UNIVERSIDADE DE LISBOA

INSTITUTO SUPERIOR TÉCNICO

DEVELOPMENT OF NEW SYNTHETIC METHODOLOGIES FOR ENZYMATIC RESOLUTIONS

Ângelo Miguel Rodrigues da Rocha

Supervisor: Doctor Carlos Alberto Mateus Afonso
Co-Supervisor: Doctor Luís Joaquim Pina da Fonseca

Thesis approved in public session to obtain the PhD Degree in Chemistry

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RESUMO

No decorrer desta tese abordaram-se dois temas distintos, sendo ambos relevantes no contexto da indústria farmacêutica. O principal tópico de investigação, resolução enzimática cinética de aminas e álcoois secundários, resulta da necessidade cada vez maior de compostos quirais opticamente puros. Das várias metodologias existentes, a resolução enzimática cinética é uma estratégia comum na Indústria para a preparação de enantiómeros puros. No sentido de resolver algumas limitações intrínsecas, tem-se procurado a sua conjugação com líquidos iónicos.

Desenvolveu-se um método para a resolução de álcoois secundários usando como agente acilante um anidrido iónico preparado *in situ* na presença do biocatalisador. Após optimização das condições reaccionais, o método permitiu a resolução de vários substratos com conversões e rácios enantioméricos elevados. Ambos os enantiómeros foram recuperados sem recorrer a nenhum passo de purificação por cromatografia. Tanto o ácido iónico de partida como o biocatalisador puderam ser recuperados e reutilizados até 9 vezes sem se observar perda significativa de enantiosselectividade.

Dois métodos em fluxo contínuo usando derivados de PEG_{600} também foram desenvolvidos com sucessor. Para aminas, o PEG_{600} diéster revelou-se um excelente agente acilante. Atingiram-se excessos enantioméricos e rendimentos elevados para ambos os enantiómeros usando um tempo de residência de apenas 30 minutos. Para quebrar a ligação amida do produto tivemos de recorrer a hidrólise química com ácido clorídrico em água/metanol. O biocatalisador pôde ser reutilizado durante 20 horas sem perda de enantiosselectividade.

Para álcoois, teve de se utilizar uma outra estratégia, em que o álcool racémico é primeiro esterificado ao PEG_{600} diácido, fazendo-se depois a resolução por hidrólise enzimática em fluxo contínuo. Após optimização, demonstrou-se que com este método podiam-se atingir productividades de 64 e 68 mg h^{-1} g $enzima^{-1}$ para os enantiómeros (R) e (S), respectivamente, durante um ensaio de 195 minutos (13 ciclos) em acetona/água usando um tempo de residência de apenas 15 minutos.

A resolução cinética enzimática em fluxo contínuo de α-hidroxiciclopentenona-aziridinas também foi estudada. Após optimização, o método desenvolvido foi aplicado na resolução de dois substratos. Partindo da aziridina de alilo, usando um tempo de residência de 10 minutos, conseguiu-se para o produto um excesso enantiomérico de 93% e um rendimento de 19%. Partindo da aziridina de butilo, usando um tempo de residência de 25 minutos, conseguiu-se para o produto um excesso enantiomérico de 96% e um rendimento de 46%. Finalmente, efectuou-se um ensaio de estabilidade do biocatalisador e a enzima manteve-se enantiosselectiva durante 350 minutos com conversões até 47%.

A resistência de bactérias a antibióticos representa uma ameaça muito séria para a saúde pública. Com tal em mente, desenvolveu-se uma metodologia sintética para a preparação de líquidos iónicos baseados em componentes de óleos essenciais com actividade anti-microbiana, tais como carvacrol, timol e eugenol.

Os líquidos iónicos sintetizados e os seus ácidos precursores foram testados contra um painel de bactérias Gram-negativas e Gram-positivas. Ao contrário do cravacrol e do timol, todos os líquidos iónicos não têm actividade contra Gram-negativas. No entanto, contra Gram-positivas, dois líquidos baseados em carvacrol ou timol demonstraram ser pelo menos 50 e 25 vezes mais potentes do que o carvacrol ou o timol sozinhos. Até os respectivos ácidos precursores se revelaram várias vezes mais potentes do que os produtos naturais de partida. Infelizmente o líquido iónico baseado em eugenol não demonstrou qualquer actividade contra todas as bactérias testadas.

Palavras-chave: resolução cinética enzimática, lipase, líquidos iónicos, química em fluxo, actividade anti-microbiana.

ABSTRACT

Two distinct subjects of interest were studied in the course of this thesis, both extremely significant for the pharmaceutical industry. The main topic of research, enzymatic kinetic resolution of secondary amines and alcohols, comes from the ever-growing demand for optically pure chiral compounds. Of the many methods available to Industry for preparation of pure enantiomers, enzymatic kinetic resolution is one of the more common ones. In order to solve some major drawbacks, its combination with ionic liquids is an important area of research.

We developed a method for the resolution of secondary alcohols using an ionic anhydride acylating agent prepared directly in the reaction medium containing the biocatalyst. After optimization of the reaction conditions, the method allowed the resolution of a number of substrates in very high conversions (46–48%) and enantiomeric ratios (E>170) along with an easy recovery of both enantiomers without the need for preparative chromatographic separation. Additionally, both the starting ionic acid and the biocatalyst could be recovered and reused up to nine cycles without significant loss of enantioselectivity.

Two continuous flow methods were also successfully developed using PEG_{600} derivatives. For amines, PEG_{600} diester revealed to be an excellent acylating agent. Very good ees (above 95%) and yields (above 35%) could be achieved for both enantiomers with a short residence time (30 minutes). For breakage of the amide groups from the product we had to resort to chemical hydrolysis with HCl in water/methanol. The biocatalyst could be used for 20 hours without any loss of enantioselectivity.

A different strategy was developed for alcohols in which the alcohol was bonded to PEG_{600} diacid and then an enzymatic hydrolysis process under flow conditions was employed. After optimization, we demonstrated that with this method we could obtain productivities of 64 and 68 mg h^{-1} g $enzyme^{-1}$ for the (R) and (S) enantiomer, respectively, during a 195 minutes trial (13 cycles) in acetone/water and with a residence time of only 15 minutes.

The enzymatic kinetic resolution in flow of α-hydroxycyclopentenone-aziridines was studied as well. After optimization, the method was successfully applied in the resolution of two substrates. For allyl aziridine, using a residence time of 10 minutes, a product ee of 93% and a conversion of 19% was obtained. For butyl aziridine, using a residence time of 25 minutes, a product ee of 96% and a conversion of 46% was attained. Finally, a biocatalyst stability study was performed, using butyl aziridine as substrate. The enzyme remained enantioselective for 350 minutes, with conversions as high as 47%.

Bacterial resistance to antimicrobial drugs poses a serious and rapidly growing threat to public health. A synthetic methodology was developed for preparation of ionic liquids based on essentials oils components with antimicrobial activity, like carvacrol, thymol and eugenol.

The synthesized ionic liquids and its respective precursor acids were tested against a panel of Gram-negative and Gram-positive bacteria. All ionic liquids, unlike pure carvacrol and pure thymol, were ineffective versus Gram-negative bacteria. On the other hand, against Gram positive bacteria, two ionic liquids based on carvacrol and on thymol were at least 50 and 25 times more potent than pure carvacrol and thymol, respectively, which are outstanding results. Even the respective precursor acids were several times more potent than carvacrol and thymol. The eugenol-based ionic liquid was ineffective against all bacteria.

Keywords: enzymatic kinetic resolution, lipase, ionic liquids, flow chemistry, antimicrobial activity

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ABBREVIATIONS

^{1}H	–	Hydrogen-1
^{13}C	–	Carbon-13
^{o}C	–	Celsius
δ	–	Chemical shift
Acetone-d_6	–	Deuterated acetone
ACN	–	Acetonitrile
API	–	Active pharmaceutical principle
BF_4	–	Tetrafluoroborate
BMIM	–	1-Butyl-3-methylimidazolium
CALB	–	*Candida antarctica* lipase B (reclassified in 2013 as *Pseudozyma antarctica* lipase B)
$CDCl_3$	–	Deuterated chloroform
D_2O	–	Deuterated water
DCC	–	Dicyclohexylcarbodiimide
DES	–	Deep eutectic solvent
DIC	–	Diisopropylcarbodiimide
DMAP	–	4-(Dimethylamino)pyridine
DMSO	–	Dimethyl sulfoxide
DMSO-d_6	–	Deuterated dimethyl sulfoxide
E	–	Enantiomeric ratio
EDC	–	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
ee	–	Enantiomeric excess
EKR	–	Enzymatic kinetic resolution
FID	–	Flame ionization detector
g	–	Gram
GLC	–	Gas-Liquid chromatography
HCl	–	Hydrogen chloride

He	–	Helium
Hz	–	Hertz
J	–	Coupling constant
M	–	Molar
m	–	Multiplet
MeOD-d_6	-	Deuterated methanol
MHz	–	Megahertz
min	–	Minute
mL	–	Milliliter
mmol	–	Millimoles
MRSA	–	Methicillin-resistant *Staphylococcus aureus*
MTBE	–	Methyl tert-butyl ether
$NaHCO_3$	–	Sodium bicarbonate
NMR	–	Nuclear magnetic resonance
PALB	–	*Pseudozyma antarctica* lipase B (formerly known as *Candida antarctica* lipase B)
PEG	–	Poly(ethylene glycol)
PF_6	–	Hexafluorophosphate
PLU/g	–	Activity expressed in mol of propyl laurate synthesized from lauric acid and 1-propanol, at 60ºC, per minute per gram of immobilized enzyme
ppm	–	Parts per million
q	–	Quartet
RTIL	–	Room-temperature ionic liquid
s	–	Singlet
t	–	Triplet
TLC	–	Thin layer chromatography
t_r	–	Retention time
UV	–	Ultraviolet
wt%	–	Weight percent

CHAPTER 1

IONIC LIQUIDS: AN OVERVIEW

1.1

INTRODUCTION

Ionic liquids are substances made entirely of ions that, as a general rule, melt below the boiling point of water. Even more interesting are the ones that are actually liquid at room temperature, called room-temperature ionic liquids (RTILs). The low melting points, in comparison with regular salts, arise from the asymmetry, in terms of size and structure, between the cations and/or the anions. These asymmetries obstruct the orderly packing of the ions and prevent the formation of a crystalline network. An efficient way to achieve this is the use of bulky non-symmetrical ions, which have conformational flexibility and delocalized charge distribution.[1, 2]

The vast majority of ionic liquids share many physicochemical properties that are critical to their popularity as neoteric solvents and to their growing employment in other applications. Among these properties, negligible vapor pressure, low flammability, high thermal and electrochemical stability, high conductivity (ionic and/or thermal), wide electrochemical potential windows and huge liquid range (the span of temperatures between the freezing point and the boiling point) are the most recognizable. As a general rule, these compounds have high viscosities, similar to glycerol. There are, however, several exceptions which possess relatively low viscosities, typically ionic liquids based on dicyanamide or hexafluorophosphate anions. In terms of polarity, this class of compounds, despite their ionic nature, usually displays moderate values, similar to ethanol. Most ionic liquids are soluble in polar solvents, such as water or methanol, and immiscible with nonpolar solvents, such as *n*-hexane or diethyl ether.[2-4]

The popularity of ionic liquids stems not only from their unique physicochemical properties but also from the ability to change and tune these properties according to the needs of the user. Such tailoring is achievable because both the anion and the cation can be modified independently, yielding an unimaginable amount of possible combinations. For example, as stated before, most ionic liquids are immiscible

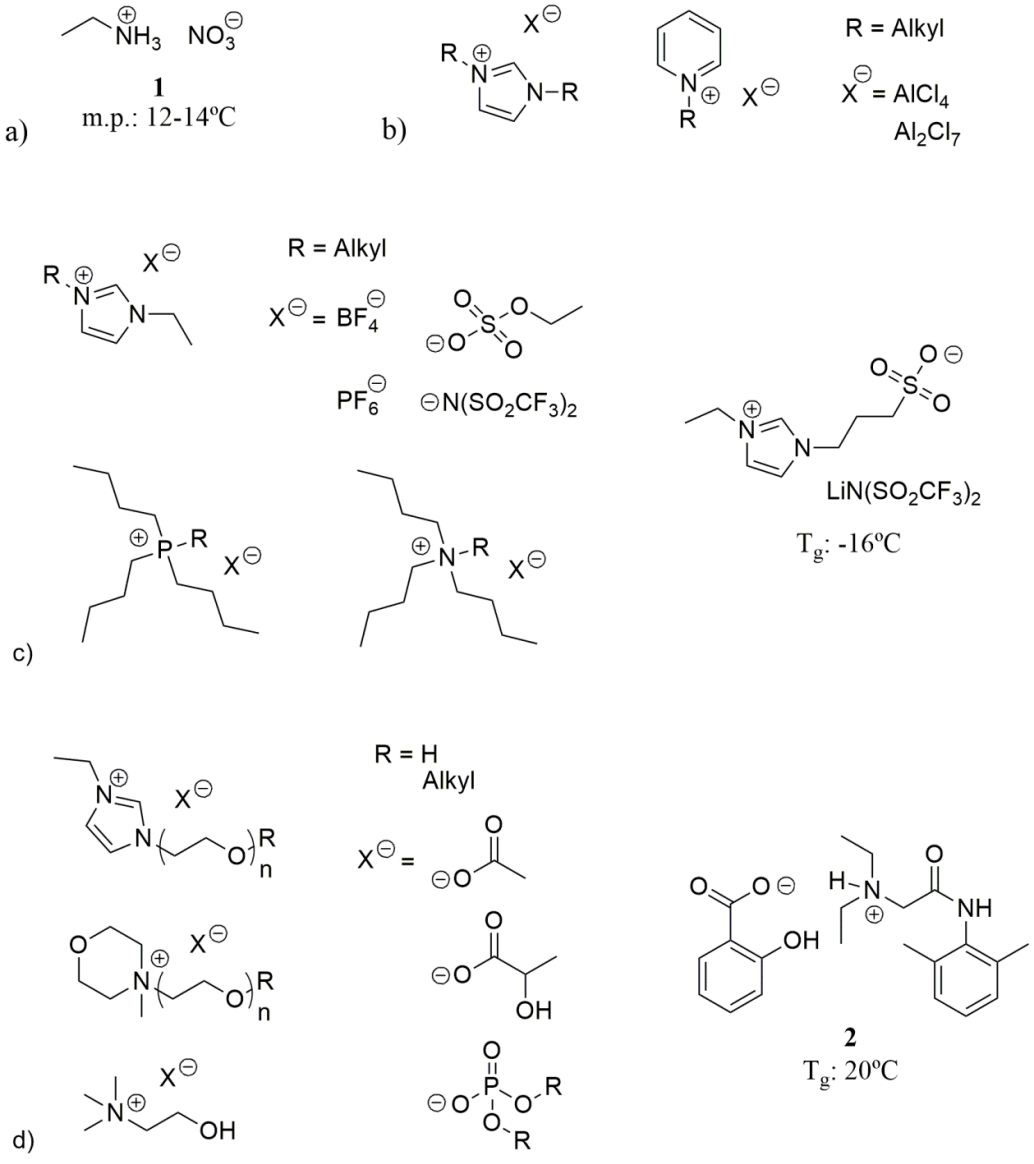
with nonpolar organic solvents, however taking advantage of the ability to vary the cation and/or the anion it is possible to create hydrophobic ionic liquids soluble in organic solvents.[2-4]

1.1.1 A bit of history

Although their history effectively started in 1914, when the properties of ethylammonium nitrate (Scheme 1.1a)) were first described by Walden *et al*,[5] only in the last thirty years of that century did this class of compounds really caught the attention of academics, and the accepted use of the term "ionic liquid" is actually quite recent. Between 1960 and 1990 several studies based on salts of dialkylimmidazolium or alkylpyridinium with metal halides as counter-ions were published (Scheme 1.1b)). Despite their interesting electrochemical properties, the selected anions were air and water sensitive, and had to be handled under inert conditions which severely limited the applicability of these compounds. Later in the 90s, a second generation of ionic liquids was developed (Scheme 1.1c)). The key difference was the use of a wide variety, in terms of structure and functional groups, of counter-anions with greater stability. Common examples are simple halides (Cl^-, Br^-, I^-) and weakly coordinating anions (PF_6, $N(SO_2CF_3)$). Dialkylimmidazolium and alkylpyridinium cations were still prevalent, but new ammonium- and phopshonium-based cations were also explored with success. By then, it was clear that this class of compounds had unmatched versatility and that the studies conducted so far were only a scratch in the surface in terms of possible combinations. This versatility allowed the exploration and exploitation of ionic liquids in countless fields beyond electrochemistry (e.g. biocatalysis), which along with the (premature) label of "green solvents" (due to negligible vapor pressure and low flammability) made their popularity skyrocket.[2-4, 6]

However, in the last decade, the knowledge about ionic liquids has increased tremendously and further attention has been given to their fate at the end of their life cycle. All this information has shown that a good number of elements from this family of compounds are not so "green" as advertised. Many studies have exposed the biodegradability issues of dialkylimidazolium cations, by far the most common type of cation used, which translates into higher bioaccumulation and high toxicity. The actual

synthetic protocols also pose environmental and health problems since most employ strong alkylating agents, such as dialkyl sulfates, sultones or alkyl halides. These alkylating agents are known carcinogenic compounds because they are able to alkylate DNA. Another critical shortcoming for the dissemination of ionic liquids is the high costs associated with their preparation, from the starting materials to the purification methods.[1-3, 7, 8]



Scheme 1.1. Representative structures of each generation of ionic liquids: a) one of the first examples, ethylammonium nitrate was reported in 1914; b) the first generation of ionic liquids employed air and water sensitive chloroaluminate anions; c) the second generation, with sturdier and more appealing anions, was responsible for the explosion in interest and popularity; d) the third generation arose with environmental sustainability and low/known toxicity as foremost features.[1-6, 8-13]

Nowadays the third generation of ionic liquids is in full swing (Scheme 1.1d)). Always with the drawbacks of the previous generations in mind, the latest ionic liquids are composed by low cost, biocompatible anions, such as amino acids, natural carboxylic acids (e.g. lactic acid), and benign cations (e.g. choline). Any building block that does not follow these principles should be redesigned to facilitate its biodegradability at the end of its life cycle. Examples of such engineering are the introduction of alcohols or ester groups, or the replacement of aliphatic chains by polyethylene glycol chains.[2, 3, 8-11]

Of all last generation ionic liquids developed so far the ones that are composed of active pharmaceutical ingredients (APIs) are of particular significance. Despite the considerable amount of APIs that are formulated and sold as a salt, only in the past few years did the employment of counter-ions with useful or complementary properties attract significant interest. The huge amount of possible combinations allows the modulation of the properties of the API-based ionic liquid in order to overcome frequent limitations, namely low solubility in physiological conditions, reduced bioavailability or solid-phase polymorphisms. One remarkable example is compound **2** (Scheme 1.1d)), a RTIL composed by two complementary APIs, salicylic acid (an anti-inflammatory) and lidocaine (a local anesthetic).[13-16]

Ionic liquids as bulk solvent phases, but not restricted to as shown above, offer new opportunities as recyclable alternatives to conventional organic solvents in a growing number of applications (organic catalysis, gas separations, heat transfer fluids, liquid crystals, etc.),[17] most notably for a more sustainable biocatalytic production of commercially important chemicals, with enhancement in many cases of the reaction rate and product selectivity.

1.1.2 Biocatalysis in ionic liquids

Enzymes are nature's catalysts and have been exploited for millennia by Mankind in the manufacture of food and beverage products. With the advent of genetic engineering and advanced biotechnology processes, their use in Industry for the synthesis of important pharmaceuticals, agrochemicals and fine chemicals has steadily increased in the last decades. Due to their excellent catalytic properties, low toxicity,

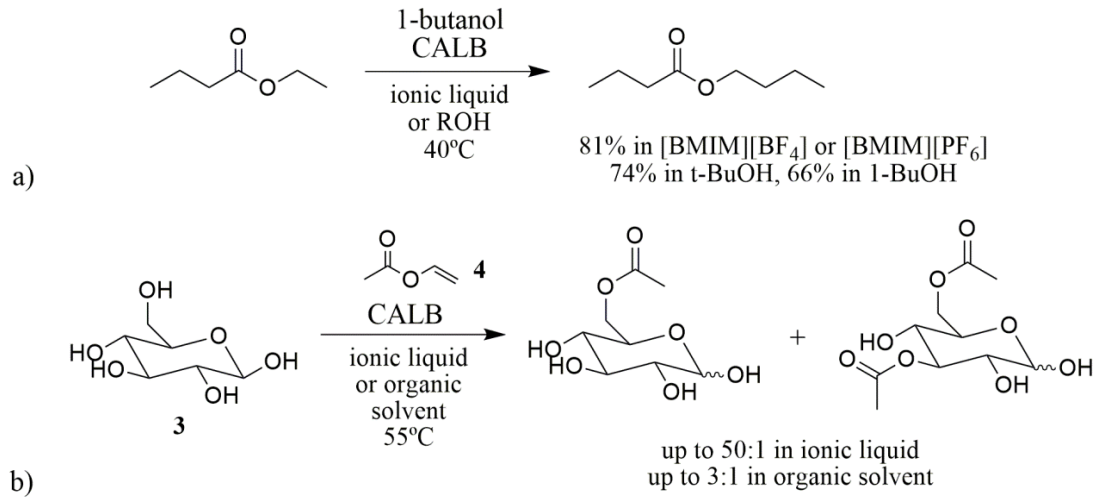
high biodegradability and efficiency under mild reaction conditions, enzymes are viewed as excellent green catalysts. Whilst some enzymes need to be associated with biological membranes to function properly, most enzymes exist in nature in an aqueous environment.[18-20]

In spite of the advantages in using water for biocatalysis, namely natural environment, high abundance and zero environmental impact, this solvent is not appealing for industrial processes. Not many organic compounds are fully soluble in water and they are prone to degradation or to unwanted parallel reactions in aqueous medium. In addition, due to the high boiling point and heat of vaporization of water the cost to remove it from reaction media is extremely high. The use of organic solvents in biocatalysis is one solution to overcome such limitations, with the added benefits of easier enzyme recovery, higher reaction rates and higher yields. Even more important is the broader scope of enzyme-catalyzed reactions available in non-aqueous medium. Such reactions are thermodynamically unfavorable when using water due to competition with hydrolytic processes.[21-23]

Obviously, the use of organic solvents has several drawbacks, such as lower enzymatic activities (when compared with the ones in natural media), and environmental and health issues due to high volatility, flammability and toxicity. As stated before, ionic liquids were heralded as "green solvents" and an alternative to organic solvents exactly because they have negligible vapor pressures and are non-flammable. Their unmatched versatility allows the tailoring of their properties according to the needs of a given process, for example improved solubilization of difficult substrates. In addition, it is known that some anions and/or cations promote enzyme stability, and improve its catalytic activity and its selectivity. All these features have prompted chemists to further explore the combination of enzymes in ionic liquids since it has the potential to provide more sustainable approaches for organic transformations to be carried out.[2, 3, 19, 24, 25]

The year 2000 was full of breakthroughs regarding the exploration of ionic liquids for biocatalysis. In the first successful report, Erbeldinger *et al* described the synthesis of Z-aspartame in [BMIM][PF_6] (5% water content), using the protease thermolysin as biocatalyst.[26] Later that year, Lye *et al* demonstrated the first biotransformation in ionic liquids using whole cells.[27] More prominently, Sheldon *et al* studied the catalytic activity of CALB (free from) in several types of reactions and observed that the results in anhydrous ionic liquids were similar to the ones in organic

solvents (Scheme 1.2a)).[28] Since then, an extraordinary amount of studies have explored the use of lipases in ionic liquids and have shown that these enzymes have particularly good stabilities, high activities and enhanced selectivities in this class of solvents.[19, 29] A clear example of the latter is displayed in Scheme 1.2b). The CALB-catalyzed acetylation of D-glucose (**3**) was significantly more regioselective in ionic liquids than in organic solvents.[30]

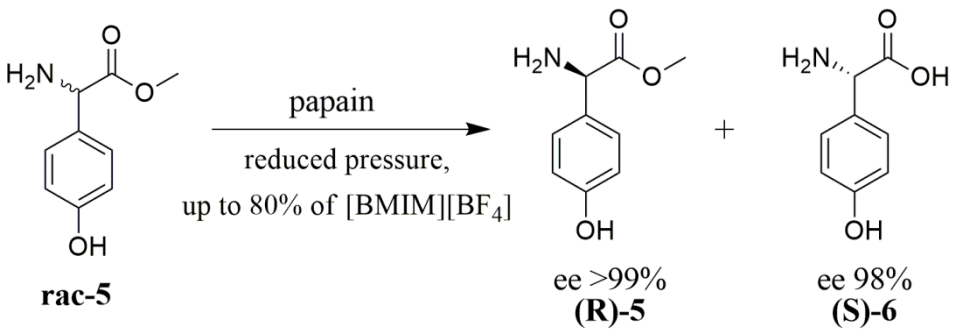


Scheme 1.2. Examples of the application of ionic liquids as non-aqueous solvents: a) transesterification reaction used by Sheldon *et al* to show comparable results between ionic liquids and organic solvents;[28] b) Acetylation of D-glucose (**3**) with vinyl acetate (**4**) catalyzed by CALB. Since the substrate is up to one hundred times more soluble in ionic liquid, the enzymatic acetylation is much more regioselective in that solvent.[30]

The employment of ionic liquids in biocatalysis can be categorized into six types of processes: i) as co-solvents in aqueous solutions; ii) as solvents in biphasic systems; iii) as non-aqueous media; iv) as substrate anchoring point; v) as agents for enzyme coating or encapsulation; vi) ionic liquid-based supported liquid membranes.

In the first case, ionic liquids can be added to aqueous solutions to promote the solubilization of reagents, that otherwise would not dissolve significantly in water, leading to higher reaction rates. The main advantage of replacing organic solvents, such as dimethyl sulfoxide or ethanol, with ionic liquids is that enzymes, generally, have higher activity and/or stability in the latter.[2, 3, 31, 32] Scheme 1.3 shows a typical example of such application. The rate of hydrolysis of the racemic ester **5**, catalyzed by papain, is up to ten times higher when using [BMIM][BF_4] as co-solvent in comparison with using just water.[33]

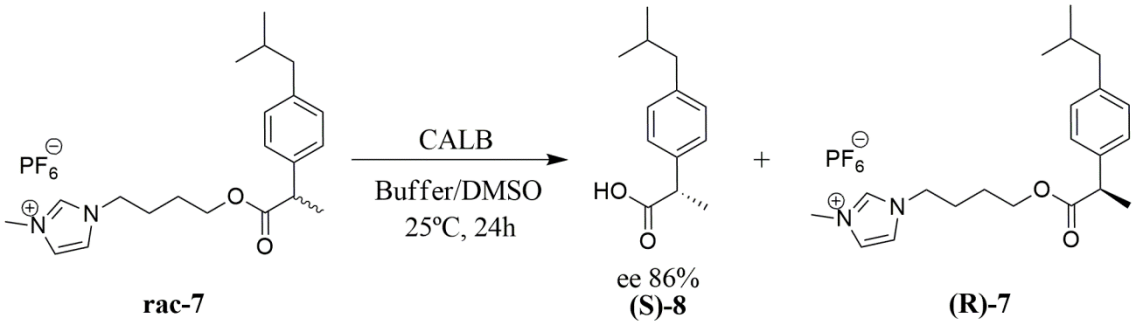
The second group of processes involves the employment of hydrophobic ionic liquids as extraction solvents in biphasic systems. As stated before, ionic liquids possess advantageous properties (low volatility and non-flammability) over common organic solvents. More importantly, in biotransformations using whole cells (some) ionic liquids have the upper hand because they do not affect the integrity of cell membranes, thus preserving cell viability. Another example is the combination of ionic liquids with supercritical fluids. Due to their ionic nature, ionic liquids are not soluble in supercritical fluids, as such the latter can be used to deliver substrates to and/or extract products from the ionic phase. A critical feature of such systems is efficient mass transfer of substrates and products between the supercritical fluid and ionic liquids, which is dependent of the ionic liquid hydrophobicity and viscosity. However these properties can be suitably tuned hence the uniqueness and utility of ionic liquids.[2, 3, 18, 32]



Scheme 1.3. Kinetic resolution of the methyl ester of (p-hydroxyphenyl)glycine (**5**) by hydrolysis in water/[BMIM][BF_4] catalyzed by papain. The water/ionic liquid mixture allows a better solubilization of the substrate, which translates into a reaction rate up to ten times higher than the one observed in water.[33]

The most common strategy is the use of ionic liquids as non-aqueous media (Scheme 1.2). This allows a broad scope of enzyme-catalyzed reactions (e.g. esterification, transesterification or aminolysis) that could not be achieved in the native aqueous media because hydrolytic processes would be thermodynamically favorable. An ample number of ionic liquids have been explored for this type of application, predominantly using hydrolases but also with other types of enzymes and microorganisms. The main advantages over organic solvents have already been mentioned, namely less environmental and health issues, and the fact that, generally, enzymes are more stable in ionic liquids.[2, 3, 18]

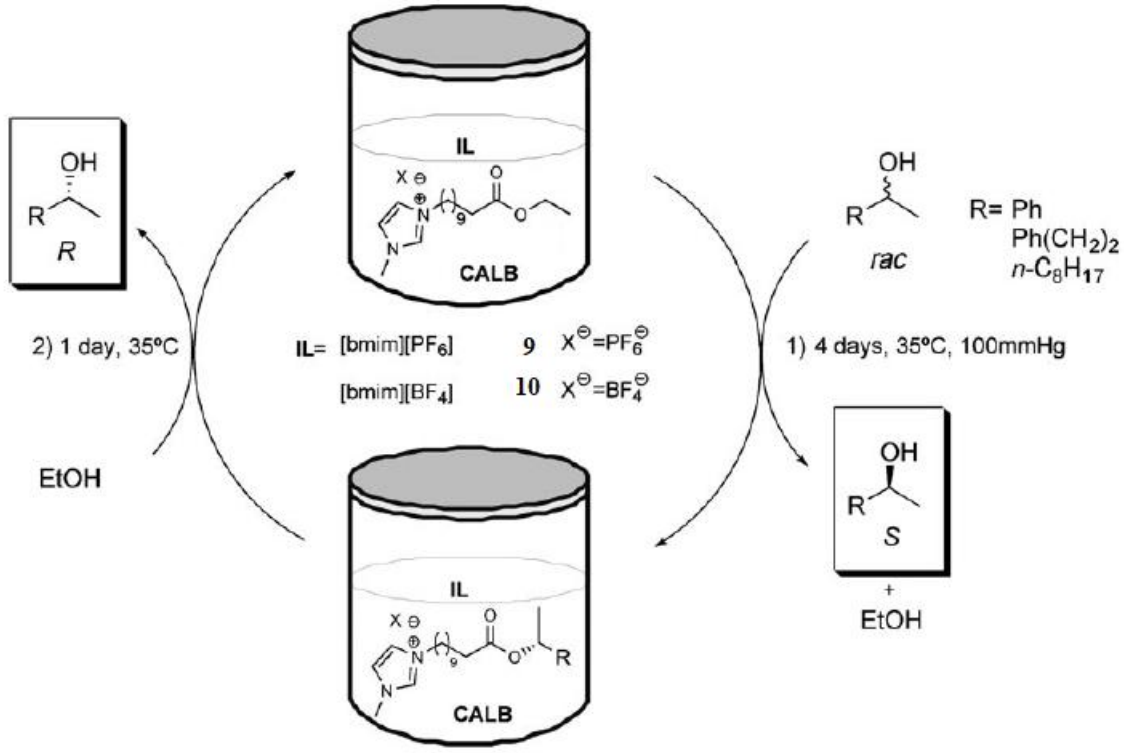
The fourth type of application was developed in order to overcome a key limitation of enzymatic kinetic resolutions, especially when applied to alcohols. Such shortcoming is the separation of the product from the unreacted substrate, usually one as an alcohol and the other as an ester (for more details see section 2.1.4, page 27). Almost always a chromatographic step is required which is a serious limitation at industrial scale. One strategy to tackle such problem is to anchor the substrate directly to an ionic liquid before or during an enzymatic resolution. Naik *et al*, in 2007, were the first to report an example of such strategy.[34] The authors synthesized an ester between racemic ibuprofen and an immidazolium ionic liquid with an alcohol at the end of an alkyl chain (Scheme 1.4). After CALB-catalyzed hydrolysis in buffer/DMSO, the free acid could be removed by simple extraction with diethyl ether since the unreacted enantiomer is still attached to the ionic liquid and is immiscible with the organic solvent. This enantiomer could be recovered after chemical hydrolysis and a second organic extraction. The ionic liquid could then be recovered and reused.



Scheme 1.4. Kinetic resolution of the ionic ester of ibuprofen (**7**) by hydrolysis in buffer/DMSO catalyzed by CALB. The separation between the product (**8**) and the unreacted substrate (**7**) is achievable by simple extraction with diethyl ether since **7** is insoluble in the organic solvent.[34]

In that same year, Lourenço *et al*, described a similar process but for the resolution of secondary alcohols (Scheme 1.5).[35] The authors prepared two ionic liquid with an ethyl ester group at the end of a long alkyl chain. These ionic acylating agents (**9** and **10**) allowed the resolution by transesterification, catalyzed by CALB, of several secondary alcohols. The first step had to be performed under reduced pressure to remove the ethanol byproduct, thus shifting the thermodynamic equilibrium in favor of the products without using a large excess of acylating agent. Similar to the procedure of Naik *et al*, after the first enzymatic reaction the product could be removed by simple extraction with diethyl ether. Both the substrate and the solvent (another ionic liquid)

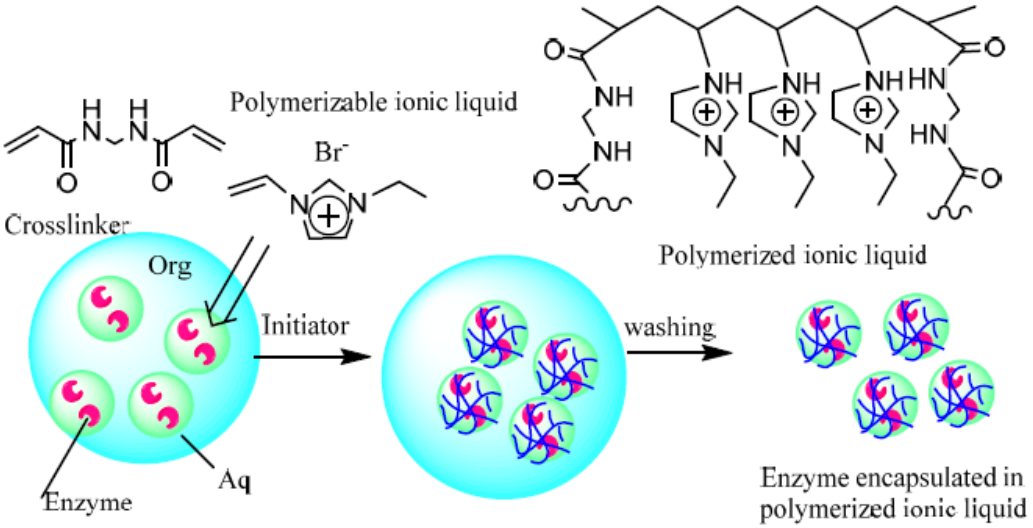
are insoluble in the organic solvent. The other enantiomer was recovered in similar fashion after a second transesterification reaction with ethanol as nucleophile. This way the ionic acylating agent is fully regenerated and can be immediately reused in another cycle.[35, 36]



Scheme 1.5. Methodology described by Lourenço *et al* based on ionic acylating agents (**9** and **10**) for the EKR of secondary alcohols catalyzed by CALB. The products can be separated from the ionic species by extraction with diethyl ether.[35]

The fifth group of methods involves coating the enzyme with ionic liquids (covalently or by adsorption). This modification not only facilitates the recovery of the enzyme from the reaction media but also enhances enzymatic activity and selectivity. Studies suggested that these improvements over native and other commercial coatings/matrixes is due to easier access of the substrate to the more porous ionic liquid-coated enzyme and favorable conformational changes and enzyme flexibility. Encapsulation of enzymes in polymers made by ionic liquids monomers has also been explored (Scheme 1.6). Again, the versatility of ionic liquids is critical since pendant functional groups can be incorporated into the cation or the anion for polymerization. The main goals are to improve enzyme stability, and facilitate its recovery and reutilization.[2, 18]

The final category of strategies takes advantage of ionic liquid-based supported liquid membranes. These materials are composed of a solvent (the ionic liquid) immobilized in the porous structure of a polymeric or ceramic membrane. The objective of supported liquid membranes is to separate a feedstock solvent and a receiving solvent while the immobilized ionic liquid selectively mediates solute transport across the membrane. Application of supported liquid membranes to enzymatic kinetic resolutions allows the conversion and separation of the desired product in a single continuous process, which is very appealing from an industrial and environmental point of view. A make-or-break feature of this type of membranes is a negligible loss of the immobilized solvent to either of the solvent phases and to the atmosphere. The low volatility and the ability to tune the miscibility make ionic liquids a fitting option as immobilized solvent.[2, 18]



Scheme 1.6. Enzyme encapsulation in an ionic liquid-based polymer.[18]

CHAPTER 2

ENZYMATIC KINETIC RESOLUTION OF SECONDARY ALCOHOLS USING AN IONIC ANHYDRIDE GENERATED *IN SITU*

2.1

INTRODUCTION

In the last decades the demand for enantiomerically pure compounds has increased tremendously. This growth has been stimulated mostly by the pharmaceutical industry, due to tighter health and safety regulations that spawned from the rising awareness of the critical role that chirality plays in the biological activity of molecules.

In Nature, most organic compounds are chiral and, usually, only one of the optical isomers is synthesized and incorporated in biological systems. As expected, each enantiomer of given molecule will interact in a singular way with any biological system which translates in different outcomes. In most cases, only one of the isomers induces a response while the other is essentially inert (e.g. vitamin C), however there are examples where both enantiomers have activity but with distinct results (e.g. each limonene isomer has a very characteristic smell). Within this last category, it is of particular importance the cases where the optical isomers have opposite activity, that is, one induces a benign response while the other is toxic. Tragically, we became aware of one of such cases with Thalidomide, a drug used by the end of the 50's decade. Thalidomide was a sedative given to pregnant women that was administered as a racemic mixture. However only after commercialization it was discovered that one of the isomers is solely responsible for the therapeutic effect while the other is a powerful teratogenic agent. In order to avoid similar accidents, nowadays regulatory pharmaceutical agencies strongly advise for the formulation of optically pure compounds, as shown by the data from 2003 when 80% of the drugs approved by the FDA for commercialization contained a single enantiomer. Another driving force for the development of novel methodologies for obtaining enantiomerically pure compounds is the growing need for more efficient and, particularly, more environmentally friendly processes.[37-40]

2.1.1 Preparation of pure enantiomers

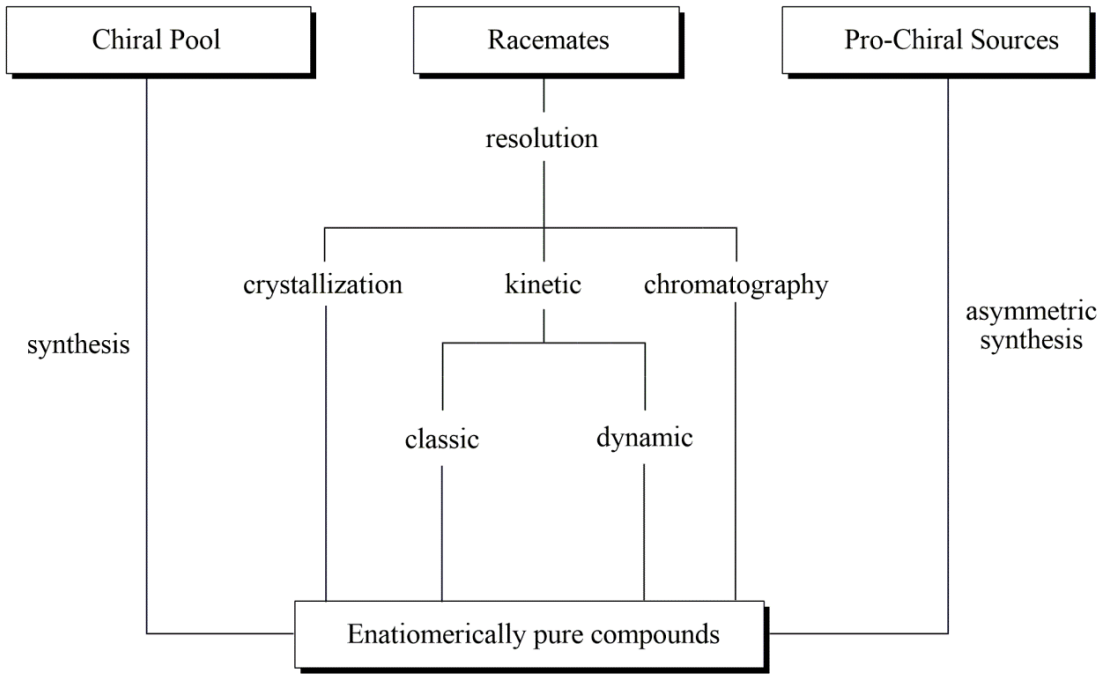
All currently available methodologies for obtaining pure optical isomers can be divided in three main categories (Scheme 2.1): synthesis using the chiral pool as source, asymmetric synthesis starting from pro-chiral compounds and resolution of racemic mixtures.[22, 38]

The first group comprises a wide variety of chiral compounds that occur naturally as pure enantiomers and that can harnessed for subsequent chemical modification(s). Important examples of the chiral pool are sugars, terpenes, alkaloids, vitamins and antibiotics, just to name a few. As stated before, in the vast majority of the cases, only one enantiomer is biosynthesized and, obviously, just that one can be extracted and used subsequently, for example only (-)-morphine is present in Nature. Nonetheless, there are some examples in which both enantiomers are obtainable, that is, when each isomer is produced by different organisms, as is the case of (+)-carvone (from *Carum carvi L.*) and (-)-carvone (from *Mentha spicata*). Within the chiral pool we can include microbial fermentation processes which use low cost substrates to easily obtain chiral products like L-amino acids, penicillins and other antibiotics, and hormones. The major drawback of exploiting the chiral pool is its limited supply, both in terms of quantity and variety, as such in many cases the industrial demands can only be fulfilled through synthetic chemistry.[38, 41, 42]

The second group of methodologies, usually named asymmetric synthesis, consists in the transformation of pro-chiral starting materials into one of the possible enantiomers exclusively or with a very high enantiomeric excess. Typical reactions of this category are reduction of ketones to secondary alcohols,[40, 41] transaminations,[41, 43] reductive aminations,[43, 44] epoxidations,[38, 45] Mannich reactions,[44, 46] among others. Ubiquitous to all these reactions is the employment of chiral catalysts, usually transition metal complexes, since it is their unique geometry that dictates which enantiomer is created. When both enantiomers are on demand, one catalyst for each isomer needs to be developed. Taking this in account the major shortcomings of asymmetric chemistry are the cost, complexity and sustainability of current catalysts. Possible alternatives are iron catalysts (one of the more abundant elements in Earth's crust), organic catalysts (due to their availability and low toxicity), or biocatalysts (the library of available

enzymes grows as we speak and the typical reaction conditions are milder and more environmentally friendly).[22, 38, 40, 41, 46-48]

The third group is based on processes that try to separate the optical isomers of racemates, that is, equimolar mixtures of two enantiomers. The separation is possible due to distinct physical and/or chemical interactions between each enantiomer and a specific chiral environment. Since the starting point is a binary mixture almost all of these procedures have the intrinsic disadvantage of a maximum theoretical yield of 50% for each enantiomer. However, in particular when both enantiomers are necessary, the resolution of racemates is still advantageous over asymmetric chemistry because is simpler (only one catalyst for each pair of isomers) and considerably less expensive (both in terms of catalyst and starting materials). Taking in account the techniques used in the actual separation of racemates, this large family of methodologies can be divided in three subsections: resolution by crystallization, separation with chromatographic techniques and kinetic resolution.[22, 23, 38]



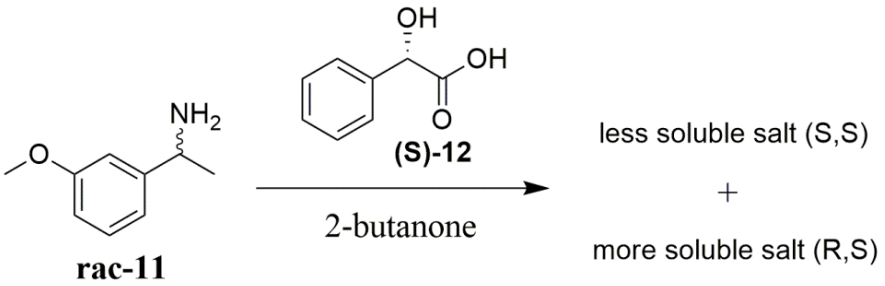
Scheme 2.1. Key starting materials and methodologies for the preparation of pure enantiomers.[22, 38]

2.1.2 Resolution processes

2.1.2.1 Crystallization

One of the oldest methods of resolution is, without doubt, the separation by crystallization. In 1848, Louis Pasteur was the first to obtain enantiomerically pure tartaric acid, after crystallization of a racemic mixture of salts of this acid. Depending on the crystallization behavior of the racemate, the protocol can be classified as direct preferential crystallization or as diastereomeric crystallization. The former applies when the starting mixture forms conglomerates that are optically pure crystals. This property can be harnessed to grow highly pure crystals of one enantiomer when a supersaturated solution of a racemate that forms conglomerates crystallizes spontaneously or by seeding with the same enantiomer. After filtration, the other isomer, present in the remaining solution, can be crystallized after concentration and seeding if necessary. As drawbacks the laborious process is also preceded by an exhaustive optimization of the experimental conditions which, throughout the crystallization, must be careful controlled. Unfortunately, the use of direct preferential crystallization is very limited since less than 20% of all racemates crystallize as conglomerates.[23, 39, 49, 50]

For "true" racemic mixtures, that is, racemates that form crystals that are also a mixture of enantiomers, we must employ diastereomeric crystallization. In this process, the enantiomers are converted in diastereoisomers after a reaction with a resolving agent or a known mixture of resolving agents. The resolving agents are optically pure enantiomers which can be linked to the starting mixture by covalent bonds, by forming complexes or by forming a salt. The obtained diastereoisomers, unlike the starting enantiomers, have distinct physicochemical properties between each other, namely different solubilities in a given solvent and, as such, can be separated by crystallization. After separation and removal of the resolving agent the pure enantiomers are recovered. Similarly to the previous case the process is laborious and the screening of resolving agents and/or solvents can be complex and time-consuming.[23, 39, 41, 49, 50] An example of a diastereomeric crystallization is displayed in Scheme 2.2. Racemic-1-(3-metoxiphenyl)ethylamine (**11**) can be resolved after an acid-base reaction with enantiomerically pure mandelic acid (**(S)-12**) forming a pair of diastereoisomers. The (S,S) salt is less soluble in 2-butanone and quickly precipitates.[51]



Scheme 2.2. Resolution of racemic 1-(3-metoxiphenyl)ethylamine (**11**) by diastereomeric crystallization, using (S)-mandelic acid (**12**) as resolving agent and 2-butanone as solvent. The (S,S) salt is considerable less soluble than the (R,S) diastereoisomer, so the former precipitates after a few minutes.[51]

2.1.2.2 Chiral chromatography

Another way to resolve racemates is through chromatographic techniques, such as gas chromatography, supercritical fluid chromatography, capillary electrochromatography, and liquid chromatography, the last of which includes thin-layer chromatography and simulated moving bed chromatography. They are based on separating systems with stationary chiral phases, usually composed of polysaccharide derivatives like cellulose or cyclodextrins. Due to the chiral nature of the stationary phase each enantiomer will interact differently with it, leading to different affinities for each stationary phase/eluent setting and, concomitantly, different retention times. The key advantages of these techniques are high selectivity, simplicity and quickness of the method (particularly when no derivatization is necessary). For some of the techniques, namely simulated moving bed chromatography and supercritical fluid chromatography there is the possibility to perform the separation in continuous flow, thus allowing a continuous process operation in a preparative scale. They are, however, considerably expensive and complex. Taking into account that the results of a given synthetic protocol are usually confirmed by an analytical chromatographic technique (HPLC and/or GLC), the data collected (like selectivities, solubilities, among others) at laboratory scale can speed up the scale-up process. However the scale-up rarely occurs in such a peaceful manor because it needs large quantities of low-cost, robust stationary phases, and the optimization of the system parameters and eluting system, in order to

obtain the best selectivity on the least amount of time, can be complex and cumbersome.[37, 52-54]

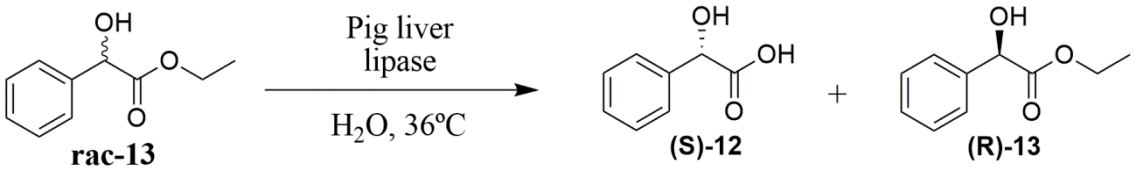
An alternative to traditional chromatography is membrane-based resolution processes. The key advantages of membrane technology are its high efficiency, low energy usage, simplicity, convenience for up- and/or downscaling, and continuous operability. Membrane-based chiral resolution can be achieved using either enantioselective or non-enantioselective membranes. Enantioselective membranes are able to separate optical isomers because they contain chiral recognition sites (e.g. chiral side chains, chiral backbones, or chiral selectors). They act as selective barriers in the resolution process, and selectively transport one enantiomer due to the stereospecific interaction between the enantiomer and the chiral recognition sites, thereby producing a permeate solution enriched with one enantiomer. The different binding affinities of two enantiomers may be the result of different hydrogen bonding, hydrophobic, Coulombic, van der Waals interactions and steric effects with the chiral sites. The non-enantioselective membranes have no enantioselectivity themselves and often assist in the separation of enantiomers, for instance, by acting as ultrafiltration membranes. Therefore, non-enantioselective membrane-assisted processes are generally combined with other chiral recognition approaches such as enzymatic kinetic resolution or systems using chiral selectors as complexing agents.[18, 53, 54]

2.1.2.3 Kinetic resolution

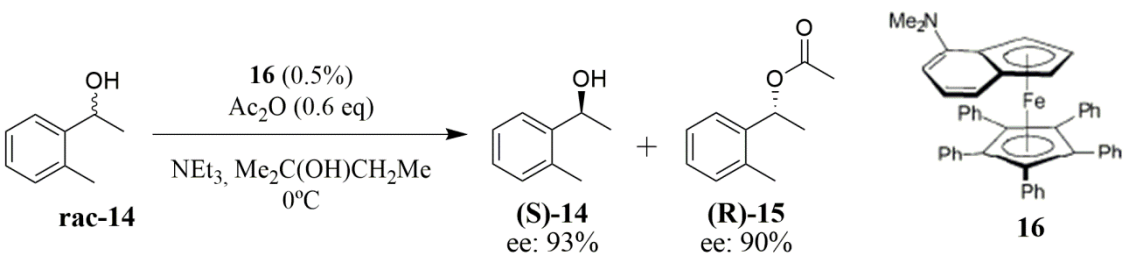
The first kinetic resolution dates back to 1858, when the famous chemist Louis Pasteur observed that the fungus *Penicillium glaucum* consumed faster the natural isomer (R;R) of tartaric acid than the (S,S) isomer. The first resolution with a lipase was performed in 1903 by H. Dakin, who reported the enantioselective hydrolysis of esters of mandelic acid catalyzed by pig liver lipase (Scheme 2.3). The first case of a non-enzymatic resolution was reported in 1899, when Marckwald and McKenzie performed the esterification of racemic mandelic acid (**12**) with optically active (-)-menthol. They observed that the (R)-ester is formed faster than the (S)-ester, which could be isolated from the unreacted (S)-mandelic acid by extraction. Yet only in this century have non-enzymatic processes become efficient enough to attract more attention from both academic and industrial communities. Despite the growing number of examples of non-

enzymatic methodologies (Scheme 2.4), they are less common in comparison with enzymatic ones. The latter benefit from the wide variety of available biocatalysts at reasonable costs, have oustanding enantioselectivities and are performed under milder and "greener" reactional conditions.[22, 23, 41, 55-57]

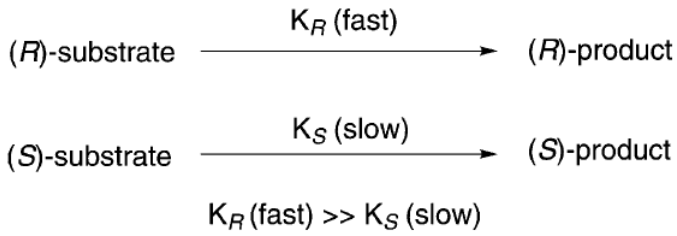
Kinetic resolution is any process in which the enantiomers of a racemic mixture are transformed by a chiral agent into products at different reaction rates (Scheme 2.5). Commonly the chiral agent is a biocatalyst, e.g. a lipase, but it can also be a chemical catalyst, such as a metallic complex. The interaction of the chiral agent with each enantiomer of the starting binary mixture results in two transitions states with distinct configurations and, subsequently, with different associated energies. The more stable configuration establishes which enantiomer has the highest reaction rate while the gap between the associated energies determines the difference in reaction rates and the respective ratio of products. To be considered efficient a kinetic process must have a substantial difference in reaction rates, which means that one isomer must be completely transformed while the other remains practically untouched. In theory, by the end of the process each enantiomer is obtained with a maximum yield of 50%, which is a considerable shortcoming when only one of the isomers is required. Nonetheless, classic kinetic resolution is one of most important methodologies in industry, due to its simplicity, low cost, robustness and applicability to most functional groups.[21, 23, 55]



Scheme 2.3. Enzymatic kinetic resolution by hydrolysis of racemic ethyl mandelate (**13**) catalyzed by pig liver lipase.[58]



Scheme 2.4. Kinetic resolution by acetylation of 1-(2-methylphenyl)ethanol (**14**) catalyzed by an iron planar-chiral complex (1**6**).[59]



Scheme 2.5. In conventional kinetic resolution a chiral agent transforms one of the enantiomers of a racemic mixture into a product while the other isomer remains unscathed. The higher the difference between the reaction rates the more efficient a kinetic resolution is.

In a kinetic resolution there are two key parameters to evaluate the efficiency of the process and the purity of substrates and products: the enantiomeric excess (ee) and the enantiomeric ratio (E). The enantiomeric excess (Equation 1) varies between 0-100% and describes the amount of each enantiomer in a sample. For example, an ee of 99% means that the compound has 99.5% of one enantiomer and 0.5% of the other. The conversion (Equation 2) and the enantiomeric ratio (Equation 3) are calculated using the enantiomeric excesses of substrates and products. The kinetic parameter E can be established as a relative proportion of the rate constants of the two enantiomers at the initial stage of the reaction. In practice, it is a classification of the enantioselectivity of the process. Reactions with values lower than 15 have very low enantioselectivity, between 15 and 200 are somewhat enantioselective and, ideally, above 200 are considered very efficient resolutions, that is, each enantiomer was isolated with very high ee.

$$\%ee = \frac{|R-S|}{|R+S|} \times 100$$

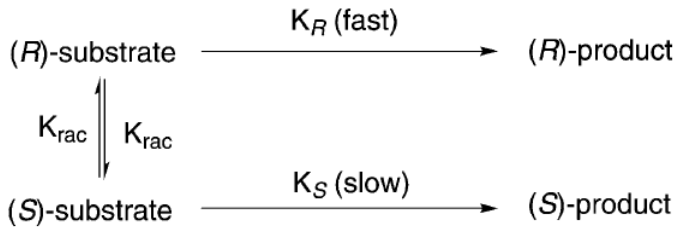
Equation 1. Formula used to determine the enantiomeric excess (ee). R: concentration of the (R)-enantiomer; S: concentration of the (S)-enantiomer.

$$c = \frac{ee_S}{ee_S + ee_p} \times 100$$

Equation 2. Formula used to determine the conversion (c) of the reaction. ee_s: enantiomeric excess of the substrate; ee_p: enantiomeric excess of the product.

$$E = \frac{Ln[\ 1 - c\ (1 + ee_P)]}{Ln[\ 1 - c\ (1 - ee_P)]}$$

Equation 3. Formula used to determine the enantiomeric ratio (E). c: conversion; ee_p: enantiomeric excess of the product.



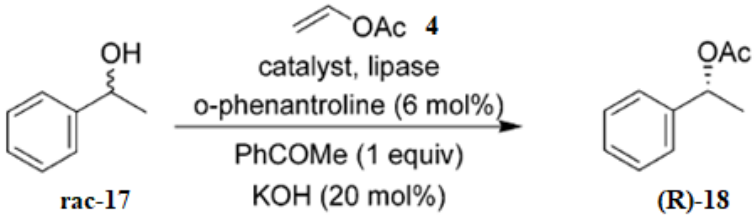
Scheme 2.6. The dynamic kinetic resolution is an evolution of the conventional kinetic resolution, to which an *in situ* racemization process is added. As the reaction takes place, there is a thermodynamic imbalance between the two isomers, which is compensated by the racemization of the less reactive enantiomer. As such, a maximum theoretical yield of 100% is possible.

As stated before, the kinetic resolution of racemates is extremely convenient when both enantiomers are on demand. However, frequently that is not the case, which means that the maximum achievable yield will be 50% even before anything is done. In order to avoid the waste of 50% of the starting material, after separation of the enantiomers, the undesired isomer must be stereoinverted (e.g. Mitsunobu reaction) or repeatedly racemized and resolved. This shortcoming is particularly costly for complex and high-value substrates, such as those employed in the pharmaceutical industry. For this reason more efficient methods have been developed, being the dynamic kinetic resolution the most important one. In this methodology, the classic kinetic resolution is combined with an *in situ* step of racemization (Scheme 2.6). As the reaction takes place, one of the enantiomers is quickly transformed into a product while the other remains untouched. In order to equalize the thermodynamical equilibrium between the starting isomers, the one that doesn't react is racemized continuously. If the rate of racemization is higher than the reaction rate of the less reactive enantiomer is possible to convert the starting racemate into only one of the enantiomers, that is, a maximum theoretical yield of 100%. For a dynamic kinetic resolution process to be successful i) the kinetic resolution must have a very high enantiomeric ratio, ii) the racemization conditions and

kinetic resolution conditions must be compatible with each other and iii) the reaction product must be inert to the racemization conditions in order to avoid a decrease of the optical purity.[22, 55, 60-62]

The adequate racemization technique depends to a large extent on the target compound. These techniques can be organized in several categories: base-catalyzed racemization, acid-catalyzed racemization, Schiff base-mediated racemization, racemization via reduction-oxidation reactions, racemization via radical reactions, thermal racemization or enzyme-catalyzed racemization. Generally the racemization step is carried out using a metallic complex as catalyst, however they have some limitations namely: they need high reaction temperatures which may lead to unwanted byproducts or can be incompatible with the biocatalysts commonly used as chiral agents; they are composed of rare transition metals which are very expensive and have issues about their sustainability and toxicity. A better alternative is the enzymatic racemization, that is, employing racemazes (EC 5.1.X.X) under milder conditions. Unfortunately, at the moment, the library of racemases is still quite narrow which hampers their exploitation. As an example, there is no available racemase capable of racemization of alcohol groups.[22, 55, 61-63]

The first chemoenzymatic dynamic kinetic resolution was accomplished in 1996 by Williams and co-workers. They performed the resolution of racemic 1-phenylethanol using a lipase for the acyl-transfer reaction, rhodium/o-phenantroline as catalyst for the racemization and a ketone as hydrogen mediator (Scheme 2.7). One year later was reported by Backvall and co-workers a notable catalytic system for the dynamic kinetic resolution of 1-phenylethanol and indan-1-ol based on the combination of Shvo's complex as a racemization catalyst and a lipase for the kinetic resolution step. These promising findings were the driving force for the development and application of more efficient dynamic kinetic methodologies, even at an industrial level.[62]

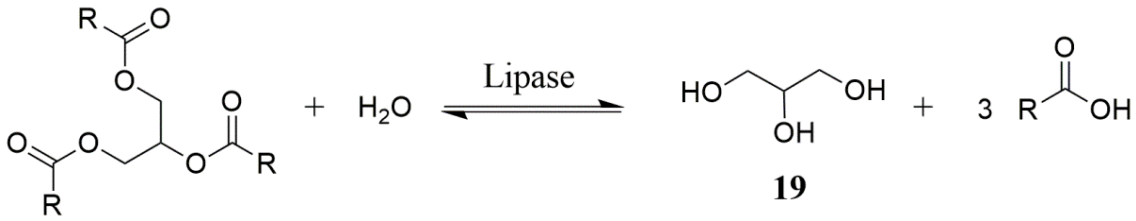


Scheme 2.7. Dynamic kinetic resolution of 1-phenylethanol (**17**) using lipase as acyl-transfer agent, vinyl acetate (**4**) as acylating agent, rhodium/o-phenantroline as racemization catalyst and acetophenone as hydrogen mediator.[62]

2.1.3 Lipases

Within enzymatic kinetic resolution processes the use of lipases clearly stands out from the pack. This subfamily of enzymes is the most commonly employed since it is particularly suited for the resolution of molecules with alcohols, carboxylic acids and its derivatives, or amines, which are key functional groups in organic chemistry.[21, 23]

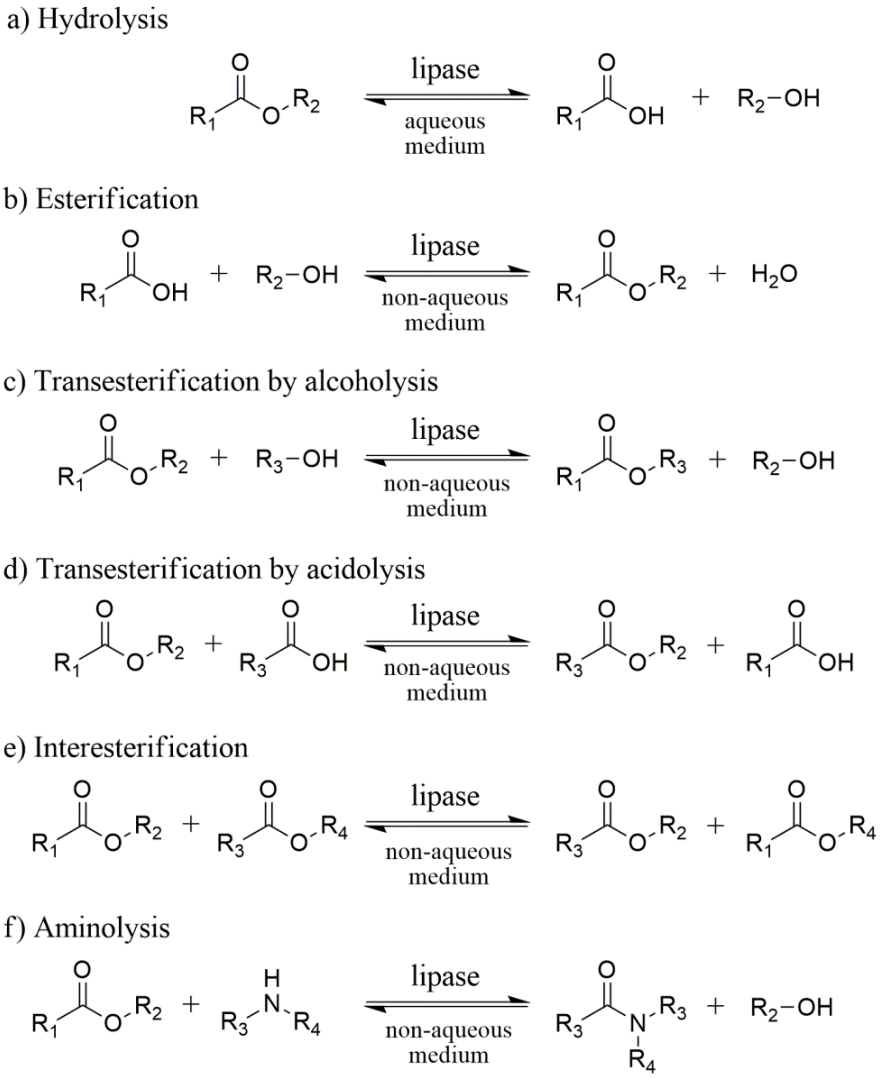
Lipases (EC 3.1.1.3), as esterases (EC 3.1.1.1), belong to the serine hydrolases family. The main difference between them is their natural substrates: esterases accept hydrophilic compounds while lipases have higher activity for triglycerides. In biological systems lipases catalyze the digestion of triglycerides into glycerol (**19**) and fatty acids at the water-oil interface (Scheme 2.8). In organic synthesis they can be used for a variety of reactions, depending on the reactional conditions employed. In aqueous medium they catalyze the hydrolysis of esters (and amides) into carboxylic acids (Scheme 2.9a)), in much milder conditions than a chemical protocol. Since the hydrolysis reaction is reversible, in non-aqueous systems these enzymes can be exploited for the synthesis of esters (and amides), by esterification, transesterification or interesterification (or aminolysis) (Scheme 2.9b-f)).[22, 23, 60, 62]



Scheme 2.8. Hydrolysis of triglycerides into glycerol (**19**) and fatty acids catalyzed by lipases at the water-oil interface. The hydrolysis reaction is reversible, as such in non-aqueous medium lipases can be used for the synthesis of esters.

Lipases are the biocatalyst of excellence for kinetic resolution processes because: i) they are available in high quantities and at low cost; ii) they are particularly stable in organic solvents; iii) can be used in free form or, more frequently, immobilized in a variety of matrixes, aiding their recovery and reuse, and increasing its stability and robustness under extreme conditions; iv) don't need any co-factors; v) have good to exceptional enantionselectivities under mild conditions. It should be pointed out that the

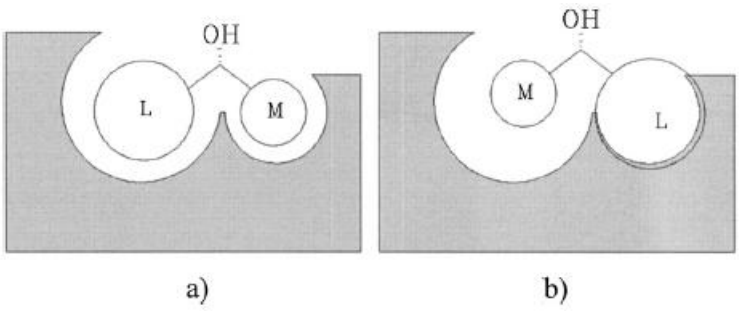
use of this subfamily of enzymes in other synthetic methods keeps growing because they easily accept non-natural substrates and catalyze alternative reactions (Scheme 2.9.f), a phenomenon termed catalytic promiscuity.[22, 23, 60, 62]



Scheme 2.9. Most common reactions catalyzed by lipases: a) hydrolysis of esters (in aqueous medium); b) esterification of carboxylic acids (in non-aqueous medium); c) transesterification by alcoholysis; d) transesterification by acidolysis; e) interesterification; f) aminolysis.

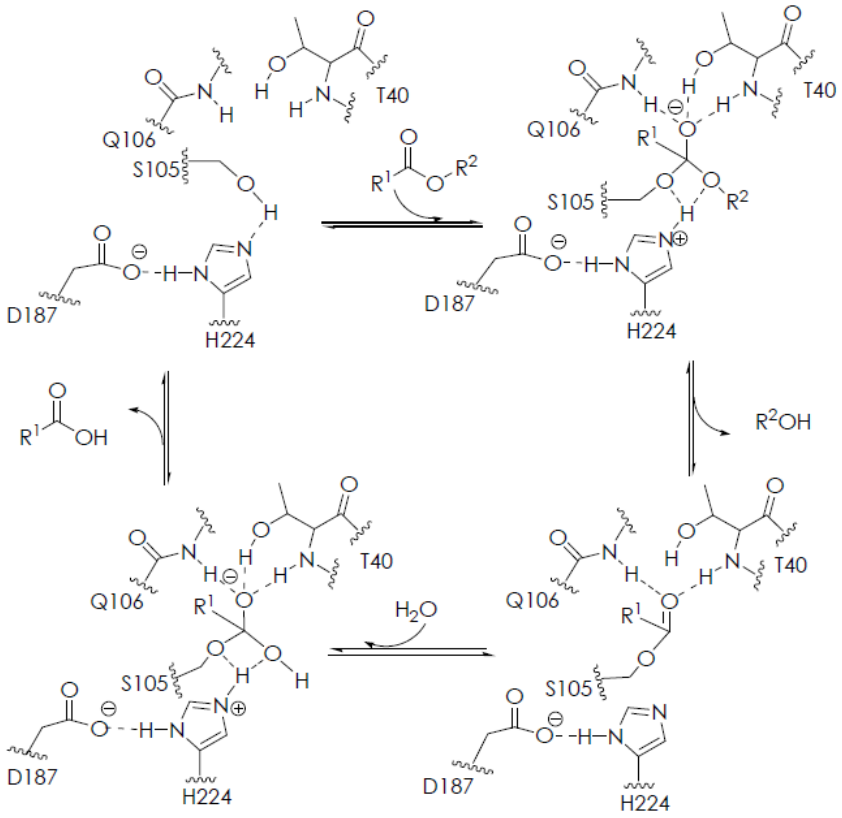
Currently, the most important member of the lipase subfamily is *Pseudozyma antarctica* lipase B (PALB), formerly known as *Candida antarctica lipase B* (CALB) before reclassification in 2013. It universally used both in academy and in industry. It is a very sturdy enzyme, available at low cost, which is selective in many levels and accepts distinct natural and unnatural substrates. PALB remains active between pH of 3.5 and 9.5 and its denaturation occurs at temperatures between 50 and 60ºC,

conditional of the pH. In terms of selectivity, it is a particularly valuable enzyme due to its chemoselectivity, regioselectivity and enantioselectivity. Chemoselectivity is defined as the ability to distinguish between different functional groups in a molecule and react first with just one of them. PALB, for example, reacts more quickly with alcohols than with thiol groups. Regioselectivity is defined as the ability to react mainly with one of several functional groups of the same type according to their position in a certain molecule. PALB performs transacylations in sugars only in the primary alcohol leaving the more abundant secondary alcohols untouched. Finally, but not least, enantionselectivity is defined as the ability to recognize preferably one enantiomer over the other. PALB owns outstanding enantioselectivity which is due to its restrictive pocket for interaction with secondary alcohols in the active site (Scheme 2.10).[64-67]



Scheme 2.10. The fast reacting enantiomer a) and the slow reacting one b) in the active site model for lipases. M: medium-sized substituent, L: large substituent.[22, 23]

As a member of the serine hydrolases, the active site of PALB comprises a serine (S105), a histidine (H224) and an aspartic acid (D187), the so called catalytic triad. This enzyme acts according to a ping pong bi bi mechanism (Scheme 2.11). First the ester group enters the active and is activated by the T40 and Q106 amino acids in the oxonium pocket. Then the catalytic triad goes into action with the serine, activated by the histidine and the aspartic acid, attacking the substrate activated carbonyl group and forming a tetrahedral intermediate. After release of the alcohol the acyl-enzyme is formed. In order to regenerate the biocatalyst, a molecule of water binds to the acyl-enzyme originating a new tetrahedral intermediate, which then breaks to liberate the enzyme from the carboxylic acid. Alternatively, in non-aqueous medium, a transesterification (or aminolysis) can occur if other nucleophile is present, such as an alcohol (or an amine).[62, 64]



Scheme 2.11. General model for the hydrolysis of an ester, catalyzed by PALB according to a ping pong bi bi mechanism. This mechanism comprises two tetrahedral intermediates and an acyl-enzyme intermediate.[62, 64]

2.1.4 Enzymatic kinetic resolution of alcohols

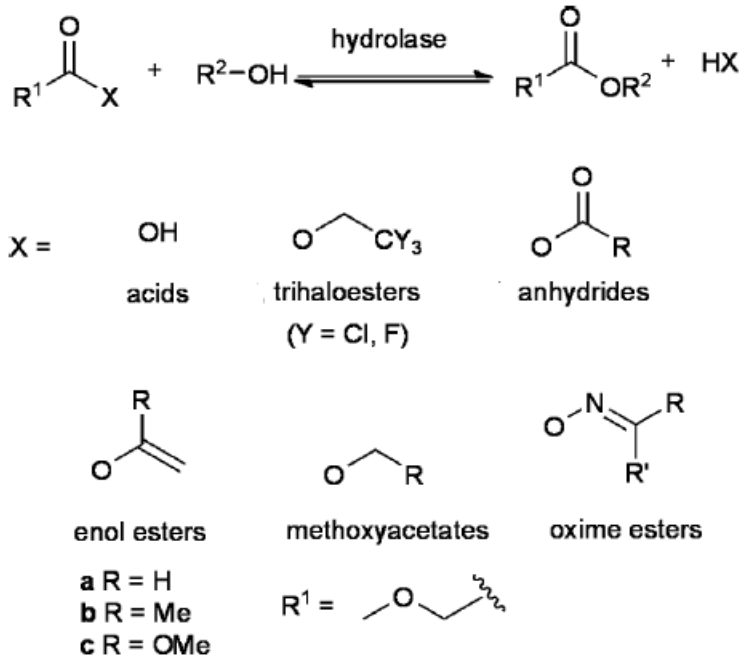
Enantiomerically pure alcohols are very important in synthetic chemistry because they are key intermediates in the production of fine chemicals, pharmaceutical compounds or agrochemicals. The most common strategies for obtaining optically pure alcohols are enzymatic resolution, ketone reduction, epoxide resolution and fermentation. Enzymatic kinetic resolution is usually the most inexpensive and practical method for production on an industrial scale, particularly if both enantiomers are necessary. A major advance in biocatalysis involving alcohols occurred when, in 1984, Klibanov *et al* explored the application of lipases in organic medium. In such conditions, the acyl-enzyme intermediate may be subjected to nucleophilic attack by different nucleophiles, such as alcohols, amines and thiols. These findings were extensively explored for the preparation of enantiomeric pure alcohols, primary,

secondary or even tertiary alcohols. Secondary alcohols are by far the most frequently used targets in lipase-catalyzed resolutions. This is due to their importance in organic synthesis but also since lipases usually show much higher enantioselectivity in their resolution when compared to primary and tertiary alcohols. This can be explained by a more efficient enantiomeric recognition by the enzyme of the hydroxyl group bonded to the chiral center. In primary alcohols, the reaction at the hydroxyl group is more distant from the stereocenter, decreasing the interaction of the latter with the active site. The efficiency of resolution of tertiary alcohols is affected by their lower reactivity due to steric hindrance.[23, 29, 64]

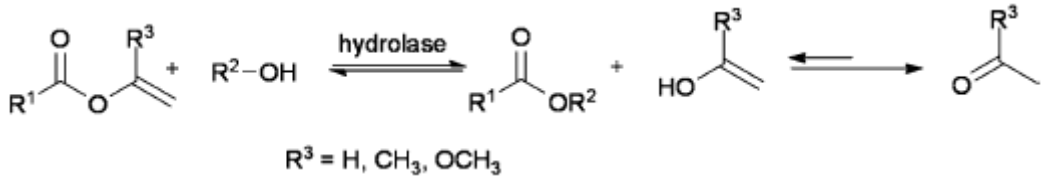
Of the many reactions catalyzed by lipases (Scheme 2.9), secondary alcohols are commonly resolved by transesterification using an acylating agent in non-aqueous medium and, to a less extent, by hydrolysis of a respective ester in aqueous medium. In both cases, one enantiomer is isolated as an alcohol and the other as an ester. The reagents and conditions of the transesterification reaction must be carefully chosen in order to achieve a good conversion. Since the reaction is reversible the leaving group of the acylating agent may attack the newly formed ester (the backward reaction), if is not removed somehow.[23, 68, 69]

Several acylating agents have been developed to help shift this equilibrium to product formation (Scheme 2.12), but the most useful are acylating agents who render the acylation reaction irreversible, such as enol esters and carboxylic anhydrides.[68] In the case of enol esters, the leaving group quickly tautomerises to the more stable ketoform, thus no nucleophile remains in solution (Scheme 2.13). With anhydrides, not only the acid that is released after acylation is a weak nucleophile but more importantly the reformation of the anhydride is entropically unfavorable.

Nonetheless, there are still some limitations, which require the development of more cost-effective and environmentally friendly acylating agents. Common drawbacks are the need of a large excess of acylating agent to achieve good conversions, formation of reactive byproducts that may deactivate the biocatalyst and the generation of toxic and/or explosive byproducts (e.g. vinyl acetate (**4**)). Also, as stated before, at the end of the kinetic resolution one enantiomer is isolated as an alcohol and the other as an ester, which usually requires a laborious separation. Typically this separation involves a chromatographic step, which is especially undesirable for large-scale processes.[68-70]



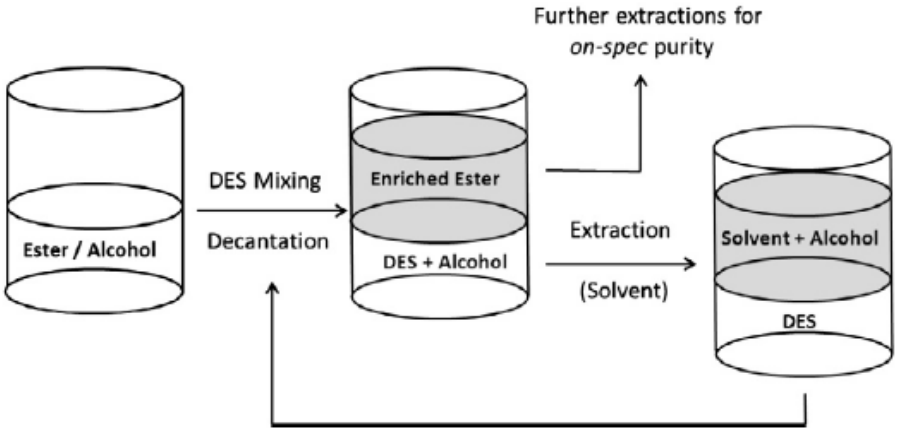
Scheme 2.12. Main acylating agents described in literature.[68]



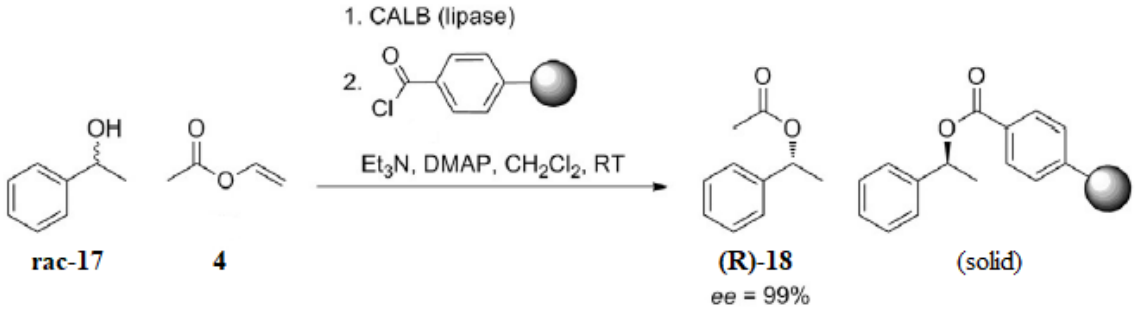
Scheme 2.13. Enol esters as acylating agents and the tautomerization that follows after the release of the leaving group rendering the acyl transfer reaction irreversible.[68]

Numerous alternative methods have been reported to overcome such drawbacks, such as the use of nonconventional solvents (e.g. fluorous solvents,[71, 72] ionic liquids (Scheme 1.5),[32, 35, 73, 74] eutectic mixtures (Scheme 2.14),[70] poly(ethylene glycols),[75] and supercritical CO_2[32, 74-76]), polymer-supported substrates[77, 78] or polymer anchoring to products or unreacted starting materials (Scheme 2.15),[78-80] and distillation processes.[81]

Despite the success achieved using these methodologies, there is still room for improvement. These methods are based either on expensive/complex reaction medium (e.g. ionic liquids, fluorous-phase solvents), or technically complex and energy-demanding processes (e.g. distillation, supercritical CO_2), or non-recyclable reaction medium and/or acylating agents.



Scheme 2.14. Concept for the deep-eutectic solvent (DES) based extraction. After the enzymatic kinetic resolution, the obtained ester and alcohol mixture was extracted with a DES. These solvents can efficiently dissolve molecules containing hydrogen-bond-donors (e.g. alcohols), while esters remain as a second phase.[70]

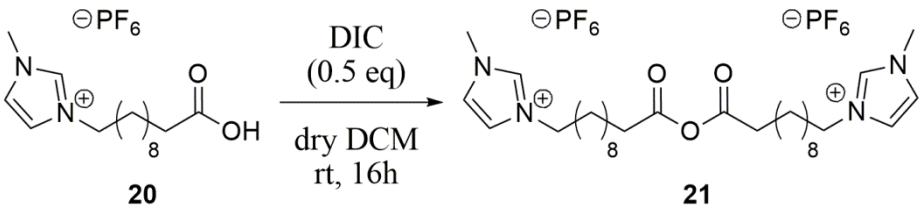


Scheme 2.15. After the EKR, a solid polymer support was used as scavenger of unreacted (S)-enriched enantiomer while the ester remains in solution.[80]

2.2

RESULTS AND DISCUSSION

In a previous work, our group described the synthesis of an ionic liquid bearing an anhydride moiety (**21**), derived from the acid 1-(10-carboxydecyl)-3-methylimidazolium hexafluorophosphate (**20**) (Scheme 2.16), as well as the application of this novel acyl donor on the enzymatic resolution of secondary alcohols.[82] The use of **21** combines the advantages of using anhydrides as acylating agents, specifically the irreversibility of the reaction given that the backwards reaction is unfavorable, with the advantages of task-specific ionic liquids, such as distinct solubility in different organic solvents. Since all ionic species obtained after the enzymatic acylation, namely (*R*)-enriched ester, liberated ionic acid and unreacted anhydride, are insoluble in several organic solvents the untouched (*S*)-enriched alcohol can be easily separated from the ionic entities by means of a simple organic extraction. Hydrolysis of the ionic ester followed by a second extraction allows the recovery of the other enantiomer.



Scheme 2.16. Coupling reaction between ionic acid **20** and diisopropylcarbodiimide (DIC) to give ionic anhydride **21**.

Nevertheless, the method has some inconveniences, namely the time consuming anhydride synthesis/purification before each EKR, and in some cases the need to prepare fresh anhydride in order to minimize potential degradation. Therefore, we explored methods for a practical regeneration of the ionic anhydride **21**. In the following sections we describe our findings regarding to the *in situ* preparation of the ionic anhydride, from the respective acid and a dehydrating agent (diisopropylcarbodiimide),

directly in the EKR reaction medium containing the biocatalyst PALB. Based on these findings, a method for the enzymatic resolution of secondary alcohols using the *in situ* generated **21** as acylating agent was successfully developed and optimized.

2.2.1 Study and comparison of EKRs using *in situ* generated anhydride versus other acylating agents

In the search of a more convenient regeneration protocol for the ionic anhydride, we discovered that the reaction between the ionic acid **20** and diisopropylcarbodiimide (DIC), giving the ionic anhydride **21**, could be performed successfully in the presence of a racemic secondary alcohol and PALB. Since the ionic acid **20** is not an efficient acylating agent,[36] the successful resolution that was observed subsequently could only be achieved due to anhydride formation and concomitant enzymatic resolution using **21** as acylating agent.

Following this observation, a comprehensive study about the *in situ* coupling/enzymatic resolution was deemed necessary. For simplification, acetic acid instead of the ionic acid **20** was used as starting material, and 1-phenylethanol (**17**) was used as model substrate. First, we wanted to compare the *in situ* method versus commercial acetic anhydride and commercial acetic acid as acylating agent. As can be seen in Figure 2.1, the reaction profiles of the ERKs using commercial or *in situ* generated acetic anhydride are very similar up to the 8 hours mark (conversion of 35-36%). After this point the *in situ* method's conversion slows down, reaching 41% after 30 hours of reaction, while the EKR using commercial anhydride achieves a maximum conversion of 45%. Hence, mixing all the components together at the same time neither interferes with the coupling reaction nor hampers significantly the catalytic activity of PALB. This study also confirmed that using free acid, as acylating agent, yields a much slower enzymatic resolution, since after 30 hours a conversion of 17% was achieved, less than half of the conversion of the EKRs using an anhydride. As such, in the *in situ* method the key acylating agent is the anhydride and not the free acid.

Another important remark taken from Figure 2.1, is that, despite the known reactivity of anhydrides, when commercial or *in situ* generated acetic anhydride were mixed with 1-phenylethanol without the presence of PALB, only a residual amount of

ester was formed (< 1.3%) even after 30 hours. These observations are critical because any ester formation that bypasses the biocatalyst will not be enantioselective and will erode the enantiomeric excess of the product, particularly in enzymatic resolutions with long reaction times.

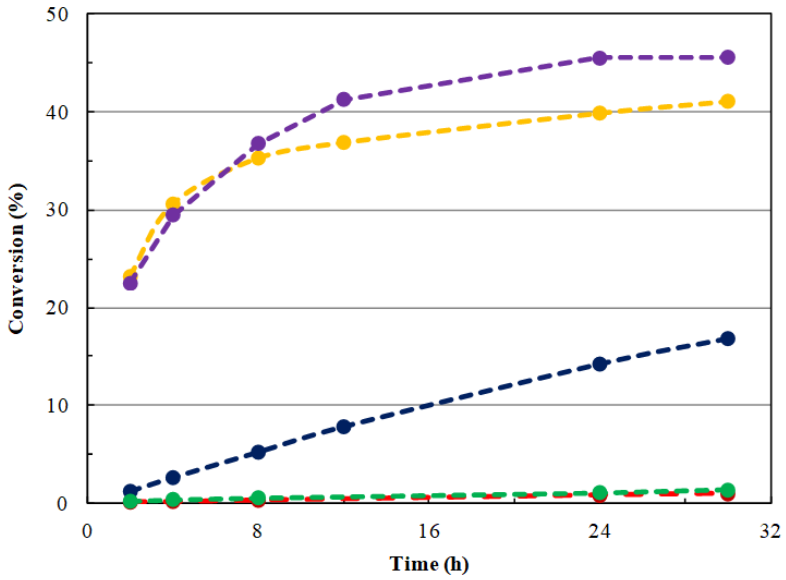


Figure 2.1. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 35ºC in acetone using different acylating agents (1 equivalent): (-●-) using commercial acetic anhydride, (-●-) using *in situ* generated acetic anhydride, (-●-) using commercial acetic acid. Reaction profiles of 1-phenylethanol (**17**) with different anhydrides (1 equivalent) at 35ºC in acetone without biocatalyst: (-●-) using commercial acetic anhydride, (-●-) using *in situ* generated acetic anhydride.

The next step was to study the reactions by ^{1}H-Nuclear Magnetic Resonance. Once more, for simplification, acetic acid was used as starting material, and 1-phenylethanol (**17**) was used as model substrate. First, we followed the coupling reaction alone. As can be observed in Figure 2.2, the acetic anhydride formation is very fast. In 5 minutes more than 2/3 of the carboxylic acid was converted to the anhydride and the reaction is almost complete after 60 minutes. Afterwards, we performed the coupling reaction *in situ* with the enzymatic resolution (Figure 2.3). Similarly to the previous experiment, the anhydride formation is very fast and at practically the same rate (about 2/3 of acetic acid converted after 5 minutes). However, unlike the previous trial, a complete conversion was not achieved. In fact, 120 minutes after the addition of DIC and onwards we observe an increase of the acetic acid signal which is due to

enzyme-catalyzed anhydride opening and subsequent formation of an ester and acetic acid. These experiments demonstrate that the enzymatic resolution is the rate-limiting step and confirms that the presence of enzyme does not interfere significantly with the coupling reaction.

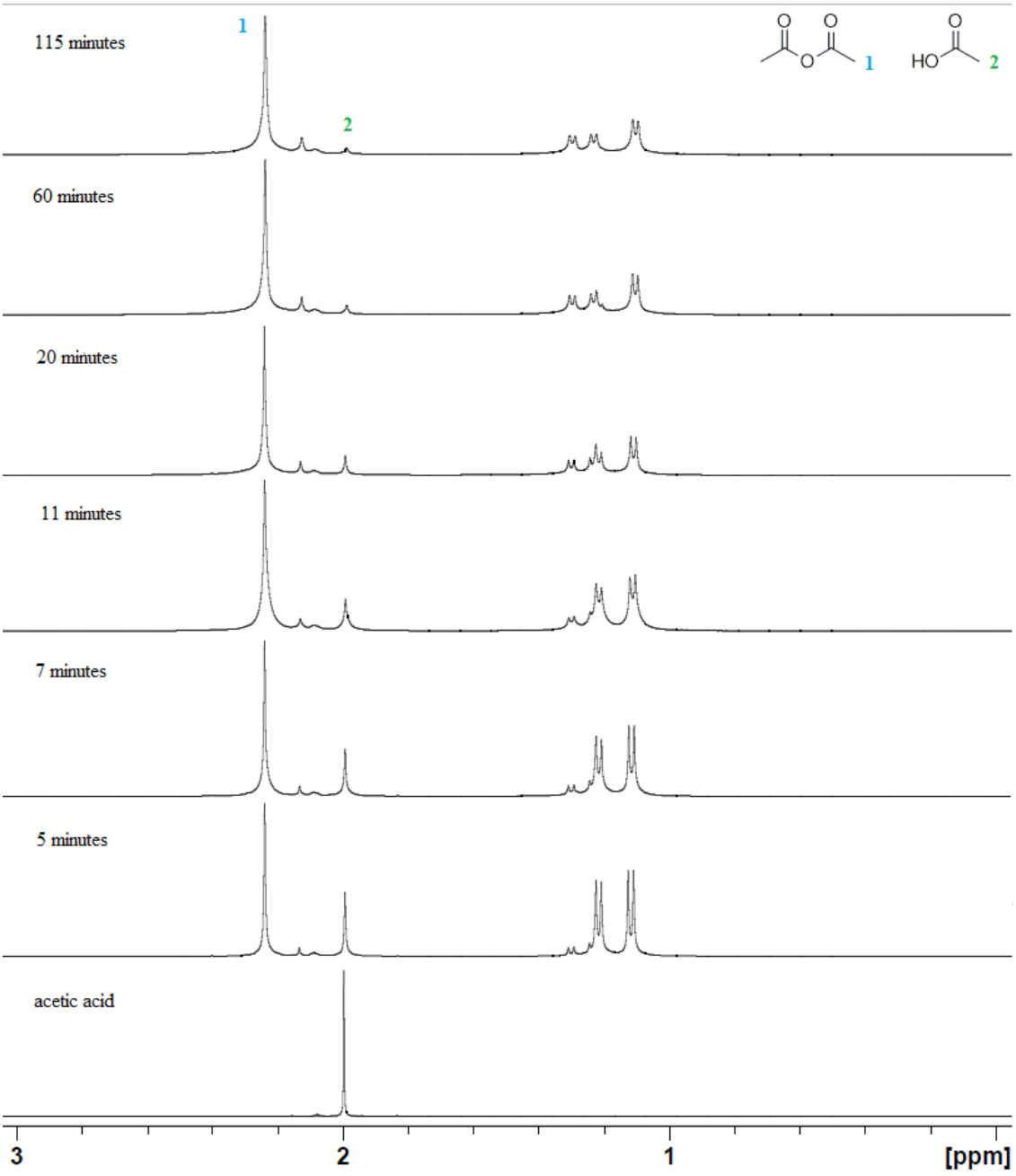


Figure 2.2. Evolution by ^{1}H NMR (400 MHz) of the formation of acetic anhydride (δ = 2.25 ppm) after addition of DIC (0.205 mmol) to acetic acid (0.41 mmol) at 35ºC in acetone-d_6 (0.400 mL). From bottom to top: spectrum of commercial acetic acid, spectra at 5, 7, 11, 20, 60 and 115 minutes after addition of DIC.

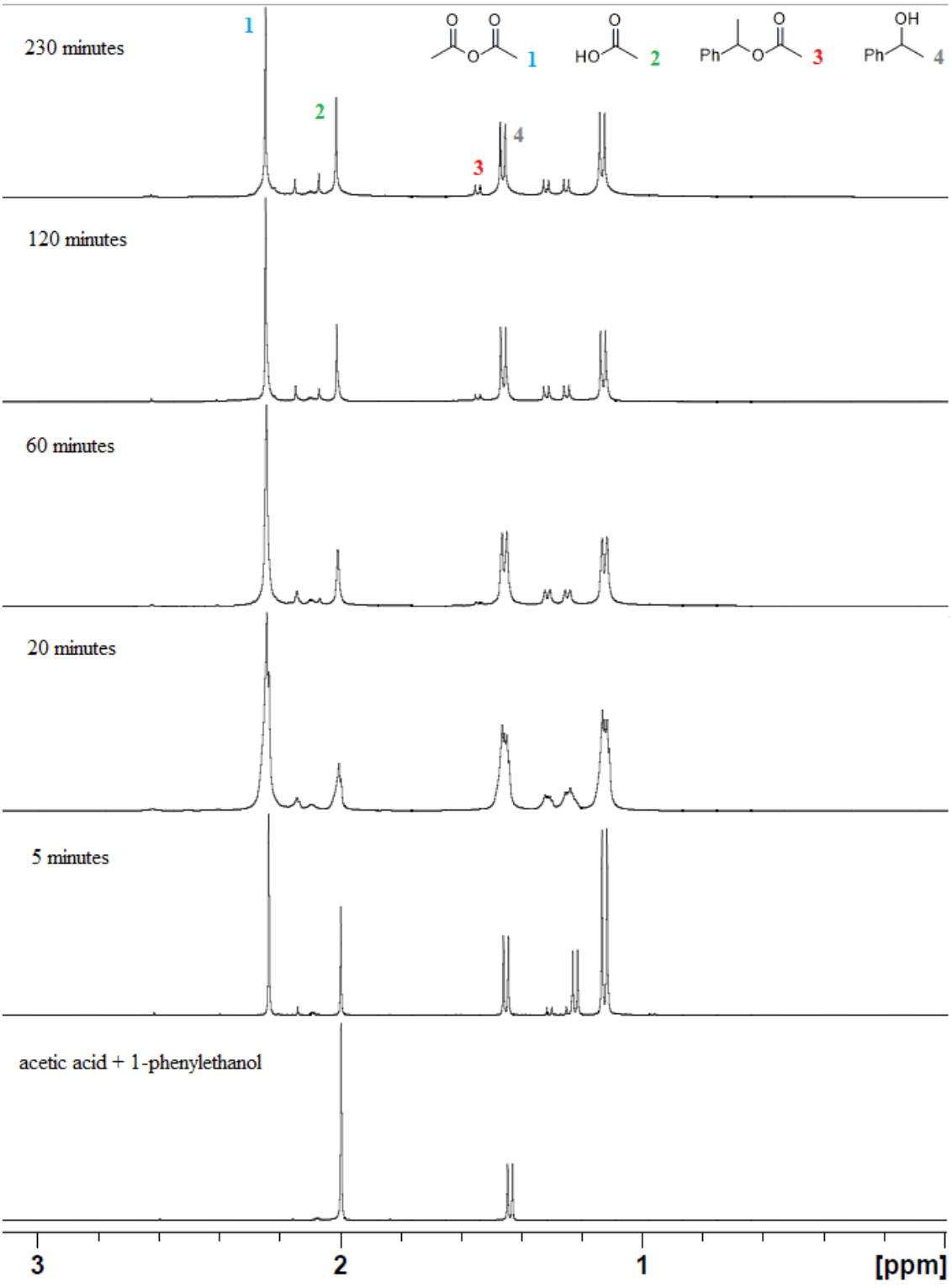


Figure 2.3. Evolution by ^{1}H NMR (400 MHz) of the coupling reaction/enzymatic resolution using acetic acid (0.41 mmol), 1-phenylethanol (0.205 mmol), PALB (10 mg) and DIC (0.205 mmol) at 35ºC in acetone-d_6 (0.400 mL). From bottom to top: spectrum of acetic acid and 1-phenylethanol, spectra at 5, 20, 60, 120 and 230 minutes after addition of PALB and DIC.

Following these preliminary studies, an assessment of the performance of the *in situ* procedure against other common acylating agents was deemed necessary. Using 1-phenyethanol (**17**) as model substrate, enzymatic resolutions were performed employing vinyl esters (acetate and butyrate), commercial anhydrides (acetic and butyric) and those same anhydrides prepared *in situ* from the respective acid, in similar fashion to the ionic anhydride (Figure 2.4). This study showed that the difference in conversion (%) between the vinyl esters based EKRs (48 and 47% for acetate and butyrate, respectively) and the one using the *in situ* ionic anhydride **21** (44%) was less than 4%, after 12 hours of reaction. The difference to commercial butyric anhydride was even smaller, less than 2%, while the EKR starting with commercial acetic anhydride actually produced a lower conversion (41%). These results demonstrate that the *in situ* ionic anhydride methodology can achieve results almost as good as the ones with common acylating agents, while providing a more advantageous recovery procedure for both enantiomers. The comparative study also showed that i) the *in situ* protocol can be used with linear carboxylic acids, despite being less efficient, and ii) a better conversion, in comparison with acetic acid (37%), was achieved with the longer butyric acid (41%).

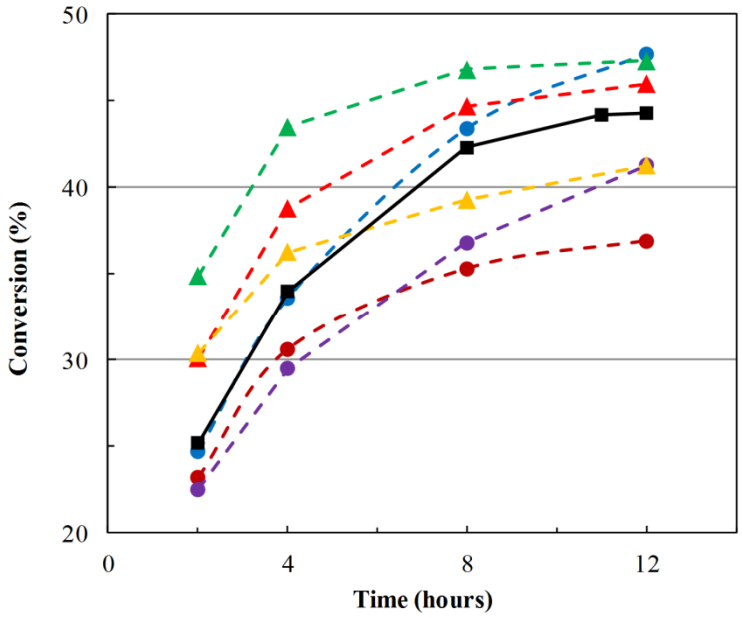


Figure 2.4. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 35ºC in acetone using different acylating agents (1 equivalent): (-■-) using *in situ* generated ionic anhydride **21**, (-●-) using vinyl acetate, (-●-) using commercial acetic anhydride, (-●-) using *in situ* generated acetic anhydride, (-▲-) using vinyl butyrate, (-▲-) using commercial butyric anhydride, and (-▲-) using *in situ* generated butyric anhydride.

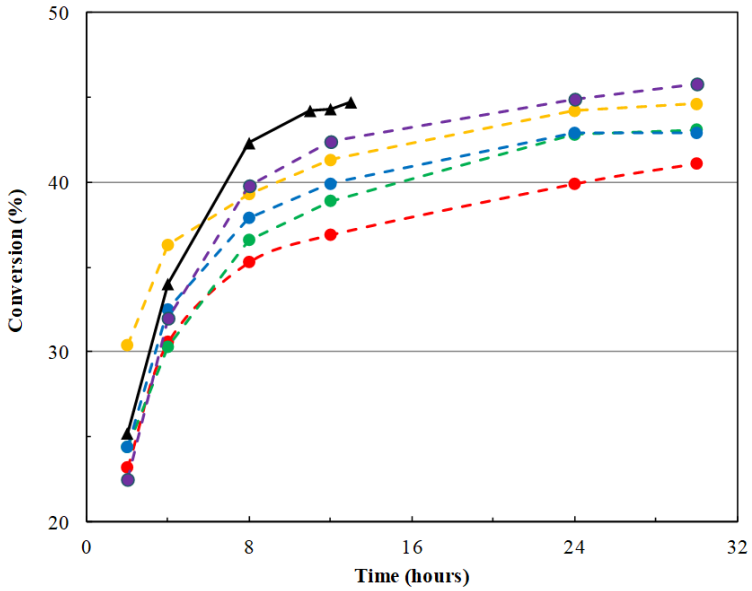


Figure 2.5. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 35ºC in acetone using different *in situ* generated linear acid anhydrides as acylating agent (1 equivalent): (-▲-) using ionic acid **20**, (-●-) using acetic acid, (-●-) using butyric acid, (-●-) using hexanoic acid, (-●-) using octanoic acid and (-●-) using dodecanoic acid.

To determine if even longer carboxylic acids maintained this trend and how they compared against the ionic acid, trials using hexanoic, octanoic and dodecanoic acid as starting material were performed (Figure 2.5). Indeed a better conversion was achieved with dodecanoic acid (42% after 12 hours), the longest of the five linear acids studied, while octanoic and hexanoic acids gave conversions, after 12 hours, of 40 and 39%, respectively. However, the results were inferior to the ones obtained with **20** (44%), confirming that the latter generates a better acylating agent. In addition, it should be pointed out that anhydrides from medium and long chain carboxylic acids are not easily available therefore the enzymatic *in situ* method described herein is an interesting alternative to the preparation of esters of these acids by the standard DMAP-catalyzed method.[83]

2.2.2 Method optimization

The next step was to optimize the reactions conditions for EKR via *in situ* ionic anhydride formation. Four parameters were selected for the study, namely equivalents

of ionic acid **20**, substrate concentration, reaction temperature and coupling agent. The EKRs were followed by chiral GLC in order to determine the conversion and the enantiomeric ratio. The results are summarized in Table 2.1 (more details are available in annex). Usually, the reaction solvent is another parameter which demands consideration, however the low solubility of the ionic acid in organic solvents limits the choice of solvent to dichloromethane, acetonitrile and acetone. We selected acetone due to lower cost, lower environmental impact and to good results obtained using this solvent in previously reported PALB-catalyzed reactions.[82, 84] It must be pointed out that acetone was distilled prior to use but was not dried (≈1.3 wt% of water). This is important not only to reduce costs associated with solvents but also to preserve the water-shell around the lipase, which is essential for a full catalytic activity, since it is known that hydrophilic solvents have a tendency to strip these water molecules from enzymes.[85]

Table 2.1. Screening of experimental conditions for the EKRs using *in situ* generated ionic anhydride **21** as acylating agent.

Entry[a]	Eq. Ionic Acid **20**	[ROH] (M)	T (ºC)	Coupling agent[b]	Conversion[c] (%)	E[d]
1	2.0	0.50	35	DIC	44	619
2	2.4	0.50	35	DIC	48	901
3	3.0	0.50	35	DIC	49	365
4	2.4	0.25	35	DIC	33	582
5	2.4	0.75	35	DIC	49	554
6	2.4	0.50	30	DIC	48	610
7	2.4	0.50	40	DIC	49	698
8	2.4	0.50	40	EDC	11	125
9	2.4	0.50	40	DCC	43	523

[a] All enzymatic reactions were carried out at the described temperature in acetone (0.55, 0.80 or 1.65 mL according to the desired substrate concentration) using ionic acid **20** (0.82, 0.984 or 1.23 mmol according to the desired number of equivalents), 0.41 mmol of rac-1-phenylethanol, 20 mg of PALB pre-treated with n-hexane and coupling agent (0.41, 0.492 or 0.615 mmol according to the number of equivalents of **20**) for at least 12 hours. [b] DIC – diisopropyl carbodiimide, EDC – N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, DCC – dicyclohexylcarbodiiimide. [c] $c = ee_s / (ee_s+ee_p)$. [d] $E = \ln[1-c(1+ee_p)] / \ln[1-c(1-ee_p)]$.

Regarding the number of equivalents of ionic acid **20**, trials were performed using 2.0, 2.4 and 3.0 equivalents. Similar conversions were obtained using 3.0 and 2.4 equivalents. However, the latter was chosen as the best value onward because a significantly better enantiomeric ratio (E) was obtained (901 vs. 365, Table 2.1, entry 2 *vs* entry 3), which means that the resolution with 2.4 equivalents of **20** was more enantioselective.

Next, the effect of the secondary alcohol starting concentration was studied, namely 0.25, 0.50 and 0.75 M concentrations. A notably slow resolution was obtained with the lowest starting concentration, with a difference in conversion to the other concentrations of almost 15%. The conversions achieved using 0.5 M and 0.75 M concentrations were basically the same. Nevertheless, 0.5 M was chosen over 0.75 M because provides higher E (901 *vs* 554, Table 2.1, entry 2 *vs* entry 5). Additionally, the use of 0.75 M generates a thick and opaque reaction medium, which could be problematic for the solubilization of other substrates.

Regarding to the effect of the temperature, resolutions were performed at 30, 35 and 40ºC. As expected, the conversion improved concomitantly with higher reaction temperatures. To our delight, the enantioselectivity of the enzyme was not disturbed by the increase in energy available in the system, so at 40ºC was possibly to achieve a conversion of almost 50% with a very high E value (698, Table 2.1, entry 7).

Finally, a survey with other similar coupling agents, such as dicyclohexylcarbodiimide (DCC) and N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), was carried out to assess if they could be applied in this method. Unfortunately, both coupling agents proved to be not as efficient and practical as DIC (Table 2.1, entries 8 and 9 *vs* entry 7). EDC, a salt, was mostly insoluble in acetone and consequently the yield of the coupling reaction was low which led to a poor conversion (11%, Table 2.1, entry 8). DCC, on the other hand, is almost as soluble in acetone as DIC so the anhydride formation was successful and a good conversion was achieved, although not as high as the one with DIC (43% *vs* 49%, Table 2.1, entries 9 and 7). DCC has the disadvantage of being a waxy solid, making it difficult to transfer it to the reaction medium. In addition, the dicyclohexylurea that is formed during the coupling reaction is not soluble in acetone, unlike the diisopropylurea byproduct which is partially soluble. The high amount of dicyclohexylurea in suspension increases the viscosity of the reaction medium, probably limiting the mass transfer mechanisms and consequently slowing down the enzymatic resolution rate.

Concerning to the isolation procedure, since the diisopropylurea formed during the coupling reaction is partially soluble in acetone and ether, a way to further purify the recovered enantiomers was indispensable. Fortunately it was observed that the byproduct is insoluble in alkanes, so after each organic extraction it was only necessary to evaporate the solvent and extract the obtained mixture with *n*-hexane in order to obtain pure alcohols.

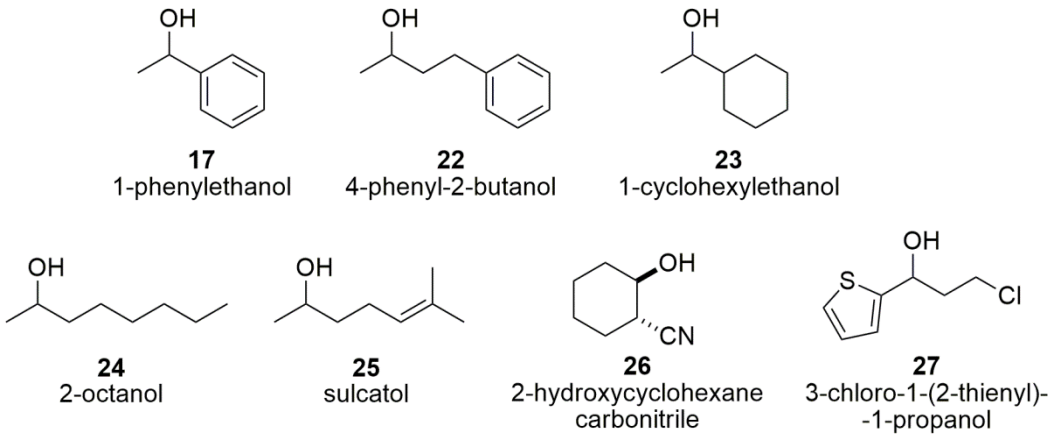
Another important feature of the method described herein is the possibility to recover the ionic acid **20** (after hydrolysis and extraction of the *(R)*-enriched enantiomer) and reuse it straight away. In order to maximize the amount recovered different protocols for the removal of **20** from the aqueous acidic medium were explored, namely: i) extraction with dichloromethane, which provided a yield of 73%; ii) saturation of the aqueous medium with NaCl followed by extraction with dichloromethane, which gave 82% of **20** and; iii) evaporation of aqueous medium under reduced pressure followed by solid-liquid extraction with dichloromethane, which allowed the isolation of **20** in a modest yield of 60%, most likely due to increased degradation of the ionic acid **20** during the concentration step.

2.2.3 Substrate scope and enzyme reutilization

The optimized method was applied to various secondary alcohols in which both enantiomers are valuable (Scheme 2.17). 1-Phenylethanol (**17**), 4-phenyl-2-butanol (**22**), 1-cyclohexylethanol (**23**) and 2-octanol (**24**) are important chiral building blocks for agrochemicals, pharmaceuticals and natural products.[73, 81] Sulcatol (6-methylhept-5-en-2-ol; **25**) is a pheromone used for pest control of *Gnathotrichus retusus* and *Gnathotrichus sulcatus* as the pure *(S)* enantiomer and as a controlled mixture of both enantiomers in a *(S):(R)* ratio of 65:35, respectively.[86] 2-Hydroxycyclohexane carbonitrile (**26**) is a key precursor to an androgen receptor antagonist that is being developed for the treatment of alopecia and excess amounts of sebum (oily skin).[87] 3-Chloro-1-(2-thienyl)-1-propanol (**27**) is a precursor of (S)-duloxetine and (R)-norduloxetine, which are important antidepressant drugs.[88] For these substrates the reaction scale was increased, from 0.41 mmol to 0.82 mmol of alcohol (approx. 100 mg of substrate). For all alcohols the resolution was previously followed by GLC in order to

determine the specific time to achieve close to 50% in conversion (14 h for **17**, 7 h for **22**, 21 h for **23**, 2 h for **24** and **25**, 10 h for **26**) and this information was used for the EKRs presented in Table 2.2.

The resolutions resulted in very high enantiomeric ratios ($321 \leq E \leq 711$) for all substrates studied, except for alcohols **26** and **27**. The enantiomeric ratio obtained for **26** (E = 170) was below the optimal value (E > 200), but, nonetheless, it is still one of the best enantiomeric ratios achieved so far for this substrate.[36, 79, 87] On the other hand, for **27** a successful resolution could not be achieved at all. After 45 hours the (S)-enantiomer was recovered with an enantiomeric excess of only 5% and a yield of 90%. These values show a very slow reaction with poor enantioselectivity. We believe this can be explained by the bulkiness of the chloride at the end of the alkyl chain. This prevents the recognition of the large-sized substituent from the medium-sized one by the active site (Scheme 2.10, page 25), hence the low enantioselectivity, and also hinders the bonding of the substrate to the active site, under the conditions used, hence the exceptional slow reaction time. As support to this statement, when 1-(2-thienyl)-1-propanol (a byproduct obtained during the preparation of **27**) was used as substrate, under the exact same conditions, the ee of the (S)-enantiomer was 91% after 45 hours.



Scheme 2.17. Secondary alcohols employed in the EKRs using *in situ* generated ionic anhydride **21** as acylating agent.

Very high conversions, between 46 and 48%, were achieved for substrates **17**, **22**-**26**. The enantiomeric excess of the *(S)*-enantiomers, recovered after the first extraction with ether, were good (87% on average), the best one being for sulcatol (**25**) with 92%. The enantiomeric excess of the *(R)*-enantiomers, recovered after hydrolysis and a second organic extraction, were all above 97%. The isolated yields, which were

obtained without the need of any chromatographic step, were very good (between 48 and 53%) for the *(S)*-enantiomer, and moderate to good (31-40%) for the *(R)*-enantiomer. Regarding the recovery of starting ionic acid **20**, it was possible to isolate it in the range of 73-88%, corresponding to an average of 79%.

The fastest EKRs were achieved with the linear alcohols **24** and **25**, both within 2 hours. The resolution of **23**, containing a bulky cyclohexane moiety adjacent to the hydroxyl group, was the more sluggish one, taking 21 hours to reach the maximum ee. The influence of bulky groups next to the hydroxyl group can be observed by comparing the results obtained for alcohols 1-phenylethanol (**17**) and 4-phenyl-2-butanol (**22**). While the resolution for **17** needed 14 hours, the EKR of **22**, where the hydroxyl is separated from the phenyl group by two carbons, only took 7 hours.

Table 2.2. Results obtained for EKRs using *in situ* generated ionic anhydride **21** as acylating agent and secondary alcohols **17**, **22-27** as substrate.

Entry[a]	ROH	Time (h)	(S)-enantiomer		(R)-enantiomer[d]		c[e] (%)	E[f][g]	Recovered Acid (%)
			ee[b] (%)	Yield[c] (%)	ee[b] (%)	Yield[c] (%)			
1	**17**	14	88	51	99	32	47	711 (1191)	88
2	**22**	7	87	53	99	40	47	390 (144)	82
3	**23**	21	86	52	98	33	47	321 (427)	81
4	**24**	2	88	51	99	35	47	568 (179)	73
5	**25**	2	92	48	99	32	48	532 (157)	74
6	**26**	10	82	50	97	31	46	170 (-)	76
7	**27**	45	5	90	-	-	-	-	-

[a] All enzymatic reactions were carried out at 40ºC in acetone (1.60 mL) using 1.97 mmol of ionic acid **20**, 0.82 mmol of racemic alcohol, 40 mg of PALB pre-treated with n-hexane and 0.98 mmol of DIC. [b] Enantiomeric excess was determined by chiral GLC. [c] Isolated yield. [d] Chemical hydrolyses were carried out in 2.4 mL of a 2.5 M solution of KOH in MeOH/H_2O (2:1) at 40ºC for 60-90 minutes. [e] conversion = ee_s / (ee_s+ee_p). [f] E = ln[1-c(1+ee_p)] / ln[1-c(1-ee_p)] [g] In is provided parentheses enantiomeric ratio obtained using the method described in reference 82.

When we compare the *in situ* method with the previous work using pure ionic anhydride **21**,[82] we can conclude that the *in situ* strategy provides similar results in terms of enantiomeric excess, enantiomeric ratio and isolated yields. For some substrates the results are actually better, in particular the enantiomeric ratios achieved for the EKRs of alcohols **22**, **24** and **25** (respectively 390, 568 and 532 *vs* 144, 179 and 157 with pure ionic anhydride, Table 2.2, entries 2, 4 and 5).[82] These results undoubtedly demonstrate that the enzymatic acylation is not affected by the *in situ* coupling reaction and its by-products, despite the presence of a carboxylic group in the catalytic triad of the active site. This fact is not only critical for the success of the method described herein but could also lead to other applications since it is one unheard of example of reagents and catalysts with conflicting functional groups that work effectively under the proper reaction conditions. Another feature of the *in situ* method is the speed of the resolutions which are much faster than the ones observed in the previous method, most notably the EKRs of substrates **22**, **23** and **24** which were performed in half the time or less (respectively 7, 21 and 2 hours *vs* 14, 43 and 3 hours with pure ionic anhydride).[82] The differences can be explained by fine optimization of the reaction conditions, namely higher reaction temperature and higher amount of equivalents of acylating agent.

As stated before, a noticeable feature of the *in situ* methodology is the possibility to recover the starting ionic acid **20** and reuse it straight away. This feature coupled with the recycling of the biocatalyst could potentially reduce costs significantly in multi batch campaigns. To support this possibility, consecutive EKRs were performed using fresh ionic acid **20** and PALB only in the first batch. In subsequent EKRs only previously used ionic acid **20** (from the preceding cycle and from earlier experiments to complete the amount needed) was employed, while PALB was recovered from the previous cycle and washed with acetone before use in the following EKR experiment. Sulcatol (**25**) was used as model substrate. As can be observed in Table 2.3, PALB could indeed be recycled for 8 cycles before a significant loss in catalytic activity in the 9th EKR. High enantiomeric ratios (>200) were achieved in all EKRs, demonstrating that the biocatalyst remains enantioselective despite the gradual loss in catalytic activity. The isolated yields were very good (47-55%) for the *(S)*-enantiomer and moderate to good (24-41%) for the *(R)*-enantiomer. On average, it was possible to recover around 77% of the starting ionic acid **20**.

Table 2.3. Results obtained for EKRs using *in situ* generated ionic anhydride **21** as acylating agent, recycled PALB as biocatalyst and sulcatol (**25**) as substrate.

Cycle[a]	Time (h)	(S)-enantiomer		(R)-enantiomer[d]		c[e] (%)	E[f]	Recovered Acid (%)
		ee[b] (%)	Yield[c] (%)	ee[b] (%)	Yield[c] (%)			
1	2	98	47	98	41	50	537	74
2	2	90	51	99	32	48	421	52
3	2	68	53	99	25	41	321	75
4	2	69	52	99	24	41	315	76
5	2.5	75	54	99	30	43	416	81
6	2.5	75	55	99	32	43	321	77
7	2.5	78	53	99	29	44	524	79
8	2.5	72	54	99	29	42	402	78
9	2.5	65	65	99	22	33	257	78

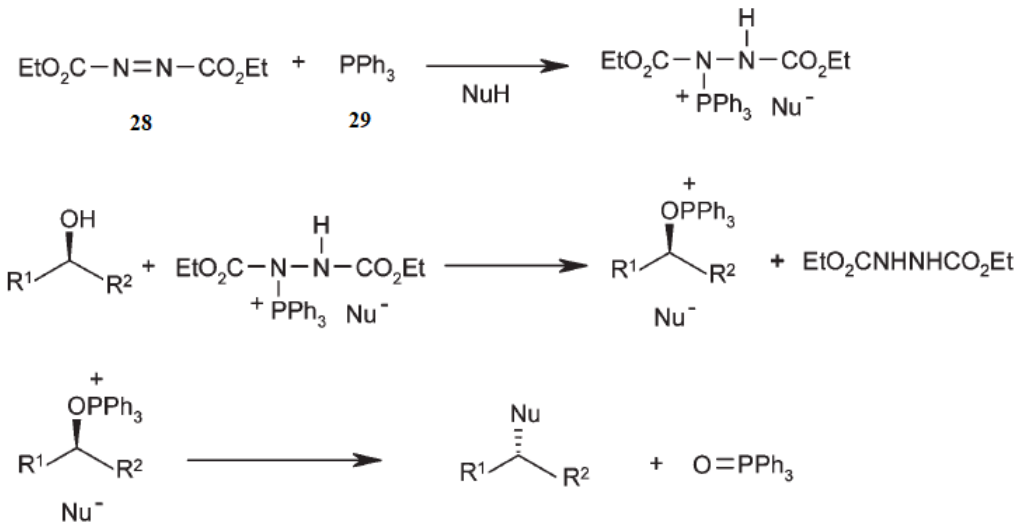
[a] All enzymatic reactions were carried out at 40ºC in acetone (1.60 mL) using 0.82 mmol of racemic sulcatol (**25**) and 0.98 mmol of DIC. In the 1st cycle was used 1.97 mmol of fresh ionic acid **20** and 40 mg of PALB pre-treated with n-hexane, while in the following was employed only used **20** (from the previous cycle and from earlier experiments to complete the required 1.97 mmol) and enzyme recovered from the preceding cycle, washed with acetone prior to use. [b] Enantiomeric excess was determined by chiral GLC. [c] Isolated yield. [d] Chemical hydrolyses were carried out in 2.4 mL of a 2.5 M solution of KOH in MeOH/H_2O (2:1) at 40ºC for 60-90 minutes. [e] conversion = $ee_s / (ee_s+ee_p)$. [f] $E = \ln[1-c(1+ee_p)] / \ln[1-c(1-ee_p)]$.

While the work described herein displays several features that contribute to a safer and more sustainable chemical process (such as a non-flammable acylating agent, separation without any chromatographic method, and recycling of both acylating agent and biocatalyst) there is one aspect that nevertheless fouls the entire procedure. That aspect is the use of hazardous solvents, namely *n*-hexane, diethyl ether, petroleum ether and dichloromethane, in the purification steps. To demonstrate that the method can successfully employ more desirable options,[89] an EKR was performed in which the abovementioned solvents were replaced by *n*-heptane, ethyl acetate or mixtures of thereof (see experimental section for more details). Using 1-phenylethanol (**17**) as model substrate, it was possible to isolate, after 14 hours, the (S) and the (R) enantiomers with an ee of 94 and 99%, respectively, versus 88 and 99% using the

"standard" protocol (Table 2.2, entry 1). As expected, due to the use of higher boiling point solvents, the yields were lower, 41 and 30% vs 51 and 32% using the "standard" protocol, but were good nonetheless. For volatile alcohols, such as sulcatol (**25**), distillation could be a better alternative for separation from diisopropylurea. Finally, it was possible to recover 78% of ionic acid **20**, a value in the range obtained with the "normal" procedure (73-88%).

2.2.4 Inversion of configuration via O-alkylisoureia

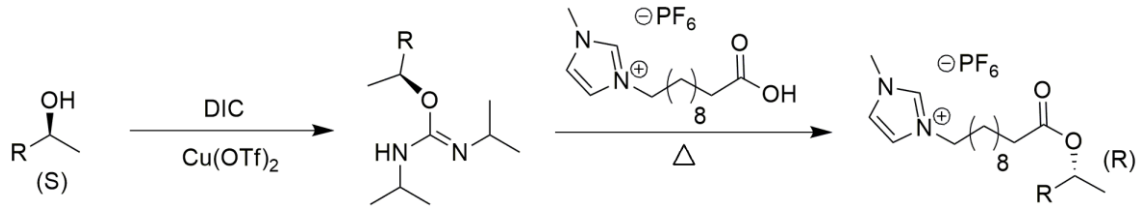
As mentioned earlier, the kinetic resolution of racemates is extremely convenient when both enantiomers are on demand. However, often that is not the case, which means that the maximum achievable yield is 50%. In order to avoid the waste of 50% of the starting material, after separation of the enantiomers, the undesired isomer must be repeatedly racemized and resolved, or stereoinverted. In laboratory the most common way to perform the stereoinversion of an alcohol is through Mitsunobu reaction (Scheme 2.18). However the industrial applications of this reaction are very limited because it needs stoichiometric amounts of azodicarboxylate (**28**) and triphenylphosphine (**29**) to transform the hydroxyl group into a good leaving group. This is a highly atom-inefficient process and, in addition, the removal of the by-products requires additional processing, such as chromatography, increasing even more the waste produced.[90]



Scheme 2.18. Depiction of the Mitsunobo reaction.

A very interesting alternate approach is the O-alkylisourea mediated ester formation. O-Alkylisoureas are easily prepared by equimolar addition of a carbodiimide (e.g. DIC) to an alcohol, under Cu(I) or Cu(II) catalysis. If this product is allowed to react with carboxylic acids, the respective esters are formed, with inversion of configuration if a secondary alcohol was the starting material. With this approach in mind, we speculated that the reagents that were used in EKR methodology described above (ionic acid **20** and DIC) could be used also for the stereoinversion process (Scheme 2.19).

Following the protocols described in literature,[91, 92] first an O-alkylisourea was prepared using sulcatol (**(S)-25**, ee: 79%) as model secondary alcohol, DIC as carbodiimide and copper triflate $(CuOTf)_2$ as catalyst. Then the ionic acid **20** in acetonitrile was added to the mixture. After 20 hours at 100ºC the ionic ester was recovered and hydrolyzed. The obtained alcohol was almost fully inverted (**(R)-25**, ee: 76%) but the yield was very low (12%). Trials under lower temperatures (90, 50ºC) and with different solvents (acetone, dioxane) were performed but unfortunately the obtained ees were never as good (< 10%) and the yields were still low (< 20%). We believe that the ionic acid **20**, under the conditions used, enhances a frequent side reaction, the elimination reaction with formation of olefins,[91] which is the reason for the low yields. A possible solution is to perform the second step under microwave irradiation, reducing the reaction time from hours to minutes,[92] which, hopefully, limits the occurrence of side reactions.



Scheme 2.19. Strategy for the stereoinversion of secondary alcohols. First, formation of O-alkylisourea, using diisopropylcarbodiimide and copper triflate, followed by reaction with ionic acid **20**.

2.3

CONCLUSIONS

A simple and straightforward method was developed for enzymatic kinetic resolution of secondary alcohols which is based on the *in situ* preparation of an anhydride-bearing ionic acylating agent **21** in the presence of PALB and the substrate. The results demonstrated that the *in situ* coupling reaction of ionic acid **20** with DIC does not inhibit the enzymatic acylation. Indeed, very high conversions and enantiomeric excesses were achieved for several aliphatic, allylic and benzylic alcohols. The methodology allows the recovery of both enantiomers through simple organic extractions and no chromatographic process was necessary. It was also demonstrated that both the starting ionic acid **20** and the biocatalyst could be recovered and reused up to eight cycles before a significant loss in catalytic activity was observed. Nonetheless, PALB under the conditions described preserved its enantioselectivity and high enantiomeric ratios ($E > 200$) were obtained in all nine cycles.

In conclusion, the work described herein greatly improves the practical application and sustainability of the method depicted previously, and it is a clear evidence that there is no need to always isolate/purify all the starting materials in order to obtain good results, saving time and resources.

2.4

EXPERIMENTAL SECTION

2.4.1 General remarks

All reagents were obtained commercially (purity >95%) and used as received, unless otherwise noted. All solvents were obtained commercially and, when necessary, purified appropriately before use. All aqueous solutions were prepared using distilled water.

All lipase-catalyzed acylations were performed in distilled acetone (≈1.3 wt% of water). Water content was measured by Karl-Fischer titration using a Methrom 831 KF coulometer. Immobilized *Pseudozyma antarctica* lipase B (PALB, formerly known as *Candida antactica* lipase B (CALB); Novozym® 435 with 1-2 wt% of water and 7000 PLU/g) was a gift from Novozymes Co. (Denmark), and was pretreated with *n*-hexane (1 hour at 35-40ºC) just before use. Secondary alcohol *trans*-2-hydroxycyclohexanecarbonitrile (**26**), synthesized following a protocol described in literature,[79] was available in the lab from previous studies.

2.4.2 Detection, isolation and purification of reaction products

Thin layer chromatography (TLC) was applied, when adequate, in the analysis of reaction mixtures and was performed in ALUGRAM® Xtra SIL G/UV_{254} silica-gel plates (Macherey-Nagel, Germany) with detection by UV irradiation or by immersion in an ethanolic solution of phosphomolybdic acid, followed by heating.

Flash chromatography was performed on silica-gel 60 (0.04 – 0.06 mm, 230 – 400 mesh ASTM) (Scharlau, Spain) under pressure and with the appropriate eluent or system of eluents (mentioned in the experimental procedures).

2.4.3 Characterization and analysis

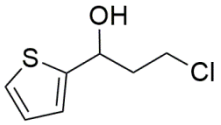
The structure of all synthesized compounds was confirmed by Nuclear Magnetic Resonance (NMR). NMR spectra were recorded at room temperature, unless otherwise noted, on Bruker Avance II+ 300 (^{1}H 300 MHz, ^{13}C 75 MHz) or 400 (^{1}H 400 MHz, ^{13}C 100 MHz) spectrometers, using the residual solvent signal as reference, unless otherwise noted. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in hertz (Hz).

The enantiomeric excess (ee) was calculated by GLC analysis and the enantiomers were identified by comparison to standards. GLC analysis was performed in a GC-2010-Plus Shimadzu with FID detection, using a Varian CP-CHIRASIL-DEX-CB (25 m x 0.25 mm x 0.25 µm) capillary column, injector at 250ºC, detector at 250ºC, 1:100 split ratio and a column flow (He) of 0.95 mL/min. 1-Phenylethanol (**17**) – oven: 120ºC for 14 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(R) = 9.6 min, t_R(S) = 10.3 min. 1-Phenylethyl acetate (**18**) – oven: 120ºC for 14 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 6.8 min, t_R(R) = 7.6 min. 1-Phenylethyl butanoate – oven: 120ºC for 18 min, ramp 15ºC/min and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 15.0 min, t_R(R) = 15.5 min. 1-Phenylethyl hexanoate – oven: 120ºC for 15 min, ramp 5ºC/min to 125ºC, 125ºC for 25 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 38.3 min, t_R(R) = 39.2 min. 1-Phenylethyl octanoate – oven: 120ºC for 15 min, ramp 1 ºC/min to 140ºC, 140ºC for 30 min, ramp 15ºC/min to 180ªC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 62.1 min, t_R(R) = 63.1 min. 1-Phenylethyl dodecanoate – oven: 120ºC for 15 min, ramp 15ºC/min to 155ºC, 155ºC for 100 min, ramp 1ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 132.5 min, t_R(R) = 133.4 min. 4-Phenyl-2-butanol (**22**) – oven: 115ºC for 30 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 7.2 min, t_R(S) = 25.9 min, t_R(R) = 27.3 min. 1-Cyclohexylethanol (**23**) derivatized as 1-cyclohexylethyl propionate – oven: 115ºC for 16 min, ramp 15ºC/min to 180ºC and 180ºC for 10 min; t_R(dodecane) = 7.2 min, t_R(S) = 12.5 min, t_R(R) = 13.4 min. 2-Octanol (**24**) derivatized as 2-octyl propionate – oven: 110ºC for 14 min, ramp 15ºC/min to 180ºC and 180ºC for 10 min; t_R(dodecane) = 8.7 min, t_R(S) = 9.5 min, t_R(R) = 10.4 min. Sulcatol (**25**) – oven: 90ºC for 24 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(S) = 13.6 min, t_R(R) = 14.5 min,

t_R(dodecane) = 21.6 min. *trans*-2-Hydroxycyclohexanecarbonitrile (**26**) – oven: 120ºC for 38 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(S) = 34.1 min, t_R(R) = 35.4 min. 3-Chloro-1-(2-thienyl)-1-propanol (**27**) – oven: 135ºC for 47 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 4.0 min, t_R(R) = 43.3 min, t_R(S) = 44.8 min.

2.4.4 Experimental procedures

2.4.4.1 Preparation of 3-chloro-1-(2-thienyl)-1-propanol (27).



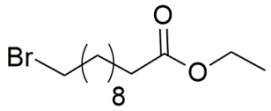
To a suspension of aluminium trichloride (1.22 g, 9.1 mmol) in dry dichloromethane (10 mL), under inert atmosphere, was added dropwise 3-chloropropionyl chloride (0.8 mL, 8.3 mmol). The suspension was cooled in an ice bath and stirred for 10 minutes before dropwise addition of tiophene (0.6 mL, 7.5 mmol). After stirring at room temperature for 4 hours, the resulting orange solution was slowly poured into an Erlenmeyer with crushed ice and stirred for 10 minutes. The mixture was transferred into a separatory funnel and the aqueous phase was discarded. The organic phase was washed with saturated aqueous solution of $NaHCO_3$ (3 x 10 mL), dried with anhydrous sodium sulfate and filtered. Activated charcoal was then added and the suspension was left under stirring overnight. After filtration and evaporation of the solvent under reduced pressure, 3-chloro-1-(2-thienyl)-1-propanone was obtained as a yellow oil (1.17 g, 89%).

To a solution of the ketone (1.17 g, 6.7 mmol) in absolute ethanol (27 mL), cooled in an ice bath, was added in small portions sodium borohydride (0.253 g, 6.7 mmol). After stirring at room temperature for 3 hours, saturated aqueous solution of ammonium chloride (55 mL) was added followed by stirring for 20-30 minutes. The aqueous solution was extracted with dichloromethane (4 x 20 mL). The organic phases were combined, dried with sodium sulfate, filtered and concentrated under reduced pressure. The obtained oil is a mixture of **27** and 1-(2-thienyl)-1-propanol that can't be efficiently separated by chromatography or distillation. However their respective acetates can be easily separated by flash chromatography. As such, to a solution of the

mixture in acetonitrile (3.5 mL) was added acetic anhydride (1.25 mL, 13.4 mmol), triethylamine (2.8 mL, 20 mmol) and DMAP (0.082 g, 0.67 mmol), followed by heating at 40ºC for 24 hours. Dichloromethane (25 mL) was added before washing with HCl (2 M, 2 x 15 mL) and distilled water (2 x 15 mL). The organic phase was dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure. The residue was purified by flash chromatography (eluent: hexane/MTBE 9:1), yielding the desired acetate. This compound was then hydrolyzed with a solution of KOH (2 M) in MeOH/H_2O (9:1) at 40ºC for 60-90 minutes. After acidification with HCl (2 M), the alcohol was extracted with dichloromethane (3 x 15 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure. The residue was purified by flash chromatography (eluent: hexane/MTBE 4:1), giving compound **27** as a colorless to pale yellow oil (0.95 g, 80%)

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.20 (d, 1H), 6.94 (d, 1H), 6.91 (t, 1H), 5.14 (s, 1H), 3.68 (m, 1H), 3.51 (m, 1H), 2.21 ppm (m, 3H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 147.6, 126.9, 125.1, 124.2, 67.3, 41.6, 41.5 ppm.
NMR data in agreement with literature.[79]

2.4.4.2 Preparation of ethyl 11-bromoundecanoate (30).



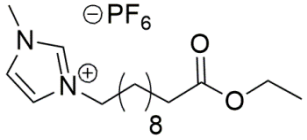
A solution of 11-bromoundecanoic acid (2.74 g, 10.3 mmol) and sulfuric acid (5-6 drops) in absolute ethanol (10 mL) was heated at 85ºC, in a pressure tube (15 mL), for 6 hours. After concentration at reduced pressure, the mixture was diluted with MTBE (20 mL) and washed with saturated aqueous solution of $NaHCO_3$ (2 x 10 mL). The organic phase was dried with anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure, giving compound **30** as a pale yellow liquid (2.82 g, 96%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.12 (q, 2H), 3.40 (t, 2H), 2.28 (t, 2H), 1.84 (m, 2H), 1.60 (m, 2H), 1.27 ppm (m, 15H).

^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.0, 60.3, 34.5, 34.2, 33.0, 29.49, 29.45, 29.3, 29.2, 28.9, 28.3, 25.1, 14.4 ppm.

NMR data in agreement with literature.[36]

2.4.4.3 Preparation of 1-(11-ethoxy-11-oxoundecyl)-3-methyl imidazolium hexafluorophosphate (9).



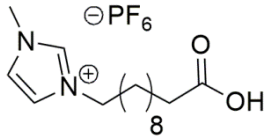
A solution of ethyl 11-bromoundecanoate (2.06, 7.0 mmol) and 1-methylimidazole (0.670 mL, 8.4 mmol) in diisopropyl ether (4 mL) was heated at 70-75ºC, in a pressure tube (15 mL), for 72 hours. After removing the upper layer, the ionic phase was washed with diisopropyl ether (3 x 5 mL) and dried at reduced pressure. The obtained viscous liquid (2.57g) was dissolved in dichloromethane (14 mL) and KPF_6 (1.88 g, 10.2 mmol) was added. The suspension was vigorously stirred at room temperature for 48 hours. The precipitate was filtered and then the organic phase was washed with distilled water (3 x 10 mL), dried with anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred at room temperature for 60-90 min. After filtration in a small column filled with Celite 577 and silica-gel, the solvent was evaporated under reduced pressure. The resulting liquid was dried under vacuum at room temperature for 24 hours to give compound **9** as a pale yellow viscous liquid (2.05 g, 67%).

^{1}H NMR (400 MHz, DMSO-d6): δ = 9.09 (s, 1H), 7.75 (s, 1H), 7.69 (s, 1H), 4.14 (t, 2H), 4.03 (q, 2H), 3.84 (s, 3H), 2.26 (t, 2H), 1.77 (m, 2H), 1.50 (m, 2H), 1.24 (s, 12H), 1.17 ppm (t, 3H).

^{13}C NMR (100 MHz, DMSO-d6): δ = 172.9, 136.5, 123.6, 122.3, 59.7, 48.8, 35.7, 33.5, 29.4, 28.8, 28.7, 28.44, 28.35, 25.5, 24.5, 14.1 ppm.

NMR data in agreement with literature.[36]

2.4.4.4 Preparation of 1-(10-carboxydecyl)-3-methyl imidazolium hexafluorophosphate (20).



To a mixture of 1-(11-ethoxy-11-oxoundecyl)-3-methyl imidazolium hexafluorophosphate (5.15 g, 11.7 mmol) in water (7 mL) was added 14 mL of a methanolic solution of KOH (4 M, 55 mmol). The mixture was stirred at 40ºC for 60-90 min. The solution was acidified with HCl (2M) and, after saturation with NaCl, the product was extracted with dichloromethane (4 x 10 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred at room temperature for 60-90 min. After filtration in a small column filled with Celite 577 and silica-gel, the solvent was evaporated under reduced pressure. The resulting solid was dried under vacuum at room temperature overnight to give compound **20** as a white powder (4.15 g, 86%).

^{1}H NMR (400 MHz, DMSO-d6): δ = 9.09 (s, 1H), 7.75 (s, 1H), 7.68 (s, 1H), 4.14 (t, 2H), 3.84 (s, 3H), 2.18 (t, 2H), 1.77 (m, 2H), 1.47 (m, 2H), 1.24 ppm (s, 12H).
^{13}C NMR (100 MHz, DMSO-d6): δ = 174.6, 136.5, 123.6, 122.3, 48.8, 35.7, 33.7, 29.4, 28.8, 28.7, 28.7, 28.5, 28.3, 25.5, 24.5 ppm.
NMR data in agreement with literature.[36]

2.4.4.5 General procedure for the enzymatic kinetic resolutions using acetic acid, commercial anhydrides or vinyl esters as acylating agent.

Novozym® 435 beads (20 mg) were first embedded in n-hexane (1 mL) for 1 hour at 35ºC. The solvent was decanted and the beads were washed with acetone (3 x 1 mL) prior to use. The enzymatic reactions began after addition of 1-phenylethanol (0.41 mmol, 0.050 mL) to a solution of acylating agent (0.41 mmol; glacial acetic acid: 0.023 mL; acetic anhydride: 0.039 mL; butyric anhydride: 0.067 mL; vinyl acetate: 0.038 mL; vinyl butyrate: 0.052 mL) in distilled acetone (0.8 mL) with the enzyme (for acetic anhydride a similar trial without enzyme was also performed). The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at

35ºC for at least 12 hours. The EKRs were followed by analysis of aliquots (0.100 mL), withdrawn from the reaction after 2, 4, 8 and 12 hours, in a chiral GLC to determine the conversion and enantiomeric ratio. Each aliquot was filtered and concentrated under reduced pressure prior to analysis.

2.4.4.6 General procedure for the enzymatic kinetic resolutions using *in situ* generated anhydrides as acylating agent.

Novozym® 435 beads (20 mg) were first embedded in n-hexane (1 mL) for 1 hour at 35ºC. The solvent was decanted and the beads were washed with acetone (3 x 1 mL) prior to use. The enzymatic reactions began after addition of DIC (0.41 mmol, 0.063 mL) to a solution of acid (0.82 mmol; glacial acetic acid: 0.047 mL; butyric acid: 0.075 mL; hexanoic acid: 0.103 mL; octanoic acid: 0.130 mL; dodecanoic acid: 0.165 g; ionic acid **20**: 0.340 g) and 1-phenylethanol (0.41 mmol, 0.050 mL) in distilled acetone (0.8 mL) with the enzyme (for glacial acetic acid a similar trial without enzyme was also performed). The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at 35ºC for at least 12 hours. The EKRs were followed by analysis of aliquots (0.100 mL), withdrawn from the reaction after 2, 4, 8, and 12 hours (also at 24 and 30 hours for all acids except **20**), in a chiral GLC to determine the conversion and enantiomeric ratio. Each aliquot was filtered and the solvent removed under reduced pressure. For EKRs using an acid other than **20**, the obtained mixture was extracted with n-hexane and then the organic fractions were filtered, combined and concentrated under reduced pressure prior to analysis. In the case of ionic acid **20**, the (S)-enriched alcohol was extracted with diethyl ether (5 x 1 mL) and then the organic fractions were filtered in a pipette size column with silica, combined and the solvent evaporated. Residual urea was removed by filtration after extraction of the mixture with n-hexane (3 x 1 mL). The alcohol was concentrated under reduced pressure prior to analysis. The ionic fraction was dissolved in water (0.5 mL) and a solution of KOH in methanol (4M, 1 mL) was added. The hydrolysis was carried out at 40ºC for 60-90 minutes. Then the solution was diluted with water (1 mL) and the (R)-enriched enantiomer was extracted with diethyl ether (4 x 1 mL). The organic fractions were passed through a pipette sized column of silica, dried with anhydrous sodium sulfate, filtered and concentrated. Residual urea was removed by filtration after

extraction of the mixture with n-hexane (3 x 1 mL). The alcohol was concentrated under reduced pressure prior to analysis.

2.4.4.7 Determination of the best extraction method to recover ionic acid 20.

The basic media, obtained after hydrolysis of the (R)-enriched enantiomer, from two independent EKRs were combined and acidified with HCl (2M). It was divided in three portions and each part was treated in a different way as described next. One portion was extracted with dichloromethane (4 x 5 mL) then the organic phases were combined and dried with anhydrous sodium sulfate. After filtration in a small column filled with Celite 577 and silica-gel, the solvent was evaporated under reduced pressure. The resulting solid was dried under vacuum at room temperature overnight (0.198 g, 73%). Another portion was first saturated with NaCl and then extracted with dichloromethane (4 x 5 mL). The organic phases were combined and treated as described in the first portion. The resulting solid was dried under vacuum at room temperature overnight (0.222 g, 82%). For the third portion the aqueous medium was evaporated under reduced pressure followed by solid-liquid extraction with dichloromethane (4 x 5 mL). The organic phases were combined and treated as described in the first portion. The resulting solid was dried under vacuum at room temperature overnight (0.163 g, 60%).

2.4.4.8 General procedure for the enzymatic kinetic resolutions to determine the ideal time to reach the maximum conversion using *in situ* generated ionic anhydride 21 as acylating agent and alcohols 17, 22-27 as substrate.

Novozym® 435 beads (40 mg) were first embedded in n-hexane (1 mL) for 1 hour at 40ºC. The solvent was decanted and the beads were washed with acetone (3 x 1 mL) prior to use. The enzymatic reactions began after addition of DIC (0.152 mL, 0.98 mmol) to a solution of ionic acid **20** (0.812g, 1.97 mmol) and sec-alcohol (0.82 mmol) in distilled acetone (1.6 mL) with the enzyme. The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at 40ºC, for the time necessary to reach a conversion of ≈50%. The EKRs were followed by analysis in a chiral GLC, to determine the conversion and enantiomeric ratio, of aliquots (0.100 mL)

withdrawn from the reaction at different time intervals. Each aliquot was filtered and concentrated under reduced pressure. The (S)-enriched enantiomer was extracted with diethyl ether (5 x 1 mL), passed through a pipette size column of silica and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-hexane (3 x 1 mL). The alcohol was concentrated under reduced pressure prior to analysis. The ionic fraction was dissolved in water (0.5 mL) and a solution of KOH in methanol (4M, 1 mL) was added. The hydrolysis was carried out at 40ºC for 60-90 minutes. Then the solution was diluted with water (1 mL) and the (R)-enriched enantiomer was extracted with diethyl ether (4 x 1 mL). The organic fractions were passed through a pipette size column of silica, combined, dried with anhydrous sodium sulfate, filtered and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-hexane (3 x 1 mL). The alcohol was concentrated under reduced pressure prior to analysis.

2.4.4.9 General procedure for the preparative enzymatic kinetic resolutions using *in situ* generated ionic anhydride 21 as acylating agent and alcohols 17, 22-27 as substrate.

Novozym® 435 beads (40 mg) were first embedded in n-hexane (1 mL) for 1 hour at 40ºC. The solvent was decanted and the beads were washed with acetone (3 x 1 mL) prior to use. The enzymatic reactions began after addition of DIC (0.152 mL, 0.98 mmol) to a solution of ionic acid **20** (0.812g, 1.97 mmol) and sec-alcohol (0.82 mmol) in distilled acetone (1.6 mL) with the enzyme. The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at 40ºC, for the time necessary to reach a conversion of ≈50% (determined in parallel following the procedure described above). Then the solvent was decanted and the enzyme washed with acetone (4 x 1 mL). All organic fractions were filtered and then combined, followed by evaporation of the solvent under reduced pressure. The untouched (S)-enriched enantiomer was extracted with diethyl ether (5 x 4 mL), passed through a pipette size column of silica and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-hexane (3 x 1 mL; petroleum ether when sulcatol was the substrate). The alcohol was dried under reduced pressure. The ionic phase, containing the (R)-enriched ionic ester was dissolved in distilled water (0.8 mL) and a

solution of KOH in methanol (4M, 1.6 mL) was added. The hydrolysis was carried out at 40ºC for 60-90 minutes. Then the solution was diluted with water (2 mL) and the (R)-enriched enantiomer was extracted with diethyl ether (4 x 2 mL). The organic fractions were passed through a pipette size column of silica, combined, dried with anhydrous sodium sulfate, filtered and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-hexane (3 x 1 mL; petroleum ether when sulcatol was the substrate). The alcohol was dried under reduced pressure. To recover the ionic acid **20**, the basic solution obtained after extraction of the (R)-enriched enantiomer was acidified with HCl (2M), saturated with sodium chloride and extracted with dichloromethane (4 x 10 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred at room temperature for 60-90 min. After filtration in a small column filled with Celite 577 and silica-gel, the solvent was evaporated under reduced pressure. The resulting white solid was dried under vacuum at room temperature overnight.

2.4.4.10 General procedure for the preparative enzymatic kinetic resolution employing more desirable solvents and using *in situ* generated ionic anhydride 21 as acylating agent and 1-phenylethanol (17) as substrate.

Novozym® 435 beads (40 mg) were first embedded in n-heptane (1 mL) for 1 hour at 40ºC. The solvent was decanted and the beads were washed with acetone (3 x 1 mL) prior to use. The enzymatic reactions began after addition of DIC (0.152 mL, 0.98 mmol) to a solution of ionic acid **20** (0.817g, 1.97 mmol) and 1-phenylethanol (0.099 mL, 0.82 mmol) in distilled acetone (1.6 mL) with the enzyme. The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at 40ºC, for 14 hours. Then the solvent was decanted and the enzyme washed with acetone (4 x 1 mL). All organic fractions were filtered and then combined, followed by evaporation of the solvent under reduced pressure. The untouched (S)-enriched enantiomer was extracted with n-heptane/ethyl acetate (2:1) (5 x 4 mL), passed through a pipette size column of silica and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-heptane (3 x 1 mL). The alcohol was dried under reduced pressure (0.041 g of **(S)-17**, 94% ee). The ionic phase, containing the (R)-enriched ionic ester was dissolved in distilled water (0.8 mL) and a solution of

KOH in methanol (4M, 1.6 mL) was added. The hydrolysis was carried out at 40ºC for 60-90 minutes. Then the solution was diluted with water (2 mL) and the (R)-enriched enantiomer was extracted with n-heptane/ethyl acetate (2:1) (4 x 2 mL). The organic fractions were passed through a pipette size column of silica, combined, dried with anhydrous sodium sulfate, filtered and concentrated. Residual urea was removed by filtration after extraction of the mixture with n-heptane (3 x 1 mL). The alcohol was dried under reduced pressure (0.030 g of **(R)-17**, 99% ee). To recover the ionic acid **20**, the basic solution obtained after extraction of the (R)-enriched enantiomer was acidified with HCl (2M), saturated with sodium chloride and extracted with ethyl acetate (4 x 10 mL). The organic fractions were combined, dried with anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred at room temperature for 60-90 min. After filtration in a small column filled with Celite 577 and silica-gel, the solvent was evaporated under reduced pressure. The resulting white solid was dried under vacuum at room temperature overnight (0.640 g of **20**).

2.4.4.11 Procedure for the consecutive enzymatic kinetic resolutions of sulcatol (25) using *in situ* generated ionic anhydride 21 as acylating agent and recycled PALB as biocatalyst.

Novozym® 435 beads (40 mg) were first embedded in n-hexane (1 mL) for 1 hour at 40ºC (only before the 1st cycle). The solvent was decanted and the beads were washed with acetone (3 x 1 mL). The enzymatic reactions began after addition of DIC (0.152 mL, 0.98 mmol) to a solution of ionic acid **20** (0.812 g, 1.97 mmol) and sulcatol (0.82 mmol) in distilled acetone (1.6 mL) with the enzyme. In the first cycle was used fresh ionic acid **20** and PALB, while in subsequent EKRs was employed only used **20** (from the preceding batch and from earlier experiments to complete the required 1.97 mmol) and PALB recovered from the previous cycle. The reactions were carried out in 4 mL glass screw-cap vials placed under orbital stirring in an incubator at 40ºC for 2 hours (1st to 4th cycles) or 2.5 hours (5th to 9th cycles). Then the solvent was decanted and the beads washed with acetone (4 x 1 mL), before storage at 4ºC in acetone (1 mL) until the following cycle. For recovery of the enantiomers and ionic acid **20** the method was exactly the same as described above for the preparative EKRs: 1st cycle: 0.049 g of **(S)-25**, 0.043 g of **(R)-25**, 0.601 g of **20**; 2nd cycle: 0.053 g of **(S)-25**, 0.034 g of **(R)-25**,

0.422 g of **20**; 3rd cycle: 0.056 g of **(S)-25**, 0.026 g of **(R)-25**, 0.615 g of **20**; 4th cycle: 0.055 g of **(S)-25**, 0.026 g of **(R)-25**, 0.613 g of **20**; 5th cycle: 0.057 g of **(S)-25**, 0.032 g of **(R)-25**, 0.659 g of **20**; 6th cycle: 0.058 g of **(S)-25**, 0.034 g of **(R)-25**, 0.623 g of **20**; 7th cycle: 0.056 g of **(S)-25**, 0.030 g of **(R)-25**, 0.640 g of **20**; 8th cycle: 0.057 g of **(S)-25**, 0.030 g of **(R)-25**, 0.630 g of **20**; 9th cycle: 0.068 g of **(S)-25**, 0.023 g of **(R)-25**, 0.636 g of **20**.

2.4.4.12 General procedure for esterification of alcohols 23 and 24 for GLC analysis.

After separation of the enantiomers, 1-cyclohexylethanol (**23**) and 2-octanol (**24**) were esterified with propionic anhydride in order to improve the separation of their peaks in the GC. The alcohols were mixed with 1.5 equivalents of propionic anhydride (10 uL, 0.075 mmol), triethylamine (11 uL, 0.080 mmol) and DMAP (0.002g) in acetonitrile (0.400 mL) for 3-4 hours at 40ºC. The crude was then dissolved in dichloromethane (2 mL) and washed with HCl (1M, 2 x 1 mL), saturated aqueous solution of $NaHCO_3$ (2 x 1 mL) and water (1mL). After being dried with anhydrous sodium sulfate, the sample was concentrated under reduced pressure.

2.4.4.11 Procedure for the stereoinversion of (S)-sulcatol.

Copper triflate (0.003 g, 0.0074 mmol) was dried at 120-130ºC, under vacuum, for 1 hour. After addition of DIC (0.025 mL, 0.155 mmol) and sulcatol (**(S)-25**, ee: 79%, 0.019 g, 0.148 mmol) the mixture was left under stirring at room temperature overnight. A solution of ionic acid 20 (0.074 g, 0.178 mmol) in acetonitrile (0.300 mL) was added and the mixture heated at 100ºC for 20 hours. After being washed with diethyl ether (4 x 5 mL), the ionic phase was dissolved in distilled water (0.300 mL) and a solution of KOH in methanol (4M, 0.700 mL) was added. The hydrolysis was carried out at 40ºC for 60-90 minutes. Then the solution was diluted with water (1 mL) and the (R)-enriched enantiomer was extracted with diethyl ether (4 x 1 mL). The organic fractions were passed through a pipette size column of silica, combined, dried with anhydrous sodium sulfate and filtered. The alcohol was concentrated under reduced pressure (0.0024 g of **(R)-17**, 76% ee).

CHAPTER 3

ENZYMATIC KINETIC RESOLUTION IN CONTINUOUS FLOW

3.1

INTRODUCTION

In flow chemistry tubing or channels are used to perform a reaction in a continuous stream rather than in a traditional flask. Flow equipment provides chemists with unique control over reaction parameters enhancing reactivity, selectivity and efficiency, or enabling new reaction protocols that are not adequate to be carried out in flask. In the past decade this technology has become increasingly popular in the field of organic chemistry with many reports extending the diversity of synthetic transformations from batch to flow.

Flow conditions are advantageous for many transformations; however, they are not the solution for every shortcoming in chemistry. In addition, developing a flow process can be time-consuming. As such, a flow versus batch analysis should be always performed to determine which one is the proper methodology to achieve the overall goal.

3.1.1 Batch versus Flow

Safety is an excellent starting point to compare flow versus batch. In a conventional chemical environment, the hazardous nature (reactivity, toxicity, explosiveness) of specific materials can limit or even prohibit their use. These dangerous materials, however, display interesting reactivity, their employment offer greater atom economy and their alternative routes are expensive and require longer reaction sequences. With all these features in mind, a safe use of such materials is of paramount interest. The small dimensions of flow reactors, particularly chip reactors, allow chemists to work with minute quantities of hazardous materials, thus minimizing safety and exposure risks. Additionally, built in quenches avoid equipment manipulations, eliminating human error which can result in spills. Unstable or

dangerous synthetic intermediates can be generated *in situ* inside a closed, pressurized system and converted directly into a more advanced, safe intermediate or product by combining multiple reagent streams. Moreover, changing the reaction temperature along the reactor channel allows uninterrupted, continuous multistep reactions to be performed and eliminates the need to handle or store excessive amounts of potentially problematic intermediates. Another issue arises from reactions that are highly exothermic and extremely fast, which have the tendency to accumulate heat and to form hot spots, resulting eventually in uncontrollable side- or even runaway reactions. Once more, with the small dimensions, and concomitant high surface area-to-volume ratio, of flow reactors allow heat can be applied and removed very efficiently, allowing exquisite control of the reaction temperature and minimizing safety hazards from runaway reactions.[93-95]

Generally, flow reactions outperform batch reactions when one of the reagents is a gas. The headspace to solvent ratio is lower and pressurizing the reactor increases the solubility of the gas in solution. Additionally, the fraction of gas volume in a closed, pressurized liquid-filled system is significantly reduced. This is critical to avoid evaporation of low-boiling reagents or formation of explosive gas mixtures. While small-scale batch reactions in closed vessels are feasible, preparative scales are not possible or are dangerous. The stability of small-diameter reactors to pressurized conditions also enables access to safe operation at high temperatures. The most straightforward way to increase reaction rate is via an increase of temperature. In a batch reactor, the atmospheric boiling point of the solvent or reagents is the frequent limiting factor. Higher temperatures can be accessed using an autoclave or microwave, which, in turn, allow faster rates. This, however, makes scale-up challenging. In a flow reactor, pressure and temperature can be safely manipulated far beyond atmospheric conditions. Since a fast reaction will require less operating time, an increased rate of reaction allows efficient utilization of energy and time. Economical use of space is also important, and fast reactions allow the utilization of smaller reactors. Analogous to microwaves synthesis, reactions done in flow conditions are often faster than the corresponding batch reactions, which gives improved energy, time, and space efficiency.[93-96]

For nonhazardous reactions that are known in batch, a chemist must decide whether or not the described conditions are satisfactory to achieve the desired goals. If it is not broken, do not fix it. Nonetheless, the operator must also take into account that

some discovery scale procedures may not be adequate to preparative scales. For new transformations, it is more convenient to screen reagents, solvents, and additives in batch. All of these parameters can be tested simultaneously, while in flow they must be done sequentially. One key exception might be reactions that require scarce or expensive starting materials. In these cases, the small dimensions of low reactors enable a chemist to perform and analyze a large number of trials using minimal quantities of a reagent. Additionally, temperature and time optimizations are usually simpler in flow because the reactor temperature can easily be changed and precise control of the reaction time can be varied via flow rates.[93, 97]

The yield and/or selectivity of a given reaction are features that demand special consideration. Mixing is critical for extremely fast reactions. In these cases, usually reagents have to be added slowly to the reaction mixture or the mixture has to be diluted or cooled to reduce reaction rates and to guarantee adequate mixing and avoid heat accumulation. While batch is adequate for small scale preparations, for preparative scales, however, some reactions are lower yielding due to poorer mixing and/or heat transfer. Typically, faster mixing and more efficient heat transfer are achieved under flow conditions, which greatly improve the yield of fast reactions. Selectivity can also be enhanced in flow, since flow reactors normally have a narrower temperature profile than batch reactors. As such, side reactions close in energy to the desired reaction can be minimized. For extremely slow reactions, intensification of reaction conditions can produce compounds in a timely fashion. As mentioned before, preparative scale high-temperature, high-pressure reactions are much safer in flow.[93, 95, 96]

The physical state of the starting materials or the products must also be taken in account. The delivery of suspensions remains a challenge for laboratory scales. In this scenario, batch reactions are more convenient and reliable. When precipitation of the product is essential to drive the reaction to completion, batch is also more convenient. Precipitation in flow conditions typically leads to clogging of the tubing or of the pressure regulator. While sophisticated technologies for the handling of solids in continuous flow processes have been recently developed, there is no universal solution to this problem and technical advances are still needed. For reactions with heterogeneous catalysts, on the other hand, flow conditions are preferred. Packed beds reactors allow higher catalyst loadings which, in turn, decrease the time to achieve full conversion. Reactions with immiscible liquids are another topic. In batch, vigorous stirring can efficiently produce emulsions and the setup is also simpler. These

emulsions, however, are less homogeneous in terms of droplet size. Therefore, when homogeneous, highly reproducible emulsions are required, flow conditions are ideal. Additionally, in flow, there are tortured path reactors which maintain emulsions via turbulent mixing.[93, 97, 98]

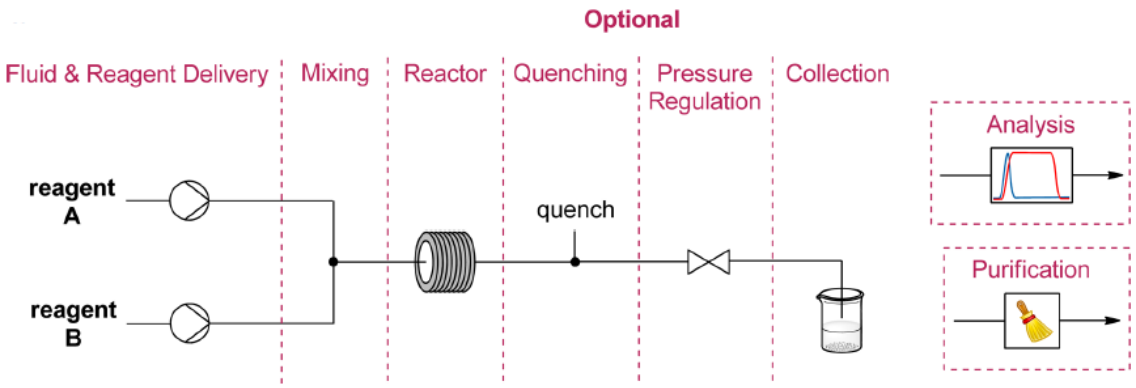
Finally, reactions which are photochemically or electrochemically driven greatly benefit from flow conditions. The Lambert-Beer law describes the attenuation of light as path length increases. Therefore, reaction mixtures will experience more uniform irradiation in flow due to the small diameter of the channels. Since all molecules are exposed to similar amounts of heat and light, photochemical reactions performed in flow are often found to be orders of magnitude faster than the corresponding batch reactions. Electrochemistry also benefits from the small dimensions of flow conditions. Since reactions can be carried out without supporting electrolytes, the cost may be reduced and the purification simplified. Continuous removal of products and improved mass transfer can also benefit the product quality. Additionally, scaling the reaction to multiple grams can be more convenient for flow by simply extending the operation time of the flow process. This is equally the case for photochemistry, where the attenuation of light is problematic for large dimension reaction vessels.[93, 96]

3.1.2 Flow reaction setup

Flow chemistry is a modular technique which provides a toolbox for synthetic chemists. A basic continuous flow setup (Scheme 3.1) for synthetic applications can be broken down into eight basic zones: fluid and reagent delivery, mixing, reactor, quenching, pressure regulation, collection, analysis, and purification.[93]

First, a fluid and reagent delivery system is necessary to accurately feed the respective substances into the flow system. Precise control over the movement of fluids is important for a continuous flow process. It not only regulates the residence time but also influences the stoichiometry if two or more reagent streams are combined in a subsequent mixing unit. Typically, the reagents are in the liquid phase but the apparatus can also be adapted for a biphasic gas-liquid mixture. Depending on the flow rate, the system pressure, and the nature of the liquid phase, three different types of pumps are commonly utilized: HPLC pumps, single shot syringe pumps and peristaltic pumps.[93, 95]

In the next module the reagent streams are combined by a dedicated mixing device before entering the reactor unit where the chemical reaction occurs. This core unit, discussed in more detail in the next subsection, is directly connected to a quenching module, which allows for accurate control of the residence time. Elevated pressure regimes are easily achieved with a pressure regulator, usually located immediately before the final collection of the product stream. In addition, several tools for analysis, as well as continuous purification modules can be implemented. Similar to analytical procedures, most purification steps rely on conventional methods following collection of the reaction mixture from the flow system. Importantly, all of these individual parts can be arranged interchangeably and repetitively, resulting in an infinite number of possible modifications. Highly complex multistep sequences can be applied to natural product synthesis or on-demand production of pharmaceuticals.[93, 95-97]

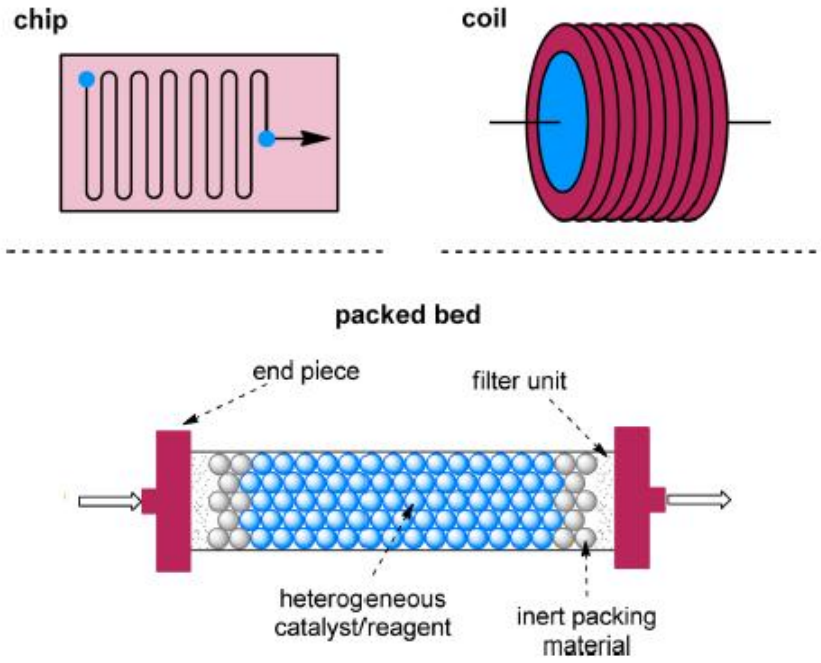


Scheme 3.1. Diagram of a standard two-feed continuous flow setup.

Standardized connections between zones make interchangeability a quality of flow chemistry. Generally, the connections between the different basic zones consist of channels and nonwetted parts, such as nuts and ferrules used to securely attach the tubing to each respective unit. In most cases, all the components required for connecting the modules are identical to those used in standard HPLC devices and are therefore readily available. The dimensions and composition of the tubing are crucial since it is in direct contact with the reagent stream. Physical parameters like the desired system pressure and chemical compatibility must be considered. In general, for low and medium pressure applications inert perfluorinated polymers are adequate. High pressure processes (e.g., reactions far above the boiling point of the reaction medium or reactions using supercritical solvents) require more robust materials such as stainless steel.[93, 95]

3.1.2.1 Types of Reactors

The reactor is the core unit of every flow system, since it is where the chemical reaction occurs. The reactors can generally be divided into three main types: chip, coil, and packed-bed reactors (Scheme 3.2). The nature of the respective transformation (exo- or endothermic, electrochemical, photochemical, multiphasic, etc.) determines the reactor type and material. In general, heating and cooling of all these units can be reached either by conventional means, such as submersion of the reactor unit in a dedicated cooling/heating bath, or by using specialized technologies such as cryogenic cooling units, microwave irradiation, or incubators. Photochemical applications require the reagents stream to be pumped through transparent polymer tubing or a transparent chip microreactor which is irradiated with a light source.[93, 95, 99]



Scheme 3.2. Main types of reactors for continuous flow.

Among all three reactor types, chip-based reactors offer the best heat transfer characteristics due to the extremely high surface-to-volume ratios. Thermal reactions can be controlled by an otherwise unreachable accuracy, making these reactors an ideal tool for process development, despite their low throughput and tendency to clog. Chip reactors are usually machined from silicon, glass, ceramics, or stainless steel by specialized techniques and, in strong contrast to coil-based reactor systems, such

systems often incorporate a mixing section within one microfabricated unit. Recent advances in 3D printing have made such integrated reactor design fast, convenient, and easy. The choice of material depends both on chemical compatibility and type of chemistry. Photochemical transformations can be performed when the chip is constructed from a light permeable material such as glass. Additionally, certain materials allow for the immobilization of a catalyst on the channel wall, providing access to reactions which are heterogeneous.[93, 95, 99]

Due to the high cost of chip-based reactors and their inherent limitations, coil reactors have emerged as the most widely used alternative in synthetic flow chemistry. Coil reactors are usually made out of simple, commercially available tubing made either from inert perfluorinated polymers or stainless steel. The selection of the right material depends of the respective application. The temperature can be easily controlled by submerging the coil in a cooling or heating bath or by mounting it on a dedicated thermostatic unit. Similarly, photochemical activation can easily be carried out by wrapping the coil around a light source or by placing the coil reactor adjacent to a lamp. Importantly, thermal and photochemical techniques can be combined resulting in variable-temperature flow photoreactors.[93, 99]

If heterogeneous catalysts or reagents are required in a continuous chemical transformation, packed-bed reactors are generally utilized. These units are defined as a volume of solid material(s) embedded between filter units through which the reaction solution is passed at a specific position of the flow path. Common packed-bed reactors involve columns or cartridges made from glass, polymeric materials, or stainless steel with dedicated, re-sealable end pieces. There are several advantages of heterogeneous catalysis in a packed bed as opposed to a batch reactor. First, this reactor type affords a significantly higher effective molarity of the catalyst/reagent, decreasing the time to achieve full conversion. Second, as the catalyst/reagent is contained in the reactor the reaction and separation are thus combined in a single step. It facilitates the recyclability of the catalyst as well. Third, the catalyst may have improved lifetime due to continuous removal from the reaction environment of undesired byproducts that may poison/deactivate the catalyst. However continuous heterogeneous catalysis in a packed bed reactor is not always trivial. In particular, for immobilized transition-metal catalysis, leaching of the catalytic material can occur, resulting in contamination of the product and deactivation of the column.[93, 96, 99, 100]

3.1.3 Key parameters

One of the most fundamental differences between chemistry in a flask and in a continuous environment is related to concentration changes. For instance, the substrate concentration decreases over time and is uniformly distributed throughout the flask. In a flow reaction, the concentration of the starting material decreases along the reactor unit reaching a minimum at its end. If ideal plug-flow behavior is assumed, the length dependency leads to a constant concentration of substrate and product at a certain position under steady state conditions. This position is reflected in the so-called residence time which is the time between initiation and termination of a continuous transformation and is often incorrectly compared with the reaction time of a batch process. The residence time can be varied either via changing the flow speed or the length/volume of the flow path (Equation 4).[93, 100]

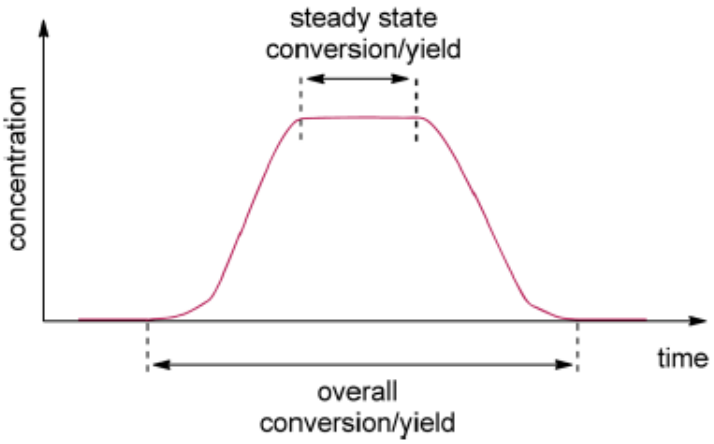
$$residence\ time\ = \frac{Volume}{flow\ speed}$$

Equation 4. Formula used to determine the residence time.

Prediction of the residence time is therefore relatively simple for single-phase transformations since the reactor volume as well as the flow rate is set by the user. For liquid/solid reactions in packed bed reactors and reactions involving a gas, this is less trivial since it depends on several factors such as the dead volume of the packed bed reactor, the solubility of the respective gas in the liquid phase, and the system pressure. It is, therefore, difficult to calculate and easier to simply measure the residence time manually by injecting a dye solution. Regulating the residence time is a nontrivial task since this strongly depends on the respective chemical transformation. The key steps for accurate residence time control are the precise initiation and termination of the reaction. Initiation is carried out by the mixing of reactive reagents with the respective substrate or physical activation by heating or irradiation. In packed bed applications, initiation is carried out at the moment the liquid substrate stream gets in contact with the solid catalyst/reagent. Termination, on the other hand, occurs when the stream leaves the reactor or is carried out via an appropriate quenching technique.[93, 100]

Overall conversion/yield means that the entire reaction mixture was collected and analyzed/isolated, while the conversion/yield under steady state conditions reflects

the values under stable conditions (Scheme 3.3). When communicating yields, it is often useful to report productivity (amount of generated product per time (per amount of catalyst if applicable)) and space-time-yield (amount of generated product per volume per time) to compare different flow and batch approaches.[93]



Scheme 3.3. Difference between overall and steady state values for conversion and yield.

3.1.4 Biocatalysis in continuous flow

The application of continuous flow techniques can favorably influence the sustainability of manufacturing processes. As stated before, performing reactions in continuous flow mode can increase yields, optimize resource utilization, minimize waste generation, and improve process safety. This has not gone unnoticed by the fine chemicals and pharmaceutical industries in its drive to employ more cost-effective and environmentally-friendly processes. As such, the application of continuous-flow techniques in industrial production is currently the focus of growing attention. Not surprisingly, this interest in continuous-flow operation also applies to the implementation of bioconversion processes. Although several large-scale industrial biocatalytic processes are operated in continuous mode (in a series of stirred tanks or in packed beds), most are still operated in fed-batch mode, mainly because of the necessity of using multipurpose plants. Nonetheless, recent developments in continuous flow chemistry are motivating its implementation in biocatalytic processes.[20, 98, 100]

As mentioned before, for reactions with heterogeneous catalysts flow conditions are preferred. Packed-beds reactors allow higher catalyst loadings which, in turn,

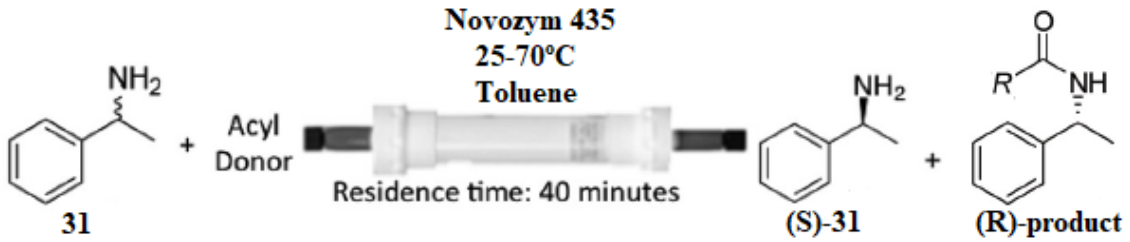
decrease the time to achieve full conversion. In addition, continuous removal of products minimizes enzyme inhibition or deactivation, the biocatalyst can be recycled in a straightforward fashion, and, high conversion and yields are frequently obtained. All these features make this technique an interesting tool for process intensification and/or developing novel reaction methodologies. For obvious reasons, the implementation of flow conditions to biocatalytic reactions is somewhat dependent of the success in biocatalyst (enzyme or whole cells) immobilization. Immobilization may cause slight distortions in the enzyme structure affecting the final properties of the enzyme, namely, activity, specificity, and selectivity. When immobilization is not desirable, reactors integrated with membrane separators, to remove the free enzyme from the output stream, can be employed. Nonetheless, enzyme immobilization is preferable in biochemical reactions because it usually enhances their stability, and facilitates its reutilization. Many different immobilization types and protocols have been developed in the last decade. When working under continuous flow conditions, protein lixiviation is always a concern and should be taken into account when choosing the immobilization protocol/method and during the process development.[96, 98-101]

The number of reports employing enzymatic processes in flow conditions grows every day. Many examples that employ hydrolases, mainly lipases, can be found in the literature. De Souza *et al*, described the resolution, using CALB as biocatalyst, of 1-phenylethylamine (Scheme 3.4), a completely un-natural substrate for a lipase. Even more interesting, was the fact that ethyl acetate could be used as acylating agent, giving similar or better results than more expensive acyl donors such as isoprenyl acetate or methyl 2-methoxyacetate. This transformation could be an alternative to traditional batch production, since the short residence time and the high space-time yield obtained make the process very competitive.[102]

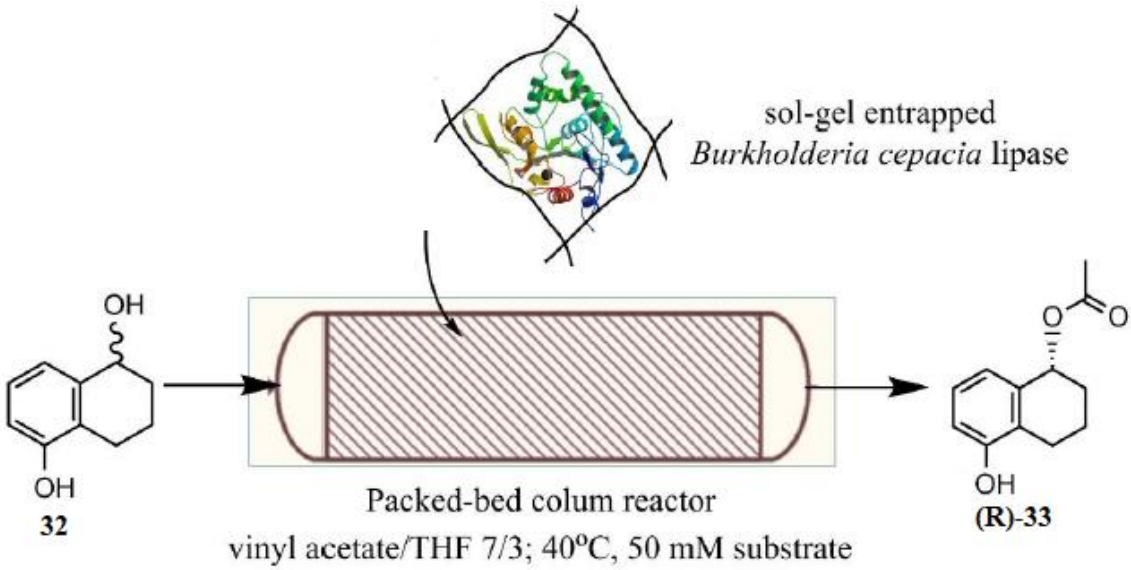
Hunt and co-workers reported the EKR of arylpropionic acids, also catalyzed by Novozym 435, under more sustainable conditions, namely using bio-based solvents like *p*-cymene. More importantly they demonstrated typical advantages of flow over batch. Under flow conditions the authors were able to perform the transformation for three weeks without loss of activity. On the other hand, in batch they observed lower conversions immediately after the first cycle, which the authors attributed to degradation of the Novozym 435 beads due to mechanical stirring. Another important difference was the time necessary to achieve good conversions. While in flow the transformation was performed with a substrate to catalyst ratio of 21 mg mg^{-1}, which

allowed the reaction to be completed after 3 hours, in batch they could only use a ratio of 6 mg mg^{-1} and it was necessary 40 hours to attain a similar result.[103]

Another example of a resolution in continuous flow catalyzed by a lipase is displayed in Scheme 3.5. The authors studied the immobilization of the lipase of *Burkholderia cepacia* in a sol-gel matrix and its application in the resolution of alcohol **32** using vinyl acetate (**4**) as acylating agent and co-solvent. Employing the specified immobilized biocatalyst the authors showed that the transformation could be performed for at least 4 days without significant loss of enantioselectivity (E > 200 during the whole process).[104]



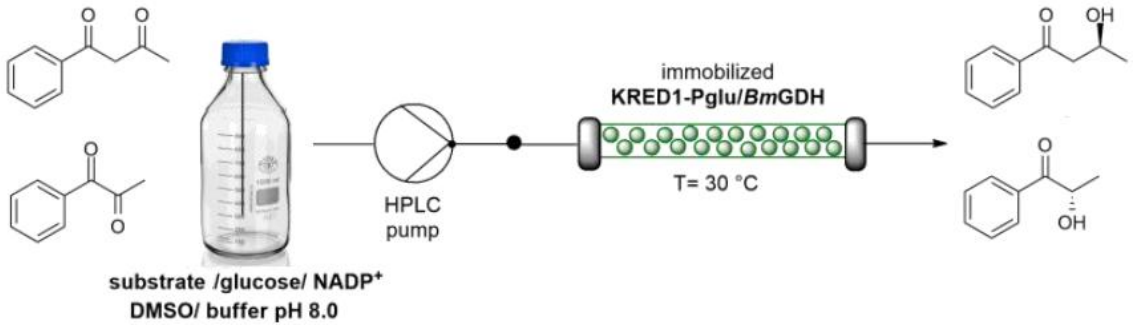
Scheme 3.4. Reported enzymatic kinetic resolution in flow conditions of 1-phenyethylamine (**31**) using CALB as biocatalyst. Several acylating agents (isoprenyl acetate, methyl 2-methoxyacetate or ethyl acetate) were tested and ethyl acetate produced similar or better results than the other more expensive acyl donors.[102]



Scheme 3.5. Reported enzymatic kinetic resolution in flow conditions of 1,5-dihydroxi-1,2,3,4-tetrahydronaphatalene (**32**) by transesterification catalyzed by immobilized *Burkholderia cepacia* lipase. The enzymatic reaction could be performed for at least 4 days without loss of enantioselectivity (E > 200 during the entire process).[104]

Other types of enzymes are used as well. Paradisi *et al*, reported the immobilization of a transaminase from *Halomonas elongata* and the employment of this biocatalyst in the continuous flow transformation of several aldehydes into the respective amines in good to excellent yields. Another interesting feature was the integration of an in-line purification process step, which allowed the collection of pure amines immediately from the output stream.[105]

Oxireductases are industrially relevant enzymes, however they rely on cofactors which are expensive and often not spontaneously regenerated in the catalytic cycle, a critical issue to consider when using these enzymes in flow reactors. Co-immobilization of enzymes, for example, can assemble a coupled enzymatic system that addresses the cofactor regeneration within the same reactor. Dall'Oglio and co-workers reported the efficient reduction of bulky ketones in flow by combining a ketoreductase from *Pichia glucozyma* with a glucose dehydrogenase (Scheme 3.6), which were compatible under the selected reaction conditions and catalytically active for several weeks.[106]



Scheme 3.6. Reported preparation in flow of enantiomerically pure alcohols by reduction of ketones. Two biocatalysts, a ketoreductase from *Pichia glucozyma* and a glucose dehydrogenase, were immobilized together and are responsible for reduction of the substrate and co-factor regeneration in one reactor.[106]

Further applications of continuous-flow biocatalysis are expected to be forthcoming, as this mode of operation results in smaller production units, significant productivity increases, and reduced inventories, affording more sustainable processes.

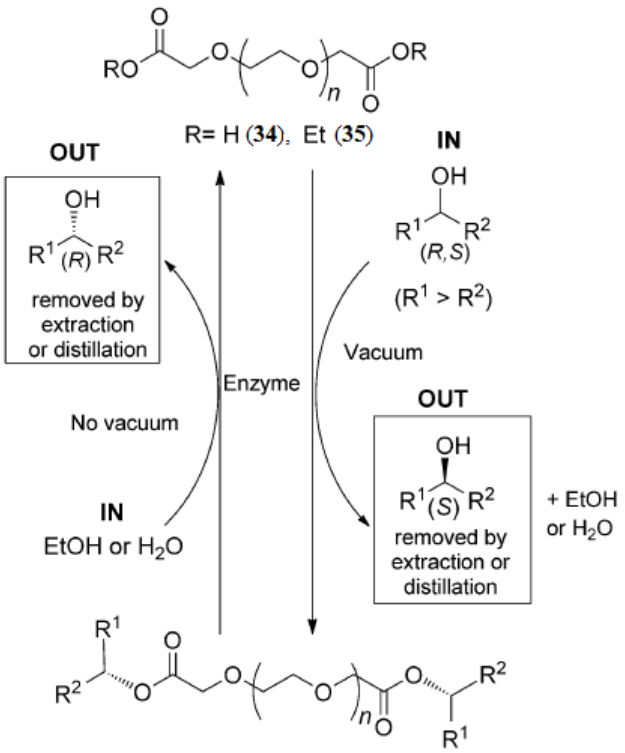
3.2

RESULTS AND DISCUSSION

3.2.1 EKR in continuous flow using PEG_{600} derivatives as acylating agents

In a previous work, our group described the successful application of PEG_{600} (poly(ethylene glycol) with average molecular weight of 600 g/mol) diacid (**34**) or diester (**35**) derivatives as acylating agents in EKR of secondary alcohols (Scheme 3.7).[79] The key advantages of this two-step methodology are i) the use of liquid PEG derivatives as both acylating agent and reaction medium, ii) the easy separation of the enantiomers by organic extraction (the PEG derivatives have higher affinity for aqueous solutions than non-polar solvents like hexane) or by distillation (high molecular weight PEG has extremely high boiling points), and iii) the easy regeneration and recyclability of the acylating agent/reaction medium. The main drawbacks are the long reaction times, between 1 and 6 days for the first step plus another 24 hours for the second step, and the need to execute the first transesterification under vacuum, to remove the water or ethanol that is formed, making the reaction irreversible.

In order to improve the methodology, we decided to explore the utilization of the above mentioned acylating agents in a continuous flow process. The rational behind this is that a higher concentration of biocatalyst by volume is achievable in a packed bed reactor in comparison with a flask in batch, which should decrease significantly the time necessary to attain similar conversions. Another point of interest in such process is the possibility to perform the EKR without vacuum since the water or ethanol that is formed is constantly removed from the reactor, thus minimizing enzyme inhibition. In order to expand the scope of the methodology, secondary alcohols and secondary amines were tested as substrates. This work was developed in collaboration with Prof. Rodrigo de Souza and his group at Universidade Federal do Rio de Janeiro, due to their immense know-how on continuous flow chemistry.



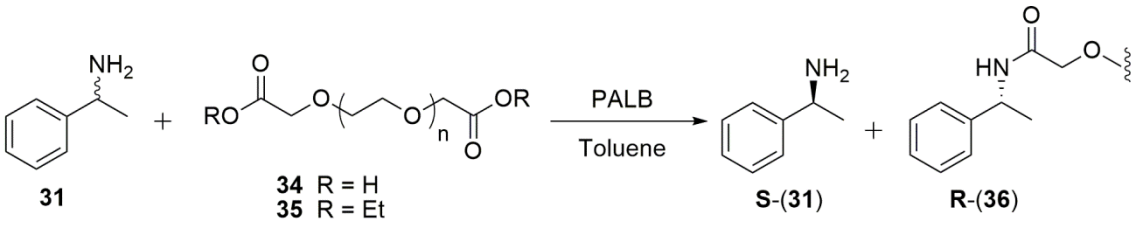
Scheme 3.7. Methodology described by Monteiro *et al* based on the use of PEG_{600} diacid (**34**) or diester (**35**) derivatives as acylating agents and reaction medium for the EKR of secondary alcohols catalyzed by CALB. The products can be separated from the PEG derivatives by organic extraction or by distillation.[79]

3.2.1.1 Secondary amines as substrate

First, a preliminary study was performed using 1-phenylethylamine (**31**) as model substrate, PEG_{600} diester or PEG_{600} diacid as acylating agent and PALB as biocatalyst (Scheme 3.8). Unlike the batch process, where the resolutions were performed in neat conditions, the flow experiments had to be carried out in toluene due to the viscosity of the PEG derivatives, which did not allow an efficient pumping. The reaction conditions (solvent, starting materials concentrations and temperature) were chosen following the procedure described previously by Prof. Rodrigo de Souza's goup.[102] At this early stage we analyzed only the enantiomeric excess of the unreacted enantiomer, since the hydrolysis of the product is not an easy task.

Very good results were obtained (Table 3.1). Enantiomeric excesses of at least 95% were achieved with residence times equal to or higher than 20 minutes. The best EKR was achieved with 30 minutes of residence time, which produced an ee of 98%

(entry 3). As such, this value was chosen for subsequent experiments. Residence times shorter than 20 minutes were not enough to reach full conversion, as such enantiomeric excesses were lower. As observed in previous studies,[79] PEG diester is a more reactive acylating agent than PEG diacid, since for similar residence times better ees were obtained with PEG diester, 25% vs 96% (entry 1 vs. entry 2).



Scheme 3.8. EKR in continuous flow of 1-phenylethylamine (**31**) using PEG_{600} diacid (**34**) or PEG_{600} diester (**35**) as acylating agent and PALB as biocatalyst.

Table 3.1. Results for EKR of racemic 1-phenylethylamine (**31**) using PEG_{600} diacid (**34**) or PEG_{600} diester (**35**) as acylating agent and PALB as biocatalyst.

Entry[a]	Acylating agent	Residence Time (min)	Flow rate (μL/min)	(**S**)-**31** ee[b] (%)
1	**34**	40	30	25
2	**35**	40	30	96
3	**35**	30	40	98
4	**35**	20	60	95
5	**35**	10	120	92
6	**35**	5	240	53

[a] All enzymatic reactions were carried out in a packed-bed reactor (1.2 mL) containing Novozym 435 (0.5 g) at 70ºC using a solution of PEG diacid (**34**) or PEG diester (**35**) ([acyl donor]=0.15 M) and rac-1-phenylethylamine ([amine]=0.15 M) in toluene. [b] Enantiomeric excess was determined by chiral GLC.

Next, employing the ideal flow conditions determined before, an experiment was performed to study the recovery of both enantiomers. After the EKR, the (S)-amine was recovered, by organic extraction, with a yield of 49% and an ee of 98%. In order to isolate the (R)-enantiomer different methodologies were tested for the hydrolysis of the amide produced in the first step. For simplification, these methodologies, namely

enzymatic hydrolysis, enzymatic ethanolysis, and chemical hydrolysis under acidic medium, were performed in batch.

As mentioned before, enzymes are considered green catalysts because, among other features, they are efficient under mild reaction conditions. With this in mind, we tried to perform the cleavage of the amide in aqueous medium using PALB as biocatalyst. Unfortunately, even after 72 hours of reaction at 30-40ºC no (R)-enantiomer was observed. Next we decided to perform the enzymatic ethanolysis using toluene as solvent. This would allow the immediate regeneration of the acylating agent. As in previous works,[36, 79] to avoid enzyme inhibition only 2.5 equivalents of ethanol were used. Yet again, no significant amount of (**R**)-**31** was detected even after 96 hours of reaction at 30-40ºC. Due to the failure of the enzymatic methodologies a classical chemical hydrolysis had to be employed. Using a solution of HCl (3 M) in H_2O/methanol at 80ºC for 14 hours, the cleavage of the product was successful and (**R**)-**31** could be recovered with a yield of 35% and an ee of 95%.

Table 3.2. Results for EKR of racemic 1-phenylethylamine (**31**) using PEG_{600} diester (**35**) as acylating agent and PALB as biocatalyst during 1200 minutes (20 hours).

Reaction[a] time (min)	(**S**)-**31** ee[b] (%)
150	98
300	98
450	99
600	99
750	99
900	99
1050	99
1200	99

[a] The reaction was carried out in a packed-bed reactor (0.830 mL) containing Novozym 435 (0.30 g) at 70ºC using a solution of PEG diester (**35**) ([acyl donor]=0.15 M) and rac-1-phenylethylamine ([amine]=0.15 M) in toluene, and a residence time of 30 minutes (flow rate of 28 µL/min). The output stream was collected in portions of 4.2 mL (equivalent to 5 cycles). [b] Enantiomeric excess was determined by chiral GLC.

The next step was to study the recyclability of PALB in the packed-bed reactor. Using the ideal conditions, racemic 1-phenylethylamine (0.15 M), PEG diester (0.15M) in toluene at 70ºC, the EKR was performed continuously for 20 hours, which is equivalent to 40 cycles of 30 minutes. As can be seen in Table 3.2, the ees of (**S**)-**31** remained above 98%, which means the enzyme maintained its enantioselectivity during the whole process.

As future work, to strengthen the methodology described herein, the procedure should be applied to commercially important substrates, such as the precursors of the drugs rotigotine and cinacalcet.

3.2.1.2 Secondary alcohols as substrate

The methodology developed for amines was then employed in the resolution of alcohols, using 1-phenylethanol (**17**) as model substrate. This task would be much more complicated than expected.

As can be seen in Table 3.3, we started the screening using PEG diester (**35**), since it is a better acylating agent than PEG diacid (**34**). Unfortunately, unlike the previous experiments with amines, we could not achieve good enantiomeric excesses. The values were always below 25% even when the residence time was increased (entries 1-3). From these results we can conclude that the transesterification reaction quickly achieves equilibrium between the direct and the inverse reaction, that is, the ethanol that is formed attacks the newly formed ester at the same speed that the labile 1-phenylethanol ester is formed. In order to favor the direct side of the reaction we performed the EKR using an [1-phenylethanol] / [acyl donor] ratio of 1:10. Given the utilization of diluted solutions of substrate, to maximize productivity the concentrations were increased, that is, 0.025 M and 0.25 M were used instead of 0.015 and 0.15 M. The concentration of PEG diester could not be higher than 0.25 M because above this threshold the solution became too viscous and the pump stalled. As expected, an increase in ee was observed (39% vs 25%, entry 4 vs entry 2) but nonetheless it remains unattractive.

So, just like in the batch methodology, we must remove the byproduct that is formed in the transesterification reaction. For ethanol that means using vacuum, but at the moment we do not possess the apparatus to do so under flow conditions. However if

we use PEG diacid instead of PEG diester, water is formed during the EKR. Water can be removed from solution using drying agents such as molecular sieves or anhydrous magnesium sulfate. These drying agents could be packed with the biocatalyst in the same reactor, in alternate layers, or in another reactor, immediately after the reactor with PALB, and perform several cycles in loop until full conversion is achieved, assuming that the enantiomeric excess of the product is not degraded between each cycle.

Table 3.3. Results for EKR of racemic 1-phenylethanol (**17**) using PEG_{600} diester (**35**) as acylating agent and PALB as biocatalyst.

Entry[a]	[substrate] (M)	[acyl donor] (M)	Residence Time (min)	Flow rate (µL/min)	(**S**)-**17** ee[b] (%)
1	0.15	0.15	20	27	25
2	0.15	0.15	30	18	25
3	0.15	0.15	60	9	25
4	0.025	0.25	30	18	39

[a] All enzymatic reactions were carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 55ºC using a solution of PEG diester (**35**) ([acyl donor]=0.15 or 0.25 M) and rac-1-phenylethanol ([OH]=0.15 or 0.025 M) in toluene. [b] Enantiomeric excess was determined by chiral GLC.

Table 3.4. Results for EKR of racemic 1-phenylethanol (**17**) using PEG_{600} diacid (**34**) as acylating agent, PALB as biocatalyst and a residence time of 30 minutes (flow rate of 18 µL/min).

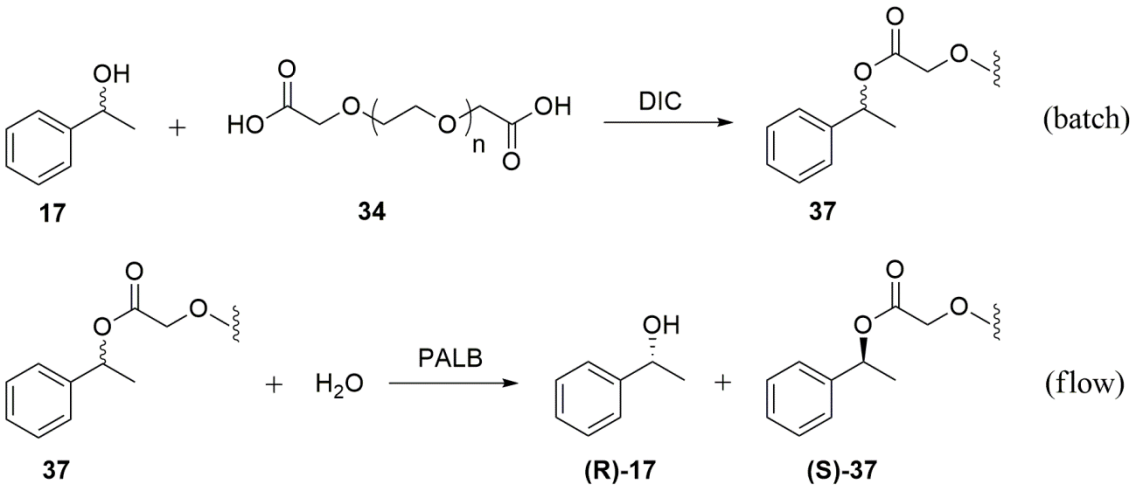
Entry[a]	[substrate] (M)	[acyl donor] (M)	Solvent	(**S**)-**17** ee[b] (%)	(**R**)-**17**[c] ee[b] (%)
1	0.025	0.25	acetone	16	-
2	0.050	0.25	acetone	19	-
3	0.25	0.25	acetone	7	-
4	0.050	0.25	tetrahydrofuran	11	-
5	0.050	0.25	acetonitrile	28	77

[a] All enzymatic reactions were carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 55ºC using a solution of PEG diacid (**34**) ([acyl donor]=0.25 M) and rac-1-phenylethanol ([OH]=0.25, 0.050 or 0.025 M) in the desired solvent (acetone, tetrahydrofuran or acetonitrile) and a residence time of 30 minutes (flow rate of 18 µL/min). [b] Enantiomeric excess was determined by chiral GLC. [c] Chemical hydrolyses were carried out in 2 mL of a 2 M solution of KOH in H_2O at 40ºC for 60-90 minutes.

We performed several EKR using PEG diacid (**34**) as acylating agent (Table 3.4). Water-miscible solvents were used instead of toluene to avoid the formation of two phases inside the packed-bed reactor. We started with acetone due to the good results obtained in PALB-catalyzed reactions described previously. We screened different [1-phenylethanol] / [acyl donor] ratios and different solvents, and determined that the best conditions were [1-phenyethanol] = 0.05M, [PEG diacid] = 0.25M using acetonitrile as solvent (entry 5). In order to advance to the multi-cycle stage, it was necessary to know the ee of the product to compare with the ones obtained in subsequent cycles. Unfortunately we observed that the ee was already low, below 77%. This result is another evidence of the lability of the ester that is formed during the EKR reaction. The ee can only decrease in the subsequent cycles and, as such, this strategy was abandoned.

We proposed another strategy (Scheme 3.9). First, PEG diacid (**34**) was esterified with racemic 1-phenylethanol (**17**) using diisopropylcarbodiimide as coupling agent. At this stage this step was done in batch. Despite tinkering with the conditions of the coupling reaction we could not achieve a full esterification of the PEG diacid (determined by NMR), hence we obtained a mixture of PEG monoester and PEG diester. However the method is reproducible because the amount of 1-phenylethanol linked to PEG was consistent from batch to batch (9-10% of the total mass, value determined by hydrolysis of the product at the end of the coupling protocol). Since we have a mixture of two compounds, it is more accurate to express the concentration of PEG ester (**37**) in mg/mL instead of molarity. The racemic ester was then hydrolyzed in continuous flow using again PALB as biocatalyst. Hydrolysis must be performed in aqueous medium however, since the ester is sparingly soluble in water, a mixture of acetone/water (3:1) was used.

Very good results were obtained with the proposed strategy (Table 3.5). Using a very short residence time, 15 minutes, excellent ees were obtained for (**R**)-**17** and (**S**)-**17**, 97 and 94% respectively (entry 1), which means an almost complete conversion (49%). Next we tried to improve productivity by increasing the concentration of racemic PEG ester. For a concentration of 160 mg/mL, the conversion was much lower, less than 23% (entry 2). This means that the biocatalyst was already working near maximum capacity at a concentration of 80 mg/mL.



Scheme 3.9. New strategy for EKR of alcohols using PEG diacid. First, esterification of PEG_{600} diacid (**34**) with 1-phenylethanol (**17**) using diisopropylcarbodiimide as coupling agent (performed in batch), followed by enzymatic hydrolysis of the racemic ester **37** (performed in flow).

Table 3.5. Results for EKR of racemic PEG_{600} ester **37** by hydrolysis using PALB as biocatalyst.

Entry[a]	[PEG ester] (mg/mL)	Residence time (min)	(**R**)-**17** ee[b] (%)	(**S**)-**17**[c] ee[b] (%)	Conversion[d] (%)
1	80	15	97	94	49
2	160	15	97	29	23

[a] All enzymatic reactions were carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 55ºC using a solution of racemic PEG ester **37** ([substrate]=80 or 160 mg/mL) in acetone/H_2O (3:1) and a residence time of 15 minutes (flow rate of 36 µL/min). [b] Enantiomeric excess was determined by chiral GLC. [c] Chemical hydrolyses were carried out in 2 mL of a 2 M solution of KOH in H_2O at 40ºC for 60-90 minutes. [d] Conversion = ee_s / (ee_s+ee_p).

The next step was to study the stability of the biocatalyst in the packed-bed reactor. Using the ideal conditions, PEG ester **37** (80 mg/mL) in acetone/water (3:1) at 55ºC, the EKR was performed continuously for 195 minutes, which is equivalent to 13 cycles of 15 minutes. As can be seen in Table 3.6, the enzyme preserved its enantioselectivity during the whole process since the ees of (**R**)-**17** remain between 96 and 97%. There is a small loss of activity, since the ee of (**S**)-**17** lowers gradually with time. Nonetheless good conversions were achieved (48-41%).

Table 3.6. Results for EKR of racemic PEG_{600} ester **37** by hydrolysis in acetone/H_2O (3:1) using PALB as biocatalyst during 195 minutes.

Reaction[a] time (min)	(**R**)-**17** ee[b] (%)	(**S**)-**17**[c] ee[b] (%)	Conversion[d] (%)
15	97	90	48
30	97	90	48
45	97	83	46
60	96	85	47
75	97	72	42
90	96	82	46
105	97	67	41
120	96	74	43
135	97	73	43
150	97	82	46
165	97	71	42
180	96	67	41
195	97	68	41

[a] The reaction was carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 55ºC using a solution of racemic PEG ester **37** ([substrate]=80 mg/mL) in acetone/H_2O (3:1) and a residence time of 15 minutes (flow rate of 36 µL/min). The output stream was collected in portions of 0.50 mL (equivalent to 1 cycle). [b] Enantiomeric excess was determined by chiral GLC. [c] Chemical hydrolyses were carried out in 2 mL of a 2 M solution of KOH in H_2O at 40ºC for 60-90 minutes. [d] Conversion = ee_s / (ee_s+ee_p).

In terms of productivity, it was 68 mg h^{-1} g $enzyme^{-1}$ for the (S) enantiomer and 64 mg h^{-1} g $enzyme^{-1}$ for the (R) enantiomer. These values are low but common for EKR reactions, due to the use of diluted solutions, and more than acceptable when you take into account the short residence time of the process.

As mentioned before, acetone as solvent has several advantages, such as low cost and low environmental impact. However it is known that acetone can deactivate enzymes due to reaction between the carbonyl group and nucleophile groups within the protein, for example amines. This could explain the loss activity seen in the enzyme reuse experiment. With this in mind and taking into account the good results obtained with acetonitrile (Table 3.4, entry 5) we decided to repeat the experiment using acetonitrile/H_2O (3:1) as solvent. Results are displayed in Table 3.7 and we can observe

two significant differences. First, while the maximum ees are similar, the loss of activity is less profound when using acetonitrile. After 120 minutes the ee of (**S**)-**17** was 87% versus 74% when using acetone. Also during the span of the first 120 minutes of reaction, the conversions in acetonitrile were always above 46% (after reaching the steady state) while in acetone the minimum was 41%. Another curious difference is that the reaction in acetonitrile needed some time to achieve the steady state (after 15-30 minutes) while in acetone it was practically immediately. In terms of productivity the results were similar, 59 mg h^{-1} g $enzyme^{-1}$ for the (S) enantiomer and 64 mg h^{-1} g $enzyme^{-1}$ for the (R) enantiomer.

Table 3.7. Results for EKR of racemic PEG_{600} ester **37** by hydrolysis in acetonitrile/H_2O (3:1) using PALB as biocatalyst during 120 minutes.

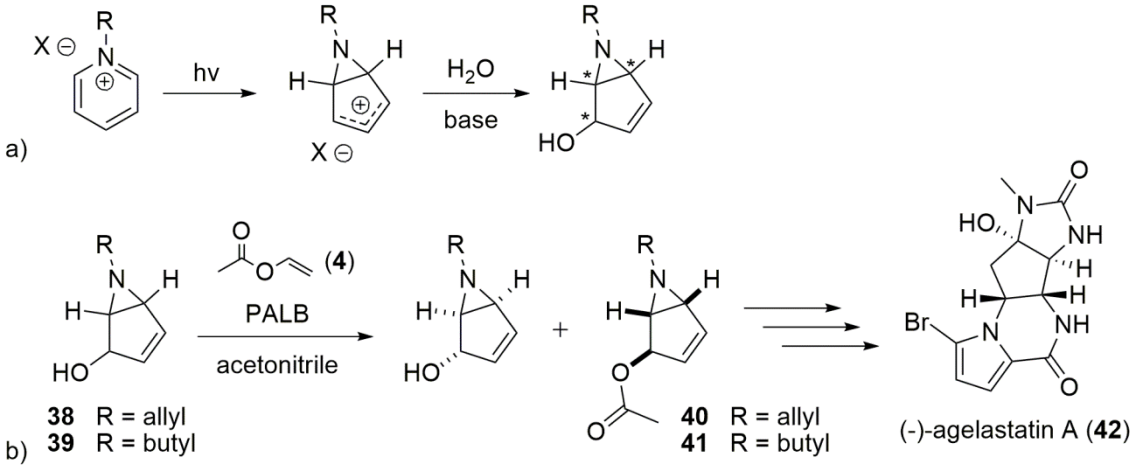
Reaction[a] time (min)	(**R**)-**17** ee[b] (%)	(**S**)-**17**[c] ee[b] (%)	Conversion[d] (%)
15	98	45	31
30	99	83	46
45	99	86	46
60	99	88	47
75	99	92	48
90	99	92	48
105	99	88	47
120	99	87	47

[a] The reaction was carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 55ºC using a solution of racemic PEG ester **37** ([substrate]=80 mg/mL) in acetonitrile/H_2O (3:1) and a residence time of 15 minutes (flow rate of 36 µL/min). The output stream was collected in portions of 0.50 mL (equivalent to 1 cycle). [b] Enantiomeric excess was determined by chiral GLC. [c] Chemical hydrolyses were carried out in 2 mL of a 2 M solution of KOH in H_2O at 40ºC for 60-90 minutes. [d] Conversion = ee_s / (ee_s+ee_p).

As future work, first of all the coupling reaction needs optimization, namely performing the reaction in continuous flow using a packed-bed reactor with immobilized DIC. This optimization will improve the yields and facilitate the purification of the ester (the urea is trapped in the reactor). Similarly to the amine protocol, this strategy should also be employed in the resolution of commercially important substrates.

3.2.2 EKR in continuous flow of α-hydroxycyclopentenone-aziridines

Agelastatins are a family of pyrrole-imidazole alkaloids exhibiting a unique tetracyclic framework with four contiguous stereogenic centers on the carbocyclic C-ring. Several members (agelastatins A to F) of this family have already been isolated from different marine sponges. Due to its potent biological activity (-)-agelastatin A (**42**) is the most interesting one, and several research groups have reported methods for the total synthesis of this alkaloid. However many of them aren't enantioselective.[107, 108]



Scheme 3.10. a) Procedure for the synthesis of valuable α-hydroxycyclopentenone-aziridines by sequential photochemical rearrangent and hydration of pyridinium salts.[109] b) Proposed EKR of two bicyclic aziridines, using vinyl acetate (**4**) as acylating agent and PALB as biocatalyst.

Our group has devoted plenty of attention to α-hydroxycyclopentenone-aziridines (Scheme 3.10a)) because they can be used to create highly functionalized cyclopentenes with biological activity by themselves or as building blocks in the synthesis of natural products.[109] We are particularly interested in studying the enzymatic kinetic resolution of some of these bicyclic aziridines (Scheme 3.10b)) with the objective of developing an enantioselective total synthesis of agelastatin A. In a previous study, performed in batch, good ees (above 93%) and good conversions (up to 50%) could be obtained. However one important problem was observed; there was significant degradation of the EKR product, up to 30%, due to opening of the aziridine ring by attack of acetic acid (itself a degradation byproduct of vinyl acetate (**4**), the

acylating agent used in the EKRs). The long reaction time, at least 20 hours, needed to achieve good conversions may possibly be a factor. Of the several bases screened as scavengers of free acetic acid, triethylamine was the best at diminishing the unwanted reaction. Nonetheless, degradation was still prevalent, particularly when acetonitrile, the solvent that allowed the best ees (above 98%), was employed. With MTBE, on the other hand, no significant degradation was observed, however the ees were far inferior for similar conversions.

With this issue in mind, we decided to study the enzymatic kinetic resolution in continuous flow conditions. As mentioned before, the higher catalyst loading allows the completion of a reaction in a much shorter time than in batch conditions. The shorter operation time followed by a quick quenching step should diminish the amount of degradation obtained. Two substrates, allyl aziridine (**38**) and butyl aziridine (**39**), were studied. Of the two, **38** is the most interesting because the allyl group can be easily removed, by hydrogenation, which is essential later for the synthesis of agelastatin A. As such, we initialized the study using this compound and the results are displayed in Table 3.8. Unfortunately, in spite of the short residence time (20 minutes), the degradation product was detected (about 20%) when acetonitrile was used as solvent (entry 1). Since the amount of degradation was significant we decided to use MTBE instead. No degradation was detected immediately after performing the EKR. However, analysis in the following day revealed the same degradation product observed in acetonitrile (despite storing the samples at -20ºC). This observation indicates that the purifications step must be executed swiftly after the EKR. The best ee, 93%, was achieved using a residence time of 10 minutes (entry 5). For longer residence times, the conversions were higher but the ees were significantly lower (entries 2-4). Similarly to the results in batch, the ees obtained using acetonitrile are higher than the ones attained with MTBE, 97% versus 93% (entry 1 vs entry 5). We also used a mixture of MTBE and acetonitrile (3:1), in an attempt to achieve the best from each world, but the results were similar to the ones obtained using pure acetonitrile.

Next, we applied the same flow conditions in the EKR of butyl aziridine (**39**). As can be seen in Table 3.9, the results obtained with this substrate were better than the ones obtained with **38**. Using a residence time of 10 minutes an ee of 96% was obtained (Table 3.9, entry 1) while for **38** the ee was 93% (Table 3.8, entry 5). The conversions were practically the same. Unlike the previous substrate, increasing the residence, to improve the conversion, did not lead to an erosion of the ee of the product. Using a

residence time of 25 minutes we obtained an excellent conversion of 46%, and ees of 95 and 81%, for the ester **41** and the untouched substrate respectively. We believe that the difference in behaviour is probably due to the longer side chain of butyl aziridine (**39**), in comparison with the allyl chain of **38**, which should fit better in the active site of the enzyme allowing a better recognition (see Scheme 2.10, page 26) and improving its enantioselectivity.

Table 3.8. Results for EKR of allyl aziridine (**38**) using vinyl acetate (**4**) as acylating agent and PALB as biocatalyst.

Entry[a]	Solvent	Residence time (min)	Flow rate (µL/min)	Ester ee[b] (%)	Ester Yield[c] (%)
1	ACN	20	27	97	20[d]
2	MTBE	60	9	69	44
3	MTBE	30	18	83	43
4	MTBE	20	27	88	29
5	MTBE	10	55	93	19
6	MTBE/ACN	20	27	98	19[d]

[a] All enzymatic reactions were carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 26.5ºC using a solution of allyl aziridine (**38**) ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene (0.3 mg/mL) in the desired solvent (acetonitrile, MTBE or MTBE/acetonitrile (3:1)). [b] Enantiomeric excess was determined by chiral GLC. [c] Yield was determined by calibration curve using GLC results. [d] Degradation product observed.

Table 3.9. Results for EKR of butyl aziridine (**39**) using vinyl acetate (**4**) as acylating agent and PALB as biocatalyst.

Entry[a]	Residence time (min)	Flow rate (µL/min)	Substrate ee[b] (%)	Ester ee[b] (%)	Conversion[c] (%)
1	10	55	20	96	17
2	20	27	50	96	34
3	25	22	81	95	46

[a] All enzymatic reactions were carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 26.5ºC using a solution of butyl aziridine (**39**) ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene (0.3 mg/mL) in MTBE. [b] Enantiomeric excess was determined by chiral GLC. [c] Conversion = $ee_s / (ee_s+ee_p)$.

The following step was to assess the enzyme stability in the flow conditions determined above. Using **39** as substrate and under ideal conditions, substrate concentration of 10 mg/mL, vinyl acetate (2 equivalents) and triethylamine (3 equivalents) in MTBE at 26.5ºC, the EKR was performed continuously for 400 minutes (6 hours and 40 minutes), which is equivalent to 16 cycles of 25 minutes. After reaching the steady state, between 150 and 200 minutes, we could achieve conversions up to 47% and ees up to 95 and 83%, for the ester **41** and the untouched substrate respectively. The enzyme started to lose activity after 350 minutes of reaction, when a significant drop in the ees was observed.

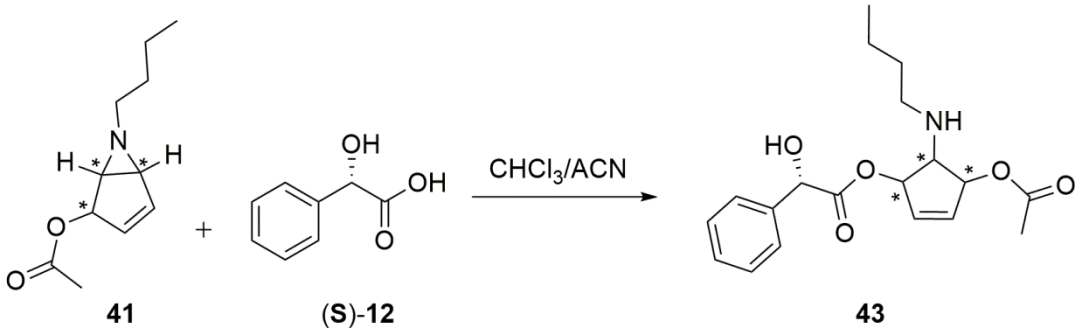
Table 3.10. Results for EKR of butyl aziridine (**39**) using vinyl acetate (**4**) as acylating agent and PALB as biocatalyst during 400 minutes.

Reaction[a] time (min)	Substrate ee[b] (%)	Ester ee[b] (%)	Conversion[c] (%)
50	28	96	23
100	53	95	36
150	74	95	44
200	65	94	41
250	68	95	42
300	83	94	47
350	80	95	46
400	63	92	41

[a] The reaction was carried out in a packed-bed reactor (0.55 mL) containing Novozym 435 (0.145 g) at 26.5ºC using a solution of butyl aziridine (**39**) ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene (0.3 mg/mL) in MTBE and a residence time of 25 minutes (flow rate of 22 µL/min). The output stream was collected in portions of 1.0 mL (equivalent to 2 cycles). [b] Enantiomeric excess was determined by chiral GLC. [c] Conversion = ee_s / (ee_s+ee_p).

The final task was the determination of the absolute configuration of the product obtained in the EKRs. Since both substrates and both esters are oils we can't employ them in X-Ray analysis. As such, derivatization is necessary to attain the mandatory solid crystal. So after the stability study ended all portions were combined and the ester **41** was purified by column chromatography. The ester **41** was chosen for derivatization because it was the compound obtained with the highest ee. Next, we opened the

aziridine ring with enantiomerically pure (S)-mandelic acid (Scheme 3.11) employing a procedure described in literature.[110] After purification by column chromatography, the ring-opening product **43** was obtained with 68% yield and, fortunately, it is indeed a solid that crystallizes. This compound has been sent to X-Ray analysis however the analysis is still pending at the time of writing.



41 (**S**)-**12** **43**

Scheme 3.11. Opening of the aziridine ring of ester **41** with enantiomerically pure (S)-mandelic acid in chloroform/acetonitrile (7:1).

3.3

CONCLUSIONS

First, two continuous flow methods were successfully developed using PEG_{600} derivatives. For amines PEG_{600} diester revealed to be an excellent acylating agent. Very good ees (above 95%) and yields (above 35%) could be achieved for both enantiomers with a short residence time (30 minutes). For breakage of the amide groups and recovery of the (R)-enantiomer, the enzymatic procedures tested using PALB were unsuccessful and we had to resort to chemical hydrolysis with HCl in water/methanol. The biocatalyst could be used for 20 hours without any loss of enantioselectivity.

Unlike amines, the transesterification reaction was not a suitable option for alcohols because the ester that is formed is quite labile and the inverse reaction quickly becomes relevant. A different strategy was developed in which the alcohol was bonded to PEG_{600} diacid and then an enzymatic hydrolysis process was employed. After optimization, we demonstrated that with this method we could obtain productivities of 64 and 68 mg h^{-1} g $enzyme^{-1}$ for the (R) and (S) enantiomer, respectively, during a 195 minutes trial (13 cycles) in acetone/water and with a residence time of only 15 minutes.. The enzyme remained enantioselective (ee of the product always above 96%) and, despite a gradual loss of activity over time, good conversions were achieved during the entire process. Due to this observed decrease in activity, we repeated the EKR in the same conditions except acetonitrile/water was used as solvent. While the productivities were similar, the biocatalyst exhibited a much slower loss of activity and, concomitantly, better conversions were obtained in the same time span.

The enzymatic kinetic resolution in flow of α-hydroxycyclopentenone-aziridines was also studied. Despite using short residence times (20 minutes), the degradation product (aziridine ring opening by acetic acid) observed in batch was also detected in flow, when acetonitrile was used as solvent. No such side reaction was observed when MTBE was used as solvent. After optimization, the method was successfully applied in the resolution of two substrates. For allyl aziridine, using a residence time of 10

minutes, a product ee of 93% and a conversion of 19% was obtained. For butyl aziridine, using a residence time of 25 minutes, a product ee of 96% and a conversion of 46% was attained. We believe that the difference in behaviour is probably due to the longer side chain of butyl aziridine, in comparison with the allyl chain, which should fit better in the active site of the enzyme allowing a better recognition and improving its enantioselectivity. Finally, a biocatalyst stability study was performed, using butyl aziridine as substrate. The enzyme remained enantioselective for 350 minutes, with conversions as high as 47%, before a significant drop in the ees of the product and the substrate was observed.

3.4

EXPERIMENTAL SECTION

3.4.1 General remarks

All reagents were obtained commercially (purity >95%) and used as received, unless otherwise noted. All solvents were obtained commercially and, when necessary, purified appropriately before use. All aqueous solutions were prepared using distilled water.

Immobilized *Pseudozyma antarctica* lipase B (PALB, formerly known as *Candida antactica* lipase B (CALB); Novozym® 435 with 1-2 wt% of water and 7000 PLU/g) was a gift from Novozymes Co. (Denmark). Allyl aziridine (**38**) and butyl aziridine (**39**) were available in the lab from previous studies.

3.4.2 Detection, isolation and purification of reaction products

Thin layer chromatography (TLC) was applied, when adequate, in the analysis of reaction mixtures and was performed in ALUGRAM® Xtra SIL G/UV_{254} silica-gel plates (Macherey-Nagel, Germany) with detection by UV irradiation or by immersion in an ethanolic solution of phosphomolybdic acid, followed by heating.

Flash chromatography was performed on silica-gel 60 (0.04 – 0.06 mm, 230 – 400 mesh ASTM) (Scharlau, Spain) under pressure and with the appropriate eluent or system of eluents (mentioned in the experimental procedures).

3.4.3 Characterization and analysis

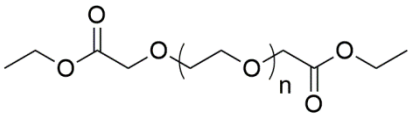
The structure of all synthesized compounds was confirmed by Nuclear Magnetic Resonance (NMR). NMR spectra were recorded at room temperature, unless otherwise noted, on Bruker Avance II+ 300 (^{1}H 300 MHz, ^{13}C 75 MHz) or 400 (^{1}H 400 MHz, ^{13}C 100 MHz) spectrometers, using the residual solvent signal as reference, unless otherwise noted. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in hertz (Hz).

The enantiomeric excess (ee) was calculated by GLC analysis and the enantiomers were identified by comparison to standards. GLC analysis of 1-phenylethanol (**17**) was performed in a GC-2010-Plus Shimadzu with FID detection, using a Varian CP-CHIRASIL-DEX-CB (25 m x 0.25 mm x 0.25 µm) capillary column, injector at 250ºC, detector at 250ºC, 1:100 split ratio and a column flow (He) of 0.95 mL/min. 1-Phenylethanol (**17**) – oven: 120ºC for 14 min, ramp 15ºC/min to 180ºC and 180ºC for 5 min; t_R(dodecane) = 6.1 min, t_R(R) = 9.6 min, t_R(S) = 10.3 min. GLC analysis of 1-phenylethylamine (**31**), allyl aziridine (**38**) and butyl aziridine (**39**) was performed in a Trace Focus Unicam with FID detection, using a HYDRODEX-β-6TBDM (25 m x 0.4 mm x 0.25 µm) capillary column, injector at 200ºC, detector at 200ºC, 1:20 split ratio and a column flow (N_2) of 10 mL/min. 1-Phenylethylamine (**31**) derivatized as N-(1-phenylethyl)acetamide – oven: 140ºC for 36 min, ramp 10ºC/min to 200ºC and 200ºC for 5 min; t_R(R) = 31.9 min, t_R(S) = 33.3 min Allyl aziridine (**38**) – oven: 100ºC for 69 min, ramp 10ºC/min to 200ºC and 200ºC for 20 min; t_R(naphtalene) = 20.3 min, t_R(acetylated enantiomer) = 42.6 min, t_R(acetylated enantiomer) = 46.3 min, t_R(substrate enantiomer) = 52.6 min, t_R(substrate enantiomer) = 56.6 min. The areas of the peaks corresponding to the substrate were not reproducible; as such the yield of the product was determined by calibration curve. Butyl aziridine (**39**) – oven: 100ºC for 89 min, ramp 10ºC/min to 200ºC and 200ºC for 20 min; t_R(naphtalene) = 20.3 min, t_R(acetylated enantiomer) = 69.0 min, t_R(acetylated enantiomer) = 71.7 min, t_R(substrate enantiomer) = 77.5 min, t_R(substrate enantiomer) = 83.6 min.

High resolution mass spectrometry was performed by Unidade Militar Laboratorial de Defesa Biológica e Química and was carried out in a Thermo Scientific Orbitrap Mass Spectrometer with ESI injection.

3.4.4 Experimental procedures

3.4.4.1 Preparation of poly(ethylene glycol)$_{600}$ diester (35).



A solution of poly(ethylene glycol)$_{600}$ diacid (10.0 g, 16.7 mmol) and sulfuric acid (15-20 drops) in absolute ethanol (125 mL) was heated at 80ºC overnight. After concentration at reduced pressure, the mixture was diluted with aqueous saturated solution of $NaHCO_3$ (100 mL) followed by extraction with dichloromethane (3 x 50 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure, giving compound **35** as a colorless viscous liquid (6.65 g, 60%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.20 (d, 4H), 4.13 (s, 4H), 3.70 (m, 8H), 3.64 (d, 32H), 1.27 ppm (t, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.1, 70.3, 70.2, 68.3, 60.4, 13.9 ppm.
NMR data in agreement with literature.[79]

3.4.4.2 General procedure for the preliminary enzymatic kinetic resolutions of 1-phenylethylamine (31) using PEG$_{600}$ derivatives as acylating agent.

A stainless-steel reactor (internal volume of 1.2 mL) filled with Novozym® 435 beads (0.50 g) was placed in an oven at 70ºC and toluene (3-4 volumes of the reactor) was pumped to wet the beads. A solution of 1-phenylethylamine ([amine]=0.15 M) and PEG diacid (**34**) or PEG diester (**35**) ([acyl donor]=0.15 M) in toluene (5 mL) was then pumped at the flow rate (240, 120, 60, 40 or 30 µL/min) necessary to achieve the desired residence time (5, 10, 20, 30 or 60 minutes). The output stream was collected in portions of 1.2 mL (equivalent to 1 cycle). Each portion was concentrated under reduced pressure before addition of HCl (1 M, 2 mL). The aqueous phase was washed with dichloromethane (3 x 1 mL) to remove PEG species, basified with NaOH (1 M) and extracted with dichloromethane (3 x 1 mL). The organic phases were combined,

dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis.

3.4.4.3 Procedure for the preparative enzymatic kinetic resolution of 1-phenylethylamine (31) using PEG_{600} diester (35) as acylating agent.

A stainless-steel reactor (internal volume of 0.830 mL) filled with Novozym® 435 beads (0.30 g) was placed in an oven at 70ºC and toluene (3-4 volumes of the reactor) was pumped to wet the beads. A solution of 1-phenylethylamine ([amine]=0.15 M) and PEG diester ([acyl donor]=0.15 M) in toluene (5 mL) was then pumped at a flow rate of 28 µL/min (residence time of 30 minutes). The output stream was collected in portions of 0.800 mL (equivalent to 1 cycle). The last three portions (2.4 mL) were combined and concentrated under reduced pressure before addition of HCl (1 M, 5 mL). The aqueous phase was washed with dichloromethane (3 x 1 mL) to remove PEG species, basified with NaOH (1 M) and extracted with dichloromethane (3 x 2 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and dried under reduced pressure (0.021 g of (**S**)-**31**, 98% ee). The dichloromethane with PEG species was concentrated under reduced pressure. The (R)-enriched amide was dissolved in methanol (3 mL) and a solution of HCl (6 M, 3 mL) was added. The hydrolysis was carried out at 80ºC for 16 hours. After concentration at reduced pressure, HCl (1 M, 5 mL) was added. The aqueous phase was washed with dichloromethane (3 x 2 mL) to remove PEG diacid, basified with NaOH (1 M) and extracted with dichloromethane (3 x 2 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and dried under reduced pressure (0.015 g of (**R**)-**31**, 95% ee).

3.4.4.4 Procedure for study of PALB stability in the enzymatic kinetic resolution of 1-phenylethylamine (31) using PEG_{600} diester (35) as acylating agent.

A stainless-steel reactor (internal volume of 0.830 mL) filled with Novozym® 435 beads (0.30 g) was placed in an oven at 70ºC and toluene (3-4 volumes of the reactor) was pumped to wet the beads. A solution of 1-phenylethylamine ([amine]=0.15 M) and PEG diester ([acyl donor]=0.15 M) in toluene (35 mL) was then pumped at a flow rate of 28 µL/min (residence time of 30 minutes) for 1200 minutes (20 hours). The

output stream was collected in portions of 4.2 mL (equivalent to 5 cycles). Each portion was concentrated under reduced pressure before addition of HCl (1 M, 8 mL). The aqueous phase was washed with dichloromethane (3 x 4 mL) to remove PEG species, basified with NaOH (1 M) and extracted with dichloromethane (3 x 4 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and dried under reduced pressure prior to analysis.

3.4.4.5 General procedure for acetylation of 1-phenylethylamine (31) for GLC analysis.

After separation of the enantiomers, 1-phenylethylamine (**31**) was derivatized to N-(1-phenylethyl)acetamide with acetic anhydride to allow the separation of peaks in the GC. The amine was mixed with acetic anhydride (2 equivalents) and triethylamine (3 equivalents mmol) in dichloromethane (1 mL). The solution was stirred for 30-60 minutes at room temperature, before washing with HCl (1M, 2 x 1 mL), saturated aqueous solution of $NaHCO_3$ (2 x 1 mL) and water (1mL). After being dried with anhydrous sodium sulfate, the sample was concentrated under reduced pressure.

3.4.4.6 General procedure for the preliminary enzymatic kinetic resolutions of 1-phenylethanol (17) using PEG_{600} diester (35) as acylating agent.

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 55ºC and toluene (3-4 volumes of the reactor) was pumped to wet the beads. A solution of 1-phenylethanol ([alcohol]=0.15 or 0.025 M) and PEG diester ([acyl donor]=0.15 or 0.25 M) in toluene (4 mL) was then pumped at the flow rate (27, 18 or 9 µL/min) necessary to achieve the desired residence time (20, 30 or 60 minutes). The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle). Each portion was diluted with water (2 mL) and the (S)-enriched enantiomer was extracted with hexane/diethyl ether ((3:1), 3 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis.

3.4.4.7 General procedure for the preliminary enzymatic kinetic resolutions of 1-phenylethanol (17) using PEG_{600} diacid (34) as acylating agent.

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 55ºC and the desired solvent (3-4 volumes of the reactor) was pumped to wet the beads. A solution of 1-phenylethanol ([alcohol]=0.25, 0.050 or 0.025 M) and PEG diacid ([acyl donor]=0.25 M) in 4 mL of the desired solvent (acetone, tetrahydrofuran or acetonitrile) was then pumped at a flow rate of 18 µL/min (residence time of 30 minutes). The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle). Each portion was diluted with water (2 mL) and the (S)-enriched enantiomer was extracted with hexane/diethyl ether ((3:1), 3 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. To recover the (**R**)-**17**, KOH was added to the aqueous phase ([KOH]=2 M) and the hydrolysis was carried out at 40ºC for 60-90 minutes. The (R)-enantiomer was extracted with hexane/diethyl ether ((3:1), 3 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis.

3.4.4.8 Preparation of PEG_{600} ester of racemic 1-phenylethanol (37).

To a solution of PEG diacid (1.50 g, 2.5 mmol) in acetonitrile (7.5 mL) was added diisopropylcarbodiimide (0.930 mL, 6.0 mmol). After stirring at room temperature for 15 minutes, 1-phenylethanol (0.605 mL, 5.0 mmol) and DMAP (0.031 g, 0.25 mmol) were added. The suspension was stirred at 50ºC for 24 hours, and then the precipitate was filtered. After concentration at reduced pressure, the mixture was dissolved in water (100 mL) and washed with hexane/diethyl ether ((3:1), 4 x 20 mL). After acidification with HCl (2 M), the product was extracted with dichloromethane (4 x 20 mL). The organic phases were combined, dried with anhydrous sodium sulfate, filtered and concentrated at reduced pressure. Another filtration was performed to remove residual urea. Afterwards, the product was dissolved in water (250 mL), washed with hexane/diethyl ether ((3:1), 4 x 40 mL) to remove residual 1-phenylethanol, and extracted with dichloromethane (3 x 40 mL). The organic phases were combined, dried

with dried with anhydrous sodium sulfate, filtered and concentrated at reduced pressure. After another filtration was necessary to remove residual urea, the product was dried under vacuum at room temperature for 24 hours to give compound **37** as a colorless viscous liquid (1.41 g, 70%). The product is a mixture of PEG monoester and PEG diester. Hydrolysis, with KOH in water, revealed a consistent binding of 1-phenylethanol (9-10% of the total mass) from batch to batch.

3.4.4.9 General procedure for the preliminary enzymatic kinetic resolutions of racemic PEG_{600} ester 37 by hydrolysis.

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 55ºC and acetone/water ((3:1), 3-4 volumes of the reactor) was pumped to wet the beads. A solution of racemic PEG ester **37** ([substrate]=80 or 160 mg/mL) in acetone/water ((3:1), 4 mL) was then pumped at a flow rate of 36 µL/min (residence time of 15 minutes). The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle). Each portion was diluted with water (2 mL) and the (R)-enriched enantiomer was extracted with hexane/diethyl ether ((3:1), 3 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. To recover the (**S**)-**17**, KOH was added to the aqueous phase ([KOH]=2 M) and the hydrolysis was carried out at 40ºC for 60-90 minutes. The (S)-enantiomer was extracted with hexane/diethyl ether ((3:1), 3 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis.

3.4.4.10 Procedure for study of PALB stability in the enzymatic kinetic resolution of racemic PEG_{600} ester 37 by hydrolysis in acetone/water (3:1).

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 55ºC and acetone/water ((3:1), 3-4 volumes of the reactor) was pumped to wet the beads. A solution of racemic PEG ester **37** ([substrate]=80 mg/mL) in acetone/water ((3:1), 8.5 mL) was then pumped at a flow rate of 36 µL/min (residence time of 15 minutes) for 195 minutes. The output stream

was collected in portions of 0.5 mL (equivalent to 1 cycle). Each portion was diluted with water (2 mL) and the (R)-enriched enantiomer was extracted with hexane/diethyl ether ((3:1), 4 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. To recover the (**S**)-**17**, KOH was added to the aqueous phase ([KOH]=2 M) and the hydrolysis was carried out at 40ºC for 60-90 minutes. The (S)-enantiomer was extracted with hexane/diethyl ether ((3:1), 4 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. After GLC analysis, the aliquots of each enantiomer were combined, concentrated, dissolved in *n*-hexane, filtered to remove residual urea and dried at reduced pressure (0.030 g of (**R**)-**17**, 0.032 g of (**S**)-**17**).

3.4.4.11 Procedure for study of PALB stability in the enzymatic kinetic resolution of PEG_{600} ester 37 by hydrolysis in acetonitrile/water (3:1).

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 55ºC and acetonitrile/water ((3:1), 3-4 volumes of the reactor) was pumped to wet the beads. A solution of racemic PEG ester **37** ([substrate]=80 mg/mL) in acetonitrile/water ((3:1), 5.5 mL) was then pumped at a flow rate of 36 µL/min (residence time of 15 minutes) for 120 minutes. The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle). Each portion was diluted with water (2 mL) and the (R)-enriched enantiomer was extracted with hexane/diethyl ether ((3:1), 4 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. To recover the (**S**)-**17**, KOH was added to the aqueous phase ([KOH]=2 M) and the hydrolysis was carried out at 40ºC for 60-90 minutes. The (S)-enantiomer was extracted with hexane/diethyl ether ((3:1), 4 x 2 mL). The organic fractions were dried with anhydrous sodium sulfate, filtered and concentrated under reduced pressure prior to analysis. After GLC analysis, the aliquots of each enantiomer were combined, concentrated, dissolved in *n*-hexane, filtered to remove residual urea and dried at reduced pressure (0.018 g of (**R**)-**17**, 0.017 g of (**S**)-**17**).

3.4.4.12 General procedure for the preliminary enzymatic kinetic resolutions of allyl aziridine (38) using vinyl acetate (4) as acylating agent.

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 26.5ºC and the desired solvent (3-4 volumes of the reactor) was pumped to wet the beads. A solution of allyl aziridine ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene (0.3 mg/mL) in 4 mL of the desired solvent (acetonitrile, MTBE, MTBE/acetonitrile (3:1)) was then pumped at the flow rate (55, 27, 18 or 9 µL/min) necessary to achieve the desired residence time (10, 20, 30 or 60 minutes). The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle), followed by GLC analysis.

3.4.4.13 General procedure for the preliminary enzymatic kinetic resolutions of butyl aziridine (39) using vinyl acetate (4) as acylating agent.

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 26.5ºC and MTBE (3-4 volumes of the reactor) was pumped to wet the beads. A solution of butyl aziridine ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene (0.3 mg/mL) in MTBE (4 mL) was then pumped at the flow rate (55, 27 or 22 µL/min) necessary to achieve the desired residence time (10, 20 or 25 minutes). The output stream was collected in portions of 0.5 mL (equivalent to 1 cycle), followed by GLC analysis.

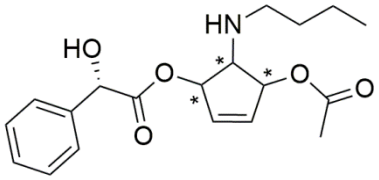
3.4.4.14 Procedure for study of PALB stability in the enzymatic kinetic resolution of butyl aziridine and purification of the acetylation product (41).

A stainless-steel reactor (internal volume of 0.55 mL) filled with Novozym® 435 beads (0.145 g) was placed in an incubator at 26.5ºC and MTBE (3-4 volumes of the reactor) was pumped to wet the beads. A solution of butyl aziridine ([substrate]=10 mg/mL), vinyl acetate (2 equivalents), triethylamine (3 equivalents) and naphthalene

(0.3 mg/mL) in MTBE (10 mL) was then pumped at a flow rate of 22 µL/min (residence time of 25 minutes) for 400 minutes. The output stream was collected in portions of 1.0 mL (equivalent to 2 cycles). After GLC analysis, the aliquots were combined and concentrated under reduced pressure. The mixture was purified by column chromatography (eluent: ethyl acetate/*n*-hexane (2:1)). The solvent was evaporated under reduced pressure, to give compound **41** as a yellow oil (0.032 g, 32%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 6.37 (d, 1H), 5.81 (d, 1H), 5.43 (d, 1H), 2.50 (m, 2H), 2.37 (m, 1H), 2.26 (m, 1H), 2.07 (s, 3H), 1.55 (m 2H), 1.33 (m, 2H), 0.90 ppm (t, 3H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 170.3, 138.1, 133.6, 76.8, 58.1, 47.9, 46.9, 31.7, 21.2, 20.6, 14.2 ppm.

3.4.4.15 Preparation of (2S)-4-acetoxy-5-(butylamino)cyclopent-2-enyl 2-hydroxy-2-phenylacetate (43).



To a solution of acetylated butyl aziridine (**41**, 0.032 g, 0.16 mmol) in chloroform/acetonitrile (7:1) was added (S)-mandelic acid (0.029 g, 0.19 mmol). The suspension was stirred at room temperature for 48 hours. After concentration under reduced pressure, the mixture was purified by column chromatography (eluent: ethyl acetate/*n*-hexane/triethylamine (40:20:1)). The product was concentrated under reduced pressure and left to crystallize by slow evaporation of the solvent, to give compound **43** as white to pale yellow crystals (0.038 g, 68%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.36 (m, 5H), 5.98 (t, 2H), 5.38 (d, 1H), 5.22 (d, 1H), 5.17 (s, 1H), 3.06 (t, 1H), 2.15 (m, 2H), 2.05 (s, 3H), 1.18 (m, 4H), 0.81 ppm (t, 3H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.5, 171.2, 138.2, 133.9, 132.7, 128.73, 128.69, 126.6, 84.4, 82.5, 73.0, 70.8, 47.7, 32.0, 21.2, 20.3, 14.1 ppm.
HRMS(ESI) m/z: 348.1892 $[M+H]^+$.

CHAPTER 4

IONIC LIQUIDS BASED ON ESSENTIAL OILS COMPONENTS WITH ANTIMICROBIAL ACTIVITY

4.1

INTRODUCTION

As mentioned in a previous chapter, environmental, health and cost issues have prompted researchers to develop more environmentally-friendly ionic liquids from inexpensive, renewable sources. One excellent alternative to frequently used cations, like 1-methylimidazolium, is N,N,N-trimethyl-2-hydroxyethylammonium, commonly known and henceforth described as choline.

4.1.1 Choline, an essential molecule

Choline is a quaternary ammonium salt that can be extracted from natural sources like eggs, peanuts and soy. Choline is found in its free form and as phosphoesters (phosphocholine, glycerophosphocholine or phosphatidylcholine). However, nowadays, it is industrially produced, as choline chloride (**44**), on the million metric scale annually as an additive for chicken feed, among other applications. In addition to being inexpensive, choline chloride is considered to be biocompatible and biodegradable.[111-114]

Choline is, in fact, an essential molecule to all organisms, in particular for animals and plants. Choline is fundamental in the biosynthesis of the most predominant phospholipids found in animal and plant cell membranes. In humans, it is needed for signaling functions of cell membranes, for normal cholinergic neurotransmission, for normal muscle function, for lipid transport from liver and it is the major source of methyl groups for S-adenosylmethionine biosynthesis, which, in turn, is the methyl donor for transmethylation reactions in the organism. While humans have a small pool of methyl donors that allow de novo synthesis of choline, it must be obtained from diet as well to replenish such pool. For all these reasons, choline is considered an essential nutrient in human diet since 1998.[111, 112, 115]

4.1.2 Synthesis of choline-based ionic liquids

By now, it is clear that choline is an excellent building block for the preparation of inexpensive, biodegradable ionic liquids. The synthetic methods to obtain ionic liquids based on choline or choline-derivatives can be divided in four groups: alkylation of tertiary amines, metathesis reactions, neutralization reactions and hydroxyl group modifications.

The first group involves the alkylation of tertiary amines, like trimethylamine or N,N-dimethyl-2-hydroxyethylamine (**45**), with alkyl halides, such as 2-chloroethanol or iodomethane. Such strong alkylating agents raise health concerns and, therefore, alternative alkylating protocols have been developed. Alkyl sulfonates (e.g. methyl tosylate), dialkylic carbonates (e.g. dimethyl carbonate) and even alkyl carboxylates (e.g. methyl salicylate) are more environmentally friendly reagents for such reactions. They also allow the preparation of halide-free ionic liquids, which is important for certain applications because even minute amounts of halide can influence the outcome of the experiment (e.g. deactivation of enzymes) or influence the desired physicochemical properties of the ionic liquids. These methods are the only ones that employ exclusively non-ionic starting materials for preparation of ionic compounds, which, frequently, translates into simpler purification steps.[116-121]

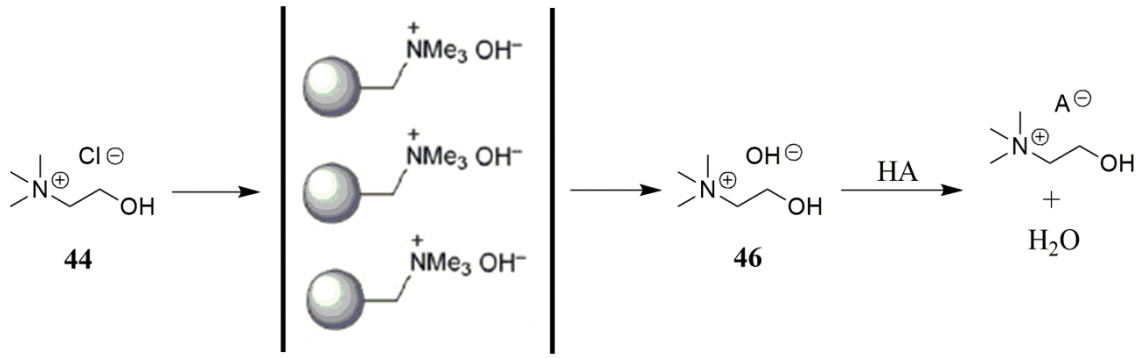
In a metathesis reaction, equimolar amounts of a choline halide, usually **44**, and an alkali metal salt of the desired anion are mixed in a proper solvent. Both salts exchange their counter-ions, originating the desired ionic liquid and the respective alkali metal halide salt. For hydrophilic ionic liquids, organic solvents, like ethanol or acetonitrile, should be used because the alkali metal halide salt is insoluble in such solvents, leading to its precipitation and shifting the reaction to product formation. For hydrophobic ionic liquids, water should be used. Unreacted starting materials and the inorganic salt remain in the aqueous phase, while the hydrophobic product originates another phase. The main drawback of this type of method is the significant amount of halide impurities in the product, which can be problematic as explained above.[19, 116, 122, 123]

Of all the procedures available for ionic liquid preparation, the neutralization method is the simplest one. An equimolar amount of choline hydroxide (**46**) or bicarbonate, in water or methanol, is added to the acid form of the desired anion. Water

is the only byproduct (and carbon dioxide for choline bicarbonate) and can be easily removed under reduced pressure. This type of methods is halide-free as well. The key limitations are the high cost and instability (mainly choline hydroxide) of the choline bases. These solutions can't be stored for long periods because with time a significant amount of choline is attacked by the base, originating trimethylamine and acetaldehyde.[10, 123]

The third type of methods is actually a combination of the two previous groups. An anion-exchange resin, with hydroxide as counter-anion, is used to exchange the halide anions of the starting choline with hydroxide anions from the resin (Scheme 4.1). This way, choline hydroxide (**46**) is prepared *in situ* and in the exact amount that is necessary to carry out the neutralization reaction that follows. This method is very useful because it combines the advantages of the two previous methods. Low-cost choline chloride (**44**) can be used as starting material and the neutralization reaction is simple and straightforward, originating water as only byproduct. The degradation of choline hydroxide is insignificant because this base is immediately used after its preparation. In addition the halide impurities are very low because the anions are trapped by the resin.[9, 113, 120]

The final group encompasses modifications made in the hydroxyl group. Generally, these reactions are esterifications, either directly in choline or in a precursor amine followed by quaternization. The objective of such modifications is the introduction of other functional groups or structures in the ionic liquid, or to modulate a desired property.[117, 120]

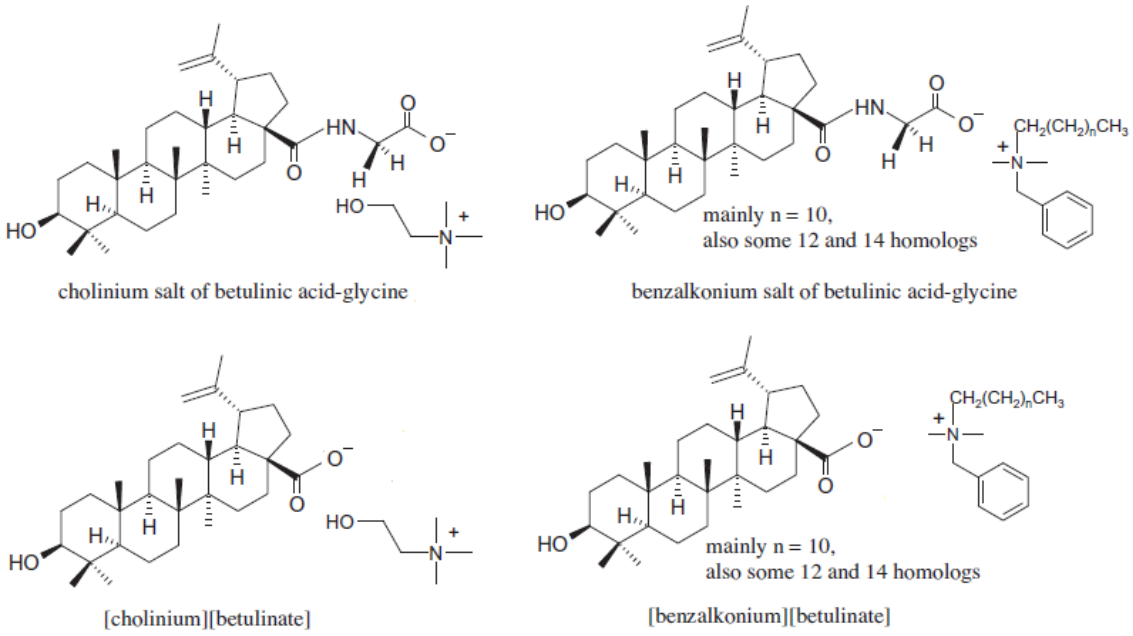


Scheme 4.1. An anion-exchange resin is employed to replace the counter-anions of choline chloride (**44**) with hydroxide anions, forming choline hydroxide (**46**) *in situ*. This base is immediately used in the neutralization of the desired anion acid form, producing the ionic liquid and water.

4.1.3 Applications of choline-based ionic liquids

A plethora of examples of choline-based ionic liquids with many applications in mind are found in the literature. Bose and co-workers prepared several ionic derivatives of betulinic acid, a potent cytotoxic agent, in order to improve its water solubility (Scheme 4.2). Curiously, the straightforward cholinium betulinate was the most potent of the four prepared, with an IC_{50} of 36 and 25 µM against A375 and MCF7 cell lines, respectively. For comparison, the acid form of betulinic acid had IC_{50} of 154 and 122 µM against the same cell lines.[15]

Branco *et al*, prepared several ionic liquids based on ampicillin, a common antibiotic, employing an anion exchange resin method. The objective was to improve its water solubility and, concomitantly, its antimicrobial activity against pathogenic bacteria. Different cations were employed, including choline. Only one combination improved the biological activity of ampicillin.[124]



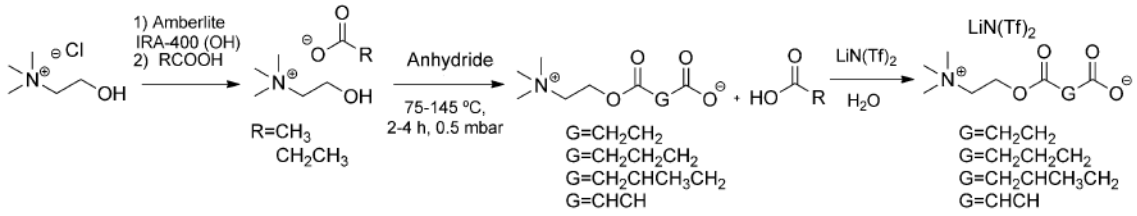
Scheme 4.2. Ionic derivates prepared by Bose and co-workers to improve the water solubility of betulinic acid, a potent cytotoxic agent. Cholinium betulinate was 3-4 times more potent than its parent acid.[15]

Many researchers have described ionic liquids based on choline and inexpensive anions for the development of novel biodegradable, low-cost solvents. Ohno *et al*, also employing an anion-exchange resin method, combined choline with natural carboxylic acids, such as benzoic acid, glycolic acid, fumaric acid, among others. Some of them

were room-temperature ionic liquids and two were actually liquid at temperatures below 0ºC. Another interesting feature was that these ionic liquids exhibited greater hydrogen bonding abilities than conventional ionic liquids.[9] A similar work was performed by Zhang and co-workers, using choline hydroxide and more complex naphtenic acids as starting materials. Biodegradability tests revealed that 8 out of the 10 ionic liquids prepared would be rapidly and completely biodegraded in aquatic environments under aerobic conditions. Common ionic liquids like [BMIM][PF_6] and [BMIM][BF_4] failed such tests.[123]

Lourenço and co-workers, developed two zwitterionic ionic liquids by esterification of choline salts with a range of anhydrides (Scheme 4.3). After their conjugation with lithium bis(trifluoromethylsulfonyl)imide two stable room-temperature ionic liquids were obtained. The use of choline and the introduction of the internal ester bond were deliberate ways to increase the biodegradability of the molecules. These ionic liquids showed excellent conductivities and could be used as electrolytes in lithium batteries.[120]

The examples selected are only a few of the many applications described so far that rely on choline-based ionic liquids.[114] The number and spectrum of such applications will only increase in the future due to the rising pressure to develop more environmentally friendly processes, in conjugation with the growing awareness that choline is a key building block to achieve such goals.

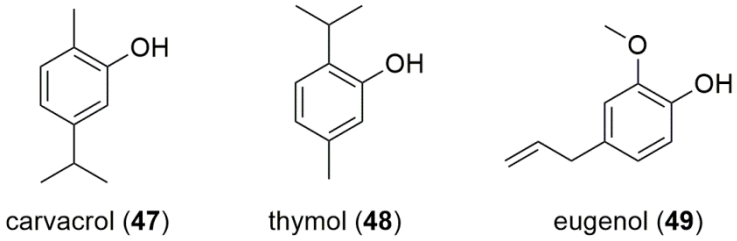


Scheme 4.3. Strategy developed by Lourenço and co-workers for the development of room-temperature zwitterionic ionic liquids.[120]

4.1.4 Antimicrobial drugs-resistance and essential oils

Bacterial resistance to antimicrobial drugs poses a serious and rapidly growing threat to public health and successful antibacterial treatment. In recent years, the

emergence of bacteria with resistance to multiple commonly used and even last resort antibiotics has accelerated dramatically. One strain of multidrug resistant bacteria which is particularly prevalent in hospital-acquired infections is methicillin-resistant *Staphylococcus aureus*, also known as MRSA. The ongoing increase in MRSA infections results in longer hospital stays and, more importantly, an increase in patient mortality. Therefore, there is a pressing need for the development of new antimicrobial agents with activity against MRSA and other Gram-positive bacteria, such as *Listeria* and *Enterococcus hirae*, which are also known to be responsible for severe food poisoning and hospital-acquired infections. Several known essential oils, which are safe to be used in humans and animals, have been shown to have potential in the treatment of antimicrobial infections. Carvacrol (**47**), thymol (**48**) and eugenol (**49**) are major active components in key essential oils (Scheme 4.4). These compounds are known for their wide variety of biological activities such as antimicrobial, anti-inflammatory, antioxidant, antihepatotoxic and antimutagenic activities. Their antimicrobial activity has a wide spectrum extended to pathogenic bacteria, mold and yeast, including drug-resistant and biofilm-forming microorganisms. While their hydrophobicity is important for their mechanism of action (interaction with the lipids in bacterial cell membrane and mitochondria, disturbing the cells structures and making them more permeable), it is also a critical shortcoming because they are poorly soluble in water which limits their use for disinfectant purposes.[124-127]



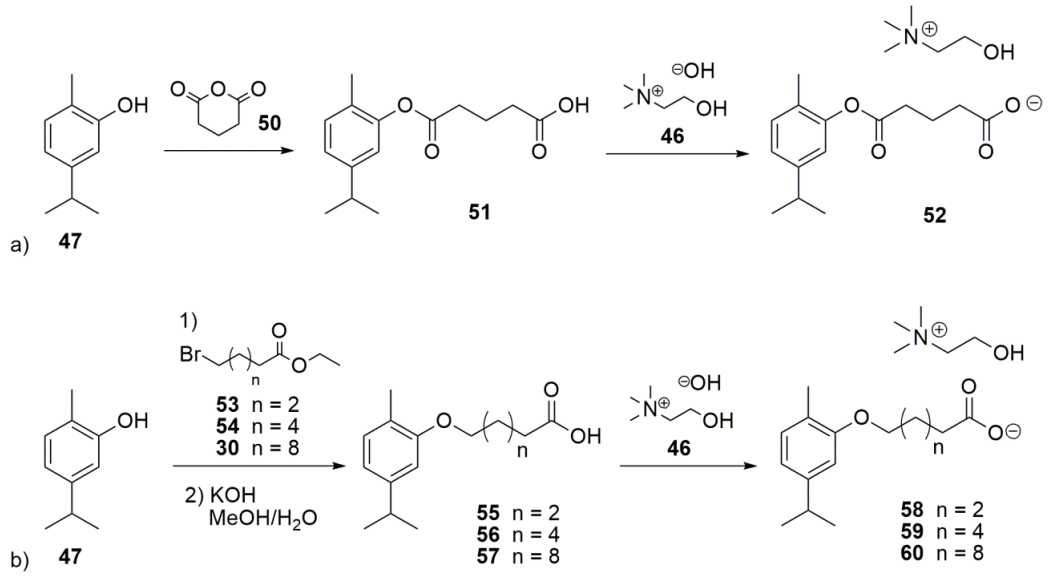
Scheme 4.4. Structures of carvacrol, thymol and eugenol, major active components in key essential oils.

4.2

RESULTS AND DISCUSSION

The work described in this chapter was performed in collaboration with Dr. João Anes and Professor Seamus Fanning from Centre for Food Safety of University College Dublin. Their group is focused on understanding the mechanisms of antibiotic resistance in bacteria in the pursuit to develop novel antibiotics or to enhance the performance of current antibacterial protocols. The employment of essential oils and their pure components, such as carvacrol (**47**) and thymol (**48**), to improve antibacterial action is one of their areas of research. However their employment is often crippled by their low solubility in water.

As mentioned before, transforming a given compound into an ionic liquid can improve its properties, namely its solubility in water. Our group decided to apply such strategy to carvacrol. Since this compound lacks an easily ionizable group, it must be derivatized first. A linker must be introduced between the molecule and an ionizable group, such as a carboxylic acid. This carboxylic acid would later be neutralized to introduce choline as counter-ion. Choline was chosen as counter-ion because i) it is a cheap, non-toxic cation, ii) it is more hydrophilic than other cations, such as sodium or imidazolium, due to its hydroxyl group. Two possible routes were envisioned (Scheme 4.5). We could perform an esterification with glutaric anhydride (**50**), creating a labile bond between the linker and carvacrol (Scheme 4.5a)). This ester bond would be easily cleaved near cell membrane by hydrolases. However this bond may be vulnerable enough to be hydrolyzed by water, destroying the derivatization before the synthesized compounds actually reach cell vicinity. With the other strategy (Scheme 4.5b)), a more robust ether bond is created which will not be easily cleaved in aqueous medium.



Scheme 4.5. Strategies developed for derivatization of carvacrol (**47**) to produce ionic liquids based on this compound: a) esterification with glutaric anhydride (**50**) followed by neutralization with choline hydroxide (**46**) to give ionic liquid **52**; b) alkylation with ethyl ω-bromoalkanoates **30**, **53** and **54**, followed by ester hydrolysis and neutralization of the precursor acid **55**-**57** with choline hydroxide to give ionic liquids **58**-**60**.

4.2.1 Study of carvacrol derivatization and biological activity of carvacrol-based ionic liquids

After opening glutaric anhydride with carvacrol, the resulting acid was neutralized with choline hydroxide. This step was performed in an ice bath to limit the amount of ester hydrolysis. After purification, unfortunately, we observed extensive degradation of the product. Unexpectedly, the major side product was choline monoester, due to attack to the carvacrol ester by the choline hydroxyl group, showing that carvacrol is a very good leaving group. Because of its lability, this strategy was not continued.

The second strategy was much more successful. Carvacrol was alkylated with three linkers (**30**, **53** and **54**) with a different chain length (5, 7 and 11 carbons) between the bromide (the leaving group) and the carboxylic acid. At this stage the carboxylic acid was protected as an ester group. Preliminary trials using free acid resulted in poor yields, probably due to dimerization instead of alkylation of carvacrol. After introduction of the linker, the ester was hydrolyzed yielding the necessary free

carboxylic acid. The precursor carboxylic acids **55**-**57** were then neutralized with choline hydroxide to obtain the desired ionic liquids. Compounds **58** and **59** are liquid at room temperature while compound **60** was obtained as a very viscous mass. All three compounds are completely soluble in water. Next, the ionic liquids and respective precursor acids were tested for biological activity against Gram-negative and Gram-positive bacteria (Tables 4.1 and 4.2).

Table 4.1. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) obtained for precursor acids **55**-**57** against Gram-negative and Gram-positive bacteria.

Strain	Carvacrol		**55**		**56**		**57**	
	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
1	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
2	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
3	0.13	0.13	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
4	2.03	2.03	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
5	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
6	0.51	0.51	-	-	-	-	>4.06	>4.06
7	2.03	2.03	-	-	-	-	0.13	0.25
8	1.02	2.03	2.03	4.06	0.25	1.02	0.13	0.13
9	1.02	1.02	4.06	>4.06	0.51	2.03	0.13	0.13
10	1.02	1.02	2.03	>4.06	0.25	2.03	0.06	0.13

Gram-negative strains: 1) *Escherichia coli* O157 NCTC 12900, **2)** *Salmonella enterica* subspe. Typhymurium 4/74, **3)** *Acinetobacter baumannii* sp3 RUH01163, **4)** *Pseudomonas aeruginosa* PAO1, **5)** *Klebsiella pneumoniae* MGH 78578, **6)** *Enterobacter aerogenes* ATCC 13048.

Gram-positive strains: 7) *Enterococcus hirae* NCTC, **8)** *Listeria monocytogenes* ATCC 13372. **9)** Methicillin susceptible *Staphylococcus aureus* (MSSA) 83254/4, **10)** Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

First of all, unlike carvacrol which is more active against Gram-negative than Gram-positive bacteria, both the precursor acids and ionic liquids are completely ineffective against Gram-negative bacteria. On the other hand, against Gram-positive bacteria, to our delight, the precursor acids **56** and **57** (with chains of 7 and 11 carbons)

and the respective ionic liquids **59** and **60** are more potent than carvacrol. Ionic acid **60**, in particular, is at least 50 times more potent than carvacrol against all four strains tested, including MRSA, which are outstanding results. These results indicate that the mechanism of action of these compounds is different from carvacrol and there is insignificant liberation of carvacrol from the parent ionic liquid. Ionization is very important because ionic liquid **60** is at least 6 times more effective in killing bacteria cells than its precursor acid **57**. It can also be observed that the potency of the compounds, in both the precursor acids and the ionic liquids, is higher the longer the chain is. From ineffective with a chain of 5 carbons (compounds **55** and **58**), to somewhat better than carvacrol with 7 carbons (compounds **56** and **59**), and finally to very potent with 11 (compounds **57** and **60**).

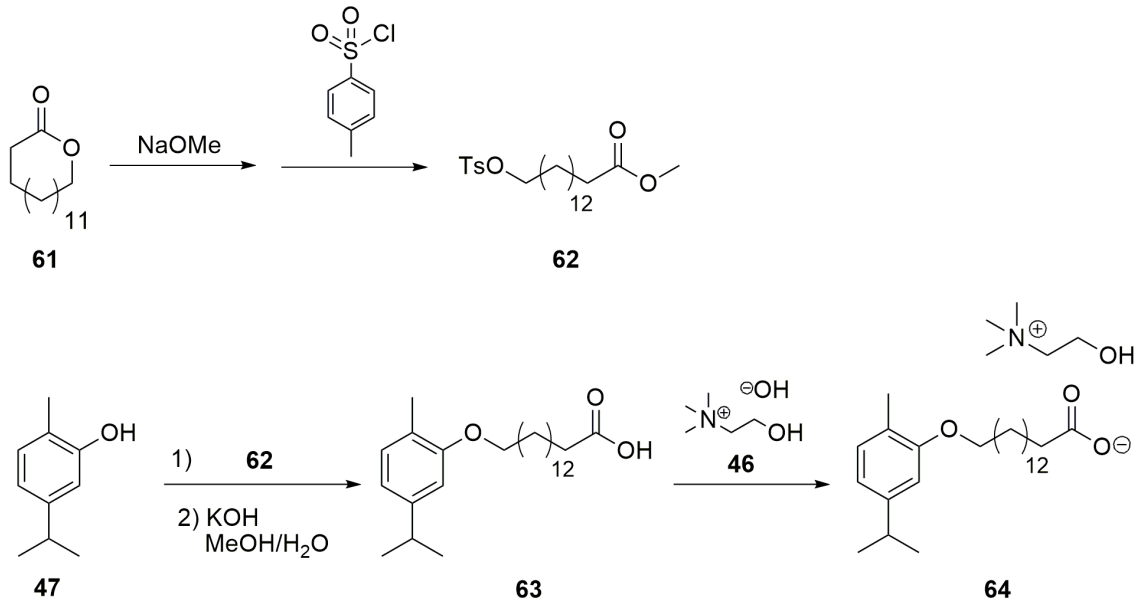
Table 4.2. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) obtained for ionic liquids **58**-**60** against Gram-negative and Gram-positive bacteria.

Strain	Carvacrol		**58**		**59**		**60**	
	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
1	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
2	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
3	0.13	0.13	>4.06	>4.06	4.06	>4.06	>4.06	>4.06
4	2.03	2.03	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
5	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
6	0.51	0.51	-	-	-	-	>4.06	>4.06
7	2.03	2.03	-	-	-	-	0.03	0.03
8	1.02	2.03	4.06	4.06	0.51	2.03	0.02	0.03
9	1.02	1.02	4.06	>4.06	1.02	2.03	0.02	0.02
10	1.02	1.02	4.06	>4.06	0.51	2.03	0.02	0.02

Gram-negative strains: 1) *Escherichia coli* O157 NCTC 12900, **2)** *Salmonella enterica* subspe. Typhymurium 4/74, **3)** *Acinetobacter baumannii* sp3 RUH01163, **4)** *Pseudomonas aeruginosa* PAO1, **5)** *Klebsiella pneumoniae* MGH 78578, **6)** *Enterobacter aerogenes* ATCC 13048.

Gram-positive strains: 7) *Enterococcus hirae* NCTC, **8)** *Listeria monocytogenes* ATCC 13372. **9)** Methicillin susceptible *Staphylococcus aureus* (MSSA) 83254/4, **10)** Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

Next, we wanted to verify if a longer chain would originate even better results. Since longer ethyl ω-bromoalkanoates were not easily available, we decided to synthesize a linker with 15 carbons from the low-cost lactone pentadecanolide (**61**) (Scheme 4.6). First, the lactone was opened with sodium methoxide to produce methyl 15-hydroxypentadecanoate. Then the hydroxyl group was transformed into a good leaving group by tosylation and this compound was used in the alkylation of carvacrol following the exact same procedure employed in the synthesis of the previous precursor acids. Acid **63** was neutralized with choline hydroxide giving ionic liquid **64** as a very viscous mass. Unlike the previous ionic liquids, this compound is not completely soluble in water; its aqueous solution is slightly opaque.



Scheme 4.6. Strategy used for synthesis of a 15-carbon linker (**62**). This molecule was then employed in the alkylation of carvacrol. After ester hydrolysis and neutralization of the precursor acid **63** with choline hydroxide, ionic liquid **64** was obtained.

Another feature that we wanted to confirm was that the pendant carvacrol is indeed important for the observed biological activity. Since higher activities were registered for long-chain compounds, the potency observed could be simply due to these compounds being more effective surfactants. As such, we prepared choline dodecanoate (**65**) and choline hexadecanoate (**66**), two known soaps,[128] which are identical to ionic liquids **60** and **64**, except they have a methyl group instead of carvacrol at the end of the linker.

The results for these four compounds are displayed in Table 4.3. The precursor acid with a chain of 15 carbons and its respective ionic liquid are ineffective against either Gram-negative or Gram-positive bacteria. We believe that these results are due to the mediocre solubilities of these compounds, particularly the acid, in water. A similar result was observed for soap **66** the longer of the two soaps tested. More importantly, we observe that choline dodecanoate (**65**) has a biological activity similar to carvacrol, which is interesting, but is nowhere near the potency observed for the equivalent ionic liquid with a pendant carvacrol moiety. This results shows that the pendant carvacrol is important for the overall activity of the synthesized molecules.

Table 4.3. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) obtained for precursor acid **63** and ionic liquids **64-66** against Gram-negative and Gram-positive bacteria.

Strain	Carvacrol		**63**		**64**		**65**		**66**	
	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
1	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
2	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
3	0.13	0.13	>4.06	>4.06	>4.06	>4.06	4.06	4.06	>4.06	>4.06
4	2.03	2.03	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
5	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
6	0.51	0.51	-	-	-	-	>4.06	>4.06	-	-
7	2.03	2.03	-	-	-	-	2.03	4.06	-	-
8	1.02	2.03	>4.06	>4.06	>4.06	>4.06	0.25	2.03	>4.06	>4.06
9	1.02	1.02	>4.06	>4.06	>4.06	>4.06	1.02	1.02	>4.06	>4.06
10	1.02	1.02	>4.06	>4.06	>4.06	>4.06	0.51	1.02	>4.06	>4.06

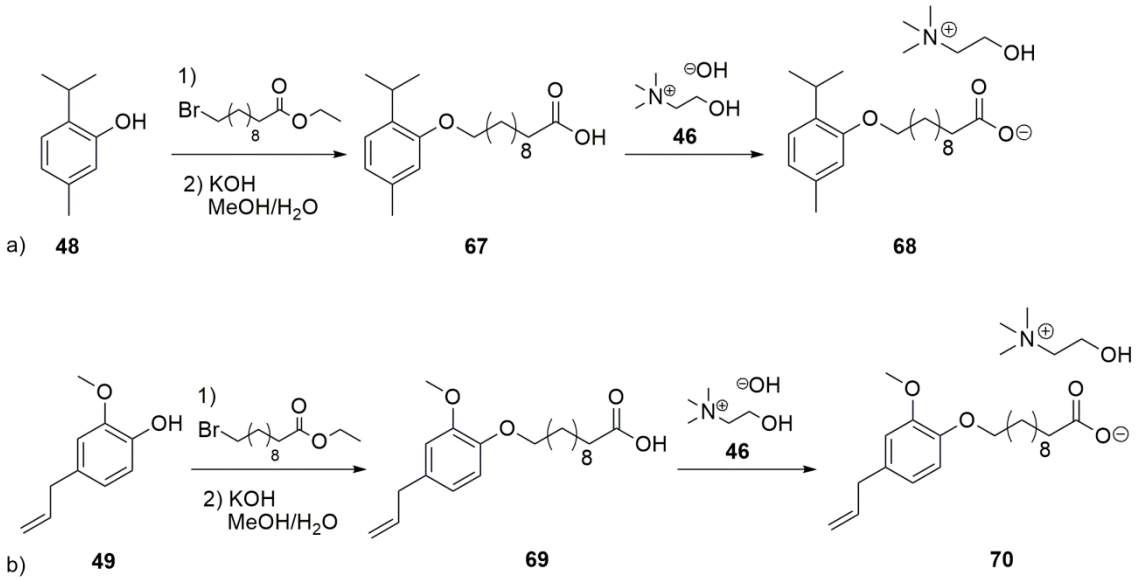
Gram-negative strains: 1) *Escherichia coli* O157 NCTC 12900, **2)** *Salmonella enterica* subspe. Typhymurium 4/74, **3)** *Acinetobacter baumannii* sp3 RUH01163, **4)** *Pseudomonas aeruginosa* PAO1, **5)** *Klebsiella pneumoniae* MGH 78578, **6)** *Enterobacter aerogenes* ATCC 13048.

Gram-positive strains: 7) *Enterococcus hirae* NCTC, **8)** *Listeria monocytogenes* ATCC 13372. **9)** Methicillin susceptible *Staphylococcus aureus* (MSSA) 83254/4, **10)** Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

Ionic liquid **60** was also tested for toxicity in fungal microorganisms and a human cell line (THP-1 cell line). No toxicity was observed in either case. Apparently, eukaryotic cells are not affected by this ionic liquid, but further testing is mandatory.

4.2.2 Biological activity of thymol- and eugenol-based ionic liquids

With the optimal chain length determined, the same methodology was applied to thymol and eugenol (Scheme 4.7). Ionic liquid **68** is a liquid at room temperature while **70** was obtained as a very viscous mass. Both are completely soluble in water.



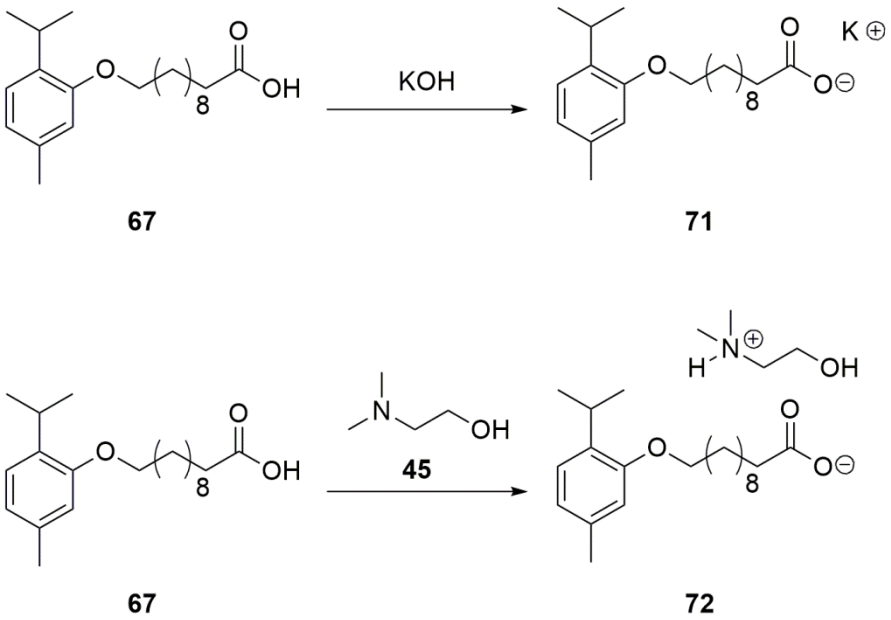
Scheme 4.7. The optimized methodology developed for carvacrol was applied to thymol (**48**) and eugenol (**49**).

For thymol-based compounds, the antimicrobial activities of the precursor acid and the ionic liquid (Table 4.4) are very similar to the ones observed for the equivalent carvacrol-based compounds (Tables 4.1 and 4.2). Ionic liquid **68** is also at least 50 times more potent than carvacrol. Since carvacrol is slight less effective than thymol, the results are "only" at least 25 times more potent when compared against the latter. The ionic liquid **68** was tested against fungal microorganisms as well. Similar to its

equivalent carvacrol-based ionic liquid, no toxicity was observed. Apparently, this type of ionic liquids is only toxic to Gram-positive bacteria.

For eugenol-based compounds the results were disappointing. Eugenol, precursor acid (**69**) and ionic liquid (**70**) had no significant activity (MIC and MBC > 4.06 mmol) against any of the bacterial strains tested.

To finalize this study, we wanted to verify if choline, as counter-ion, was vital for the observed activities. Two new ionic liquids were prepared (Scheme 4.8), one with potassium as counter-ion and the other with hydrogen N,N-dimethyl-2-hydroxyethylammonium. The first was prepared by neutralization of acid **67** with potassium hydroxide, the latter by simple acid-base reaction between acid **67** and amine **45**, forming a more labile protic ionic liquid. Ionic liquid **71** was obtained as an almost solid mass, completely soluble in water. The protic ionic liquid **72** is a colorless liquid at room temperature. It is, however, not completely soluble in water, forming a slightly opaque solution. The antimicrobial results are displayed in Table 4.4. It is evident that the cation is not critical for the observed activities, since the compounds with choline, potassium or hydrogen N,N-dimethyl-2-hydroxyethylammonium have the exact same potency. We can conclude from these last results that what is crucial is that the compound must be ionized.



67 **71**

67 **72**

Scheme 4.8. Ionic liquids prepared from acid **67**, with other cations (potassium and N,N-dimethyl-2-hydroxyethylammonium) to determine if choline is essential to the observed activities.

Table 4.4. Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) obtained for precursor acid **67** and ionic liquids **68, 71** and **72** against Gram-negative and Gram-positive bacteria.

Strain	Thymol		**67**		**68**		**71**		**72**	
	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)	MIC (mM)	MBC (mM)
1	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
2	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
3	0.25	0.25	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
4	1.02	1.02	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
5	0.51	0.51	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
6	1.02	1.02	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06	>4.06
7	1.02	1.02	0.06	0.25	0.02	0.03	0.02	0.06	0.03	0.03
8	1.02	1.02	0.02	0.06	0.02	0.02	0.02	0.03	0.02	0.03
9	0.51	1.02	0.03	0.06	0.02	0.02	0.02	0.02	0.02	0.03
10	0.51	1.02	0.13	0.25	0.02	0.02	0.02	0.02	0.02	0.03

Gram-negative strains: 1) *Escherichia coli* O157 NCTC 12900, **2)** *Salmonella enterica* subspe. Typhymurium 4/74, **3)** *Acinetobacter baumannii* sp3 RUH01163, **4)** *Pseudomonas aeruginosa* PAO1, **5)** *Klebsiella pneumoniae* MGH 78578, **6)** *Enterobacter aerogenes* ATCC 13048.

Gram-positive strains: 7) *Enterococcus hirae* NCTC, **8)** *Listeria monocytogenes* ATCC 13372. **9)** Methicillin susceptible *Staphylococcus aureus* (MSSA) 83254/4, **10)** Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300.

4.3

CONCLUSIONS

A synthetic methodology was developed for preparation of ionic liquids based on essentials oils components like carvacrol, thymol and eugenol. This methodology involves the alkylation of the desired molecule with an ethyl ω-bromoalkanoate, followed by hydrolysis of the ethyl ester and subsequent neutralization of the carboxylic acid with choline hydroxide. The alkylation step is necessary to introduce a linker between the desired molecule and an easily ionizable carboxylic acid. Several linkers with different chain lengths (5, 7, 11 and 15 carbons) were studied and the highest activity was observed for the ionic liquid with a linker of 11 carbons. All ionic liquids synthesized were completely soluble in water, the only exception being of the one with a chain length of 15 carbons.

The synthesized ionic liquids and its respective precursor acids were tested against a panel of Gram-negative and Gram-positive bacteria. All ionic liquids, unlike pure carvacrol and pure thymol, were ineffective versus Gram-negative bacteria. On the other hand, versus Gram-positive bacteria, the ionic liquids (with a linker of 11 carbons) based on carvacrol and on thymol displayed MIC and MBC at least 50 and 25 times lower than pure carvacrol and thymol, respectively, which are outstanding results. Even the respective precursor acids were several times more potent than carvacrol and thymol. The eugenol-based ionic liquid was ineffective against all bacteria. The pendant carvacrol (or thymol) moiety is fundamental for the observed activities since the equivalent ionic liquid without such moiety (choline dodecanoate) had higher MIC and MBC, similar to the ones observed for pure carvacrol and pure thymol. The cation used is not important (the same anion with three different cations gave similar results), what is important is the compound being ionized to increase its solubility in water.

4.4

EXPERIMENTAL SECTION

4.4.1 General remarks

All reagents were obtained commercially (purity >95%) and used as received, unless otherwise noted. All solvents were obtained commercially and, when necessary, purified appropriately before use. All aqueous solutions were prepared using distilled water.

4.4.2 Detection, isolation and purification of reaction products

Thin layer chromatography (TLC) was applied, when adequate, in the analysis of reaction mixtures and was performed in ALUGRAM® Xtra SIL G/UV_{254} silica-gel plates (Macherey-Nagel, Germany) with detection by UV irradiation or by immersion in an ethanolic solution of phosphomolybdic acid, followed by heating.

Flash chromatography was performed on silica-gel 60 (0.04 – 0.06 mm, 230 – 400 mesh ASTM) (Scharlau, Spain) under pressure and with the appropriate eluent or system of eluents (mentioned in the experimental procedures).

4.4.3 Characterization and analysis

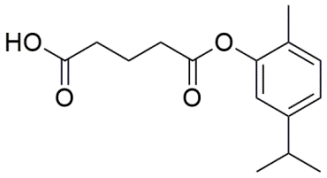
The structure of all synthesized compounds was confirmed by Nuclear Magnetic Resonance (NMR). NMR spectra were recorded at room temperature, unless otherwise noted, on Bruker Avance II+ 300 (^{1}H 300 MHz, ^{13}C 75 MHz) or 400 (^{1}H 400 MHz, ^{13}C 100 MHz) spectrometers, using the residual solvent signal as reference, unless

otherwise noted. Chemical shifts (δ) are reported in parts per million (ppm) and coupling constants (J) in hertz (Hz).

High resolution mass spectrometry was performed by Unidade Militar Laboratorial de Defesa Biológica e Química and was carried out in a Thermo Scientific Orbitrap Mass Spectrometer with ESI injection.

4.4.4 Experimental procedures

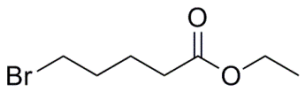
4.4.4.1 Preparation of 5-(5-isopropyl-2-methylphenoxy)-5-oxo-pentanoic acid (51).



A solution of glutaric anhydride (0.450 g, 3.9 mmol), carvacrol (0.840 mL, 5.5 mmol), DMAP (0.023 g, 0.2 mmol) and triethylamine (1.1. mL, 7.8 mL) in acetonitrile (4 mL) was heated at 40ºC for 20 hours. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with ethyl acetate (4 x 10 mL). After drying with anhydrous sodium sulfate, filtration and solvent removal, the crude was purified by column chromatography (eluent: *n*-hexane/ethyl acetate (3:1)). The solvent was removed under reduced pressure, to give compound **51** as a pale yellow liquid (0.420 g, 41%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.15 (d, 1H), 7.02 (d, 1H), 6.86 (s, 1H), 2.88 (m, 1H), 2.69 (t, 2H), 2.55 (t, 2H), 2.10 (m, 5H), 1.23 ppm (d, 6H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.0, 171.3, 149.3, 148.2, 131.0, 127.2, 124.3, 119.8, 33.7, 33.2, 33.1, 24.0, 20.0, 15.9 ppm.

4.4.4.2 Preparation of ethyl 5-bromopentanoate (53).

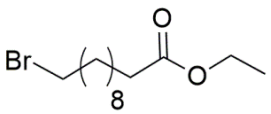


A solution of 5-bromopentanoic acid (1.85 g, 10.2 mmol) and sulfuric acid (5-6 drops) in absolute ethanol (10 mL) was heated at 85ºC, in a pressure tube (15 mL), for 6

hours. After concentration at reduced pressure, the mixture was diluted with MTBE (20 mL) and washed with saturated aqueous solution of $NaHCO_3$ (2 x 10 mL). The organic phase was dried with anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure, giving compound **53** as a pale yellow liquid (1.973 g, 93%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.12 (q, 2H), 3.40 (t, 2H), 2.32 (t, 2H), 1.83 (m, 4H), 1.24 ppm (t, 3H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 173.3, 60.5, 33.4, 33.2, 32.1, 23.6, 14.4 ppm.
NMR data is in agreement with literature.[129]

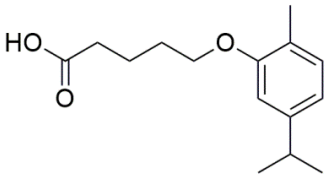
4.4.4.3 Preparation of ethyl 11-bromoundecanoate (30).



A solution of 11-bromoundecanoic acid (2.74 g, 10.3 mmol) and sulfuric acid (5-6 drops) in absolute ethanol (10 mL) was heated at 85ºC, in a pressure tube (15 mL), for 6 hours. After concentration at reduced pressure, the mixture was diluted with MTBE (20 mL) and washed with saturated aqueous solution of $NaHCO_3$ (2 x 10 mL). The organic phase was dried with anhydrous sodium sulfate, filtered and the solvent evaporated under reduced pressure, giving compound **30** as a pale yellow liquid (2.82 g, 96%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 4.12 (q, 2H), 3.40 (t, 2H), 2.28 (t, 2H), 1.84 (m, 2H), 1.60 (m, 2H), 1.27 ppm (m, 15H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 174.0, 60.3, 34.5, 34.2, 33.0, 29.49, 29.45, 29.3, 29.2, 28.9, 28.3, 25.1, 14.4 ppm.
NMR data in agreement with literature.[36]

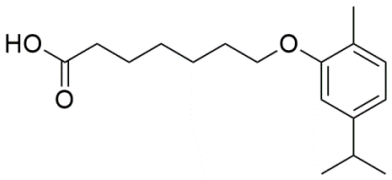
4.4.4.4 Preparation of 5-(5-isopropyl-2-methylphenoxy)pentanoic acid (55).



To a solution of carvacrol (0.308 mL, 2.0 mmol) and ethyl 5-bromopentanoate (0.465 g, 2.2 mmol) in acetonitrile (4 mL) was added potassium iodide (0.033 g, 0.2 mmol) and potassium carbonate (1.109 g, 8.0 mmol). The suspension was heated at 90ºC in a pressure tube (15 mL) for 24 hours. 1-Methylimidazole (0.080 mL, 1.0 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (15 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). The solvent was evaporated under reduced pressure, followed by addition of water (1.2 mL) and a methanolic solution of potassium hydroxide (4 M, 2.5 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 10 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. After solvent removal under reduced pressure, the obtained solid was recrystallized from warm *n*-hexane. After drying under vacuum, at room temperature for at least 24 hours, compound **55** was obtained as white crystals (0.352 g, 70%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.06 (d, 1H), 6.74 (d, 1H), 6.68 (s, 1H), 4.00 (t, 2H), 2.87 (m, 1H), 2.48 (t, 2H), 2.20 (s, 3H), 1.88 (m, 4H), 1.25 ppm (d, 6H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.8, 157.1, 148.0, 130.5, 124.2, 118.1, 109.5, 67.3, 34.3, 33.8, 28.9, 24.3, 21.7, 16.0 ppm.
HRMS(ESI) m/z: 249.1493 $[M-H]^-$.

4.4.4.5 Preparation of 7-(5-isopropyl-2-methylphenoxy)heptanoic acid (56).

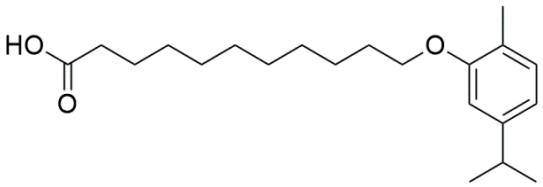


To a solution of carvacrol (0.308 mL, 2.0 mmol) and ethyl 7-bromoheptanoate (0.494 g, 2.2 mmol) in acetonitrile (4 mL) was added potassium iodide (0.035 g, 0.2 mmol) and potassium carbonate (1.107 g, 8.0 mmol). The suspension was heated at

90ºC in a pressure tube (15 mL) for 24 hours. 1-Methylimidazole (0.080 mL, 1.0 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (15 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). The solvent was evaporated under reduced pressure, followed by addition of water (1.2 mL) and a methanolic solution of potassium hydroxide (4 M, 2.5 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 10 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. The crude was purified by column chromatography (eluent: hexane/ethyl acetate 6:1 to 3:1). After solvent removal under reduced pressure and drying under vacuum, at room temperature for at least 24 hours, compound **56** was obtained as a pale yellow oil (0.481 g, 86%).

^{1}H NMR (300 MHz, $CDCl_3$): δ = 7.06 (d, 1H), 6.73 (d, 1H), 6.69 (s, 1H), 3.98 (t, 2H), 2.87 (m, 1H), 2.39 (t, 2H), 2.19 (s, 3H), 1.81 (m, 2H), 1.70 (m, 2H), 1.50 (m, 4H), 1.25 ppm (d, 6H).
^{13}C NMR (75 MHz, $CDCl_3$): δ = 180.2, 157.2, 148.0, 130.5, 124.2, 118.0, 109.6, 67.8, 34.3, 34.1, 29.4, 28.9, 26.0, 24.7, 24.3, 16.0 ppm.
HRMS(ESI) m/z: 277.1807 $[M-H]^-$.

4.4.4.6 Preparation of 11-(5-isopropyl-2-methylphenoxy)undecanoic acid (57).

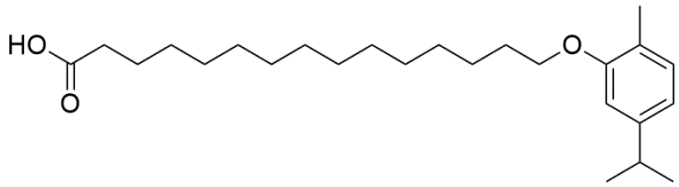


To a solution of carvacrol (0.308 mL, 2.0 mmol) and ethyl 11-bromoundecanoate (0.652 g, 2.2 mmol) in acetonitrile (4 mL) was added potassium iodide (0.033 g, 0.2 mmol) and potassium carbonate (1.104 g, 8.0 mmol). The suspension was heated at 90ºC in a pressure tube (15 mL) for 24 hours. 1-Methylimidazole (0.080 mL, 1.0 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (15 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). The solvent was evaporated under reduced pressure, followed by addition of water (1.2 mL) and a methanolic

solution of potassium hydroxide (4 M, 2.5 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 10 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. The crude was purified by column chromatography (eluent: hexane/ethyl acetate 6:1 to 3:1). After solvent removal under reduced pressure and drying under vacuum, at room temperature for at least 24 hours, compound **57** was obtained as a pale yellow oil (0.524 g, 78%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.05 (d, 1H), 6.72 (d, 1H), 6.68 (s, 1H), 3.96 (t, 2H), 2.86 (m, 1H), 2.35 (t, 2H), 2.19 (s, 3H), 1.79 (m, 2H), 1.64 (m, 2H), 1.48 (m, 2H), 1.32 (s, 10H), 1.24 ppm (d, 6H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.2, 157.3, 147.9, 130.5, 124.3, 117.9, 109.6, 68.0, 34.3, 34.2, 29.7, 29.6, 29.5, 29.4, 29.2, 26.3, 24.8, 24.3, 16.0 ppm.
HRMS(ESI) m/z: 333.2430 $[M-H]^-$.

4.4.4.7 Preparation of 15-(5-isopropyl-2-methylphenoxy)pentadecanoic acid (63).



To a suspension of pentadecanolide (1.240 g, 5.0 mmol) in methanol (15 mL) was gradually added a methanolic solution of sodium methoxide (5.4 M, 5 mL). The suspension was heated at 40ºC for 72 hours. After acidification with HCl (1M, 30 mL), the crude was extracted with MTBE (3 x 15 mL). The organic fractions were combined, washed with saturated solution of $NaHCO_3$ (3 x 10 mL), dried with anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred for 1-2 hours. After filtration, the solvent was removed under reduced pressure (0.900 g).

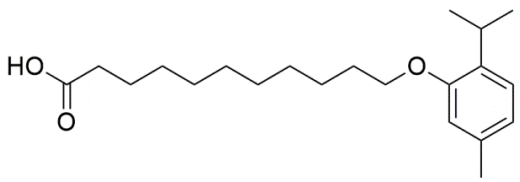
To a solution of methyl 15-hydroxypentadecanoate (0.900 g, 3.3 mmol), triethylamine (0.925 mL, 6.6 mmol) and trimethylamine hydrochloride (0.035 g, 0.33 mmol) in acetonitrile (8 mL), cooled in an ice bath, was added a solution of tosyl chloride (0.985 g, 5.1 mmol) in acetonitrile (2 mL). The solution was allowed to warm to room temperature and stirred for 1 hour. After addition of MTBE (20 mL), the solution was washed with HCl (1 M, 2 x 10 mL) and water (2 x 10 mL), dried with

anhydrous sodium sulfate and filtered. Activated carbon was added and the suspension stirred for 1-2 hours. After filtration, the solvent was removed under reduced pressure (1.328 g).

To a solution of carvacrol (0.475 mL, 3.1 mmol) and methyl 15-(tosyloxy)pentadecanoate (1.328 g, 3.1 mmol) in acetonitrile (6.2 mL) was added potassium iodide (0.052 g, 0.31 mmol) and potassium carbonate (1.720 g, 12.4 mmol). The suspension was heated at 90ºC in a pressure tube (15 mL) for 24 hours. 1-Methylimidazole (0.125 mL, 1.6 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (20 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). The solvent was evaporated under reduced pressure, followed by addition of water (2 mL) and a methanolic solution of potassium hydroxide (4 M, 4 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 15 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. After solvent removal under reduced pressure, the obtained solid was recrystallized from warm acetonitrile. After drying under vacuum, at room temperature for at least 24 hours, compound **63** was obtained as white crystals (0.739 g, 38%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.05 (d, 1H), 6.72 (d, 1H), 6.68 (s, 1H), 3.96 (t, 2H), 2.86 (m, 1H), 2.35 (t, 2H), 2.19 (s, 3H), 1.79 (m, 2H), 1.63 (m, 2H), 1.46 (m, 2H), 1.25 ppm (m, 24H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 179.6, 157.3, 147.9, 130.5, 124.3, 117.9, 109.6, 68.0, 34.3, 34.1, 29.8, 29.7, 29.59, 29.56, 29.4, 29.2, 26.3, 24.8, 24.3, 16.0 ppm.
HRMS(ESI) m/z: 389.3056 $[M-H]^-$.

4.4.4.8 Preparation of 11-(2-isopropyl-5-methylphenoxy)undecanoic acid (67).

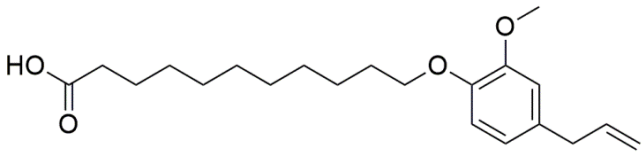


To a solution of thymol (0.455 g, 3.0 mmol) and ethyl 11-bromoundecanoate (0.995 g, 3.3 mmol) in acetonitrile (6 mL) was added potassium iodide (0.050 g, 0.3

mmol) and potassium carbonate (1.708 g, 12.0 mmol). The suspension was heated at 90ºC in a pressure tube (15 mL) for 48 hours. 1-Methylimidazole (0.120 mL, 1.5 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (25 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). The solvent was evaporated under reduced pressure, followed by addition of water (3 mL) and a methanolic solution of potassium hydroxide (4 M, 12 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 15 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. After solvent removal under reduced pressure, the obtained solid was recrystallized from warm acetonitrile. After drying under vacuum, at room temperature for at least 24 hours, compound **67** was obtained as a white powder (0.725 g, 72%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.09 (d, 1H), 6.73 (d, 1H), 6.66 (s, 1H), 3.95 (t, 2H), 3.30 (m, 1H), 2.36 (t, 2H), 2.33 (s, 3H), 1.80 (m, 2H), 1.65 (m, 2H), 1.48 (m, 2H), 1.32 (s, 10H), 1.21 ppm (d, 6H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.3, 156.3, 136.3, 134.2, 125.9, 121.0, 112.3, 67.9, 34.2, 29.64, 29.57, 29.50, 29.45, 29.4, 29.2, 26.8, 26.3, 24.8, 22.9, 21.5 ppm.
HRMS(ESI) m/z: 333.2428 $[M-H]^-$.

4.4.4.9 Preparation of 11-(4-allyl-2-methoxylphenoxy)undecanoic acid (69).

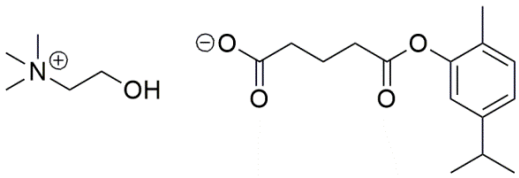


To a solution of eugenol (0.450 mL, 2.9 mmol) and ethyl 11-bromoundecanoate (0.933 g, 3.2 mmol) in acetonitrile (5 mL) was added potassium iodide (0.049 g, 0.29 mmol) and potassium carbonate (1.620 g, 11.6 mmol). The suspension was heated at 90ºC in a pressure tube (15 mL) for 72 hours. 1-Methylimidazole (0.120 mL, 1.5 mmol) was added and the suspension heated at 90ºC for another 24 hours. The suspension was diluted in MTBE (20 mL) followed by washing with water (2 x 10 mL) and HCl (1 M, 2 x 5 mL). After drying with anhydrous sodium sulfate, filtration and removal of the solvent, the crude was purified by column chromatography (eluent: *n*-hexane/ethyl acetate (10:1)). The solvent was evaporated under reduced pressure, followed by

addition of water (1 mL) and a methanolic solution of potassium hydroxide (4 M, 3 mL). The hydrolysis was carried out at 40ºC for 60-90 minutes. The solution was diluted with water (20 mL), acidified with HCl (2 M) and extracted with MTBE (3 x 15 mL). The organic phases were combined, dried with anhydrous sodium sulfate and filtered. After solvent removal under reduced pressure, the obtained solid was recrystallized from warm acetonitrile. After drying under vacuum, at room temperature for at least 24 hours, compound **69** was obtained as a white powder (0.724 g, 72%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 6.80 (d, 1H), 6.70 (d, 2H), 5.96 (m, 1H), 5.07 (m, 2H), 3.98 (t, 2H), 3.85 (s, 3H), 3.33 (d, 2H), 2.35 (t, 2H), 1.82 (m, 2H), 1.62 (m, 2H), 1.44 (m, 2H), 1.30 ppm (s, 10H).
^{13}C NMR (100 MHz, $CDCl_3$): δ = 180.2, 149.5, 147.0, 137.8, 132.8, 120.6, 115.7, 113.3, 112.5, 69.3, 56.1, 39.9, 34.2, 29.6, 29.47, 29.45, 29.33, 29.31, 29.1, 26.1, 24.8 ppm.
HRMS(ESI) m/z: 347.2220 $[M-H]^-$.

4.4.4.10 Preparation of choline 5-(5-isopropyl-2-methylphenoxy)-5-oxo-pentanoate (52).

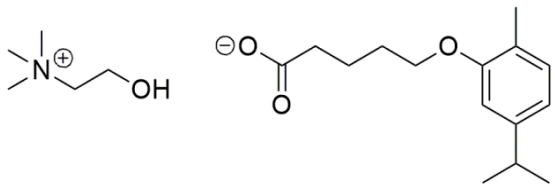


To a solution of acid 5-(5-isopropyl-2-methylphenoxy)-5-oxo-pentanoic (0.225 g, 0.84 mmol) in acetonitrile (3 mL), cooled in an ice bath, was added dropwise a methanolic solution of choline hydroxide (45 wt%, 0.240 mL, 0.84 mmol). The solution was stirred at 0ºC for 30 minutes, then at room temperature for another 30 minutes. Activated carbon was added and the suspension was stirred for 1 hour before being filtered in a small column filled with Celite® 577, aluminum oxide and silica-gel. After solvent removal under reduced pressure, the product was dried under vacuum at room temperature overnight. NMR analysis showed significant degradation (above 30%) of the ester.

4.4.4.11 General procedure for the preparation of choline-based ionic liquids 58-60, 64-66, 68 and 70.

To a solution of the respective precursor acid (0.65-1.0 mmol) in methanol (2 mL) was added dropwise a methanolic solution of choline hydroxide (45 wt%, 0.65-1.0 mmol). The solution was stirred at room temperature for 2-4 hours, then activated carbon was added. The suspension was stirred for 1-2 hours before being filtered in a small column filled with Celite® 577, aluminum oxide and silica-gel. After solvent removal under reduced pressure, the product was dried under vacuum at room temperature for at least 24 hours,

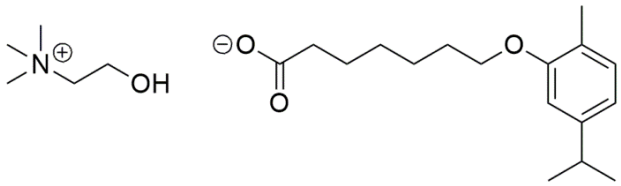
4.4.4.11.1 Choline 5-(5-isopropyl-2-methylphenoxy)pentanoate (58).



From acid 5-(5-isopropyl-2-methylphenoxy)pentanoic (0.163 g, 0.65 mmol) and choline hydroxide (0.185 mL, 0.65 mmol), compound **58** was obtained as a pale yellow, viscous liquid (0.201 g, 87%).

^{1}H NMR (400 MHz, D_2O): δ = 7.13 (d, 1H), 6.92 (s, 1H), 6.84 (d, 1H), 4.05 (m, 4H), 3.48 (m, 2H), 3.17 (s, 9H), 2.85 (m, 1H), 2.24 (t, 2H), 2.16 (s, 3H), 1.73 (m, 4H) 1.18 ppm (d, 6H).
^{13}C NMR (100 MHz, D_2O): δ = 183.4, 156.5, 148.9, 130.8, 124.7, 119.0, 111.7, 69.0, 67.4, 55.6, 53.8, 48.9, 37.2, 33.5, 28.5, 23.3, 22.5, 14.9 ppm.

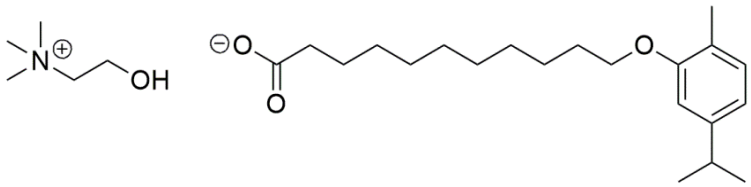
4.4.4.11.2 Choline 7-(5-isopropyl-2-methylphenoxy)heptanoate (59).



From acid 7-(5-isopropyl-2-methylphenoxy)heptanoic (0.210 g, 0.75 mmol) and choline hydroxide (0.215 mL, 0.75 mmol), compound **59** was obtained as a pale yellow, viscous liquid (0.226 g, 79%).

^{1}H NMR (300 MHz, D_2O): δ = 6.90 (d, 1H), 6.65 (s, 1H), 6.56 (d, 1H), 4.00 (m, 2H), 3.80 (t, 2H), 3.44 (m, 2H), 3.13 (s, 9H), 2.64 (m, 1H), 2.13 (t, 2H), 2.05 (s, 3H), 1.56 (m, 4H), 1.31, (m, 4H), 1.04 ppm (d, 6H).
^{13}C NMR (75 MHz, D_2O): δ = 183.0, 156.6, 147.7, 130.4, 123.8, 118.1, 110.2, 68.4, 67.3, 55.5, 53.8, 48.8, 37.7, 33.6, 29.0, 28.9, 26.0, 25.6, 23.6, 15.3 ppm.

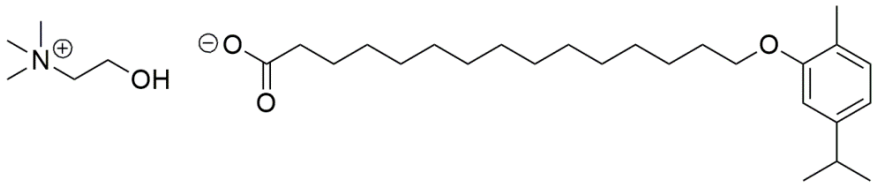
4.4.4.11.3 Choline 11-(5-isopropyl-2-methylphenoxy)undecanoate (60).



From acid 11-(5-isopropyl-2-methylphenoxy)undecanoic (0.345 g, 1.0 mmol) and choline hydroxide (0.285 mL, 1.0 mmol), compound **60** was obtained as a yellow, viscous mass (0.414 g, 95%).

^{1}H NMR (400 MHz, D_2O): δ = 6.76 (d, 1H), 6.44 (s, 2H), 3.99 (m, 2H), 3.62 (t, 2H), 3.44 (m, 2H), 3.14 (s, 9H), 2.55 (m, 1H), 2.13 (t, 2H), 1.99 (s, 3H), 1.52 (m, 4H), 1.20 (m, 12H), 0.99 ppm (d, 6H).
^{13}C NMR (100 MHz, D_2O): δ = 182.5, 156.7, 147.2, 130.2, 123.4, 117.7, 109.2, 67.6, 67.4, 55.5, 53.8, 37.9, 37.7, 29.73, 29.68, 29.6, 29.3, 26.4, 26.1, 23.8, 15.5 ppm.

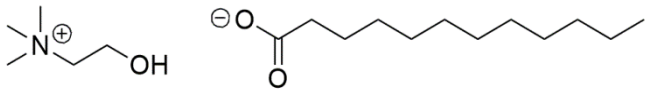
4.4.4.11.4 Choline 15-(5-isopropyl-2-methylphenoxy)pentadecanoate (64).



From acid 15-(5-isopropyl-2-methylphenoxy)pentadecanoic (0.259 g, 0.65 mmol) and choline hydroxide (0.185 mL, 0.65 mmol), compound **64** was obtained as a yellow, viscous mass (0.286 g, 89%).

^{1}H NMR (400 MHz, D_2O): δ = 6.75 (d, 1H), 6.44 (s, 2H), 3.99 (m, 2H), 3.62 (t, 2H), 3.44 (m, 2H), 3.14 (s, 9H), 2.55 (m, 1H), 2.13 (t, 2H), 2.00 (s, 3H), 1.54 (m, 4H), 1.21 (m, 20H), 0.98 ppm (d, 6H).
^{13}C NMR (100 MHz, D_2O): δ = 182.3, 156.8, 147.1, 130.1, 123.2, 117.5, 108.9, 67.5, 67.4, 55.6, 53.8, 37.9, 33.8, 30.1, 29.9, 29.8, 29.6, 29.4, 26.5, 26.2, 23.8, 15.6 ppm.

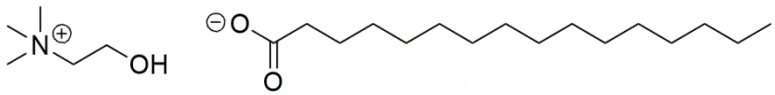
4.4.4.11.5 Choline dodecanoate (65).



From acid dodecanoic (0.340 g, 1.65 mmol) and choline hydroxide (0.470 mL, 1.65 mmol), compound **65** was obtained as a yellow, viscous mass (0.416 g, 83%).

^{1}H NMR (300 MHz, D_2O): δ = 4.04 (m, 2H), 3.51 (m, 2H), 3.20 (s, 9H), 2.15 (t, 2H), 1.54 (m, 2H), 1.29 (s, 16H), 0.88 ppm (t, 3H).
^{13}C NMR (75 MHz, D_2O): δ = 183.0, 67.4, 55.6, 53.9, 37.9, 31.8, 29.64, 29.57, 29.4, 29.3, 26.3, 22.5, 13.8 ppm.
NMR data in agreement with literature.[128]

4.4.4.11.6 Choline hexadecanoate (66).



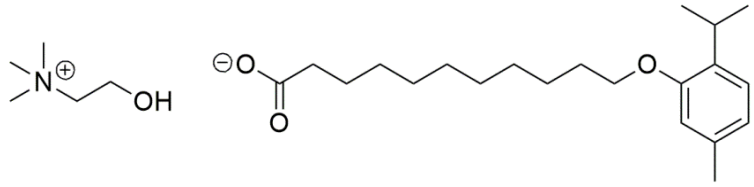
From acid hexadecanoic (0.429 g, 1.65 mmol) and choline hydroxide (0.470 mL, 1.65 mmol), compound **66** was obtained as a yellow, viscous mass (0.463 g, 78%).

^{1}H NMR (300 MHz, D_2O): δ = 4.05 (m, 2H), 3.53 (m, 2H), 3.22 (s, 9H), 2.16 (t, 2H), 1.56 (m, 2H), 1.32 (s, 24H), 0.90 ppm (t, 3H).

^{13}C NMR (75 MHz, D_2O): δ = 182.5, 67.5, 55.6, 53.9, 38.0, 32.0, 30.1, 30.0, 29.9, 29.8, 29.7, 29.6, 26.5, 22.7, 13.8 ppm.

NMR data in agreement with literature.[128]

4.4.4.11.7 Choline 11-(2-isopropyl-5-methylphenoxy)undecanoate (68).

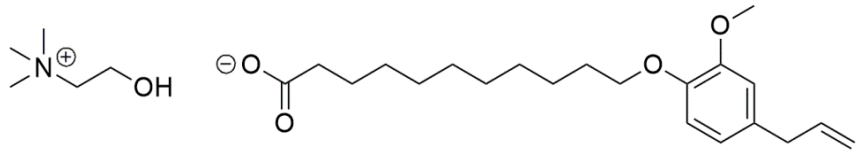


From acid 11-(2-isopropyl-5-methylphenoxy)undecanoic (0.279 g, 0.83 mmol) and choline hydroxide (0.235 mL, 0.83 mmol), compound **68** was obtained as a pale yellow, viscous liquid (0.332 g, 91%).

^{1}H NMR (400 MHz, D_2O): δ = 6.83 (d, 1H), 6.48 (d, 1H), 6.42 (s, 1H), 4.01 (m, 2H), 3.62 (t, 2H), 3.45 (m, 2H), 3.15 (s, 10H), 2.15 (t, 2H), 2.06 (s, 3H), 1.54 (m, 4H), 1.22 (m, 12H), 1.02 ppm (d, 6H).

^{13}C NMR (100 MHz, D_2O): δ = 182.4, 155.8, 135.7, 133.4, 125.4, 121.0, 112.0, 67.6, 67.4, 55.6, 53.8, 37.8, 29.8, 29.7, 29.6, 29.5, 29.4, 26.4, 26.1, 22.5, 20.9 ppm.

4.4.4.11.8 Choline 11-(4-allyl-2-methoxylphenoxy)undecanoate (70).

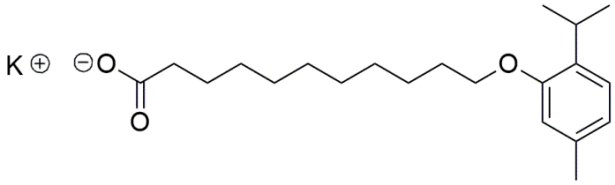


From acid 11-(4-allyl-2-methoxylphenoxy)undecanoic (0.349 g, 1.0 mmol) and choline hydroxide (0.285 mL, 1.0 mmol), compound **70** was obtained as a yellow, viscous mass (0.332 g, 93%).

^{1}H NMR (300 MHz, D_2O): δ = 6.55 (d, 2H), 6.46 (d, 1H), 5.76 (m, 1H), 4.90 (m, 2H), 4.03 (m, 2H), 3.68 (t, 2H), 3.61 (s, 3H), 3.49 (m, 2H), 3.18 (s, 9H), 3.09 (d, 2H), 2.12 (t, 2H), 1.54 (m, 4H), 1.16 ppm (m, 12H).

^{13}C NMR (75 MHz, D_2O): δ = 183.0, 149.0, 146.5, 137.7, 132.4, 120.3, 115.2, 112.8, 112.3, 68.8, 67.4, 55.6, 55.3, 53.8, 39.4, 37.9, 29.5, 29.3, 29.0, 26.2, 25.8 ppm.

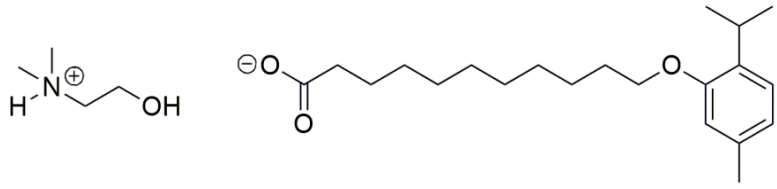
4.4.4.12 Preparation of potassium 11-(2-isopropyl-5-methylphenoxy)undecanoate (71).



A solution of acid 11-(2-isopropyl-5-methylphenoxy)undecanoic (0.334 g, 1.0 mmol) and potassium hydroxide (0.058 g, 1.0 mmol) in methanol (2 mL) was stirred at room temperature for 60-90 minutes. After solvent removal under reduced pressure and drying under vacuum, at room temperature for at least 24 hours, compound **71** was obtained as an opaque, almost solid mass (0.390 g, 99%).

^{1}H NMR (300 MHz, D_2O): δ = 6.82 (d, 1H), 6.47 (d, 1H), 6.40 (s, 1H), 3.61 (t, 2H), 3.13 (m, 1H), 2.16 (t, 2H), 2.05 (s, 3H), 1.55 (m, 4H), 1.22 (m, 12H), 1.01 ppm (d, 6H).
^{13}C NMR (75 MHz, D_2O): δ = 183.2, 155.7, 135.6, 133.4, 125.4, 121.0, 112.2, 67.7, 37.9, 29.74, 29.69, 29.6, 29.5, 29.4, 26.3, 26.1, 22.5, 20.8 ppm.

4.4.4.13 Preparation of hydrogen N,N-dimethyl-2-hydroxyethylammonium 11-(2-isopropyl-5-methylphenoxy)undecanoate (72).



Acid 11-(2-isopropyl-5-methylphenoxy)undecanoic (0.341 g, 1.0 mmol) was added in small portions to N,N-dimethyl-2-hydroxyethylamine (0.092 g, 1.0 mmol). Only after achieving a homogeneous liquid was the next portion added to the mixture. After addition of the final portion, the mixture was placed under orbital stirring for 15-20 minutes, giving compound **72** as a clear, colorless liquid (0.432 g, 100%).

^{1}H NMR (300 MHz, MeOD-d4): δ = 7.01 (d, 1H), 6.67 (m, 2H), 3.93 (t, 2H), 3.79 (t, 2H), 3.25 (m, 1H), 3.02 (t, 2H), 2.72 (s, 6H), 2.27 (s, 3H), 2.18 (t, 2H), 1.78 (m, 2H), 1.55 (m, 4H), 1.33 (m, 10H), 1.17 ppm (d, 6H).
^{13}C NMR (75 MHz, MeOD-d4): δ = 182.0, 157.5, 137.3, 134.9, 126.6, 121.9, 113.3, 68.9, 60.8, 57.5 44.1, 38.3, 30.73, 30.70, 30.62, 30.59, 30.4, 27.8, 27.4, 23.2, 21.4 ppm.

4.4.4.14 Antibacterial activity assay methodology (determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)).

The MICs for the compounds were determined by the two-fold broth microdilution method in 96-well microtiter plates. Briefly, bacterial overnight cultures were diluted in sterilized PBS to approximately 10^5 CFU/mL and 5 µL were inoculated into a 96-well of the plate containing 100 µL of each compound at the concentrations prepared, from two-fold serial dilutions in MH (for Gram-negative bacteria) or BHI (for Gram-positive bacteria) broth medium. The concentration ranges [high-to-low] used for compounds were 4.06-0.008 mM. Plates were incubated at 37°C for 16-18 h and the MIC values recorded thereafter. Measurement of the MBC values was performed in MH or BHI broth. Five microliters were aliquoted from the latter 96-well plate and re-inoculated into a fresh sterile 96-well plate containing fresh MH or BHI media. These plates were incubated at 37°C and the results recorded after 16–18 h.

List of stains used	
Gram-negative bacteria	Gram-positive bacteria
Escherichia coli O157 NCTC 12900	*Enterococcus hirae* NCTC
Salmonella enterica subsp. Typhymurium 4/74	Methicillin susceptible *Staphylococcus aureus* (MSSA) 83254/4
Acinetobacter baumannii sp3 RUH01163	Methicillin resistant *Staphylococcus aureus* (MRSA) ATCC 43300
Pseudomonas aeruginosa PAO1	*Listeria monocytogenes* ATCC 13372
Klebsiella pneumoniae MGH 78578	-
Enterobacter aerogenes ATCC 13048	-

4.4.4.15 Antifungal activity assay methodology (determination of minimum inhibitory concentration (MIC)).

The antifungal assay was carried by the broth microdilution method in 96-well microtiter plates. Briefly, compounds **60** or **68** were diluted by the two-fold method in 96-well microtiter plates in yeast extract peptone dextrose media. The final concentration ranges [high-to-low] used for compounds were 4.06 - 0.008 mM. Fungal overnight cultures were diluted in sterilized PBS to approximately 0.1 OD_{600} and 100 µL were inoculated into a 96-well of the plate containing 100 µL of each compound at twice the concentrations mentioned. Plates were incubated at 37°C for 24 h and the MIC values recorded thereafter.

Fungal isolates used: *Candida kruseii* CBS573, *Candida albicans* SC5314, *Candida albicans* 3035 and *Candida parapsilosis* CLIB214.

4.4.4.16 Monocyte toxicity assay methodology (determination of minimum inhibitory concentration (MIC)).

To test the toxicity of compound **60**, the THP-1 cell line was used and checked for malformations or differentiations in the presence of the compounds. The cell line was rapidly defrosted and centrifuged 10 minutes at 1400 rpm. The pellet was resuspended in 1 mL of complete media (RPMI 1640 + 10% FBS heat inactivated) previously warmed up to 37°C. The cell suspension was added to 9 mL of complete media in a T25 cell culture flask and incubated at 37°C under 5% CO2 and 95% humidified atmosphere for 3 days. The THP-1 cells were sub-cultured every 3 days, to a maximum of 15 passages, to maintain a high viability. The cell number and morphology were checked microscopically at the time of each passage. For each passage, cells were washed twice by centrifuging 10 minutes at 1400 rpm and resuspending the pellet in 50 mL of Hank ’s Balanced Salt Solution. The cells were centrifuged 10 minutes at 1400 rpm a third time and the pellet resuspended in 2 mL of previously warmed up complete media. To determine the toxicity of the compounds, these were resuspended in complete media at their MBC concentration and 2x MBC concentration, and incubated for 24 hours at 37°C under 5% CO2 and 95% humidified atmosphere. Trypan blue exclusion method was used to check cell numbers and viability in the TC20® Automated Cell

Counter. The compounds were compared versus their equivalent essential oil. The cells were tested for the solvents used as well. This assay was performed in duplicate.

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ANNEXES

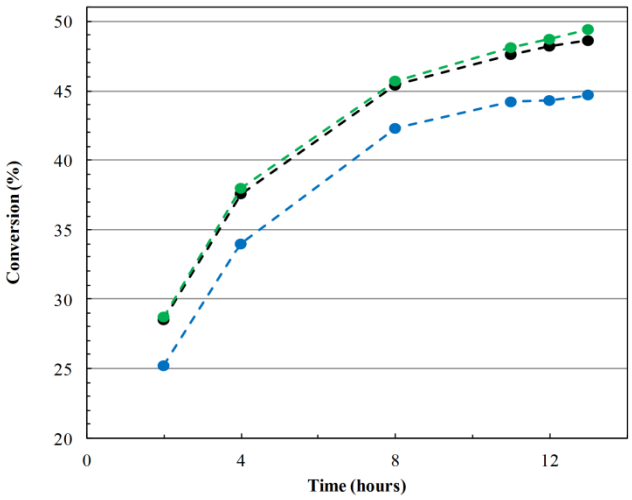

Figure A1. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 35ºC in acetone, using *in situ* generated ionic anhydride **21** as acylating agent, starting with different amounts of equivalents of ionic acid (**20**): (-●-) using 3.0 equivalents, (-●-) using 2.4 equivalents and (-●-) using 2.0 equivalents.

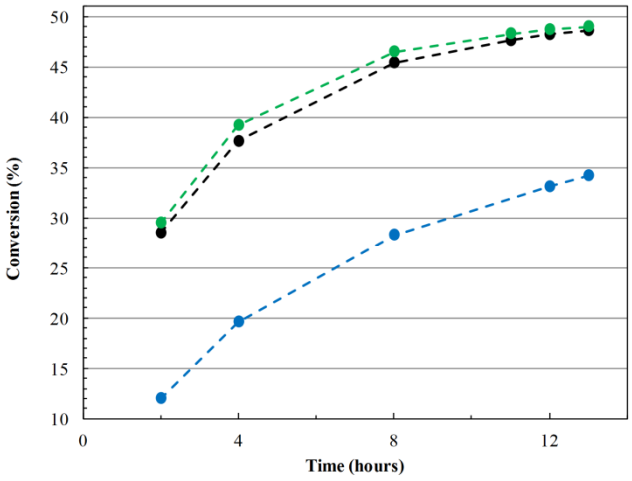

Figure A2. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 35ºC in acetone, using *in situ* generated ionic anhydride **21** as acylating agent, starting with different concentrations of substrate: (-●-) starting with a concentration of 0.75 M, (-●-) starting with a concentration of 0.50 M and (-●-) starting with a concentration of 0.25M.

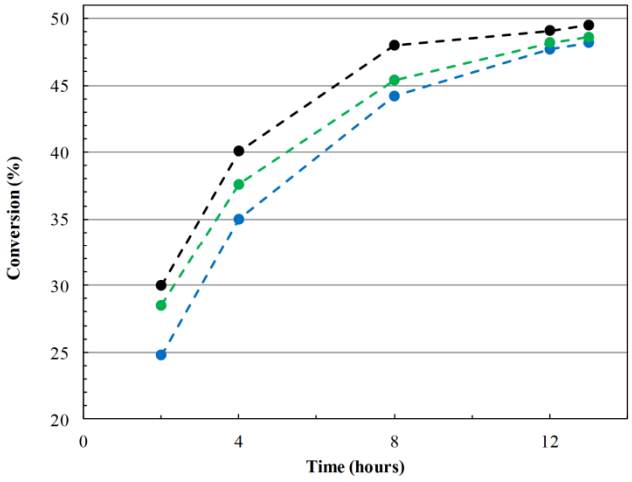

Figure A3. Reaction profiles of EKRs for 1-phenylethanol (**17**) in acetone, using *in situ* generated ionic anhydride **21** as acylating, performed at different temperatures: (-●-) at 40ºC, (-●-) at 35ºC and (-●-) at 30ºC.

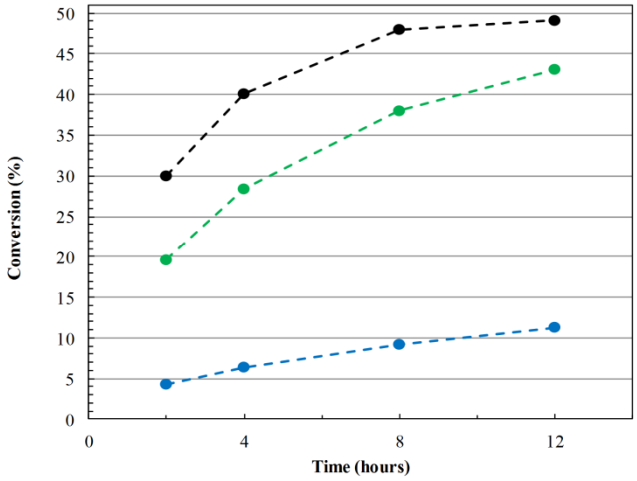

Figure A4. Reaction profiles of EKRs for 1-phenylethanol (**17**) at 40ºC in acetone, using *in situ* generated ionic anhydride **21** as acylating agent, employing different carbodiimides as coupling agent: (-●-) DIC, (-●-) DCC and (-●-) EDC.