The two-phases were then left to separate for 24 hours and the upper organic solvent layer from each concentration transferred to glass vials for HPLC analysis, as described in Section 2.5.1. By determining the organic solvent concentration of SB-235349 in each phase at equilibrium, the organic solvent-ionic liquid partition coefficient, K, can be determined as the ratio between the equilibrium concentration of solute in the organic solvent, C_{org}, and the equilibrium concentration of solute remaining in the ionic liquid, C_{IL}, using Equation 2.9. These results are shown in Appendix II (Figure II.4) together with the HPLC calibration curves (Figure II.3).

$$K = \frac{C_{org}}{C_{IL}} \tag{2.9}$$

2.5.2 Confirmation of Chiral Purity of SB-240101

The chiral purity (enantiomeric excess, e.e.) of the product SB-240101 was determined by chiral HPLC as described by Atkins et al., (2003). The column used was a 5 μm CHO-3981 (250 x 4.6 mm) operated at 25 °C. The mobile phase consisted of 98 % hexane and 2 % IPA which was pumped isocratically at 0.7 $mL.min^{-1}$ with UV detection at 215 nm. Typical retention times of the *R*-ester (SB-240098) and *S*-ester were 32.2 and 30.2 minutes respectively (the *S*-acid product is not detected as it binds irreversibly to the column). Samples from t-butanol reactions (100 µl) were injected directly onto the column. Samples from ionic liquid reactions were first extracted into an equal volume of the HPLC mobile phase before injection.

2.5.3 Quantification of thymine and thymidine

Bioconversion samples in either Tris HCl buffer or ionic liquids (100 µl) were analysed by HPLC (Dionex PeakNet system) using a Phenomenex 5 µm C18 column (150 x 4.6 mm), as described in the European Patent (EP 1179598A1 2003). The eluent used was 10mM phosphoric acid: acetonitrile 95:5 (v/v). The mobile phase flow rate was 1 $mL.min^{-1}$ over 10 minutes with UV detection at 260 nm, with a column temperature of 40 °C. Typical retention times for thymine and thymidine were 3.6 and 5.8 minutes respectively. The UV response was quantified as described in Section 2.5.1 and calibration curves (Figs: III.3 and III.4) and sample chromatograms (Figs: III.1 and III.2) are shown in Appendix III. The maximum coefficient of variance for the quantification of SB-235349 and SB-240101 concentrations was 1.5 % and 2 % respectively.

2.5.4 Analysis of ionic liquids

2.5.4.1 NMR spectroscopy

NMR samples were prepared by placing a drop of the ionic liquid into an NMR tube and half filling the tube with either d-chloroform or acetone. The ^1H NMR shifts for ionic liquids are available in the literature: for [BMIM][PF_6] and [BMIM][BF_4] see Park and Kazlauskas, (2001), for [BMIM][$N(SO_2CF_3)_2$] and [BMIM][Cl] see Deetlefs and Seddon, (2003). The ionic liquids synthesised in this case displayed the same NMR results as in the literature and no organic impurities were observed.

2.5.4.2 Determination of water content

The water content of the ionic liquids was determined by Karl Fischer titration. This method has already been used for the determination of the water content of ionic liquids (Wong et al., 2002). The water content of the ionic liquids tested was monitored using a 701 Karl Fisher Titrino (Metrohm, Switzerland). Measurements

were performed at the GSK Ulverston site according to standard methods. The maximum coefficient of variance of this assay was 4 %.

Table 2.1: Water content of the ionic liquids used for reactions determined by Karl Fisher.

Ionic Liquid	% H_2O [w/w]
[BMIM][PF_6]	3.073
[BMIM][$N(CF_3SO_3)$]	0.275
[BMIM][BF_4]	0.613
[BMIM][$N(SO_2CF_3)_2$]	0.167

2.5.4.3 Determination of chloride content

The principle of this method is to precipitate all chloride ions present, as silver(I) chloride by adding a known excess of silver(I) nitrate. The precipitate is filtered off, and the indicator iron(III) nitrate added. The excess of silver present in the sample is then back titrated with a solution of thiocyanate. Excess thiocyanate, in turn forms the thiocyanatoiron(III) complex with the iron(III) nitrate, which is reddish-brown. The endpoint of the titration is determined visually by the first permanent appearance of a red colour:

$$Cl^- + Ag[NO_3] \Rightarrow AgCl + [NO_3]^- + Ag^+_{(excess)}$$

$$Ag^-_{(excess)} + [SCN]^- \Rightarrow Ag[SCN]$$

$$[SCN]^-_{(excess)} + Fe^{3+} \Rightarrow [Fe(SCN)]^{2+}$$

The aqueous potassium thiocyanate solution used was 0.0981M and the aqueous silver(I) nitrate solution was 0.1M. In a flask (250 cm^3), the sample solution (25 cm^3) was acidified with nitric acid (6M, 5 cm^3). Silver nitrate (1M, 1.5 cm^3) was added to precipitate the chloride. After shaking the mixture, the white precipitate was filtered off, and the filtrate washed through three times with dilute nitric acid. The indicator was added to the combined washings, and the solution titrated with 0.0981M potassium thiocyanate solution until the first permanent reddish colour occurred. The

endpoint was read from the burette (5 cm^3). This procedure was repeated three times, and the average used as the final result. The chloride content of the [BMIM][BF_4] was 0.032 % [Cl^-] w/w.

2.5.5 Physical measurements

2.5.5.1 Density measurements

Gravimetric analysis was used to determine the liquid density (ρ); 10 mL liquid volumes were measured into density bottles and weighed on a Mettler-Toledo AB54 electronic balance (Leicester, UK). All measurements were preformed in triplicate. The values for pure [BMIM][PF_6], ρ = 1380 kg. m^{-3} and t-butanol (88 % v/v), ρ = 795 kg. m^{-3} were in line with literature values (Mantz and Trulove, 2003). The water saturated [BMIM][PF_6] had a density of 1316 kg. m^{-3}.

2.5.5.2 Viscosity measurements

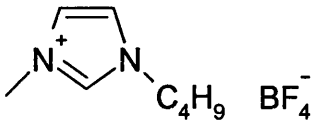
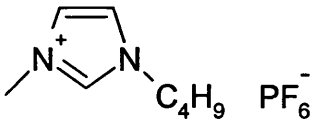
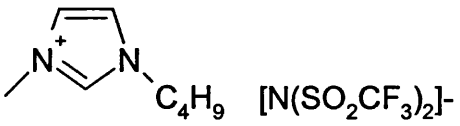
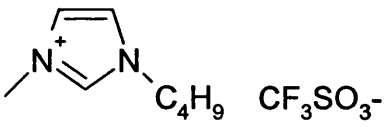
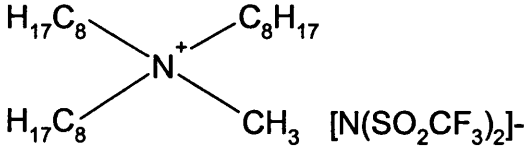
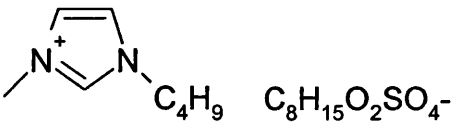
Liquid viscosity measurements were made using a Contraves Rheomat 115 rheometer (Contraves AG, Zurich, Switzerland). All measurements were preformed in triplicate. The values defined for pure [BMIM][PF_6], μ = 207 mPas and t-butanol (88 % v/v), μ = 5 mPas were in line with literature values (Mantz and Trulove, 2003). The water saturated [BMIM][PF_6] had a viscosity of 85.05 mPas. The [BMIM][PF_6] was shown to have Newtonian rheology by a straight-line plot of shear stress against shear rate when both pure and saturated with water (Appendix IV).

3.0 Kinetics of Lipase Catalysed Resolutions in Ionic Liquids

3.1 Aims

The aim of this chapter is to illustrate a generic approach to the design and optimisation of a lipase-catalysed resolution reaction in ionic liquids, and to elucidate novel findings using ionic liquid media compared to conventional organic solvents. The particular reaction considered is the hydrolytic resolution of racemic 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester to (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid (SB-240101) by immobilised *Candida antarctica* lipase B, CALB (Novozyme 435). As shown in Figure 1.3, this reaction is a key step in the synthesis of Lotrafiban. Firstly the reaction kinetics were determined in t-butanol, the solvent used in the industrial process, under previously optimised conditions. Subsequently the resolution was examined in a limited range of ionic liquids as shown in Table 3.1, to determine which could be used as alternative reaction media, and if so, what advantages the use of ionic liquids may provide over conventional organic solvents. The reaction in the ionic liquid medium was then examined by the variation of key parameters in order to identify optimum reaction conditions for the ionic liquid medium so that the results might be compared to those for the optimised t-butanol process. The majority of the results presented in this Chapter have been published: Roberts N.J., Seago A., Carey J.S., Freer R., Preston C. and Lye G.J., 2004. Lipase Catalysed Resolution of the Lotrafiban Intermediate 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester in Ionic Liquids: Comparison to the Industrial t-Butanol Process. Green Chemistry. Vol.6, p475-482.

Table 3.1- Structure and physical properties of the various ionic liquids screened for use in the lipase catalysed resolution of SB-235349. Ionic liquids synthesised and prepared as described in Section 2.2

Ionic Liquid Structures and Nomenclature	Summary of Physical Properties	References
 [BMIM][BF_4]	Hydrophilic Melting point –71.0 °C Miscible with water Miscible with acetonitrile	Branco et al., (2002) Huddleston et al., (2001) Holbrey et al., (1999)
 [BMIM][PF_6]	Hydrophobic Melting point 6.0 °C Immiscible with water Miscible with acetonitrile	Huddleston et al., (2001) Dzyuba et al., (2002) Holbrey et al., (2002)
 [BMIM][$N(SO_2CF_3)_2$]	Hydrophobic Melting point –6.0 °C Immiscible with water Miscible with acetonitrile	Dzyuba et al., (2002) Huddleston et al., (2001)
 [BMIM][CF_3SO_3]	Hydrophilic Melting point 17.0 °C Miscible with water Miscible with acetonitrile	Huddleston et al., (2001) Wasserscheid et al., (2000)
 [Oc_3MeN][$N(SO_2CF_3)_2$]	Hydrophobic Melting point –70.0 °C Immiscible with water Miscible with acetonitrile	Patent No. EP 1178050, (2002) www.solventinnovation.com
 [BMIM][$MDEGSO_4$]	Hydrophilic Miscible with water Miscible with acetonitrile	www.solventinnovation.com

3.2 Characterisation of the Reaction Substrate and Product

3.2.1 Properties of the Substrate and Product Compounds

The initial step in characterising the bioconversion was to obtain physico-chemical property data on the reaction substrate and product, either from literature data, or experimentally. The racemic methyl ester substrate (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349) is converted to the reaction product, (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid (SB-240101), leaving the un-reacted methyl ester (2R)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-240098). The substrate is a solid at room temperature with the molecular formula $C_{13}H_{16}N_2O_3$ and a molecular weight of 248. The SB-240101 product is also a solid at room temperature with the molecular formula $C_{12}H_{14}N_2O_3$, with a molecular weight of 233. The boiling points of the racemic esters SB-235349 and SB-240098 were 174-176 °C and 132-134 °C respectively; the boiling point of the SB-240101 product was 148-150 °C (Atkins et al., 2003) so no loss of either compound by evaporation was expected.

The racemic substrate and the product were analysed throughout this work by HPLC, as described in Section 2.5.1, and a sample HPLC chromatogram is shown in Appendix I. Neither of the compounds are commercially available and were supplied as purified components by GlaxoSmithKline (Tonbridge, UK). The racemic methyl ester substrate, (a mixture of both the S form SB-235349 and the R form SB-240098) had a retention time on the HPLC column of 6.1 minutes, and appeared as a single peak on the chromatogram. The SB-240101 product was also visualised as a single peak with a retention time of 4.4 minutes. To confirm the correct substrate enantiomer was being converted by the enzyme the substrates (SB-235349 and SB-240098) were analysed by chiral HPLC as described in Section 2.5.2, and a sample HPLC chromatogram is shown in Appendix V. The SB-235349 and SB-240098 had retention times of 30.2 and 32.2 minutes, respectively, and both were visualised as a single peak on the chromatogram.

3.2.2 Substrate Solubility and Stability

The solubility of the SB-235349 substrate in [BMIM][PF_6] was 42.9 ± 0.1 g.L^{-1} at 50 °C as described in Section 2.3.1.2. The solubility of the SB-240101 product in [BMIM][PF_6] was 28.5 ± 0.1 g.L^{-1} measured at 50 °C as described in Section 2.3.1.2.

The stability of the solutes in ionic liquids at elevated temperatures was also assessed by monitoring changes in HPLC chromatograms (peak retention time and area) over time. The concentration of SB-235349 and SB-240101 in [BMIM][PF_6] at 50 °C was measured over a six-hour period, as described in Section 2.3.1.3. It was observed that both compounds were stable over this time period. In a concurrent experiment the stability was tested at 75 °C as [BMIM][PF_6] is known to degrade at these temperatures, producing acidic species and potentially HF (Swatloski, et al. 2003); no degradation of [BMIM][PF_6] was observed by HPLC analysis.

The stability of the enzyme was also investigated, and after heating at 100 °C for 8 hours in water the Novozyme 435 was found to still be active for the conversion of SB-235349 to SB-240101.

3.3 Characterisation of the Novozyme 435 Catalysed Resolution of SB-235349 in t-butanol

Initial experiments in t-butanol examined the activity of the batch of Novozyme 435 lipase used throughout this work in order to provide a basis for comparison with subsequent ionic liquid studies. Experiments were performed under the optimum conditions previously established for the industrial resolution of SB-235349, as described in Section 2.3.1 (Atkins et al., 2003). Typical bioconversion results are shown in Figure 3.1. Measurements of the liquid phase solute concentration showed a rapid initial decrease in SB-235349 concentration from 5 to 3 g.L^{-1}. This was attributed primarily to the adsorption of substrate onto the Novozyme 435 resin on which the CALB is immobilised. By comparison there is a steady, almost linear increase in the concentration of SB-240101 up to 180 min, which best represents the apparent rate of product synthesis. The final SB-240101 concentration of 2.3 g.L^{-1}

after 8 hours corresponds to a 47 % conversion, as reported for the industrial process (Atkins et al., 2003). The liquid phase mass balance (Figure 3.1) indicates an initial drop to ~60 % corresponding to the decrease in SB-235349 concentration due to adsorption. This is followed by a steady increase to ~100 %, in line with SB-240101 formation, which implies that there is no significant binding of the acid product to the resin.

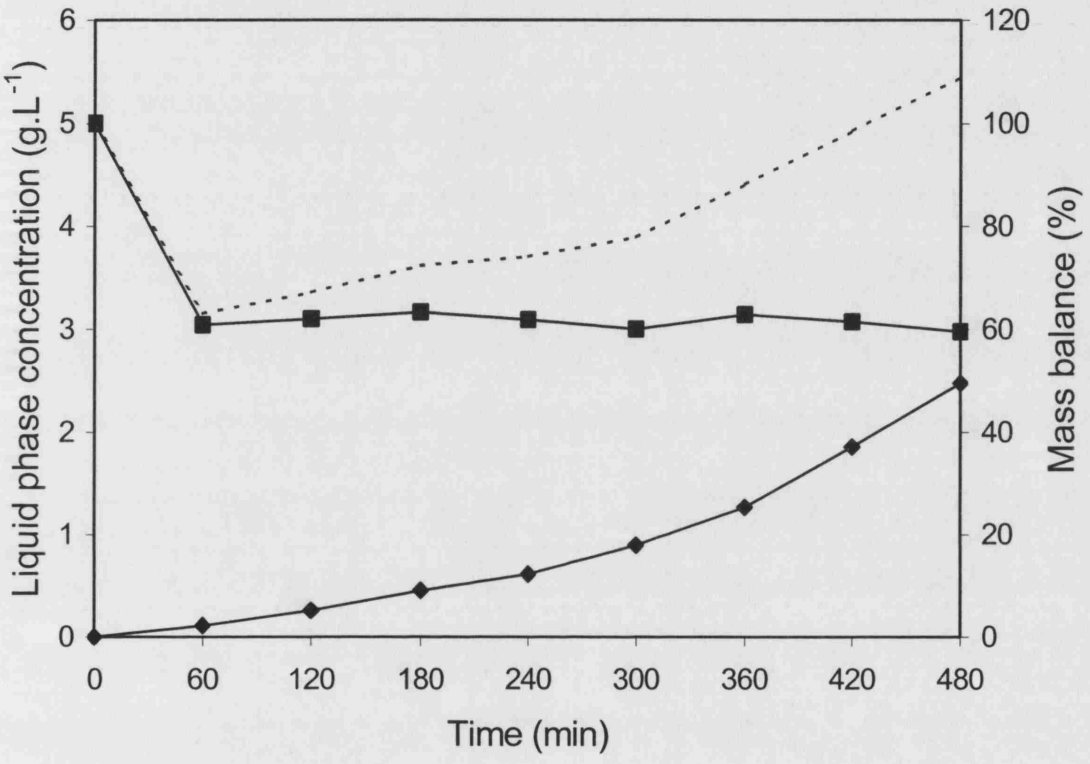


Figure 3.1- Kinetics of CALB catalysed resolution of SB-235349 in t-butanol: (♦) SB-240101 concentration, (■) SB-235349 concentration, (---) liquid phase mass balance. Reaction conditions as described in Section 2.3.1: solvent = t-butanol (88 % v/v), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control.

Optimisation of the t-butanol process had shown control of pH was not a quality critical parameter, however, at low pH a degree of enzyme inhibition was observed (Atkins et al., 2003). Therefore, experiments were performed with and without pH control, as shown in Table 3.2. The pH change measured over the course of the t-butanol process without pH control was pH 6.39 to pH 4.74. These results showed no significant increase in the apparent initial rate of product formation in t-butanol, and

no change in the overall yield of SB-240101. Any pH change induced by the hydrolytic resolution was thus considered to be insignificant under these conditions, and it is well known that the Novozyme 435 CALB preparation has a reasonably broad pH optimum of between 5 – 8 (Atkins et al., 2003).

Table 3.2- Effect of pH control (pH 7) on the initial rate of SB-240101 formation in t-butanol and various ionic liquids (12 % H_2O w/w). Reaction conditions as described in Section 2.3.1: [Novozyme 435] = 1.4 $g.L^{-1}$, initial [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C.

Reaction Medium	Apparent Initial Rate ($g.L^{-1}.min^{-1}$)	pH Control
t-butanol	4.6×10^{-3}	No
t-butanol	4.7×10^{-3}	Yes
[BMIM][$N(CF_3SO_2)_2$]	1.0×10^{-2}	No
[BMIM][$N(CF_3SO_2)_2$]	1.0×10^{-3}	Yes
[BMIM][PF_6]	3.8×10^{-3}	No
[BMIM][PF_6]	5.3×10^{-4}	Yes

3.4 Novozyme 435 Catalysed Resolution of SB-235349 in Ionic Liquids

3.4.1 Ionic Liquid Screening

Initial experiments with ionic liquids aimed to establish if the CALB catalysed resolution of SB-235349 would actually occur in an ionic liquid, and if so, which type of ionic liquid was most suitable for the reaction. For these experiments a limited range of ionic liquids were chosen that exhibited different physico-chemical properties, as shown in Table 3.1. All were tested under conditions identical to those used in Figure 3.1, apart from the replacement of t-butanol with the appropriate ionic liquid. The ionic liquids were synthesised and purified before use as described in Section 2.2.

The first ionic liquid tested was [BMIM][BF_4] which is considered hydrophilic, and is miscible with water (Holbrey et al., 1999; Huddleston et al., 2001; Branco et al., 2002). As shown in Figure 3.2 the substrate rapidly decreased, however, no SB-240101 could be detected, the results suggest an alternate product was produced which displayed a different retention time from SB-240101 by HPLC analysis. With [BMIM][PF_6], which is considered to be more hydrophobic (Huddleston et al., 2001; Dzyuba et al., 2002; Holbrey et al., 2002), a relatively slow decrease in the substrate concentration was found with the corresponding production of SB-240101. After six hours the SB-240101 concentration was 0.7 $g.L^{-1}$, approximately half that produced in the t-butanol system. The results for [BMIM][$N(SO_2CF_3)_2$] which is hydrophobic, and [BMIM][CF_3SO_3] which is hydrophilic (Table 3.1), are similar to those for [BMIM][PF_6] with SB-240101 concentrations of 1 $g.L^{-1}$ and 0.65 $g.L^{-1}$, respectively, being attained after 6 hours. Other ionic liquids tested (results not shown in Figure 3.2) include methyl-trioctyl-imidazolium bis(trifluormethansulfonimide) [Oc_3MeN][$N(CF_3SO_2)_2$], which is water immiscible, and 1-butyl-3-methyl-imidazolium diethyleneglycol monomethylethersulphate [BMIM][$MDEGSO_4$] which is also water miscible (Table 3.1). The [BMIM][$MDEGSO_4$] showed a rapid decrease in SB-235349 concentration, however there was no SB-240101 formation. The HPLC traces of the reaction in [Oc_3MeN][$N(SO_2CF_3)_2$] showed no peaks of any kind. In summary, screening of a very limited range of ionic liquids yielded a number that could act as useful media for the CALB catalysed resolution, but at rates and conversions below those for the previously optimised t-butanol system.

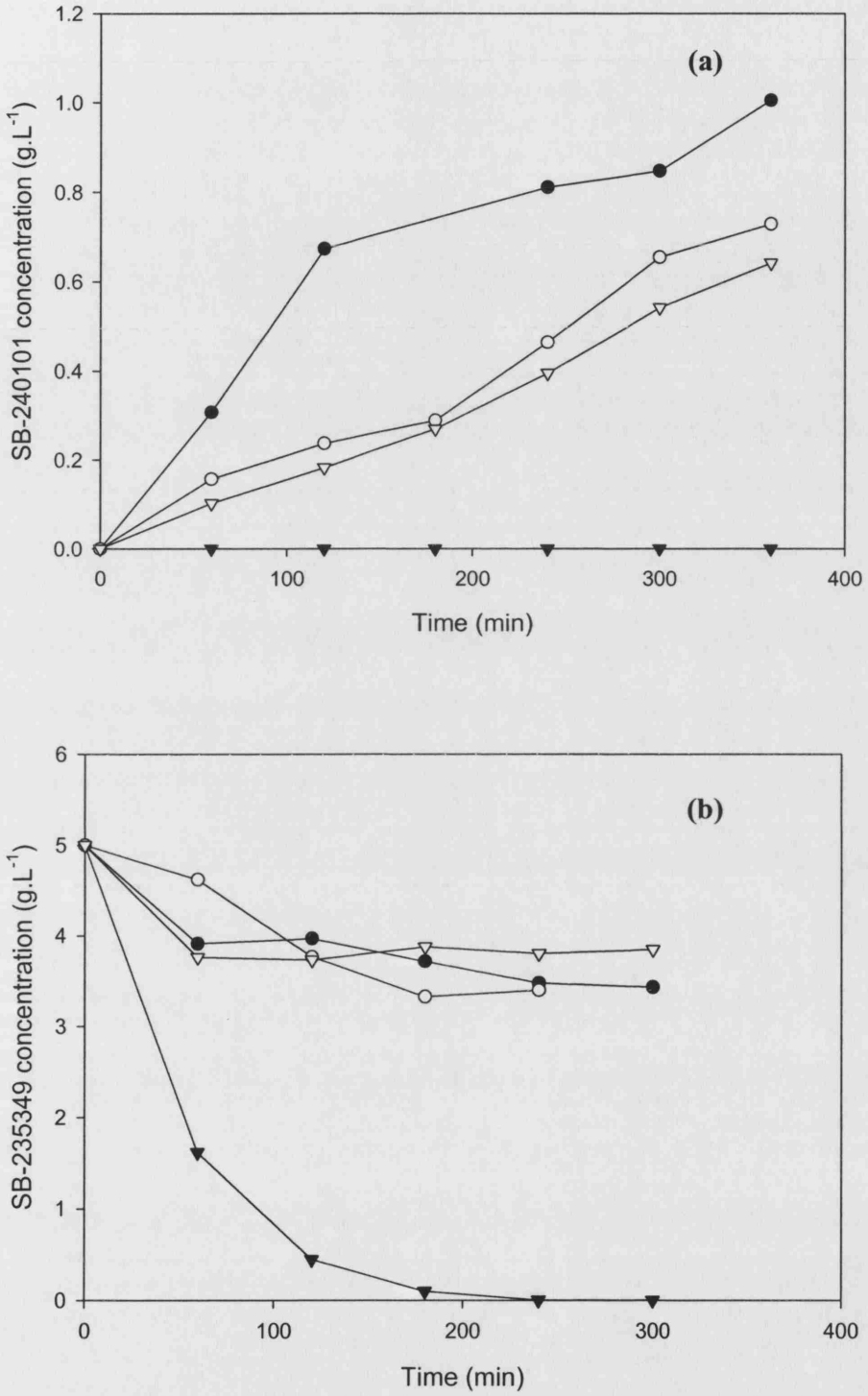


Figure 3.2- Kinetics of CALB catalysed resolution of SB-235349 in a range of ionic liquids, (a) SB-240101 concentration, (b) SB-235349 concentration: (○) [BMIM][PF_6], (▼) [BMIM][BF_4], (●) [BMIM][$N(SO_2CF_3)_2$], (▽) [BMIM][$N(CF_2SO_3)$]. Reaction conditions as described in Section 2.3.1: Solvent = ionic liquid (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control.

3.4.2 Influence of pH Control and Agitation on Reaction Rate

As with the t-butanol process, a number of the promising ionic liquid bioconversions were performed with and without pH control. The meaning of a pH value determined in an ionic liquid is clearly different to that measured in aqueous solutions (King et al., 1996; Olivier-Bourbigou et al., 2002; Thomazeau et al., 2003), however, we were interested to see what benefits, if any, there might be of operating in pH-stat mode. Table 3.2 shows the apparent initial rates of reaction with and without control of pH at pH 7. In contrast to the results obtained for t-butanol, there is a seven-fold decrease in the rate of reaction with [BMIM][PF_6], and a ten-fold decrease with [BMIM][$N(SO_2CF_3)_2$]. These marked decreases were attributed to the large initial volume of ammonia solution added to the ionic liquids compared to the volume required in t-butanol. Ammonia solution was used to provide a direct comparison to the t-butanol process. Large volumes of ammonia would have a significant negative effect on the activity of the CALB enzyme, increased acidity would cause the enzyme to denature. All subsequent experiments in ionic liquids were thus carried out without pH control.

Experiments were also performed in [BMIM][PF_6] with increasing stirrer speeds from 1000 – 3000 rpm. The results shown in Figure 3.3 indicated an increase in the initial rate of SB-240101 formation with stirrer speeds up to 2000 rpm. This suggests that the measured activity of the CALB is diffusion controlled at the lower agitation rates (Gordon et al., 2003). Compared to the t-butanol system, the initial rate of reaction in [BMIM][PF_6] (3000 rpm) was ~28 % slower. This decrease is probably explained by the difference in solvent viscosity as described in Section 2.5.5.2. The viscosity of the saturated ionic liquid was noticeably less than that of the pure anhydrous ionic liquid, but much greater than the t-butanol (88 % v/v), as will be discussed further in Chapter 4. All subsequent experiments were performed at 3000 rpm.

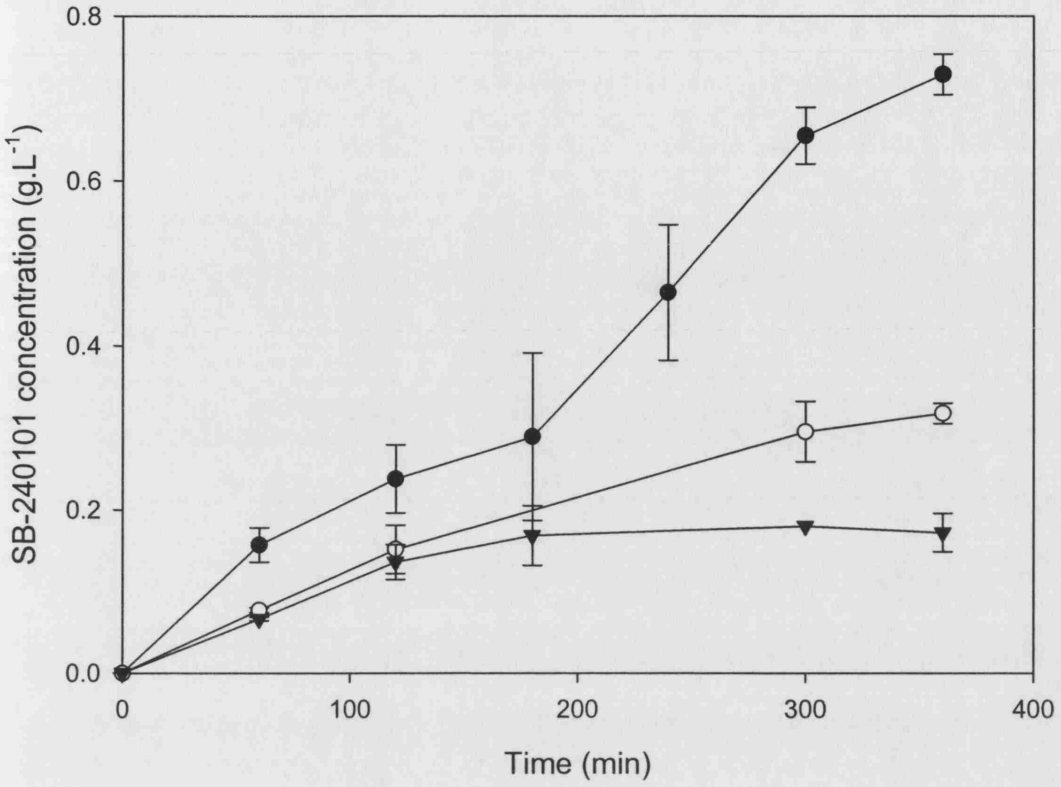


Figure 3.3- Effect of stirrer speed on the initial rate of SB-240101 formation in [BMIM][PF_6]: (●) 3000 rpm, (○) 2000 rpm, (▼) 1000 rpm, Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control. Error bars indicate one standard deviation.

3.4.3 Effect of Substrate Concentration on SB-240101 Formation in [BMIM][PF_6]

Having established that the resolution of SB-235349 was possible in ionic liquids, the solvent properties of selected ionic liquids were explored further as were the conditions under which the resolution was performed. An eight-fold increase in substrate solubility was observed at 50 °C in the widely used [BMIM][PF_6] (Huddleston et al., 2001; Dzymba et al., 2002; Holbrey et al., 2002), compared to t-butanol (88 % v/v) under the same conditions (as described in Section 2.3.1.2). As

shown in Figure 3.4, Novozyme 435 catalysed resolutions were subsequently performed in [BMIM][PF_6] at 50 °C and various initial SB-235349 concentrations of up to 40 $g.L^{-1}$ (0.16 Molar). The apparent initial rate of the reaction increased dramatically as the substrate concentration was increased from 5 $g.L^{-1}$, to around 25 $g.L^{-1}$ where it became relatively constant. At 40 $g.L^{-1}$ there is a three-fold increase in the apparent initial rate of SB-240101 formation in [BMIM][PF_6] at 50 °C, compared to that obtained under optimal conditions in the t-butanol system. For all the substrate concentrations investigated in [BMIM][PF_6] there was no evidence of substrate inhibition at these high solute concentrations.

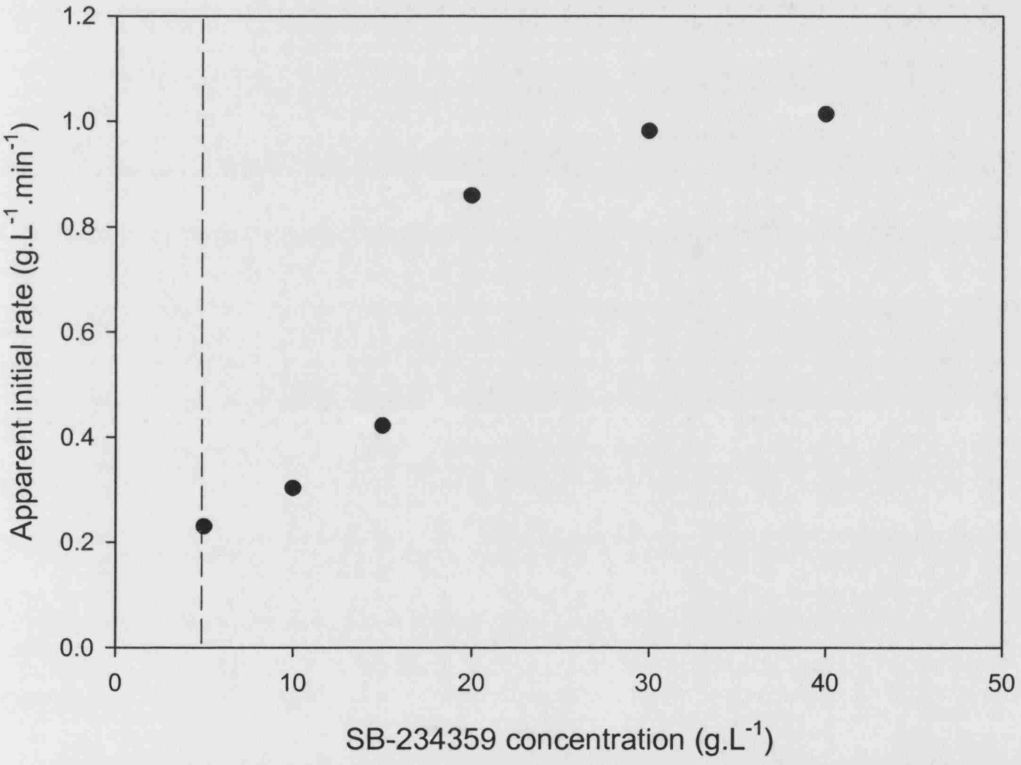


Figure 3.4- Effect of SB-235349 concentration on the apparent initial rate of SB-240101 formation in [BMIM][PF_6]. Dashed line indicates solubility limit of SB-235349 in t-butanol at 50 °C. Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 - 40 $g.L^{-1}$, temp = 50 °C, no pH control.

3.4.4 Effect of Temperature on SB-240101 Formation in [BMIM][PF_6]

Although the previous experiments were performed at 50 °C, the kinetics of the resolution were also explored at a range of temperatures from 25 – 100 °C. For the t-butanol system a reaction temperature of 50 °C was necessary to achieve acceptable substrate solubilities, however, this is at the expense of adverse affects on the kinetics and stability of the Novozyme 435 biocatalyst (Atkins et al., 2003). As shown in Table 3.3 with the ionic liquid [BMIM][PF_6], SB-240101 was produced at temperatures up to 75 °C with an associated increase in the apparent initial rate of product formation. At 75 °C there is a four-fold enhancement in the initial rate of product formation when compared to the t-butanol system at 50 °C. In each case the final conversion obtained was 47 % w/w. Above 75 °C [BMIM][PF_6] is known to degrade producing acidic species and potentially HF (Swatloski et al., 2003). Not surprisingly experiments performed at temperatures above 75 °C showed a significant decrease in initial reaction rate (final conversion yield not determined). What is remarkable, however, is sustained enzymatic activity over a period of four hours seen at the elevated temperatures of 75 °C and above (Table 3.3). This suggests that the CALB has a significantly greater thermal stability in [BMIM][PF_6], compared to aqueous or organic solvents (Schofer et al., 2001; Eckstein et al., 2002). The stability of the substrate and product molecules does not seem to be an issue at elevated temperatures in this ionic liquid, shown by control experiments as described in Section 3.2.2. Stability of the enzyme at elevated temperatures was also investigated and Novozyme 435 was found to still active after heating at 100°C in water as described in Section 3.2.2.

Table 3.3- Effect of temperature on the apparent initial rate of SB-240101 formation. Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w) or t-butanol (88 % v/v), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, no pH control.

	Apparent initial rate of SB-240101 formation				
Time (min)	**t-butanol at 50 °C**	**[BMIM][PF_6] at 25 °C**	**[BMIM][PF_6] at 50 °C**	**[BMIM][PF_6] at 75 °C**	**[BMIM][PF_6] at 100 °C**
0	**0**	**0**	**0**	**0**	**0**
60	**0.1130**	**0.0379**	**0.1568**	**0.8987**	**0.0086**
120	**0.2577**	**0.1286**	**0.2375**	**1.3375**	**0.1379**
240	**0.6170**	**0.1581**	**0.2890**	**1.9166**	**0.1396**

3.4.5 Effect of Added Water Content on SB-240101 Formation in [BMIM][PF_6]

As the resolution of SB-235349 is a hydrolytic reaction (Figure 1.3), water is an important reactant and must be present in order for the reaction to occur. The t-butanol process used the commercial source of the solvent, t-butanol (88 % v/v), contained sufficient water to drive the reaction to completion (Atkins et al., 2003). The ionic liquids used in this work are supplied as anhydrous, or with very low water contents. Experiments with ionic liquids were therefore performed utilising a range of added water concentrations. As described in Section 2.2.2, the preparation of the hydrophobic ionic liquid [BMIM][PF_6] involved multiple extraction stages, and was therefore saturated with water before use. The saturation levels of water in the various ionic liquids used were determined by Karl Fisher titration as described in Section 2.5.4.2. The saturation water content of [BMIM][PF_6] was determined as 3.07 % H_2O w/w (which is in good agreement with the value of 3.18 % H_2O w/w reported by

Wong et al., 2001). For [BMIM][$N(CF_3SO_3)$], [BMIM][BF_4], and [BMIM][$N(SO_2CF_3)_2$] the concentrations were 0.28, 0.61, 0.17 % H_2O w/w, respectively, as shown in Table 2.1. Due to the requirement for water in the resolution studied here, higher initial water contents were evaluated compared to those reported by other groups, who frequently dry the ionic liquids after washing to levels around 0.03 % H_2O w/w (Laszlo et al., 2001; Lau et al., 2000; Berberich et al., 2003).

Figure 3.5 shows the effect of added water content in [BMIM][PF_6] on the initial rate of reaction. For these experiments the overall water content was calculated to include the saturation level of 3.07 % H_2O w/w. As the water content is increased up to 8 % H_2O w/w, the apparent initial rate of product formation increases significantly. At higher water contents of approximately 15 % H_2O w/w, the rate significantly decreases to a value below that observed with 4 % H_2O w/w water. For water concentrations of 4 - 8 % H_2O w/w, the final conversion obtained was 47 % w/w (final conversions were not determined, for the water contents of 15 % H_2O w/w and 3 % H_2O w/w due to the low rates of reaction). These results with varying water contents are comparable to previous studies on the hydration of 1,3-dicyanobenzene in [BMIM][PF_6] using a whole cell nitrile hydratase biocatalyst (Roberts and Lye, 2002), and suggest that there is an optimum water concentration for hydrolytic reactions in ionic liquids. Laszlo and Compton (2001) have obtained similar trends with the free form of α-chymotrypsin in [BMIM][PF_6], showing that the enzyme activity increased up to a water content of 0.5 % (v/v) and then decreased at higher levels.

Although the saturation level of water in [BMIM][PF_6] was determined as 3.07 % H_2O w/w, it was observed that bioconversions containing up to 4 % H_2O w/w water existed as a single liquid phase. This suggests some water could be adsorbing onto the Novozyme 435 resin or the immobilised CALB. When the water content was increased further a separate water phase was observed which rapidly dispersed in the form of small droplets when mixed. Scale-down studies (Cull et al., 2002) on the hydrodynamics of ionic liquid-aqueous dispersions, and the implications for solute mass transfer rates are discussed in Chapter 4.

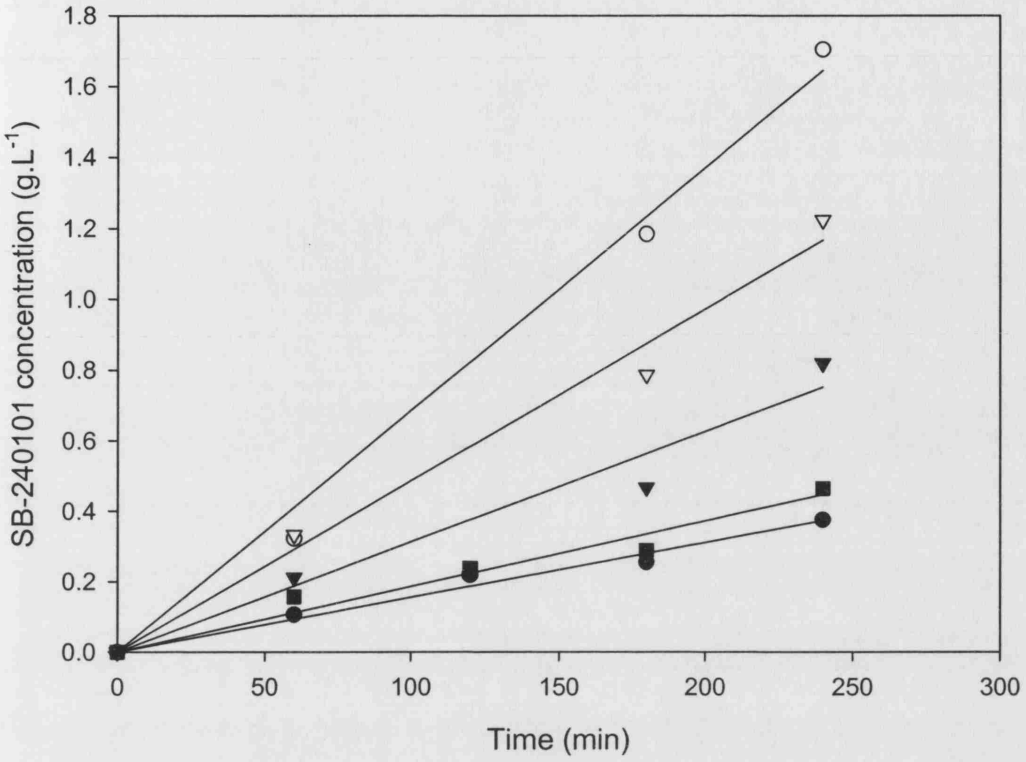


Figure 3.5- Effect of initial total water content of [BMIM][PF_6] on the apparent initial rate of SB-240101 formation: (■) 15 % H_2O w/w, (○) 8 % H_2O w/w, (▽) 6 % H_2O w/w, (▼) 4 % H_2O w/w, (●) 3 % H_2O w/w. Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control. Solid lines fitted by linear regression for calculation of apparent initial reaction rate.

3.4.6 Evaluation of Optimised Bioconversion Conditions in [BMIM][PF_6]

Having investigated separately the effects of substrate concentration, temperature and water content on the CALB-catalysed resolution of SB-235349 in ionic liquids, the final experiments investigating reaction kinetics aimed at identifying optimum overall reaction conditions. As shown in Figure 3.6, at a substrate concentration of 25 $g.L^{-1}$ in [BMIM][PF_6], a temperature of 75 °C and with a total water content of 8 % H_2O w/w, a remarkable twenty seven-fold increase in the concentration of SB-240101 was obtained in the first hour, compared to the optimised t-butanol system. The final conversion obtained was 47 % w/w, and the overall increase in the rate of conversion was calculated to be four-fold greater. Analysis of the chiral purity of the SB-240101

produced by the CALB-catalysed resolution in [BMIM][PF_6] showed that the product had an e.e. of 99 %, identical to that obtained in the industrial t-butanol process (Atkins et al., 2003).

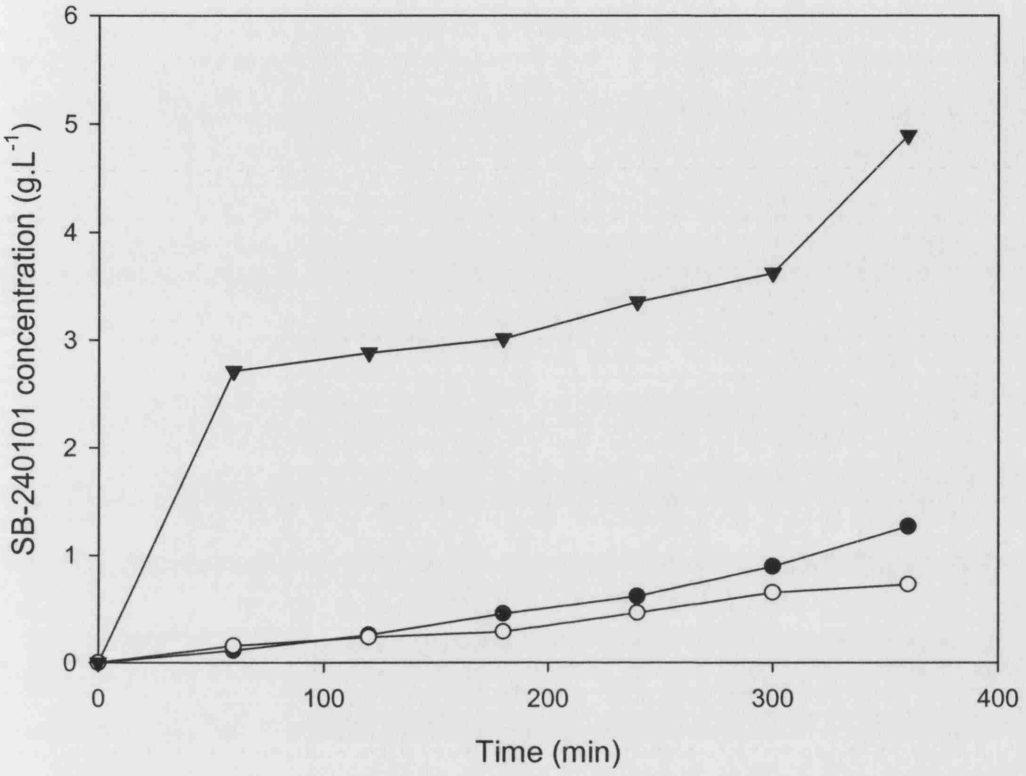


Figure 3.6- Kinetics of SB-240101 formation under optimised conditions in various solvent systems: (●) t-butanol (88 % v/v), [SB-235349] = 5 $g.L^{-1}$, temp 50 °C, (○) [BMIM][PF_6] (12 % H_2O w/w), [SB-235349] = 5 $g.L^{-1}$, temp 50 °C, (▼) [BMIM][PF_6] (8 % H_2O w/w), [SB-235349] = 25 $g.L^{-1}$, temp 75 °C. Other reaction conditions as described in Section 2.3.1: [Novozyme 435] = 1.4 $g.L^{-1}$, no pH control.

3.4.7 Evaluation of Enzyme Re-use

As stated in Section 1.3, the high cost of the Novozyme 435 CALB preparation requires that there is recycling of the enzyme over a number of batches. In the industrial t-butanol process the use of Novozyme 435 over 10 cycles has been reported (Atkins et al., 2003). Experiments here examined if the Novozyme 435 could be re-used over 10 cycles in [BMIM][PF_6]. Table 3.4 shows that recycling of the enzyme was possible in the ionic liquid, however, the initial reaction rate dropped

significantly to ~ 40 % of it's initial value after six reaction and wash cycles. This was followed by a further gradual decline where the Novozyme 435 preparation retained ~ 20 % of it's initial activity after 10 cycles. In contrast results for enzyme re-use in the t-butanol system showed a greater retention in activity with 48 % activity achieved over 10 cycles (Atkins et al., 2003). It is assumed that due to the affect of water on the enzyme, the residual activity after each cycle could be improved by avoiding washing the Novozyme 435 preparation with water between each reaction in the ionic liquid, however, this would however require further investigation. Although the initial rate of reaction decreased after each cycle, an overall conversion of around 47 % w/w could be obtained in both ionic liquid and t-butanol by simply increasing the reaction time (data not shown).

Table 3.4- Biocatalyst recycle and re-use in [BMIM][PF_6]. Values in brackets are for the industrial t-butanol system and represent the average rate of product formation (calculated from Atkins et al., (2003)). Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, initial [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control. The Novozyme resin was recovered and cleaned after each recycle as described in Section 2.3.1.1.

Reaction Cycle	Apparent Initial Rate of SB-240101 formation ($g.L^{-1}.min^{-1}$)	Fraction of Original Rate (%)
1	3.8×10^{-2}	100 (100)
2	3.7×10^{-3}	97 (68)
3	3.3×10^{-3}	87 (70)
4	2.2×10^{-3}	58 (78)
5	2.3×10^{-3}	60 (69)
6	1.5×10^{-3}	39 (63)
7	1.2×10^{-3}	32 (74)
8	1.3×10^{-3}	34 (74)
9	1.4×10^{-3}	37 (69)
10	7.4×10^{-4}	19 (48)

3.4.8 Comparison of Immobilised and Free Enzyme

Although the use of immobilised CALB is preferable in order to facilitate recovery and re-use of the enzyme, the resolution of SB-235349 in [BMIM][PF_6] was also performed using a lyophilised preparation of the free enzyme. As shown in Figure 3.7 at the same biocatalyst concentration the free lipase exhibited a 2.5-fold higher rate of

reaction, with 1.85 $g.L^{-1}$ of SB-240101 formed after 6 hours compared to 0.7 $g.L^{-1}$ with the Novozyme 435. This result of higher reaction rate with the free enzyme than the immobilised Novozyme 435 is attributed to the significantly lower activity load of the immobilised form (Husum et al., 2001). The immobilised form has 10,000 $units.g^{-1}$. where as the free lipase has an activity load of 4000,000 $units.g^{-1}$. During these experiments it was observed that the free enzyme when added to the [BMIM][PF_6] formed a suspension of fine particles within the ionic liquid. At free enzyme activity loads of 2800,000 $units.L^{-1}$ and 1400,000 $units.L^{-1}$ the almost identical results observed suggest a significant amount of the enzyme activity is lost due to the denaturation of a portion of the free CALB that will dissolve in the ionic liquid. It must also be noted that with the free enzyme there was no evidence of substrate adsorption, the substrate was seen to decease at a tenth of the initial rate of that observed with the immobilised enzyme (results not shown).

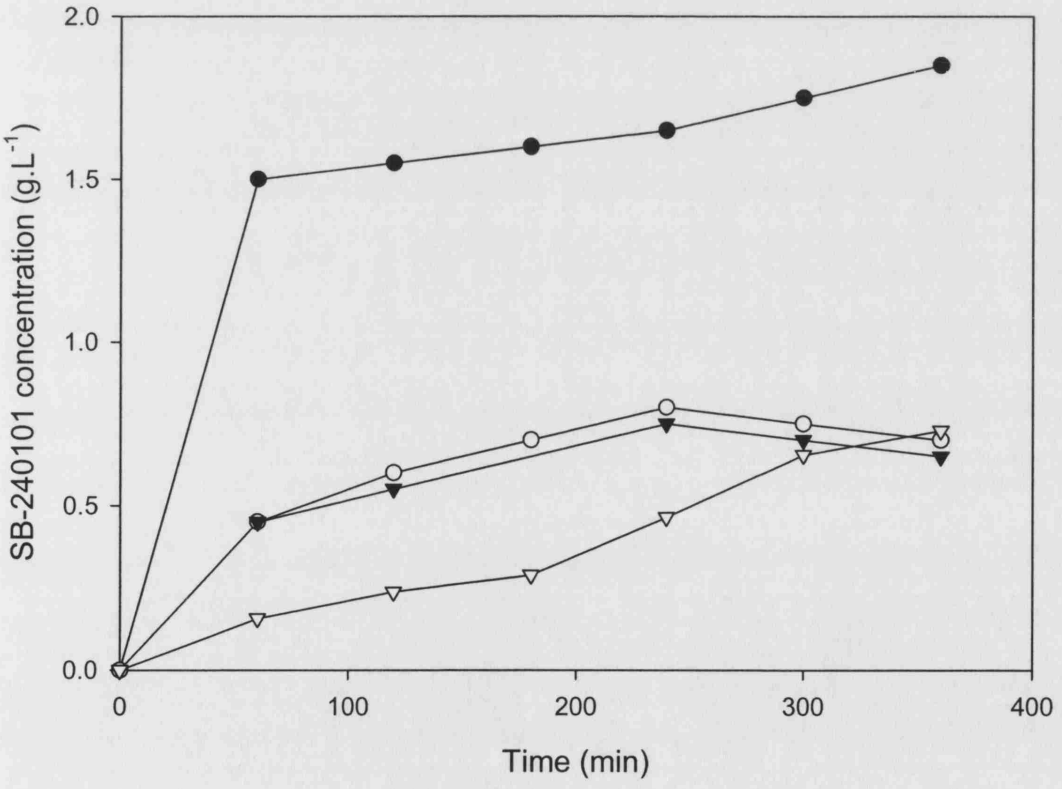


Figure 3.7- Kinetics of SB-240101 formation with free CALB in [BMIM][PF_6] and immobilised CALB: Free CALB activity load: (●) [CALB] = 5600,000 $units.L^{-1}$, (○) [CALB] = 2800,000 $units.L^{-1}$, (▼) [CALB] = 1400,000 $units.L^{-1}$. Immobilised CALB: (▽) [CALB] 14,000 $units.L^{-1}$.Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control.

3.5 Discussion

The optimised conditions for the lipase catalysed resolution of SB-235349 (Figure 1.3) require the use of the organic solvent t-butanol, due to the limited solubility of the reagents in aqueous media. The ability of ionic liquids to potentially dissolve a vast array of pharmaceutical intermediates and final drug substances at high concentrations means that they may provide alternative reaction media that could increase reaction rates or yields when compared to conventional organic solvents. The aim of the research in this chapter was to characterise an optimised industrial lipase resolution in an organic solvent, and to determine the advantages that operation in ionic liquid media might offer and demonstrate the generic design methodology. The work showed that firstly the conversion occurs in ionic liquids, and that operation in ionic liquid offered several advantages over the organic solvent system, including increased solubility of the reagents. Finally, utilising these properties the resolution was optimised for an ionic liquid system at higher conversion rates than the optimised t-butanol process.

3.5.1 Resolution of SB-235349 to SB-240101 in Ionic Liquids

The CALB catalysed resolution of SB-235349 as described in Section 3.3 was found to occur in three of the six ionic liquids tested ([BMIM][$N(SO_2CF_3)_2$], [BMIM][PF_6] and [BMIM][CF_3SO_3]) under the optimised t-butanol conditions (Figure 3.2). The rates and conversions were lower under these conditions in ionic liquids compared to the optimised t-butanol system. The conversion of SB-235349 was shown to occur in both water miscible [BMIM][CF_3SO_3] and water immiscible [BMIM][$N(SO_2CF_3)_2$] and [BMIM][PF_6] (Table 3.1). The anions of these three ionic liquids [CF_3SO_3], [PF_6] and [$N(SO_2CF_3)_2$] show increasing hydrophobicity, with increasing rate of conversion 0.65 $g.L^{-1}$, 0.7 $g.L^{-1}$ and 1.0 $g.L^{-1}$ respectively over six hours under optimised t-butanol conditions. Only two types of cation were tested for the conversion, and the product was only observed in ionic liquids with the [BMIM] cation. The BMIM cation has been extensively used in research due to the fact that ionic liquids with this cation have a wide liquid temperature range, and are compatible with a broad group of anions. Results show that increasing the hydrophobicity of the ionic liquid increases

the rate of reaction, therefore the most hydrophobic ionic liquid [BMIM][$N(SO_2CF_3)_2$] shows the highest conversion. This ionic liquid, however, is very expensive and therefore [BMIM][PF_6] which is also hydrophobic, was utilised for the majority of experiments. [BMIM][PF_6] is commercially available, comparatively cheaper than [BMIM][$N(SO_2CF_3)_2$], and has been widely studied for research purposes.

Optimisation of the t-butanol process had shown control of pH was not a quality critical parameter, however, at low pH values a degree of enzyme inhibition was observed (Atkins et al., 2003). Under the optimised t-butanol process conditions, controlling the pH was shown to have a negative effect in ionic liquids (Table 3.2), decreasing the rate in [BMIM][PF_6] by a factor of seven and in [BMIM][$N(SO_2CF_3)_2$] by a factor of ten. It was observed that the pH of [BMIM][PF_6] during the conversion of SB-235349 without pH control remained reasonably stable ± 0.2 over six hours. This indicates that the ionic liquid has a stabilising effect, and the problems encountered at low pH would, therefore, not be an issue. The results of an investigation of this reaction at different agitation rates (1000–3000 rpm) as shown in Figure 3.3, indicate mass transfer seems to be an issue at low stirrer speeds (<2000 rpm).

Examination of enzyme re-use in the ionic liquid [BMIM][PF_6] (Table 3.4), showed that after the first 5 re-uses, the immobilised resin Novozyme 435 retained 60 % of its initial activity, compared to 68 % retained by the optimised t-butanol system. The results for the t-butanol system showed the initial activity had dropped by 48 % after 10 re-uses, which was washed with t-butanol between uses. The enzyme activity in the ionic liquid media showed a significant decrease to 20 % of its initial activity after only 10 re-uses, having been washed between each conversion with water. Due to the sensitivity of the Novozyme 435 enzyme to water. Overall, the results indicate that the expensive enzyme can be used repeatedly in ionic liquids, and that with alternative wash treatment a higher retained enzyme activity might be achieved.

Experiments using free lyophilised and immobilised preparations of the enzyme were performed in [BMIM][PF_6] (Figure 3.7), and showed that both forms of the enzyme were able to perform the conversion of SB-235349 in this medium. However, the free

lyophilized version of the enzyme displayed a 2.5-fold higher rate of conversion, which is attributed to the lower activity load of the immobilised form. From a manufacturing perspective the immobilised form of the resin is more applicable to process integration for re-use of the enzyme, and is therefore preferred to the free lyophilised form.

3.5.2 Optimisation of SB-235349 Resolution in Ionic Liquids

The solubility of the racemic substrate ester was low in the t-butanol system, and is one of the limiting factors for the conversion in this solvent (Atkins et al., 2003). The ionic liquid [BMIM][PF_6] was shown to have an eight-fold increased solubility for SB-235349, compared to t-butanol at 50 °C. Increasing the initial substrate concentration for the conversion of SB-235349 (Figure 3.4), showed a significant increase in the apparent initial rate of product formation from 5 $g.L^{-1}$ to 25 $g.L^{-1}$.Overall these results indicate the use of an ionic liquid as reaction media could remove substrate solubility as a limiting factor for the conversion of SB-235349.

With the t-butanol process, the reaction required a high temperature to dissolve large quantities of the substrate, however, at elevated temperatures the enzyme became unstable. The optimum temperature was determined as 50 °C for sufficient substrate to dissolve in the t-butanol, while maintaining a high enzyme activity (Figure 3.1). Temperature was therefore examined for the conversion in [BMIM][PF_6] at a range from 25 – 100 °C (Table 3.3). The conversion was shown to occur at all temperatures tested, the apparent initial rate of product formation increased as the temperature was increased from 25 - 75 °C. At 75 °C there is a four-fold enhancement in the initial rate of product formation compared to the t-butanol system at 50 °C. As [BMIM][PF_6] is known to degrade at high temperatures, the fact that product formation was still observed under these extreme enzyme conditions (temperatures > 75 °C), indicates that [BMIM][PF_6] may have a stabilising effect on the enzyme. If ionic liquids were indeed having a stabilising effect on the enzyme, then operation of the reaction at temperatures above 75 °C could be an option in more thermally stable ionic liquids such as [BMIM][$N(SO_2CF_3)_2$]. The [$N(SO_2CF_3)_2$] anion unlike the [PF_6] anion is not subject to hydrolysis at high temperatures, however at present it is expensive.

Investigation of water content on the rate of reaction in [BMIM][PF_6] reaction media shows that the water content of the ionic liquid plays an important role. As shown in Figure 3.5 increasing the water content from 3 % up to 8 % H_2O w/w increased the apparent initial rate of product formation. Above 8 % H_2O w/w the rate dropped significantly, indicating there is an optimum water content for the hydrolytic conversion of SB-235349 in [BMIM][PF_6]. The additional advantage offered by the use of a water saturated ionic liquid is the effect it has on the viscosity, which is significantly less than the pure anhydrous form (discussed further in Chapter 4).

The final step was to utilise all these properties offered by the use of the ionic liquid [BMIM][PF_6], to give an example of how this conversion can be optimised in an ionic liquid. The highest reaction rate was observed at a temperature of 75 °C with no apparent degradation observed, consequently this was selected as the reaction temperature. As the substrate concentration had greatest impact up to 25 $g.L^{-1}$ this was selected as the optimum concentration. Finally the optimum water content selected was 8 % H_2O w/w, as this displayed the highest conversion in [BMIM][PF_6]. The initial rate of product formation at temp = 75 °C, [substrate] = 25 $g.L^{-1}$, in [BMIM][PF_6] (8 % H_2O w/w), showed a twenty seven-fold increase in the first hour (Figure 3.6), compared to the optimised t-butanol system. This suggests substrate concentration is no longer a limiting factor to the initial rate of reaction under these conditions. Results indicate the enzyme is unaffected by operation at temperatures up to 75 °C in [BMIM][PF_6], attributed to the ionic liquid providing stability to the enzyme. Overall all these reaction conditions gave an optimum reaction in an ionic liquid that increased the overall rate of reaction four-fold compared to the optimised t-butanol system, while maintaining the same high chiral purity and yield.

3.6 Summary

As stated in Section 3.1, the aim of this Chapter was to determine a generic approach to design and identify the advantages of operating the CALB catalysed resolution of SB-235349 in ionic liquid media. The key advantages identified are summarised below:

- Limited screening of ionic liquids with different physico-chemical properties identified three ionic liquids in which the resolution of SB-235349 to SB-240101 was observed to occur, but at rates and conversions below those for the previously optimised t-butanol system. The substrates and products of the bioconversion were shown to be stable in ionic liquids by HPLC examination. Results show changes in the anion to increase the hydrophobicity of the ionic liquid increases the rate of the conversion of SB-235349, in particular the hydrophobic ionic liquids [BMIM][PF_6] and [BMIM][$N(SO_2CF_3)_2$] showed the highest conversion rates.

- The substrate displayed an eight-fold increase in substrate solubility compared to t-butanol (88 % v/v) at 50 °C. The initial rate and final yield of the reaction have subsequently been determined in [BMIM][PF_6] as a function of initial substrate concentration (5 - 40 $g.L^{-1}$), the results showed that increasing the substrate concentration increased the rate of reaction. Increasing the substrate concentration up to 25 $g.L^{-1}$ had a significant effect on the initial rate of reaction, indicating that above this amount the substrate concentration is no longer a limiting factor.

- Increasing the reaction temperature from 25 - 75 °C displayed a large effect on the initial reaction rate in [BMIM][PF_6]. Above this temperature the initial rate of product formation decreased dramatically, indicating degradation of the [PF_6] anion. This is not the case with many other ionic liquids including the [$N(SO_2CF_3)_2$] anion, which could also be used as media for the conversion. Operation of the conversion in t-butanol at elevated temperatures causes denaturation of the enzyme, and increased safety concerns. The application of an ionic liquid for this conversion occurs across a wide temperature range (25 - 100 °C), and the remarkable ability of the enzyme to remain active at temperatures of 100 °C within degraded [BMIM][PF_6], indicates ionic liquids as reaction medium increase the stability of the enzyme.

- The optimum water content of the reaction medium was examined in [BMIM][PF_6], and 8 % H_2O w/w displayed the highest initial rate of conversion

of SB-235349. The initial water content was studied over the range 3 - 15 % H_2O w/w, and showed that increasing the water content increased the rate of reaction from 3 - 8 % H_2O w/w, above this content the rate of reaction dropped significantly.

- Exploiting the increased solubility of the substrate in ionic liquids, and the ability to operate at higher temperatures increased the initial rate of product formation twenty seven-fold over the first hour, when the ionic liquid [BMIM][PF_6] is used instead of t-butanol. The overall rate of reaction was increased four-fold under optimised [BMIM][PF_6] conditions compared to the optimised t-butanol system, while maintaining the same overall yield of 47%. In each case the e.e of the product was 99 %, identical to the t-butanol system.

Overall the results suggest that ionic liquids can be very favourable reaction media for industrial bioconversion processes, which also overcome many of the safety and environmental concerns of conventional organic solvents. Screening a very limited range of ionic liquids can identify a suitable ionic liquid and highlight the key reaction parameters involved in optimising lipase catalysed resolutions in ionic liquids. Having considered the reaction kinetics and yield in this chapter, in Chapter 4 the issues of product recovery and substrate and ionic liquid recycling will be examined.

4.0 Approaches to Product Recovery and Material Recycling from Lipase Catalysed Resolution Reactions in Ionic Liquids.

4.1 Aims

In the previous chapter a systematic approach to the design of biocatalytic resolutions in ionic liquids was presented. Having shown that ionic liquids offer advantages in the central bioconversion it is now necessary to consider their use within a whole manufacturing process. In this chapter a generic approach to assess product recovery and ionic liquid recycling options will be outlined. It will be illustrated using the lipase-catalysed resolution described in Chapter 3, and will address the key issues of product recovery, ionic liquid cleaning and re-use, and the recovery and re-racemisation of the un-reacted substrate enantiomer.

4.2 Evaluation of Potential Recovery Methods for SB-240101 and SB-240098

A summary of the potential methods for recovery of the product and un-reacted substrate is shown in Table 4.1. These are largely limited to examples of techniques previously described in the ionic liquid literature. Distillation as a separation technique is an ideal way of removing volatile products due to the negligible vapour pressure of most ionic liquids (Schofer et al., 2001). In this application, however, the SB-240101 product is also largely non-volatile, and thus distillation was excluded from further investigation. Supercritical CO_2 extraction is a recovery method that has been reasonably well investigated (Blanchard et al., 1999) for the extraction of non-volatile or heat labile products. Although this method is environmentally friendly and widely used in industry, the technique is limited by the solvation properties of supercritical CO_2 for the particular substrate and product molecules under

Table 4.1- Summary of the advantages and disadvantages of possible methods for product recovery from ionic liquids. Cited references specifically relate to examples of use with ionic liquid phases.

Method	Advantages	Disadvantages	Reference
Distillation	• Widely used industrially • Established design methodologies	• Limited to volatile products • Concentration of impurities in the ionic liquid	Schofer et al., (2001).
Supercritical carbon dioxide ($SCCO_2$)	• "Green" technology • Used in industry	• Technically demanding and capitally expensive • Hydrophilic ionic liquids at very low concentrations cannot be removed from water using CO_2. • Use of enzymes in CO_2 is well reported. • Limited by solvation properties of $SCCO_2$	Blanchard et al., (2003). Reetz et al., (2002).
Liquid-liquid extraction	• Widely used industrially • Applicable to a wide range of solutes	• Continued use of organic solvents • Finite ionic liquid solubility in aqueous phase could pose further down stream separation problems.	Itoh et al., (2001). Visser et al., (2000). Huddleston et al., (1998). Cull et al., (2000).
Adsorption	• Widely used industrially	• Removal of anion or cation from ionic liquids could affect ionic liquid integrity	Anthony et al., (2001).
Pervapopration/ Nanofiltration	• Energetically favourable	• No current industrial processes • Limited membrane selection • Large molecular weight products have low solubility and diffusion kinetics	Schafer et al., (2001). Dudziak et al., (1999)
Precipitation/ Crystallisation	• Widely used industrially	• Has not been investigated • Processes often product specific	

consideration. Additionally, the method is technically demanding and its use has been shown to have a deactivation effect on enzymes in ionic liquids attributed to pH changes from the CO_2 (Lozano et al., 2002), and this is why this method was not investigated following the conversion of SB-235349. For organic solutes crystallisation from the ionic liquid solvent is an alternative method of separation. Precipitation in an ionic liquid, however, is often product specific. Pervaporation is a method of separation that although being energetically favourable is limited by membrane choice. Large molecular weight products like SB-240101, which has a molecular weight of 233, have low solubility and diffusion kinetics, and therefore this method of separation was not pursued.

Liquid-liquid extraction is one of the most popular separation methods since the process is both well understood, and the characteristics of the biphasic solvent system can be easily adjusted for the specific separation task (Schweiser, 1996). Extractions with ionic liquids have been demonstrated (Huddleston et al., 1998; Visser et al., 2000; Cull et al., 2000), indicating that solute partitioning behaviour is similar to that observed with traditional organic solvents. Aqueous phase systems (Willauer et al., 1999) in combination with ionic liquids would be of particular interest from a "green" perspective. [BMIM][PF_6] in particular has been identified as an ionic liquid with suitable properties for use in liquid-liquid separations with water as the second phase. Huddleston and co-workers (1998) examined water-[BMIM][PF_6] phase separations and showed that neutral hydrophobic aromatic solutes have an affinity for the ionic liquid phase and, for the solutes studied, partitioning to the ionic liquid phase generally increased with solute hydrophobicity. For specific solutes a pH-induced ionisation may alter their partitioning to or from the ionic liquid phase (Visser et al., 2000). When considering liquid-liquid extraction there are three features of the phase that are vitally important:

- The ionic liquid and the extracting phase must be immiscible, i.e. separate to form two different phases;
- Mutual solubility, i.e. the solubility of the ionic liquid in the extraction phase and the solubility of the extraction phase in the ionic liquid phase. The solubility of the solvent in the extraction phase may have positive or negative consequences. Contamination of the extraction phase with ionic liquid represents an important

issue for downstream processing where the product must be separated from the extracting phase and the contaminating ionic liquid. Ultimately solubility of the ionic liquid in the extracting phase results in a net loss of the ionic liquid, which is quite expensive and may reduce the economic viability of the process;

- Partition of the solute must favour solute recovery into the extraction phase with regard to the model conversion used here (Figure 1.3).

Liquid-liquid extraction appears to be one of the most viable routes following the conversion of SB-240101. Therefore a variety of solvents (organic and aqueous) were screened for miscibility and extraction of the substrate and the product (Section 4.2.1). Finally, adsorption (Anthony et al., 2001), given the wide use of this separation technique in industry, and the commercial availability of resins with different properties, a limited screening of potential resins for recovery of SB-240101 was also performed (Section 4.2.2).

4.2.1 Solvent Screening

Based on the optimised bioconversion conditions described in Section 3.4.6, [BMIM][PF_6] was selected for all the downstream processing studies described in this chapter. Initial experiments aimed to establish if any of the solvents examined were firstly, immiscible with [BMIM][PF_6] and secondly, whether the racemic substrate (SB-235349/SB-240098) and product (SB-240101) could be extracted from the [BMIM][PF_6]. Evidence of any precipitate formation in any of the solvents was also monitored. The solvents examined were a range of 33 polar and non-polar organic solvents and aqueous solvents commonly used within the pharmaceutical industry (Section 2.4.1.1). Table 4.2 summarises the results of the solvent screening. No precipitate was observed in any of the solvents after 24 hours of contact. Of the 33 solvents screened only 23 were immiscible with [BMIM][PF_6], 5 of the 13 polar organic solvents were immiscible, 7 of the 8 non-polar organic solvents were immiscible and 11 of the 12 aqueous solvents were immiscible.

Of the 5 polar organic solvents, which were immiscible with the ionic liquid, ethanol and isopropyl alcohol produced the highest extraction results for SB-235349/SB-

240098 of 20 % w/w and 30 % w/w, respectively, while both showed negligible (<1 % w/w) extraction of the product. The remaining polar organic solvents, ether and isopropyl ether both showed low recovery of the substrate (<1 % w/w) and the product (<10 % w/w), and the results for phenol indicate potential 'modification' of the substrate and product judging by the change in the HPLC peak retention times (analysis as described in Section 2.5.1). All of the 7 immiscible non-polar organic solvents displayed either no extraction of the substrate and product of any kind, or that the solute retention times were altered indicating 'modification'. Most of the aqueous solvents were immiscible with [BMIM][PF_6]. 4 of these showed extraction of the product to some degree and a low extraction of the substrate, the highest extraction was measured with sodium bicarbonate of 62 % w/w removal of the product with <3 % w/w of the substrate extracted. Tartic acid, brine and citric acid showed some evidence of relative product extraction but at low levels of recovery. The remaining 7 aqueous solvents displayed 'modifications' of the substrate and product molecules as described previously. An interesting observation of the extraction results for sodium hydroxide was that the HPLC traces of the [BMIM][PF_6] phase after extraction showed very low substrate and product peaks (99 % w/w of the product was removed and 70 % w/w of the substrate was removed).

Overall aqueous solvents such as sodium bicarbonate indicate that with high pH high extraction of the product is achieved, indicating aqueous biphasic pH dependent partitioning of the product from the ionic liquid could be a viable route for product recovery. As the highest extraction of the substrate was displayed by isopropyl alcohol, with a low extraction of the product this organic solvent was selected as the route for recovery of the substrate for the re-racemisation step. This has the advantage that in the optimised industrial process this is carried out in a polar organic solvent. To recycle the [BMIM][PF_6] sodium hydroxide 0.1M showed a high removal of both the substrate and the product, but with 'modification', therefore this was considered as a clean up and recycle of the media following removal of the majority of the substrate and product. In summary, as with the bioconversion step described in Section 3.4.1, the screening of a fairly limited range of solvents has identified phases that might allow efficient product recovery and also enzyme recycling.

Table 4.2- Screening of a range of polar, non-polar and aqueous solvents to examine the extraction of SB-240101 and SB-235349 from [BMIM][PF_6]. The results highlighted in grey show where the solvent phase was miscible with [BMIM][PF_6]. Where the extraction result is described by "Modification" the HPLC peak produced for either SB-240101 and SB-235349 showed a significant change in retention time or additional peaks were formed. N/A means not available. Conditions as described in Section 2.4.1.1: solvent = [BMIM][PF_6], [SB-235349] = 2.5 $g.L^{-1}$, [SB-240101] = 2.5 $g.L^{-1}$.

Extraction solvent		Immiscible with [BMIM][PF_6]	Product extraction (% w/w)	Substrate extraction (% w/w)	Precipitate formed within 24 hours
Polar	Dichloromethane	No	N/A	N/A	No
	Ethyl acetate	No	N/A	N/A	No
	Methanol	No	N/A	N/A	No
	Ethanol	Yes	<1 %	20 %	No
	Isopropyl alcohol	Yes	<1 %	30 %	No
	Pyridine	No	N/A	N/A	No
	Ether (diethylether)	Yes	<10 %	<1 %	No
	Isopropyl ether	Yes	<1 %	<10 %	No
	Phenol	Yes	Modification	Modification	No
	Aniline	No	N/A	N/A	No
	Acetonitrile	No	N/A	N/A	No
	Dimethylformamide	No	N/A	N/A	No
	Dimethyl acetamide	No	N/A	N/A	No
Non-polar	Chloroform	No	N/A	N/A	No
	Pentane	Yes	None	None	No
	Hexane	Yes	None	None	No
	Heptane	Yes	None	None	No
	Toluene	Yes	Modification	Modification	No
	Cyclohexane	Yes	None	None	No
	Xylene	Yes	Modification	Modification	No
	Trichloroethylene	Yes	Modification	Modification	No
Aqueous	Acetic acid	No	N/A	N/A	No
	Trifluoroacetic acid	Yes	Modification	Modification	No
	Ammonium Hydroxide	Yes	Modification	Modification	No
	Sodium bicarbonate	Yes	62 %	<3 %	No
	Brine	Yes	<50 %	<10 %	No
	Sodium carbonate	Yes	Modification	Modification	No
	Sodium hydroxide	Yes	Modification	Modification	No
	Soidum thiosulphate	Yes	Modification	Modification	No
	Sodium metabisulphite	Yes	Modification	Modification	No
	HCl	Yes	Modification	Modification	No
	Citric acid	Yes	<20 %	<10 %	No
	Tartic acid	Yes	<50 %	<20 %	No

4.2.2 Adsorbent Screening

As a potential alternative to liquid-liquid extraction three different commercially available adsorbents were experimentally characterised in order to determine the most suitable product recovery route, as described in Section 2.4.1.2. The three adsorbents characterised were chosen to represent the two main classes of commercially available non-functionalised adsorbents, namely carbonaceous adsorbents and synthetic polymeric resins. Both of these classes of adsorbent are used industrially and have the required mechanical, thermal and chemical stabilities for industrial adsorption operations (Seader et al., 1998). The specific adsorbents chosen are listed in Table 4.3 with some further details on their relevant chemical and physical characteristics. The results showed that both the anion and cation exchange resins achieved results of ~60 % w/w product removal, however, the results displayed little selectivity with ~40 % w/w of the substrate also removed. Activated charcoal removes none of the product and shows ~50 % removal of the substrate. Given the poor initial results with the resins their use was not investigated further.

Table 4.3- Summary of the chemical and physical properties of the adsorbents screened in this work for the recovery of SB-240101 and SB-240098 from [BMIM][PF_6] (* additional peak produced on chromatogram could be either degradation of product or of the ionic liquid: N/A as not available).

Adsorbent	**Chemical nature**	**Mesh size (microns)**	**Total capacity (m_{eq}.mL resin bed)**	**Removal of SB-240101 (% w/w)**	**Removal of SB-235349 (% w/w)**
Anion exchange resin (Biorad-AG®1-X8)	synthetic polymeric resins	100-200	1.2	62	41
Cation exchange resin (Biorad-AG®50W-X8)	synthetic polymeric resins	20-50	1.7	61*	42
Activated charcoal	carbonaceous	20-60	N/A	0	40

4.2.3 Influence of pH on Extraction of SB-240101

The primary target for the downstream processing of the bioconversion medium (Section 3.5.2) is the recovery of the product SB-240101 in high yield with minimal solvent usage. This can be maximised by having the maximum possible equilibrium distribution coefficient for the product. Ionisable solutes such as SB-240101 are known to exhibit pH-dependent partitioning behaviour such that their affinity for the ionic liquid decreases when they carry a positive or negative charge (Visser et al., 2003). The influence of pH on the partitioning of SB-240101 was thus examined between [BMIM][PF_6] and an aqueous buffer. The carboxylic acid group within the SB-240101 would facilitate extraction into an aqueous buffer at a pH higher than the pKa of the acid group. The distribution coefficient (often product as a function of pH) was determined as described in Section 2.4.1.3, and the results shown in Figure 4.1. These experiments indicate that at low pH, the carboxylic acid group does not dissociate, and therefore with no charge on the molecules only a very small amount is extracted. At high pH, above the pKa of ~4.5 of the carboxylic acid group, the molecules are charged and are therefore extracted into the aqueous phase. Control experiments showed that the SB-240098 substrate remains in the ionic liquid phase (<0.1 $g.L^{-1}$ SB-240098 was extracted into the aqueous phase across the pH range 2-11, at pH 12 the SB-240098 concentration increases in the aqueous phase to 3 $g.L^{-1}$). From these results a pH of 10 was selected as the optimum pH for the extraction phase to extract the highest quantity of SB-240101 with the best selectivity over SB-240098.

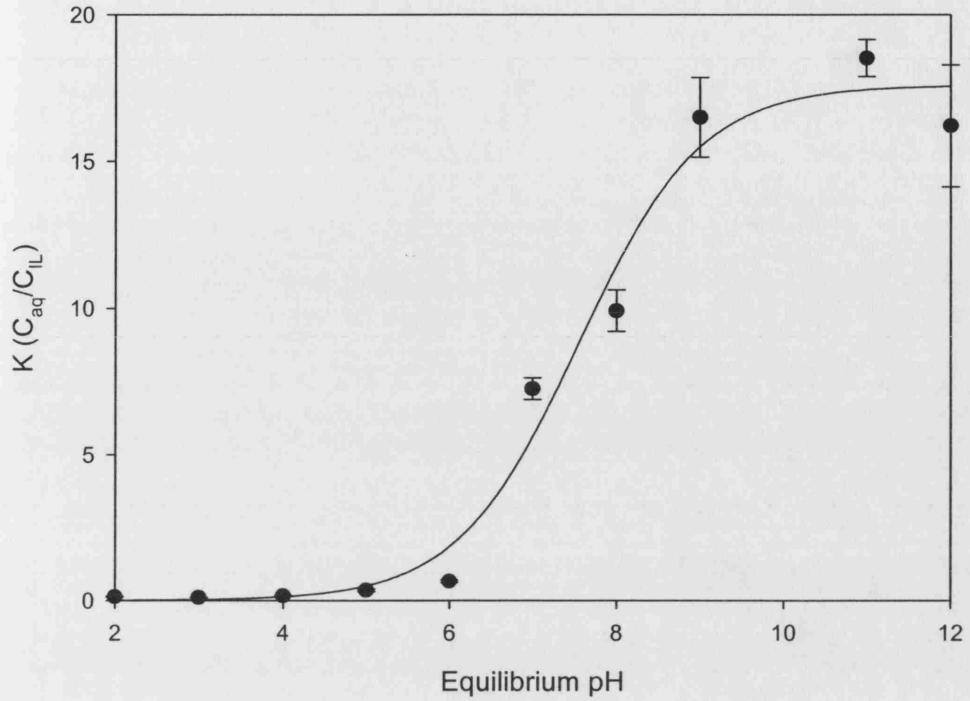


Figure 4.1- Effect of pH on the equilibrium distribution coefficient of SB-240101 between [BMIM][PF_6] and 50mM aqueous buffer V_R = 1. pH 2-3, 6-8 and 11-12: phosphate buffer, pH 4-5: acetate buffer, and pH 9-10 bicarbonate buffer. Conditions as described in Section 2.4.1.3: solvent = [BMIM][PF_6], [SB-240101] = 2.5 $g.L^{-1}$. Error bars show one standard deviation.

4.3 Design of a Liquid-Liquid Extraction Process for Product Recovery and Recycle of Ionic Liquid Media Following SB-235349 Conversion Process

In order to selectively remove SB-240101 and SB-240098 from an ionic liquid reaction medium following the resolution of SB-235349 as described in Chapter 3, important design choices need to be made. These include identification of a method or methods for product recovery, their operation and integration into a whole process. The optimum bioconversion conditions were determined as described in Section 3.4.6. In Section 4.2 consideration and screening of a number of potential recovery methods identified liquid-liquid extraction as a potential method for product recovery, substrate removal and cleaning of the ionic liquid system. Figure 4.2 illustrates a

potential downstream processing route, which would facilitate recycling of the ionic liquid. For product recovery it is considered that extraction into an aqueous buffer at pH 10 is the best approach because of the high affinity for the product and low affinity for the substrate. For substrate recovery it is considered that extraction with IPA is the best approach because of the reasonable substrate affinity and low product affinity. Finally as a means of recycling the ionic liquid, sodium hydroxide is considered as the optimum medium to remove the trace amounts of the product and substrate. The practical implementation of this downstream processing sequence is considered in the following sections.

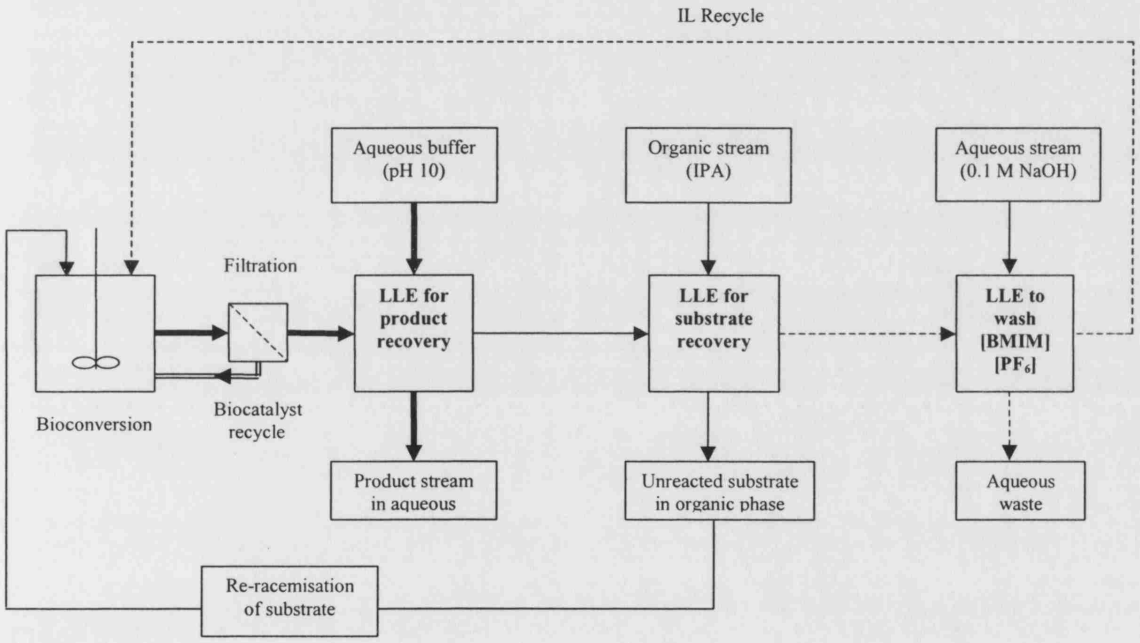


Figure 4.2- Conceptual product recovery and recycle system for the lipase catalysed resolution of SB-240101 in [BMIM][PF_6] (LLE = Liquid-liquid extraction). Bold lines indicate product stream, double lines indicate enzyme recycle, solid lines indicate recovery, re-racemisation and reuse of un-reacted enantiomer while dashed lines indicate cleaning and recycling of the ionic liquid.

4.3.1 Extraction of SB-240101 from [BMIM][PF_6]

The first stage in the downstream processing shown in Figure 4.2 following biocatalyst removal by filtration is recovery of the SB-240101 product. The SB-240101 produced by the resolution of SB-235349 can be removed by liquid-liquid

extraction with aqueous bicarbonate buffer (pH 10). As shown in Figure 4.3 the first extraction of the reaction media with aqueous buffer removed 79% w/w of the SB-240101 from the reaction, the second extraction with fresh buffer removed a further 9% w/w and the third and final extraction removed a further 5% w/w resulting in a total extraction yield of 93% w/w. HPLC analysis of the aqueous phase indicated the product purity to be >98 % w/w with respect to the un-reacted substrate.

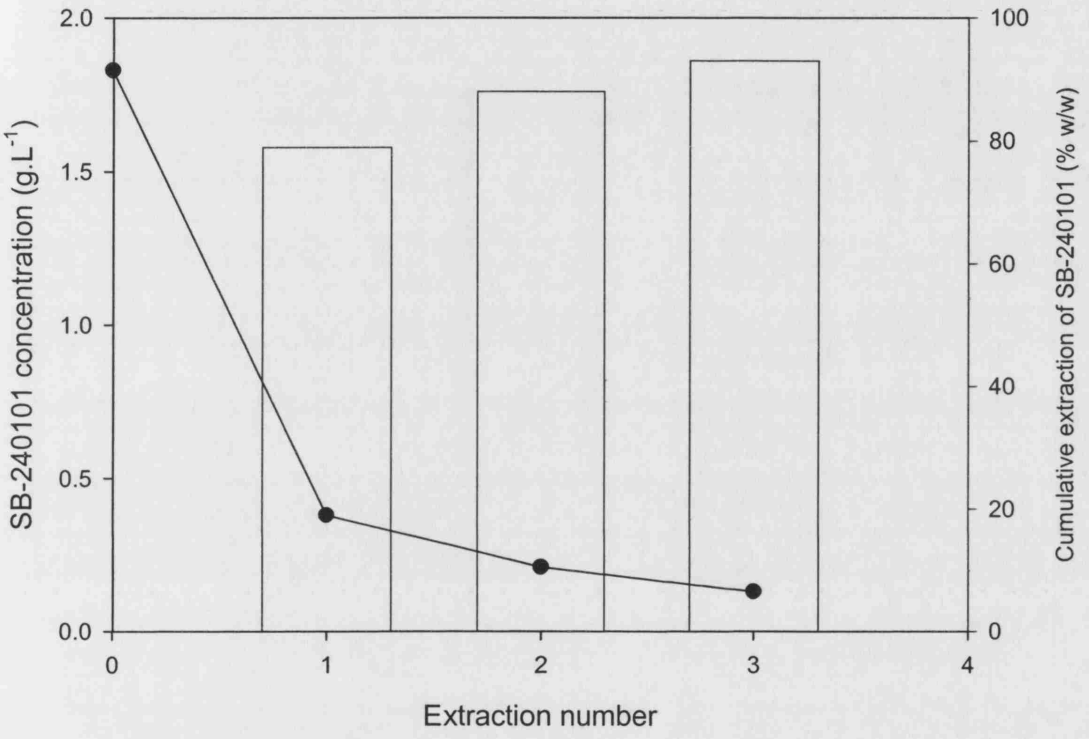


Figure 4.3- Liquid-liquid extraction of SB-240101 from [BMIM][PF_6] using 50mM bicarbonate buffer (pH10). Solid line shows concentration of SB-240101 in [BMIM][PF_6] after successive extractions and the bars show the cumulative extraction of SB-240101. V_R = 1. Experiments performed as described in Section 2.4.1.4.

4.3.2 Extraction of SB-240098 from [BMIM][PF_6] and Re-racemisation

The remaining substrate ester SB-240098 that is not hydrolysed in the bioconversion can be recovered by liquid-liquid extraction with isopropyl alcohol. Figure 4.4 shows that after eight extraction stages with fresh IPA 91% w/w of the SB-240098 was extracted from the [BMIM][PF_6]. Although it has not been experimentally investigated here, recovery of the substrate in IPA would allow ready re-racemisation

of this unused enantiomer. The re-racemisation of the unused R- enantiomer in the industrial process has been reported to show conversion of the R enantiomer to the S enantiomer with an average 88 % yield (Atkins et al., 2003). Using dimethyl carbonate at 40 °C and 30 % w/v sodium methoxide in methanol, after 5 hours the reaction is then quenched with acetic acid and diluted with water, finally IPA is added and the mixture stirred for 1 hour at 40 °C and 4 hours at 20 °C, the SB-235349 is then isolated by centrifugation (Atkins et al., 2003).

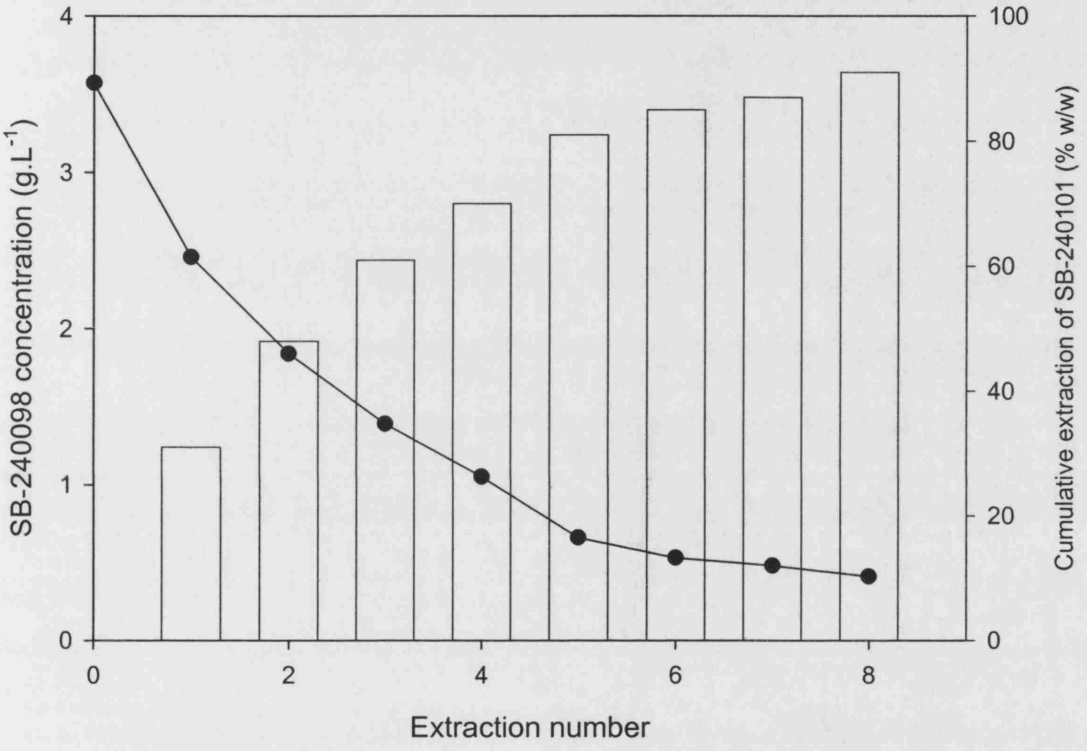


Figure 4.4- Liquid-liquid extraction of SB-240098 from [BMIM][PF_6] using isopropyl alcohol. Solid line shows the residual concentration of SB-240098 in [BMIM][PF_6] after successive extractions and the bars show the cumulative extraction of SB-240098. $V_R = 1$. Experiments performed as described in Section 2.4.1.5.

4.3.3 Cleaning and Recycling of [BMIM][PF_6]

Based on the screening experiments described in Section 4.2.1, extraction with 0.1 M sodium hydroxide was used to remove any final traces of SB-240098 and SB-240101 present in the ionic liquid. Figure 4.5 shows the efficiency of the sodium hydroxide

clean-up, as determined by HPLC, and also illustrates the performance of the whole downstream processing sequence:

(a) The first HPLC trace of the bioconversion medium displayed two peaks for both SB-240101 and SB-240098 following conversion of SB-235349 after six hours;

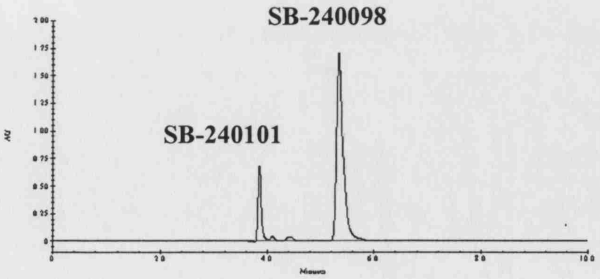
(b) The second trace shows that after three extractions with aqueous buffer (pH 10) as described in Section 4.3.1, the SB-240101 peak area reduced significantly;

(c) The third trace shows that after eight extractions with IPA as described in Section 4.3.2, the SB-240098 peak had also significantly decreased;

(d) The fourth trace shows that after a single wash with sodium hydroxide, the ionic liquid HPLC trace displayed no significant peaks;

(e) The final trace shows that the recycled [BMIM][PF_6] as bioconversion medium displayed two peaks for both SB-240101 and SB-240098 following conversion of SB-235349 after six hours;

Experimentally it was observed that the volume of the ionic liquid phase increased (8 % w/w) following extraction with IPA, indicating that a small amount of IPA dissolved in the [BMIM][PF_6]. Following liquid-liquid extraction with sodium hydroxide the volume of the ionic liquid phase reduced to the original level, indicating that the sodium hydroxide extracts not only the substrate and the product but also the dissolved IPA from the [BMIM][PF_6].



(a)

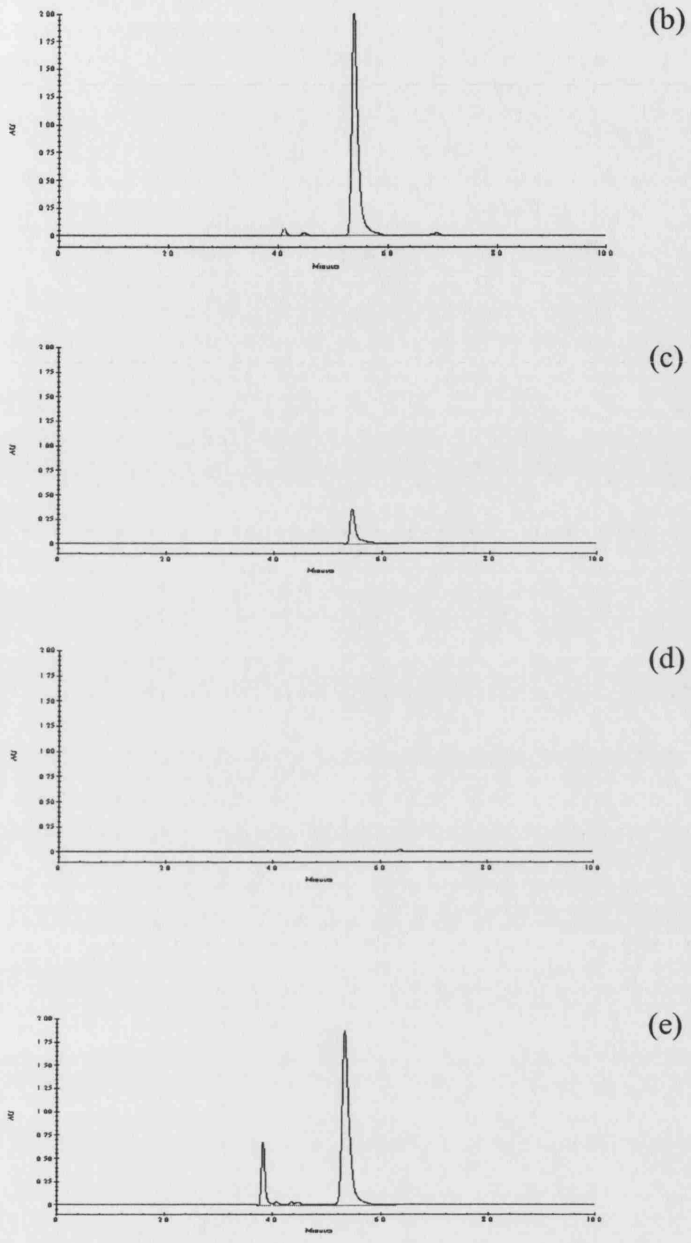


Figure 4.5- HPLC analysis of the [BMIM][PF_6] phase following the initial conversion, and successive product and substrate extraction cleaning and re-use; (a) Reaction mixture after bioconversion (6 hours) of SB-235349 in fresh [BMIM][PF_6], (b) [BMIM][PF_6] after 3 extractions with aqueous bicarbonate buffer (pH10) to remove SB-240101, (c) [BMIM][PF_6] after eight extractions with IPA to remove SB-240098, (d) [BMIM][PF_6] after a single sodium hydroxide wash, (e) Reaction mixture after bioconversion (6 hours) of SB-235349 using the recycled [BMIM][PF_6]. Conditions as described in Section 2.4.1.

4.3.4 Resolution of SB-235349 to SB-240101 in [BMIM][PF_6]

Finally, to show that the cleaned up ionic liquid can be re-used in the bioconversion process the entire downstream processing sequence was operated over two reaction cycles, using fresh enzyme each time, however, as described in Section 3.4.7, this could be re-used. The dashed lines in Figure 4.6 show the bioconversion kinetics in freshly prepared ionic liquid as described in Section 3.4.1. Results show that there is a rapid initial decrease in SB-235349 concentration attributed to the adsorption of the substrate to the Novozyme 435 resin on which the lipase is immobilised. This is followed by a steady increase in the concentration of SB-240101 to a final concentration of 1.85g.L^{-1} after 6 hours. Following this the product was recovered as outlined in Figure 4.2. Data points in Figure 4.6 show results with the re-used ionic liquid displayed almost identical reaction rates and yields, indicating the extraction route used did not alter the utility of the ionic liquid as reaction media. The similarity between the bioconversion results with both fresh and recycled ionic liquid is also shown in Figure 4.5 (e) which shows an almost identical HPLC trace with the recycled ionic liquid as Figure 4.5 (a) for reaction in fresh ionic liquid.

4.4 Liquid-Liquid Extraction Kinetics with Ionic Liquid Phases

Having shown a route of product recovery and ionic liquid recycle (Figure 4.2) it is necessary to consider the solute mass transfer kinetics, and the controlling mass transfer process in order to enable selection of the most appropriate phase contactor and operating conditions. This work will examine the mass transfer kinetics for both SB-240101 extraction and SB-240098 extraction in a Lewis cell.

4.4.1 K_0 Determination in a Lewis Cell

A Lewis cell provides a defined interfacial area between two phases and allows accurate measurements of the change in solute concentration with time to be made under precisely defined hydrodynamic conditions (Savastano et al., 1991). Operation of the Lewis cell used here is described in Section 2.4.3. The physical properties of

the phases and the equilibrium distribution coefficient for SB-235349 and SB-240101 are given in Table 4.4.

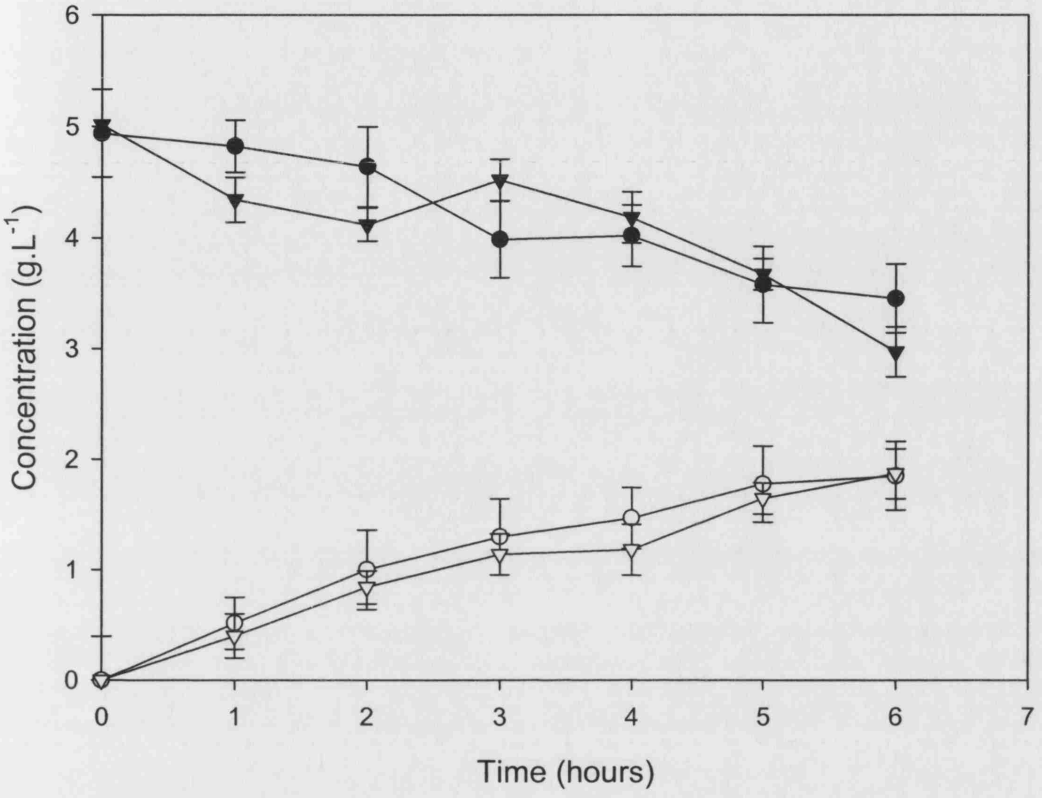


Figure 4.6- Lipase catalysed resolution of SB-235349 in fresh and recycled [BMIM][PF_6]. SB-240101 concentration in recycled [BMIM][PF_6] (○), SB-235349 concentration in recycled [BMIM][PF_6] (●). Dashed lines indicate original bioconversion kinetics in fresh ionic liquid. Reaction conditions as described in Section 2.3.1: solvent = [BMIM][PF_6] (12 % H_2O w/w), [Novozyme 435] = 1.4 $g.L^{-1}$, [SB-235349] = 5 $g.L^{-1}$, temp = 50 °C, no pH control. Error bars indicate one standard deviation.

Table 4.4- Physical properties of the phases used for liquid-liquid extraction and equilibrium distribution coefficients of both SB-2235349 and SB-240101. Physical properties were determined as described in Section 2.5.5. Equilibrium distribution coefficients determined as described in Section 2.5.1.1 and Section 2.5.1.2.

Phases and equilibrated systems used in extraction experiments	**Density ($kg.m^{-3}$)**	**Viscosity x 10^{-3} ($kg.m^{-1}.s^{-1}$)**	**$m_{SB-240101}$ ($= C_{aq}/C_{IL}$)**	**$m_{SB-240098}$ ($= C_{org}/C_{IL}$)**
[BMIM][PF_6]- single phase	1387	207	N/A	N/A
50mM bicarbonate buffer (pH10) - single phase	1001	1.00	N/A	N/A
Isopropyl alcohol - single phase	785	1.37	N/A	N/A
50mM bicarbonate buffer (pH10) [BMIM][PF_6]	1002 1316	1.00 85.0	16.7	N/A
Isopropyl alcohol [BMIM][PF_6]	792 1237	1.42 36.0	N/A	5.07

Examples of the experimental extraction data for the change in product and substrate concentration with time in the ionic liquid phase are shown in Figure 4.7. The results at each Reynolds number were obtained from three separate experiments and are plotted according to Equation 2.7 for both the product and the substrate. The results fall on a straight line indicating the two-film theory of mass transfer is applicable to this experimental system.

The overall mass transfer coefficients (K_0) in the two cases were determined in the Lewis cell over the range of Reynolds numbers for which a flat, non-perturbed, interface could be maintained were in the range 1.0 - 3.5 x 10^{-6} $m.s^{-1}$ for product extraction over the Reynolds number range 60 - 145, and 1.0 – 2.5 x 10^{-6} $m.s^{-1}$ for substrate extraction over the Reynolds number range 50 - 85. In both extraction experiments there was a linear increase in K_0 with Reynolds number as shown in Figure 4.8, indicating that the mass transfer was controlled by molecular diffusion from the ionic liquid as would be expected for the transport of a non-reacting solute across a stable interface (Lye et al., 2001). The difference between the substrate and

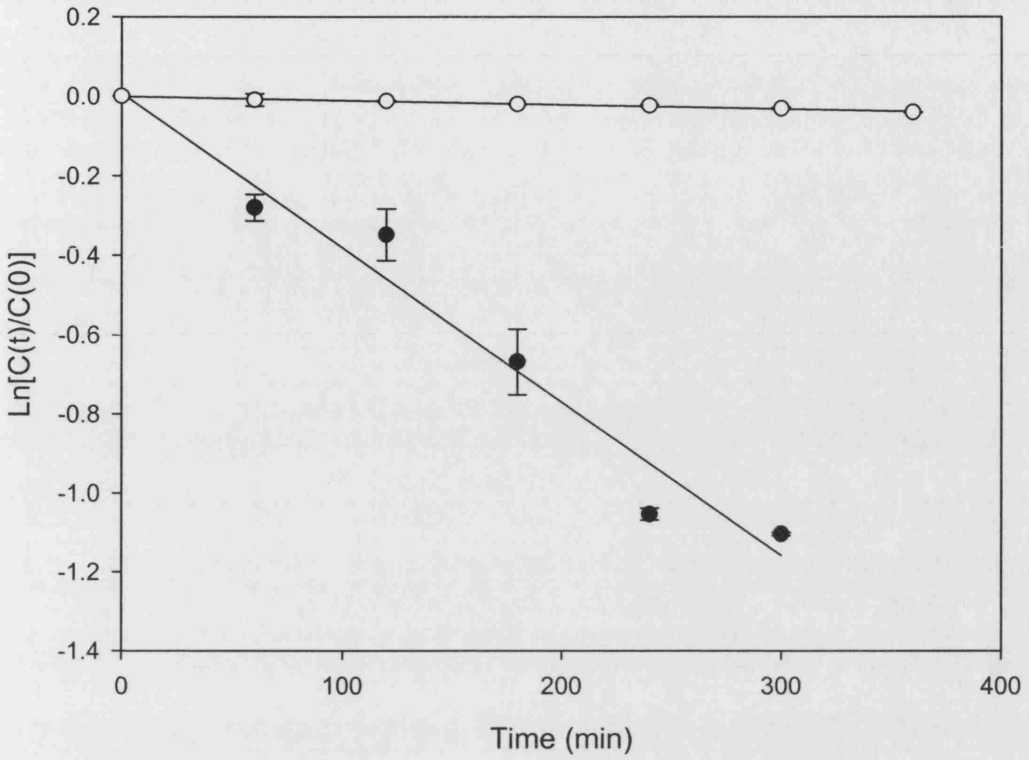


Figure 4.7- Kinetics of SB-240101 and SB-240098 extraction in the Lewis cell, (●) extraction of 2.5 $g.L^{-1}$ SB-240101 from [BMIM][PF_6] into 50mM aqueous bicarbonate buffer (pH10), Re = 145, (○) extraction of 2.5 $g.L^{-1}$ SB-240098 from [BMIM][PF_6] into IPA, Re = 54. Data plotted according to Eq. (2.7) and solid lines fitted by linear regression (error bars indicate one standard deviation). Experiments performed as described in Section 2.4.3.

product K_0 values shows the same gradient in the two systems however, the SB-240098 extraction shows a higher magnitude and is attributed to the large difference in viscosity between the two systems as shown in Table 4.4. The viscosity of the saturated [BMIM][PF_6] with IPA is less than half that of the [BMIM][PF_6] saturated with aqueous bicarbonate buffer. Using the Wilke-Chang correlation (Bailey et al., 1986) used to estimate the diffusion coefficient of small molecules in low-molecular-weight solvents, the influence of viscosity on diffusivity can be seen.

$$D = 7.4\times10^{-8}\frac{T(x_a M)^{1/2}}{\mu_l V_m^{0.6}} \qquad (4.1)$$

where M is the solute molecular weight, V_m is the molecular volume of solute at boiling point, and μ_l is the liquid viscosity, T is the temperature and x_a is the association factor of solvent. The molecular weight of SB-240101 is only slightly higher than that of SB-240098 and it is therefore likely that the molecular volume of each solute is similar. The temperature of both systems is the same and the association factor of the solvent is assumed to be similar for each system, therefore the factor considered to produce the largest changes in diffusivity would be the viscosity. The overall mass transfer rate from an ionic liquid is in a similar range to the transport of a solute through an aqueous-organic interface 1.0 - 3.5 x 10^{-6} $m.s^{-1}$ (Lye et al., 2001), however, the Reynolds number range for the ionic liquid system is much lower 60 - 145 compared to 200 - 650 for the conventional solvent system.

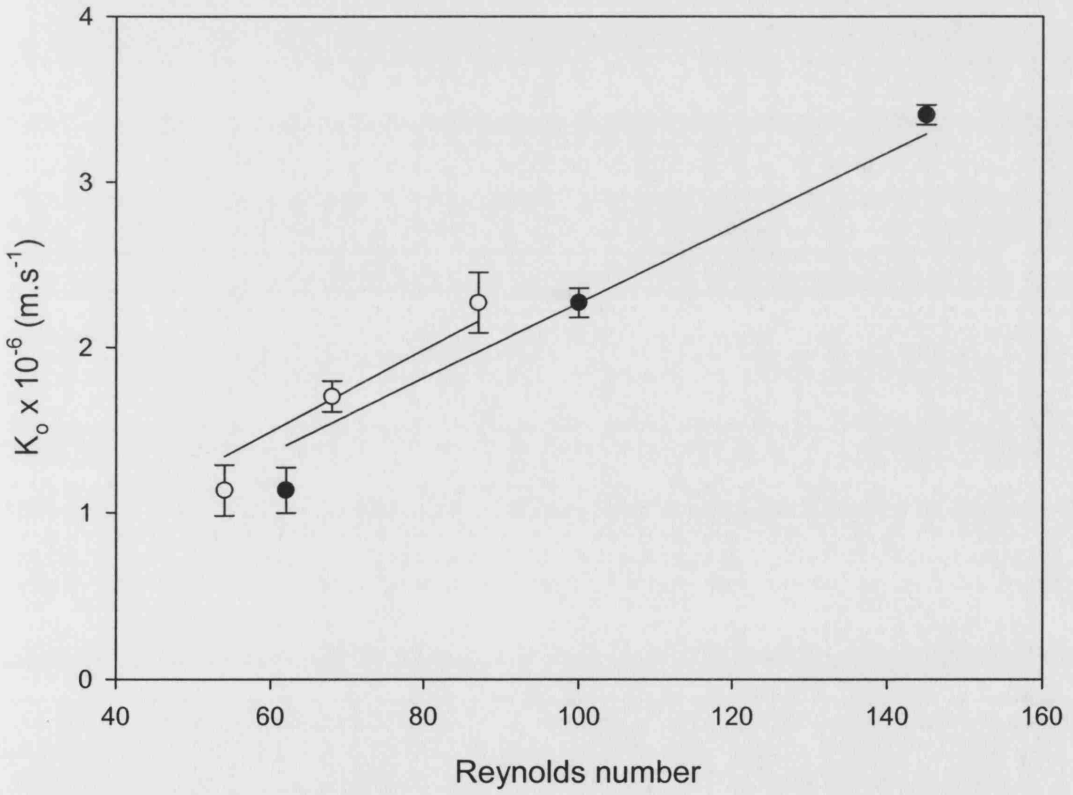


Figure 4.8- Effect of Reynolds number on the overall SB-240101 and SB-240098 mass transfer coefficients; (●) extraction of 2.5 $g.L^{-1}$ SB-240101 from [BMIM][PF_6] into 50mM aqueous bicarbonate buffer (pH 10); (○) extraction of 2.5 $g.L^{-1}$ SB-240098 from [BMIM][PF_6] into IPA. Solid lines fitted by linear regression (error bars indicate one standard deviation). Experiments performed as described in Section 2.4.3.

4.5 Discussion

The optimised industrial lipase-catalysed resolution of Lotrafiban (Figure 1.3) has been shown to have increased reaction rates or yields when performed in ionic liquids compared to the optimised organic solvent system. In order to exploit the potential of ionic liquids as alternative reaction media certain key issues still need to be resolved before ionic liquids can be implemented in an industrial process. The experiments performed in this chapter demonstrate the application of ionic liquids as alternative reaction media, extraction of the product and substrate from the media and recycling of the ionic liquid reaction media for reaction. The results present a positive initial indication to their implementation; firstly 90 % w/w removal of the product was achieved into aqueous media and 90 % w/w removal of the racemic substrate into organic media. Finally, re-use of the ionic liquid following reaction, extraction and subsequent reaction again showed almost identical results to those produced in the fresh ionic liquid.

4.5.1 Determination of Product Recovery and Recycle Route of Ionic Liquid

Several methods of product recovery were eliminated at an early stage due to the properties of the product and substrate. Two routes that were examined for product recovery options were liquid-liquid extraction and adsorption due to their wide spread industrial application and availability. Screening of adsorbents (Table 4.3) which were either anionic or cationic exchange resins produced low selectivity, and activated charcoal showed no removal of the product and therefore due to the poor initial results with adsorbents they were excluded from further investigation.

To determine two of the critical parameters for liquid-liquid extraction 33 polar, non-polar and aqueous solvents were examined for miscibility with [BMIM][PF_6] and extraction of either SB-240101 or SB-240098 from the ionic liquid phase to the extracting phase (Table 4.2).

From the results of the solvent screening and supporting literature the method of pH dependent partitioning of the solute from the ionic liquid seemed an ideal way of

removing the charged product. First the distribution coefficient of the SB-240101 extracted from [BMIM][PF_6] into aqueous buffer over the pH scale 2-12 was determined to find the optimum pH (Figure 4.1), which was pH 10 for the highest extraction of SB-240101 and the lowest extraction of SB-240098. This method not only extracted high concentrations of the product ~80 % w/w with the first extraction but also avoids the use of organic solvents.

Overall these results show the best route determined for the extraction of SB-240101 from [BMIM][PF_6] is by liquid-liquid extraction with aqueous bicarbonate buffer (pH 10). The route for SB-240098 extraction from [BMIM][PF_6] was determined as liquid-liquid extraction with IPA. Finally to remove the remaining SB-240101 and SB-240098 still present in the [BMIM][PF_6] liquid-liquid extraction with aqueous sodium hydroxide (50mM) was shown to display the highest extraction for both the substrate and the product. The final route used for the product recovery and recycle of [BMIM][PF_6] following conversion of SB-235349 is shown in Figure 4.2.

Results for the extraction of SB-240101 following conversion of SB-235349 are shown in Figure 4.3 and show that pH-partitioning of SB-240101 from [BMIM][PF_6] with aqueous buffer (pH 10) removes ~90 % after three extractions with fresh buffer. Subsequent down stream processing steps of the product for the optimised industrial process show removal of the product from an organic phase into an aqueous phase as shown here. Extraction of SB-240098 with IPA is shown in Figure 4.4, following eight extractions with fresh IPA ~90 % of the SB-240098 was removed into the organic solvent phase. Although the use of IPA introduces an organic solvent back into the process this does not negate the use of the ionic liquid due to the advantages (outlined in Chapter 3) offered by the use of ionic liquids. Finally sodium hydroxide was used to extract the remaining SB-240101 and SB-240098 present in the ionic liquid phase. Results of the extraction steps on the HPLC traces are shown in Figure 4.5 and show that following extraction with aqueous buffer (pH 10), IPA, and sodium hydroxide there are no significant peaks present in the ionic liquid phase. An additional observation was that the volume of the ionic liquid increased following extraction with IPA, indicating a small amount of IPA dissolved in the [BMIM][PF_6]. This was then subsequently removed by liquid-liquid extraction with sodium hydroxide where the volume of the ionic liquid phase reduced again, indicating that

the sodium hydroxide extracts not only the substrate and the product but also the dissolved IPA from the [BMIM][PF_6]. Following reaction and extraction the conversion of SB-235349 was again performed in the [BMIM][PF_6], the results shown in Figure 4.6 show that the results with fresh and recycled [BMIM][PF_6] are almost identical and indicate that the extraction methods used have had no significant negative effect on the composition of the ionic liquid.

4.5.2 Kinetics of Liquid-Liquid Extraction Steps

The kinetics of the extraction steps for the removal of SB-240101 and SB-240098 from [BMIM][PF_6] were then examined in more detail in the Lewis cell to determine the overall mass transfer coefficients for liquid-liquid extraction from [BMIM][PF_6]. In Figure 4.7 results show that at a specific Reynolds number the change in either product or substrate concentration with time in the ionic liquid phase fall on a straight line indicating the application of the two-film theory of mass diffusion to this experimental system. The values of K_0 determined in the Lewis cell (Figure 4.8) were in the range 1.0 - 3.5 x 10^{-6} $m.s^{-1}$ for product extraction over the Reynolds number range 60 - 145, and 1.0 – 2.5 x 10^{-6} $m.s^{-1}$ for substrate extraction over the Reynolds number range 50 - 85. Rates were found to be similar to conventional solvents. Both show a linear increase in K_0 with Reynolds number indicating that the process is limited by diffusion, most likely through ionic liquid film at ionic liquid-aqueous/solvent interfaces. The difference in magnitude between substrate and product K_0 values is attributed to the large difference in viscosity and equilibrium coefficient between the two systems as shown in Table 4.4. Results suggest for liquid-liquid extraction steps the use of conventional mixer/settler (or centrifugal contactors) phase contactors in which the ionic liquid phase is dispersed (solvent/aqueous continuous) in order to minimise diffusion distances and optimise ionic liquid interfacial area. To increase the interfacial area when mixing the two phases the volume fraction of the ionic liquid should be less than 50 %, ideally 20-30 %. An additional consideration is the high power input that would be required in order to produce the small droplet sizes for mixing (Harnby et al., 1985).

In summary, the results in this chapter have shown how liquid-liquid extraction is an attractive technique to facilitate product recovery and clean-up/recycling of ionic liquids. In the next chapter the application of the knowledge and design procedures developed for the lipase catalysed resolution (Chapter 3) and associated product recovery (Chapter 4) will be evaluated on an alternate bioconversion process.

5.0 Application of Process Design Principles to the Thymidine Phosphorylase Catalysed Synthesis of Thymidine

5.1 Aims

In the previous chapters approaches to the design of enzyme catalysed reactions in ionic liquids and subsequent product recovery options were demonstrated. These showed the key experimental variables relevant to the use of ionic liquids. The aim of this chapter is to illustrate the generic nature of the design procedure to a phosphorylase-catalysed synthesis reaction in ionic liquids. As shown in Figure 1.4 the particular reaction considered is the thymidine phosphorylase-catalysed synthesis of thymidine from thymine and a phosphate salt. Firstly, the reaction kinetics were determined in aqueous media at optimised conditions. Subsequently the reaction was examined in a limited range of ionic liquids as shown in Table 5.1, to determine which could be used as alternative reaction media, and if so, what advantages the use of ionic liquids may provide over conventional aqueous solvents. Finally, product recovery options for the ionic liquid based process were considered in order to examine the feasibility of implementing ionic liquid media into an industrial process. Given the expense of the 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt, and the limited availability of one of the ionic liquids used, the experiments were limited in number and scale of operation.

5.2 Characterisation of the Substrate and Products of the Thymidine Phosphorylase Conversion

5.2.1 Properties of the Substrate and Product Compounds

The initial step in characterising the thymidine phosphorylase conversion is to obtain physico-chemical property data on the reaction substrates and product, either from literature data or experimentally. The substrates for this conversion were thymine and the di(monocyclohexylammonium) salt of 2-deoxyribose-1-phosphate, while the reaction products are thymidine and dicyclohexylamine. Nitric acid and Calcium phosphate are also produced due to reaction in the presence of calcium nitrate (Figure 1.4). The thymine substrate is a solid at room temperature and has the molecular

formula of $C_5H_6N_2O_2$ and a molecular weight of 126.1. The thymidine product is also a solid and has the molecular formula of $C_{10}H_{14}N_2O_5$ with a molecular weight of 242.2. All the substrates and products of this conversion are commercially available. The boiling points of thymine and thymidine are 316-317 °C and 185-190 °C respectively, so loss of either compound by evaporation is not expected.

The substrates and products were analysed throughout this work by HPLC, as described in Section 2.5.3. A sample HPLC chromatogram is shown in Appendix III with each species being identified by the injection of known standards. The substrate thymine had a retention time of 3.6 minutes, and was visualised as a single peak on the chromatogram. The thymidine product was also visualised as a single peak with a retention time of 5.8 minutes.

5.2.2 Solubility Screening and Stability

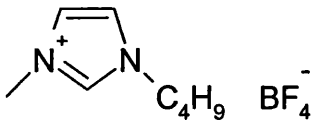
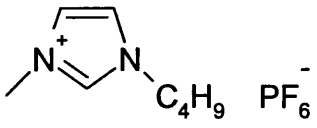
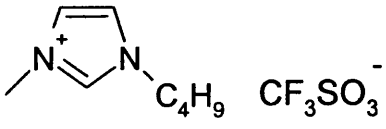
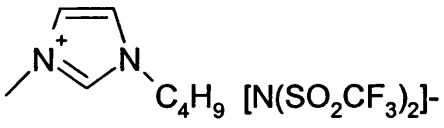
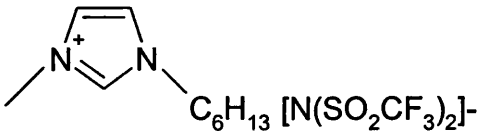
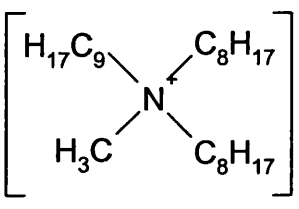
In order for ionic liquids to be usefully applied as reaction media for the conversion shown in Figure 1.4, the solubility of both the substrate and product is an important criterion. As shown in Chapter 3, screening of a limited range of ionic liquids generally yields a number that can be considered good solvents. The solubility of thymine and thymidine were determined as described in Section 2.3.2.1, and the ionic liquids used were prepared as described in Section 2.2. The first ionic liquid tested was the hydrophobic 1-butyl-3-methyl imidazolium hexafluorophosphate ([BMIM][PF_6]), in which the solutes dissolved poorly as shown in Table 5.2. The next ionic liquids tested were the hydrophilic 1-butyl-3-methyl imidazolium tetrafluoroborate ([BMIM][BF_4]) and 1-butyl-3-methyl imidazolium trifluoromethane sulfonate ([BMIM][CF_3SO_3]), which displayed higher solubility for both thymine and thymidine compared to [BMIM][BF_4] of < 3 g.L^{-1} and > 20 g.L^{-1} respectively. The results for 1-butyl-3-methyl imidazolium bis(trifluoromethylsulfonyl)amide ([BMIM][$N(SO_2CF_3)_2$]) and 1-hexyl-3-methyl imidazolium bis(trifluoromethylsulfonyl)amide ([HMIM][$N(SO_2CF_3)_2$]) both considered hydrophobic ionic liquids, showed that increasing the hydrophobicity of the ionic liquid by increasing the alkyl chain length on the imidazolium cation decreased the solubility of thymidine in the ionic liquid from < 5 g.L^{-1} to < 4 g.L^{-1}. The next ionic liquid tested was methyl-trioctyl-imidazolium bis(trifluoromethylsulfonyl)amide

([Oc_3MeN][$N(SO_2CF_3)_2$]), which is water immiscible. This ionic liquid showed that substitution of the [HMIM] cation with the [Oc_3MeN] cation only increased the solubility of thymine slightly. The ionic liquid ECOENG™ 500 (PEG-5 cocomonium methosulfate) ([CABHEM][$MeSO_4$]), which is immiscible with water, showed a reasonably high solubility for thymine and thymidine of < 9 $g.L^{-1}$ and < 15 $g.L^{-1}$ respectively compared to the solubility levels previously observed in hydrophobic ionic liquids.

As the ionic liquids generally considered to be hydrophilic displayed the highest solubility for both thymine and thymidine, the ionic liquids 1-butyl-3-methyl-imidazolium octylsulphate ([BMIM][$OcSO_4$]), 1-butyl-3-methyl-imidazolium diethyleneglycol monomethylethersulphate ([BMIM][$MDEGSO_4$]) and 1-ethyl-3-methyl imidazolium tosylate ([EMIM][tosylate]) were also examined. As shown in Table 5.2 all these ionic liquids displayed high solubility for thymidine; The ionic liquids [BMIM][$MDEGSO_4$], [EMIM][tosylate] and in particular [BMIM][OMs]) showed high solubility for both thymine and thymidine. Overall these preliminary solubility values give a general idea of what kind of ionic liquids are most suitable to the solubility of thymine and thymidine and therefore which ionic liquids should be tested for the conversion of thymine.

As described in Section 1.4 another group have been investigating nucleoside chemistry in ionic liquids, and have also reported the solubility of thymidine in ionic liquids (Uzagare et al., 2003). The solubility limit determined for thymidine in [BMIM][PF_6], [BMIM][BF_4] and [MOEMIM][OMs] is similar to that reported here in Table 5.2. This group also tested several other ionic liquids containing the OMs anion and the solubility was found to be lower than that observed in [MOEMIM][OMs].

Table 5.1 - Structure and physical properties of the various ionic liquids screened for use in the thymidine phosphorylase catalysed synthesis of thymidine. Ionic liquids were synthesised and prepared as described in Section 2.2.

Ionic liquid structures and nomenclature	Summary of physical properties	References
 [BMIM][BF_4]	Hydrophilic Melting point –71.0 °C Viscosity 145 mPa.s Density 1210 $kg.m^{-3}$	Branco et al., (2002) Huddleston et al., (2001) Holbrey et al., (1999)
 [BMIM][PF_6]	Hydrophobic Melting point 6.0 °C Viscosity 207 mPa.s Density 1387 $kg.m^{-3}$	Huddleston et al., (2001) Dzyuba et al., (2002) Holbrey et al., (2002)
 [BMIM][CF_3SO_3]	Hydrophilic Melting point 17.0 °C	Huddleston et al., (2001) Wasserscheid et al., (2000)
 [BMIM][$N(SO_2CF_3)_2$]	Hydrophobic Melting point –6.0 °C Immiscible with water Miscible with acetonitrile	Dzyuba et al., (2002) Huddleston et al., (2001)
 [HMIM][$N(SO_2CF_3)_2$]	Hydrophobic	www.merck.com
 [Oc_3MeN][$N(SO_2CF_3)_2$]	Hydrophobic Immiscible with water Density 1387 $kg.m^{-3}$	www.solventinnovation.com

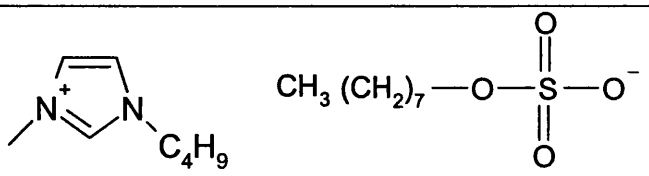
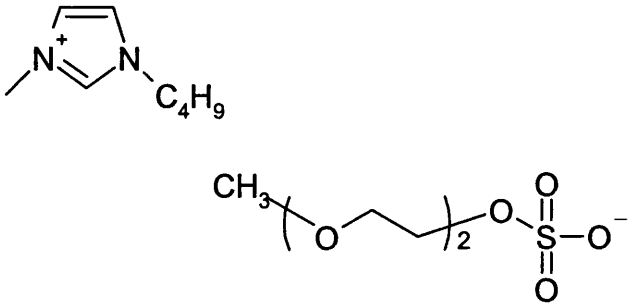
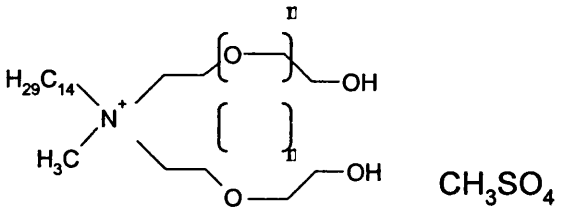
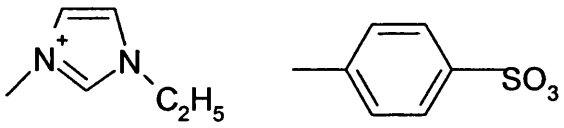
 [BMIM][OcSO4]	Hydrophilic Miscible with water Density 940 kg.m^{-3}	www.solvent innovation.com
 [BMIM][MDEGSO4]	Hydrophilic Viscosity 340 mPa.s Density 1060 kg.m^{-3}	www.solvent innovation.com
 [CABHEM][MeSO4]	Hydrophilic Viscosity 3000 mPa.s Density 825 kg.m^{-3}	www.solvent innovation.com
 [EMIM][tosylate]	Hydrophilic Viscosity 477.8 mPa.s Density 930 kg.m^{-3}	www.solvent innovation.com
 [MOEMIM][OMs]	Hydrophilic	www.solvent innovation.com

Table 5.2- Solubility of thymine and thymidine in a selection of ionic liquids and aqueous buffer. Ionic liquids were prepared and synthesised as described in Section 2.2 and solubilities determined as in Section 2.3.2.1. (N/A means not applicable).

Ionic liquid	Physical properties	Solubility limit ($g.L^{-1}$)	
		Thymine	Thymidine
[BMIM][PF_6]	Hydrophobic	< 1	< 2
[BMIM][$N(SO_2CF_3)_2$]	Hydrophobic	< 1	< 5
[HMIM][$N(SO_2CF_3)_2$]	Hydrophobic	< 1	< 4
[Oc_3MeN][$N(SO_2CF_3)_2$]	Hydrophobic	< 2	< 4
[BMIM][$OcSO_4$]	Hydrophobic	< 3	> 20
[CABHEM][$MeSO_4$]	Hydrophobic	< 9	< 15
[BMIM][BF_4]	Hydrophilic	< 1	> 10
[BMIM][CF_3SO_3]	Hydrophilic	< 9	> 20
[BMIM][$MDEGSO_4$]	Hydrophilic	< 9	> 20
[EMIM][tosylate]	Hydrophilic	> 10	> 20
[MOEMIM][OMs]	Hydrophilic	> 10	> 20
10mM Tris HCl buffer	N/A	4	> 10

5.3 Characterisation of the Thymidine Phosphorylase Catalysed Synthesis of Thymidine in Aqueous Media

Initial experiments in aqueous 10mM Tris HCl buffer examined the activity of the batch of thymidine phosphorylase used throughout this work in order to provide a basis for comparison with subsequent ionic liquid studies. These also provide a basis against which to later assess the performance of the bioconversion in ionic liquid media. Experiments were performed as described in Section 2.3.2, under the conditions previously shown for the production of thymidine (EP 1179598A1, 2002). Typical bioconversion results are shown in Figure 5.1. Measurements of the liquid phase solute concentration show conversion of thymine to thymidine, the reaction reached 50 % conversion within 5 hours and the concentration of thymine decreased from 2.5 mM to 1.25 mM, with the formation of a precipitate which indicated the presence of calcium phosphate. Although this reaction has been reported as achieving 90 % conversion (EP 1179598A1, 2002), the reaction described with this method is an equilibrium reaction with K_{eq} ~1. The calcium nitrate is present to shift equilibrium toward product formation which has been reported by others to result in ~90 % yield,

however, this was not achieved in this case. The limitation of the use of aqueous media is the low solubility of thymine (4 $g.L^{-1}$/32 mM).

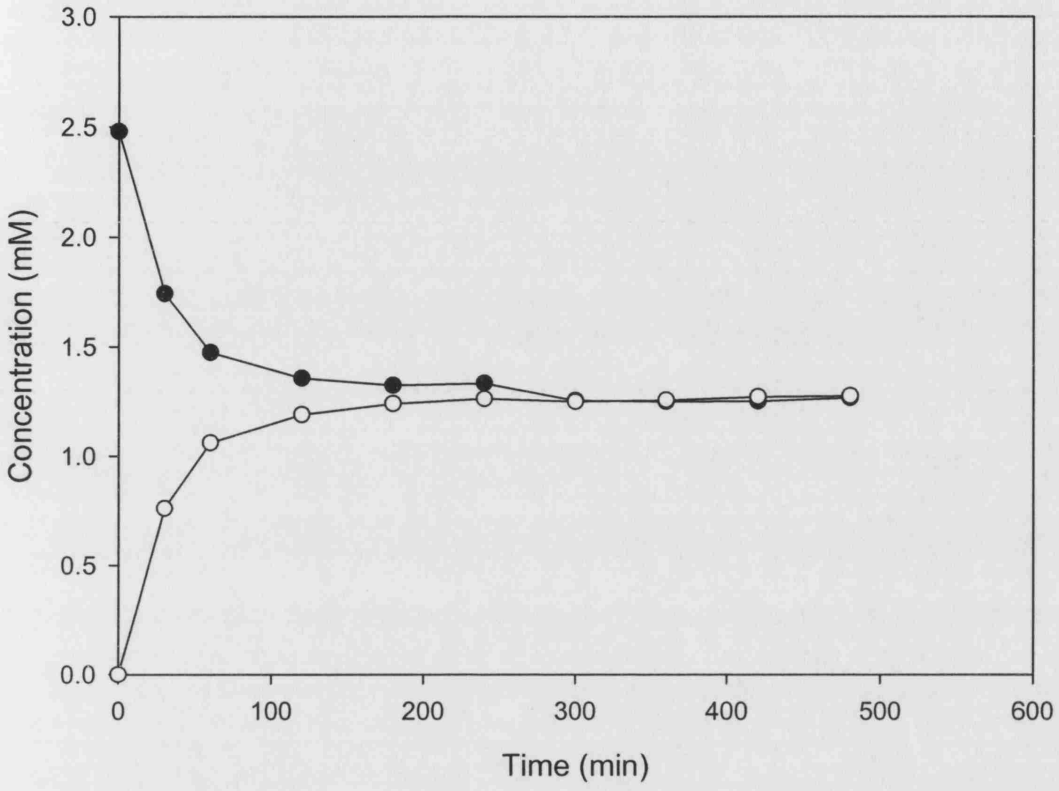


Figure 5.1- Kinetics of the thymidine phosphorylase catalysed synthesis of thymidine in 10mM Tris HCl buffer (pH 7.4): (●) thymine concentration, (○) thymidine concentration. Reaction conditions as described in Section 2.3.2: Solvent = 10mM Tris HCl buffer (pH 7.4), [thymidine phosphorylase] = 12 $units.mL^{-1}$, initial [thymine] = 2.5mM (0.30 $g.L^{-1}$), [calcium nitrate] = 10mM added at the start of the reaction, temp = 30 °C.

5.4 Thymidine Phosphorylase Catalysed Synthesis of Thymidine in Ionic Liquids

5.4.1 Ionic Liquid Screening

As for the lipase catalysed resolution described in Chapter 3, initial experiments with ionic liquids aimed to establish if the thymidine phosphorylase catalysed synthesis of thymidine would actually occur in an ionic liquid, and if so, which type of ionic liquid was most suitable for the reaction. For these experiments a limited range of ionic

liquids were chosen that exhibited different physico-chemical properties, as shown in Table 5.1, and different solubilities for thymine and thymidine (Table 5.2). All were tested under conditions identical to those used in Figure 5.1 apart from the replacement of the Tris HCl buffer with the appropriate ionic liquid. The ionic liquids were synthesised and purified before use as described in Section 2.2. All reactions were performed in triplicate as described in Section 2.3.2.

As shown in Table 5.3, the first ionic liquids tested were a widely used range with different properties which although having low solubility for thymine and thymidine would provide an idea of the type of ionic liquids suited to this type of conversion.

Table 5.3- Reaction of thymine to thymidine in a selection of ionic liquids and aqueous buffer. Ionic liquids were prepared and synthesised as described in Section 2.2 and reacted as determined as in Section 2.3.2.

Ionic liquid	Thymidine detected	Formation of a precipitate
[BMIM][PF_6]	Yes	Yes
[BMIM][BF_4]	No	Yes
[BMIM][CF_3SO_3]	No	No
[BMIM][$MDEGSO_4$]	No	Yes
[EMIM][tosylate]	No	Yes
[CABHEM][$MeSO_4$]	No	Yes
[MOEMIM][OMs]	No	No
10mM Tris HCl buffer	Yes	Yes

[BMIM][PF_6], which is considered to be hydrophobic and is water immiscible (Huddleston et al., 2001; Dzyuba et al., 2002; Holbrey et al., 2002) was one of the first ionic liquids tested. As shown in Figure 5.2 the conversion of thymine to thymidine is very slow and only goes to 30 % completion after 6 hours compared to the 50 % conversion in 10mM Tris HCl. After three hours the thymidine concentration was 1.39 mM almost half that produced in the aqueous system. The increased solubility of thymidine in the hydrophilic ionic liquids such as [BMIM][BF_4] and [BMIM][CF_3SO_3] suggested that conversion in these ionic liquids would offer more advantages as reaction media. Results with the hydrophilic [BMIM][BF_4] did not show any conversion, i.e. the concentration of the thymine did

not change over time; there was, however, the production of a precipitate upon addition of calcium nitrate. HPLC analysis showed a small peak at an early retention time of 2.54 minutes, this early peak could represent the deoxyribose group cleaved or the phosphate group cleaved from the 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt, which would explain the production of the precipitate. The absence of thymidine in the reaction mixture indicates the enzyme is unable to perform the coupling of the phosphate group with thymine to produce thymidine in this medium. The reaction in [BMIM][CF_3SO_3] showed no conversion of thymine and no production of a precipitate with the addition of calcium nitrate, indicating the reaction does not occur or that the enzyme is inactive in this ionic liquid.

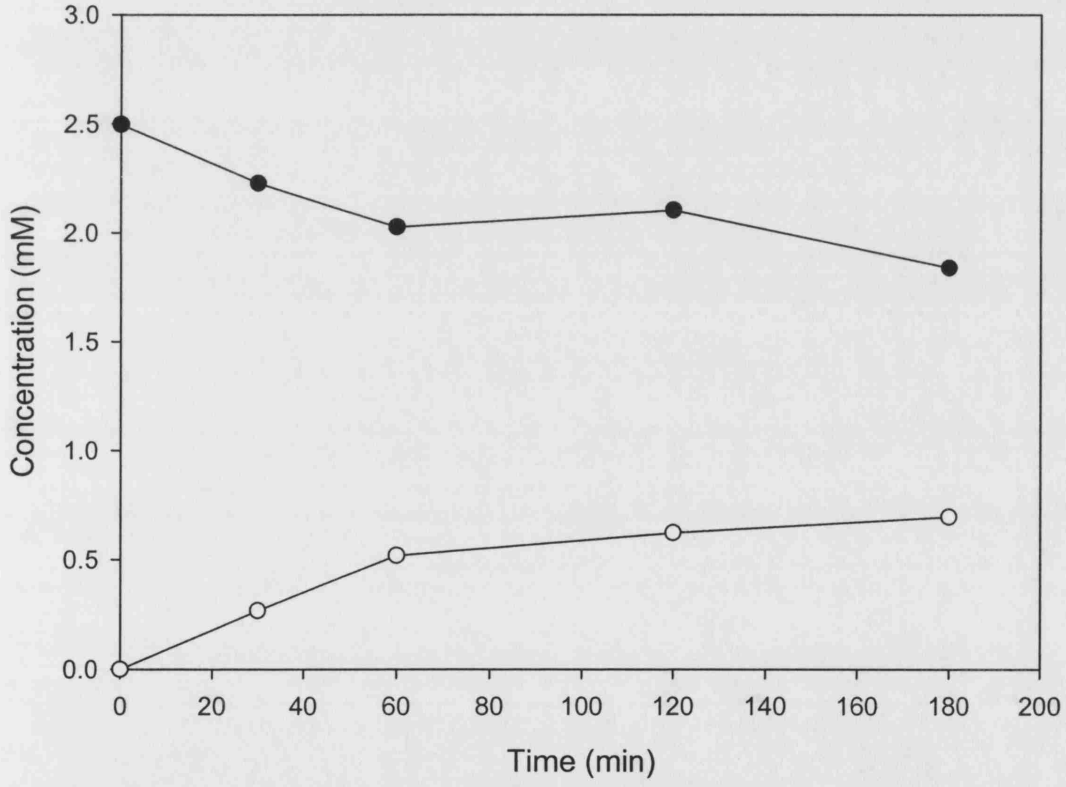


Figure 5.2- Kinetics of the thymidine phosphorylase catalysed synthesis of thymidine in [BMIM][PF_6]: (●) thymine concentration, (○) thymidine concentration. Reaction conditions as described in Section 2.3.2: Solvent = [BMIM][PF_6], [thymidine phosphorylase] = 12 units.mL^{-1}, initial [thymine] = 2.5mM (0.30 g.L^{-1}), [calcium nitrate] = 10mM added at the start of the reaction, temp = 30 °C.

Due to the high solubility of thymine and thymidine in [EMIM] [tosylate], [BMIM][MDEGSO$_4$], [CABHEM][MeSO$_4$] and [MOMIM][OMs] these ionic liquids were also investigated. Results for [EMIM][tosylate], [BMIM][MDEGSO$_4$], and [CABHEM][MeSO$_4$] were similar to [BMIM][BF$_4$], with no change in the substrate concentration with time, but produced a precipitate with the addition of calcium phosphate. These ionic liquids, however, have much higher viscosity than [BMIM][BF$_4$] as shown in Table 5.1, and as a result the analysis was difficult and the peaks observed by HPLC were very small. The ionic liquid [BMIM][OMs], also showed high solubility for thymine and thymidine, comparatively low viscosity and displayed no sign of reaction by HPLC analysis or precipitation of calcium phosphate.

In summary, the screening of a very limited range of ionic liquids yielded a single ionic liquid [BMIM][PF$_6$] in which the reaction was shown to occur, but at a lower reaction rate than that observed in Tris HCl buffer. This ionic liquid has a limited solubility of the substrate and product, significantly less than that attainable in Tris HCl buffer. A number of ionic liquids were identified which have high levels of solubility for the substrate and product, however, these were unsuitable for quantification of the reaction by HPLC, due to the high viscosity of these ionic liquids.

5.4.2 Effect of Water Content on Thymidine Formation in Ionic Liquids

The ionic liquids used in this work are supplied as anhydrous or with very low water contents, and subsequent investigation of the solubility of thymine and thymidine showed that the more hydrophilic ionic liquids displayed the highest solubility (Table 5.2). As described in Section 5.4.1, however, reactions in the ionic liquids with high solubility of thymine and thymidine are not suitable for thymidine synthesis, due to the high viscosity displayed by these ionic liquids and subsequent difficulty in analysis. The only ionic liquid that did show conversion clearly was [BMIM][PF$_6$] which was prepared as described in Section 2.2 and contained 3 % H_2O w/w after preparation. Increasing the water content of an ionic liquid as shown in the previous chapter usually decreases the viscosity of the ionic liquid, however this would also have an impact on the solubility of both thymine and thymidine.

The ionic liquids [EMIM][tosylate] and [BMIM][$MDEGSO_4$] both display high solubility for thymine and thymidine, high viscosity and formation of a precipitate under reaction conditions. Therefore, these were tested for conversion at various water contents. Both ionic liquids are miscible with water and therefore formed a single phase when mixed with water. Ionic liquids were prepared as described in Section 2.2. Firstly the effect of added water content was examined on the solubility of thymine and thymidine in [EMIM][tosylate] as described in Section 2.3.2.1. As shown in Table 5.4 increasing the water content of the ionic liquid decreased the solubility of both thymine and thymidine, however, the solubility levels are still higher than achievable in the Tris HCl buffer. The optimum would therefore be the ionic liquid with a minimal water content in order to maintain a high solubility for thymine and thymidine, but high enough to sufficiently decrease the viscosity for conversion.

Table 5.4- Solubility of thymine and thymidine in a selection of hydrophilic ionic liquids as a function of added water content. Ionic liquids were prepared and synthesised as described in Section 2.2 and solubilities determined as in Section 2.3.2.1.

Ionic liquid and water content	**Solubility limit ($g.L^{-1}$)**	
	Thymine	**Thymidine**
[EMIM][TOSYLATE] (no added water)	> 10	> 20
[EMIM][TOSYLATE] (10 % H_2O w/w)	> 10	> 100
[EMIM][TOSYLATE] (20 % H_2O w/w)	< 10	< 100
[EMIM][TOSYLATE] (30 % H_2O w/w)	< 10	< 100
[EMIM][TOSYLATE] (40 % H_2O w/w)	< 10	< 100
[EMIM][TOSYLATE] (50 % H_2O w/w)	< 10	< 100
[BMIM][$MDEGSO_4$] (no added water)	< 9	> 20
[BMIM][$MDEGSO_4$] (10 % H_2O w/w)	< 8	> 20
[BMIM][$MDEGSO_4$] (20 % H_2O w/w)	< 7	> 20

Control experiments analysing the ionic liquid without substrates and products present showed [EMIM][tosylate] at water contents of 5 % H_2O w/w and 10 % H_2O w/w produced a wide peak at a retention time of 5.2 minutes, this unidentified compound in the ionic liquid with water present produces a peak which overlaps the thymidine peak and elutes at a retention time of 5.8 minutes in [EMIM][tosylate] without added water present. Consequently concentrations of thymidine could not be accurately determined in [EMIM][tosylate] with water present. The 2-deoxyribose-1-phosphate di(monocyclohexylammonium) salt substrate was undetected either before or after reaction. Examination of thymine concentrations, which were clearly visible as a single peak, are shown in Figure 5.3. Results show that without added water to the reaction in [EMIM][tosylate] (as described in Section 2.3.2) there is no change in substrate concentration, with a water content of 5 % H_2O w/w there is a slow decrease in substrate concentration, however, when the water content is increased to 10 % H_2O w/w the decrease in the thymine concentration is at a similar rate as in the Tris HCl buffer. Although the thymidine concentration cannot be determined accurately in this ionic liquid, the rate of decrease in substrate concentration and the formation of a precipitate suggest that the reaction is occurring within this ionic liquid.

Based on the method used for [EMIM][tosylate], [BMIM][$MDEGSO_4$] was subsequently investigated for the conversion of thymine with increasing water content. The solubility of thymine and thymidine in [BMIM][$MDEGSO_4$] with 10 % and 20 % H_2O w/w present was investigated and showed that at increased water levels the solubility is reduced as with [EMIM][tosylate] (Table 5.4). Control experiments in [BMIM][$MDEGSO_4$] with added water present were carried out and when analysed by HPLC (as described in Section 2.5.3), showed a peak produced at a retention time of 6.9 minutes from the ionic liquid, however, this does not interfere with substrate and product calibration times. Reaction with thymidine phosphorylase in [BMIM][$MDEGSO_4$] with 10 % H_2O w/w present (data not shown) displayed a peak at 6.4 minutes, which does not correspond to the retention times for thymidine or for the other products of the reaction under the same conditions. This indicates that this peak represents the formation of an alternative product. The conversion was also examined in [BMIM][$MDEGSO_4$] with 20 % H_2O w/w present, and again a peak from the ionic liquid at 6.9 minutes was observed without reagents present. Results suggest (data not shown) that both thymine and thymidine are unstable in this media

as indicated by HPLC analysis, which displayed two peaks produced for thymine and two peaks for thymidine when dissolved in this ionic liquid.

Overall these results indicate that the effect of added water content on the conversion in [BMIM][$MDEGSO_4$] not only had a negative effect on solubility, but also on the stability of the thymine and thymidine in this ionic liquid. The conversion of thymine to thymidine in [EMIM][tosylate] with added water present is, however, indicated to occur. Although the thymidine concentration could not be quantified, reaction is indicated by the change in thymine concentration and by the formation of a precipitate with calcium nitrate. The increase in the rate of reaction with increasing water content is attributed to the change in viscosity of [EMIM][tosylate] with water content.

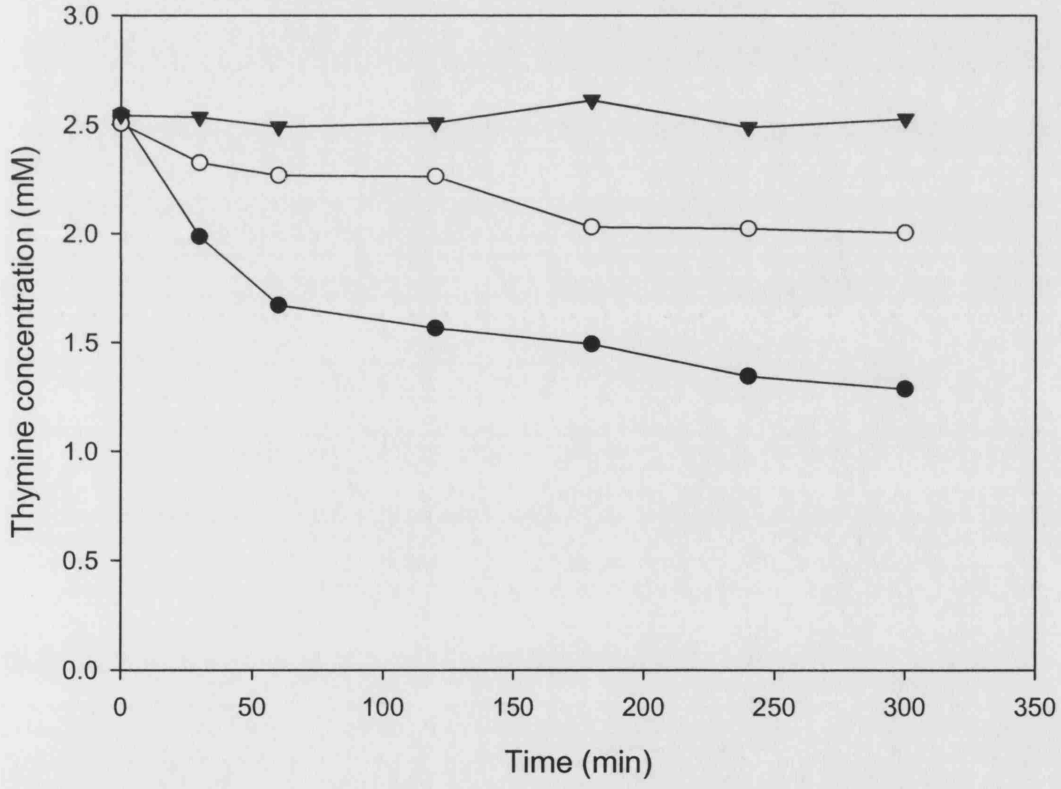


Figure 5.3- Kinetics of the thymidine phosphorylase catalysed synthesis of thymidine in [EMIM][tosylate] as a function of water content: (▼) thymine concentration in [EMIM][tosylate] (no added water), (○) thymine concentration in [EMIM][tosylate] (5 % H_2O w/w), (●) thymine concentration in [EMIM][tosylate] (10 % H_2O w/w). Reaction conditions as described in Section 2.3.2: [thymidine phosphorylase] = 12 units.mL^{-1}, initial [thymine] = 2.5mM (0.30 g.L^{-1}), [calcium nitrate] = 10mM added at the start of the reaction, temp = 30 °C.

5.4.3 Effect of Substrate Concentration on Thymidine Synthesis in [EMIM][tosylate]

The conversion of thymine in [EMIM][tosylate] (10 % H_2O w/w) is shown to occur, although the thymidine concentration cannot be quantified due to the overlapping peak from the ionic liquid which has the same retention time (as shown by control experiments in which only the ionic liquid phase was analysed by HPLC). This ionic liquid shows a progressive decrease in thymine concentration and formation of a precipitate upon the addition of calcium nitrate at equilibrium (as described in Section 5.4.2). The conversion of thymine by thymidine phosphorylase was therefore performed in [EMIM][tosylate] (10 % H_2O w/w) at a high substrate concentration (30 mM/ 3.86 $g.L^{-1}$) as described in Section 2.3.2. As shown in Figure 5.4 results displayed the same trend of a 50 % decrease in substrate concentration and the rate of reaction was shown to increase ten-fold at higher substrate concentrations. Thus far experiments have suggested that the conversion of thymine is indicated in [EMIM][tosylate] (10 % H_2O w/w), and that this ionic liquid has the ability to dissolve higher concentrations of thymine and thymidine than attainable in aqueous media. The next step in evaluating the use of this ionic liquid for the conversion of thymine is an investigation of product recovery routes, in order to isolate and quantify the thymidine produced.

5.5 Evaluation of Product Recovery Routes for the Conversion of Thymine by Thymidine Phosphorylase in Ionic Liquids

5.5.1 Liquid-liquid extraction experiments

Initial experiments using [EMIM][tosylate] aimed to establish if any of the solvents examined were firstly, immiscible with [EMIM][tosylate], secondly, whether thymine and thymidine could be extracted from the [EMIM][tosylate] into the solvent, and finally whether the substrate or product could be recovered as a precipitate in any of the solvents. The solvents examined were a range of 21 polar and non-polar solvents commonly used within the pharmaceutical industry as described in Section 2.4.2.1. As [EMIM][tosylate] is miscible with water, aqueous solvents were ruled out of the

solvent screening. Table 5.5 shows the results of the solvent screening. No precipitate formation was observed in any of the solvents screened over 24 hours. Of the 21 solvents screened only 10 were immiscible with [EMIM][tosylate] (10 % H_2O w/w), 3 of the 13 polar organic solvents were immiscible, 7 of the 8 non-polar organic solvents were immiscible. However all of the immiscible solvents tested showed no extraction of thymine or thymidine from the ionic liquid upon analysis by HPLC as described in Section 2.5.3.

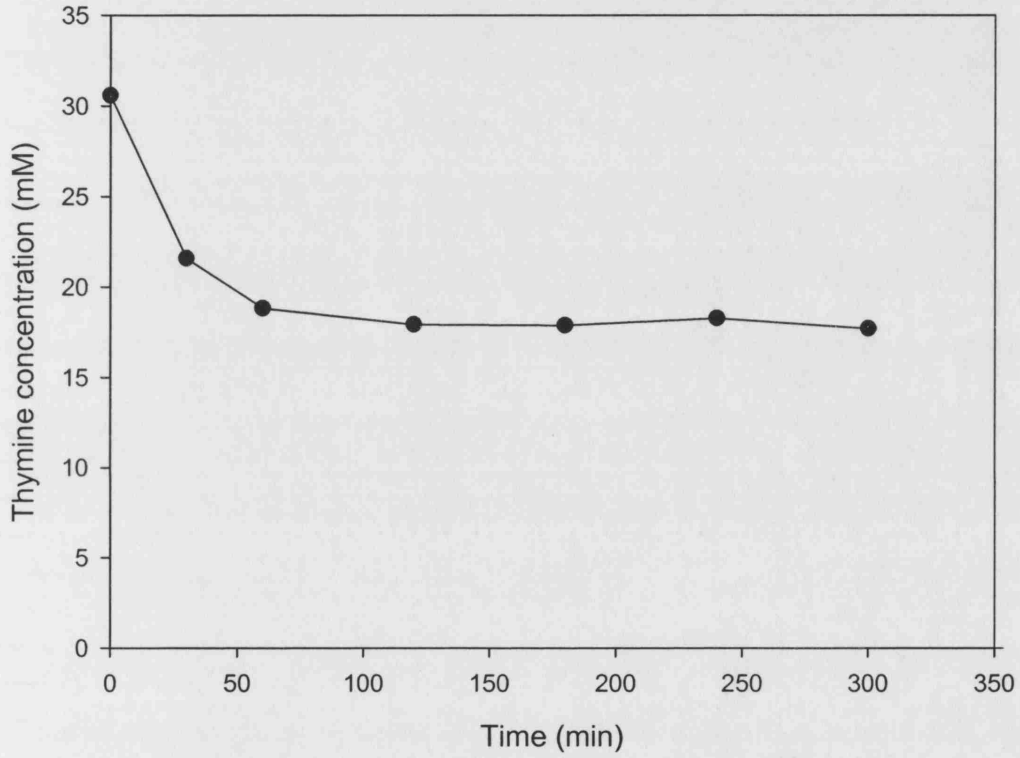


Figure 5.4- Kinetics of the thymidine phosphorylase catalysed synthesis of thymidine in [EMIM][tosylate] (10 % H_2O w/w): Reaction conditions as described in Section 2.3.2: Solvent = [EMIM][tosylate] (10 % H_2O w/w), [thymidine phosphorylase] = 12 units.mL^{-1}, initial [thymine] = 30mM (3.86 $g.L^{-1}$), [calcium nitrate] = 10mM added at the start of the reaction, temp = 30 °C

Table 5.5- Screening of a range of polar and non-polar solvents to examine the extraction of thymine and thymidine from [EMIM][tosylate] (10 % H_2O w/w). The results highlighted in grey show where the solvent phase was miscible with the ionic liquid (N/A means not applicable). Experimental conditions as described in Section 2.4.2.1: solvent = [EMIM][tosylate] (10 % H_2O w/w), [Thymine] = 5mM (0.63 $g.L^{-1}$), [Thymidine] = 5mM (1.21 $g.L^{-1}$).

Extraction Solvent		Immiscible with [EMIM][tosylate]	Thymidine extraction	Thymine extraction	Precipitate formed within 24 hours
Polar	Dichloromethane	No	N/A	N/A	No
	Ethyl acetate	Yes	N/A	N/A	No
	Methanol	No	N/A	N/A	No
	Ethanol	No	N/A	N/A	No
	Isopropyl alcohol	No	N/A	N/A	No
	Pyridine	No	N/A	N/A	No
	Ether (diethylether)	Yes	No	No	No
	Isopropyl ether	Yes	No	No	No
	Phenol	No	N/A	N/A	No
	Aniline	No	N/A	N/A	No
	Acetonitrile	No	N/A	N/A	No
	Dimethylformamide	No	N/A	N/A	No
	Dimethyl acetamide	No	N/A	N/A	No
Non-polar	Chloroform	No	N/A	N/A	No
	Pentane	Yes	No	No	No
	Hexane	Yes	No	No	No
	Heptane	Yes	No	No	No
	Toluene	Yes	No	No	No
	Cyclohexane	Yes	No	No	No
	Xylene	Yes	No	No	No
	Trichloroethylene	Yes	No	No	No

5.5.2 Adsorbent Screening

In this work six different commercially available adsorbents were characterised in order to determine the most suitable adsorbent to be applied following conversion of thymine, as described in Section 2.4.2.2. The specific adsorbents chosen are listed in Table 5.6 with additional details of their relevant chemical and physical characteristics. As with reaction samples the unidentified compound coming from the ionic liquid made thymidine quantification in [EMIM][tosylate] (10 % H_2O w/w) a problem, however, the thymine concentrations and adsorption could be quantified. The adsorption of thymine from [EMIM][tosylate] (10 % H_2O w/w) was screened in

addition to the thymine and thymidine adsorption from the Tris HCl buffer. As shown in Figure 5.5 the results show that the cation exchange resin IRA-458 had low affinity for thymine and thymidine in the Tris HCl buffer. The binding of thymine in [EMIM][tosylate] (10 % H_2O w/w) was also low. Results for the cation exchange resin 50W-X8 are similar to those of IRA-458 in the Tris HCl buffer. The results for the anion exchange resin 1-X8 (Figure 5.5) indicate this adsorbent has very high affinity for both the substrate and product with 100 % of the thymine and 99.5 % of the thymidine adsorbed from the Tris HCl buffer following batch adsorption with the resin. In the ionic liquid the resin showed much lower adsorption with only 20 % of the thymine removed from the [EMIM][tosylate] (10 % H_2O w/w). Overall screening of ion exchange resins showed that anion exchange resins produce the highest extraction of thymine and thymidine from both the Tris HCl buffer and the ionic liquid.

Table 5.6- Chemical and physical properties of the commercially available adsorbents investigated in this work. (N/A means not available). Data complied from manufacturers data sheets.

Adsorbent	Chemical nature	Mesh size	Total ion exchange capacity	Surface Area
Biorad-AG®1-X8	synthetic anion exchange polymeric resins	100-200	3.2 m_{eq}/dry g 1.2 m_{eq}/ml resin bed	N/A
Biorad-AG®50W-X8	synthetic cation exchange polymeric resins	20-50	5.1 m_{eq}/dry g 1.7 m_{eq}/ml resin bed	N/A
Amberlite IRA-458	synthetic cation exchange polymeric resins	N/A	N/A	N/A
Activated charcoal	carbonaceous	20-60	N/A	N/A
Amberlite XAD7	hydrophobic acrylic ester	20-60	N/A	450 m^2/g
Amberlite XAD16	hydrophobic polyaromatic	20-60	N/A	800 m^2/g

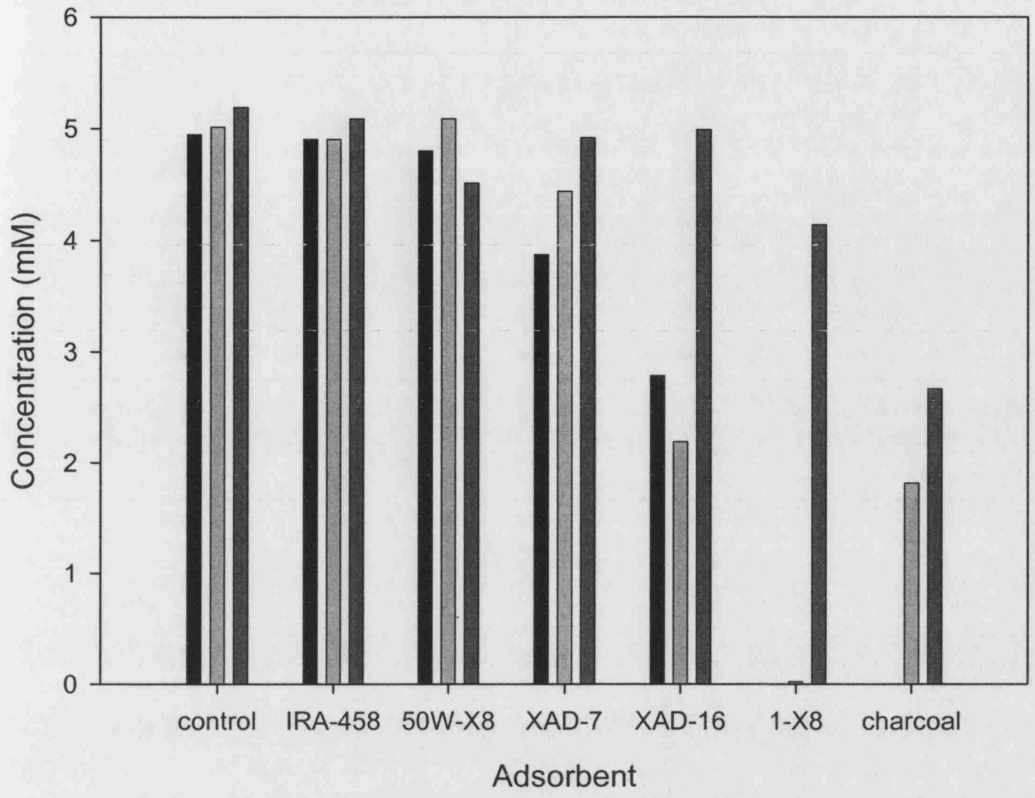


Figure 5.5- Screening of a range of commercially available adsorbents to investigate their ability to remove both thymine and thymidine from 10mM Tris HCl buffer and thymine from [EMIM][tosylate] (10 % H_2O w/w); (■) thymine concentration in 10mM Tris HCl buffer, (■) thymidine concentration in 10mM Tris HCl buffer, (■) thymine concentration in [EMIM][tosylate] (10 % H_2O w/w). Batch adsorption conditions as described in Section 2.4.2.2: Initial [thymine] = 5mM (0.63 $g.L^{-1}$), initial [thymidine] = 5mM (1.21 $g.L^{-1}$).

The results for hydrophobic resins indicate XAD-7 provides limited adsorption of thymine and thymidine from the Tris HCl buffer, however, there was decreased removal from the [EMIM][tosylate] (10 % H_2O w/w) with only 5 % of the thymine adsorbed onto the resin. The results for XAD-16 showed higher adsorption from the Tris HCl buffer with ~ 50 % of the thymine and thymidine adsorbed. In the ionic liquid the XAD-16 resin showed much lower adsorption with only 4 % of the thymine bound to the resin from the [EMIM][tosylate] (10 % H_2O w/w), indicating the ionic liquid is interfering with adsorption. Activated charcoal again shows high selectivity for the thymine from the Tris HCl buffer with 100 % adsorbed following mixing with the activated charcoal; selectivity for the thymidine is lower with only 76 % adsorbed from the Tris HCl buffer. The activated charcoal when mixed with the

[EMIM][tosylate] (10 % H_2O w/w) shows the highest removal of thymine from the ionic liquid with 49 % extracted from the [EMIM][tosylate] (10 % H_2O w/w).

Overall the adsorbents which show the highest removal from both the Tris HCl buffer and the [EMIM][tosylate] (10 % H_2O w/w) were the 1-X8 resin and activated charcoal, the adsorption kinetics of these adsorbents were then investigated further in order to try and isolate thymidine from the [EMIM][tosylate] (10 % H_2O w/w). Both these adsorbents show high adsorption for the substrate (thymine) with some adsorption of the product (thymidine). The options for recovery of thymidine in a purified form are;

(i) To run the bioconversion to 100 % completion (i.e. no thymine present), but conversion over 50 % as discussed earlier in Section 5.3 poses a problem.

(ii) To examine selective elution and binding kinetics of thymidine

5.5.3 Adsorption kinetics

From the adsorbent screen as described in Section 2.4.2.2, the anion exchange resin 1-X8 and hydrophobic activated charcoal were identified as having the potential to remove both thymine for recycling and thymidine from [EMIM][tosylate] (10 % H_2O w/w). In order to determine the adsorption kinetics for these adsorbents the change in thymine and thymidine concentration with time was examined from the Tris HCl buffer, and thymine adsorption kinetics were examined from [EMIM][tosylate] (10 % H_2O w/w). The results (Figure 5.6) show that thymine and thymidine bind to the 1-XA resin quickly and almost at the same rate in the Tris HCl buffer. In contrast, the binding of thymine in the [EMIM][tosylate] (10 % H_2O w/w) is ten-fold slower, presumably due to viscosity differences between the two media and mass transfer limitations to the surface of the resin.

The binding kinetics for activated charcoal are shown in Figure 5.7, and again show that in the Tris HCl buffer the thymine and thymidine bind at the same rate. Results show fast binding with a large initial drop in both concentrations followed by a small increase, indicating desorption. This trend was also observed with thymine binding in

[EMIM][tosylate] (10 % H_2O w/w), but the rate of binding was two-fold slower in the ionic liquid. These results show that the binding of thymine in the ionic liquid media is similar to that observed in the Tris HCl buffer, however, the rate of binding is slower in the ionic liquid attributed to the viscosity difference between the two media. Given that thymine and thymidine bind with the same kinetics to both resins it can be assumed that the same is true when the resins are used with ionic liquids.

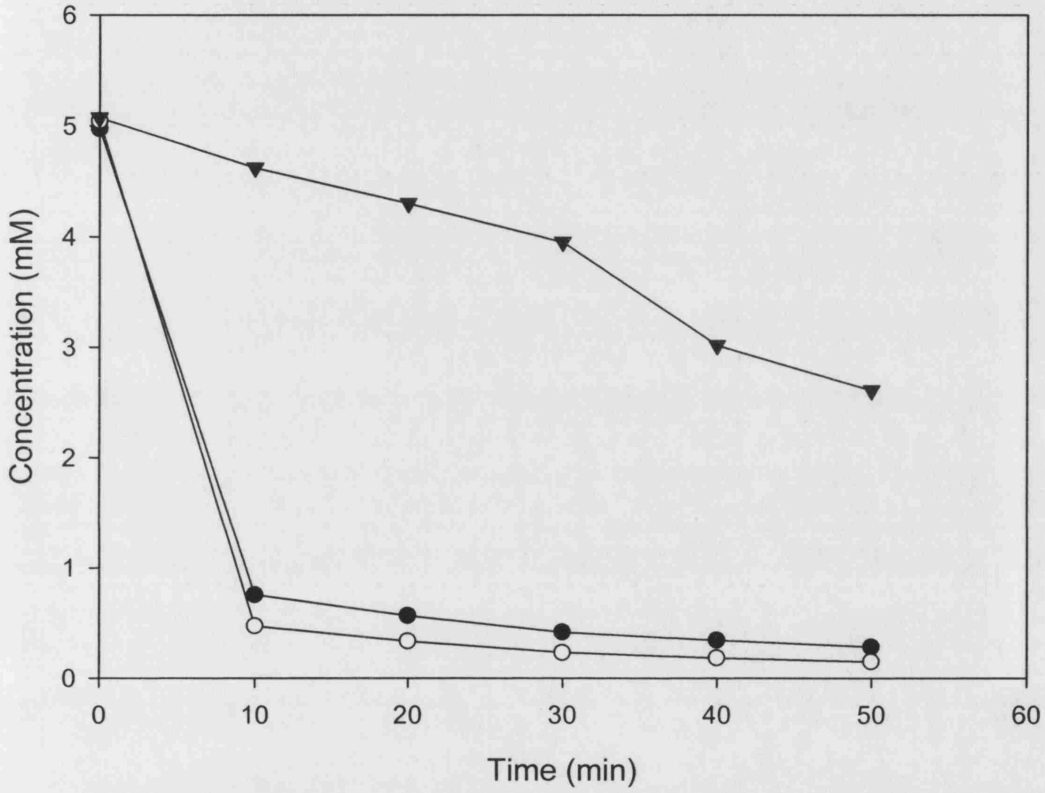


Figure 5.6- Adsorption kinetics of thymine and thymidine with the anion exchange resin 1-X8 in different media; (●) thymine concentration in 10mM Tris HCl buffer, (○) thymidine concentration in 10mM Tris HCl buffer, (▼) thymine concentration in [EMIM][tosylate] (10 % H_2O w/w). Batch adsorption conditions as described in Section 2.4.2.3: initial [thymine] = 5mM (0.63 $g.L^{-1}$), initial [thymidine] = 5mM (1.21 $g.L^{-1}$).

5.5.4 Product Recovery Following Conversion of Thymine by Thymidine Phosphorylase

In the previous section a means of product removal from ionic liquid was established. In order to isolate the thymidine produced from the conversion of thymine in

[EMIM][tosylate] (10 % H_2O w/w) the product would need to be eluted from either the 1-X8 resin or from the activated charcoal. Following conversion of thymine in [EMIM][tosylate] (10 % H_2O w/w) with thymidine phosphorylase using the conditions described in Section 2.3.2, and the removal of the calcium phosphate precipitate by filtration, the reaction mixture was then mixed with either the 1-X8 resin or activated charcoal, filtered and the thymine and thymidine eluted from the 1-X8 resin with 50 mM sodium chloride as described in Section 2.4.2.4, or from the activated charcoal with ethanol as described in Section 2.4.2.4 (The use of sodium chloride and ethanol as eluents was determined from literature). In a concurrent experiment the same investigation of both adsorbents was examined in the Tris HCl buffer (pH 7.4).

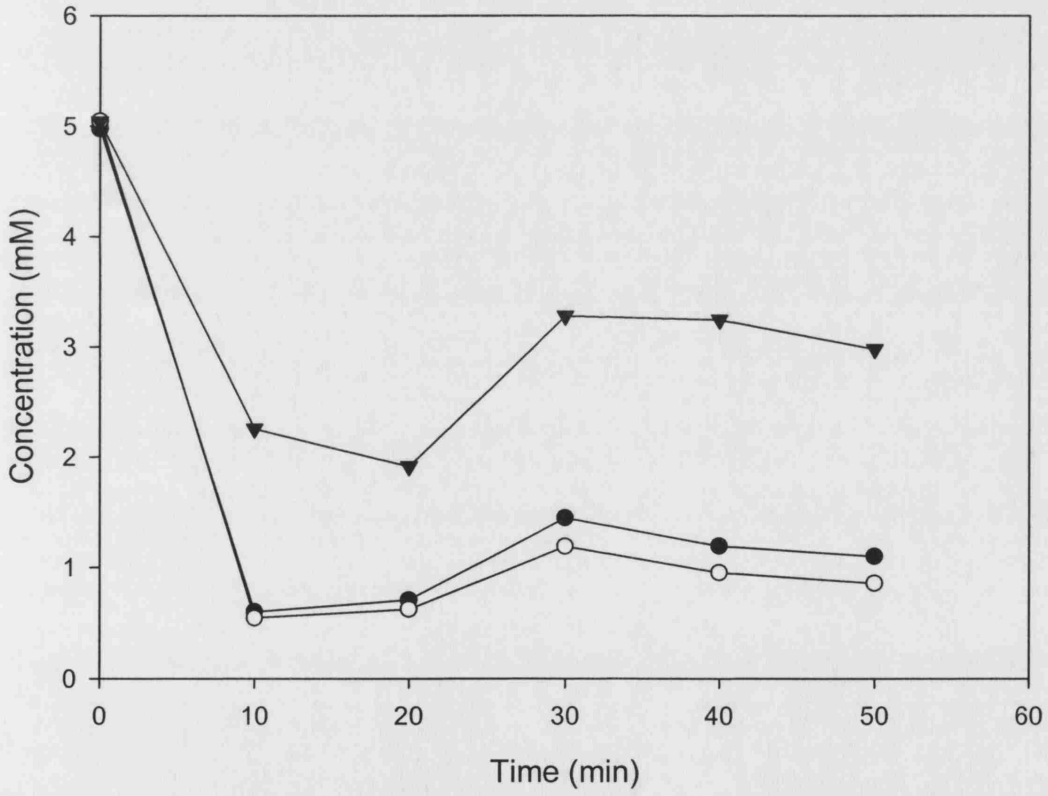


Figure 5.7- Adsorption kinetics of thymine and thymidine with activated charcoal in different media; (●) thymine concentration in 10mM Tris HCl buffer, (○) thymidine concentration in 10mM Tris HCl buffer, (▼) thymine concentration in [EMIM][tosylate] (10 % H_2O w/w). Batch adsorption conditions as described in Section 2.4.2.3: Initial [thymine] = 5mM (0.63 $g.L^{-1}$), initial [thymidine] = 5mM (1.21 $g.L^{-1}$).

As shown in Table 5.7 following conversion in the Tris HCl buffer with 0.32 g (2.5mM) thymine present, 0.30 g (1.25mM) thymidine was produced with 0.16 g (1.25mM) un-reacted thymine remaining. When this reaction mixture was mixed with the 1-XA resin 99.96 % (0.16 g) of the thymine and 99.98 % (0.30 g) of the thymidine bound to the resin in the Tris HCl buffer. In the [EMIM][tosylate] (10 % H_2O w/w), 0.16 g (1.25mM) thymine also remained following reaction and subsequent binding to the 1-XA resin removed 60 % (0.095 g) of the thymine from the ionic liquid. The thymidine again could not be quantified due to the co-eluting ionic liquid peak (as described in Section 5.4.2), however, it is assumed that a similar amount of thymidine bound to the resin (as described in Section 5.5.3). Extraction from the resin was then carried out with 50 mM sodium chloride as described in Section 2.4.2.4. Following binding to the 1-XA resin in the Tris HCl buffer, of the 99.96 % of the thymine that bound to the resin, a total of 55 % (0.086 g) was released from the resin into the sodium chloride within four washes. A total of 52 % (0.157 g) of the thymidine bound to the resin was recovered with sodium chloride within four washes. Following binding to the 1-XA resin in [EMIM][tosylate] (10 % H_2O w/w) (Table 5.7), a total 72 % (0.068 g) of the thymine, which bound to the resin, was recovered with sodium chloride within four washes. Comparing yields of thymine recovered from the 1-XA resin showed that although much higher quantities bound to the resin in the Tris HCl reaction system around 100 % (0.16 g) only 55 % (0.086 g) was recovered following extraction from the resin. In the ionic liquid, binding to the resin was a lot slower and only 60 % (0.095 g) of the thymine present bound to the resin, however, 72 % (0.068 g) was recovered, indicating that although binding is hindered with the use of ionic liquids elution from the 1-XA resin is faster. The first elution from the resin showed the thymine peak clearly, but the thymidine peak was not detectable when the sodium chloride was analysed due to the co-eluting ionic liquid peak (as described in Section 5.4.2). This indicates that either small amounts of the ionic liquid were present still coating the resin when it was washed with sodium chloride which then dissolved in the sodium chloride or that the ionic liquid is binding to the resin. Subsequent elutions, however, did show the presence of thymidine in the sodium chloride showing thymidine was formed in the ionic liquid. Contamination of the product with the ionic liquid is a key issue for the implementation of ionic liquids into industrial processes, therefore this product recovery step would require further investigation in order to determine if the resin could be dried before elution of the product or an

additional wash step could be added to remove the residual ionic liquid around the resin without extraction of the product.

Table 5.7- Batch elution of thymine and thymidine from Biorad 1-X8 resin with 50mM sodium chloride following the thymidine phosphorylase catalysed bioconversion of thymine and then subsequent binding to the 1-X8 resin in both 10mM Tris HCl buffer and [EMIM][tosylate] (10 % H_2O w/w). Bioconversion conditions as described in Section 2.3.2. Adsorption and elution conditions as determined in Section 2.4.2.4. (N/A means not applicable).

	Concentrations within 10mM Tris HCl buffer		**Concentrations within [EMIM][tosylate] (10 % H_2O w/w)**	
	Thymine (g)	Thymidine (g)	Thymine (g)	Thymidine (g)
Concentration measured before bioconversion	0.32	0	0.32	N/A
Concentration measured after bioconversion	0.16	0.30	0.16	N/A
Concentration measured following adsorption to 1-X8 resin	0.0006	0.0005	0.06	N/A
	Concentration within sodium chloride eluent			
First elution	0.057	0.104	0.058	N/A
Second elution	0.020	0.036	0.006	0.012
Third elution	0.006	0.012	0.003	0.005
Fourth elution	0.003	0.005	0.001	0.002
Total measured	0.086	0.157	0.068	0.019
Total still bound	0.072	0.145	0.026	N/A

As shown in Table 5.8 following conversion in the Tris HCl buffer with 0.32 g (2.5mM) thymine present and thymidine phosphorylase, 0.30 g (1.25mM) thymidine was produced with 0.16 g (1.25mM) of un-reacted thymine present. When this reaction mixture was mixed with the activated charcoal 99.6 % (0.16 g) of the thymine and 99.84 % (0.30 g) of the thymidine bound from the Tris HCl buffer. In the [EMIM][tosylate] (10 % H_2O w/w), 0.16 g (1.25mM) thymine was present following reaction and subsequent binding to the activated charcoal removed 59 % (0.092 g) of the thymine from the ionic liquid. Elution from the activated charcoal was then carried out with ethanol as described in Section 2.4.2.4. Results following binding to activated charcoal in the Tris HCl buffer, show that 99.6 % (0.16 g) of the thymine bound to the activated charcoal, and a total of 62 % (0.097 g) was released into the

ethanol within four washes. A total of 0 % of the thymidine bound to the resin was recovered with ethanol within four washes, indicating that the thymidine is tightly bound to the activated charcoal, requiring another solvent for elution. Following binding to the activated charcoal in [EMIM][tosylate] (10 % H_2O w/w) (Table 5.8), a total of 59 % (0.054 g) of the thymine, which bound to the activated charcoal, was recovered with ethanol. Comparing yields of thymine recovered although much higher quantities bound to the activated charcoal in the Tris HCl reaction system around 100 % (0.16 g) only 62 % (0.097 g) was recovered. In the ionic liquid binding to the activated charcoal was a lot slower and only 59 % (0.092 g) of the thymine present bound to the resin and the percentage recovered was similar with 59 % (0.054 g) recovered in the ethanol wash. Results indicate the activated charcoal behaved in a similar way with both media but rates were a lot slower in the ionic liquid attributed to the viscosity difference between the two systems.

Table 5.8- Batch elution of thymine and thymidine from activated charcoal with ethanol following the thymidine phosphorylase catalysed bioconversion of thymine and then subsequent binding to the activated charcoal in both 10mM Tris HCl buffer and [EMIM][tosylate] (10 % H_2O w/w). Bioconversion conditions as described in Section 2.3.2. Adsorption and elution conditions as determined in Section 2.4.2.4. (N/A means not applicable).

	Concentrations within 10mM Tris HCl buffer		**Concentrations within [EMIM][tosylate] (10 % H_2O w/w)**	
	Thymine (g)	Thymidine (g)	Thymine (g)	Thymidine (g)
Concentration measured before bioconversion	0.32	0	0.32	N/A
Concentration measured after bioconversion	0.16	0.30	0.16	N/A
Concentration measured following adsorption to activated charcoal	0.0006	0.0005	0.06	N/A
	Concentration within ethanol eluent			
First elution	0.035	0	0.025	N/A
Second elution	0.028	0	0.014	N/A
Third elution	0.019	0	0.010	N/A
Fourth elution	0.015	0	0.005	N/A
Total eluted	0.097	0	0.054	N/A
Total still bound	0.061	0.30	0.037	N/A

The fact that no thymidine was eluted from the activated charcoal in both reaction media indicates that the thymidine is tightly bound to the activated charcoal. Overall results suggest activated charcoal is not a suitable method for thymidine recovery from [EMIM][tosylate] (10 % H_2O w/w). The results for thymidine recovery with 1-XA resin show much more positive results and with further investigation could provide a method of product recovery following conversion in [EMIM][tosylate] (10 % H_2O w/w).

5.6 Discussion

The conditions for the thymidine phosphorylase catalysed synthesis of thymidine (Figure 1.4), requires the use of an aqueous 10mM Tris HCl buffer (pH 7.4), but this conversion is limited by the low solubility of the reagents. The ability of ionic liquids to potentially dissolve a vast array of pharmaceutical intermediates and final drug substances at high concentrations, means that they may provide alternative reaction media that could increase reaction rates or yields when compared to conventional aqueous and organic solvents. The aim of the research in this chapter was to characterise the phosphorylase reaction in aqueous media (Figure 5.1), and to determine the advantages that operation in ionic liquid media might offer. The work showed that firstly the conversion occurs in ionic liquids, and that operation in ionic liquid potentially offers several advantages over the aqueous solvent system, including increased solubility of the reagents.

5.6.1 Reaction of Thymine to Thymidine in Ionic Liquids

Initial screening of six different ionic liquids (Table 5.1) yielded one [BMIM][PF_6] that showed conversion of thymine by thymidine phosphorylase (Figure 5.2), however this ionic liquid had poor solubility for thymine and thymidine (Table 5.2). In order to avoid the problem of high viscosity the water content was varied for two ionic liquids that showed high viscosity, high solubility and formation of a precipitate when calcium nitrate is present in the reaction media. Increasing the water content was found to have a significant effect on reducing the viscosity of the ionic liquid. Analysis of the hydrophilic ionic liquid [EMIM][tosylate] with water present

however, resulted in a co-eluting ionic liquid peak (as described in Section 5.4.2), which had the same retention time as thymidine; therefore this could not be quantified. The thymine concentration could be quantified and indicated conversion due to the decrease in the thymine concentration by 50 % (Figure 5.3) and by the formation of a precipitate with calcium nitrate. The increase in the rate of reaction with increasing water content is attributed to the change in viscosity of [EMIM][tosylate] with water content. The rate of reaction was also shown to increase at higher substrate concentrations (Figure 5.4). Further investigation is required to obtain higher conversions of ~90 % which have been determined (EP 1179598A1, 2002) as discussed in Section 1.4.

5.6.2 Product Recovery Approached for Thymidine in Ionic Liquids

Liquid-liquid extraction results yielded no solvents, which would extract either thymine or thymidine from the ionic liquid [EMIM][tosylate] (10 % H_2O w/w) or the formation of a precipitate (Table 5.5). This lead to the next most viable route for product extraction of adsorbent screening being investigated as discussed in Section 5.5.2. The adsorbent screen identified two types of adsorbent the anion exchange resin 1-X8 and activated charcoal, which could recover both the substrate and product from the reaction media (Figure 5.5). The kinetics of the thymine and thymidine adsorption with these adsorbents was subsequently investigated and showed that the binding of thymine and thymidine in the ionic liquid was similar to that in the aqueous buffer but the rate of adsorption was much lower, attributed to the viscosity differences between the two media (Figure 5.6 and 5.7) Finally the elution of the product and substrate from both the adsorbents was investigated (Table 5.7 and 5.8). These results showed that the anion exchange resin 1-XA with further investigation could provide a method of product recovery following conversion of thymine to thymidine in [EMIM][tosylate] (10 % H_2O w/w).

In conclusion it has been shown, for the first time, that enzyme catalysed phosphorylations can be performed in ionic liquids. The general design procedures outlined in Chapter 3 and 4 were shown to be applicable and led to the rapid establishment of initial reaction and product recovery conditions. In this particular case, however, the ionic liquids that demonstrated high substrate and product

solubility, were high viscosity ionic liquids (without water present) in which the initial rate of reaction was considerably reduced. The issue of viscosity was addressed by the addition of water, which reduces the viscosity although the solubility of the substrate and product is slightly reduced. After the addition of 10 % H_2O w/w the ionic liquid displayed the same final yields and initial rates of reaction as those achieved under the same conditions in the aqueous buffer. The issue of product recovery was also investigated and the initial results suggest adsorption is one of the more viable routes for product recovery in this case.

6.0 General Discussion

The original experimental work described in Chapters 3 to 5 of this thesis has been discussed in detail at the end of each chapter. Here the key findings of this research are highlighted together with the wider concerns related to the use of ionic liquids in industrial bioconversion processes.[∞]

6.1 Considerations related to this thesis

Biocatalysts are widely used in many forms within the pharmaceutical industry as discussed in Section 1.1. There are, however still several drawbacks to their current application, which frequently relate to the need to use organic solvents. Such reaction media have several disadvantages associated with their use as described in Section 1.1.1 (Table 1.2), most notably their toxicity to the environment, and the volatile and flammable nature of most organic solvents that make them a potential explosion hazard. The lipase catalysed resolution of SB-235349, as described in Section 1.3 is an example of an industrial bioconversion process which, due to the low aqueous solubility of the substrate is performed in the organic solvent t-butanol. This places many limitations upon the bioconversion, which are common among many industrial applications of biocatalysis. An alternative reaction medium to organic solvents has been suggested in this work in the form of ionic liquids. These offer many potential advantages for use in industrial bioconversions as discussed in Section 1.2 (Table 1.3).

In this research a generic approach to the application of ionic liquids as reaction media was developed and exemplified for the lipase catalysed conversion of SB-235349 (Figure 1.3). The lipase catalysed conversion of SB-235349 is a key chiral intermediate in the production of Lotrafiban, a drug which made it through to Phase III clinical trials as an anti-thrombiotic agent and was routinely produced at scales of

[∞] Part of the discussion presented in this chapter has been previously published as: Roberts, N.J. and Lye, G.J. (2002). Application of room temperature ionic liquids in biocatalysis: Opportunities and

up to 3000L (Section 1.3). The approach was taken to characterise the bioconversion on a small scale in a series of ionic liquids and compare these results with the optimised organic process (Figure 3.1, 3.2). The bioconversion in the ionic liquid was then optimised to maximise the bioconversion utilising the unique properties offered by the use of ionic liquids (Figure 3.7), namely the ability to dissolve high concentrations of the substrate and the ability to operate at elevated temperatures due to the stability of the biocatalyst in the ionic liquid phase.

Subsequent investigations, as described in Chapter 4, exemplified a generic method of product recovery from an ionic liquid, in order to determine the effects of ionic liquids on down stream processing. One of the reasons highlighted hindering the implementation of ionic liquids in the pharmaceutical industry is the feasibility of ionic liquid recycle due to the present high cost of ionic liquids (Freer et al., 2000). In this study (Chapter 4), the extraction of the SB-240101 product and un-reacted SB-240098 substrate is achieved and the ionic liquid recycled with no change in reaction rates and yields observed. This investigation demonstrates that the application of established techniques such as liquid-liquid extraction and adsorption can be applied with ionic liquids. Also, that in the instance of liquid-liquid extraction the standard approaches to design of an extraction process also work in the case of ionic liquids i.e. examining K as a function of pH and measuring of mass transfer coefficients. The two-film theory of mass transfer was shown to be applicable and that mass transfer was controlled by molecular diffusion from the ionic liquid, the mass transfer rates achieved however, are comparable to those using conventional organic solvents (Lye et al., 2001). This work represents the first instances of an entire bioconversion process involving reaction and downstream processing steps. Given that ionic liquids are considered as 'green' solvents, the use of the organic solvent IPA was selected as the substrate extraction route, as it provided effective recovery and was a suitable solvent for the re-racemisation of the un-reacted enantiomer. The use of an organic solvent does not negate the use of the ionic liquid due to the advantages (outlined in Chapter 3) offered by the use of ionic liquids.

challenges. In *Ionic Liquids: Industrial Applications for Green Chemistry* (ACS Symposium Series 818), Rogers, R.D. and Seddon, K.R. Eds., Pp 347-359. American Chemical Society, Washington, DC

The generic approach produced from investigation of the lipase catalysed conversion of SB-235349, was then applied to a very different type of reaction the thymidine phosphorylase-catalysed synthesis of thymidine from thymine (Figure 1.4). Like the lipase-catalysed conversion this is a bioconversion which is limited by the solubility of the substrates and products; low solubility of reactants limits the productivity and cost effectiveness of a bioconversion. Unlike the lipase-catalysed conversion, however, the reaction occurs in aqueous media and uses a free enzyme form of thymidine phosphorylase instead of the immobilised lipase. The thymidine phosphorylase catalysed synthesis of thymidine from thymine is an example of nucleoside chemistry, a variety of nucleosides and their analogues are used as a starting material for synthesis or drug formulation in production of an antiviral, anticancer or antisense agents (Section 1.4). The generic design procedures outlined in Chapters 3 and 4 were applied to this bioconversion. The synthesis of thymidine was examined in a series of ionic liquids and the results compared with those performed in aqueous media (Figure 5.1, 5.2 and 5.3). Within a limited range of ionic liquids, two were identified in which the enzyme-catalysed phosphorylation was shown to occur. The free enzyme thymidine phosphorylase was shown to be active in the ionic liquid. The ionic liquid [BMIM][PF_6] showed conversion of thymidine, however, rates and yields were lower than in aqueous media due the low solubility of the solutes in this media. The ionic liquid [EMIM][tosylate] produced no conversion as a pure solvent, however, with 10 % H_2O w/w present similar rates and yields compared to aqueous media were attainable in the ionic liquid. Product recovery with adsorption was possible from this ionic liquid but further investigation is required to improve this method. Overall the general approaches to design as illustrated for the Lotrafiban reaction were also applicable to the synthesis of thymidine.

Operation of both the conversions described in this work are limited by the solubility of the substrates and the products. One of main features for the implementation of ionic liquids is their ability to readily dissolve a wide range of pharmaceutical intermediates. To date, however, there has been limited data on the application of ionic liquids to industrial processes. The substrates and products of both the conversions studied here showed the advantage of much higher solubility of the substrate in the ionic liquid media. The solubility of the SB-235349 substrate showed an eight-fold increase in solubility compared to the organic solvent; at this high

substrate concentration there is a three-fold increase in the apparent initial rate of reaction (Figure 3.4). The solubility of thymine substrate showed a three-fold increase in solubility compared to the traditional aqueous solvent used for the conversion and the thymidine product showed a ten-fold increase in solubility (Table 5.2). Overall ionic liquids have excellent solvation properties for both hydrophilic and hydrophobic solutes.

Having identified solute solubility as a key factor when considering the application of ionic liquids, the next factor to be identified was the water content of the ionic liquid (particularly with hydrolase reactions). The water content of the ionic liquid has been shown to have a significant effect on other factors influencing the reaction such as the ionic liquid viscosity and the substrate/product solubility. The influence of water content on reaction rate was shown in Figure 3.5, and shows water plays an important role for a reaction in ionic liquids. When investigating the solubility of thymine and thymidine as a function of the added water content, the water miscible [EMIM][tosylate] showed that as the water content of the ionic liquid is increased the solubility of both thymine and thymidine decreases (Table 5.4). This again highlights how important water content is for application of ionic liquids to biocatalysis.

Therefore, it follows that the structure of the ionic liquid would be an important factor to consider when applying an ionic liquid to a bioconversion, as structure determines the properties of the ionic liquid, such as water miscibility (or immiscibly) and viscosity of the ionic liquid. In particular the anion of the ionic liquid has a significant effect on the water miscibility as described in Section 1.2, the ionic liquids [BMIM][PF_6] (water immiscible) and [BMIM][BF_4] (water miscible) although having the same cation display very different properties. They also show very different results when applied as reaction media, as shown in Figure 3.2, [BMIM][PF_6] displayed the correct product on analysis and [BMIM][BF_4] although showing conversion displayed a different product than that required.

One of the key claims of ionic liquids is that they offer significant advantages over conventional solvent systems. This has been shown in the case of the optimised lipase catalysed conversion of SB-235349, with a 28 % increase in yield compared to the optimised organic process (Figure 3.6). An additional factor, which was identified

during the optimisation of the lipase catalysed conversion in ionic liquids, was the potential to operate at elevated temperatures as shown in Table 3.3, compared to conventional organic solvents. The high solubility of solutes in ionic liquids offered the advantage of operation at a range of temperatures from room temperature to above boiling point.

The bioconversions studied here are very different examples and show how ionic liquids can be applied across a wide area of biocatalysis. This approach to the selection of an ionic liquid as reaction media demonstrates a series of experiments, which can be performed, to highlight the most likely ionic liquid and the benefits, which can be offered by their application to a given bioconversion.

6.2 Considerations of the wider use of ionic liquids for industrial biocatalysis

There are still a number of issues that need to be addressed if the potential of ionic liquids in bioconversion processes is to be fulfilled. These relate to the basic science involved (Table 7.1) together with a number of engineering and industrial concerns (Table 7.2).

One important concern for control and optimization of isolated enzymes in ionic liquids is an understanding of how they interact with the structure and function of enzymes. This also applies to the role of water within an ionic liquid. The term 'designer solvents' has been used to describe ionic liquids (Freemantle, 1998), but for this to become a reality a rational basis for these interactions is required, in order to avoid screening experiments for each new application investigated. Similar considerations apply to the influence of ionic liquids on the cell membranes of bacterial systems. For example a suitable toxicity scale would be required as with organic solvents, such as the LogP scale (Laane et al., 1987).

Product recovery is another key issue, which requires further investigation. A number of methods have been investigated to date, such as liquid-liquid extraction with water (Erbeldinger et al., 2000), or organic solvents (Lau et al., 2000) as well as solid phase

adsorption (Anthony et al., 2001). Other potential product recovery methods used include distillation (Schofer et al., 2001) and supercritical carbon dioxide (Blanchard et al., 2003, Reetz et al., 2002). Overall a greater understanding of the relationship between the structure of an ionic liquid and key physico-chemical properties, such as density, viscosity, heat capacity and thermal conductivity, would aid the rational design of biocatalytic reactors and product recovery operations.

Table 7.1- Basic science issues to address

Whole cell biocatalysis	Isolated enzyme biocatalysis
Generality of results to date	Generality of results to date
Membrane structure and function	Structure and dynamics
Role of water	Mechanism and kinetics
Definition of toxicity scale	"Memory effects"
Effect on intracellular enzymes	Role of water
Stability	Stability
	Reaction equilibria

Table 7.2- Engineering and industrial issues to address

Biochemical engineering	Industrial and regulatory
Mixing and phase separation	Cost and supply
Diffusion and mass transfer	Safety and toxicity
Product recovery	Long term stability
Ionic liquid selection criteria	Disposal
Design and optimisation	Regulatory approval
Cleaning and recycling	

A major industrial concern for the application of ionic liquids is the lack of precedent with their use. There is currently one industrial process employing ionic liquids for the manufacture of alkoxyphenylphosphines. The company BASF use N-methylimidazole to scavenge acid formed in the process. The reaction results in the production of N-methylimidazolium (Freemantle, 2003). Although the cost of ionic liquids is still comparatively high, to organic solvents, there are now an increasing number of suppliers who supply ionic liquid screening kits and offer production of large volumes > 100 L. The increased demand will ultimately bring down the cost of ionic liquids. There is still only limited data on the long term stability and toxicity of

many ionic liquids and issues such as recycling and disposal would also need to be addressed. Regulatory approval and validation of ionic liquid removal from the final product will be crucial for their application to the pharmaceutical sector.

7.0 Overall Conclusions and Future Work

7.1 Overall Conclusions

In this work the hydrolytic resolution of racemic 2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid methyl ester (SB-235349) to (2S)-2,3,4,5-tetrahydro-4-methyl-3-oxo-1H-1,4-benzodiazepine-2-acetic acid (SB-240101) by immobilised *Candida antarctica* lipase B, CALB (Novozyme 435), was characterised in an ionic liquid and compared to the optimised industrial process performed in an organic solvent. The overall aim was to identify the key factors involved in the operation of bioconversions in ionic liquids, including product recovery options, in order to establish a generic and rapid design procedure for the study of future conversions. To show the generic nature of the design procedure it was tested on a completely different industrial bioconversion, the thymidine phosphorylase catalysed synthesis of thymidine from thymine. The most important results from this investigation are summarised below:

- It has been shown that ionic liquids act as excellent solvents capable of dissolving high concentrations of substrate and/or product molecules. Substrate and/or product solubility is a key parameter for the application of an ionic liquid for a particular bioconversion, as in most industrial processes substrate/product solubility is a limiting factor. Therefore in order to justify the application of ionic liquids significant benefits such as high substrate/product solubility are required as demonstrated here.

- The structure of the ionic liquid has been shown to be critical to their application to bioconversions, as properties (such as substrate and product solubility) within an ionic liquid are determined by the structure. The structure of an ionic liquid also affects other key issues such as miscibility with water. Although the choice of ionic liquid for a particular bioconversion has been identified as important, in the examples studied here, and those in

the literature (Table 1.4, 1.5 and 1.6) only a small range of ionic liquids need to be screened in order to find one suitable for a particular bioconversion.

- Water content of the ionic liquid has been identified as an important factor as a minimum amount of water is necessary to maintain the enzyme activity, particularly with hydrolytic reactions. Therefore identification of the optimum water content for a particular reaction is often necessary for the bioconversion under investigation. The water content of an ionic liquid is also known to have a strong influence on viscosity and solubility of both the substrate and the product, whether the ionic liquid is water miscible or immiscible.

- Under certain conditions ionic liquids have been shown to act as better reaction media (compared to aqueous or organic phases), in terms of both rate and yield of the conversion. Operation in ionic liquids has also been shown to increase the long-term stability of the biocatalyst.

- The use of immobilised rather than free enzymes would appear to be an advantage in terms of ease of enzyme recovery and re-use. The type of enzyme used for the particular conversion in an ionic liquid also plays a role on the conditions of the conversion. Immobilisation of an enzyme offers additional advantages such as increased stability over a wide temperature and pH range.

- Product recovery has been shown to be possible using techniques widely used in industry such as liquid-liquid extraction and adsorption. Product recovery routes available for a particular conversion in an ionic liquid is dependent upon the type of ionic liquid used for the conversion. As described previously the structure of the ionic liquid is a key factor which influences the properties of the ionic liquid, and therefore the product recovery routes available down stream. In this work the water immiscible ionic liquid [BMIM][PF_6] used for the lipase catalysed conversion of SB-235349, is very suitable for liquid-liquid extraction (Section 4.2). The ionic liquid

[EMIM][tosylate] used for the thymidine phosphorylase catalysed synthesis of thymidine, is water miscible and unsuitable for liquid-liquid extraction, in this case adsorption was more suitable (Section 5.5).

7.2 Future work

Although the work described in this thesis has demonstrated a generic approach to the application of ionic liquids to industrial bioconversions, there remain a number of areas worthy of further investigation.

- In this work a limited number of ionic liquids were screened for the ability to act as alternative reaction media for the lipase catalysed conversion of SB-235349. The ionic liquid identified as the most suitable with high conversion rates was not used for subsequent optimisation experiments, due to the high price of this ionic liquid. Although if higher yields and rates are attainable in a more expensive ionic liquid these benefits could out weigh the high initial outlay for the ionic liquid. Therefore optimisation of the lipase catalysed conversion of SB-235349 in [BMIM][$N(SO_2CF_3)_2$] still needs to be investigated.

- Due to the high costs of enzymes their re-use following a bioconversion is a key issue, although re-use of Novozyme 435 following the lipase catalysed bioconversion of SB-235349 was demonstrated. To clearly show if the ionic liquid has a significant effect on the re-use of the enzyme, their re-use within recycled ionic liquid should be investigated as discussed in Section 3.4.7.

- Although in this work the product recovery of SB-240101 was investigated from the ionic liquid [BMIM][PF_6], the purity and amount were quantified only by HPLC. Purity of the product is a key issue, particularly when it comes to pharmaceutical production. Therefore in order to validate the product recovery route shown in Figure 4.2, the SB-240101 product should be isolated from the aqueous phase and quantified by mass spectrometry, to determine if the ionic liquid contaminates the product in any way.

- It therefore follows that contamination of the [BMIM][PF_6] ionic liquid with either the substrate (SB-235349), product (SB-240101) or extraction medium, is also an important issue and should also be confirmed. Therefore further investigation in order to determine the purity of the recycled ionic liquid should be analysed by NMR spectroscopy and compared to results with fresh ionic liquid.

- Although two ionic liquids were identified in which the conversion of thymine was indicated to occur, one of these was very viscous (Table 5.1), and the other displayed low solubility of the substrates and products. Therefore if further research were carried out, investigation of a wider range of ionic liquids with differing water contents would be recommended, because as demonstrated in this work, the ionic liquids which display the highest solubility for the substrate (thymine) and product (thymidine) have the highest viscosity, and addition of an aqueous phase reduces the viscosity significantly making the ionic liquid more suitable for reaction and analysis.

- When the thymidine phosphorylase catalysed conversion of thymine was investigated initial results of product recovery routes showed limited success with a range of adsorbents. Given more time to investigate this route and other possible routes of product recovery (Table 4.1), confirmation of product recovery and analysis by mass spectroscopy should be carried out. In general, product recovery routes that do not use organic solvents in order to move toward 'greener' overall processes are preferable.

- As discussed in Chapter 6 there are still many issues that need to be resolved before ionic liquids can be implemented into industrial processes. These issues, which require further investigation, include a better understanding of the structure and function of enzymes within ionic liquids and determination of techniques for the rapid identification of biocompatible ionic liquids. Finally before ionic liquids could be given regulatory approval key issues such as toxicity, recycling and disposal of ionic liquids need to be addressed.

8.0 References

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Zubay G.L. 1998. Biochemistry 4^{th} Ed. Wm. C. Brown Publishers, p629-665.

Appendix I: HPLC Analysis of the Bioconversion Substrate and Product

I.1 Example HPLC Chromatograms

Figure I.1 shows a typical HPLC chromatogram of the SB-235349 substrate and SB-240101 product of the Lotrafiban bioconversion in t-butanol. Figure I.2 shows SB-235349 and SB-240101 in [BMIM][PF_6].

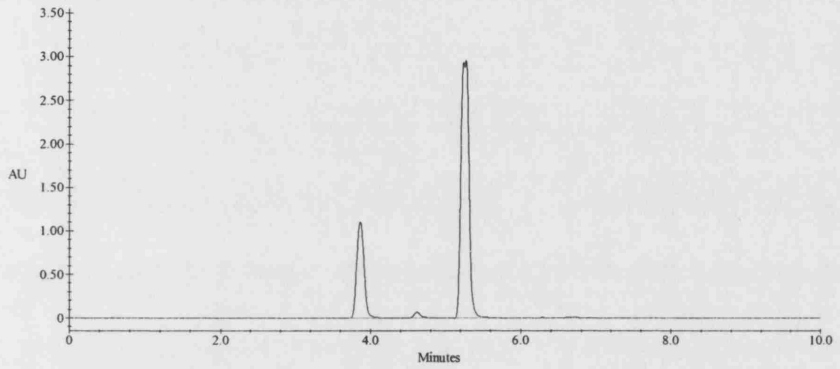


Figure I.1. HPLC chromatogram showing UV response against time. The sample from the t-butanol system was analysed as described in Section 2.5.1. The SB-235349 substrate has a retention time of 5.5 minutes and the SB-240101 products have a retention time of 3.9 minutes.

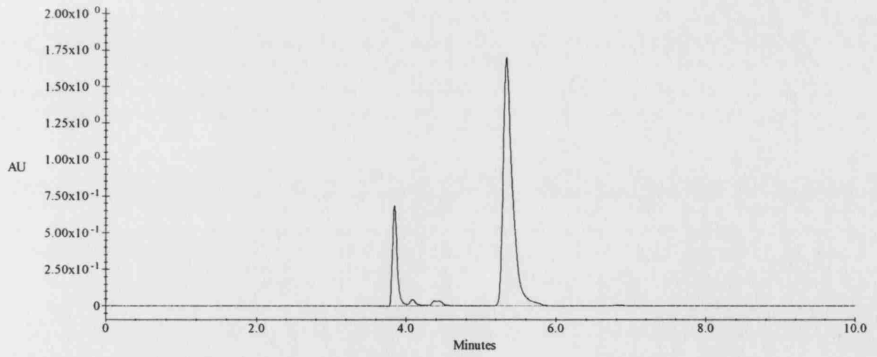


Figure I.2. HPLC chromatogram showing UV response against time. The sample from the [BMIM][PF_6] system was analysed as described in Section 2.5.1. The SB-235349 substrate has a retention time of 5.5 minutes and the SB-240101 products have a retention time of 3.9 minutes.

I.2 Calibration Curves

External calibration curves were used to quantify the SB-235349 substrate and SB-240101 product of the bioconversion. Figures I.3 and I.4 show typical standard curves of solute concentration against UV response (integrated peak area) for these compounds.

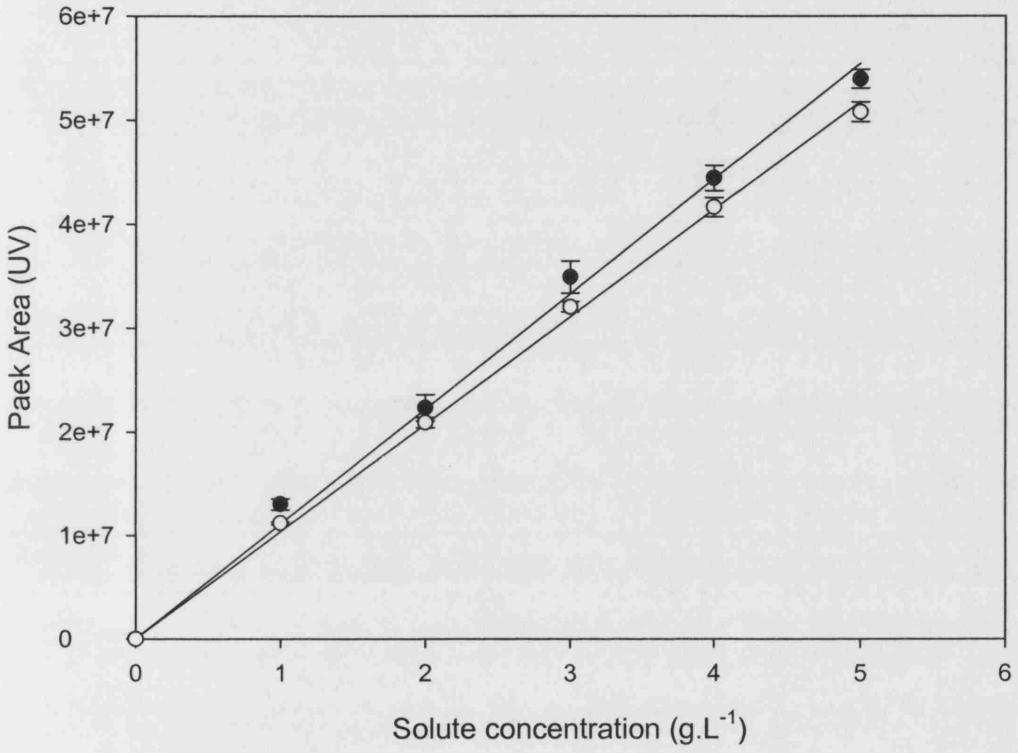


Figure I.3. Typical calibration curve for SB-235349 (●) and SB-240101 (○) in t-butanol. Samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

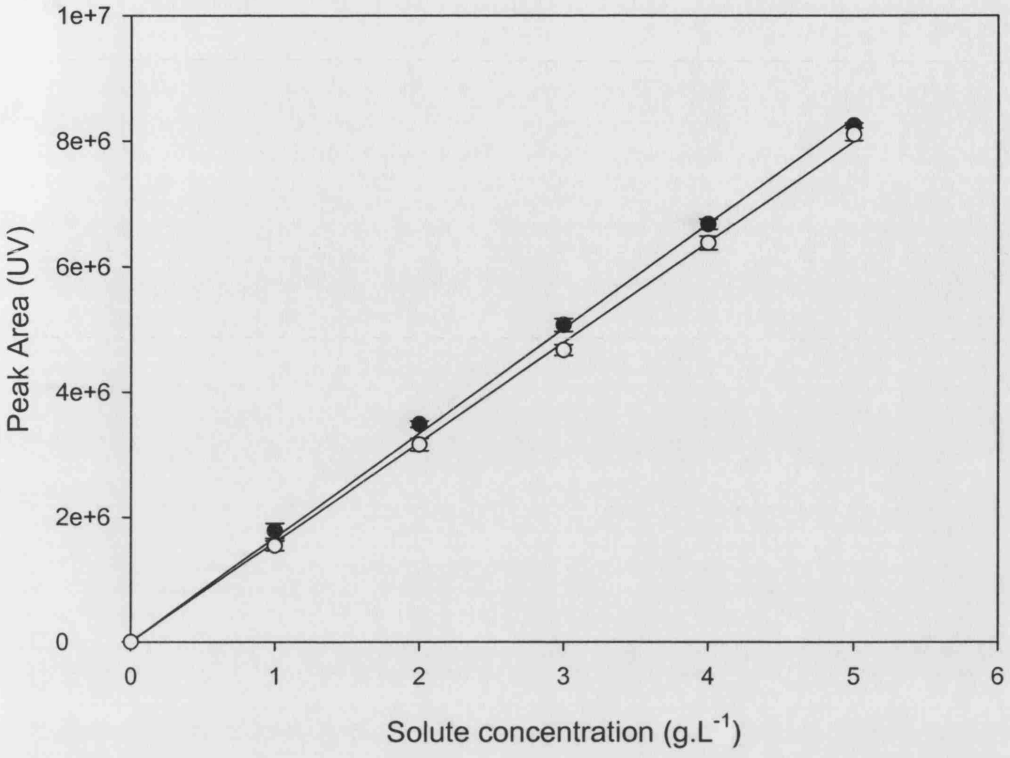


Figure I.4. Typical calibration curve for SB-235349 (●) and SB-240101 (○) in [BMIM][PF_6]. Samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

Appendix II: Determination of Equilibrium Coefficients for SB-235349 and SB-240101.

II.1 Aqueous Phosphate Buffer (pH 10) Partition Coefficient Determination

In order to quantify the amounts of SB-235349 and SB-240101 in the [BMIM][PF_6] samples, which had been extracted with aqueous buffer (pH 10), it was necessary to determine the equilibrium aqueous buffer/ionic liquid partition coefficient for these compounds. This was carried out as described in Section 2.5.1.1. Figure II.1 shows the amounts of SB-235349 and SB-240101 present in the aqueous buffer phase at equilibrium after contact with ionic liquid solutions. The partition coefficient for the SB-240101 was calculated to be 16.7 ± 0.1.

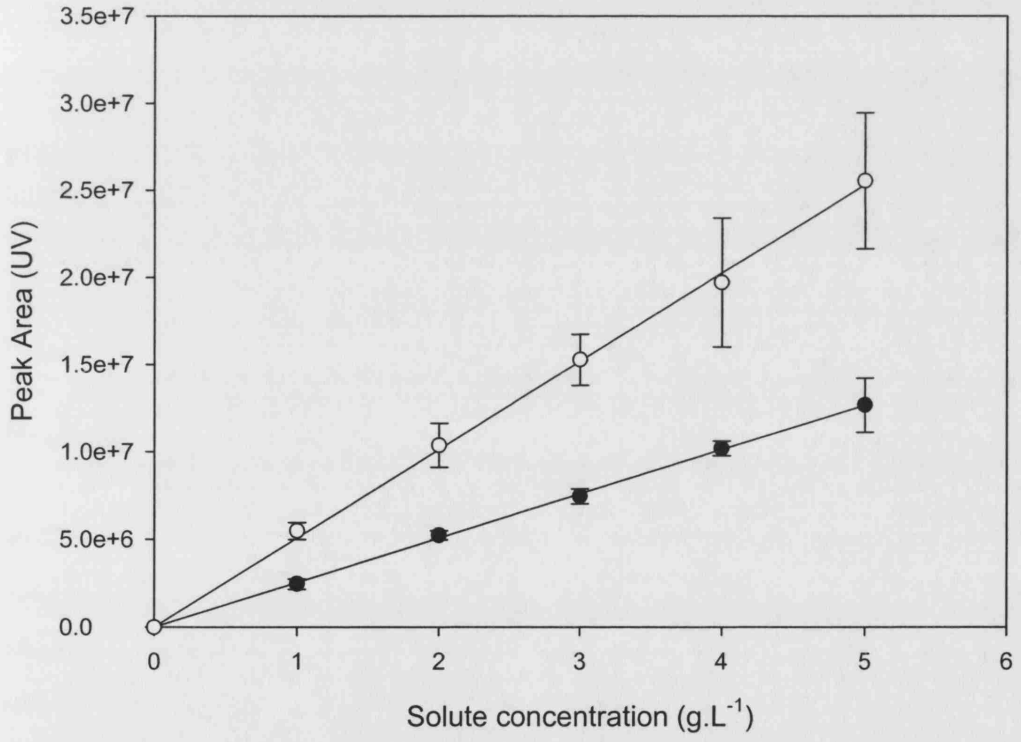


Figure II.1. Typical calibration curve for SB-240101 (○) and SB-235349 (●) in aqueous phosphate buffer (pH 10). The samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

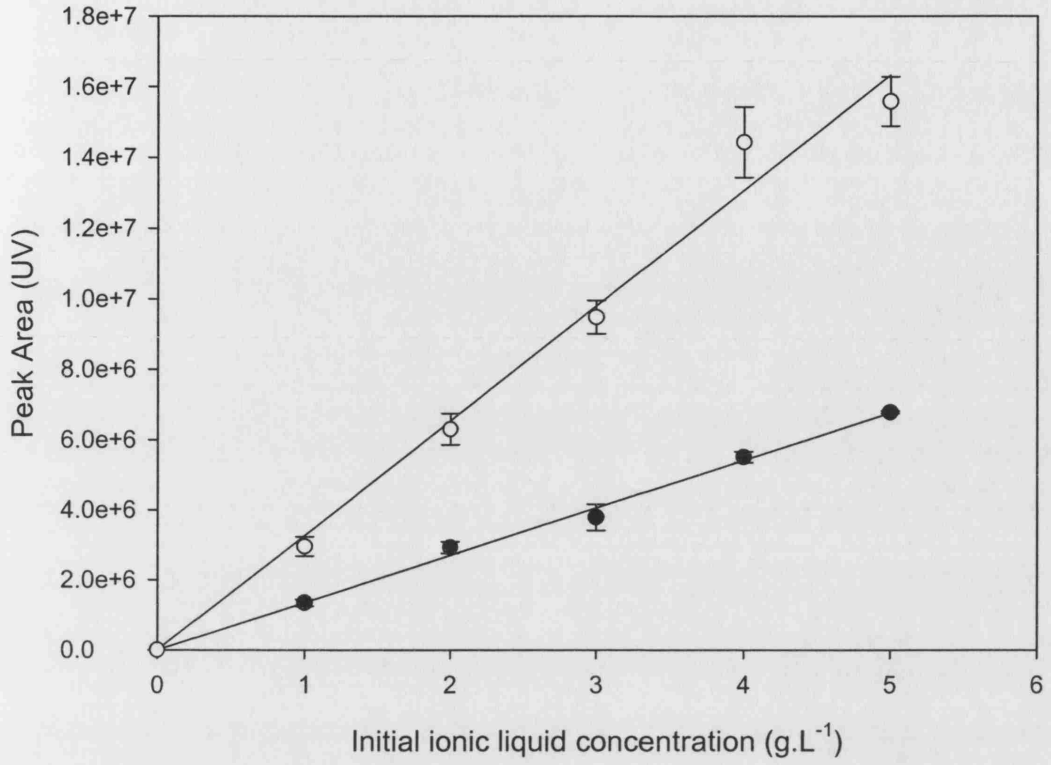


Figure II.2. The amount of SB-240101 (○) and SB-235349 (●) in aqueous phosphate buffer (pH 10) at equilibrium with ionic liquid solutions of variable initial concentration. This experiment to determine the solute equilibrium partition coefficients in ionic liquid-aqueous biphasic systems is described in Section 2.5.1.1. Samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

II.2 Isopropyl Alcohol Partition Coefficient Determination

In order to quantify the amounts of SB-235349 and SB-240101 in the [BMIM][PF_6] samples, which had been extracted with IPA, it was necessary to determine the equilibrium IPA/ionic liquid partition coefficient for these compounds. This was carried out as described in Section 2.5.1.2. Figure II.2 shows the amounts of SB-235349 and SB-240101 present in the phase at equilibrium after contact with ionic liquid solutions. The partition coefficient for the SB-240101 was calculated to be 5.07 ± 0.1.

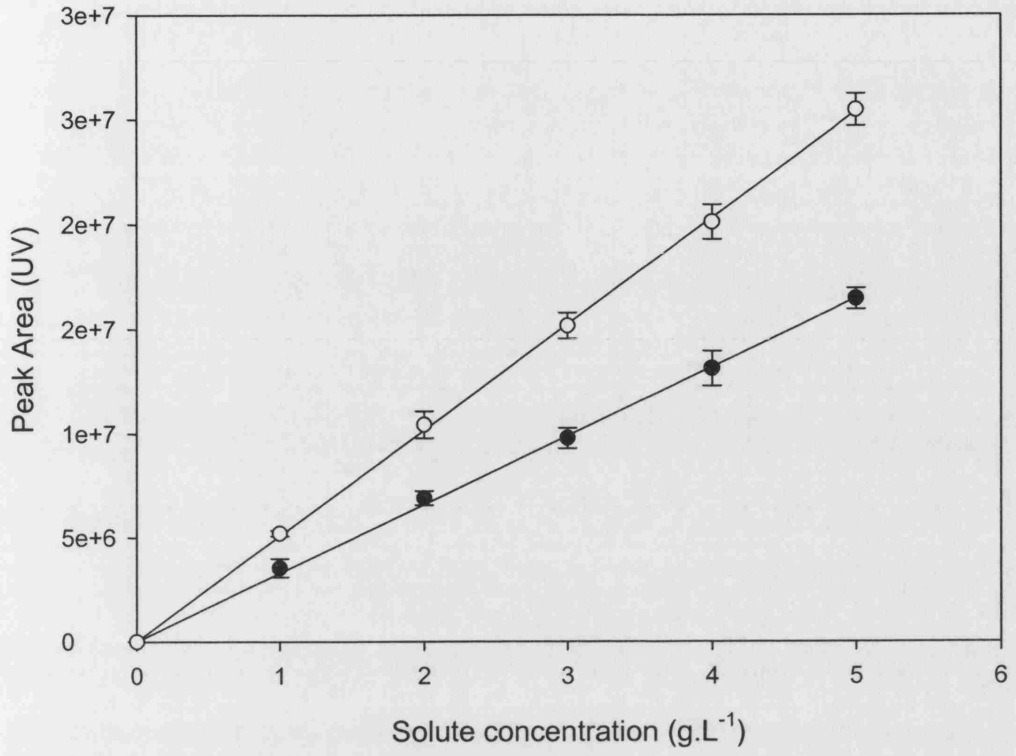


Figure II.3. Typical calibration curve for SB-240101 (●) and SB-235349 (○) in IPA. The samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

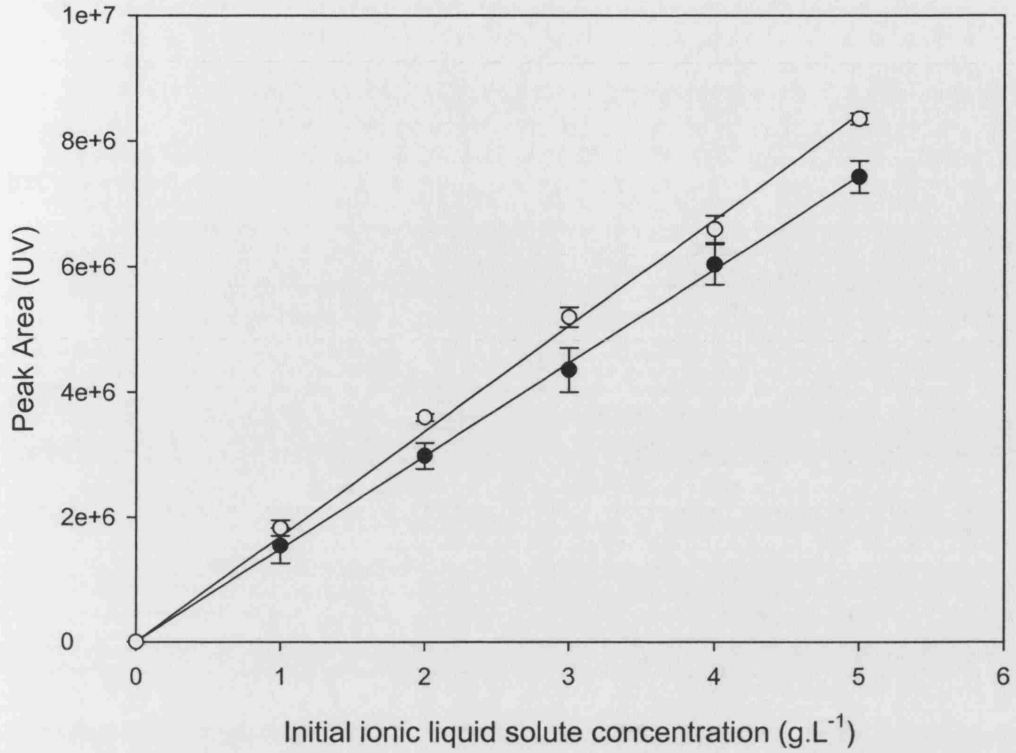


Figure II.4. The amount of SB-240101 (●) and SB-235349 (○) in IPA at equilibrium with ionic liquid solutions of variable initial concentration. This experiment to determine the solute equilibrium partition coefficients in ionic liquid-aqueous biphasic systems is described in Section 2.5.1.2. Samples were analysed as described in Section 2.5.1. Error bars show standard deviation between samples.

Appendix III: HPLC Analysis of the Bioconversion Substrate and Product

III.1 Example HPLC Chromatogram

Figure III.1 shows a typical HPLC chromatogram of the thymine substrate and thymidine product of the thymidine phosphorylase catalysed bioconversion in aqueous 10mM Tris HCl buffer. Figure III.2 shows a sample HPLC chromatogram in [EMIM][tosylate] (10 % H_2O w/w).

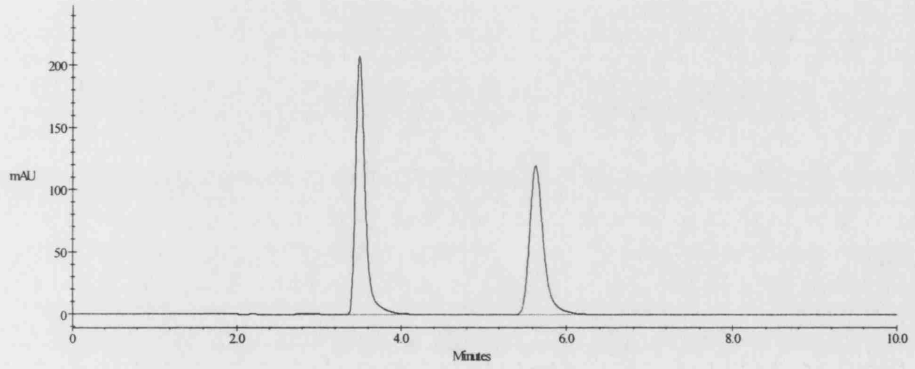


Figure III.1. HPLC chromatogram showing UV response against time. The sample from the Tris HCl buffer system was analysed as described in Section 2.5.3. The thymine substrate has a retention time of 3.6 minutes and the thymidine product has a retention time of 5.8 minutes.

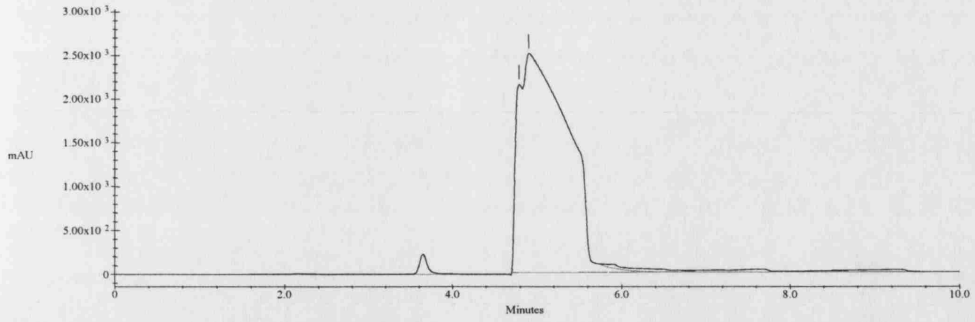


Figure III.2. HPLC chromatogram showing UV response against time. The sample from the [EMIM][tosylate] (10 % H_2O w/w) was analysed as described in Section 2.5.3. The thymine substrate has a retention time of 3.6 minutes and the thymidine product was undetectable as described in Section 5.4.2.

III.2 Calibration Curves

External calibration curves were used to quantify the thymine substrate and thymidine product of the bioconversion. Figures I.2 and I.3 show typical standard curves of solute concentration against UV response (integrated peak area) for these compounds.

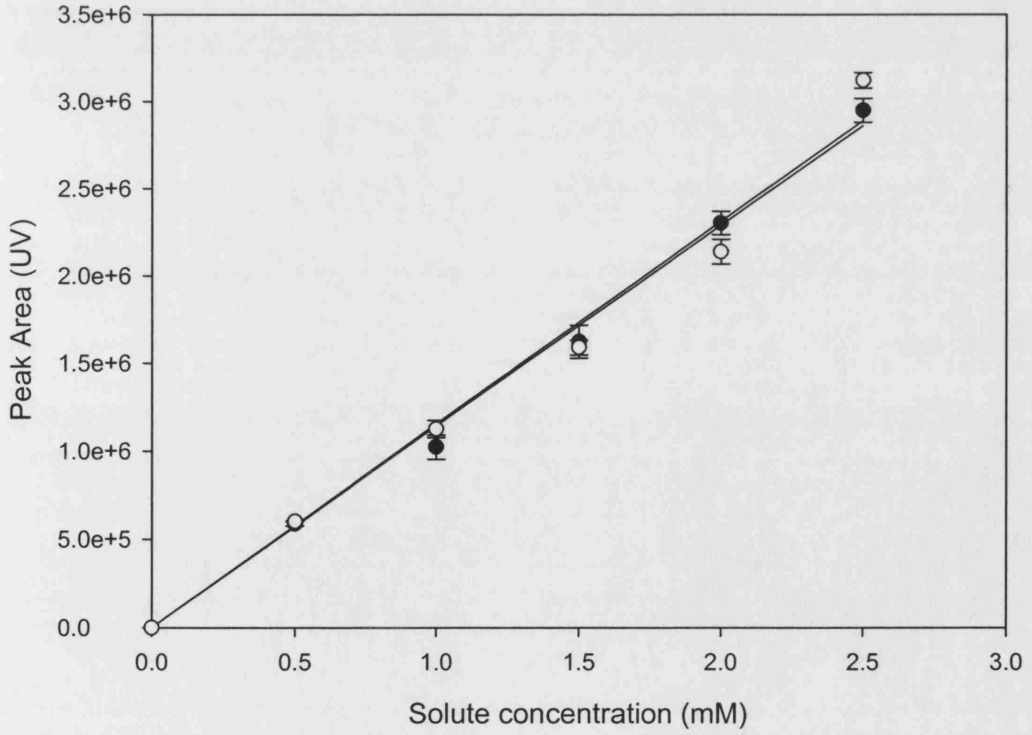


Figure III.3. Typical calibration curve for thymine (●) and thymidine (○) in 10mM Tris HCl buffer. Samples were analysed as described in Section 2.5.3. Error bars show standard deviation between samples.

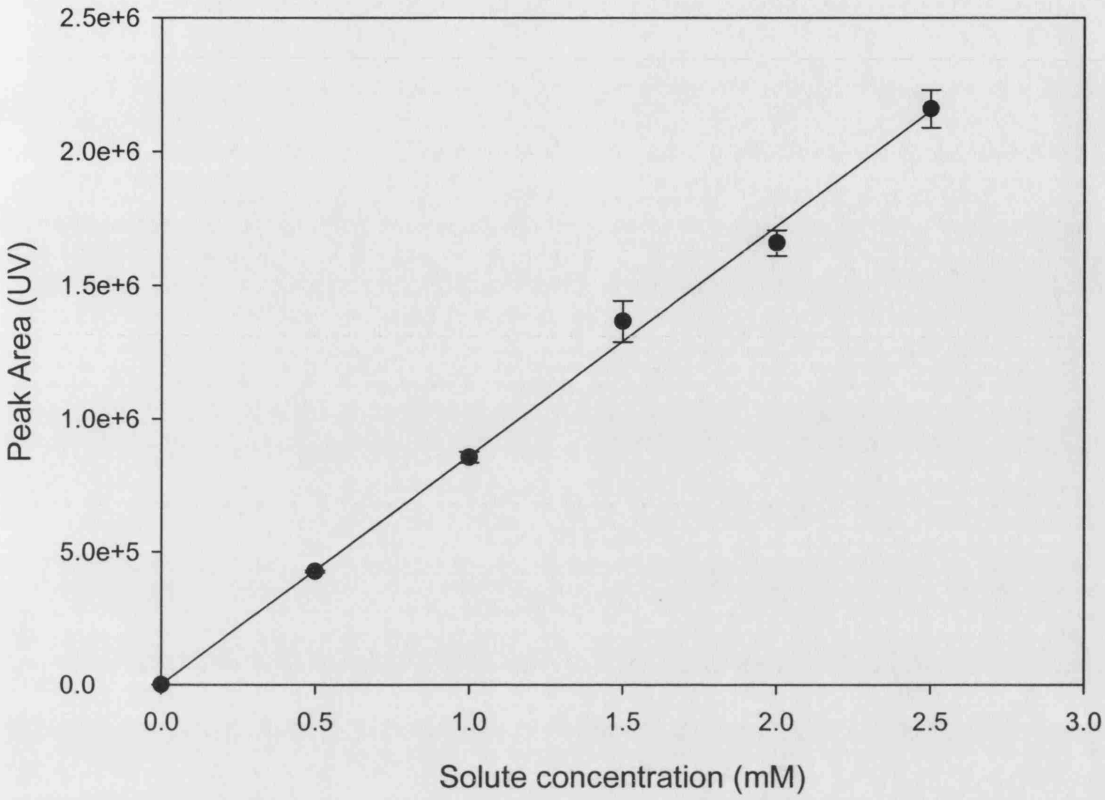


Figure III.4. Typical calibration curve for thymine (●) in [EMIM][tosylate] (10 % H_2O w/w). Samples were analysed as described in Section 2.5.3. Error bars show standard deviation between samples.

Appendix IV: Shear Stress Against Shear Rate

IV.1 Example of Shear Stress Against Shear Rate for [BMIM][PF_6]

Straight line plot of shear stress against shear rate shows Newtonian rheology of saturated [BMIM][PF_6].

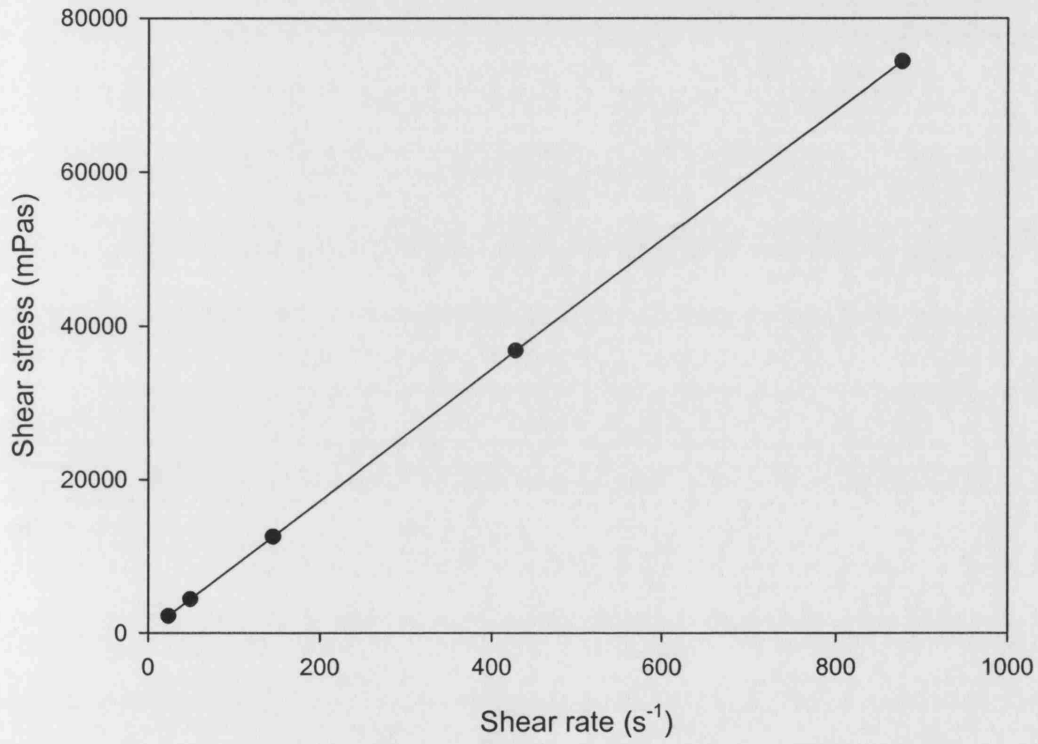


Figure IV.1 Shows a plot of shear stress vs. shear rate for saturated [BMIM][PF_6]. The methods for measuring shear stress and shear rate are described in Sections 2.5.5.2.

Appendix V: HPLC Analysis of the Chiral Substrate

V.1 Example HPLC Chromatogram

Figure V.1 shows a typical HPLC chromatogram for the chiral substrate *R*-ester (SB-240098) and *S*-ester (SB-235349). Figure V.2 shows a sample HPLC chromatogram of the chiral substrate following conversion.

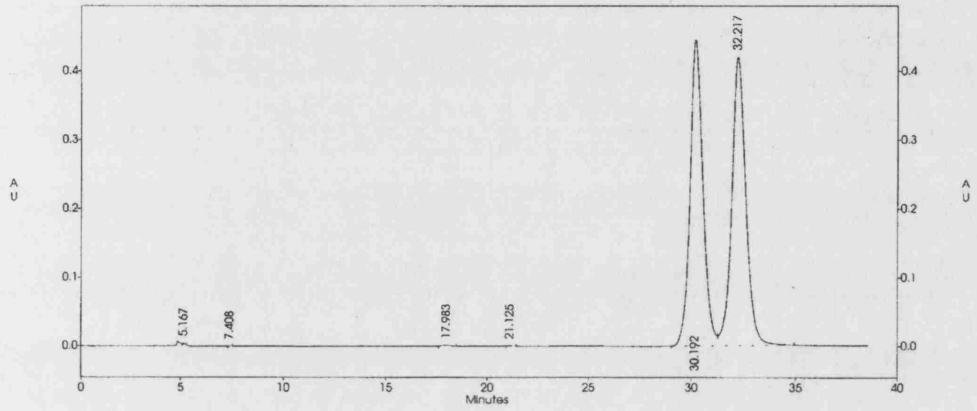


Figure V.1. HPLC chromatogram showing UV response against time. The sample was analysed as described in Section 2.5.2 before reaction. The *S*-ester (SB-235349) has a retention time of 30.2 minutes and the *R*-ester (SB-240098) has a retention time of 32.2 minutes.

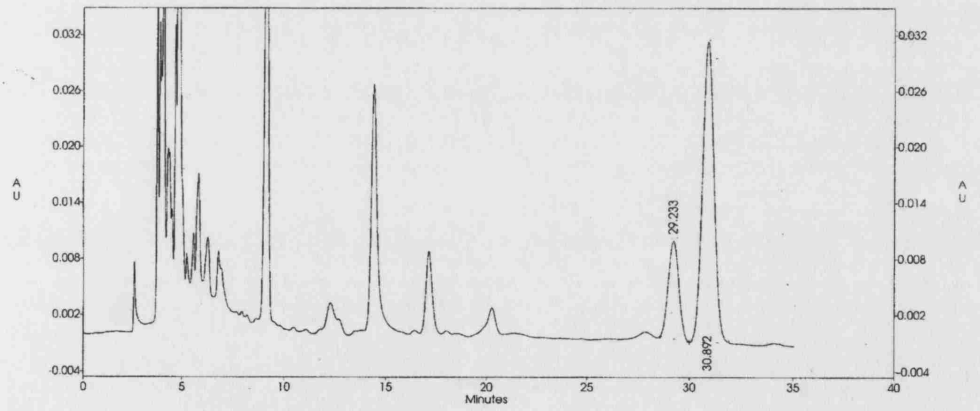


Figure V.2. HPLC chromatogram showing UV response against time. The sample was analysed as described in Section 2.5.2 following conversion. The *S*-ester (SB-235349) has a retention time of 30.2 minutes and the *R*-ester (SB-240098) has a retention time of 32.2 minutes (the *S*-acid product is not detected as it binds irreversibly to the column).

Appendix VI: Business Report

Ionic Liquids

New Technology Ventures
December 9, 2002

by:

Eugenia Christakis
Angela De Martini
Robert Helszajn
Jon Postlethwaite
Nicola Roberts
Ruslan Severin

1 Executive summary

The project investigates the feasibility of using ionic liquid technology as a replacement for commonly used organic solvents in the manufacture of pharmaceuticals. Unlike organic solvents, ionic liquids are environmentally friendly, non-flammable, and non-toxic. Recent environmental legislation makes the use of organic solvents increasingly expensive. Moreover, the use of ionic liquids has been proven to increase yields in laboratory experiments. Commercial realisation of a 5% yield increase in one process step of one blockbuster drug would result in annual savings of circa $1 million.

There are currently certain limitations in applying the technology, such as product recovery, disposal of ionic liquids and purity of the final product issues, as well as cost. Nevertheless, the consensus in the research community is that these limitations will be overcome in the next few years. As a result of recent developments in ionic liquid research, major pharmaceutical companies are actively evaluating this technology as well. As companies start to implement this technology we expect costs to come down due to economies of learning and scale.

The pharmaceutical industry and in particular the chiral pharmaceutical segment have been and are expected to experience strong growth. The annual sales of the pharmaceutical industry are approximately $400 billion, and the annual sales of organic solvents are approximately $4 billion. Our analysis concludes that the market is attractive for players with the right expertise.

The business model of choice has two phases: phase 1 is a consultancy business, which will generate low risk revenue and establish our credibility within the pharmaceutical

industry; phase 2 is to establish a laboratory to conduct R&D and patent high value processes in ionic liquids.

2 Technology review

2.1 Introduction

The production of pharmaceuticals involves numerous steps, or unit operations. Often, some of the latter steps in the manufacturing process involves using organic solvents to facilitate a reaction, during which a chemical conversion occurs. The product is then extracted from the organic solvent and passed further down the manufacturing and purification chain, leaving pharmaceutical companies with the problem of recycling the organic solvent.

The current worldwide usage of organic solvents to synthesise chemicals (petrochemical and pharmaceutical) is £4 billion[1]. However, the environmental impact of these solvents is significant, and the Montreal protocol[2] has resulted in a compelling need to re-evaluate many well-established chemical manufacturing processes[3]. Ionic liquids, although more expensive than organic solvents, have the potential to be *green*[4] alternatives to conventional organic solvents currently utilised by the pharmaceutical industry.

Organic solvents are currently used in the production of pharmaceuticals because higher concentrations of reactants can be dissolved in them. Chemical transformations occur more rapidly when reactants are dissolved; the more reactants that are dissolved the less solvent is required and the more product can be produced. However there are many disadvantages to using organic solvents: they are toxic to both process operators and the environment and

[1] The pros and cons of using ionic liquids in the pharmaceutical industry. R.Freer and A.Curzons (in press).
[2] The Montreal Protocol is an international agreement signed in 1987 and was designed to protect the stratospheric ozone layer by limiting the production and consumption of compounds that deplete the ozone layer.
[3] The pros and cons of using ionic liquids in the pharmaceutical industry. R.Freer and A.Curzons (in press).
[4] Ionic liquids are currently believed to be non-polluting and therefore defined green. However, detailed studies of environmental effects have not yet been commissioned.

their flammable nature makes them an explosion hazard. Organic solvents have also been shown to damage and ultimately destroy the cell walls of the bacteria used as catalysts during the manufacture of pharmaceuticals. The consequence of this is the additional cost of constantly replacing the bacteria.

The recycling of organic solvents means that 30% is generally lost and mostly to the atmosphere. This is not significant financially (EUR 1 per litre), but it is significant environmentally. Recycling costs of organic solvents are EUR 300 per tonne, this is half the cost of replacing the solvent. Incineration of non-organic solvents costs around EUR 4000 per tonne.

At present most pharmaceutical manufacturing processes involve at least one step in an organic solvent. Organic solvents have the advantage of being cheaper to purchase when compared to the total cost of manufacturing the pharmaceutical, and they are well established within the industry. Special precautions need to be taken however, when working with organic solvents and the waste products created must be treated appropriately before they can be disposed of or recycled.

2.2 Ionic liquids

The use of ionic liquids has the potential of being a disruptive technology and is likely to replace organic solvents in the future. We believe this to be the case, as ionic liquids overcome some of the problems associated with organic solvents as well as offering other benefits.

2.2.1 Description

Ionic Liquids are molten salts, solutions composed entirely of ions and are stable at room temperature in the presence of air[5]. A wide variety of chemical transformations have been performed in ionic liquids and in many cases significant yield improvements compared to conventional organic solvents have been observed. Most ionic liquids exhibit a high solubility for molecules used in the pharmaceutical industry. Ionic liquids can be tailor made for a particular reaction to give the greatest yield, unlike organic solvents. Additionally, because of the non-volatile and non-flammable nature of ionic liquids, they offer the advantages of being greener, safer alternatives to organic solvents. Ionic liquids could be implemented into existing drug manufacturing processes, and have been shown not to compromise the reaction occurring.

2.2.2 What still needs to be resolved

At present there are many issues still to be resolved regarding the use of ionic liquids in the pharmaceutical industry. These are:

- **Product recovery** - In the pharmaceutical industry, as with all industries, it is important to recover as much product as possible. However, if large amounts of organic solvents or energy are required to extract the product from the ionic liquid, then their use counteracts their benefits. Many new methods of product recovery are being investigated worldwide[6].
- **Disposal of ionic liquids** - To date there has been no detailed study on the environmental impact of ionic liquids. How to dispose of waste containing ionic

[5] Roberts, N.J., and Lye, G.J., Application of Room temperature ionic liquids in` biocatalysis: Opportunities and challenges. In *Ionic liquids as solvents: The current state of the art*; Rogers, R. and Seddon, K., Eds. ACS Publishing, **2002**.
[6] Blanchard, L.A., Hancu, D., Beckman, E.J. and Brennecke, J.F. Green processing using ionic liquids and CO2. Nature, Vol. 399, 6 May 1999.

liquids could be an issue. However, generally researchers believe that the recycling of ionic liquids after reactions would be possible and the best approach in industry.

- **Purity** – In the pharmaceutical industry, products need to be 100% pure to satisfy industry regulators. Therefore, evidence needs to be shown that ionic liquids do not contaminate the product in any way.

- **Yield** - In order to justify the expense of changing an existing and well-established process, the increase in product yield would need to be significant. In the pharmaceutical industry if there is a greater than 5% increase in yield, then the cost-benefit analysis is generally positive[7].

The lack of data regarding ease of recovery, recycling and re-use of ionic liquids compared to the tried and tested tradition of organic solvents, means their adaptation into the pharmaceutical industry will take time. It should be noted that due to the early stages of ionic liquid development, no ionic liquid process is yet in place. We believe that all work in the field is currently still at the feasibility stage. Nevertheless, the consensus in industry and the research community is that these issues will be resolved in the next couple of years. We believe that it is worthwhile for pharmaceutical companies to incorporate the use of ionic liquids in their new product development processes, which take approximately 5 years in the UK. Within this time many of the remaining issues with the use of ionic liquids will have been resolved. To convert an existing process typically into ionic liquids takes approximately 2 years. We advise pharmaceutical companies to carry out cost-

[7] The pros and cons of using ionic liquids in the pharmaceutical industry. R.Freer and A.Curzons (in press).

benefit analysis on existing drugs to determine the feasibility on a case-by-case basis. This could open up opportunities for the consultancy model discussed in the Enterprise Options Section.

2.3 Competing technologies

Competing with ionic liquids for the removal of organic solvents from the pharmaceutical manufacture are:

2.3.1 Water-based reactions

New molecules are being designed that use water as the reaction media. However, there will only ever be limited success with this approach due to the polar nature of water, which means that most molecules will not dissolve in water.

2.3.2 Solvent free systems

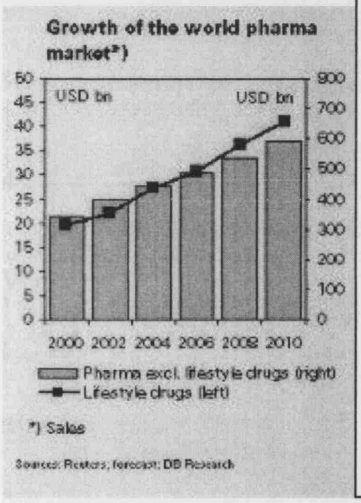
A new area of research is reactions that don't use a solvent media. This involves mixing powders together and letting reactions occur in the solid state. The idea however, has poor conversion rates, because collision of reactants is slow in solid form.

3 Macro-level market assessment (Pharmaceutical Industry)

The worldwide market for pharmaceuticals is not only significantly large, but its growth forecasts make it an attractive industry to operate in. The discovery and market introduction of new blockbuster drugs fuels further growth[8].

[8] "Mating and waiting", The Economist, Jul 18th 2002

According to Deutsche Bank research, worldwide sales of pharmaceutical products are set to increase from about $400 billion to nearly $600 billion between 2002 and 2010. This cumulative growth rate of circa 50% reinforces the attractiveness of the industry. We estimate that organic solvents currently make up a negligible amount of the cost of sales. This is clearly not a significant expense to the industry but increases in product yield present an exciting opportunity to be exploited by alternative, disruptive technologies, such as the use of ionic liquids.



Pfizzing

Worldwide pharmaceutical sales, $bn

	1995		2001
Glaxo Wellcome	12.5	Pfizer/Pharmacia*	37.5
Merck	11.3	GlaxoSmithKline	24.8
HMR	8.4	Merck	20.2
Novartis	8.1	AstraZeneca	16.5
Bristol-Myers Squibb	7.4	Aventis	15.7
Pfizer	7.1	Bristol-Myers Squibb	15.3
Roche	6.9	Johnson & Johnson	14.8
Johnson & Johnson	6.3	Novartis	11.9
AHP	6.1	Wyeth†	10.9
SmithKline Beecham	6.1	Eli Lilly	10.8

*Combined sales, pro forma †Was AHP

Sources: Lehman Brothers; company reports

Environmental issues are becoming more and more important with legislation increasingly restricting or taxing the use of pollutants and dangerous substances. For example, in Switzerland, there is a tax on organic solvents (or volatile organic compounds – VOCs) of EUR 1.24 per kg VOC in 2000-2002, rising to EUR 1.86 per kg VOC in 2003[9].

[9] The Eco-Tax Database of Forum for the Future, Switzerland 2000

Further environmental restrictions are imposed by a European Council Directive[10], which prohibits what it terms as "substantial changes in emissions or discharge of organic substances" of >10% for large installations and >25% for small installations[11]. The Consequences of non-compliance are sanctions including having to stop using the plant.

The rising importance of corporate social responsibility for listed companies exerts further pressure to "clean-up" and switch to safe, clean production methods.

These environmental and legislative trends increase costs and make the use of alternative methods, such as ionic liquids, more attractive. We believe that there is vast scope to benefit from these trends, particularly in the areas of outsourcing and licensing of cheaper, cleaner and greener methods and products.

4 Micro-level market assessment (Chiral)

Within the pharmaceutical industry, a rapidly growing subset of drugs exists. These are known as chiral (also known as single-enantiomer) drugs[12]. Though chemically identical, many drugs take one of two mirror-image forms, or chiral forms. These chiral molecules are said to be either "left-handed" or "right-handed" depending upon the configuration of the molecule in space. The effectiveness of pharmaceuticals is often based on the precise matching of the drug's molecular structure with the structure of receptors inside the body. As a result, it is frequently only one of the two mirror-image forms of a molecule that provides the desired therapeutic result.

[10] www.europa.eu: Council Directive 1999/13/EC of 11 March 1999

[11] The scope of the Directive, where it relates to the manufacturing of pharmaceutical products, covers "the chemical synthesis, fermentation, extraction, formulation and finishing of pharmaceutical products and where carried out at the same site, the manufacture of intermediate products".

[12] Erb. S.E. (2002). Single-enantiomer markets and technologies, Gen. Engng. News, 22(9) 47-68.

Chiral pharmaceuticals have become big business in recent years: In 2001, chiral drugs accounted for $147 billion of sales in America, or 36% of the total drug market, according to Technology Catalysts International (TCI)[13]. In 2002, industry sources report that over 80% of new medicines currently in development are chiral and that the demand for chiral ingredients has risen[14]. The UK's Department of Trade and Industry puts this figure even higher at 90%.

As a result of this, technologies associated with chiral drugs are also experiencing strong growth. A recent Frost & Sullivan report estimates that revenues for chiral technology will reach EUR 18.34 billion in 2007, up from EUR 5.73 billion in 2000 and EUR 7.59 billion in 2001. Chiral manufacture is expected to maintain strong growth during the period to 2007, mainly due to growing use in pharmaceutical applications, which already accounts for 80% of consumption. This represents a compound annual growth rate of 13.4% in the period 2000-2007[15]. A sub-sector – the global market for chiral technology-enabling products - is forecast to cross $3 billion by 2004[16].

This explosion of growth makes chiral drugs one of the most attractive parts of the pharmaceutical industry. Techniques, such as the use of ionic liquid technology to produce more of the useful molecular configuration required for chiral manufacture have the ability to increase the efficiency and lower the cost of the drug manufacturing process.

An example of the benefits of the yield-enhancing qualities of substituting ionic liquids for organic solvents has been estimated based on cost data provided by GSK on their Paroxet

[13] "Telling right from left", Economist, Sep 19th 2002.
[14] "Chiral chemistry is an essential pharma kit component, Specialty Chemicals Magazine, Sep 18th 2002.
[15] "Forefront of the Chiral Boom, Specialty Chemicals Magazine, Mar 1st 2002.
[16] Research Studies-Business Communications, Inc. (magazine): Apr 24th 2000

drug, which is chiral. During stage three (of seven), a 50% yield increase would reduce cost of goods by circa 25% per year. On a blockbuster drug with sales of £1 billion, that equates to £10 million in savings per year in cost of goods sold. A more realistic yield improvement of 5% saves £1 million per year in cost of goods sold. When the current limitations associated with ionic liquids have been addressed, cost savings of this scale for just one step of one drug, make this technology an attractive business proposition.

5 Macro-level industry assessment (Competitive environment)

5.1 Buyers

Potential buyers refers to companies that can use the ionic liquid technology for the production of chiral pharmaceuticals. Pharmaceutical companies that have an existing process in organic solvents will be our main target market. These companies have enormous financial and scientific resources, and they would be able to develop ionic liquid technologies in-house or through various partnerships with universities and independent research labs. Accordingly, the huge size of pharmaceutical companies, their ability to develop the ionic liquid processes in-house and the fragmentation of the ionic liquid industry means that pharmaceutical companies would have significant bargaining power. This bargaining power would be significantly reduced if IP rights were established.

On the other hand, the actual costs of designing the process in ionic liquids is insignificant compared to the benefits involved. Accordingly, pharmaceutical companies would be primarily interested in the delivery and implementation of an efficient process in ionic liquids. Therefore, pharmaceutical companies would be prepared to pay high fees, provided that we bring something important to the table, such as patents or ionic liquid expertise. The designing of a process in ionic liquids requires significant scientific

expertise and practical experience, and companies that would be able to differentiate themselves as industry leaders in this area would be able to command premium prices.

5.2 Suppliers

There are a number of ionic liquids suppliers, including Merck, Acros, Covalent Associates, and Solvent Innovation, specialising in this area. Production of ionic liquids is a relatively straightforward process. There are no IP rights that are involved in the production of ionic liquids. Ionic liquids can be produced in any reasonably equipped chemistry lab, in a 3-6 week production run.

Currently, ionic liquids are primarily used for scientific research purposes and there are few commercial applications of them. Therefore, the market demand is satisfied by small batch producers or independent labs who at present command a high market price. If pharmaceutical processes in ionic liquids develop there will be a need of large quantity of ionic liquid. The company may chose to produce the ionic liquids itself, which means suppliers of ionic liquids would need to compete with the cost to manufacture the ionic liquids in-house. The more ionic liquid you produce the more you can benefit from the economies of scale and this should result in a decrease of prices.

5.3 Rivalry & new entrants

Although there are no commercial applications of ionic liquids in the pharmaceutical industry yet, there are dozens of companies that are trying to achieve this. Most of them are university-based, company-sponsored or both. Ionic liquid research is being done in many leading universities, and some of them, already have associated consultancy companies, which are working in the ionic liquid field.

QUILL (Queen's University Ionic Liquid Laboratories) is one of the most famous players in the ionic liquid industry. It has about 20 corporate founders including Merck and GlaxoSmithKline. Membership subscriptions range from USD $30,000 to $50,000 per annum per member, providing funds for research.

Rivalry is not particularly strong today, since the technology is still largely in the academic research phase and there are significant issues to be overcome before it can be commercialised. However, once the technology would be ready for commercialisation, we expect a large number of businesses to be formed on the basis of current academic research groups, both commercialising IP and technical knowledge.

5.4 Conclusion on industry attractiveness

Designing the pharmaceutical processes in ionic liquids is very expertise intensive and can potentially bring significant benefits to the pharmaceutical companies. Therefore, the competition in the industry is likely to be based on the quality of the services or IP provided, rather than on price. We believe that despite high bargaining power of customers, the industry is attractive.

6 Intellectual Property

There is a current drive in the industry to patent ionic processes. Utility patents are granted for inventions and discoveries of — and improvements to — a new process, machine, article of manufacture or composition of matters. In our case, improvement to an already existing process is achieved by developing and optimising a process step in which a specific reaction is run in ionic liquids instead of conventional organic solvents. The

process for running a specific reaction, using a specific catalyst, in a specific ionic liquid is patentable.

Processes for validated reactions run in ionic liquids have already been patented in the chemical domain[17].

Other substances involved in the process being patented, complicate the patentability of processes run in ionic liquids. For example, patenting a process that involves the use of a patented catalyst means that the rights of the third party have to be considered.

The ownership of the intellectual property that is developed is a key outcome of the type of enterprise model we choose to exploit the ionic liquid technology. If the work is developed with a university, the ownership and therefore the revenues, will be split between the university and ourselves. If an independent laboratory is set up, then the ownership will belong wholly to us. If the IP were developed using a consultancy model, this would tend to belong to the company that it was developed for, unless we specifically make arrangements that this will not be the case.

7 Enterprise options

A number of possible enterprise options have been identified as appropriate for us to exploit this technology.

7.1 Consultancy Business Model

[17] Examples of patents that have been extended include: Separation of olefins from paraffins using ionic liquid solutions (US patent 6,339,182) and Process for making aromatic aldehydes using ionic liquids (US patent 6,320,083).

One business opportunity we can pursue is to create a consultancy firm that would help pharmaceutical companies to set up the processes, which use ionic liquids. This could be done either as a pure consultancy, with staff using the client's labs to perform the R&D, or by using our own facilities in a development lab.

The consultancy option is the least risky of the business models proposed, as it requires minimal capital investments. Obviously, a small lab with basic equipment is required. However, more expensive equipment can be accessed either on the client's premises or rented at the client's expense. A consultancy model would also imply low staff requirements.

The disadvantage of a consultancy business model is that it captures only a small fraction of the value created. Almost all economic benefits will be captured by the pharmaceutical company, and a consulting firm would only get a relatively small fee for its services.

A potential difficulty is recruiting people with the appropriate expertise. However, given that our team has access to some of the best industry experts, we believe that this would be our competitive advantage.

The consulting business model can be used to take the business off the ground, since it is a low risk – relatively low return option. Once the firm has established credibility and generated some revenue, the business model can be switched to, or supplemented by, more risky but potentially more rewarding models, such as developing in-house patents for specific processes. Patenting of process steps in ionic liquids would require stable flow of capital investment over several years until the patenting process is finished and some form

of revenues is generated. Such capital investments can be provided by the consulting business model.

7.2 Collaboration with University

We can also commercialise the technology by carrying out the ionic liquid process development work in a university and then either sell or licence the results to pharmaceutical companies. Within the Advanced Centre for Biochemical Engineering (ACBE) at UCL, all of the equipment exists to allow us to carry out the research and development, and it is available to rent.

This model allows us the flexibility to decide upon the degree of collaboration with the university. Costs could be lowered by sponsoring PhD students and post-doctoral researchers, or the facilities could be run effectively independent of the university. Any IP arising from the project may complicate matters however as there may be a conflict of interest between the university, the pharmaceutical company whose process we are working on and ourselves. This model involves significant capital infusion.

7.3 Independent Lab

The success of setting up an independent laboratory depends on the ability to commercialise and patent one or more ionic liquid processes. These processes would be licensed to pharmaceutical companies on a royalty fee basis. Our competitive edge is based on the patented processes together with continued in-house R&D. Potential issues include government accreditation, health and safety, quality control, and scalability of the process.

This model is the most risky of the three due to the high capital requirements to establish the lab and fund the research and the uncertainty of the outcome of the patenting process.

7.4 Funding

The UK Department of Trade and Industry's Office of Science and Technology runs a number of "LINK" programmes[18] to fund research projects amongst industry, universities and other researchers, in order to further the promotion of new technologies and industry best-practices in the UK. Research into the use of ionic liquids in the production of chiral pharmaceuticals may be eligible for grants under these schemes, two of which are highlighted below:

Manufacturing Molecules:

Projects could include the following:

1. New techniques for chiral formation or separation (90% of all drugs at this stage are chiral)
2. New process technologies (new reactors using ionic liquids, microwaves, super-critical fluids)
3. Less environmental impact (better yields, less effluent)
4. Better separation techniques (less waste/effluent – especially solids separation)

Programme funding and duration:

[18] www.dti.gov.uk/ost/link

LINK provides funding of up to 50% of the eligible project costs. Government funding of £1.2 million will be available over 3 years. This will be at least matched by industry funding.

Sustainable Technologies Initiative LINK Programme

Research coverage:

1. Novel technologies
2. Better uptake of sustainable design, production and consumption
3. Innovative use of existing technologies
4. Production of less waste and pollution

Programme funding and duration:

LINK provides funding of up to 50% of the eligible project costs. Government funding of £15 million will be available over 5 years. This will be at least matched by industry funding. A parallel programme of £5 million (matched by industry) over 5 years also exists.

The existence of these and similar schemes reduce the R&D costs and provides an incentive for pharmaceutical companies to partner with us.

7.5 Overall business model chosen

The business opportunities in this area will largely depend on developments in research and generally in the ionic liquid field. The enterprise option we would most likely adopt would be setting up a firm to perform consulting projects for pharmaceutical companies.

This benefits from a reasonably stable income and does not require high initial capital investment. Once established, the business could set up an independent lab and look at outsourced R&D or manufacturing for large pharmaceutical companies.

8 Conclusion

The group is confident that ionic liquid technology will replace organic solvents in the chiral pharmaceutical industry over the next decade. Ionic liquids are non-toxic and non-flammable and have the potential to not only be "greener" alternatives to organic solvents but also increase yield. With all these benefits and with the governments putting more and more pressure on pharmaceutical companies to reduce toxic emissions into the environment, pharmaceutical companies will be sufficiently motivated to replace organic solvents with ionic liquids.

However, current drawbacks of ionic liquids technology, such as higher costs, purity and product recovery problems, do not allow immediate commercial applications of this technology. Significant progress has been made in the recent years to eliminate these drawbacks, and we expect that further developments due to continuing intensive research by universities and pharmaceutical companies would make the use of ionic liquids commercially viable. Commercial applications would start by introducing ionic liquid technology into a few manufacturing steps, where ionic liquids would give a particularly substantial increase in yield. Once the technology is implemented, we expect significant learning curve effects, which would improve the technology and gradually increase the number of processes in which it would be applied.

Currently, the most likely scenario is starting consulting projects for pharmaceutical companies. This business model does not require large capital investment or expertise in all related fields and provides a reasonably stable income. Once sufficient practical experience has been obtained and the credibility and networking contacts have been established, the business could set up an independent lab and look at outsourced R&D or manufacturing for large pharmaceutical companies.

9 Glossary

- The substrate is the reactant in any enzyme-catalyzed reaction.
- Organic solvents carbon-containing liquids that will dissolve another substance.
- Chiral molecules are in a given configuration, not symmetrical with its mirror image.
- Molten salts are liquids at a temperature greater than or equal to 100 °C and below its flash point. Flash point is the lowest temperature at which a liquid can form an ignitable mixture in air near the surface of the liquid. The lower the flash point, the easier it is to ignite the material.
- Ionic liquid are salts/salt mixtures with melting points below ambient temperature. They represent a class of novel solvents with non-molecular, ionic character.
- Ions are electrically charged atoms
- Yield total amount of product captured at the end of a process
- Product recovery method of removal of product i.e. pharmaceutical from reaction media
- Catalyst substance causing or assisting a chemical reaction without taking part in it.
- Polar molecules have a partially positive side and a partially negative side. The partial positive on one molecule will be attracted to the partial negative on a second molecule. This attraction results in a Hydrogen bond.