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Development of Novel Active Pharmaceutical Ionic Liquids and Salts Based on Antibiotics and Anti-fungal Drugs

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Resumo

Os Líquidos Iónicos (LIs) são uma classe de compostos que se popularizaram em meados da década de 90 do século XX. Apesar de os LIs serem definidos por uma propriedade física (ponto de fusão), muitas das suas aplicações actuais baseiam-se nas suas propriedades biológicas. Com efeito, o uso de um fármaco na forma líquida é susceptível de evitar alguns dos problemas inerentes ao polimorfismo, responsável por influenciar a solubilidade da droga e a sua dosagem. Adicionalmente, o arranjo de um catião ou anião com um fármaco pode: a) alterar a classificação biofarmacêutica do fármaco; b) influenciar o processo de formulação do fármaco; e c) alterar as propriedades biológicas do Ingrediente Farmacêutico Activo (IFA).

A presente Tese tem como objectivo a síntese e o estudo das propriedades físico-químicas e biológicas de LIs como IFAs a partir de antibióticos beta-lactâmicos (ampicillina, penicilina G e amoxicilina) e a partir do anti-fúngico anfotericina B. Os IFAs usados foram combinados, através de métodos de neutralização tamponados, com adequados catiões hidróxidos. Os catiões hidróxido foram obtidos a partir de halogenetos através troca iónica na resina de Amberlite (na forma OH). Os estudos das propriedades biológicas destes novos compostos foram feitos usando os métodos colorimétricos e de microdiluição.

No total, foram sintetizados 19 LIs (6 LIs baseados na ampicilina, 4 LIs baseados na amoxicilina, 6 LIs baseados na penicilina G, e 4 LIs baseados na anfotericina B) caracterizados por métodos espectroscópicos e analíticos para confirmar a sua estrutura e pureza. O estudo das propriedades biológicas dos LIs sintetizados demonstrou que estes possuem actividade antimicrobiana em bactérias e fungos, inclusive em bactérias resistentes. Este trabalho permitiu, ainda, verificar que os LIs baseados na ampicilina poderiam funcionar como agentes anti-tumorais. Estes resultados confirmam que a escolha do contra-ião pode provocar alterações nas propriedades físico-químicas e biológicas dos IFA-LIs com grande impacto, tendo em conta as suas aplicações.

Palavras-chave: Ingrediente Farmacêutico Activo, Líquidos Iónicos; Agentes antimicrobianos, Resistência a antimicrobianos; Actividade anti-tumoral.

Abstract

Ionic Liquids (ILs) are class of compounds, which have become popular since the mid-1990s. Despite the fact that ILs are defined by one physical property (melting point), many of the potential applications are now related to their biological properties. The use of a drug as a liquid can avoid some problems related to polymorphism which can influence a drug´s solubility and thus its dosages. Also, the arrangement of the anion or cation with a specific drug might be relevant in order to: a) change the correspondent biopharmaceutical drug classification system; b) for the drug formulation process and c) the change the Active Pharmaceutical Ingredients' (APIs).

The main goal of this Thesis is the synthesis and study of physicochemical and biological properties of ILs as APIs from beta-lactam antibiotics (ampicillin, penicillin G and amoxicillin) and from the anti-fungal Amphotericin B. All the APIs used here were neutralized in a buffer appropriate hydroxide cations. The cation hydroxide was obtained on Amberlite resin (in the OH form) in order to exchange halides. The biological studies of these new compounds were made using techniques like the micro dilution and colorimetric methods.

Overall a total of 19 new ILs were synthesised (6 ILs based on ampicillin, 4 ILs, based on amoxicillin, 6 ILs based on penicillin G and 4 ILs based on amphotericin B) and characterized by spectroscopic and analytical methods in order to confirm their structure and purity. The study of the biological properties of the synthesised ILs showed that some have antimicrobial activity against bacteria and yeast cells, even in resistant bacteria. Also this work allowed to show that ILs based on ampicillin could be used as anti-tumour agents. This proves that with a careful selection of the organic cation, it is possible to provoke important physico-chemical and biological alteration in the properties of ILs-APIs with great impact, having in mind their applications.

Keywords: Active pharmaceutical ingredients, Ionic Liquids; Antimicrobial agents, Antimicrobial resistance; Anti-tumoral activity.

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List of Abbreviations

Ace	Acesulfamate
Amp	Ampicillin
AmphB	Amphotericin B
Amx	Amoxicillin
API-ILs	Active Pharmaceutical Ingredients – Ionic Liquids
APIs	Active Pharmaceutical Ingredients
ATTC	American Type Culture Collection
BA	Benzalkonium
BCS	Biopharmaceutics Drug Classification System
BF_4	Tetrafluoroborate
BV	Bed Volumes
Cat	Cation
DDA	Didecyldimethylammonium
DSC	Differential Scanning Calorimetry
EMIM	1-Ethyl-3-methylimidazolium
ESI-MS	Electrospray Ionization Mass Spectrometry
FDA	Food and Drug Administration
GF	Gingival Fibroblasts
HAIs	Health Care-Associated Infections
HDPC	Hexadecylphosphocholine
HepG2	Liver Cancer Cell Lines
IC50	Half Maximal Inhibitory Concentration
ILs	Ionic Liquids

LB	Lysogeny Broth
LD50	Median Lethal Dose
m.p.	Melting Point
MBC	Minimal Bactericidal Concentration
MG63	Osteosarcoma Cancer Cell Lines
MIC	Minimal Inhibitory Concentration
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NMR	Nuclear Magnetic Resonance
PBPs	Penicillin-Binding Proteins
PC3	Prostate Cancer Cell Lines
Pen	Penicillin
PYCC	Portuguese Yeast Culture Collection
RDIC	Relative Decrease of Inhibitory Concentration
RKO	Colon Cancer Cell Lines
RTILs	Room Temperature Ionic Liquids
Sac	Saccharinate
SF	Skin Fibroblasts
T47D	Breast Cancer Cell Lines
Tdec	Decomposition Temperature
TEA	Tetraethylammonium
Tg	Glass Transition Temperature
Tm	Melting Temperature
WHO	World Health Organization
XTT	2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner Salt
α-MEM	α-Minimal Essential Medium

"Organic chemistry nowadays almost drives me mad. To me it appears like a primeval tropical forest, full of the most remarkable things, a dreadful endless jungle into which one does not dare enter for there seems to be no way out"

Friedrich Wöhler (1800-1882)

Chapter 1.
Objectives and General Plan

1 Objectives and General Plan

1.1 Study Approach

Ionic Liquids (ILs) are a new type of compounds, which have become a hot topic since the mid-1990s[1]. A literature search in Web of Science® database has revealed that the term "Ionic Liquids" in 1990 only had 3 results, in 2002, it had 485 results and in 2012, it had 3461 results (Figure 1.1). ILs have been attracting a great interest in the last decade. At the same time, there has been a growing awareness of the range of properties that ionic liquids can possess[2].

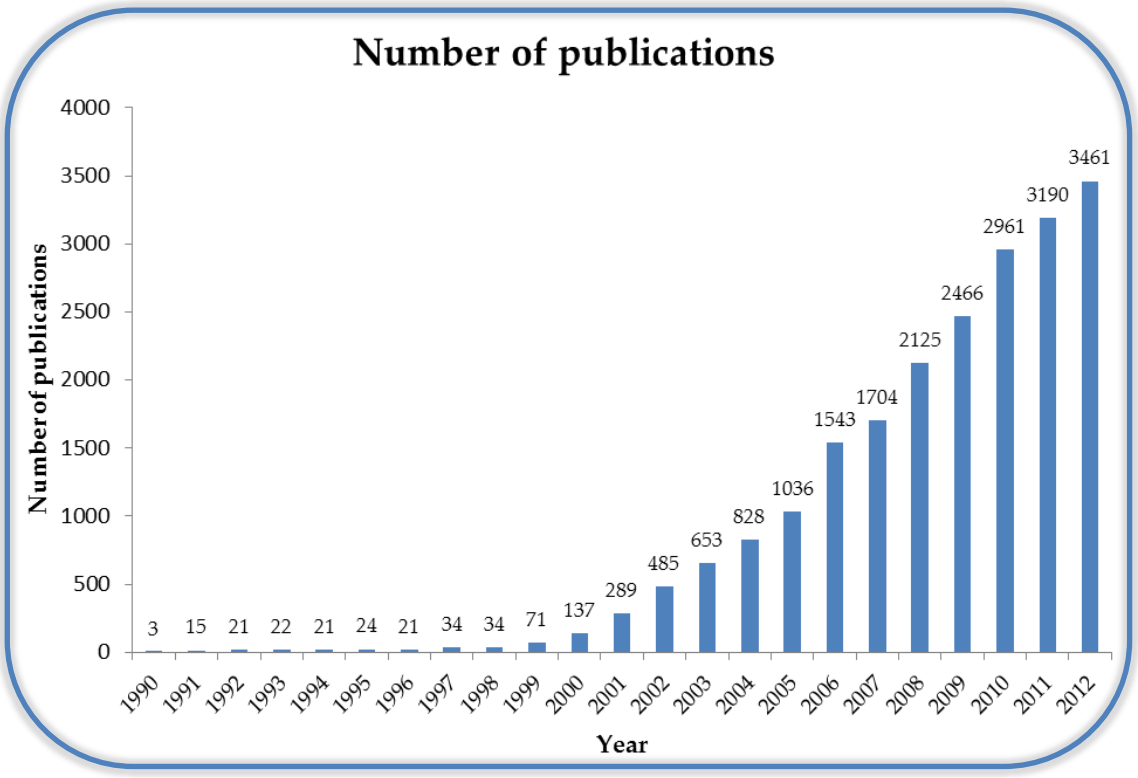


Figure 1.1 Publication frequency of the term "Ionic Liquids" obtained from Web of Science® database.

The definition of ILs, although ambiguous, is generally defined as salts with melting points below 100 °C (many are liquid at room temperature), and composed only by discrete cations and anions[3]. ILs are not new compounds, they have been known for over a century, but just quite recently they have become under intense worldwide scrutiny, not only due to the implications of their use as solvents[3,4], but mainly because of the biological applications of ILs. Biologically active ions have been used to make new ILs. However, the primary driver for these materials has been the use of ions of known low toxicity to obtain the IL physical property set[5]. They have been used as potential alternatives to the classical volatile organic solvents[2,6-9].

Recently, the attention for ILs came from other areas like biology and medicine. Despite the fact that ILs today are defined by one physical property (melting point), many of the potential applications are now using combinations of chemical or biological properties, such as in the study of energetic materials or pharmaceutical ILs[9-11]. ILs are indeed tuneable, multipurpose materials for a variety of applications, rather than just solvents[12] (Figure 1.2).

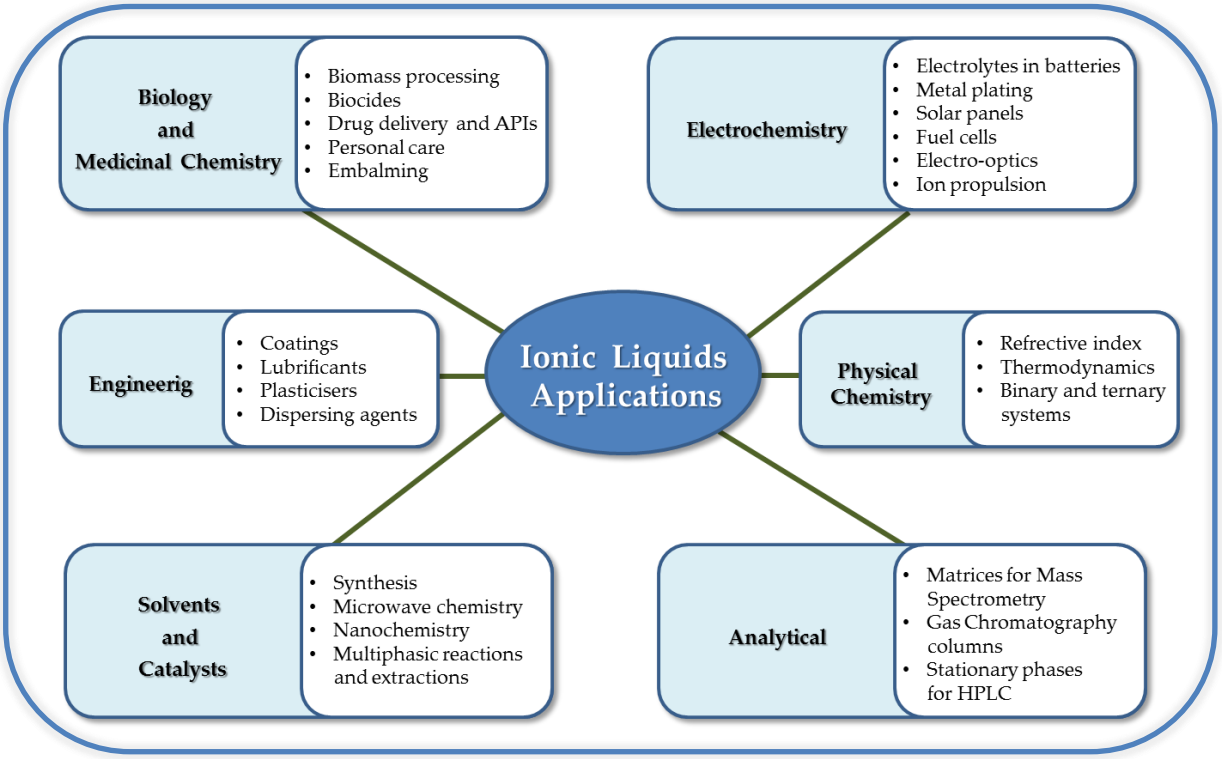


Figure 1.2 Examples of some applications of ILs (adapted from Plechkova Seddon[13]).

As described below, the present work was designed in order to develop new methods of producing ILs based on Active Pharmaceutical Ingredients (API) and the study of their biological applications. This approach could be particularly useful for the pharmaceutical industry because ILs can offer new and distinctive properties compared to the solid pharmaceutical forms.

The selected pharmaceutical drugs were divided in two classes: 1) beta-lactam antibiotics 2) anti-fungal (Amphotericin B). For all the cases, the drugs were used as anion combined with organic cations based on imidazolium, ammonium, phosphonium and pyridinium structures.

The organic cations such as cetylpyridinium, choline and imidazolium structures were selected based on their biocompatibility and recent applications in the pharmacy field[9]. Cetylpyridinium is a cation and is used as an antiseptic, present in most toothpastes[14-16]. Choline is an available metabolite of phosphatidylcholine and has an important contribution in both proliferative growth and programmed cell death[17]. Imidazolium cations are one of the most common cations in ILs field and they are being applied as antibacterial[18-21], antifungal[22] and anti-tumour[23]. There are also some studies about the toxicological effect of these cations[24,25].

A more sustainable synthetic strategy was explored for all the Active Pharmaceutical Ingredients – Ionic Liquids (API-ILs) according with the final interest and the API stability. The most conventional synthetic method for the preparation of ILs involves a metathesis reaction step by the anion exchange of halide salts with adequate metal salts. The pure IL can be obtained by the elimination of undesired inorganic salts (mainly sodium, potassium or lithium chloride or bromide) using a precipitation followed by filtration or other efficient separation processes such as ultra or nanofiltration methods. This work further explores ion exchange resin methods recently developed by Fukumoto *et al*[26].

After the synthesis and purifications of ILs, based on beta-lactam antibiotics, it was necessary to understand their biological behavior. We intended to study their

antibacterial activity against sensitive Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* (clinically isolated), as well as Gram-positive *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* and *Enterococcus faecalis*. The arising resistance developed by bacteria to antibiotics is a serious public health threat and needs new and urgent actions such as the development of new drugs that show an efficient antimicrobial activity. In this context, we also intended to study the antibacterial activity of these new compounds against a panel of resistant bacteria (clinically isolated strains): *E. coli* CTX M9, *E. coli* TEM CTX M9, *E. coli* TEM1, *E. coli* CTX M2, *E. coli* AmpC Mox2[27-29].To better understand the behaviour of these drugs in human cell line and to explore the dual activity (antibacterial and anti-tumour) of the ILs with ampicillin anion [Amp][23,30,31], we also studied anti-proliferative activity against five different human cancer cell lines, in particular T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon) and two primary human cell types, namely, skin fibroblasts (SF)and gingival fibroblasts (GF). On the one hand, the right conjugation of the cation and the anion can be relevant on modifying the correspondent biopharmaceutic drug classification system (BCS)[9,32], as well as their drug formulation process. On the other hand it increases the activity of the drug or gives the drug a new function.

This work also pretends to show the applications of the neutralization method using exchange resin in pharmaceutical industry. The applications of this method are described on the synthesis of Amphotericin B (AmphB) conjugated with different cations. AmphB is a polyene antibiotic and one of the most frequently used against fungal infections[33]. Due to the fact that AmphB is insoluble in water there are two ways of administering AmphB. One is through the addition of sodium desoxycholate to form a mixture, which provides a colloidal dispersion for intravenous infusion, following reconstitution (*Fungizone*®)[33,34]. But this approach has some inconvenient side effects such as nephrotoxicity[33,35]. The other form of administration is by single bilayer liposomal drug delivery system (*Ambisome*®)[33,36]. This allows large dose tolerance and less side effects but needs more repetitive administrations.

1.2 Objectives

The general objectives of the present work were:

a) the synthesis of ILs based on antibiotics (ampicillin, penicillin G and amoxicillin) and Amphotericin B

b) the study of the physical and chemical properties of the compounds newly synthetized;

c) the study of the biological properties of the new compounds obtained.

c1) the study of antimicrobial activity on bacteria and yeast cells

c2) the study of cellular viability and the cytotoxicity of the new compounds obtained on human cell lines

The particular objectives of each chapter are described as follows:

Chapter 4 – the objectives were to develop a method to synthesize ILs based on ampicillin and to analyze the physical and chemical properties of these new ILs.

Chapter 5 – in this chapter the aims were to study the antibacterial activity of ILs based on ampicillin against Gram-positive and Gram-negative and against resistant strains and to compare the results with sodium ampicillin.

Chapter 6 – the goal was to study the anti-proliferative activity against five different human cancer cell lines, in particular T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon) of the Ampicillin-ILs. Besides that, evaluate the toxicity on two human cell lines.

Chapter 7 – Here, the intention was to synthetize several ILs based on beta-antibiotics (penicillin G and amoxicillin) and the anti-fungal drug (amphotericin B) as well as to evaluate their biological behaviour.

1.3 General Plan

The present work is composed of 9 chapters, including the current chapter describing the objectives and the general plan of the work developed (Chapter 1). In **Chapter 2** the state of the art of the work in ILs and the importance that ILs have on the development of APIs is described. A mini review in ILs as APIs was included in this chapter (the content of this subchapter was published in *Chemmedchem*, 2011, **6**, 975-985[9]). Chapter 3 describes all the material and methods used during this work. Chapter 4, Chapter 5, 6, and 7 describe the work developed according to specific objectives. These Chapters present the results obtained, and are reproductions of the papers that have already been published, accepted or submitted.

In **Chapter 4 – Development of Novel Ionic Liquids Based on Ampicillin**, which was published in *Medchemcomm*, 2012, 3, 494-497, describes a new methodology of the synthesis of ampicillin ILs and the relevant physical properties of these novel ionic liquids based on ampicillin.

In **Chapter 5 – Antibacterial Activity of Ionic Liquids Based on Ampicillin Against Resistant Bacteria** - the antibacterial activity of new ionic liquids (ILs) containing ampicillin anion was described in comparison to sodium salt of ampicillin on resistant bacteria. This chapter was submitted to RSC Advances and is a reproduction of the paper.

On the other hand, **Chapter 6 – Anti-tumoral Activity of Ampicillin Ionic Liquids and Their Salts**, describes anti-proliferative effects against different tumour cell lines using innovative APIs. The content of this chapter is a reproduction of a manuscript ready to submit.

Chapter 8 – Discussion and Conclusion, gives a general discussion of all the work, summarizing the main conclusions. In this chapter the future perspectives are also presented.

Chapter 9 – References, this presents the bibliography used.

Chapter 2.
General Introduction

This chapter contains parts of a published review article:

Ionic Liquids as Active Pharmaceutical Ingredients

Ricardo Ferraz, Luís C. Branco, Cristina Prudêncio, João Paulo Noronha and Željko Petrovski.

Chemmedchem, 2011, 6, 975-985.

(authorized reproduction)

2 General Introduction

2.1 Active Pharmaceutical Ingredients

The World Health Organization (WHO) defines APIs as "a substance used in a finished pharmaceutical product (FPP), intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of a disease, or to have direct effect in restoring, correcting or modifying physiological functions in human beings"[37]. Therefore, the API is the active compound present in a drug while the inactive substances are the excipients. For the pharmaceutical industry, the differences between APIs and drugs are of extreme importance. Understanding these differences is fundamental for manufacturers to specialize in, for regulators to focus on resources; and for pharmacists to produce equivalents to brand name products.

2.2 Antimicrobials Agents

2.2.1 Beta-lactam Antibiotics

A compound responsible for killing or inhibiting the growth of a microorganism is called an antimicrobial agent[38]. An antibiotic compound indicates that it came from a natural source.[38]

There are many classes of antibiotics. One of the most important, due to its extensive use are the beta-lactamic antibiotics. This class is characterized by the presence of a beta-lactam ring, a highly strained and reactive cycle amide[38]. Attending to the type of ring structure, this class of antibiotics is divided into several groups, being the most important: penam, penem, carbapenem, cephem and monobactam (Figure 2.1).

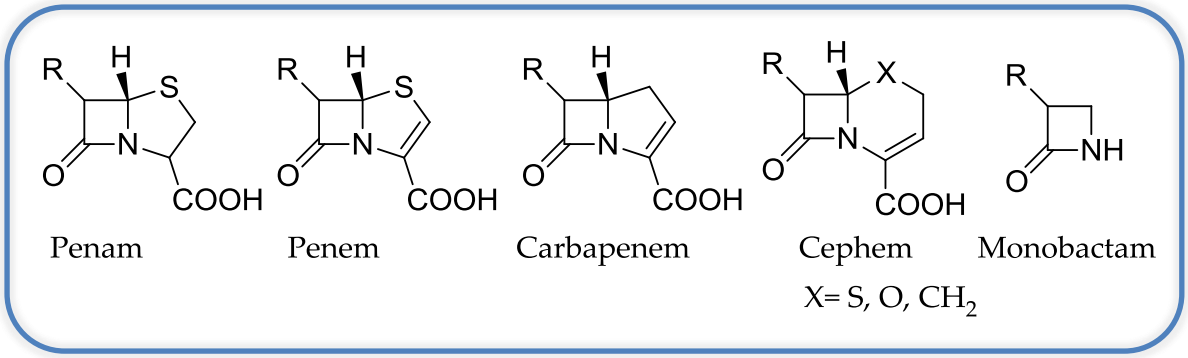


Figure 2.1 General strutures for the five relevant ring strutures of beta-lactams.

The largest group of beta-lactam antibiotics are penams. This group includes penicillin, ampicillin and amoxicillin. Penams are composed of an enclosed dipeptide formed by the condensation of L-Cysteine and D-Valine resulting in the beta-lactam ring and in the thiazolidinic ring producing the 6-aminopenicillanic acid[38,39].

Beta-lactamic antibiotics are specific for bacteria. Bacteria are prokaryotic cells, so they have many structural and metabolic pathways that are different from eukaryotic cells[38].

2.2.2 Anti-fungal Agents

Amphotericin B (AmphB) entered in clinics in 1958 (Figure 2.2) and for nearly 30 years it was almost the only drug available for controlling some severe fungal infections[40,41]. There are other types of anti-fungals; especially heterocyclic compounds like imidazole or triazole[40,42].

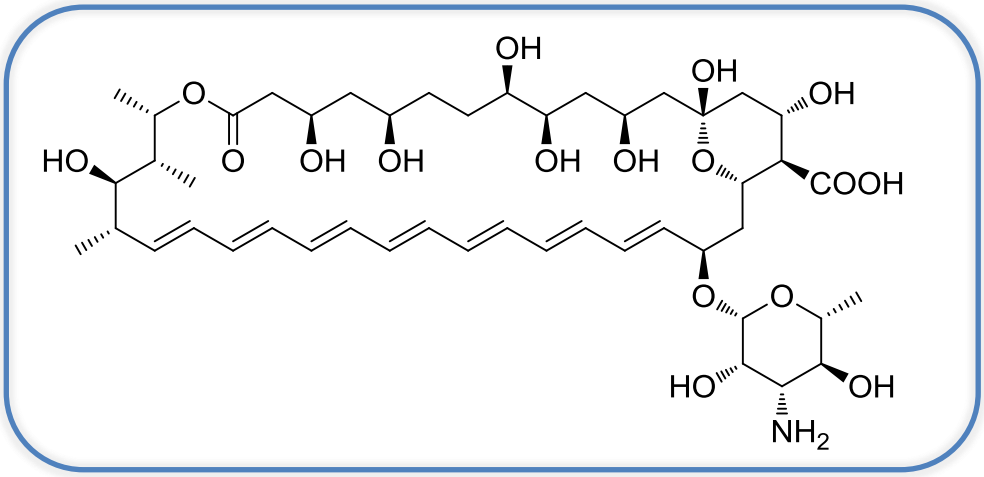


Figure 2.2 Struture of Amphotericin B.

At the present time, the most common antifungal therapies are limited because of drug's toxicity[42]. Nevertheless, AmphB, such a well-known nephrotoxic drug continues to be the drug of choice against life-threatening systemic infections with fungi, such as *Candida albicans* or *Aspergillus fumigatus*[42] and also against visceral and cutaneous leishmaniasis. To avoid and decrease the toxicity of AmphB new formulations have been made since 1990[43].

AmphB is a low-soluble polyene antifungal which is able to self-aggregate[43] and is the most important member of the mycosamine family, which includes nystatin, candidin and rimodicin. AmphB at an industrial scale is extracted from *Streptomyces nodosus*[43,44]. In 1988 the total synthesis of AmphB was described by Nicolaou *et al.*[45] but the biotechnological production was much cheaper and more efficient[44]. There have been some innovations in synthetic procedures, but the focus is in the synthesis of new analogues with improved antifungal activity and/or water solubility[44]. AmphB is a yellow product and has a polar and apolar side of the lactone ring giving the molecule its amphiphilic behaviour. Besides, AmphB has an ionisable carboxyl and amine group (Figure 2.2), which gives amphoteric properties to this drug[42-44]. These physico-chemical properties are the reason why AmphB is poorly soluble in all aqueous solvents and in many organic solvents[43]. As shown in Table 2.1, the solubility of AmphB in water is less than 1 mgL^{-1} at physiological pH, at pH below 2 or above 11 is soluble but is not stable under these conditions[43].

Table 2.1 Solubility of AmphB in different solvents from Lemke *et al.*[44]

Solvent	**Solubility (mgL^{-1})**
Water	<1 (at pH 6–7)
Methanol	2000
Ethanol	500
Chloroform	100
Petrol ether	10
Dimethyl formamide	2
Propylene glycol	1
Cyclohexane	20

Due to AmphB´s toxicity problems (nephrotoxicity) and to its poor oral absorption, it was necessary to adapt some formulations of the drug: liposomes or colloidal dispersions[33,43]. AmphB form of *Fungizone*® is a surfactant-stabilised formulation that helps in the oral solubility and is currently and exclusively administered parenterally[33,34]. However, this has been associated to some adverse side effects[33,35]. Liposomal preparations, like *Ambisome*®, allow the application of higher dose tolerance of dissolved AmphB[33,43] without inducing the adverse side effects. This formulation could be the most popular choice in the future[43].

2.2.3 Mechanisms of Anti-Microbial Action and Resistance

One of the biggest problems of ampicillin, amoxicillin, penicillin and the other penams is the reactivity of the highly strained ring that causes the degradation process of these compounds. At room temperature, the nitrogen of the beta-lactam ring is easily protonated by acids, and is followed by nucleophilic attack of the remaining lateral chain carbonyl[38,39]. Therefore, the oxazoline formed will produce a new imidazole and finally penicillic acid.

Another problem, besides chemical degradation, is that of a group of enzymes that specifically destroy and inactivate beta-lactams. Many bacteria produce these enzymes called penicillinases and the beta-lactamase is the most prevalent type of these enzymes[38,46].

Normally, antibiotics can be divided in 5 categories, according to the mechanism of action[38]: a) inhibition of cell wall synthesis; b) impairment cytoplasmic membrane; c) inhibition of nucleic acid synthesis d) inhibition of protein synthesis; and e) metabolic antagonist action. Bacteria, on the other hand, found several ways to resist antibiotics: 1) enzymatic inactivation of the drug by the presence of beta-lactamases; 2) presence of an enhanced efflux pump; 3) porin mutation that obstruct the drug entrance and 4) target modification of the drug, such as the mutation of penicillin binding proteins (PBPs). Figure 2.3 is an example of resistance mechanisms in Gram-negative bacteria.

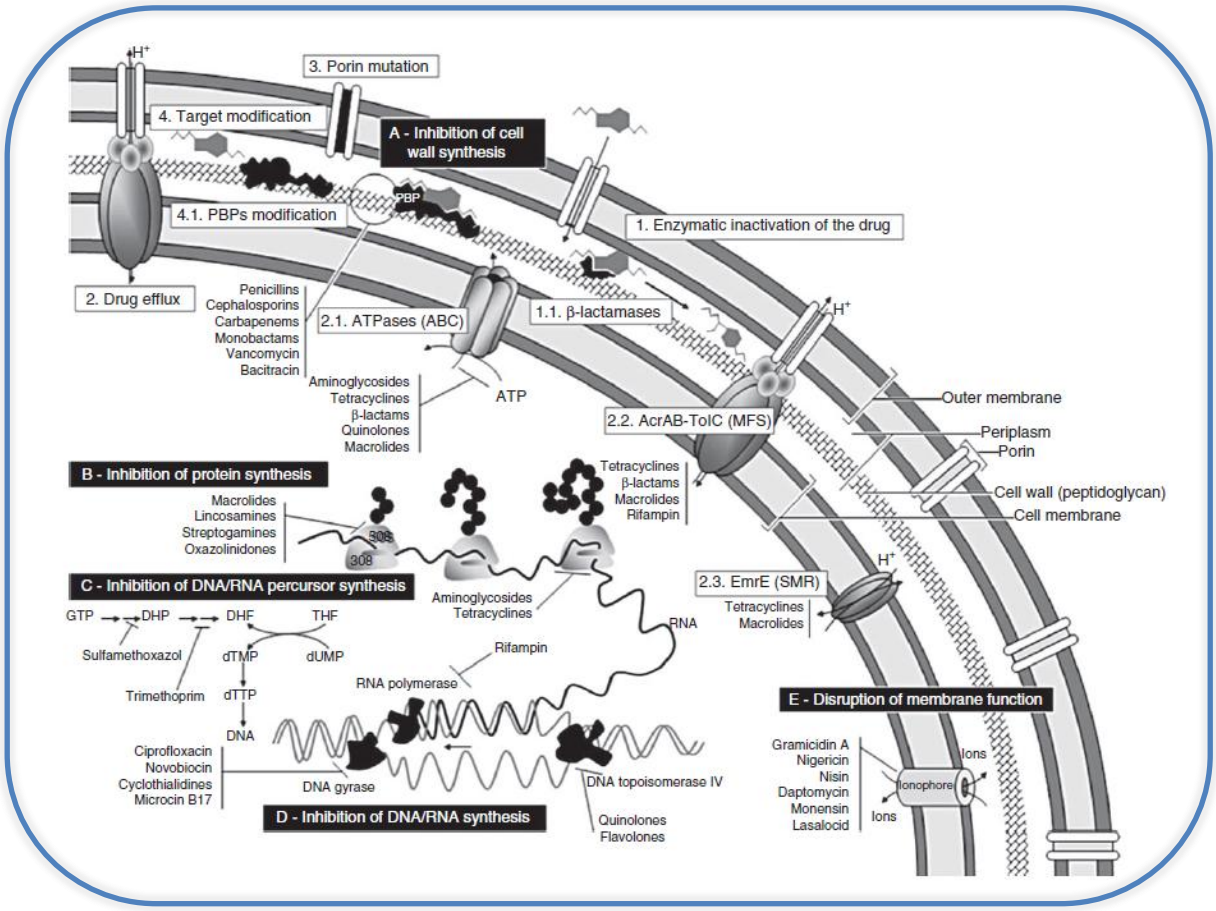


Figure 2.3 Mechanisms of antimicrobial action and resistance in Gram-negative organisms. This picture represents a Gram-negative bacteria cell. Black boxes represent mechanisms of drug action and white boxes represent mechanisms of resistance. Below or on the side of each box there are several examples of drugs presenting those types of mechanisms. The main mechanisms of antimicrobial action can be divided into five major classes: (**A**) those which act in the cell wall synthesis; (**B**) those which act in the protein translation; (**C**) those which act in metabolic precursor biosynthesis; (**D**) those which act in the molecular genetics processes (replication, transcription); and (**E**) those which disrupt membrane function and permeability. Some of the resistance mechanisms are represented here by numbers: (**1**) enzymatic inactivation of the drug by the presence of beta-lactamases (**1.1**); (**2**) presence of an enhanced efflux pump, whether it is by an active transport system involving ATPases (**2.1**) or rather if it is driven by proton motive force (**2.2**), (**2.3**); (**3**) porin mutation obstructing the drug entrance; and (**4**) target modification of the drug, such as the mutation in the penicillin binding proteins (PBPs) (Authorized reproduction)*.

Until approximately 1960, scientists produced and developed more than 20 new classes of antibiotics. Since then, only two new classes of antibiotics have appeared[47]. The problem of resistance presented by bacteria has been well known for approximately 20

* This figure was kindly offered by the author from "beta-Lactams: chemical structure, mode of action and mechanisms of resistance", *Reviews in Medical Microbiology*, R. Fernandes, P. Amador and C. Prudêncio, 2013, 24, 7-17.

years or more but the pharmaceutical industry does not invest in the development of new antibiotics[47]. This may be due to the fact that the period necessary for bacteria to develop resistance is becoming shorter[48], which consequently increases the challenges that pharmaceutical industry faces[9]. Instead of developing new antimicrobial agents that have proved to be more expensive and unsuccessful[48] ,the scientific community seems now to be opting for another approach – the modification of existing antimicrobial agents[48,49].

Nowadays, bacterial resistance to different antibiotics is a major public health problem[27,30,50,51]. Recent outbreaks that evidence this problem[27-29,52] are cases such as the one found in Germany for *E. coli* O104[53,54] as well as the emergence of multi-drug-resistant organisms, as in the of Gram-negative Enterobacteriaceae associated to the New Delhi metallo β-lactamase[55-57]. In fact, bacterial resistance, not only has public health implications, but is also a global safety threat, specifically at an economic and social level[58,59]. The latest studies have also reported the significant financial burden on health care-associated infections (HAIs) in the USA[60,61]. In the UK, approximately 9% of hospitalized patients acquire an infection after post-admission to hospital, which increases the costs in the health care system[60,62].

In this context, it is important to know and study the resistance mechanisms, and also to study non-antibiotic compounds as antimicrobial agents to find new therapeutic strategies to reduce the spread of resistant bacteria and their evolution.

2.3 Anti-cancer Agents

Anti-cancer agents are drugs used to inhibit cell cancer growth. Nowadays, cancer is one of the most important causes of morbidity and mortality worldwide[63]. In most cases, effective cancer care requires the linkage of early diagnosis to the appropriate use of therapy[63]. The research for new anti-cancer agents is focused on avoiding tumour resistance and decreasing the toxicity associated with chemotherapies[23]. In recent years, some of the compounds that have desirable drug like properties have suffered of poor solubility in water and physiological fluids[31,64] – when a chemical

entity has some pharmaceutical relevance, its bioavailability also becomes a challenge[31,64,65]. Because of that, the research for new strategies to solubilize these types of substances is essential[64,66,67]. One way to overcome the solubility and stability problems of APIs is salt formation[23,31]. There are many examples where pharmaceutical active cations and anions have been combined together. The resulting salt exhibits therapeutic effects of both its components[31] and many of these salts have some characteristics of ILs. So it is possible to prepare ILs (using their tuneable properties) as anti-cancer agents[31,68].

2.4 APIs Issues in the Pharmaceutical Industry

"The pharmaceutical industries are undoubtedly experiencing a series of challenges"[69]. This industry has always faced difficult challenges in the innovation of drugs, but normally, these difficulties can be overcome with an increase of the final product prices[70]. One of the difficulties is the amount of regulation that authorities demand to approve a new drug (particularly in the case of the US Food and Drug Administration (FDA))[70]. The complications are due to the solid form of many drugs, like low solubility, polymorphism and low bioavailability. Adding to these, there are related problems, like low solubility media of the drug molecule or the starting material for synthesis of drugs in aqueous systems and in most of the pharmaceutical acceptable organic solvents[69].

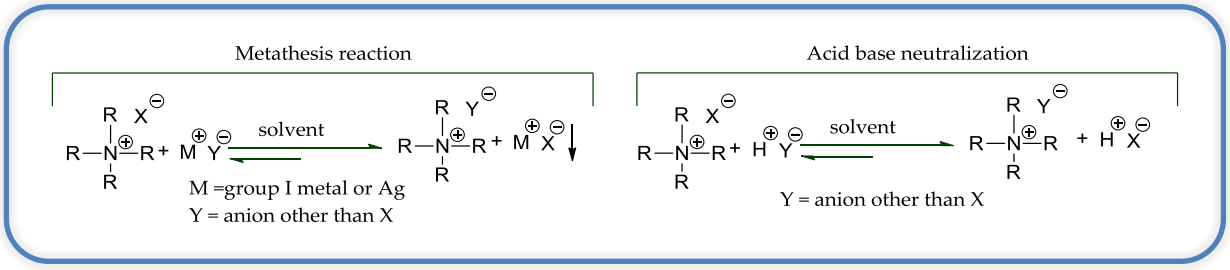
In the salt formation process of pharmaceuticals, the cation most commonly used is sodium ion and chloride ion is the most used anion. The use of solid crystalline forms for the delivery of APIs is still the most reliable for the pharmaceutical industry, in spite of the fact that many drugs exhibit multiple crystalline forms (polymorphs, solvates, etc.), which, as said before, can have a profound effect on the solubility, physical and chemical stability, dissolution rate and, in some cases, bioavailability of the compound[71-74].

These issues like polymorphism could be solved using ILs form of the API because ILs can be specially made with pre-selected characteristics by varying the cations and anions of which they are comprised.[71]

As referred before, there is another big problem that the pharmaceutical industry is facing, drug resistance. This is a problem of the pharmaceutical industry but it also affects public health. In general, antimicrobial resistance is the capacity that any microorganism, like bacteria, fungi and parasites has to neutralize or to deteriorate the drugs which become ineffective[38,75-77]. This is of major concern because drug resistance is deadly, can spread to other microorganisms, and carry out costs to individuals and society[75-82]. Many of the drug treatment breakthroughs of the last century could be lost through the spread of antimicrobial resistance. As a result, many infectious diseases may one day become uncontrollable and could rapidly spread throughout the world[82]. In terms of bacteria resistance, this problem is currently and directly responsible for 15 times as many deaths as AIDS every year in Europe[75].

2.5 Methods of Preparing Ionic Liquids

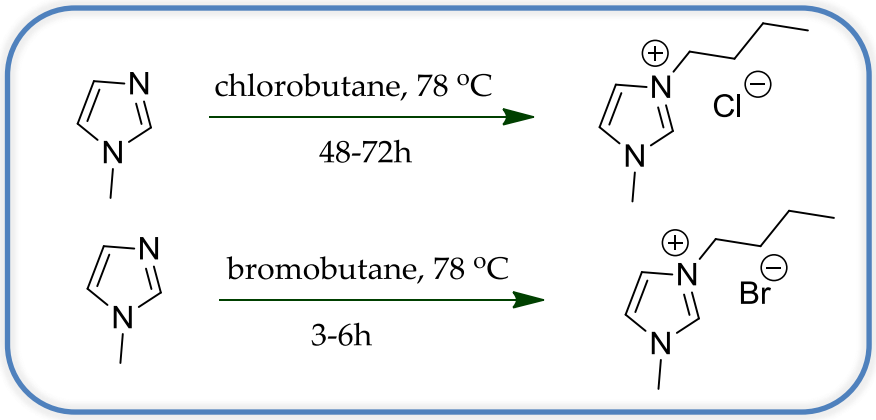
ILs are usually prepared from ammonium, phosphonium or sulphonates ions[83]. There are 2 main methods of preparing ILs: metathesis and acid base neutralization (**Erro! A origem da referência não foi encontrada.**). In the case of imidazolium cations we could consider another method, that developed by Earl and Seddon: the reaction of imidazole carbenes as strong bases[84].



Scheme 2.1 General procedure for the metathesis reaction and acid base neutralization.

Nowadays, there are many alkylammonium halides commercially available and they can be used directly in metathesis reactions[85]. In other cases, a quaternization reaction

is necessary, when an organic halide salt is formed through the alkylation of a base by a haloalkane[85]. This method can also be used to prepare pyridinium and imidazolium halides (Scheme 2.2). Currently, the use of non-conventional methods like microwave[24,86] and ultrasound techniques [24,87] is being applied in the quaternization reaction with better results[24,88].



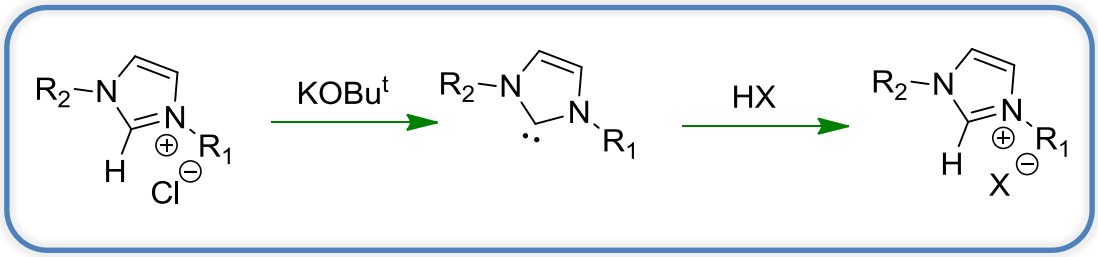
Scheme 2.2 Examples of the preparation of imidazolium halides using classic methods from Lévêque *et. al*[88].

The metathesis reaction is used to prepare ILs halide free like [EMIM][BF_4] from the halide salt ([EMIM]I) and from silver or a group 1 metal (Ag[BF_4])[85]. Other ILs have been prepared using metathesis reaction like thiocyanate, nonafluorobutanesulfonate, bis((trifluoromethyl)sulfonyl)imide, tris(trifluoromethyl)sulfonyl, methide, trifluoro-acetate, and heptafluorobutanoate. This reaction is quite simple and easy to prepare. Nevertheless, this reaction has some inconvenients, like the contamination with a small amount of halide ions, which may react with solute materials and the presence of water or silver[24,85]. Recently Srour *et al*.[24] have shown a silver and water free metathesis reaction. Another problem considering metathesis reactions is the limited variety of commercially available metal salts and also the coordination transition metal with bioactive compounds like aminoacids[26,89].

The acid base neutralization method avoids the contamination problem[90]. This procedure is also quite simple and through the use of equimolar mixing, it is possible to obtain the salts without the formation of by products[90]. This method results very well with tertiary amines with halide acids or some organic acids. The only problem is when we have weaker acids than hydrohalic acids[91]. In this case the neutralization has

to use hydroxide quaternary cations[30]. Recently ion exchange resin methods have been developed by Ohno and co-workers[26,91-93].

The use of imidazole carbenes as a strong base[84] (**Scheme 2.3**) also has the advantage of non-contaminated ILs with halide ions or metal ions, but the main advantage is the possibility of preparing ILs or salts from alcohols like methanol and propanol, from carbonic acid, acetic acid and alkyl sulphonic acid. The disadvantage is that it only works to prepare imidazolium ILs.



Scheme 2.3 General procedure for the synthesis of ILs using imidazole carbenes.

2.6 Ionic Liquids as Active Pharmaceutical Ingredients*

2.6.1 Abstract

Ionic liquids (ILs) are ionic compounds that possess a melting temperature below 100 ^{o}C. Their physical and chemical properties are attractive for various applications. Several organic materials that are now classified as ionic liquids were described as far back as the mid-19th century. The search for new and different ILs has led to the progressive development and application of three generations of ILs:

1) The focus of the first generation was mainly on their unique intrinsic physical and chemical properties, such as density, viscosity, conductivity, solubility, and high thermal and chemical stability.

2) The second generation of ILs offered the potential to tune some of these physical and chemical properties, allowing the formation of "task-specific ionic liquids" which can be used as lubricants, energetic materials (in the case of selective separation and extraction processes), and as more environmentally friendly (greener) reaction solvents, among others.

3) The third and most recent generation of ILs involves active pharmaceutical ingredients (API), which are being used to produce ILs with biological activity.

Herein, we summarize recent developments in the area of the third-generation ionic liquids that are being used as APIs, with a particular focus on the efforts to overcome current hurdles encountered by APIs. We also offer some innovative solutions in new medical treatment and delivery options.

* This subchapter is a reprint of a published review article: Ionic Liquids as Active Pharmaceutical Ingredients, Ricardo Ferraz, Luís C. Branco, Cristina Prudêncio, João Paulo Noronha, Željko Petrovski, *Chemmedchem*, 2011, **6**, 975-985 (authorized reproduction).

2.6.2 Introduction

Ionic liquids (ILs) have been a topic of great interest since the mid-1990s[1].They have attracted particularly high attention in recent years; approximately 1800 papers were published in the area of ILs in 2008 alone,[94] documenting a variety of new IL applications. The range of IL uses has broadened, and there has been a significant increase in the scope of both physical and chemical IL properties over the years[2,95].

ILs are generally defined as organic salts with melting points below 100 ^{o}C (some of them are liquid at room temperature) and composed entirely of ions[94-97]. Despite the fact that ILs were first reported in the mid-1800s, widespread interest in this compound class has occurred only recently. ILs have come under worldwide scrutiny mainly through their use as solvents[2-4,94]. In particular, the room temperature ionic liquids (RTILs), also known as "designer solvents" (because it is possible to create an IL with a given required property), have served as greener alternatives to conventional toxic organic solvents[3,94,98]. RTILs have been used for several other applications, and their development continues at a considerable rate owing to their peculiar physical and chemical properties such as high thermal and chemical stability, lack of inflammability, low volatility, and tunable solubility with several organic compounds. By taking advantage of their unique properties,[94,99] several IL applications have been described, including reaction media for many organic transformations,[94,100] in separations and extractions,[94,101,102] as electrolytes for electrochemistry,[94,103,104] in nanotechnology,[94,105,106] in biotechnology,[94,107] and in engineering processes,[94,108,109] among others.

ILs can be grouped into three generations according to their properties and characteristics[8]. The first generation includes ILs for which the accessible physical properties such as decreased vapor pressure and high thermal stability[110] are often unique (Figure 2.4, 1st Generation). The second-generation ILs have potential use as functional materials, such as energetic materials, lubricants, and metal ion complexing agents, (Figure 2.4, 2nd Generation). By taking advantage of their tunable physical and chemical properties, ILs can produce a remarkable platform on which—at least potentially—the properties of both cation and anion can be independently modified

and designed to enable the production of new useful materials while maintaining the main properties of an IL. Some RTILs have been used as reaction media to produce or improve the preparation of various pharmaceuticals[3,111,112]. Recently, the third generation of ILs[8] (Figure 2.4, 3rd Generation) has been described using active pharmaceutical ingredients (APIs) to produce ILs with biological activity.

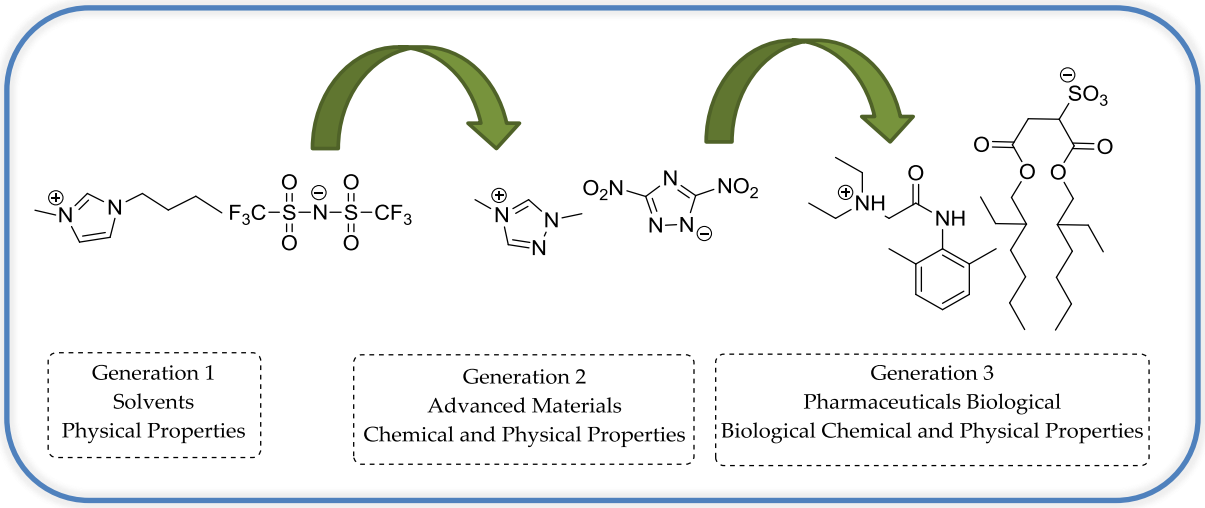


Figure 2.4 The scientific evolution of ILs, from unique physical properties (Generation 1) through the combination of chemical and physical properties (Generation 2), to the more recent studies of their biological and pharmaceutical activities (Generation 3) [adapted from Hough *et al.*][8] .

While a tremendous amount of research has focused on the physical and chemical properties of ILs, more recently, the toxicity and biological behavior of ILs have been included as two of the most highly debated topics in this field. Biologically, active ions have been used to develop novel ILs; however, the primary drive behind the research into these materials has been focused on the use of well-known low-toxicity ions to obtain ILs with the desired set of properties[5,8,113].

2.6.2.1 Historical Perspective

The first ionic liquid was described as "red oil" and was produced in the course of Friedel-Crafts reactions carried out in the mid-19th century. However, the composition of this red oil was only lately identified as a salt. For $AlCl_3$-catalyzed reactions, the structure proposed for this liquid was the heptachlorodialuminate salt shown in Figure 2.5. This IL as red oil, along with more complicated structures, were patented as useful materials, but no industrial application has been reported[3,114].

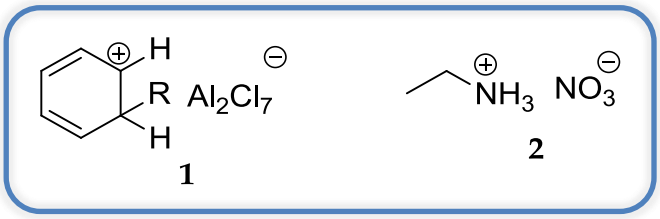


Figure 2.5 The structure proposed for the heptachlorodialuminate salt (**1**), intermediate in the Friedel-Crafts reactions. An example of alkylammonium nitrates: ethylammonium nitrate (**2**).

Modern ILs are quite different from those of the beginning of the 20th century, such as the alkylammonium nitrates **2**, shown in Figure 2.5[114]. The most common ILs containing quaternary heterocyclic cations (such as alkylpyridinium or dialkylimidazolium) and inorganic anions have an ancestry traceable to traditional high-temperature molten salts[114]. The inorganic chloroaluminates are considered examples of salts between the truly high-temperature molten salts (such as cryolite or LiCl–KCl) and the current ionic liquids.

The history behind the alkali chloroaluminate molten salts is a good example of fundamental research emerging rather quickly into practical application. In an example case, researchers at the United States Air Force Academy (Colorado Springs, CO, USA) picked up on the work carried out by Frank Hurley and Thomas Wier[115,116] about electrodeposition of aluminum using $AlCl_3$ – based molten salts. This led to the development of electrolytes for thermal batteries based on mixtures of $AlCl_3$ and 1-ethylpyridinium halides, mainly bromide (Figure 2.6).

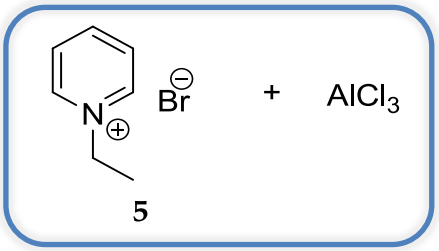


Figure 2.6 Mixture of $AlCl_3$ and 1-ethylpyridinium bromide (**5**).

One of most important breakthroughs in the history of ILs is related to the discovery of the 1-butylpyridinium chloride–aluminum chloride mixture (BPC–$AlCl_3$, Figure 2.7)[117]. This all chloride system represented a substantial improvement over the mixed

bromide–chloride ionic liquids[117], but had some disadvantages, which lead to new research and developments that brought forth the water-stable ionic liquids[114,118,119].

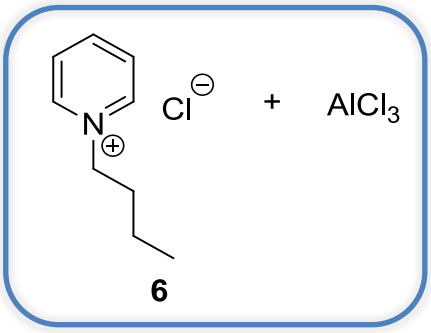


Figure 2.7 1-Butylpyridinium chloride (**6**) and aluminum chloride mixture (BPC–$AlCl_3$).

The research for novel water-soluble ILs was described by Fuller *et al.*[120,121] using a series of ILs from the traditional dialkylimidazolium cations combined with different anions (tetrafluoroborate, hexafluorophosphate, nitrate, acetate, and sulfate) along with the additional series of mostly larger anions (Figure 2.8). Over the years, new classes of cations and anions have been reported[94]. Because ILs are intrinsically safer than highly volatile and flammable organic solvents, their use as solvents improves the safety margins and the environmental performance in solution chemistry[95]. Nowadays, the interest from disciplines outside chemistry and engineering is growing. Recent IL applications include the use in sensors, solar cells, solid-state photocells and batteries, as well as thermal fluids, lubricants, hydraulic fluids, and ionogels. ILs are indeed tunable, multipurpose materials for a variety of applications[12].

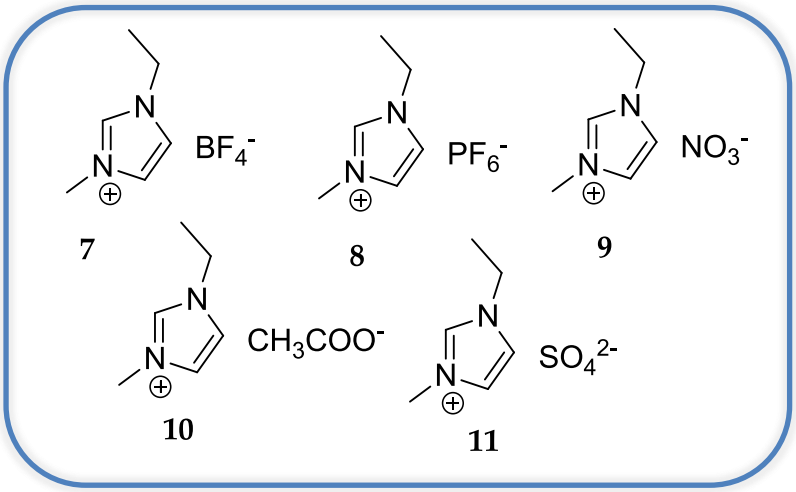


Figure 2.8 Tetrafluoroborate (**7**), hexafluorophosphate (**8**), nitrate (**9**), acetate salts (**10**), and sulfate (**11**) as anions combined with 1-ethyl-2-methylimidazolium cation ILs.

Finally, the particular interest in ILs from biological and pharmaceutical sciences is not only for use as reaction media, but as pharmaceutical solvents or co-solvents for the delivery of drugs with poor water solubility[6]. They are also applied in micro-emulsion systems, which can facilitate the dissolution of drugs that are insoluble or poorly soluble in water. Some IL micro-emulsions can be used as modern colloidal carriers for topical and transdermal delivery, while other IL systems have been used as entrapped/solubilized drug reservoirs for controlled release[122].

2.6.3 ILs as Active Pharmaceutical Ingredients (APIs)

2.6.3.1 Ionic Pharmaceuticals and the Polymorphism Problem

The pharmaceutical industry is unquestionably facing a series of challenges. While many of these challenges are related to the features of this industry and present business models, there is also an urgent need for new scientific advances that yield innovative and effective drugs and therapies. The classical strategies currently being followed are reaching the point at which it is difficult to come up with effective and acceptable new chemical entities. Very few drugs (<10%) that are evaluated in clinical tests make it to the market, decreasing the accessibility of efficient therapies for the people who need them[123].

Roughly 50% of available drugs are administrated as salts. The physicochemical and biopharmaceutical properties of a given drug can be finely tuned by pairing with various counterions. From a pharmaceutical point of view, melting temperature and solubility are relevant parameters because of their routine measurement and due to their potential influence on drug processing and bioavailability[124]. This is an easy way to adjust the properties of a drug with ionizable functional groups to overcome undesirable features present in the parent drug[74,125]. The quality, safety, and performance of a drug are related to the salt structure. The selected ion pair can significantly influence the pharmacokinetics of a drug candidate. This is one of the reasons why regulatory authorities have begun to classify novel salts of a registered drug as a new chemical entity.

The development of salts of the targeted active compounds is a suitable and well-known approach to overcome the limitations faced by the pharmaceutical industry. In spite of this, cocrystals, amorphous forms, and polymer-embedded pharmaceuticals have been tested in order to solve such classical problems as spontaneous polymorphic transformation of crystalline drug forms; this can pose significant problems for drug designers and can convert an effective dose into a lethal dose by altering the solubility of the active ingredient[123]. Pure compounds, salts, all kinds of pharmaceuticals and drug candidates can suffer polymorphism (Figure 2.9), and there are no means to predict the emergence of polymorphism in any given compound despite recent efforts toward a better understanding of crystal polymorphism in pharmaceutical compounds[126-128].

The cost of a pharmaceutical product depends directly on crystal polymorphism and solvation state. This situation has been illustrated by costly product failures and protracted patent litigation. For example, the case of the Norvir capsule product failure in 1998 was recounted and rationalized by solving the crystal structures of the Ritonavir polymorphs[129]. In theory, all solid drugs are susceptible to unpredictable polymorph formation. An unexpected metastable form of 5-fluorouracil (a well-known drug) was described as a new polymorphic structure[71,126]. The use of initial generic versions of some relevant drugs has been permitted based on patently different yet pharmaceutically equivalent polymo[130]rphs (or hydrates), which in some cases have led to legal disputes[126]. Academic researchers are currently working with both experimental (engineering) and theoretical (prediction) insight into crystal forms[128].

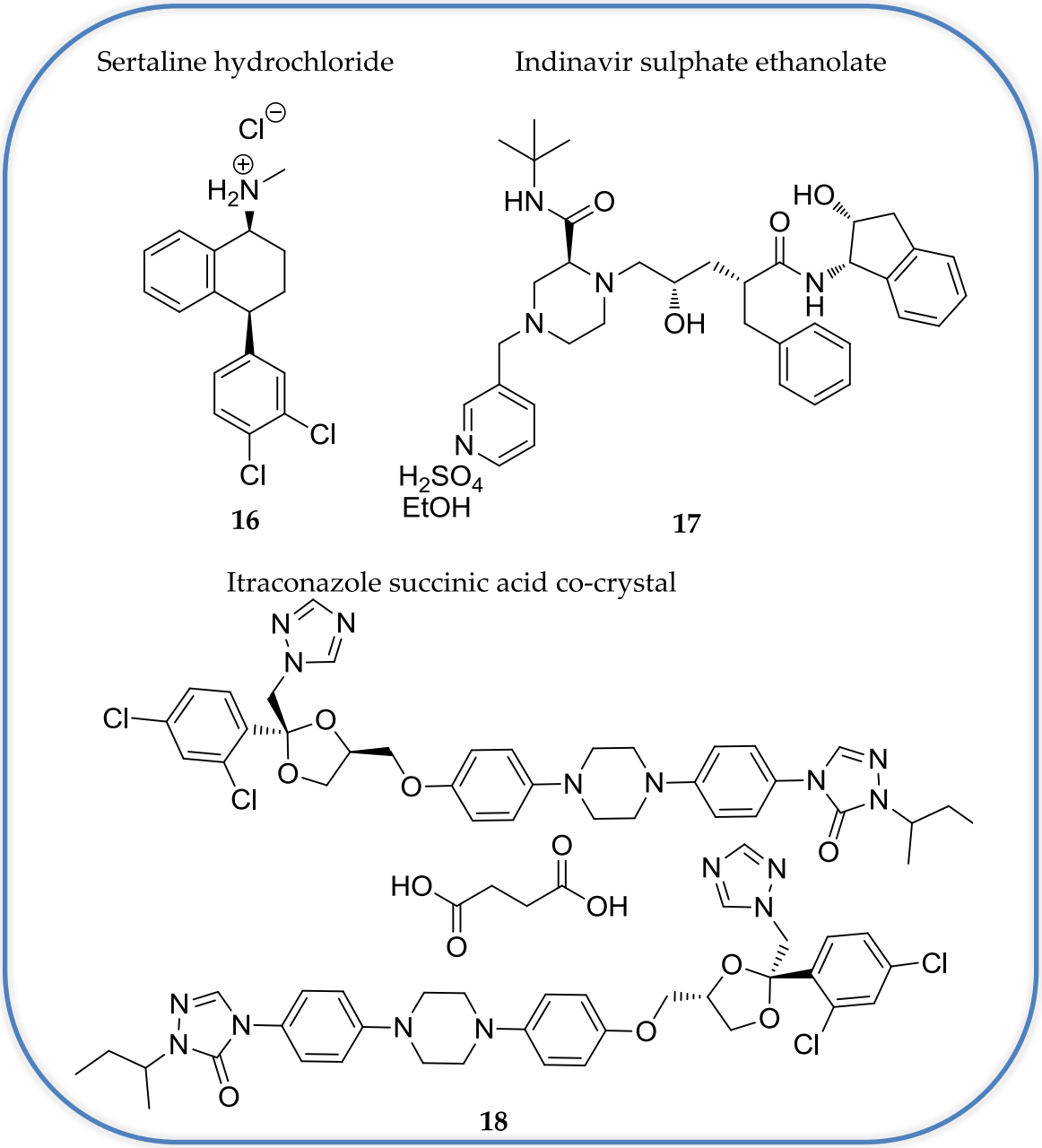


Figure 2.9 Examples of the types of crystal forms of pharmaceutical compounds that can have problems with polymorphism.

The pharmaceutical industry should be open to the potential benefits that crystal engineering can offer, but it also has to be aware of other non-crystalline forms. Drug companies mainly rely on solid, primarily crystalline forms for the delivery of APIs for reasons of purity, thermal stability, manufacturability, and ease of handling. In contrast, liquid drug formulations are much less common, and are usually based on eutectic mixtures[130,131]. However, problems associated with the solid form of many drugs have been consistently reported; issues include polymorphic conversion, low

solubility, low bioavailability for crystalline solids, and the tendency of amorphous forms to spontaneously crystallize. For these reasons, and also due to considerable financial interests brought about by legal ramifications, screening for new drug forms, including salts, solvates, and cocrystals is a continuous pursuit[126,130]. Therefore, the use of an active drug in liquid form can avoid some of polymorphism problems associated with solids. Other similar approaches have been developed with liquid drug formulations prepared as eutectic mixtures[130,132], but these can dilute the APIs owing to large quantities of inactive ballast in the formulation. In this light, pure liquid-phase APIs would provide new perspectives for drug delivery and treatment approaches.

From the point of view of the pharmaceutical industry, the use of liquid salts is relevant, preferably those with melting points below room temperature. Some synthetic strategies that have been employed to decrease the melting point of the salts include a selection of cations with a low tendency to crystallize, or ions with a more diffuse charge. For example, 3-ethyl-1-methylimidazolium chloride is an organic salt with a melting point of 77–79 ^{o}C, which can be lowered to -21 ^{o}C by simple replacement of the chloride with a dicyanamide anion[130,133].

2.6.3.2 Pharmaceutical Activity

The question of IL toxicity has delayed the entry of ILs into the biosciences. The toxicities observed toward microorganisms and cell cultures cover a wide range of biocidal potencies: from those of rather inactive molecular solvents, such as ethanol or dimethyl sulfoxide, which are biocompatible to very high aqueous concentrations, to highly active biocides. The latter have even led to the proposal for the use of some ionic liquids as wood preservatives and in a variety of other pharmaceutical applications[130,134](Figure 2.10).

The number of publications reporting antimicrobial activity for ILs is growing, and this could be very interesting for the development of new bioactive materials as antiseptics[31,130,135,136], for example (Figure 2.11).

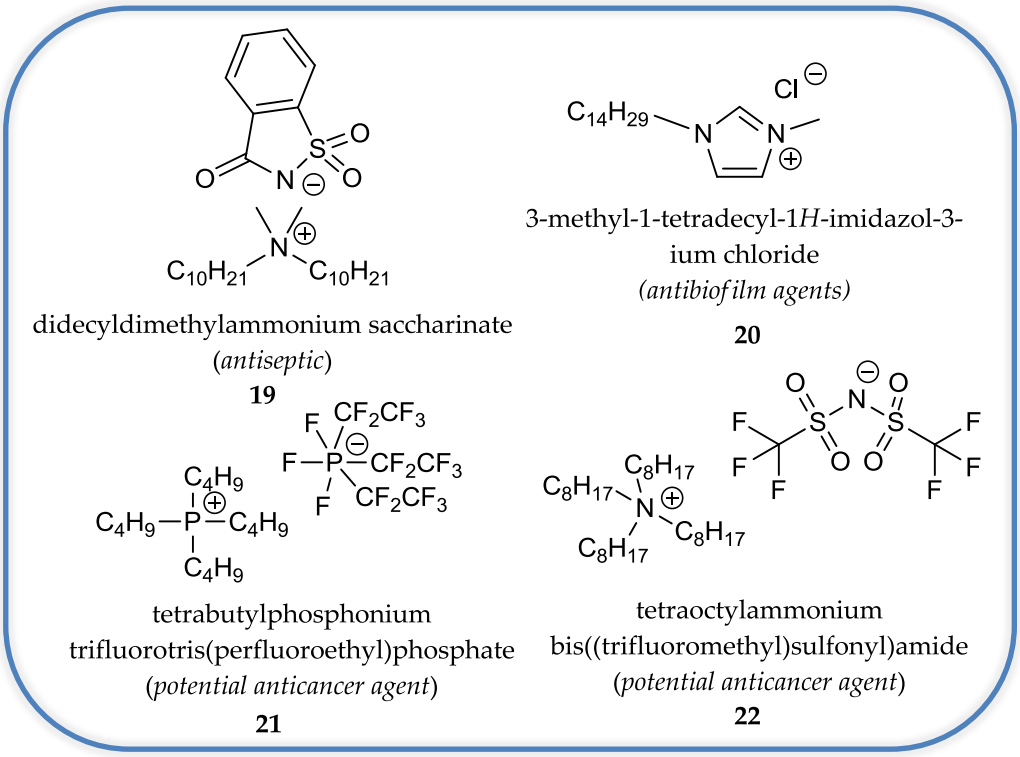


Figure 2.10 Some examples of ILs and their application.

Table 2.2 and Table 2.3 list some examples of antimicrobial activity (minimum inhibitory concentration and minimum bactericidal or fungicidal concentration, respectively) observed for ILs based on ammonium and benzalkonium cations combined with saccharinate and acesulfamate anions. These results demonstrate the potential use of ILs, in particular, against *Streptococcus mutans*.

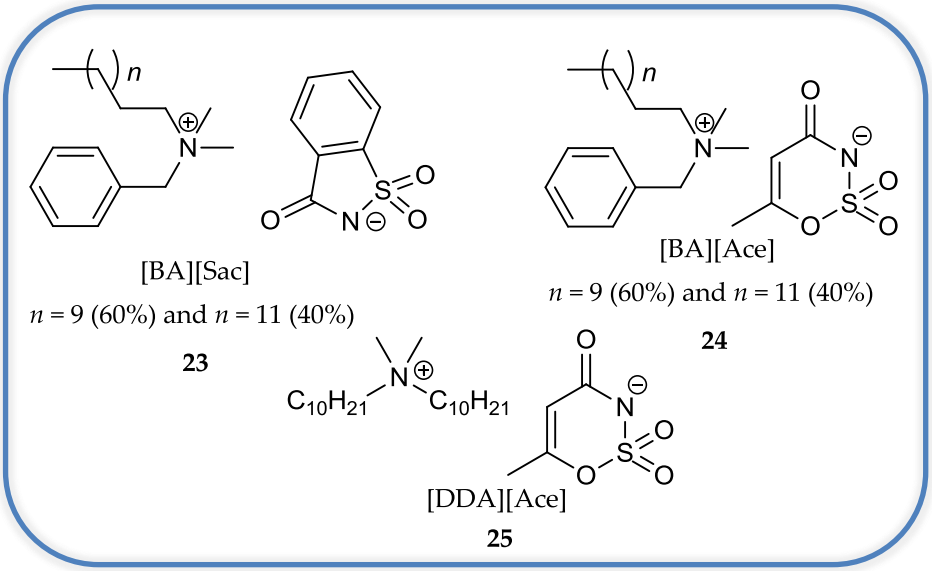


Figure 2.11 Examples of antibacterial agents.

Table 2.2 Minimum inhibitory concentrations (MIC) for various ILs and starting salts

Strain	**MIC** [ppm][a]					
	[BA][Sac][b] (**23**)	[DDA][Sac][c] (**19**)	[Ba][Ace][d] (**24**)	[DDA][Ace][e] (**25**)	[BA][Cl][f]	[DDA][Cl][f]
Staphylococcus aureus	4	4	4	8	2	2
Staphylococcus aureus (MRSA)	4	4	4	4	2	2
Enterococcus faecium	8	8	8	8	4	4
Escherichia coli	16	16	31	16	8	8
Micrococcus luteus	8	4	8	8	4	2
Staphylococcus epidermidis	4	4	4	4	2	2
Klebsiella pneumoniae	4	4	8	4	4	4
Candida albicans	16	16	16	16	8	8
Rhodotorula rubra	16	16	16	16	8	4
Streptococcus mutans	0.1	31	1	16	2	2

[a] Lowest compound concentration that inhibits visible growth of a microorganism after overnight incubation [b] Benzalkonium saccharinate. [c] Didecyldimethylammonium saccharinate. [d] Benzalkonium acesulfamate. [e] Didecyldimethylammonium acesulfamate. [f] Starting salts: benzalkonium chloride ([BA][Cl]) and didecyldimethylammonium chloride ([DDA][Cl]); data from Hough-Troutman *et al.*,[133] listed for comparison.

Table 2.3 The minimum bactericidal or fungicidal (MBC) concentrations for various ILs and starting salts.

Strain	**MBC** [ppm][a]					
	[BA][Sac][b] (**23**)	[DDA][Sac][c] (**19**)	[Ba][Ace][d] (**24**)	[DDA][Ace][e] (**25**)	[BA][Cl][f]	[DDA][Cl][f]
Staphylococcus aureus	31.2	62.5	31.2	16	62.5	31.2
Staphylococcus aureus (MRSA)	31.2	31.2	31.2	31.2	31.2	31.2
Enterococcus faecium	16	16	31.2	31.2	31.2	31.2
Escherichia coli	62.5	16	125	62.5	62.5	31.2
Micrococcus luteus	62.5	31.2	62.5	62.5	31.2	31.2
Staphylococcus epidermidis	31.2	16	62.5	31.2	16	31.2
Klebsiella pneumoniae	62.5	16	31.2	31.2	31.2	16
Candida albicans	31.2	16	31.2	31.2	16	16
Rhodotorula rubra	62.5	31.2	62.5	62.5	31.2	31.2
Streptococcus mutans	0.5	62.5	16	125	16	16

[a] Lowest compound concentration required to kill a microorganism. [b] Benzalkonium saccharinate. [c] Didecyldimethylammonium saccharinate. [d] Benzalkonium acesulfamate. [e] Didecyldimethylammonium acesulfamate. [f] Starting salts: benzalkonium chloride ([BA][Cl]) and didecyldimethylammonium chloride ([DDA][Cl]); data from Hough-Troutman *et al.*,[133] listed for comparison.

ILs could even be used as potential anticancer agents[113,130,137,138] (Figure 2.12 and Table 2.4). Recently the anti-biofilm activity of some ILs and their reported potent, broad-spectrum activity against a variety of clinically significant microbial pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA)[71], have been investigated[139] (Figure 2.4 and Table 2.5).

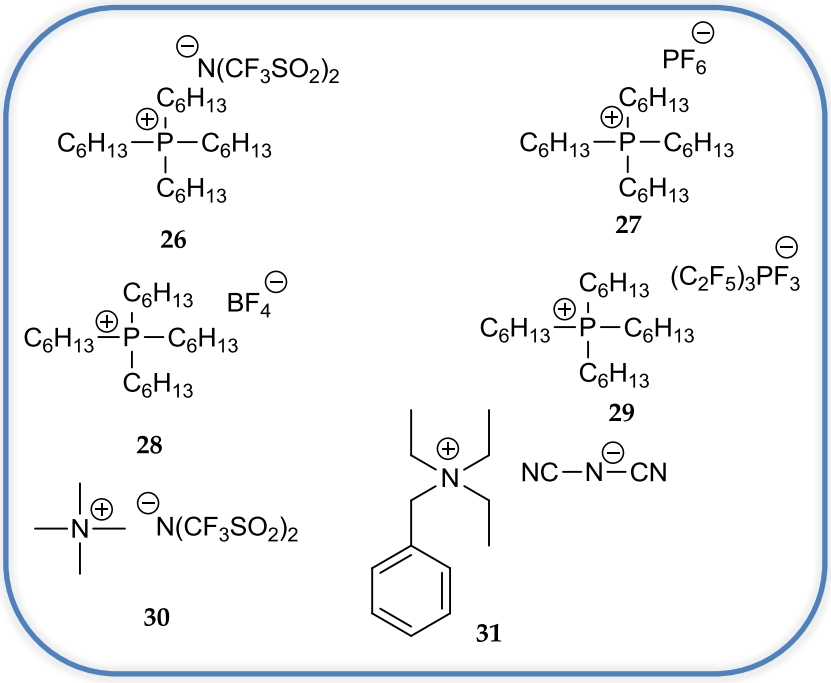


Figure 2.12 Examples of potential anticancer agents[31].

Microbial biofilms are everywhere in nature and represent the dominant mode of microorganism growth. Various types of bacteria, such as MRSA, are observed in colonies adherent to material surfaces. These colonies often form coatings, known as biofilms. A common feature of biofilm communities is their tendency to exhibit significant tolerance and resistance to antibiotics and antimicrobial or biocidal challenge, relative to planktonic bacteria of the same species[135]. One of the attractions of ionic liquids in this regard is the possibility to tailor their physical, chemical, and biological properties by building specific features into the chemical structures of the cation and/or anion components that could facilitate antibiotic entry into the biofilm.

Table 2.4 Antitumor activity (GI50 [mm][a] and LC50 [mm][b] data) of compounds **26–30** (Figure 2.12) from five dose studies with the NCI 60-cell-line[c] screen from Kumar and Malhotra[31].

	26		**27**		**28**		**29**		**30**	
	GI50	LC50	GI50	LC50	GI50	LC50	GI50	LC50	GI50	LC50
Leukaemia										
CCRF-CEM	0.026	8.458	0.038	2.140	0.034	nd	4.410	>100	0.190	2.440
HL-60(TB)	0.046	0.711	0.025	0.642	0.039	nd	2.420	>100	0.173	1.230
K-562	0.041	>100	0.087	>100	0.038	>100	1.680	>100	0.186	4.859
MOLT-4	0.069	>100	0.046	1.750	0.090	>100	10.70	>100	0.324	4.890
RPMI-8226	0.016	0.098	0.042	49.10	0.016	nd	1.640	>100	0.046	0.951
SR	0.072	8.085	0.047	3.580	0.107	>100	34.60	>100	0.162	5.134
Non-small cell lung cancer										
A549/ATCC	0.292	>100	0.377	9.840	0.391	>100	3.340	>100	1.900	8.580
EKVX	0.123	>100	0.069	4.600	0.206	nd	2.470	>100	0.215	4.310
HOP-62	0.435	>100	0.245	5.010	0.399	>100	5.170	>100	0.787	7.770
HOP-92	0.030	>100	0.025	3.350	0.038	nd	4.790	>100	0.206	4.470
NCI-H226	0.105	>100	0.088	4.230	0.189	nd	3.910	>100	0.478	4.410
NCI-H23	0.192	>100	0.143	9.260	0.236	>100	1.950	>100	0.308	6.330
NCI-H322M	0.321	>100	0.359	4.230	0.401	nd	6.120	>100	1.810	6.120
NCI-H460	0.315	>100	0.285	3.560	0.346	>100	2.840	>100	0.362	4.100
NCI-H522	0.105	>100	0.069	4.260	0.158	>100	3.420	>100	0.287	5.400
Colon cancer										
COLO 205	0.182	>100	1.730	>100	0.243	>100	1.700	9.160	0.292	>100
HCC-2998	0.240	>100	1.230	7.440	0.297	>100	2.410	>100	0.425	6.960
HCT-116	0.061	>100	0.050	6.960	0.084	>100	2.630	>100	0.319	9.900
HCT-15	0.291	>100	0.246	3.710	0.339	nd	2.880	>100	0.993	5.230
HT29	0.053	>100	0.057	6.490	0.061	>100	3.800	>100	0.305	5.170
KM12	0.049	>100	0.045	0.080	0.059	>100	2.890	>100	0.310	5.310
SW-620	0.049	>100	0.047	6.580	0.072	>100	2.870	>100	0.378	10.00
CNS cancer										
SF-268	0.072	>100	0.066	6.270	0.088	>100	7.440	>100	0.493	5.500
SF-295	0.205	>100	0.147	6.690	0.251	>100	4.070	>100	0.333	4.570
SF-539	0.174	>100	0.100	3.450	0.234	nd	5.290	>100	0.514	4.190
SNB-19	0.069	>100	0.068	7.830	0.097	>100	3.180	>100	0.587	8.250
SNB-75	0.076	>100	0.255	5.780	0.223	>100	4.510	>100	0.336	3.770
U251	0.041	5.620	0.037	3.610	0.037	nd	2.630	>100	0.334	4.320
Melanoma										
LOX IMVI	0.058	0.592	0.037	0.418	0.050		4.240	>100	0.192	1.080
MALME-3M	0.029	5.380	0.041	4.110	0.034	>100	2.000	>100	0.290	4.110
M14	0.052	>100	0.044	5.490	0.087	>100	3.050	>100	0.453	9.190
SK-MEL-2	0.027	>100	nd	nd	0.081	>100	8.250	>100	0.356	>100
SK-MEL-28	0.207	6.590	0.178	6.060	0.376	>100	1.800	>100	0.710	7.180
SK-MEL-5	0.079	0.829	0.052	0.663	0.074	nd	2.470	>100	0.177	0.677
UACC-257	0.103	>100	0.112	4.810	0.272	nd	2.380	>100	0.411	6.160
UACC-62	0.045	>100	0.043	4.180	0.061	nd	3.440	>100	0.335	4.100
Ovarian cancer										
IGROV1	0.071	>100	nd	nd	0.316	>100	7.460	>100	0.388	>100
OVCAR-3	0.051	6.760	0.036	3.100	0.118	>100	2.620	>100	0.315	4.180
OVCAR-4	0.073	>100	0.096	8.690	0.125	>100	4.340	>100	0.309	5.800
OVCAR-5	0.318	>100	0.279	7.590	0.337	nd	3.300	>100	1.240	8.170
OVCAR-8	0.086	>100	0.079	6.460	0.229	>100	5.120	>100	0.468	5.880
SK-OV-3	2.610	>100	0.254	5.000	0.331	>100	2.70	>100	0.565	5.120

Table 2.4 Antitumor activity (GI50 [mm][a] and LC50 [mm][b] data) of compounds **26–30** (Figure 2.12) from five dose studies with the NCI 60-cell-line[c] screen from Kumar and Malhotra[31] (continued).

	26		**27**		**28**		**29**		**30**	
	GI50	LC50	GI50	LC50	GI50	LC50	GI50	LC50	GI50	LC50
Renal cancer										
786-0	0.253	>100	0.086	5.890	0.211	>100	5.290	>100	0.415	5.020
A498	0.310	>100	0.451	6.080	0.472	nd	2.360	>100	1.820	6.360
ACHN	0.260	>100	0.212	3.390	0.244	nd	2.920	>100	0.630	4.520
CAK-1	0.236	>100	0.207	4.480	0.350	>100	3.130	>100	0.084	3.280
RXF 393	0.141	>100	0.179	>100	0.352	>100	7.660	>100	0.464	5.750
SN12C	0.042	4.440	0.043	3.560	0.062	nd	3.310	>100	0.367	4.060
TK-10	0.341	>100	0.145	4.490	0.234	nd	2.570	>100	0.503	4.860
UO-31	0.350	>100	0.298	5.410	0.398	>100	4.330	>100	0.686	5.950
Prostate cancer										
PC-3	0.045	5.900	0.035	0.779	0.044	>100	3.780	>100	0.282	3.990
DU-145	0.156	>100	0.117	5.290	0.357	>100	6.020	>100	0.407	4.440
Breast cancer										
MCF7	0.144	>100	0.082	3.910	0.253	>100	3.050	>100	0.317	4.020
NCI/ADR-RES	0.730	>100	0.765	8.400	0.947	nd	3.080	>100	1.680	8.130
MDA-MB-231ATCC	0.056	>100	0.047	3.220	0.107	nd	6.980	>100	0.345	3.630
HS 578T	0.053	>100	0.077	9.390	0.084	>100	1.760	>100	0.417	>100
MDA-MB-435	0.045	19.80	0.038	8.460	0.050	>100	3.560	>100	0.335	5.700
BT-549	0.066	>100	0.061	4.370	0.094	>100	3.100	>100	0.421	4.420
T-47D	0.073	>100	0.068	20.40	0.089	>100	1.150	>100	0.428	>100
MDA-MB-468	0.036	2.560	0.044	4.240	0.053	>100	2.220	>100	0.119	2.110

[a] Drug concentration that results in a 50% decrease in net protein increase relative to control cells and toxicity. [b] Drug concentration lethal to 50% of cells; nd: not determined. [c] A 60-cell-line panel used as an *in vitro* substitute for the use of transplantable animal tumours in anticancer drug screening[140].

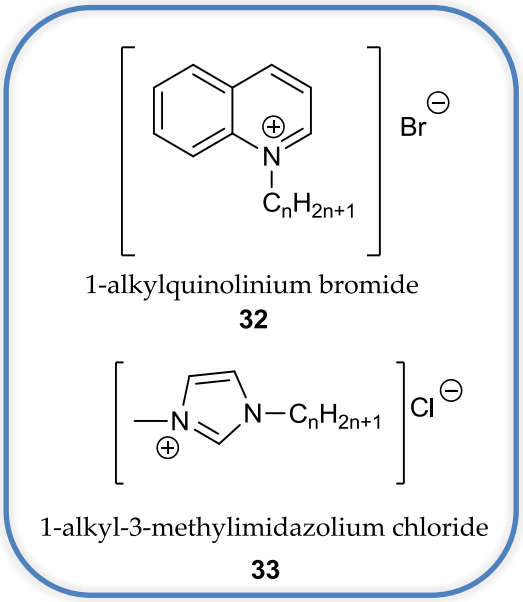


Figure 2.13 Examples of anti-biofilm agents[135].

The pharmaceutical industry is currently paying more attention to ILs because they are customizable materials that can be specially tailored with selected characteristics by varying the combination of their cations and anions. This combination results in various ILs that can offer a wide range of hydrophobicity/hydrophilicity, acidity/basicity, viscosities, among other attributes[130,137].

Table 2.5 MIC and minimum biofilm eradication concentration (MBEC)[a] in mM of 1-alkyl-3-methylimidazolium chlorides ([$C_n$mim]Cl) (**33**) from Carson *et al*[135].

Organism		*n*			
		8	10	12	14
S. aureus ATCC 29213	MIC	722	40	18	16
	MBEC	2708	2415	272	124
E-MRSA 15	MIC	722	40	18	16
	MBEC	2708	1207	272	248
MRSA	MIC	1444	160	36	16
	MBEC	21666	4829	545	124
S. epidermidis ATCC 35984	MIC	722	40	36	7.75
	MBEC	10833	4829	272	124
E. coli NCTC 8196	MIC	722	321	73	33
	MBEC	21666	9659	1089	124
P. aeruginosa PA01	MIC	5416[b]	2415[b]	580	264
	MBEC	21666	2415	1089	496
K. aerogenes NCTC 7427	MIC	1444	643	73	33
	MBEC	43331	19318	2179	248
B. cenocepacia J2315	MIC	>1444	1287	290	132
	MBEC	43331	19318	2179	496
P. mirabilis NCTC 12442	MIC	1444	1287	580	264
	MBEC	43331	9659	4357	1984
C. tropicalis NCTC 7393	MIC	1444	321	73	66
	MBEC	>43331	19318	8714	248

[a] MBEC is the concentration of an antimicrobial agent required to kill a microbial biofilm[141]. [b] MIC values determined by the chemical bath deposition (CBD) method as per manufacturer's protocol, and defined as the lowest concentration of antibiotic at which a planktonic population could not be established by shedding of bacteria from a biofilm [141]; data are included for clarification and comparison only.

The arrangement of cations and anions with few possibilities for strong attractive intermolecular hydrogen bonding interactions decreases the potential for crystallization and provides facile access to pharmaceutically active ILs[71,138]. This will naturally lead to ILs or salts that otherwise would not be explored if crystallization is the primary goal. One such example is the combination of a didecyldimethyl-

ammonium cation and saccharinate: the former is a cation with antimicrobial activity, and the latter is an anion with a sweet taste (compound **19**, Figure 2.10). Indeed, the frequent designation of "designer solvents" for ILs might be easily adapted for IL "designer drugs," as physical, chemical, and biological properties of a drug can be tuned by choice of counterion rather than by covalent modification.

Some compounds have difficulty in penetrating biological membranes because they are very hydrophilic. The correct arrangement between an active ion with another more lipophilic character could offer a solution for this problem. An elucidative example is the case of lidocaine docusate, which combines the local surface anesthetic lidocaine cation with the hydrophobic anion, docusate (an emollient) to create a novel hydrophobic IL salt. This IL demonstrates decreased or controlled water solubility, and thus should exhibit extended residence time on the skin[8,71,130] (Figure 2.14). The counterions are chosen by their inactive nature in order to give the desired physicochemical properties of a neutral drug. Recently, a small number of so-called "combination salts" have been prepared, including two active units (APIs) (compound **36**, Figure 2.14) in the same singular compound coupled as a cation and an anion[71,142]. In general, this approach tends to be influenced by the need to obtain a crystalline material, or the fixed stoichiometry of active units found in such a crystalline salt. The so called "dual functionality" has been explored as an important aspect of the IL field (for example, in dual acidic or double chiral ILs)[130,143]. In the context of APIs, these kinds of studies open new avenues for further exploration in the pharmaceutical field.

Some examples of ILs composed of two biologically active ions were recently described in which both the cation and anion were selected based on the desired physical, chemical, and biological properties[133]. Such ILs are frequently found as antimicrobials and disinfectants[144], for which the introduction of sweetness as a second functionality in the same formulation can be a desired factor for oral applications, such as mouthwashes.

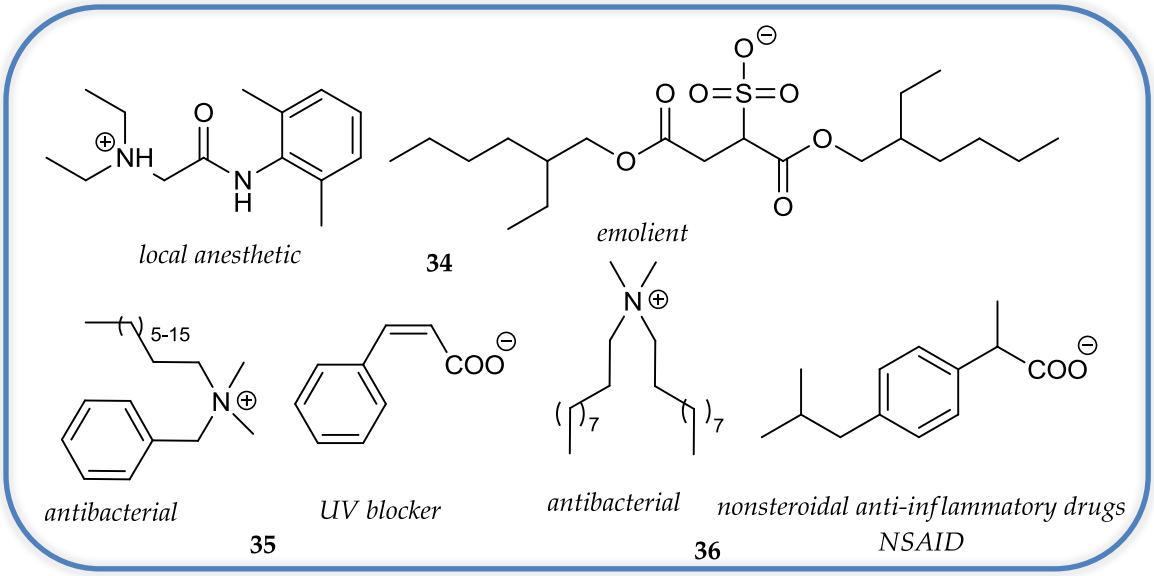


Figure 2.14 Examples of ILs with targeted biological properties combined with adequate selected physical and chemical properties[8].

In the context of APIs, a variety of approaches can be contemplated and for which the two actives can be chosen. The counterions can be selected to synergistically enhance the desired effects or to neutralize unwanted side effects of the active entity. The counterion can also be chosen to pharmacologically act in an independent way[71,130,142].

In 2007, Rogers and co-workers patented a method for the preparation of ILs containing active pharmaceutical, biological, nutritional and energetic ingredients. When a pharmaceutical activity is a desired property of the IL, one or more of the ions in the disclosed IL composition can be a pharmaceutical ingredient[10,145] (Figure 2.15).

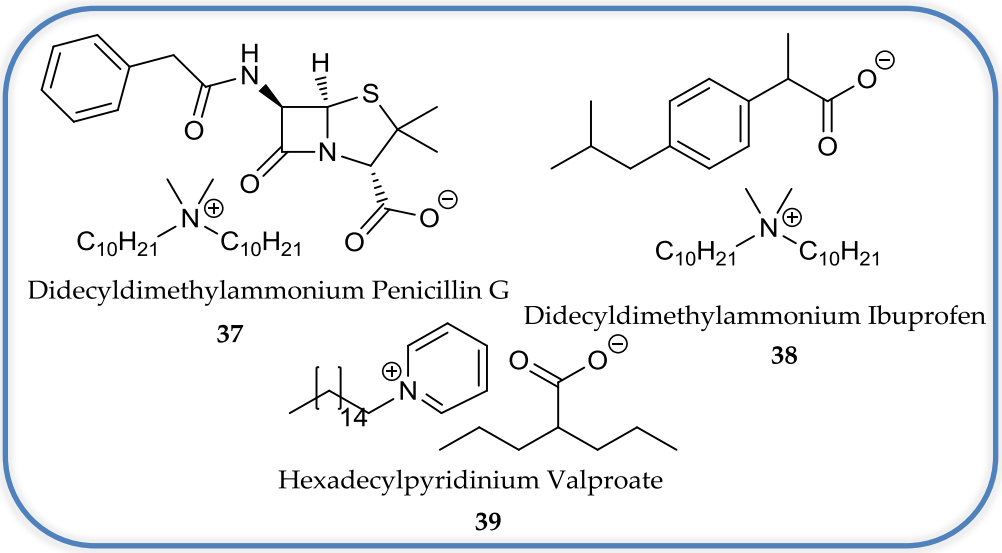


Figure 2.15 Examples of an antibiotic (**37**), nonsteroidal anti-inflammatory agent/analgesic (**38**), and an antiepileptic agent (**39**) as ILs[8,10,145].

Importantly, a co-formation of two separate solid actives in a solid dosage form is significantly different from IL formulation. The ions from an IL will dissolve in bodily fluids in exactly the same way, as one ion cannot dissolve without the other. This is not true of separate solid forms administered at the same time, as each may dissolve at quite different rates[71].

2.6.3.3 Biopharmaceutics Drug Classification System (BCS)

The development of drugs is always associated with standards and directives that also aid in drug classification. Biopharmaceutics is defined by the physical and chemical properties of a biologically active compound as well as the formulation and physiology of the route of administration. Nowadays, numerous molecules are classified through screening processes, and promising candidates are selected for additional *in vitro* and *in vivo* tests. At the end of the process, regulatory agencies make the ultimate authorization[146].

The introduction of the biopharmaceutics drug classification system (BCS)[32] (Table 2.6) into the guidelines of the US Food and Drug Administration (FDA) is a major step forward in classifying the biopharmaceutical properties of drugs and drug products. Based on mechanistic approaches to the drug absorption and dissolution processes, as well as intestinal permeability, the BCS enables regulatory bodies to simplify and improve the drug approval process. The knowledge of the characteristics of a drug in a formulation, according to BCS, can also be used by formulation scientists to develop a more optimized dosage form based on fundamental mechanistic, rather than empirical, information. In this context, the combination of the appropriate anion or cation with a drug could be a simple method to change a pharmaceutical ingredient in the BCS.

Table 2.6 BCS[a] classification of drugs and *in vitro/in vivo correlation* (IV/IVC) expectations for immediate release products based on the biopharmaceutics class, from Löbenberg *et al*[146].

Class	Solubility	Permeability	IV/IVC expectation
I	High	High	IV/IVC if the dissolution rate is slower than the gastric emptying rate, otherwise limited or no correlation
II	Low	High	IV/IVC expected if the in vitro dissolution rate is similar to the in vivo dissolution rate, unless the dose is very high
III	High	Low	Absorption (permeability) is rate determining and limited or no IV/IVC with dissolution rate
IV	Low	Low	Limited or no IV/IVC expected

[a] BCS biopharmaceutics drug classification systems.

2.6.3.4 Some Examples of Ionic APIs

There are numerous published examples in which pharmaceutically active compounds are salts of an active ion in combination with a relatively simple and inert counterion, or that can be easily transformed into cationic or anionic species. Table 2.7 illustrates some examples of drugs (or their APIs) that could be used for preparation of novel and pharmaceutically active ILs. The examples selected herein were listed in the 2009 Top-200 generic drug list by retail dollars[147]. From the IL point of view, it is possible to use some examples from Table 2.7 as the cation unit, such as Omeprazole (rank 2), a drug used to treat gastroesophageal reflux disease, or the anion unit, such as the amoxicillin antibiotic (rank 9 and 28). Some of these possess dual functionalities, so they can be used as cation or anion, such as the antiepileptic Gabapentin (rank 8), or the angiotensin-converting enzyme inhibitor Lisinopril (rank 13), which is used for hypertension.

Table 2.7 Examples of drugs (or their APIs) that could be used in ILs as listed in the 2009 Top-200 generic drugs by retail dollars[147].

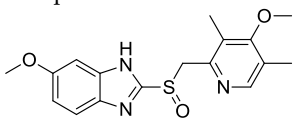
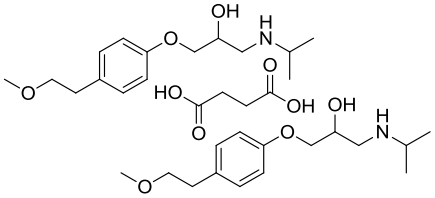
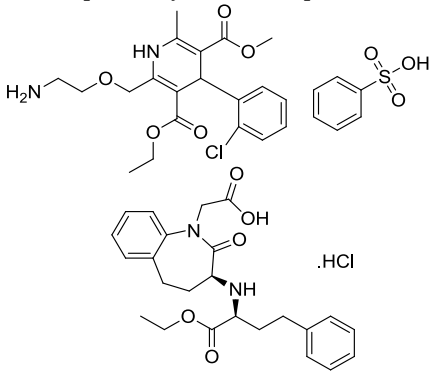
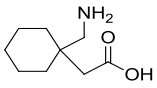
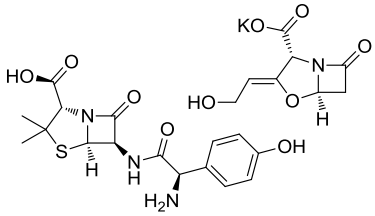
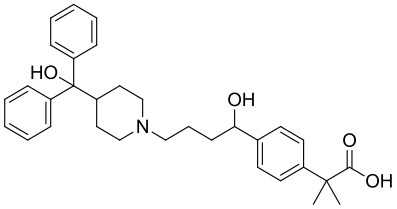
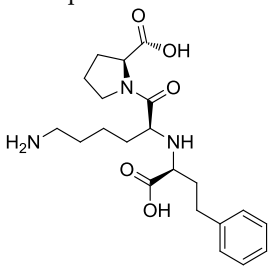
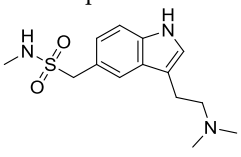
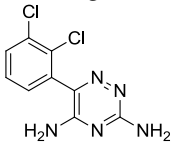
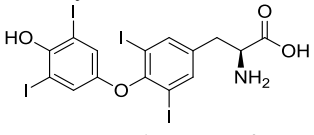
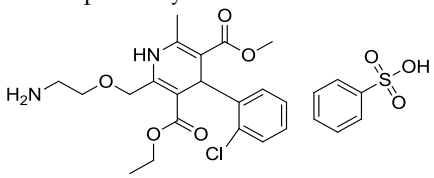
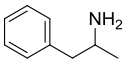
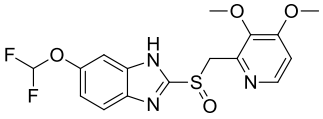
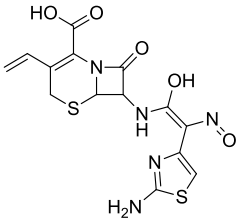
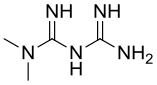
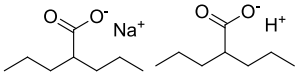
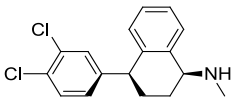
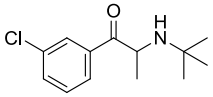
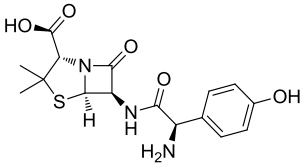
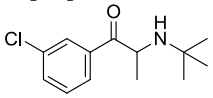
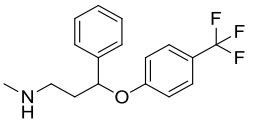
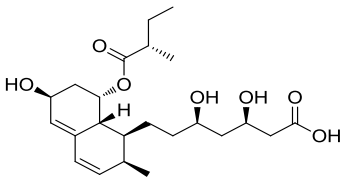
Rank	Drug[a]	Rank	Drug[a]
2	Omeprazole  *Treat symptoms of gastroesophageal reflux disease*	3	Metoprolol succinate  *For hypertension treatment*
7	Amlodipine besylate benazepril  *For hypertension treatment*	8	Gabapentin  *Treatment of epilepsy, and major depressive disorder*
9	Amoxicillin/potassium clavunate  *Antibiotic with β-lactamase inhibitor*	10	Fexofenadine  *Anti-histamine drug*
13	Lisinopril  *For hypertension treatment*	14	Sumatriptan oral  *For migraine headaches*
15	Lamotrigine  *Treatment of epilepsy*	16	Levothyroxine  *Hormone replacement for patients with thyroid problems*

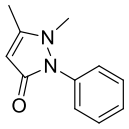
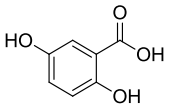
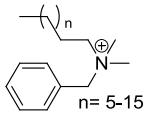
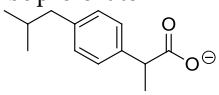
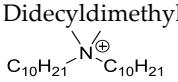
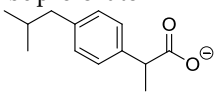
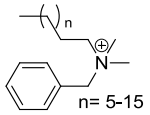
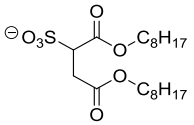
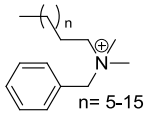
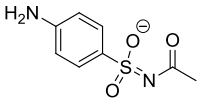
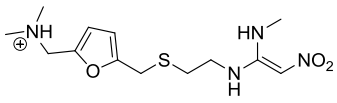
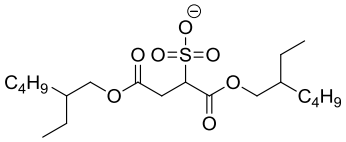
Table 2.7 Examples of drugs (or their APIs) that could be used in ILs as listed in the 2009 Top-200 generic drugs by retail dollars[147] (continued).

Rank	Drug[a]	Rank	Drug[a]
17	Amlodipine besylate  *For hypertension treatment*	18	Amphetmn. salt cmb. SR  *Increased wakefulness and focus*
21	Pantoprazole  *Treat symptoms of gastroesophageal reflux disease*	22	Cefdinir  *Antibiotic*
24	Metformin  *Anti-diabetic*	25	Divalproex sodium  *Treatment of epilepsy*
26	Sertaline  *Antidepressant*	27	Budeprion XL  *Antidepressant and smoking cessation*
28	Amoxicillin  *Antibiotic*	32	Bupropion XL  *Antidepressant and smoking cessation*
34	Fluoxetine  *Antidepressant*	35	Pravastatin  *Lowering cholesterol and preventing cardiovascular disease*

[a] The chemical formula of some compounds from the table is a rather simplified presentation of all functional groups present without analysis of acid-base reaction.

There are also numerous published examples of APIs in which both cation and anion are active pharmaceutical ingredients (Table 2.8)[142]. Following dissociation in solution, the cation and anion will each follow their independent kinetic and metabolic pathways.

Table 2.8 Some examples of ionic liquid salt pairs having both cation and anion as active component from Kumar *et al*.[142]

Cationic drug component	**Anionic drug component**	**Salt pair**
Phenazone  m.p. = 113 °C *analgesic, anti-inflammatory, antipyretic*	Gentisic acid  m.p. = 200-205 °C *analgesic, anti-inflammatory, antipyretic*	Phenazone gentisate m.p.= 87-88 °C *analgesic, anti-inflammatory, antipyretic*[148]
Benzalkonium  *Antibacterial*	Ibuprofenate  *Anti-inflammatory*	Benzalkonium ibuprofenate m.p.= -41 °C[7]
Didecyldimethylammonium  *Antibacterial*	Ibuprofenate  *Anti-inflammatory*	Didecyldimethylammonium ibuprofenate m.p.: liquid at room temperature (RT)[8]
Benzalkonium  *Anti-bacterial*	Colawet MA-80  *Wetting agent*	Benzalkonium colawet MA-80 m.p.: liquid at RT[7]
Benzalkonium  *Anti-bacterial*	Sulfacetamide  *Anti-acne*	Benzalkonium sulfacetamide m.p.: liquid at RT[7]
Ranitidine  *Histamine H2-receptor antagonist*	Docusate  *Emollient*	Ranitidine docusate[8]

2.6.4 Conclusions and Future Perspectives

The development of new synthetic strategies in organic chemistry using "eco-friendly" conditions is an issue of increasing interest. This leads to ILs, and has attracted the attention of the pharmaceutical industry. Naturally, further studies must be carried out in order to discover the full potential of their biomedical applications. The incorporation of the IL approach into pharmaceuticals will continue to open new perspectives in industry and modern society. It is particularly important to emphasize that even slight modifications of an API can significantly change a drug's physical properties as well as its classification in the BCS. This approach can provide a platform to improve the pharmaceutical activity for new treatment options or even personalized medicine. In summary, this Mini Review highlights the very recent progress in the API–IL field, and demonstrates that ILs have the potential to impart an incredible degree of flexibility in the fine tuning of physical, chemical, and biological properties without covalent manipulation of the active units. Certainly, there are associated challenges to bear in mind, including manufacture, scale-up, purification, stability, toxicity, and delivery, among others. However, the rush to obtain new drugs by molecular manipulation and discovery may have obscured the fact that many known drugs could be manipulated into more effective species by simple salt chemistry — albeit a salt chemistry unlike any other yet attempted by the industry. For that reason, the liquid state by itself should not be overlooked, but should be considered as an alternative to common solid-state techniques. In this context, new possibilities, challenges, and thrilling opportunities might be the reward.

2.7 Recent applications of ILs-APIs

From 2011 until now there have been more dissemination of novel work related to ILs-API. In the case of the synthesis of ILs with APIs, there has only been reported the synthesis of ampicillin[19,30,149] and the synthesis of IL forms of aspirin and salicylic acid in combination with different pharmaceutically active cations[149,150]. ILs-APIs are characterized by the presence of the cation or anion with pharmaceutical activities (pharmacokinetic and pharmacological).

Other approaches are being made between ILs and APIs. Rogers's group have been working in this field. Recently, his group has described the use of ILs as prodrugs (acetaminophen prodrugs paired with the docusate anion)[151], the use of ILs to improve the water solubility of APIs like amphotericin B and itraconazole[152]. This demonstrate that ionic liquids can be used as both drug delivery systems and solubilisation agents in order to improve the aqueous solubility of many drugs[152]. Another approach, ILs with herbicide, is being made not only by Rogers[153], but also by Pernak[153,154].

In the case of biological properties and toxicity of ILs, many studies have been made between 2011 and now. There are some studies of Ventura *et al.*[155,156] that describes the relation between the chemical structure and the toxicity. Pham *et al.* describes the environmental risk assessment of ILs[157].

In a recent publication, Frizzo *et al.*[149] summarize the ILs applications where it is concluded that ILs-APIs are a research area developed by few groups and that it is necessary to produce important results in this area with a complete and elaborate work, which include synthesis, physical and chemical property studies as well as pharmacological activity estimation[149].

Chapter 3.
Material and Methods

3 Material and Methods

This chapter presents a detailed description of the methods and the materials used to synthesize the new ILs as well as the methodology followed to characterize the biological, chemical and physical properties of these compounds. These procedures are partially described in the next chapters, which are presented as reproduction of accepted or submitted papers.

3.1 Synthesis

Commercially available reagents were purchased from Aldrich, BDH – laboratory reagents, Frilabo and Solchemar and were used as received. The solvents were from Valente & Ribeiro and distilled before used. Whenever necessary, the solvents were dried by standard procedures, distilled under nitrogen and stored over molecular sieves.

The basic anion-exchange resin Amberlite IRA-400-OH (ion-exchange capacity 1.4 $eq.mL^{-1}$) was purchased from Supelco. ^{1}H and ^{13}C-NMR spectra in $(CD_3)_2SO$ or CD_3OD (from Euriso-Top) were recorded on a Bruker AMX400 spectrometer at room temperature unless specified otherwise. Chemical shifts are reported downfield in parts per million (ppm). Mass spectra for Ampicillin ILs were obtained in Espectro ESI-FIA-TOF-Medida de Masas Exactas from Unidade de Espectrometria de Masas e Proteómica at Universidade de Santiago de Compostela. Mass spectra for Amoxicillin and Penicillin G ILs were obtained in API-ION TRAP(PO03MS) from Mass Spectrometry Laboratory at Instituto de Tecnologia Química e Biológica. The mass spectra of Amphotericin B ILs were made by Matrix Assisted Laser Desorption Ionization – Time Of Flight (MALDI-TOF) in voyager DE PRO ™ Biospectrometry Workstation IR at Faculdade de Ciências e Tecnologia da Universidade Nova de

Lisboa. IR spectra were measured on a Perkin Elmer 683. Optical rotations were recorded on a Perkin Elmer 241MC. The water content of the liquid [was determined by Karl Fischer titration in an 831 KF coulometer Metrohom. The Melting temperature (mT) was determined by melting point apparatus (Stuart Scientific). DSC analysis was carried out using a TA Instruments Q-seriesTM Q200 DSC with a refrigerated cooling system. The sample was continuously purged with 50 $mLmin^{-1}$ nitrogen. About 5 to 10 mg of the synthesized IL was crimped in an aluminium standard sample pan with lid.

3.1.1 Synthesis of Ampicillin ILs

3.1.1.1 Preparation of Tetraethylammonium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [TEA][Amp]

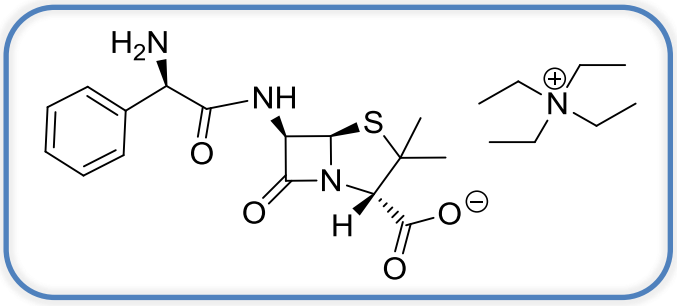


Figure 3.1 Struture of [TEA][Amp].

Tetraethylammonium bromide (0.321 g; 1.53 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh-1). Then, tetraethylammonium hydroxide solution was slowly added to ampicillin (0.549 g; 1.57 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The reaction mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL of (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of the ampicillin. Then ampicillin crystals were filtered from the solution, the solution was evaporated and dried *in vacuum* for 24 h. The desired product was obtained as a yellow solid (0.556 g; 76.0 %). m.p. 79 °C; $[\alpha]_D^{27}$= 48.7 ± 2.5 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.48 (d, 2H, *J* = 7.4Hz), 7.36 (t, 1H, *J* = 7.3Hz), 7.30 (d, 2H, *J* = 6.1Hz), 5.00 (d, 1H, *J* = 6.0Hz), 4.64 (s, 1H), 4.33 (d, 1H, *J* = 6.0Hz), 3.42, (s, 1H) 3.28 (q, 8H, *J* = 7.3 Hz), 1.45 (s, 3H), 1.28 (tt, 12H, *J* = 1.9 Hz, *J* = 7.3 Hz), 1.22 (s, 3H) ppm; ^{13}C-

NMR (100.62 MHz, CD_3OD) δ = 175.58, 174.83, 140.88, 130.06, 129.88, 129.22, 128.58, 77.13, 66.66, 60.19, 60.00, 59.54, 53.27, 53.24, 53.21, 27.79, 27.44, 7.63 ppm; IR (KBr): υ = 3390, 2978, 2929, 1674, 1598, 1488, 1456, 1394, 1304, 1253, 1185, 1174, 1130, 1069, 1029, 1002, 968, 920, 871, 787, 701, 636 cm^{-1}; (EI^+) *m/z* calcd for $C_8H_{20}N^+$: 130.1590, found 130.1590; (EI^-) *m/z* calcd for $C_{16}H_{18}N_3O_4S^-$: 348.1024, found 348.1013.

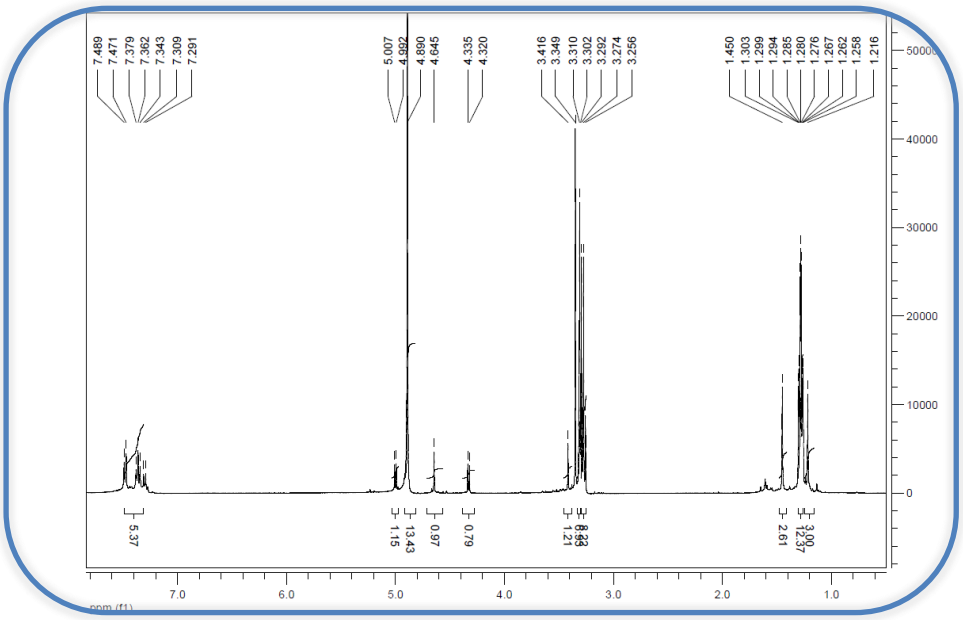


Figure 3.2. [TEA][Amp] ^{1}H-NMR spectrum in CD_3OD.

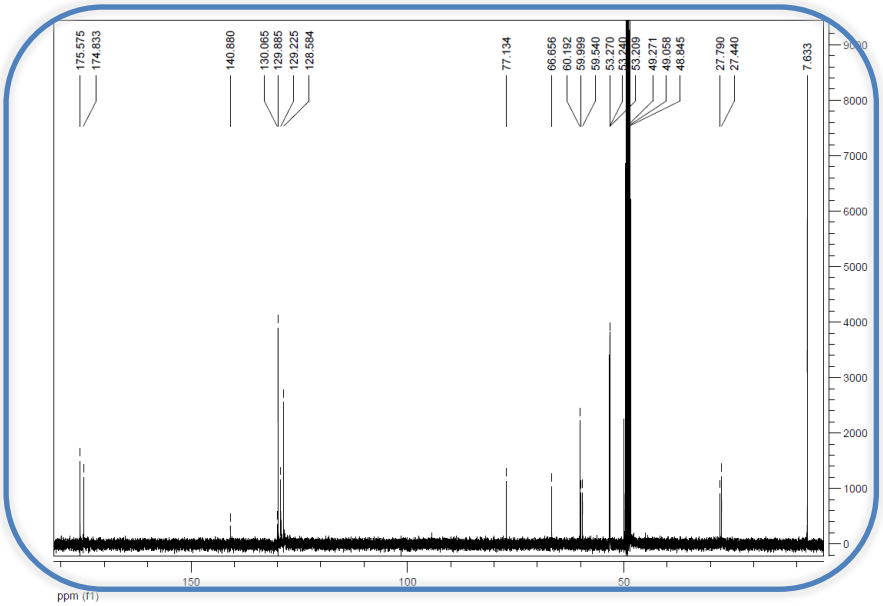


Figure 3.3. [TEA][Amp] ^{13}C-NMR spectrum in $(CD_3)_2SO$.

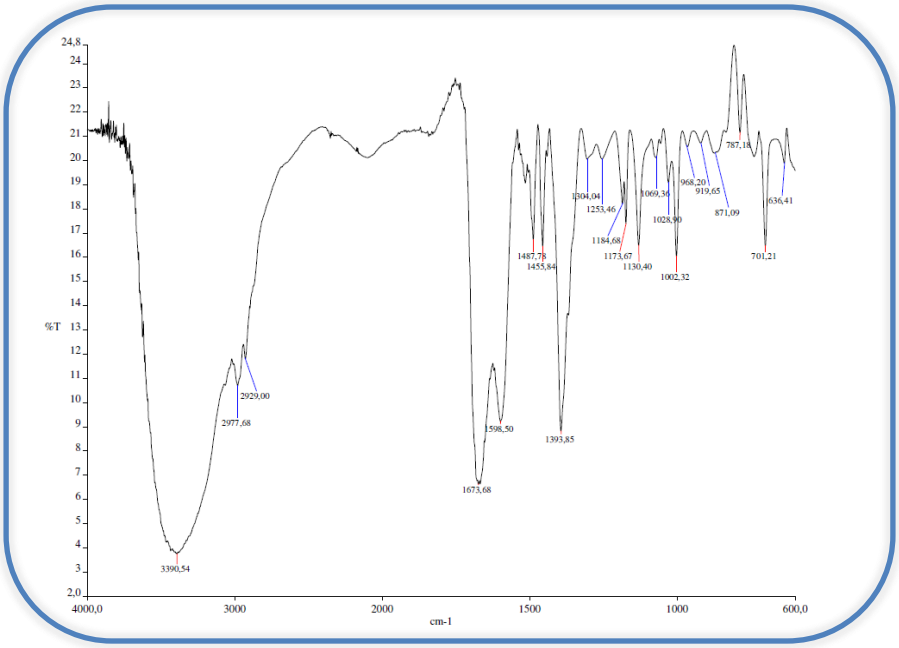


Figure 3.4. [TEA][Amp] IR spectrum in KBr.

3.1.1.2 Preparation of Trihexyltetradecylphosphonium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [$P_{6,6,6,14}$][Amp]

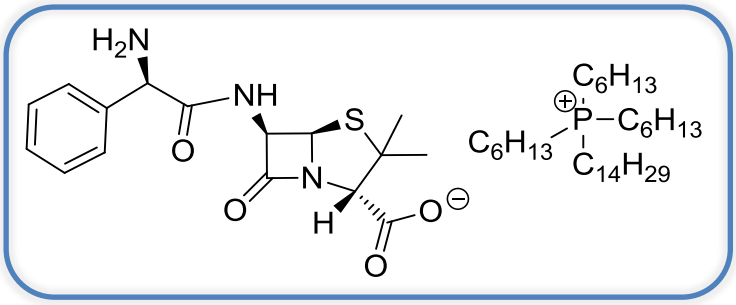


Figure 3.5 Struture of [$P_{6,6,6,14}$][Amp].

Trihexyltetradecyl phosphonium chloride (1.006 g; 1.94 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then, trihexyltetradecylphosphonium hydroxide solution was slowly added to ampicillin (0.761 g; 2.18 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL of (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[30] to induce crystallization of ampicillin excess. Then, ampicillin crystals were filtered from the solution which was evaporated and dried *in vacuum* for 24 h. The desired product was

obtained as a yellow viscous liquid (1.331 g; 80.0 %). $[\alpha]_D^{27}$= 22.3 ± 1.5 (c = 2 mgmL^{-1} in methanol; Water content = 14.7 ppm (determined by Karl Fisher titration); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.48-7.27 (m, 5H), 5.01 (d, 1H, *J* = 6.0 Hz), 4.59 (s, 1H), 4.31 (d, 1H, *J* = 6.0 Hz), 3.44 (s, 1H), 2.19 (m, 8H), 1.65-1.22 (m, 54H) 0.94 (m, 12H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.60, 174.90, 141.97, 129.76, 128.91, 128.35, 77.15, 66.59, 60.34, 60.16, 59.52, 33.12, 32.20, 31.85, 31.70, 31.63, 31.48, 30.85, 30.83, 30.81, 30.71, 30.53, 30.46, 29.92, 27.80, 27.46, 23.79, 23.51, 22.39, 22.35 22.30, 19.50, 19.44, 19.03, 18.97, 14.51, 14.38 ppm; IR (KBr): ν = 3301, 3186, 3060, 2956, 2952, 2855, 1671, 1601, 1485, 1317, 1299, 1268, 1215, 1130, 1111, 1028 cm^{-1}; (EI$^+$) *m*/*z* calcd for $C_{32}H_{68}P^+$: 483.5053, found 483.5056; (EI$^-$) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 348.1024, found 348.1013.

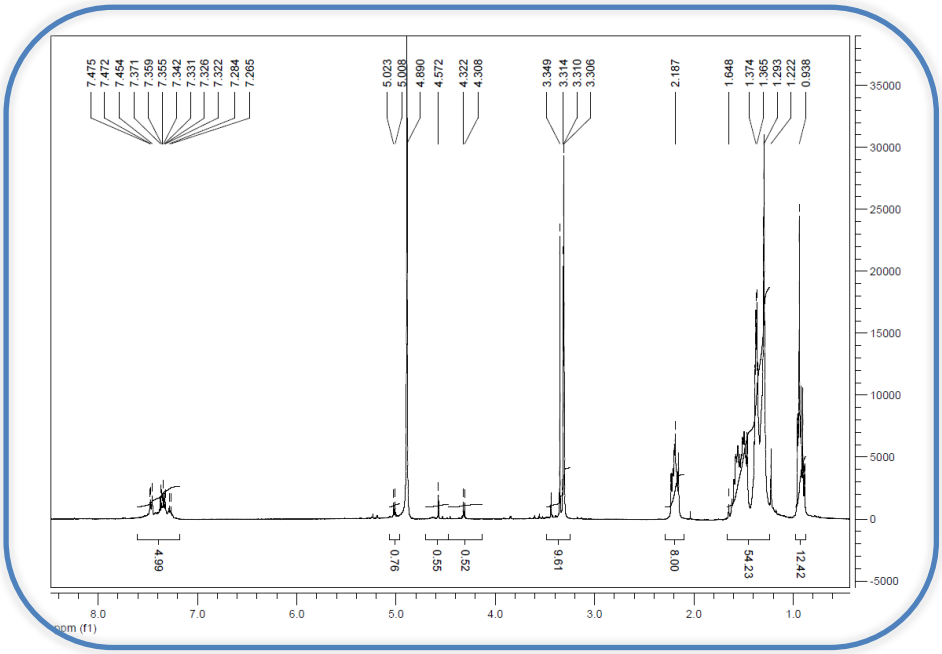


Figure 3.6. [$P_{6,6,6,14}$][Amp] ^{1}H-NMR spectrum in CD_3OD.

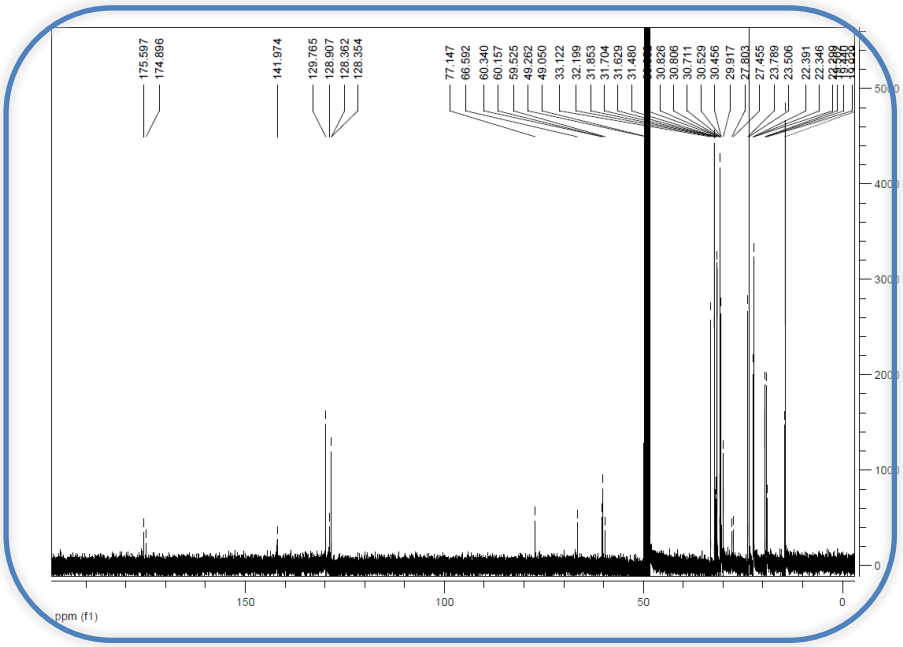


Figure 3.7. [$P_{6,6,6,14}$][Amp] ^{13}C-NMR spectrum in CD_3OD.

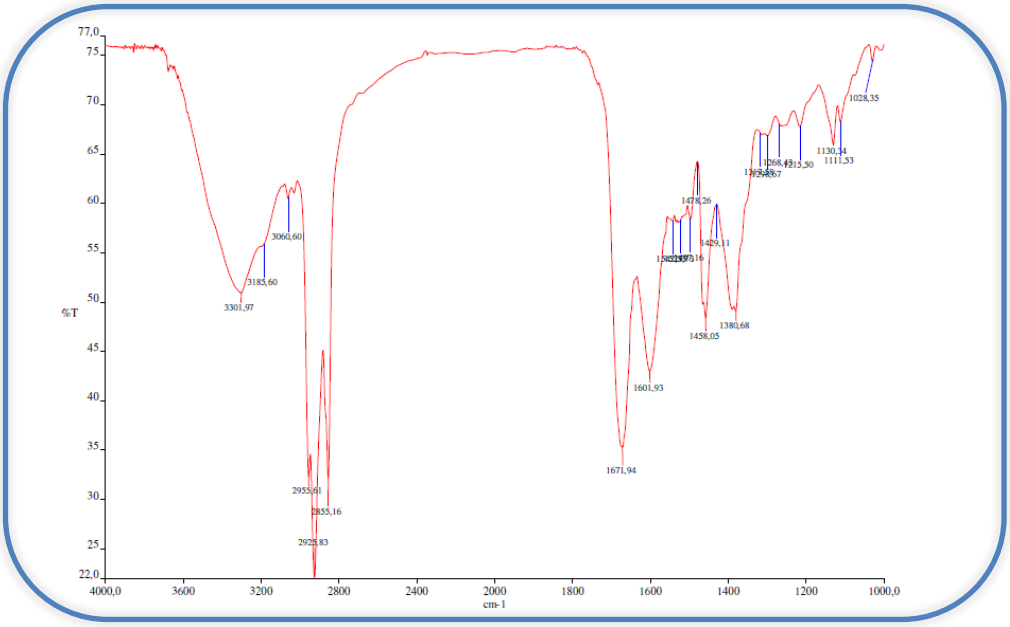


Figure 3.8. [$P_{6,6,6,14}$][Amp] IR spectrum in KBr.

3.1.1.3 Preparation of 1-Hexadecylpyridin-1-ium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C_{16}Pyr][Amp]

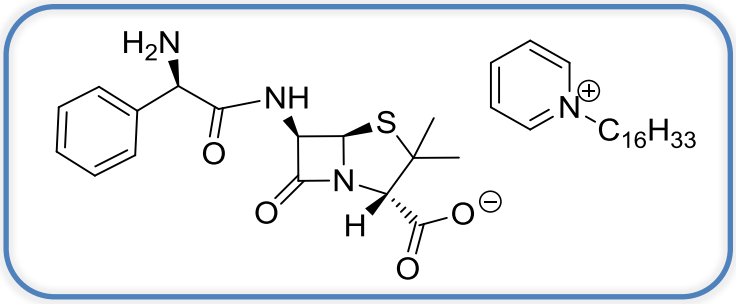


Figure 3.9 Struture of [C_{16}Pyr][Amp].

Cetylpyridinium chloride (0.694 g; 2.04 mmol), was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then, cetylpyridinium hydroxide solution was slowly added to ampicillin (0.714 g; 2.12 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation the residue was dissolved in 20 mL of (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[30] to induce crystallization of ampicillin excess. Then, ampicillin crystals were filtered from the solution which was evaporated and dried *in vacuum* for 24h. The desired product was obtained as a yellow solid (1.018 g; 76.4 %). m.p. 86^{o}C; $[\alpha]_D^{27}$= 51.7 ± 0.9 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 8.98 (2H, d, *J* = 5.5Hz), 8.58 (1H, t, *J* = 7.8Hz), 8.10 (2H, t, *J* = 6.70Hz) 7.47 (2H, d, *J* = 7.3Hz), 7.35 (2H, t, *J* = 7.4Hz), 7.28 (1H, d, *J* = 7.3Hz), 5.0 (1H, d, *J* = 6.0 Hz), 4.62 (3H,m), 4.33 (1H, d, *J* = 6.0), 3.43 (1H, s), 2.01 (2H, m), 1.65-1.11 (32H), 0.90 (3H, t, *J* = 6.7 Hz) ppm; ^{13}C-NMR (100.62MHz, CD_3OD) δ = 175.52, 174.80 , 170.23, 146.87, 145.93, 129.88, 129.78, 129.76, 129.72, 129.56, 129.02, 128.59, 77.15, 66.65, 63.15, 62.40, 61.41, 60,18, 59.55, 33.09, 32.52, 30.80, 30.78, 30.74, 30.65, 30.53, 30.49, 30.15, 27.78, 27.45, 27.73, 23.76, 14.48 ppm; IR (KBr): ν = 3419, 3061, 2923, 2852, 1688, 1593, 1483, 1456, 1385, 1176, 1130, 1029, 964, 778, 686 cm^{-1}; (EI^+) *m*/*z* calcd for $C_{21}H_{38}N^+$: 304.2999, found 304.2999; (EI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 348.1024, found 348.1013.

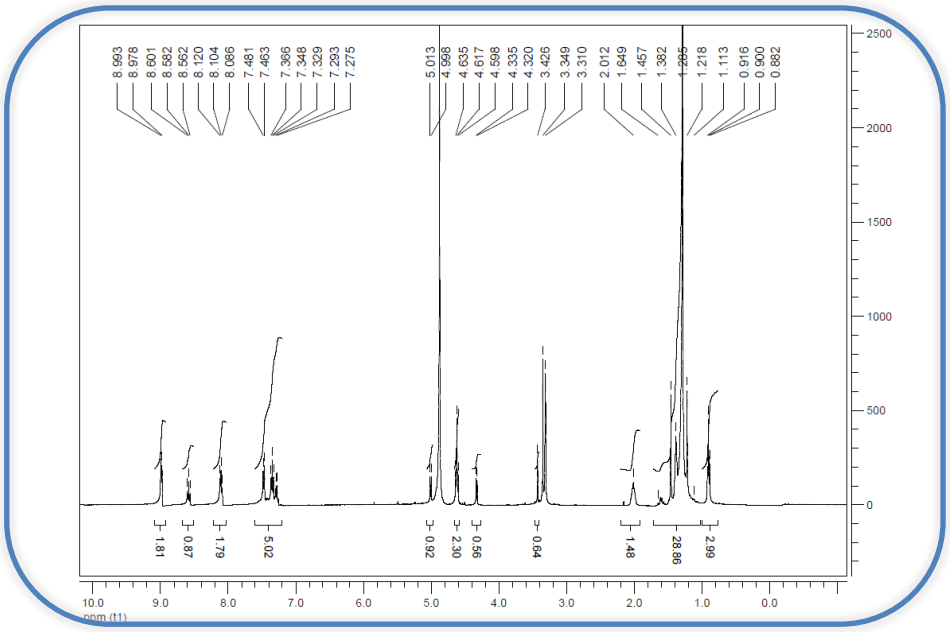


Figure 3.10. [C_{16}Pyr][Amp] ^{1}H-NMR spectrum in CD_3OD.

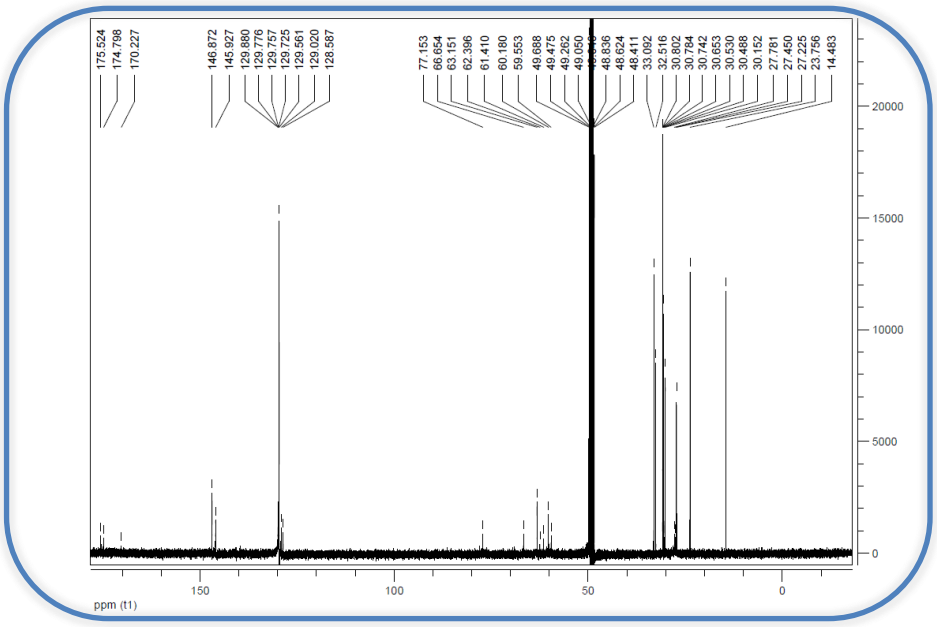


Figure 3.11. [C_{16}Pyr][Amp] ^{13}C-NMR spectrum in CD_3OD.

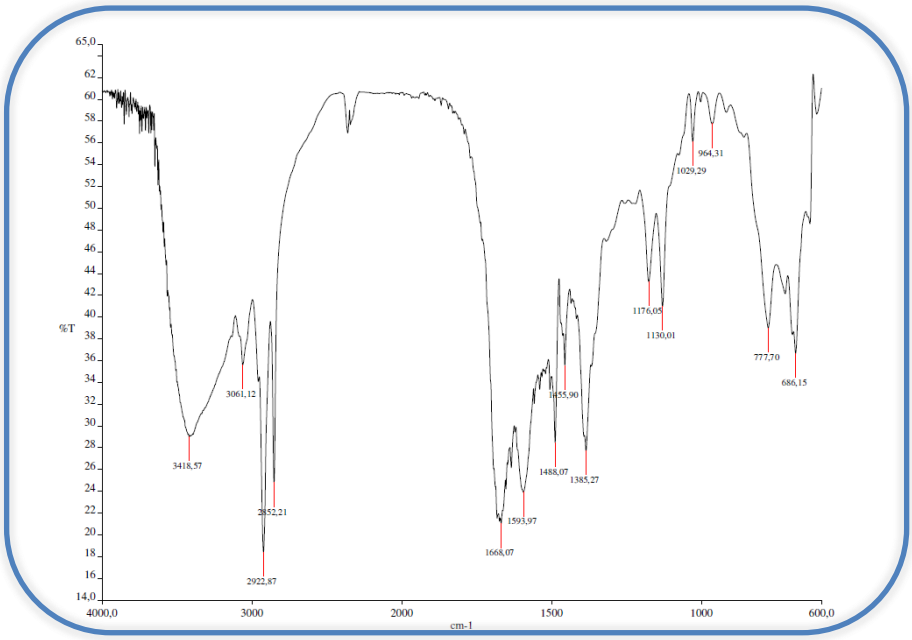


Figure 3.12. [C16Pyr][Amp] IR spectrum in KBr.

3.1.1.4 Preparation of (2-Hydroxyethyl)trimethylammonium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [Cholin][Amp]

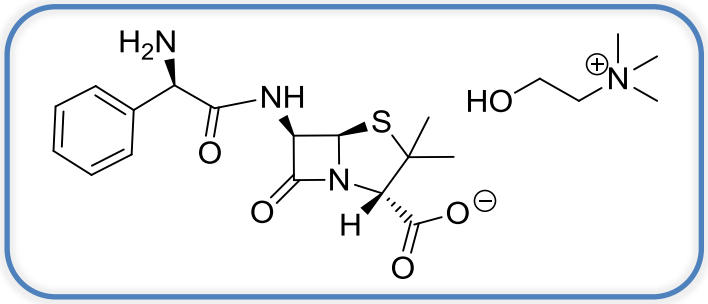


Figure 3.13 Struture of [Cholin][Amp].

(2-Hydroxyethyl)-trimethylammonium chloride (0.546 g; 3.91 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then, choline hydroxide solution was slowly added to ampicillin (1.606 g; 4.60 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL of (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of ampicillin excess. Then, ampicillin crystals were filtered from the solution which was evaporated and dried *in vacuum* for 24 h. The desired product was obtained as a yellow solid (1.252 g; 70.7 %). m.p. 58 °C; $[\alpha]_D^{27}$= 52.3 ± 0.8 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz,

(CD_3OD) δ = 7.49-7.27 (5H, m, Ar), 5.00 (1H, d, *J* = 6.0 Hz), 4.65 (1H, s), 4.34 (1H, d, *J* = 6.0Hz), 3.98 (2H, m), 3.46 (2H, m), 3.42 (1H, s), 3.19 (9H, s), 1.45 (3H, s), 1.22 (3H, s) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.59, 174.91, 174.82, 140.95, 129.88, 129.22, 128.56, 77.11, 69.06, 66.67, 60,20, 60.04, 59.53, 57.10, 54.73, 27.78, 27.47 ppm; IR (KBr): ν = 3042, 2826, 1668, 1595, 1490, 1456, 1385, 1285, 1194, 1132, 1086, 1005, 922, 866, 784, 740, 702 cm^{-1}; (EI^+) *m*/*z* calcd for $C_5H_{14}NO^+$: 104,1070, found 104.1070; (EI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 348,1024, found 348.1013.

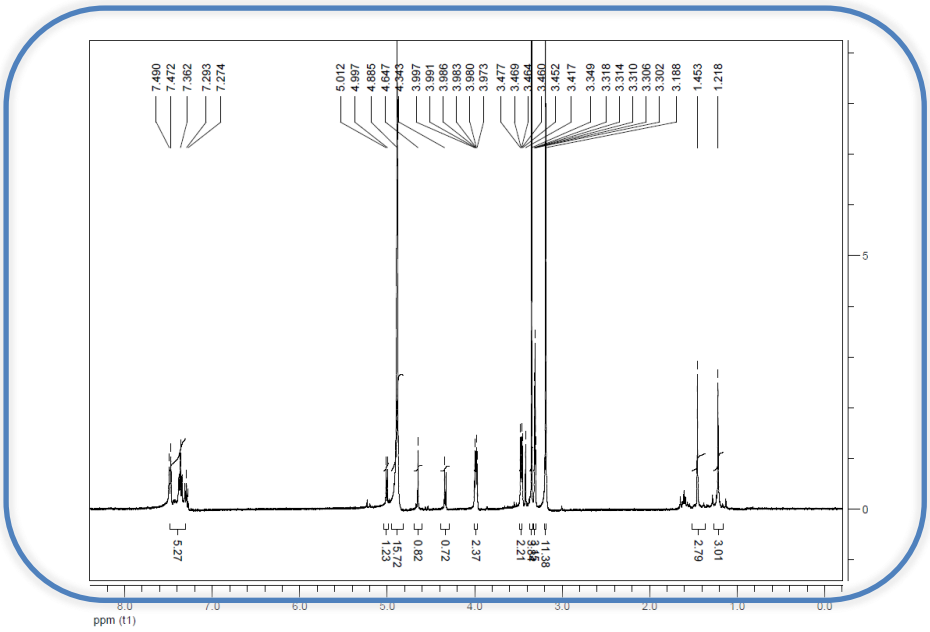


Figure 3.14. [Choline][Amp] ^{1}H-NMR spectrum in CD_3OD.

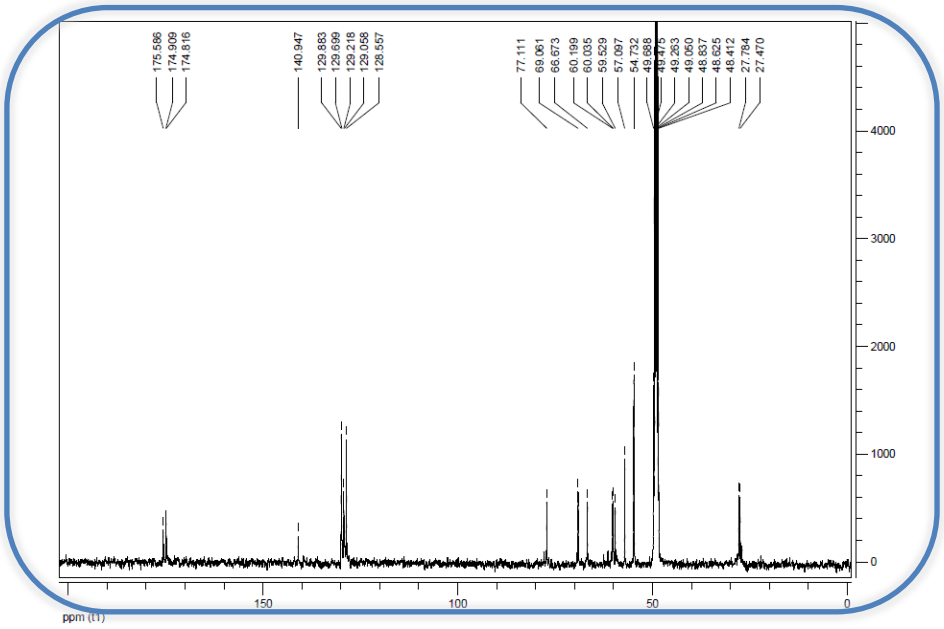


Figure 3.15. [choline][Amp] ^{13}C-NMR spectrum in CD_3OD.

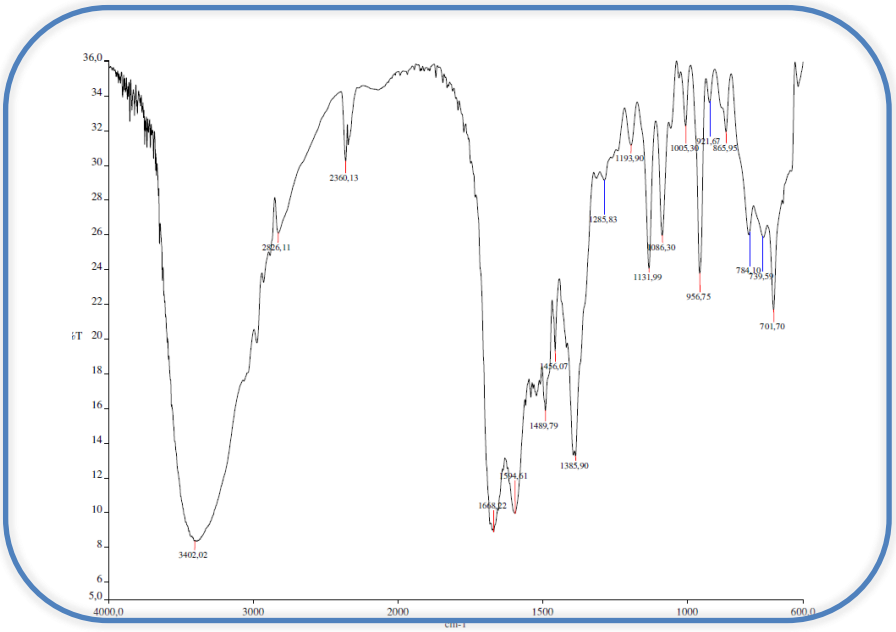


Figure 3.16. [choline][Amp] IR spectrum in KBr.

3.1.1.5 Preparation of 1-Ethyl-3-methyl-1*H*-imidazol-3-ium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [EMIM][Amp]

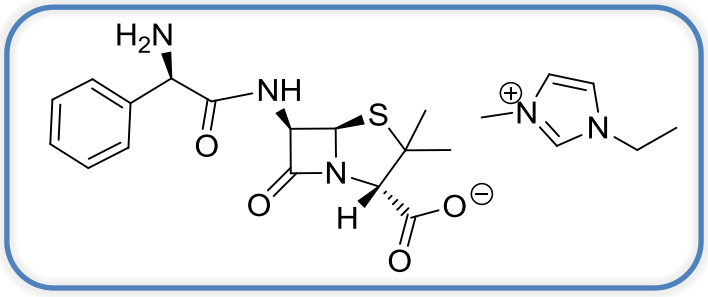


Figure 3.17 Struture of [EMIM][Amp].

1-Ethyl-3-methyl-1*H*-imidazol-3-ium chloride (0.576 g; 3.93 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the 1-ethyl-3-methylimidazolium hydroxide solution was slowly added to Ampicillin (1.606 g; 4.60 mmol) dissolved in 1M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of ampicillin. Then, ampicillin crystals were filtered from the solution which was evaporated and dried *in vacuum* for 24 h. The desired product was obtained as a yellow solid (1.709 g; 94.6 %). m.p. 70-72 °C); $[\alpha]_D^{27}$ = 89.3 ± 5.5 (c = 2

mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.63 (1H, d, *J* = 1.9Hz), 7.55 (1H, d, *J* = 1.9Hz), 7.48-7.46 (2H, m), 7.36-7.32 (2H, m), 7.27-7.25 (1H, m), 5.01 (1H, d, *J* = 6.0 Hz), 4.59 (1H, s), 4.32 (1H, d, *J* = 6.0 Hz), 4.24 (2H, q, *J* = 7.4Hz), 3.90 (3H, s), 3.43 (1H, s), 1.52 (3H, t, *J* = 7.4Hz), 1.46 (3H, s), 1.22 (s, 3H) ppm; ^{13}C-NMR (100.62, MHz, CD_3OD) δ = 175.57, 175.15, 174.84, 141.24, 129.83, 129.12, 128.50, 124.96, 123.31, 77.12, 66.67, 60,18, 60.11, 59.54, 46.03, 36.46, 27.78, 27.47, 15.63 ppm; IR (KBr): ν = 3381, 2974, 2925, 2828, 1668, 1591, 1516, 1456, 1393, 1255, 1169, 1130, 1029, 962, 877, 824, 788, 746, 702, 648 cm^{-1}; (EI^{+}) *m*/*z* calcd for $C_6H_{11}N_2^{+}$: 111,0917, found 111.0917; (EI^{-}) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^{-}$: 348,1024, found 348.1013.

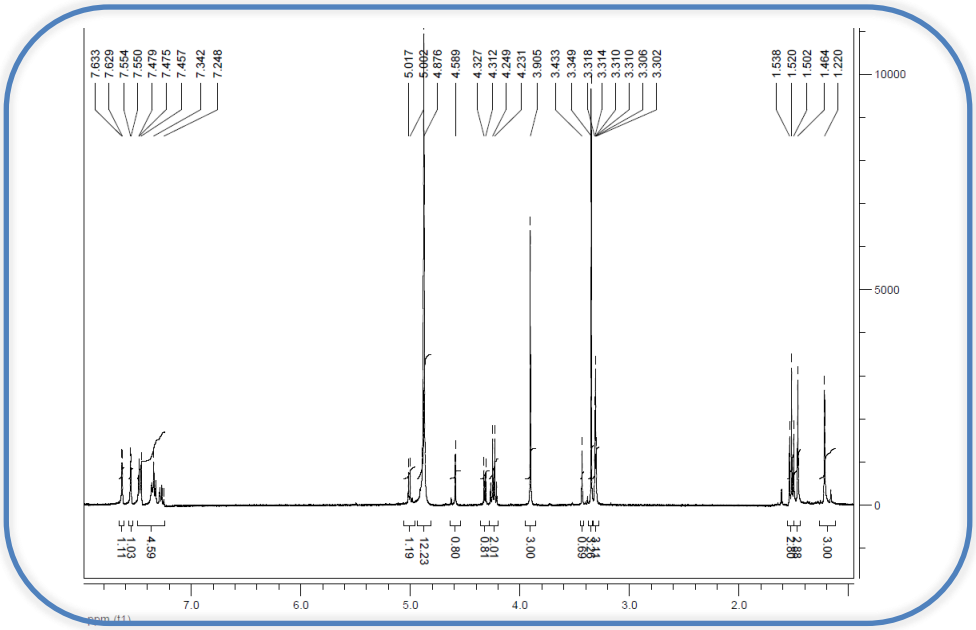


Figure 3.18. [EMIM][Amp] ^{1}H-NMR spectrum in CD_3OD.

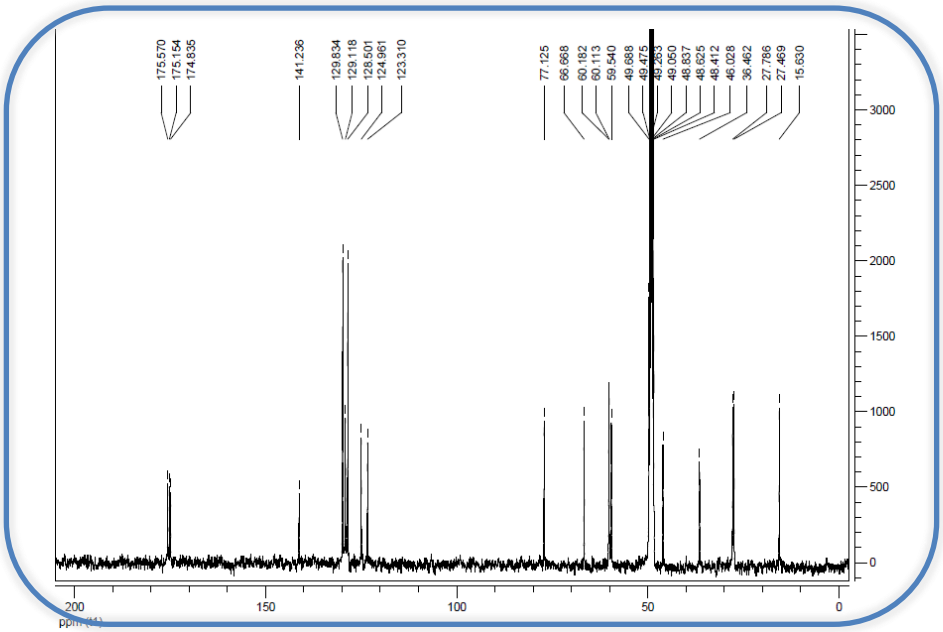


Figure 3.19. [EMIM][Amp] ^{13}C-NMR spectrum in CD_3OD.

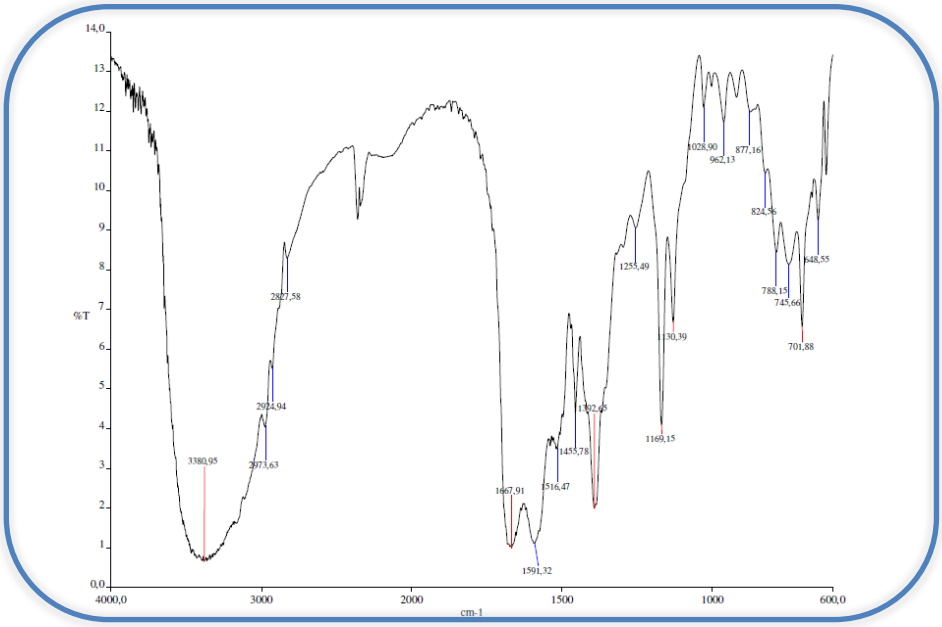


Figure 3.20. [EMIM][Amp] IR spectrum in KBr.

3.1.1.6 Preparation of 3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium (2S,5R,6R)-6-((R)-2-amino-2-phenylacetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C_2OHMIM][Amp]

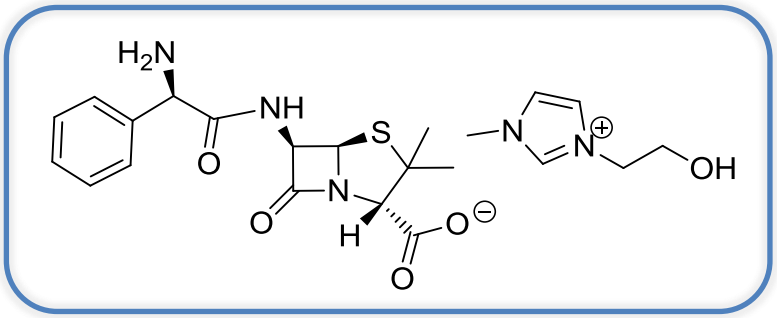


Figure 3.21 Struture of [C_2OHMIM][Amp].

3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium chloride (0.625 g; 3.86 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to Ampicillin (1.624 g; 4.65 mmol; 1.2 eq) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL of (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of ampicillin. Then, ampicillin crystals were filtered from the solution which was evaporated and dried *in vacuum* for 24 h. The desired product was obtained as a yellow solid (1.593 g; 86.8 %). m.p. 115-117 °C; $[\alpha]_D^{26}$= 86.3 ± 4.5 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.61 (1H, d, *J*= 1.8Hz, k), 7.55 (1H, d, *J* = 1.8Hz, j), 7.47 (2H, d, *J* = 7.2Hz, m), 7.35 (2H, t, *J* = 7.3Hz,l), 7.29 (1H, d, *J* = 7.2Hz, n), 5.00 (1H, d, *J* =6.0 Hz, c), 4.63 (1H, s, b), 4.33 (1H, d, *J* =6.0 Hz, d), 4.28 (2H, t, *J* =3.8 Hz, g), 3.92 (3H, s, f), 3.87 (2H, t, *J*=3.8 Hz), 3.42 (1H, s), 1.45 (3H, s), 1.22 (3H, s) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.60, 175.17, 174.85, 141.22, 129.85, 129.14, 128.51, 124.74, 124.03, 77.11, 66.67, 61.06, 60.19, 60.12, 59.53, 53.29, 36.46, 27.78, 27.48 ppm; IR (KBr): ν = 3394, 2969, 2888, 2836, 1674, 1545, 1456, 1394, 1299, 1253, 1167, 1131, 1073, 1027, 1071, 871, 784, 752, 702, 652, 622 cm^{-1}; (EI^+) *m*/*z* calcd for $C_6H_{11}N_2O^+$: 127.0866, found 127.0866; (EI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 348.1024, found 348.1013.

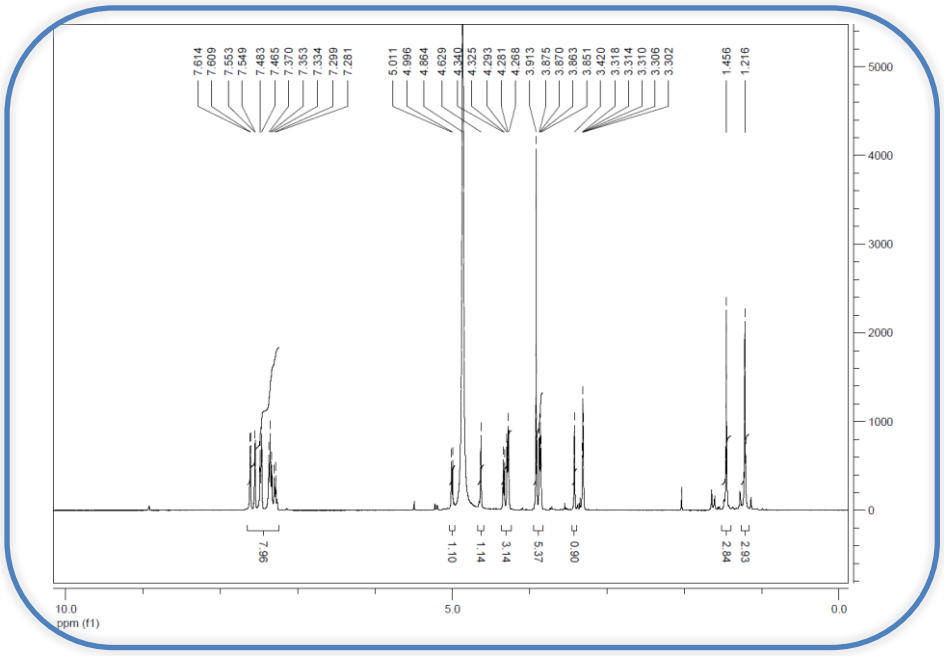


Figure 3.22. [C2OHMIM][Amp] ^{1}H-NMR spectrum in CD_3OD.

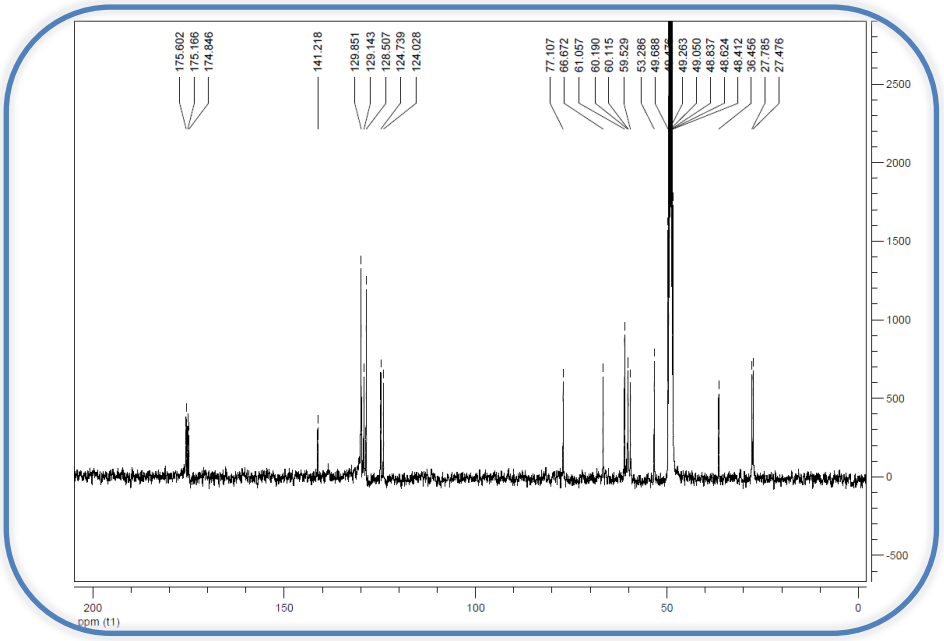


Figure 3.23. [C2OHMIM][Amp] ^{13}C-NMR spectrum in CD_3OD.

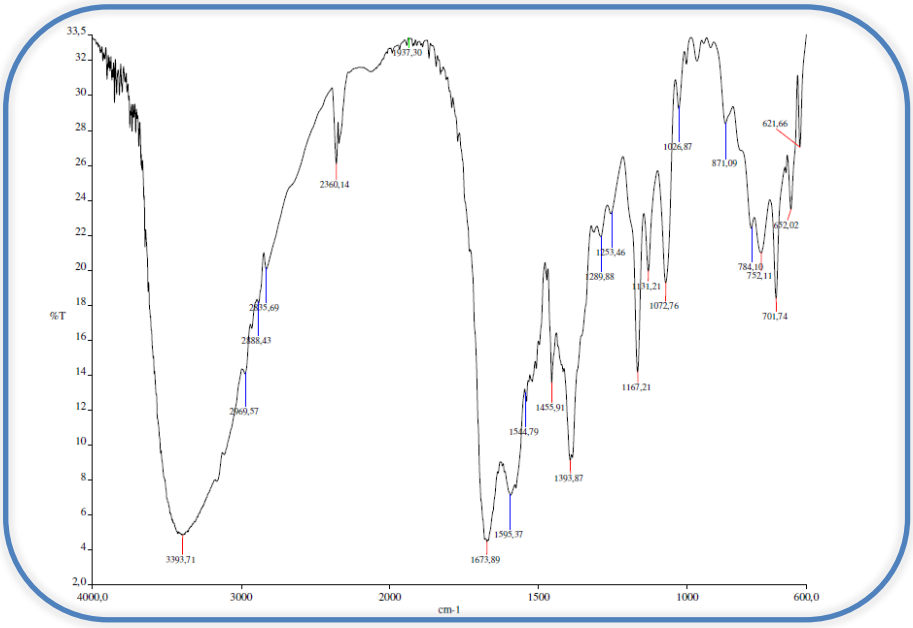


Figure 3.24. [C2OHMIM][Amp] IR spectrum in KBr.

3.1.2 Synthesis of Penicillin ILs

3.1.2.1 Preparation of Ammonium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [NH4][Pen]

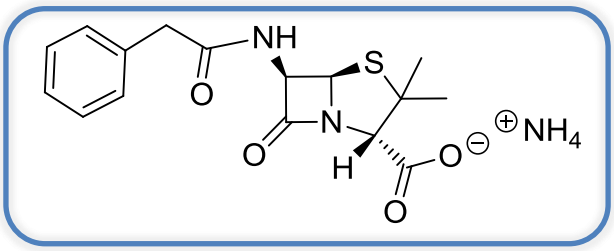


Figure 3.25 Struture of [NH4][Pen].

The potassium penicillin was transformed in ammonium penicillin before the reaction with hydroxide reactants. The ammonium penicillin was obtained by the dissolution of potassium penicillin (5.141 g, 13.80 mmol) in 6.5 mL of ammonium sulphate saturate solution at a pH = 6.5. The solution was stirred at room temperature for 24 h. After that period the solution was filtered and washed with ammonium sulphate solution (50 %).The ammonium penicillin was obtained as a white cristaline solid (4.774 g, 98 %) and dried *in vacuum* for 24 h. ^{1}H-NMR (400.13 MHz, D_2O) δ = 7.28-7.23 (m, 5H), 5.41 (s, 1H), 5.33 (s, 1H), 4.12 (s, 1H), 3.57 (m, 2H), 1.47 (s, 3H), 1.38 (s, 3H) ppm.

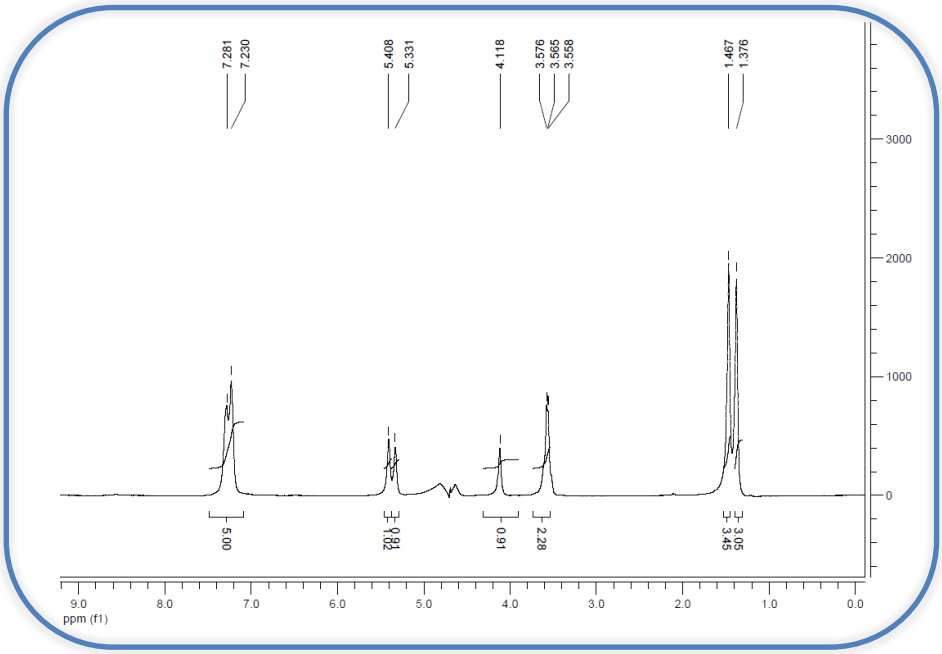


Figure 3.26. [NH_4^+][Pen] ^{1}H-NMR spectrum in D_2O.

3.1.2.2 Preparation of Tetraethylammonium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [TEA][Pen]

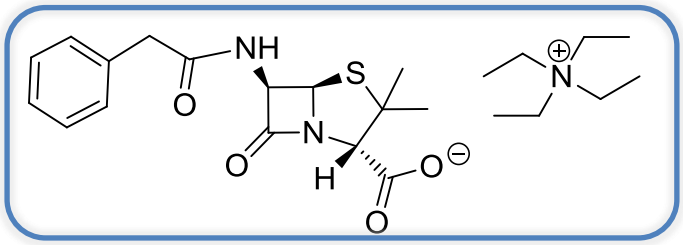


Figure 3.27 Struture of [TEA][Pen].

Tetraethylammonium bromide (0.420 g; 2.00 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the tetraethylammonium hydroxide solution formed was slowly added to the ammonium penicillin (0.751 g; 2.14 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The reaction mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^oC)[30] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide desired the product as a yellow viscous liquid (0.856 g;

92%). $[\alpha]_D^{25}$ = 104.0 ± 6.1 (c = 2 mgmL^{-1} in methanol), ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.31-7.22 (m, 5H), 5.46 (s, 1H), 4.17 (s, 1H) 3.70 (s, 1H) , 3.63-3.58 (m, 2H), 3.51 (bs, 1H), 3.27-3.26 (m, 8H), 1.63 (s, 3H), 1.55 (s, 3H), 1.30-1.24 (m, 12H); ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 174.72, 174.37, 174.15, 140.88, 136.73, 130.31, 130.25, 129.63, 127.96, 75.73, 66.68, 60.19, 59.27, 53.29, 46.65, 43.83, 29.41, 28.67, 28.38, 27.66, 7.64 ppm; IR (KBr): υ = 3420, 2981, 2924, 2862, 1840, 1736, 1721, 1648, 1560, 1543, 1490, 1459, 1432, 1396, 1367, 1173, 1130, 1053, 1027, 1001, 785, 734, 696, 619, 539 cm^{-1}; (ESI$^+$) *m*/*z* calcd for $C_8H_{20}N^+$: 130.1 found 130.0; (ESI$^-$) *m*/*z* calcd for $C_{16}H_{17}N_2O_4S^-$ 333.4 found $[M+OH]^-$ 349.8.

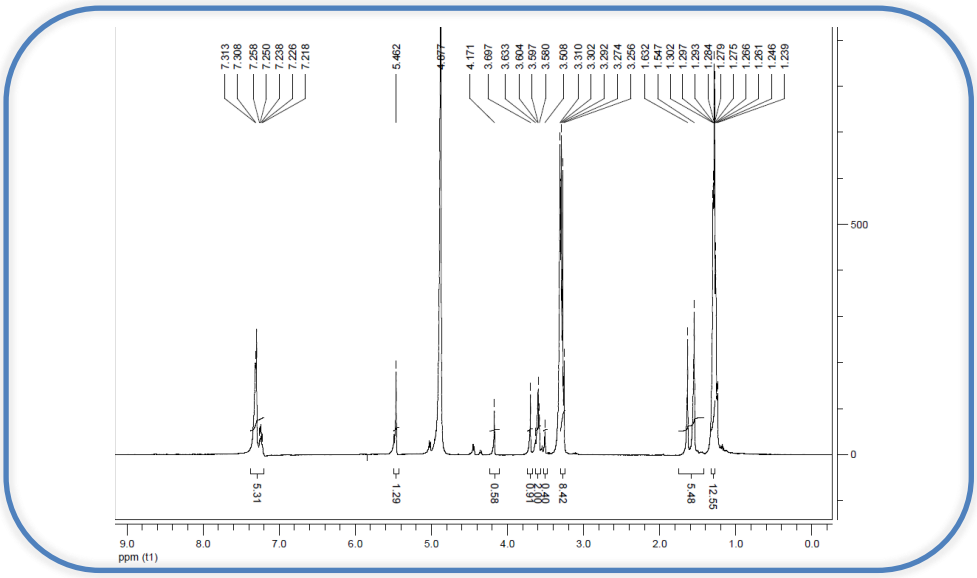


Figure 3.28. [TEA][Pen] 1H-NMR spectrum in CD_3OD.

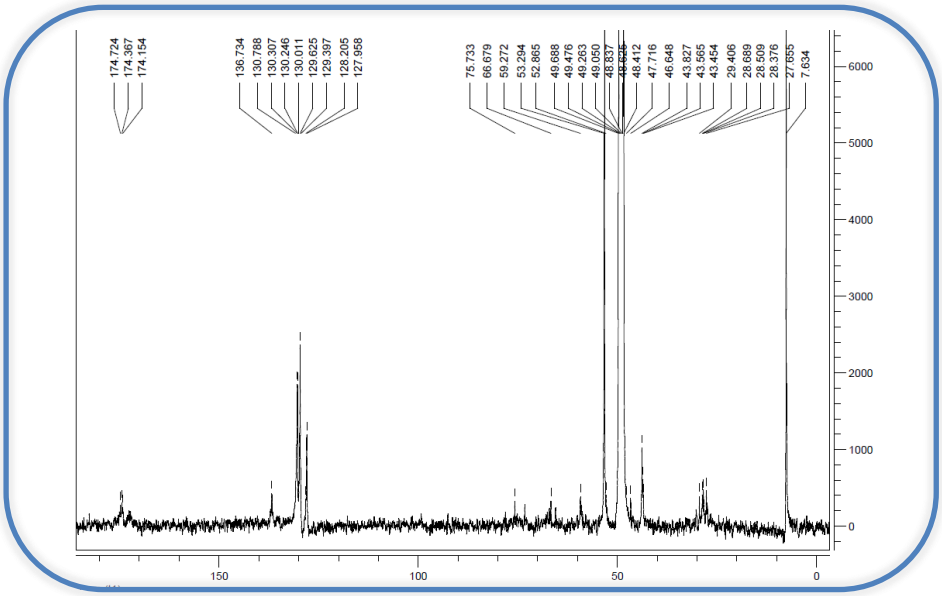


Figure 3.29. [TEA][Pen] ^{13}C-NMR spectrum in CD_3OD.

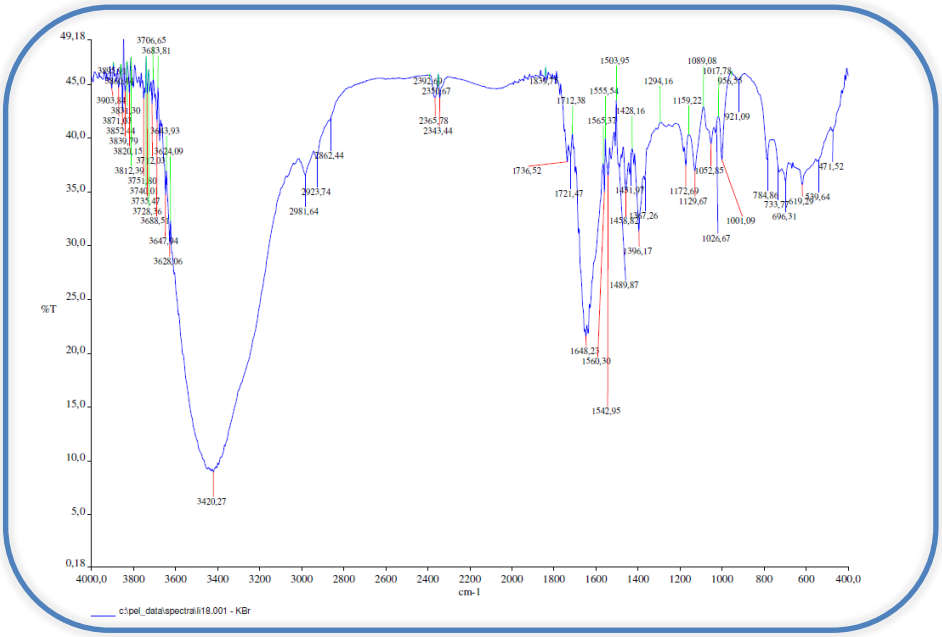


Figure 3.30. [TEA][Pen] IR spectrum in KBr.

3.1.2.3 Preparation of Trihexyl(tetradecyl)phosphonium 3 (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [$P_{6,6,6,14}$][Pen]

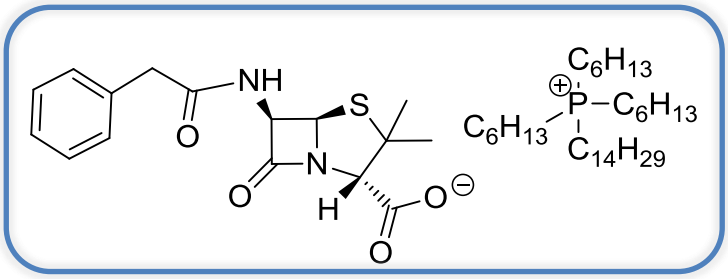


Figure 3.31 Struture of [$P_{6,6,6,14}$][Pen].

Trihexyl(tetradecyl) chloride (1.000 g; 1.92 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the trihexyl(tetradecyl)phosphonium hydroxide solution formed was slowly added to ammonium penicillin (0.853 g; 2.43 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[28] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow viscous liquid (1.560 g;

99%). $[\alpha]_D^{25}$ = 67.7 ± 3.0 (c = 2 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.34-7.22 (m, 5H), 4.97 (1H, d, *J* = 6.7 Hz), 4.34 (dd, 1H, *J* = 6.7 Hz), 3.60 (d, 2H, *J* = 8.2 Hz), 3.50 (s, 1H), 2.20 (m, 8H), 1.56-1.25 (m, 54H), 0.96-0.88 (m, 12H) ppm ; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.30, 174.83, 173.92, 136.76, 130.37, 129.59, 127.87, 76.81, 66.78, 60.14, 54.86, 43.68, 33.11, 32.19, 31.92, 31.84, 30.80, 30.51, 30.45, 29.91, 27.88, 23.77, 23.49, 22.36, 19.53, 19.05, 14.51, 14.38 ppm; IR (KBr): ν = 3308, 3028, 2951, 2923, 2853, 1737, 1669, 1607, 1536, 1496, 1456, 1418, 1379, 1262, 1201, 1113, 1031, 986, 860, 810, 761, 722, 694, 617, 454, 439, 424 cm^{-1}; (ESI$^+$) *m*/*z* calcd for $C_{32}H_{68}P^+$: 483.4 found 483.8; (ESI$^-$) *m*/*z* calcd for $C_{16}H_{17}N_2O_4S^-$ 333.4, found $[M+OH]^-$ 349.9.

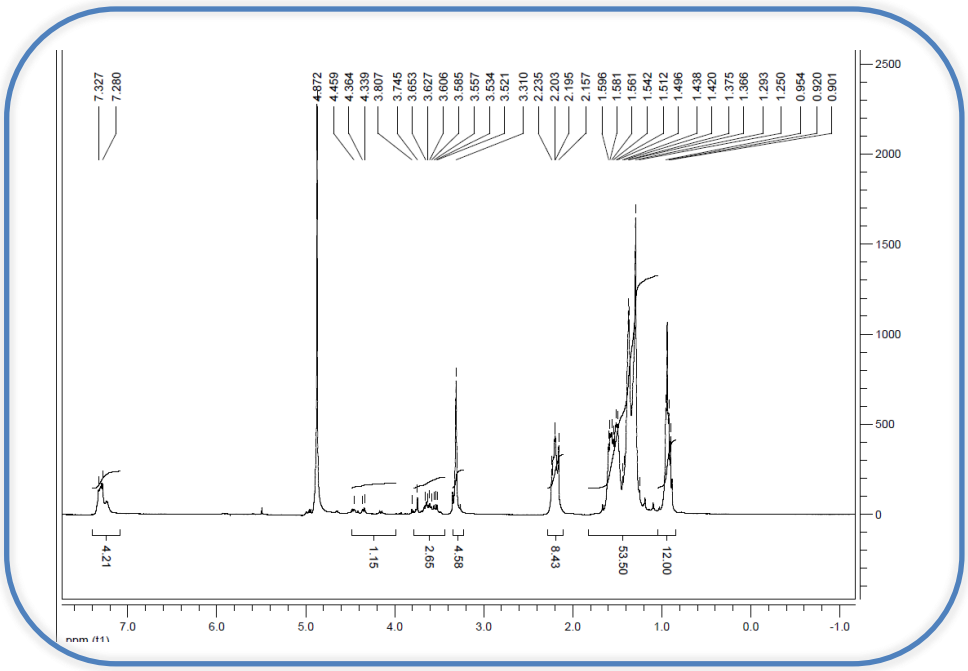


Figure 3.32. $[P_{6,6,6,14}][Pen]$ ^{1}H-NMR spectrum in CD_3OD.

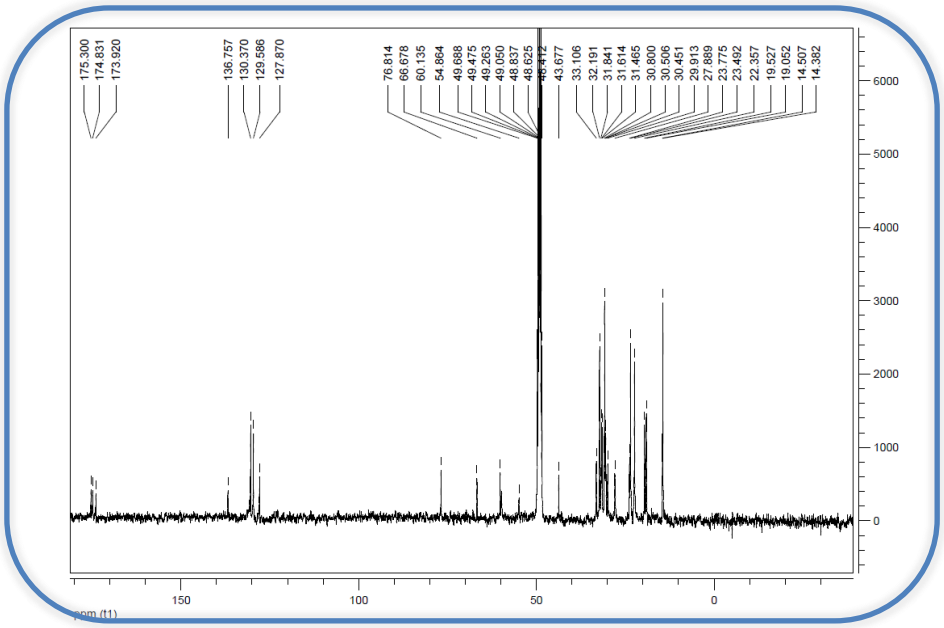


Figure 3.33. [$P_{6,6,6,14}$][Pen] ^{13}C-NMR spectrum in CD_3OD.

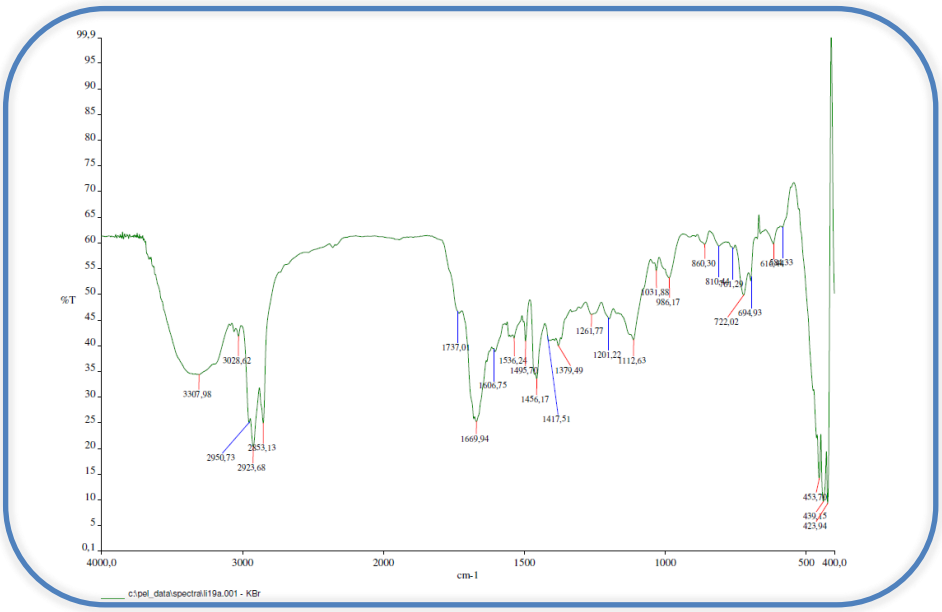


Figure 3.34. [$P_{6,6,6,14}$][Pen] IR spectrum in KBr.

3.1.2.4 Preparation of 1-Hexadecylpyridin-1-ium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C_{16}Pyr][Pen]

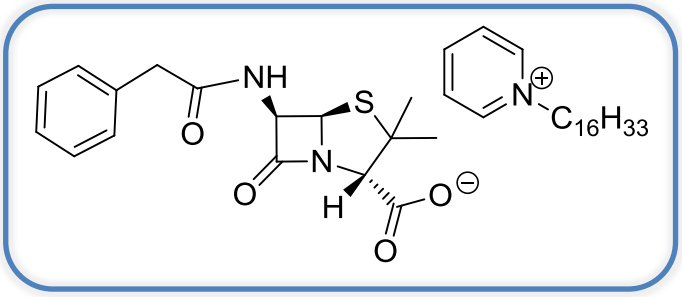


Figure 3.35 Struture of [C_{16}Pyr][Pen].

Cetylpyridinium chloride (0.822 g; 2.30 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$). Then the cetylpyridinium hydroxide solution formed was slowly added to ammonium penicillin (0.973 g; 2.77 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in a 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[30] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow solid (1.332 g; 91%). m.p. 76-78 ^{o}C; $[\alpha]_D^{25}$ = 47.3 ± 3.6 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 9.01 (d, 2H, *J* = 5.7 Hz), 8.59 (t, 1H, *J* = 7.8 Hz), 8.12 (t, 2H, *J* = 6.8 Hz), 7.33-7.21 (m, 5H), 4.95 (d, 1H, *J* = 7.1 Hz), 4.63 (t, 2H, *J* = 7.5Hz,), 4.35 (d, 1H, *J* = 7.0 Hz), 3.60 (2H, d, *J* = 7.5 Hz), 3.50 (s, 1H), 1.56 (m, 3H), 1.42-1.09 (m, 31H), 0.90 (t, 3H, *J* = 6.6 Hz) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ =175.16, 174.78, 173.98, 146.87, 146.00, 136.73, 130.40, 129.80, 129.60, 127.93, 76.34, 66.64, 63,15, 60.14, 43.67, 33.12, 32.55, 30.81, 30.67, 30.17, 27.86, 27.24, 23.78, 14.49 ppm; IR (KBr): δ = 3041, 3059, 2914, 2848, 1739, 1658, 167, 1601, 1542, 1528, 1508, 1487, 1472, 1397, 1368, 1322, 1270, 1209, 1177, 1128, 1078, 1032, 987, 960, 926, 818, 777, 716, 686, 619, 574, 475 cm^{-1}; (ESI^+) *m*/*z* calcd for $C_{21}H_{38}N^+$: 304.3 found 304.2; (ESI^-) *m*/*z* calcd for $C_{16}H_{17}N_2O_4S^-$ 333.4, found $[M+OH]^-$ 349.9.

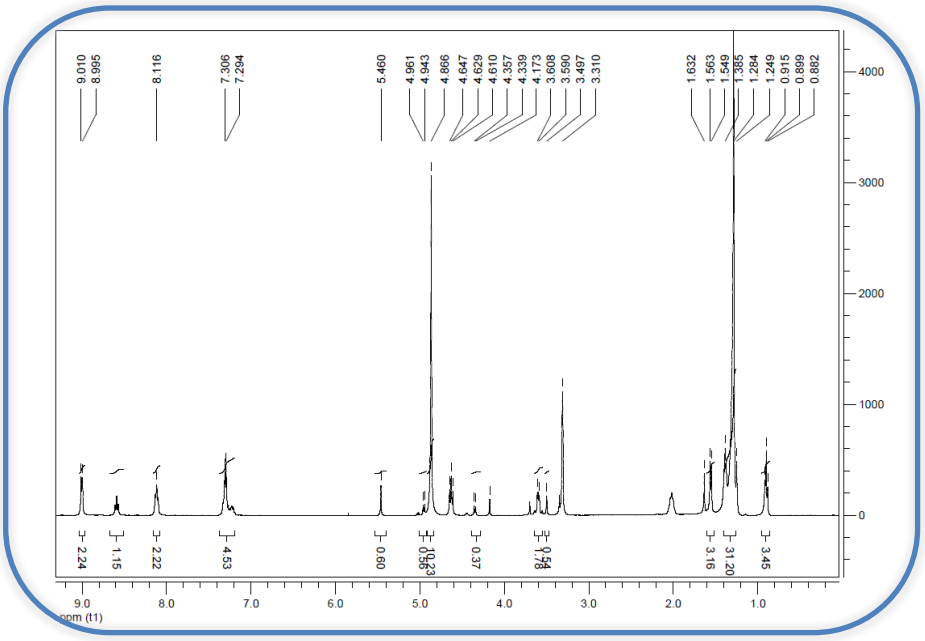


Figure 3.36. [C_{16}Pyr][Pen] ^{1}H-NMR spectrum in CD_3OD.

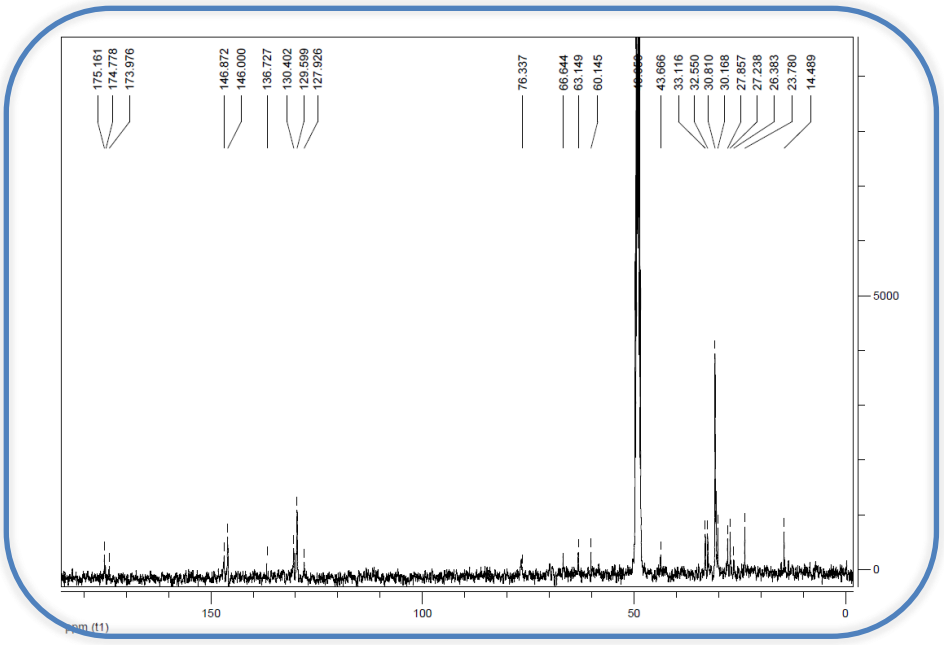


Figure 3.37. [C_{16}Pyr][Pen] ^{13}C-NMR spectrum in CD_3OD.

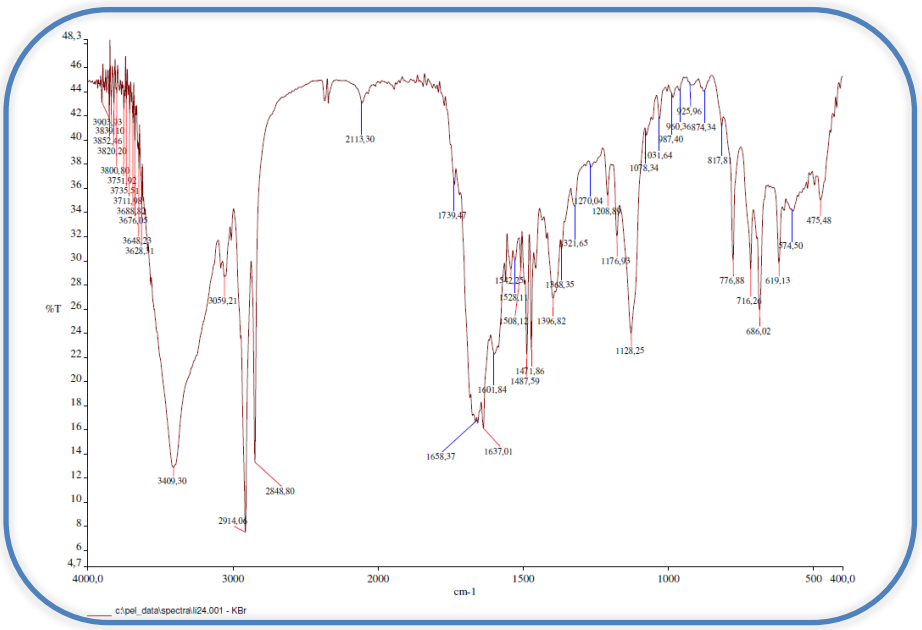


Figure 3.38. [C_{16}Pyr][Pen] IR spectrum in KBr.

3.1.2.5 Preparation of (2-Hydroxyethyl)-Trimethylammonium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [Cholin][Pen]

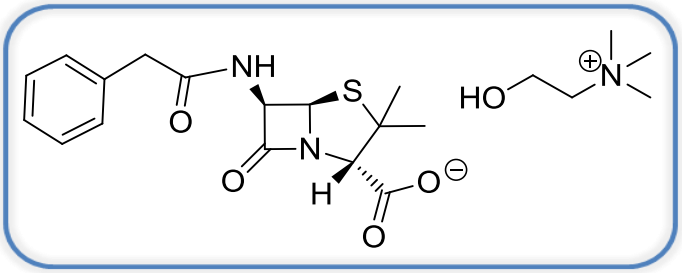


Figure 3.39 Struture of [Cholin][Pen].

(2-Hydroxyethyl)-trimethylammonium chloride (0.277 g; 1.99 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to ammonium penicillin (0.848 g; 2.41 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in a 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow solid (0.856 g; 98 %). m.p. 69-71 °C; $[\alpha]_D^{25}$ = 47.3 ± 3.6 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD)

δ = 7.33-7.29 (m, 5H), 4.95 (d, 1H, J_1 = 7.0 Hz), 4.35 (d, 1H, J_1 = 7.0 Hz), 4.02-3.98 (m, 2H), 3.66-3.56 (m, 2H), 3.50-3.47 (m, 3H), 3.20 (s, 9H) 1.56 (s, 3H), 1.25 (s, 3H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 174.72, 174.37, 174.15, 140.88, 136.73, 130.31, 130.25, 129.63, 127.96, 75.73, 69.06, 66.67, 60.20, 60.04, 59.53, 57.10, 46.65, 27.78, 27.47 ppm; IR (KBr): υ = 3468, 3074, 2966, 1652, 1496, 1479, 1461, 1396, 1356, 1279, 1204, 1131, 1083, 1054, 1010, 955, 887, 867, 796, 734, 702, 670, 620, 540, 476 cm^{-1}; (ESI^+) *m/z* calcd for $C_5H_{14}NO^+$: 104.1, found 104.1; (ESI^-) *m/z* calcd for $C_{16}H_{18}N_3O_4S^-$: 333.4, $[M+OH]^-$ 349.9.

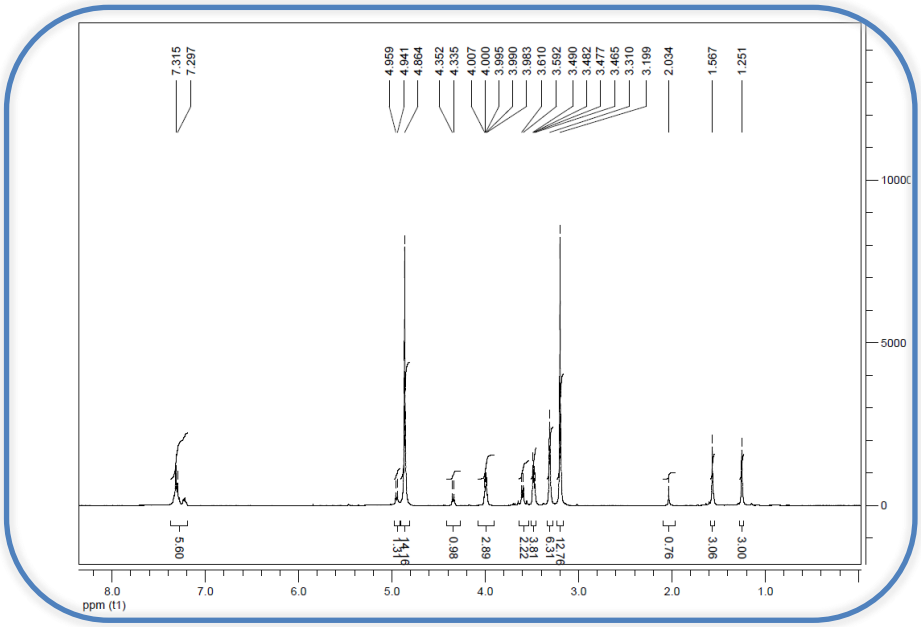


Figure 3.40. [Choline][Pen] ^{1}H-NMR spectrum in CD_3OD.

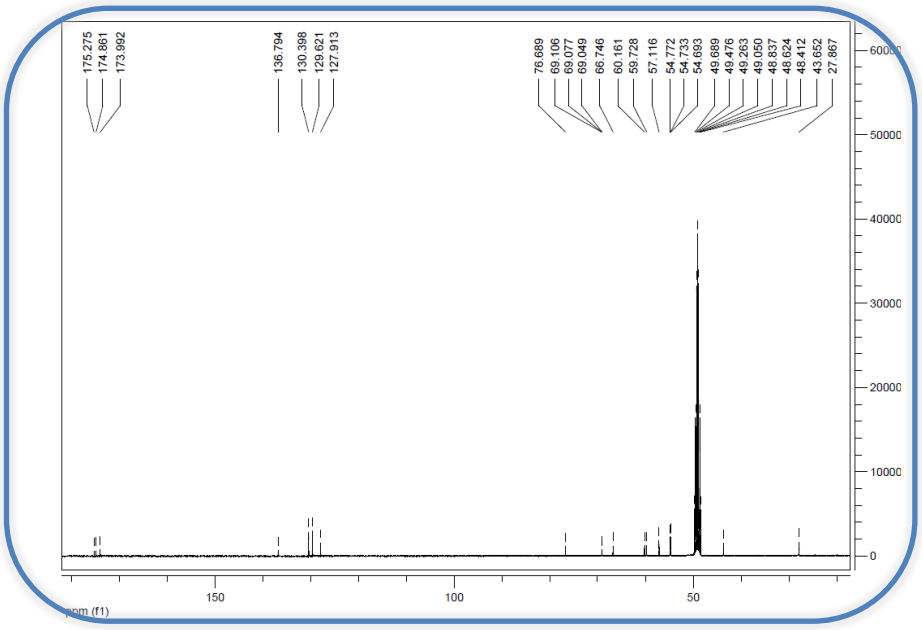


Figure 3.41. [Choline][Pen] ^{13}C-NMR spectrum in CD_3OD.

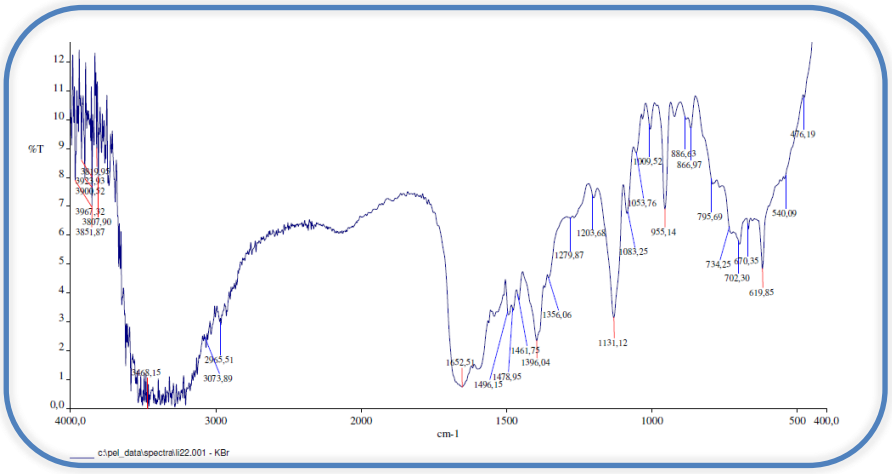


Figure 3.42. [Choline][Pen] IR spectrum in KBr

3.1.2.6 Preparation of 1-Ethyl-3-methyl-1*H*-imidazol-3-ium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [EMIM][Pen]

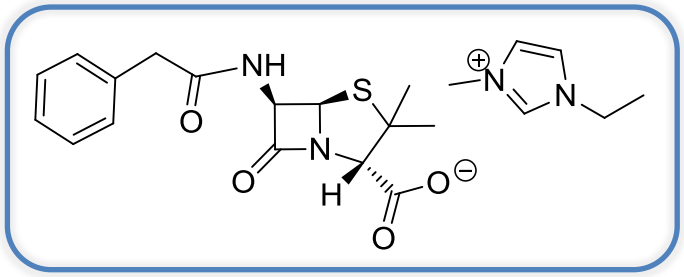


Figure 3.43 Struture of [EMIM][Pen].

1-Ethyl-3-methyl-1*H*-imidazol-3-ium bromide (0.578 g; 3.03 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to ammonium penicillin (1.11 g; 3.16 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in a 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a viscous liquid (1.136 g; 84.4%). $[\alpha]_D^{25}$ = 89.0 ± 7.0 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 8.99 (bs, 1H), 7.63 (s, 1H) 7.53 (s, 1H), 7.34-7.20 (m, 5H), 4.95 (d, 1H, *J* = 7.0 Hz), 4.35 (d, 1H, *J* = 7.0 Hz), 4.24 (q, 2H, *J* = 7.3 Hz), 3.92 (s, 3H), 3.60 (d, 2H, *J* = 7.2 Hz), 3.50 (s, 1H), 1,60-

1.50 (m, 6H), 1.25 (s, 3H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.06, 174.83, 173.99, 136.80, 130.65, 130.39, 129.60, 127.90, 124.94, 123.25, 76.51, 60.13, 60.18, 60.11, 59.54, 46.02, 43.66, 36.51, 27.81, 27.65, 15.61 pm; IR (KBr): υ = 3468, 3368, 2970, 1660, 1540, 1501, 1456, 1395, 1456, 1395, 1354, 1300, 1258, 1169, 1131, 1301, 965, 918, 862, 828, 729, 700, 651, 620, 545 cm^{-1}; (ESI^+) *m*/*z* calcd for $C_6H_{11}N_2^+$: 111.1, found 111.0; (ESI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 333.4, found $[M+OH]^-$ 349.9.

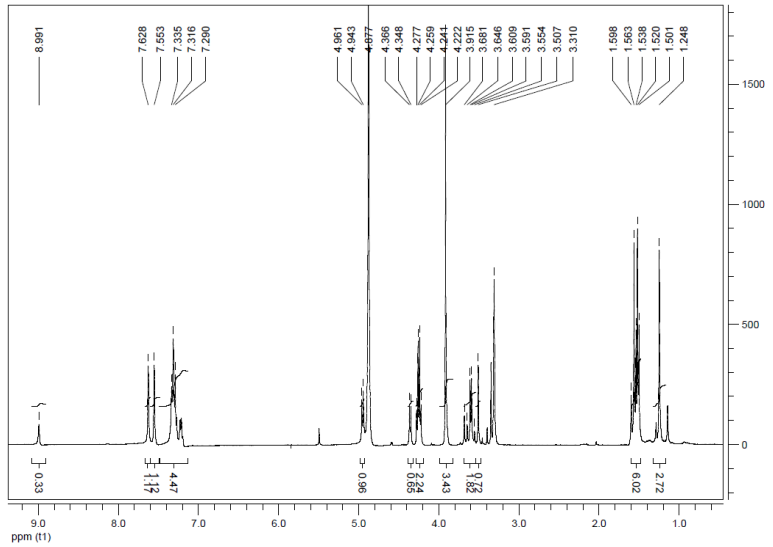


Figure 3.44. [EMIM][Pen] ^{1}H-NMR spectrum in CD_3OD.

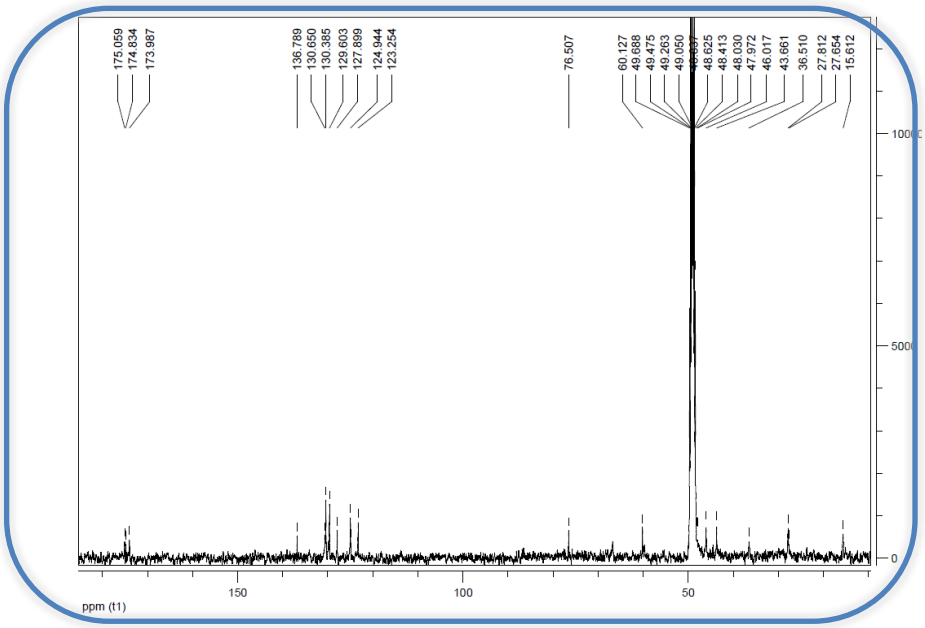


Figure 3.45. [EMIM][Pen] ^{13}C-NMR spectrum in CD_3OD.

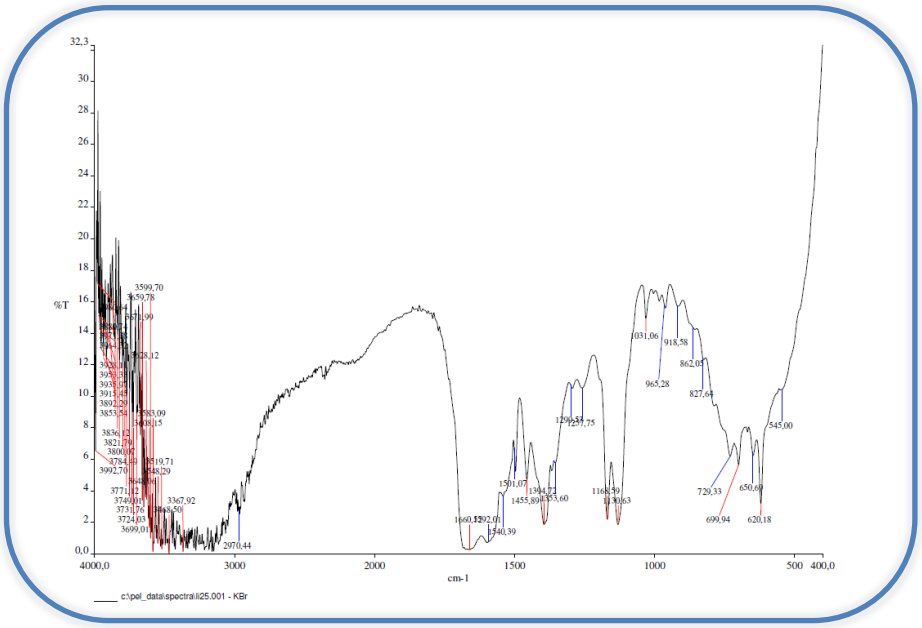


Figure 3.46. [EMIM][Pen] IR spectrum in KBr.

3.1.2.7 Preparation of 3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium (2S,5R,6R)-3,3-dimethyl-7-oxo-6-(2-phenylacetamido)-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C$_2$OHMIM][Pen]

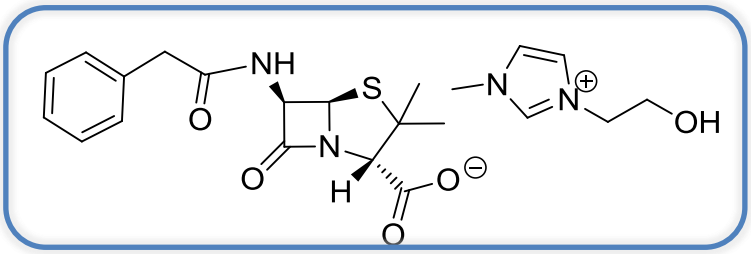


Figure 3.47 Struture of [C$_2$OHMIM][Pen].

3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium chloride (0.328 g; 2.03 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400(OH)[26,30] (5 eq., flux rate 0.133 mLmL^{-1}min^{-1} = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to ammonium penicillin (0.754 g; 2.14 mmol) dissolved in 1.0 M ammonium solution (50 mgmL^{-1}). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[30] to induce crystallization of excess of ammonium penicillin. Then ammonium penicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow solid (0.799 g; 85.6 %). m.p.

48-50 °C; $[\alpha]_D^{25}$ = 41.3 ± 6.0 (c = 2 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 8.98, (s, 1H), 7.61 (s, 1H), 7.54 (s, 1H), 7.33-7.21 (m, 5H), 4.94 (d, 1H, *J* = 7.1 Hz), 4.36 (d, 1H, *J* = 7.1 Hz), 4.29 (t, 2H, *J* = 4.9 Hz), 3.92, (s, 3H), 3.86 (t, 2H, *J* = 4.9 Hz), 3.59 (d, 2H, *J* = 7.1 Hz), 3.50 (s, 1H), 1.55 (s, 3H), 1.24 (s, 3H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.04, 174.82, 174.00, 136.78, 130.63, 130.51, 130.40, 129.61, 129.85, 129.61, 127.91, 124.68, 124.00, 76.50, 66.76, 61.10, 60.12, 59.53, 53.26, 43.65, 36.45, 27.84 ppm; IR (KBr): υ = 3418, 2965, 2931, 2108, 1644, 1585, 1499, 1455, 1398, 1356, 1260, 1167, 1127, 1076, 1034, 879, 798, 734, 704, 668, 619, 464, 445, 432, 424 cm^{-1}; (ESI$^+$) *m*/*z* calcd for $C_6H_{11}N_2O^+$: 127.2, found 127.0; (ESI$^-$) *m*/*z* calcd for $C_{16}H_{18}N_3O_4S^-$: 333.4, found $[M+OH]^-$ 349.9.

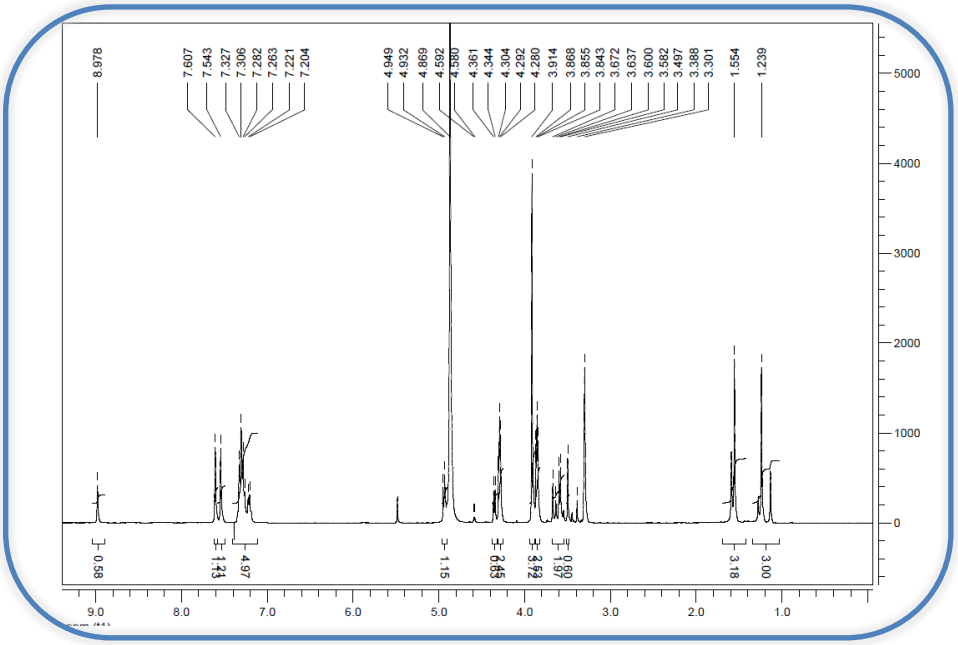


Figure 3.48. [C_2OHMIM][Pen] ^{1}H-NMR spectrum in CD_3OD.

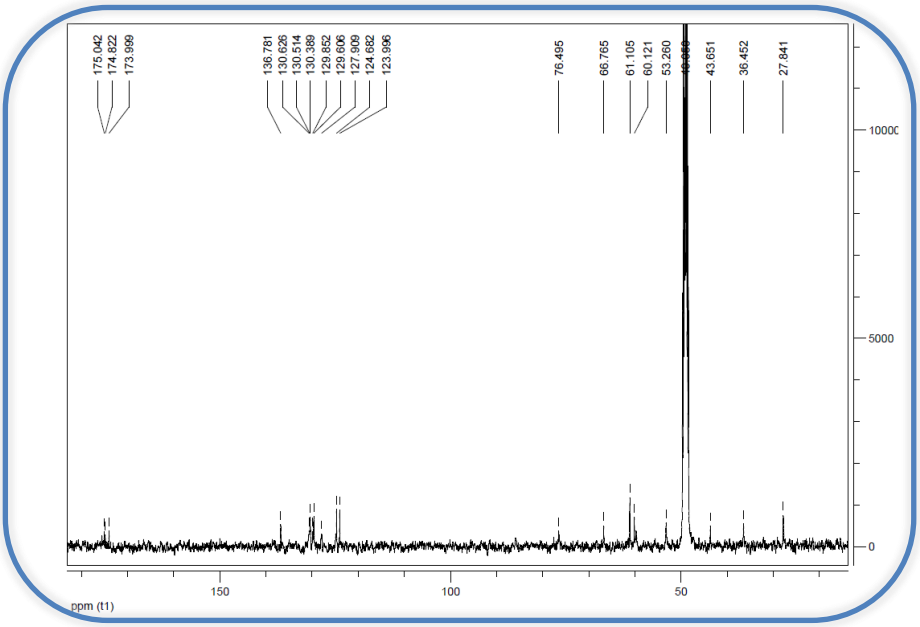


Figure 3.49. [C_2OHMIM][Pen] ^{13}C-NMR spectrum in CD_3OD.

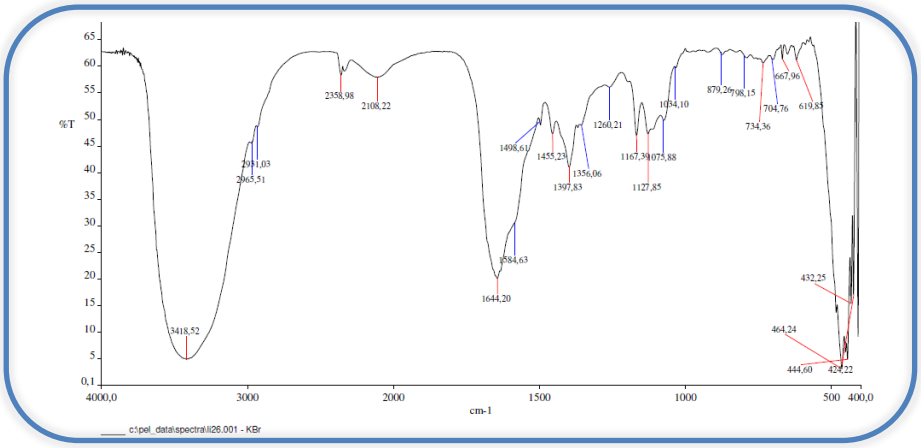


Figure 3.50. [C_2OHMIM][Pen] IR spectrum in KBr.

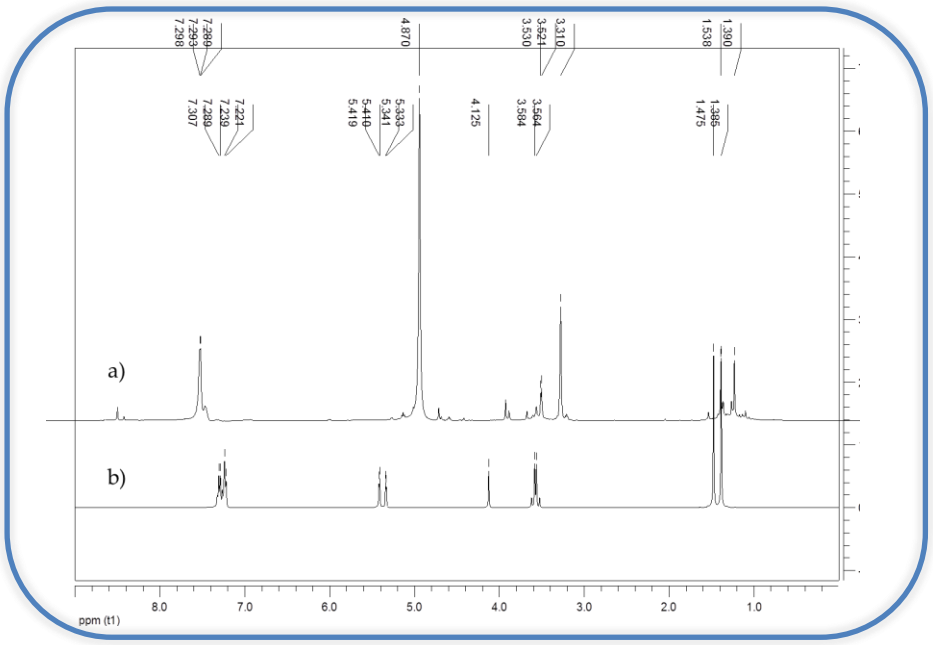


Figure 3.51. a) Sulphoxide Penicillin[2] ^{1}H-NMR spectrum in CD_3OD. **b**) Potassium Penicillin ^{1}H-NMR spectrum in CD_3OD.

3.1.3 Synthesis of Amoxicillin ILs

3.1.3.1 Preparation of 1-Ethyl-3-methyl-1*H*-imidazol-3-ium (2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [EMIM][Amx]

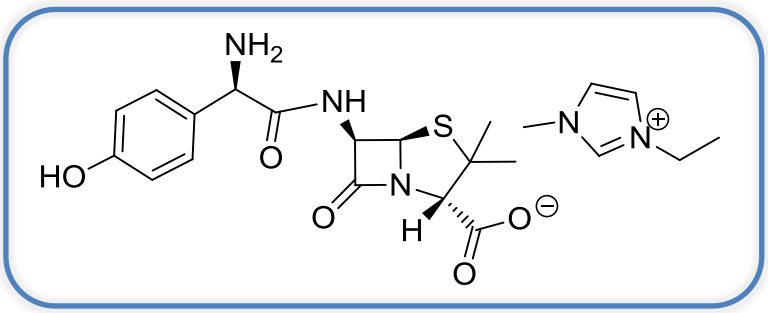


Figure 3.52 Struture of [EMIM][Amx].

1-Ethyl-3-methyl-1*H*-imidazol-3-ium chloride **(**0.385 g; 2.01 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amoxicillin trihydrate (0.930 g; 2.22 mmol) dissolved in 1.0 M

[2] Sulphoxide Penicillin was prepared according Lim *et al.*(Y.-H. Lim, D.-H. Park, Y.-Y. Youn, K.-H. Kim and H.-S. Cho, *Mass Spectrometry Letters*, 2011, 2, 16.

ammonium solution (50 mgmL^{-1}). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[30] to induce crystallization of excess of amoxicillin. Then amoxicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacum* for 24 h to provide the desired product as a yellow solid (0.768 g; 80.2 %). m.p. 84-86 ^{o}C; $[\alpha]_D^{25}$ = 48.3 ± 5.0 (c = 2 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.64 (s, 1H), 7.56 (s, 1H), 7.27 (d, 2H, J = 8.2 Hz), 6.74 (d, 2H, J = 8.4 Hz), 5.00 (d, 1H, J_1 = 5.9 Hz), 4.74 (s, 1H), 4.30 (d, 1H, J_1 = 5.9 Hz), 4.25 (1, 2H, J = 7.3 Hz), 3.77 (bs, 1H), 3.92 (s, 3H), 3.73, (bs, 1H), 3.43 (bs, 1H), 3.35 (s, 1H, s), 1.55-1.48 (m, 6H); 1.22 (s, 3H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.57 , 175.15, 174.84, 141.24, 129.83, 129.12, 128.50, 124.96, 123.31, 77.12, 66.67, 60.18, 60.11, 59.54, 46.03, 36.46, 27.78, 27.47, 15.63 ppm; IR (KBr): υ = 3461, 2921, 2852, 1706, 1688,1656, 1636, 1560, 1541, 1508, 1461, 1403, 1348, 1260, 1170, 1130, 673, 620, 474, 422 cm^{-1}; (ESI^+) m/z calcd for $C_6H_{11}N_2^+$: 111.1, found 111.0; (ESI^-) m/z calcd for $C_{16}H_{18}N_3O_5S^-$: 364.4, found $[M+OH]^-$ 380.8.

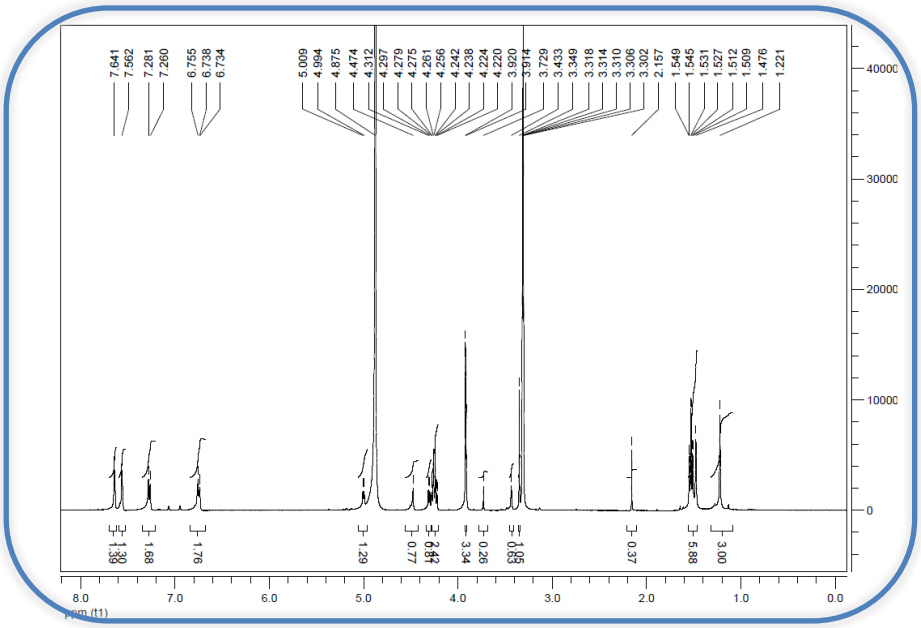


Figure 3.53. [EMIM][Amx] ^{1}H-NMR spectrum in CD_3OD.

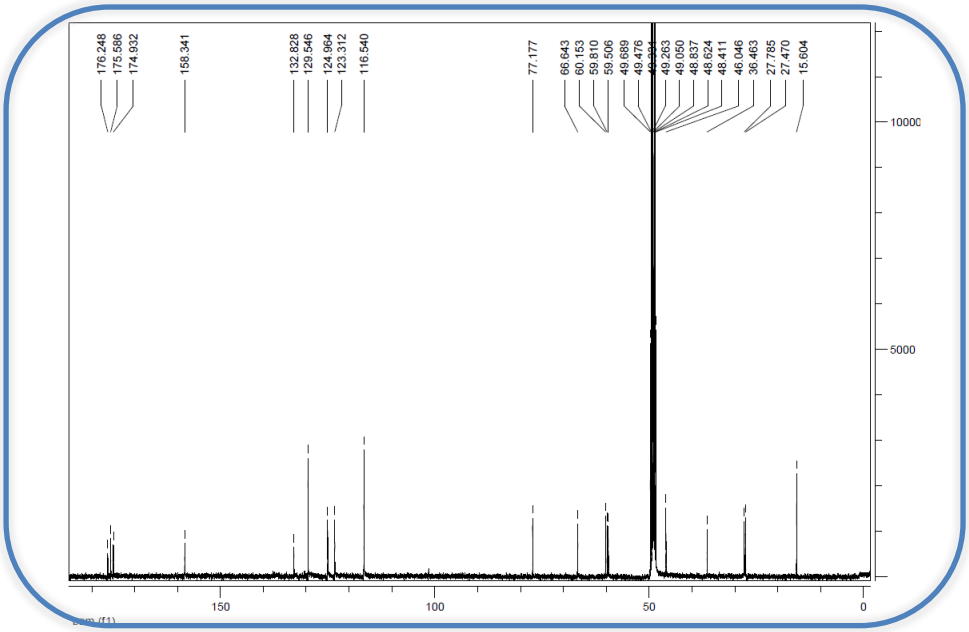


Figure 3.54. [EMIM][Amx] ^{13}C-NMR spectrum in CD_3OD.

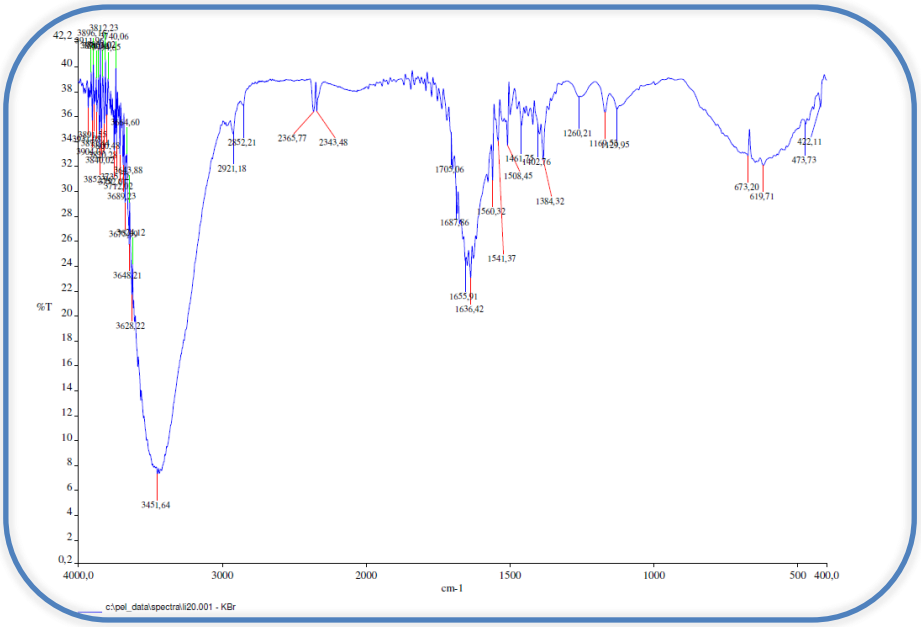


Figure 3.55. [EMIM][Amx] IR spectrum in KBr**.**

3.1.3.2 Preparation of Trihexyl(tetradecyl)phosphonium (2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [$P_{6,6,6,14}$][Amx]

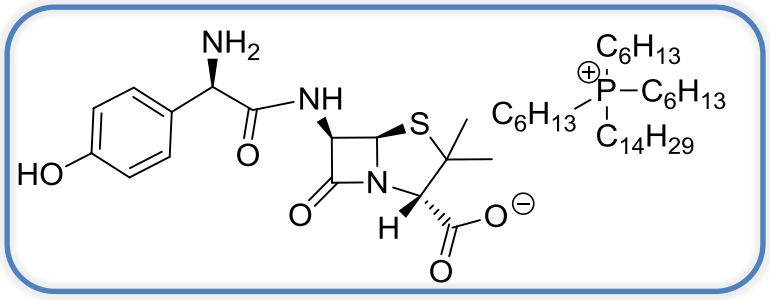


Figure 3.56 Struture of [$P_{6,6,6,14}$][Amx].

Trihexyl(tetradecyl) chloride (1.042 g; 2.01 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the trihexyl(tetradecyl)phosphonium hydroxide solution formed was slowly added to amoxicillin (0.988 g ; 2.36 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 ^{o}C)[28] to induce crystallization of excess of amoxicillin. Then amoxicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow viscous liquid (1.586 g; 93.5%). $[\alpha]_D^{25}$ = 22.0 ± 5.8 (c = 2 $mgmL^{-1}$ in methanol); (400.13 MHz, CD_3OD) δ = 7.28 (d, 2H, *J* = 8.4 Hz), 6.76 (d, 2H, *J* = 8.4 Hz), 5.00 (d, 1H, *J* = 5.9 Hz), 4.53 (s, 1H), 4.31 (d, 1H, *J* = 5.9 Hz), 3.42 (s, 1H), 2.23-2.16 (m, 8H), 1.60-1.22 (m, 54H), 0.95-0.88 (m, 12H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 175.35, 174.22, 142.01, 129.62, 128.92, 116.59, 77.12, 66.57, 60.17, 54.94, 43.76, 33.18, 32.27, 31.93, 31.56, 30.89, 30.58, 30.01, 27.96, 23.85, 23.57, 22.45, 19.62, 19.15, 14.58, 14.46 ppm; IR (KBr): ν = 3419, 3921, 2107.38, 1638, 1560, 1506, 1459, 1398, 1270, 1130, 1000, 668, 619, 570, 476, 456, 433, 412 cm^{-1}; (ESI^+) *m*/*z* calcd for $C_{32}H_{68}P^+$: 483.85 found 483.58; (ESI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_5S^-$: 364.40, found $[M+OH]^-$ 380.98.

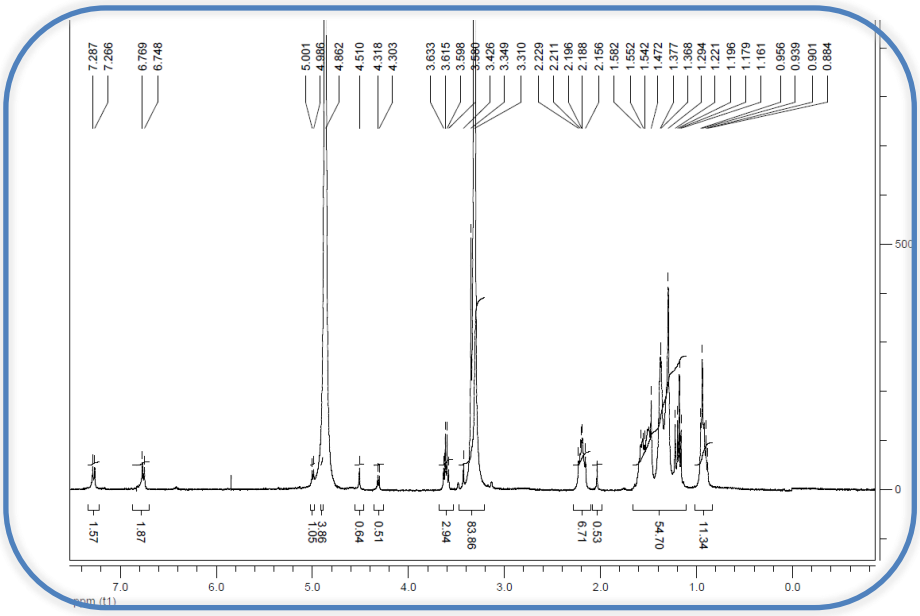


Figure 3.57. [$P_{6,6,6,14}$] [Amx] ^{1}H-NMR spectrum in CD_3OD.

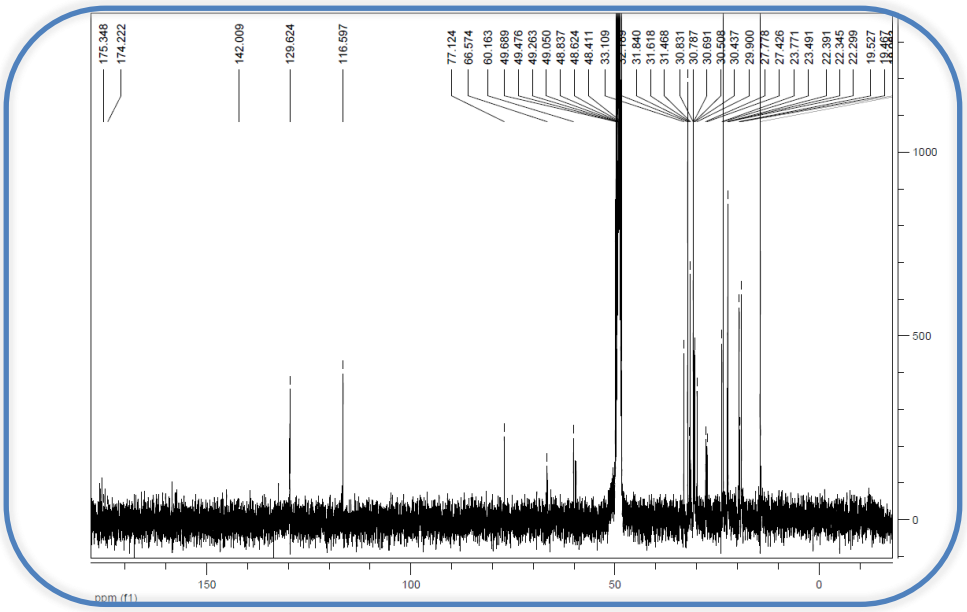


Figure 3.58. [$P_{6,6,6,14}$] [Amx] ^{13}C-NMR spectrum in CD_3OD.

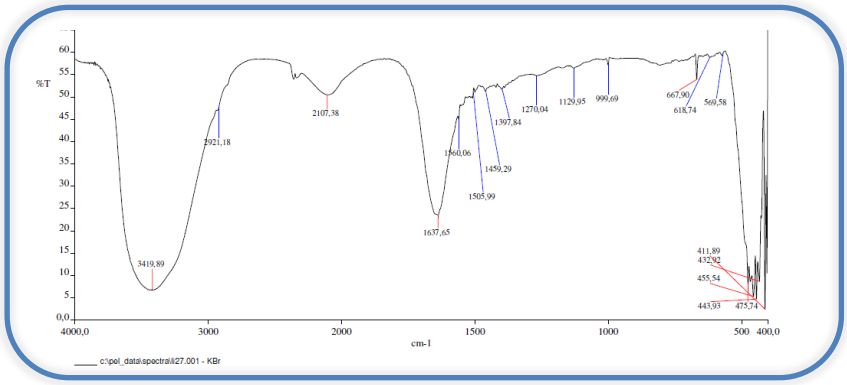


Figure 3.59. [$P_{6,6,6,14}$][Amx] IR spectrum in KBr.

3.1.3.3 Preparation of 1-Hexadecylpyridin-1-ium 6 (2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C_{16}Pyr][Amx]

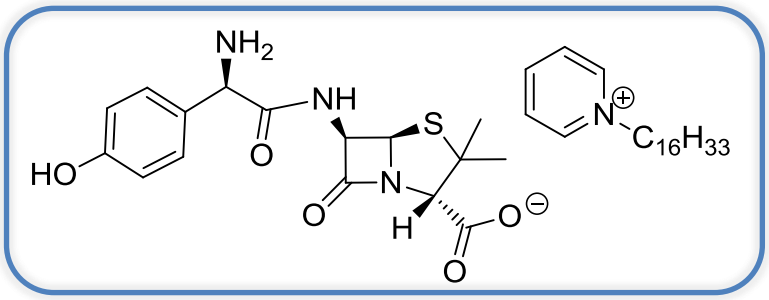


Figure 3.60 Struture of [C_{16}Pyr][Amx].

Cetylpyridinium chloride (0.456 g; 1.28 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the cetylpyridinium hydroxide solution formed was slowly added to amoxicillin (0.587 g; 1.40 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of amoxicillin. Then amoxicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide the desired product as a yellow solid (0.402 g; 47.0 %). m.p. 96-98 °C; $[\alpha]_D^{25}$ = 77.0 ± 5.8 (c = 2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 8.98 (d, 2H, *J* = 5.8 Hz), 8.58 (t, 1H, *J* = 7.74 Hz), 8.10 (t, 2H, *J* = 6.7 Hz), 7.26 (d, 2H, *J* = 8.5 Hz), 6.73 (d, 2H, *J* = 8.4 Hz), 5.01 (d, 1H, *J* = 6.0 Hz), 4.62 (t, 2H, *J* = 7.5 Hz), 4.46 (s, 1H), 4.30 (d, 1H, *J* = 6.0 Hz), 3.44 (s, 1H), 2.02, (t, 2H, *J* = 6.9 Hz), 1.48 (s, 3H), 1.38 -1.26 (m, 28H), 1.22 (s, 3H) 0.90 (3H, t, *J* = 6.7 Hz) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ =176.32, 175.60, 174.94, 158.32, 150.28, 146.87, 145.91, 132.86, 129.54, 116.55, 77.13, 66.61, 63,17, 60.14, 33.11, 32.53, 30.80, 30.67, 30.52, 30.16, 27.24, 23.77, 14.49 ppm; IR (KBr): υ = 3440, 2914, 2849, 1685, 1651, 1636, 1560, 1488, 1472, 1400, 1384, 1260, 1175, 1128, 847, 778, 720, 687, 621, 498, 476 cm^{-1}; (ESI^+) *m*/*z* calcd for $C_{21}H_{38}N^+$: 304.30 found 304.38; (ESI^-) *m*/*z* calcd for $C_{16}H_{18}N_3O_5S^-$ 364.10, found $[M+OH]^-$ 380.93.

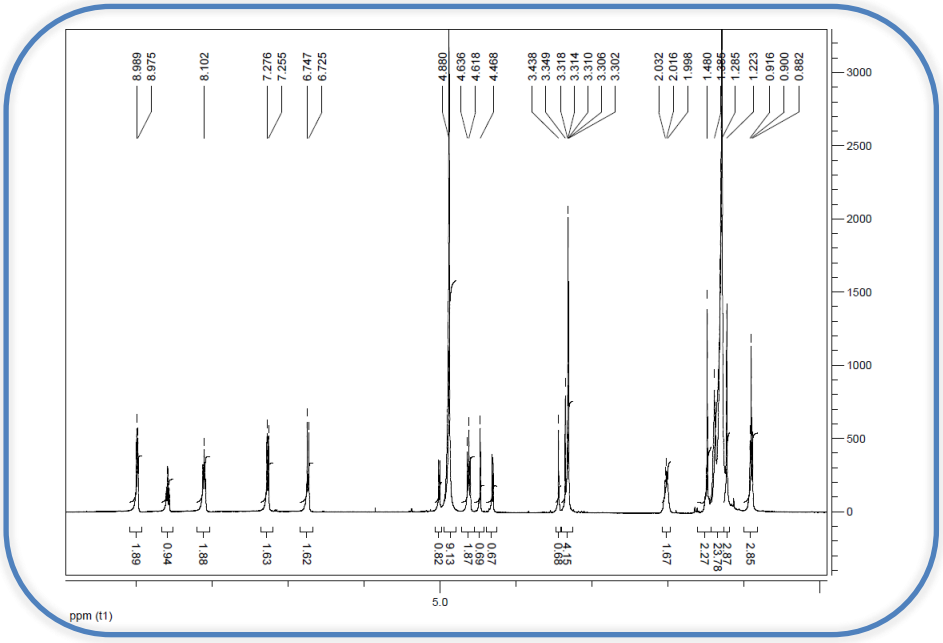


Figure 3.61. [C_{16}Pyr][Amx] ^{1}H-NMR spectrum in CD_3OD.

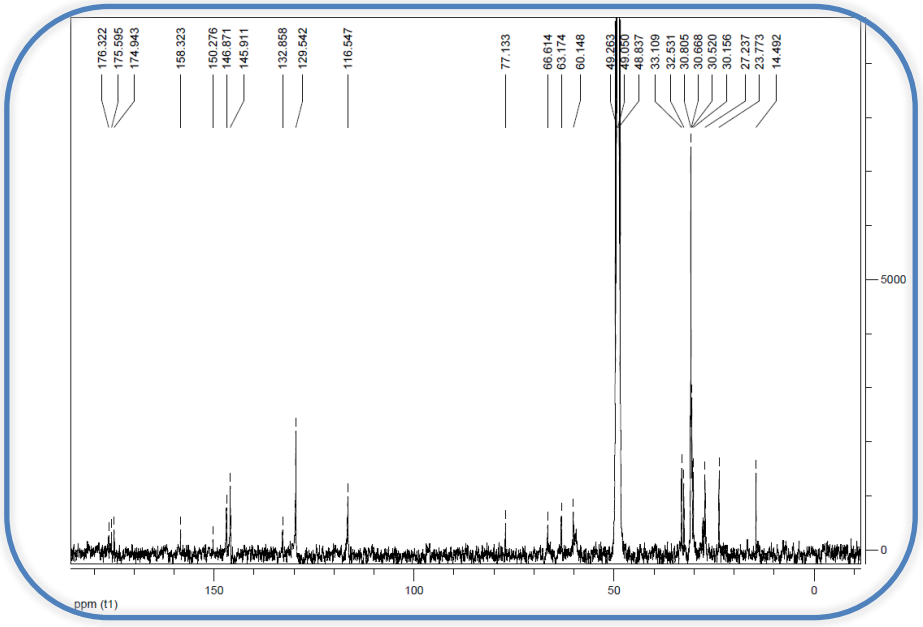


Figure 3.62. [C_{16}Pyr][Amx] ^{13}C-NMR spectrum in CD_3OD.

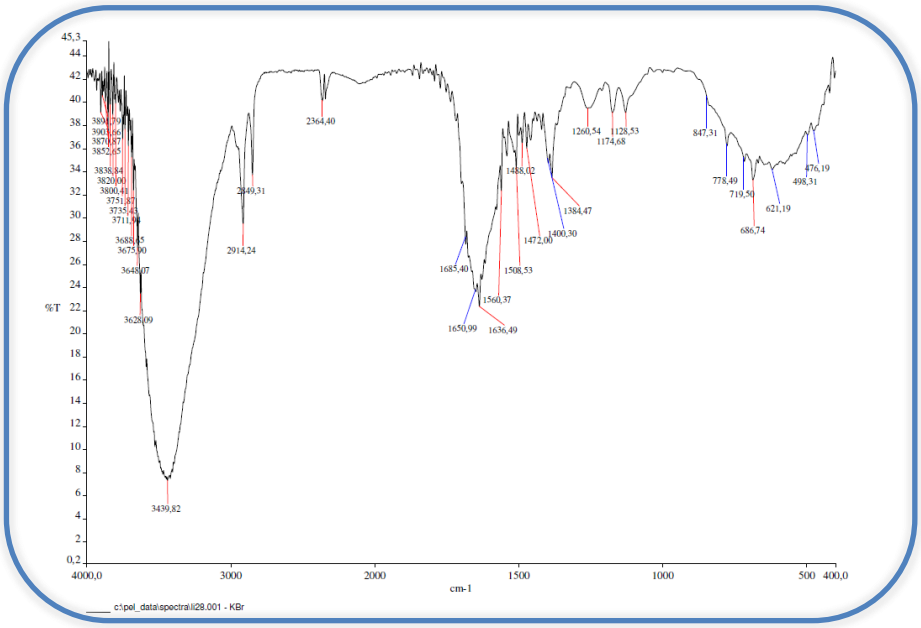


Figure 3.63. [C16Pyr][Amx] IR spectrum in KBr.

3.1.3.4 Preparation of 3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3- (2S,5R,6R)-6-((R)-2-amino-2-(4-hydroxyphenyl)acetamido)-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate [C2OHMIM][Amx]

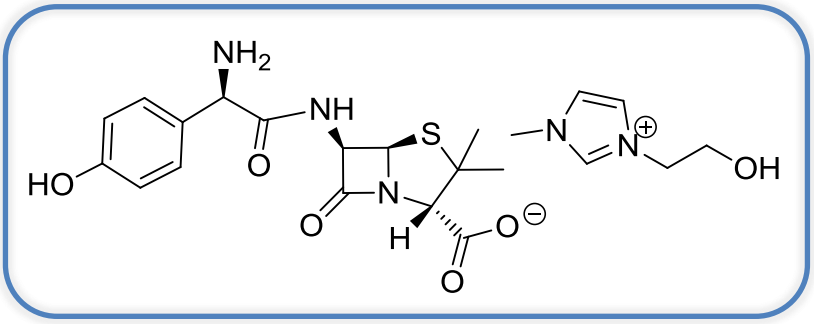


Figure 3.64 Struture of [C2OHMIM][Amx].

3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol chloride (0.456 g; 1.28 mmol) was dissolved in methanol and passed through an ion-exchange column Amberlite IRA-400-OH[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amoxicillin (0.525 g; 1.44 mmol) dissolved in 1.0 M ammonium solution (50 $mgmL^{-1}$). The mixture was stirred at room temperature for 1 h. After solvent evaporation, the residue was dissolved in 20 mL solution (methanol/acetonitrile 1:9)[26,30] and left refrigerated overnight (4 °C)[30] to induce crystallization of excess of amoxicillin. Then amoxicillin crystals were filtered from the solution, the solution was evaporated and the rest dried *in vacuum* for 24 h to provide

the desired product as a yellow solid (0.359 g; 62.1 %). m.p. 109-111 ^{o}C; $[\alpha]_{D}^{25}$ = 47.3 ± 3.6 (c = 2 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, CD_3OD) δ = 7.61 (s, 1H), 7.55 (s, 1H), 7.27 (d, 2H, *J* = 8.4 Hz), 6.74 (d, 2H, *J* = 8.4 Hz), 5.00 (d,1H, *J* = 6.0 Hz), 4.47 (s, 1H), 4.30-4.27 (m, 3H), 3.92 (s, 3H), 3.86 (t, 2H, *J* = 4.86 Hz), 3.43 (s, 1H), 1.48 (s, 3H), 1.22 (s, 3H) ppm; ^{13}C-NMR (100.62 MHz, CD_3OD) δ = 176.33, 175.62, 174.95, 158.34, 132.89, 129.54. 124.75,124.04, 116.56, 77.17 66.64, 61.11, 60.15, 59.83, 59.50, 53.81, 36.45, 27.78, 27.47 ppm; IR (KBr): υ = 3420, 2970, 2921, 1722, 1690, 1655, 1599, 1577, 1545, 1509, 1436, 1386, 1322, 1251, 1170, 1132, 1108, 1067, 877, 840, 820, 778, 652, 621, 535, 474 cm^{-1}; (ESI^{+}) *m*/*z* calcd for $C_6H_{11}N_2O^{+}$: 127.2, found 127.0; (ESI^{-}) *m*/*z* calcd for $C_{16}H_{18}N_3O_5S^{-}$ 364.10, found $[M+OH]^{-}$ 380.9.

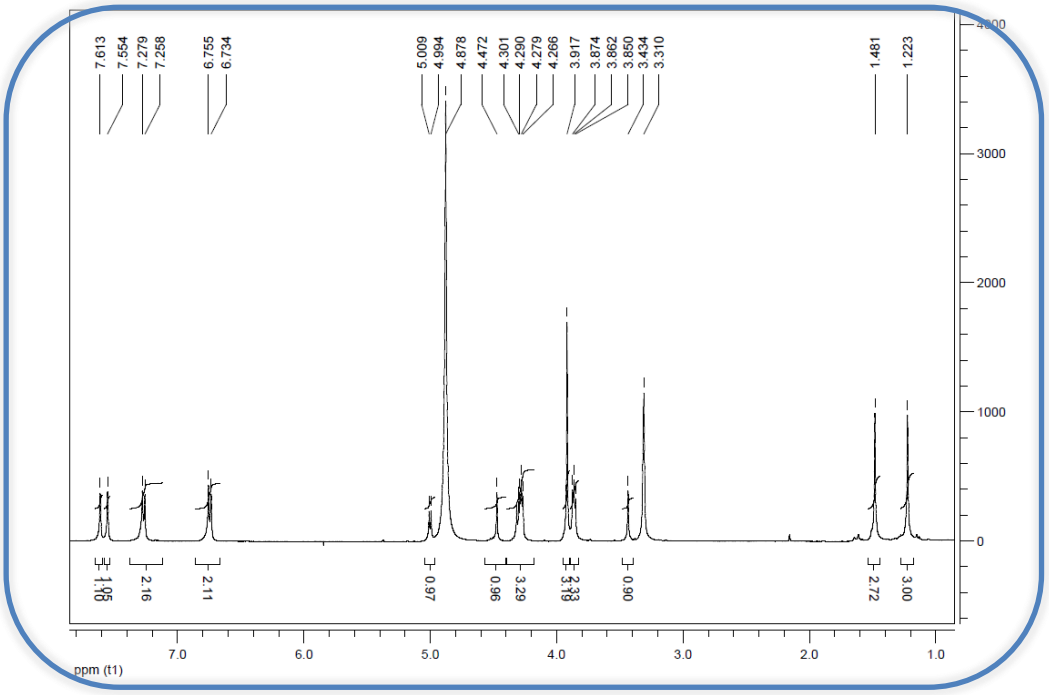


Figure 3.65. [C_2OHMIM][Amx] ^{1}H-NMR spectrum in CD_3OD.

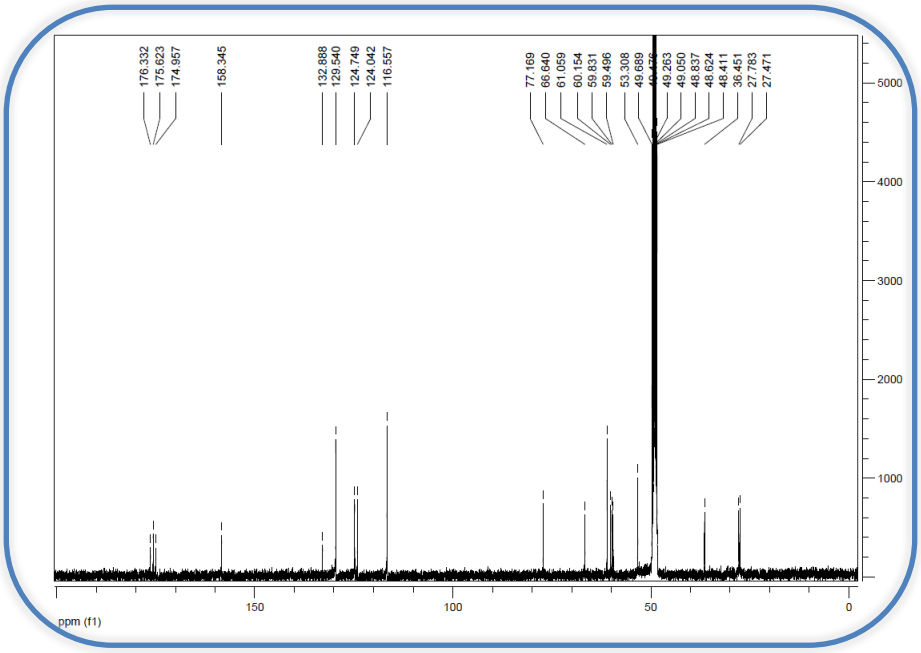


Figure 3.66. [C_2OHMIM][Amx] ^{13}C-NMR spectrum in CD_3OD.

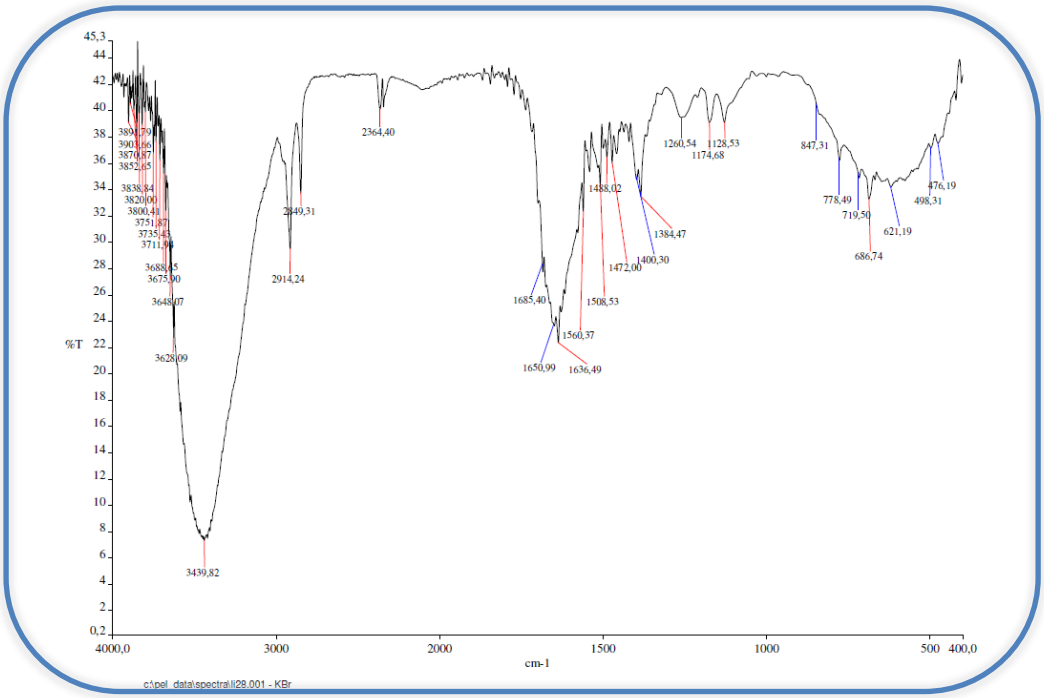


Figure 3.67. [C_2OHMIM][Amx] IR spectrum in KBr.

3.1.4 Synthesis of Amphotericin B ILs

In order to minimize the impact from Grob fragmentation[158,159] and ester hydrolysis, pure API-ILs based on Amphotericin B were prepared using dried solvents (water-free conditions at the beginning of the reaction).

3.1.4.1 Preparation of 1-Hexadecylpyridin-1-ium (1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-(((2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylate [C_{16}Pyr][AmphB]

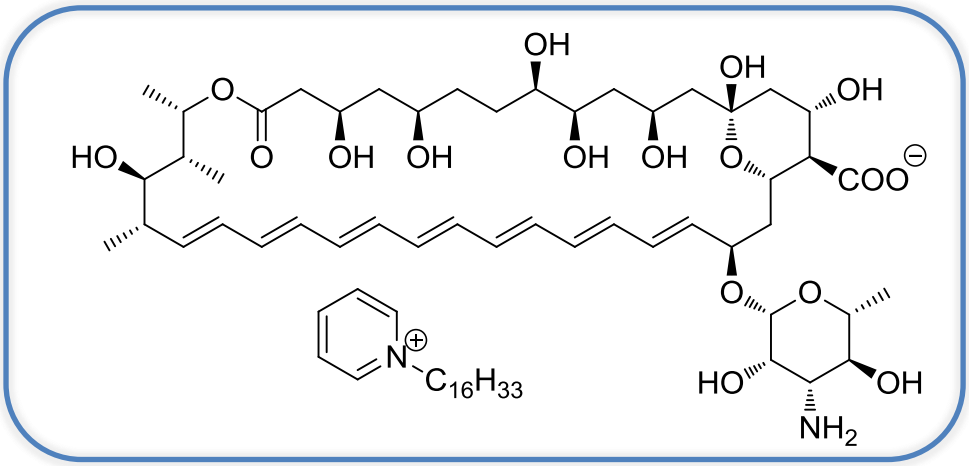


Figure 3.68 Struture of [C_{16}Pyr][AmphB].

Cetylpyridinium chloride (0.088g; 0.246 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH)[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amphotericin B (0.250 g; 0.271 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. Then the solution was filtered and recrystallizes from methanol in order to remove the amphotericin crystalline impurities insoluble in methanol and then it was treated with calcium carbonate (3.0 g; 30.0 mmol), to remove acid impurities. After solvent evaporation, it was dried *in vacuum* for 24 h to provide the desired product as an orange solid (0.215 g; 71.3 %). $[\alpha]_D^{25}$ = 49.7 ± 5.8 (c = 0.2 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 9.10 (d, 2H, *J* = 5.6 Hz), 8.60 (t, 1H, *J* = 7.6 Hz), 8.16 (t, 2H, *J* = 6.7 Hz), 6.47-5.97 (m, 14H), 5.65 (bs, 1H), 5.51-5.40 (m, 1H), 5.33-5.31 (m, 1H), 5.21-5.20 (m, 1H), 4.79-4.78 (m, 3H), 4.59 (t, 3H, *J* = 7.4 Hz) 4.34 (bs, 1H), 4.24-4.23 (m, 2H), 4.17-4.12 (m, 1H), 4.07-4.05 (m, 1H), 3.74-2.81 (m, 12H), 2.41-2.25 (m, 3H), 2.15 (d, 1H, *J* = 5.7 Hz), 1.91-1.89 (m, 2H), 1.82-1.37 (m, 16H), 1.27-1.23 (m, 28H), 1.14 (d, 3H, *J* = 5.8 Hz), 1.11 (d, 3H, *J* = 6.1 Hz), 1.03 (d, 3H, *J* = 5.9 Hz), 0.90 (d, 3H, *J* = 6.9 Hz), 0.85 (t, 3H, *J* = 6.7 Hz) ppm; ^{13}C-NMR (150 MHz, $(CD_3)_2SO$) δ = 170.45, 145.37, 144.64, 133.66, 133.63, 133.61, 133.47, 133.17, 132.70, 132.66,

132.25, 132.20, 131.99, 131.90, 131.84, 131.80, 127.99, 99.28, 96.65, 79.10, 78.88, 78.66, 73.50, 72.87, 69.18, 68.75, 67.70, 66.15, 60.68, 44.65, 44.29, 44.27, 41.96, 35.08, 31.20, 30.61, 28.95, 28.91, 28.81, 28.68, 28.61, 28.28, 25.31, 22.00, 18.41, 18.00, 16.91, 13.87, 11.99 ppm; IR (KBr): υ = 3435, 3010, 2920, 2852, 1638, 1579, 1563, 1488, 1456, 1401, 1383, 1340, 1324, 1272, 1181, 1130, 1108, 1069, 1037, 1009, 982, 908, 887, 853, 772, 719, 683 cm^{-1}. MALDI-TOF-MS analysis: *m/z* calcd for $C_{21}H_{38}N^{+}$: 304.2999 found 304.3117; *m/z* calcd for *m/z* calcd for $C_{47}H_{72}NO_{17}^{-}$ 922.4806, found $[M-2H]^{-}$ 920.5183.

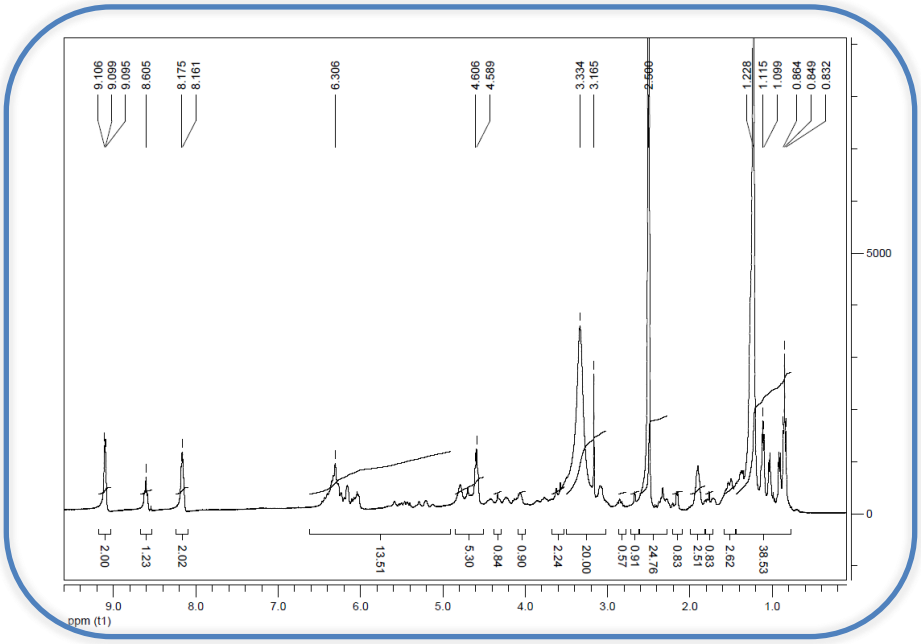


Figure 3.69. [C_{16}Pyr][AmphB] ^{1}H-NMR spectrum in $(CD_3)_2SO$.

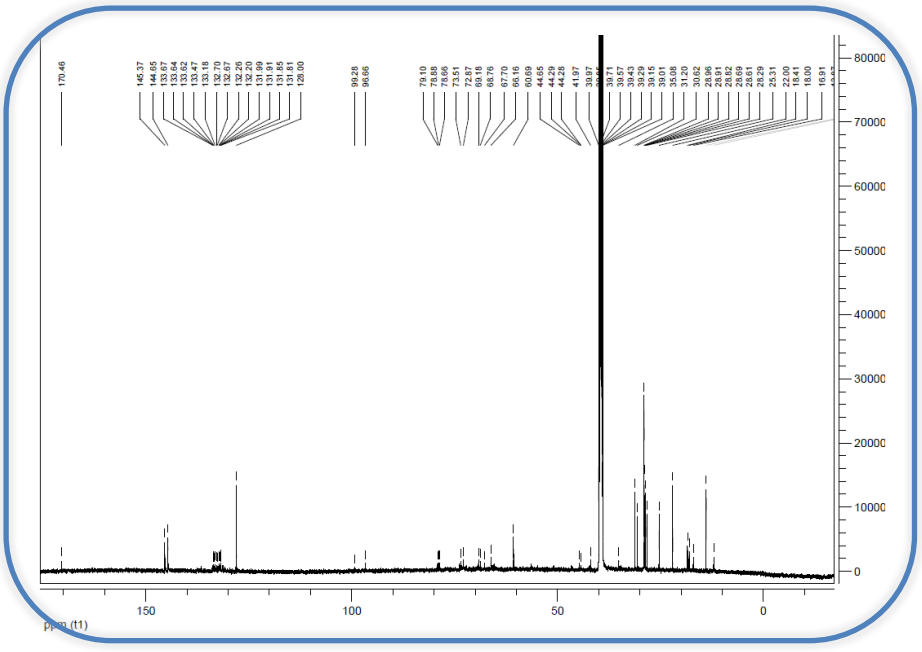


Figure 3.70. [C_{16}Pyr][AmphB] ^{13}C-NMR spectrum in $(CD_3)_2SO$.

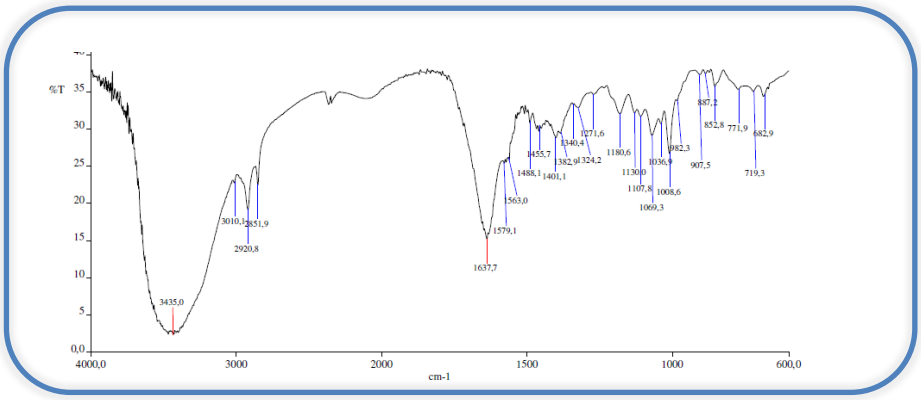


Figure 3.71. [C_{16}Pyr][AmphB] IR spectrum in KBr.

3.1.4.2 Preparation of (2-Hydroxyethyl)-trimethylammonium (1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-(((2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylate [Cholin][AmphB]

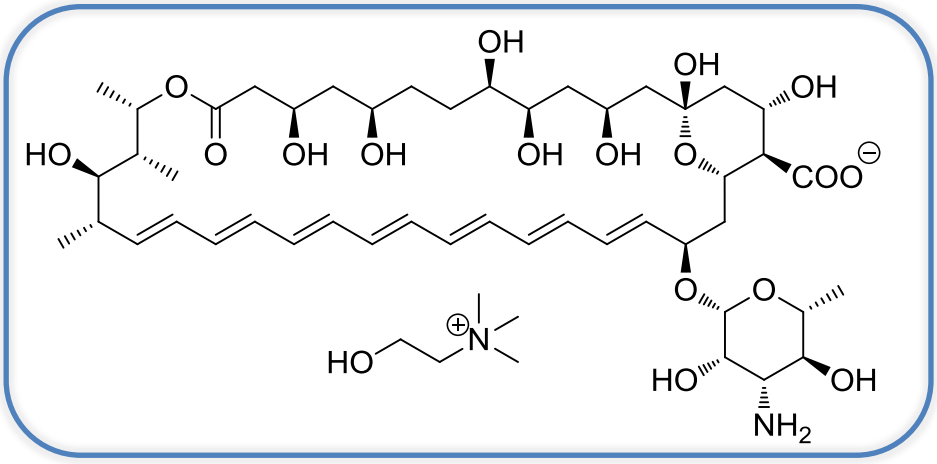


Figure 3.72 Struture of [Cholin][AmphB].

(2-Hydroxyethyl)-trimethylammonium chloride (0.0355g; 0.254 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH)[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amphotericin B (0.249 g; 0.269 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. Then the solution was filtered and recrystallizes from methanol in order to remove the

amphotericin crystalline impurities insoluble in methanol and then it was treated with calcium carbonate (3.0 g; 30.0 mmol), to remove acid impurities. After solvent evaporation, it was dried *in vacuum* for 24 h to provide the desired product as an orange solid (0.1335 g; 51.1 %). m.p. degradated.; $[\alpha]_D^{25}$ = 50.0 ± 5.8 (c = 1 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 6.47-5.97 (m, 14H), 5.68 (bs, 1H), 5.46-5.40 (m, 1H), 5.35-5.32 (m, 1H), 5.24-5.20 (m, 1H), 4.98-4.74 (m, 1H), 4.63 (bs, 1H) 4.34 (bs, 1H), 4.37-4.32 (m, 1H), 4.26-4.24 (m, 1H), 4.08-4.04 (m, 1H), 3.84-3.83 (m, 6H), 3.61-3.64 (m, 4H), 3.60-3.38 (m, 10H), 3.10 (s, 9H), 2.33-2.27 (m, 3H), 2.15 (d, 1H, *J* = 5.8 Hz), 1.91-1.70 (m, 1H), 1.65-1.31 (m, 10H), 1.23 (s, 3H), 1.14 (d, 3H, *J* = 5.6 Hz), 1.10 (d, 3H, *J* = 6.1 Hz), 1.03 (d, 3H, *J* = 6.0 Hz), 0.91 (d, 3H, *J* = 7.0 Hz), 0.83 (t, 3H, *J* = 6.7 Hz) ppm; IR (KBr): υ = 3398, 3018, 2917, 2077, 1638, 1577, 1559, 1506, 1460, 1401, 1387, 1324, 1270, 1183, 1130, 1110, 1073, 1035, 982, 956, 851, 721 cm^{-1}. MALDI-TOF-MS analysis: *m*/*z* calcd for $C_5H_{14}NO^+$: 104.1070, found 104.1080; *m*/*z* calcd for $C_{47}H_{72}NO_{17}^-$ 922.4806, found $[M-2H]^-$ 920.6717.

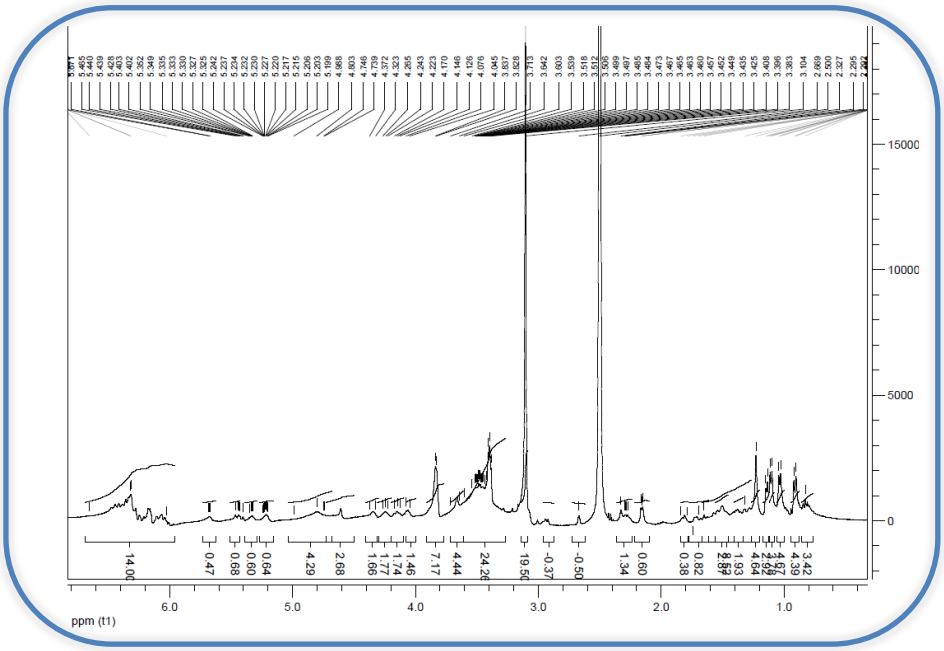


Figure 3.73. [Cholin][AmphB] ^{1}H-NMR spectrum in $(CD_3)_2SO$.

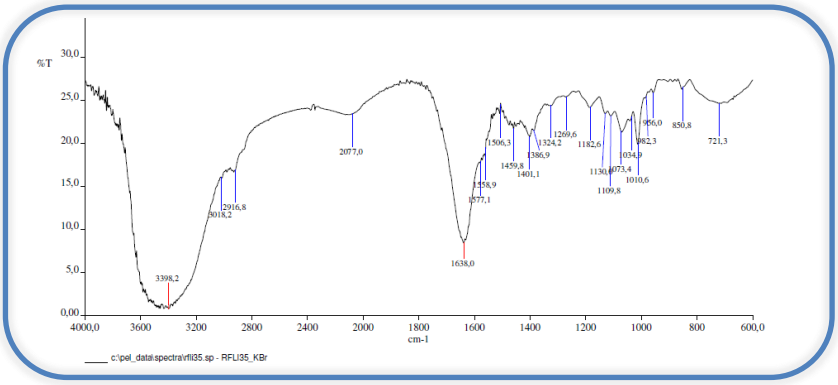


Figure 3.74. [Cholin][AmphB] IR spectrum in KBr.

3.1.4.3 Preparation of (3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium (1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-(((2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylate [C$_2$OHMIM][AmphB]

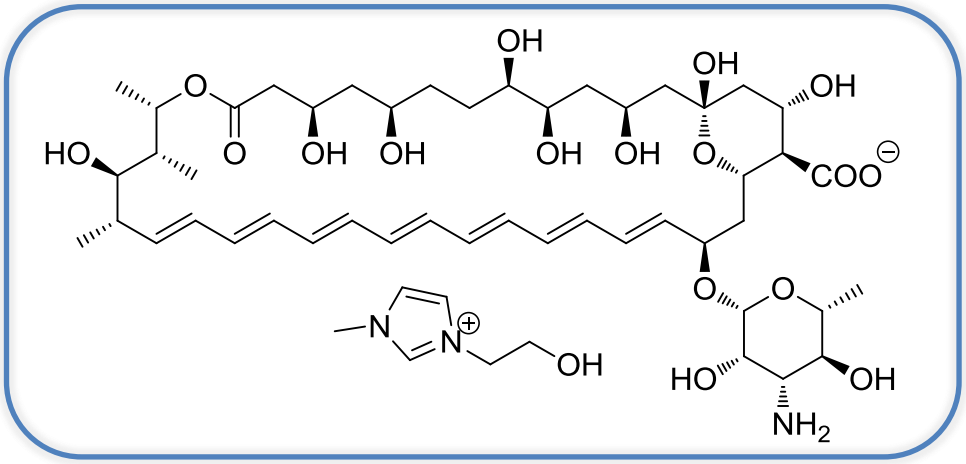


Figure 3.75 Struture of [C$_2$OHMIM][AmphB].

3-(2-Hydroxyethyl)-1-methyl-1*H*-imidazol-3-ium (0.040 g; 0.226 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH)[26,30] (5 eq., flux rate 0.133 mLmL^{-1}min^{-1} = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amphotericin B (0.251 g; 0.272 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. Then the solution was filtered and recrystallizes from methanol in order to remove the amphotericin crystalline impurities insoluble in methanol and then it was treated with

calcium carbonate (3.0 g; 30.0 mmol), to remove acid impurities. After solvent evaporation it was dried *in vacuum* for 24 h to provide the desired product as orange solid. (0.1731 g; 72.8 %). m.p. degradated.; $[\alpha]_D^{25} = 63.0 \pm 5.8$ (c = 1 mgmL^{-1} in methanol); ^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 9.10 (s, 1H), 7.72 (s, 1H), 7.69 (s, 1H), 6.48-5.96 (m, 14H), 5.69 (bs, 1H), 5.53-5.40 (m, 1H), 5.32 (bs, 1H), 5.21-5.10 (m, 1H), 4.86-4.70 (m, 2H), 4.65-4.57 (m, 2H), 4.44-4.33 (m, 2H), 4.21 (t, 3H, *J* = 4.9 Hz), 4.08-4.03 (m, 2H), 3.86 (s, 3H), 3.72 (t, 3H, *J* = 4.9 Hz), 3.64-3.17 (m, 12H), 3.13-2.84 (m, 5H), 2.36-2.28 (m, 2H), 2.16 (d, 1H, *J* = 5.9 Hz), 1.86- 1.23 (m, 16H), 1.14 (d, 3H, *J* = 5.9 Hz), 1.11 (d, 3H, *J* = 6.1 Hz), 1.04-1.01 (m, 3H), 0.91 (d, 3H, *J* = 6.9 Hz) ppm; IR (KBr): υ = 3436, 2924, 2856, 1637, 1567, 1490, 1468, 1458, 1403, 1389, 1328, 1272, 1233, 1183, 1132, 1104,1071, 1009, 978, 905, 857, 776, 721, 687 cm^{-1}. MALDI-TOF-MS analysis: *m*/*z* calcd for $C_6H_{11}N_2O^+$: 127.0866, found 127.0710; *m*/*z* calcd for $C_{47}H_{72}NO_{17}^-$ 922.4806, found $[M\text{-}2H]^-$ 920.4689.

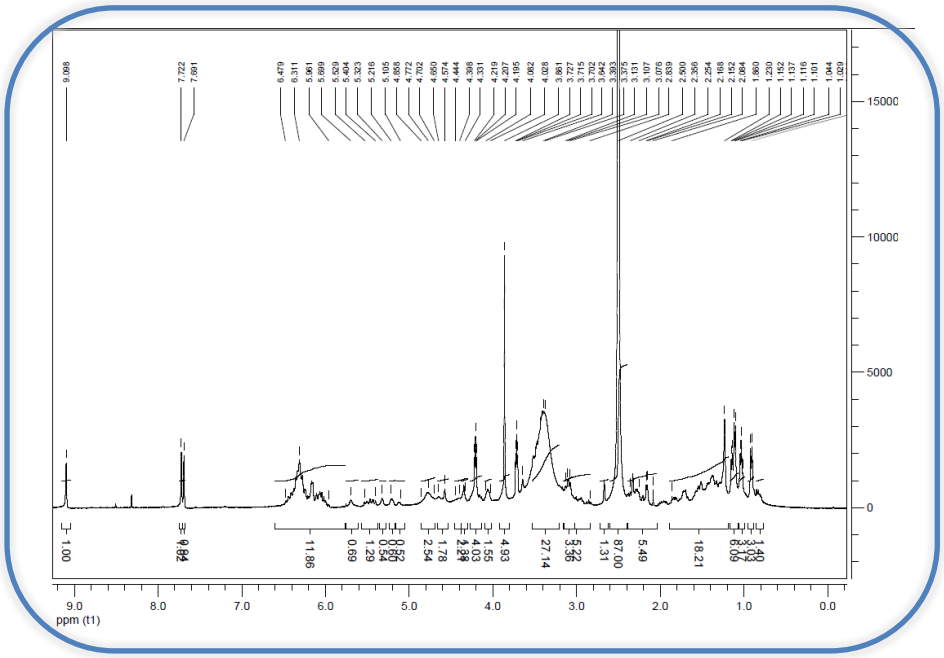


Figure 3.76. $[C_2OHMIM][AmphB]$ ^{1}H-NMR spectrum in $(CD_3)_2SO$.

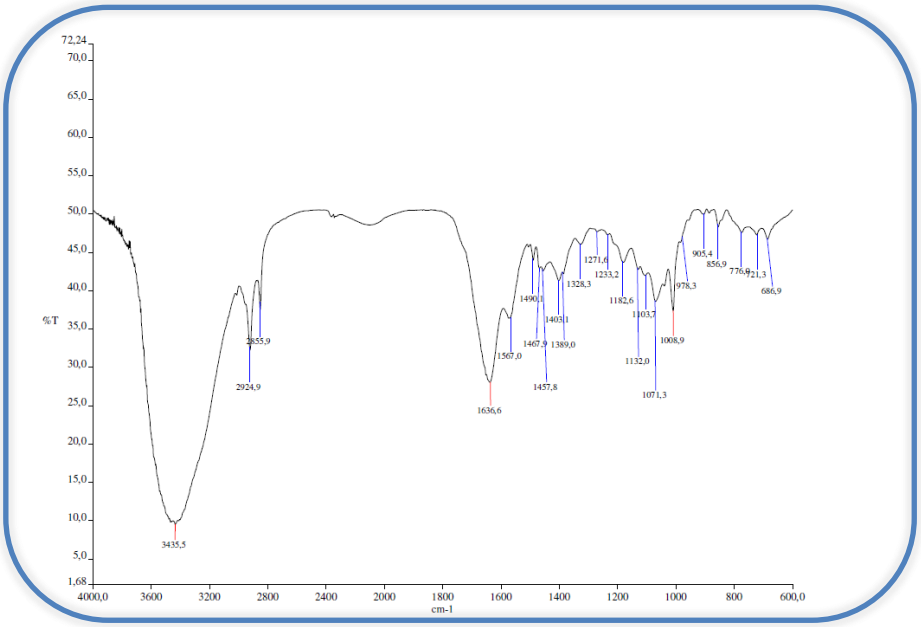


Figure 3.77. [C2OHMIM][AmphB] IR spectrum in KBr.

3.1.4.4 Preparation of (3-(2-Methoxyethyl)-1-methyl-1*H*-imidazol-3-ium (1R,3S,5R,6R,9R,11R,15S,16R,17R,18S,19E,21E,23E,25E,27E,29E,31E,33R,35S,36R,37S)-33-(((2R,3S,4S,5S,6R)-4-amino-3,5-dihydroxy-6-methyltetrahydro-2*H*-pyran-2-yl)oxy)-1,3,5,6,9,11,17,37-octahydroxy-15,16,18-trimethyl-13-oxo-14,39-dioxabicyclo[33.3.1]nonatriaconta-19,21,23,25,27,29,31-heptaene-36-carboxylate [C3OMIM][AmphB]

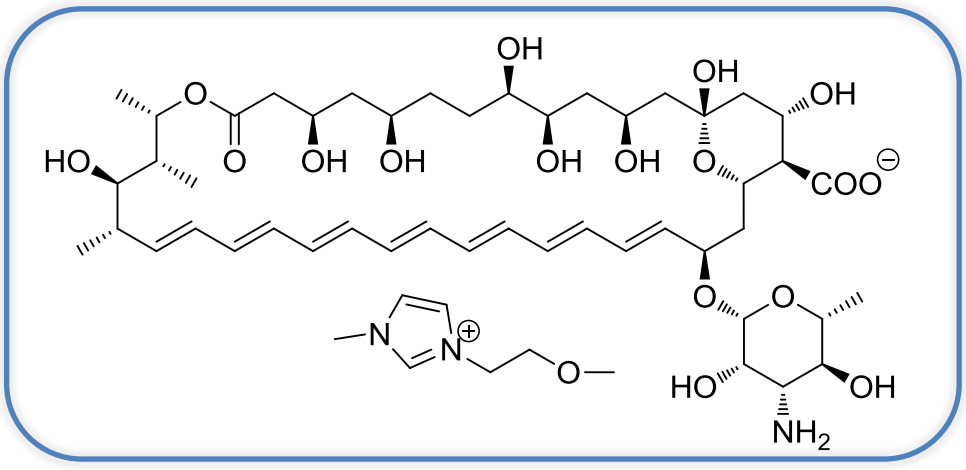


Figure 3.78 Struture of [C3OMIM][AmphB].

(3-(2-methoxyethyl)--1-methyl-1-methyl-1*H*-imidazol-3-ium (0.041 g; 0.252 mmol) was dissolved in methanol and passed through an ion-exchange Amberlite IRA-400(OH)[26,30] (5 eq., flux rate 0.133 $mLmL^{-1}min^{-1}$ = 8 BVh^{-1}). Then the hydroxide solution formed was slowly added to amphotericin B (0.251 g; 0.272 mmol) dissolved in 1.0 M dried triethylamine methanolic solution. The mixture was stirred at room temperature for 1 h. Then the solution was filtered and recrystallizes from methanol in order to remove the amphotericin crystalline impurities insoluble in methanol and then it was treated with calcium carbonate (3.0 g; 30.0 mmol), to remove acid impurities. After solvent evaporation, it was dried *in vacuum* for 24 h to provide the desired product as orange solid. (0.1592 g; 59.5 %). m.p. degradated.; $[\alpha]_D^{25}$ = 41.0 ± 7.1 (c = 1 $mgmL^{-1}$ in methanol); ^{1}H-NMR (400.13 MHz, $(CD_3)_2SO$) δ = 9.11 (s, 1H), 7.73 (s, 1H), 7.70 (s, 1H), 6.51-5.96 (m, 14H), 5.71 (bs, 1H), 5.53-5.40 (m, 2H), 5.35 (bs, 1H), 5.21-5.11 (m, 1H), 4.85-4.63 (m, 5H), 4.57-4.56 (s, 2H), 4.36-4.34 (m, 4H), 4.26-4.22 (m, 2H), 4.19-4.14 (m, 2H), 4.07-4.04 (m, 2H), 3.58-3.36 (m, 12H), 3.26 (s, 3H), 2.33-2.25 (m, 2H), 2.16 (d, 1H, *J* = 5.6 Hz), 1.91- 1.23 (m, 16H), 1.15 (d, 3H, *J* = 5.7 Hz), 1.11 (d, 3H, *J* = 6.2 Hz), 1.04 (d, 3H, *J* = 5.9 Hz), 0.91 (d, 3H, *J* = 6.9 Hz) ppm; IR (KBr): υ = 3435, 2921, 2848, 1656, 1648, 1579, 1561, 1490, 1480, 1456, 1385, 1322, 1262, 1179, 1130, 1106,1069, 1039, 1009, 901, 853, 776, 719, 685 cm^{-1} MALDI-TOF-MS analysis: *m*/*z* calcd for $C_6H_{11}N_2O^+$: 127.0866, found 127.0710; *m*/*z* calcd for $C_{47}H_{72}NO_{17}^-$ 922.4806, found $[M-2H]^-$ 920.4689.

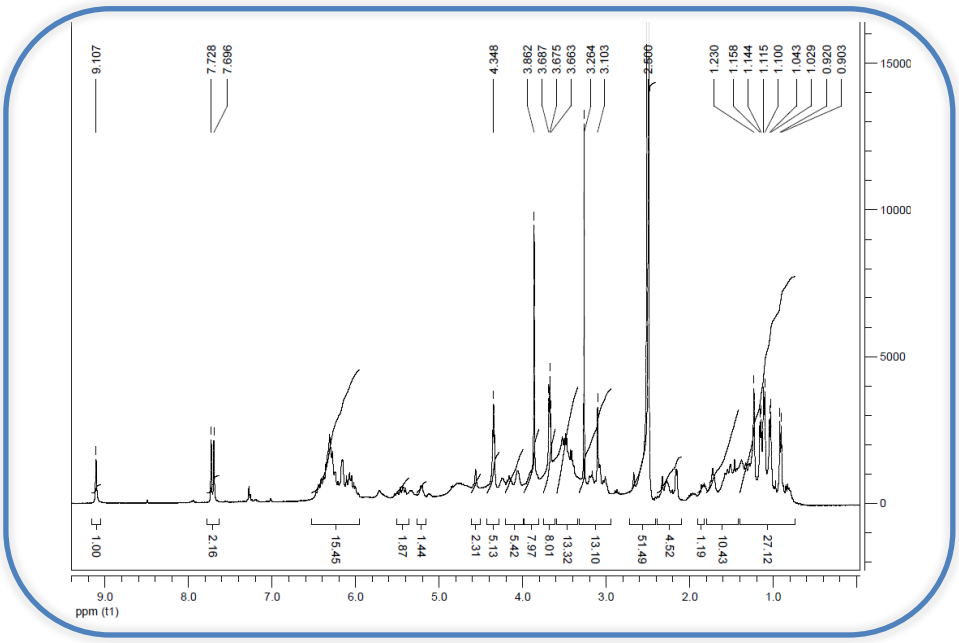


Figure 3.79. [C_3OMIM][AmphB] ^{1}H-NMR spectrum in $(CD_3)_2SO$.

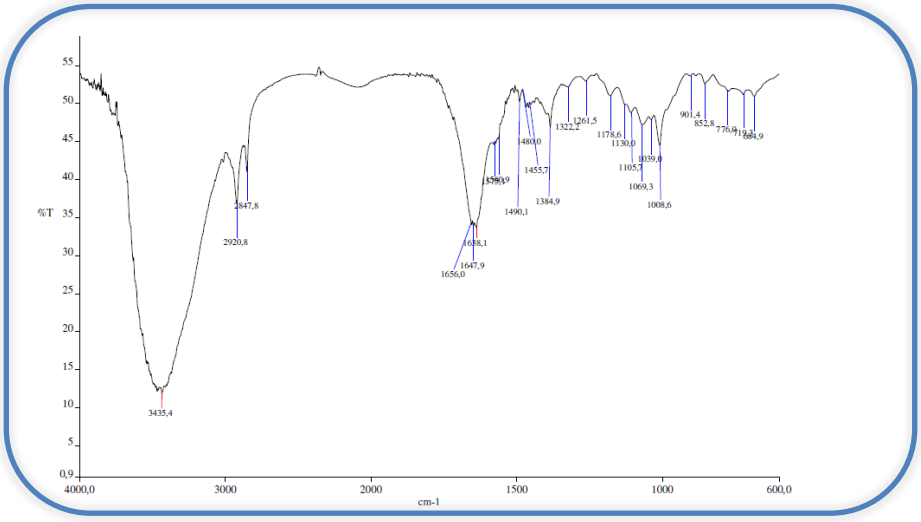


Figure 3.80. [C_3OMIM][AmphB] IR spectrum in KBr.

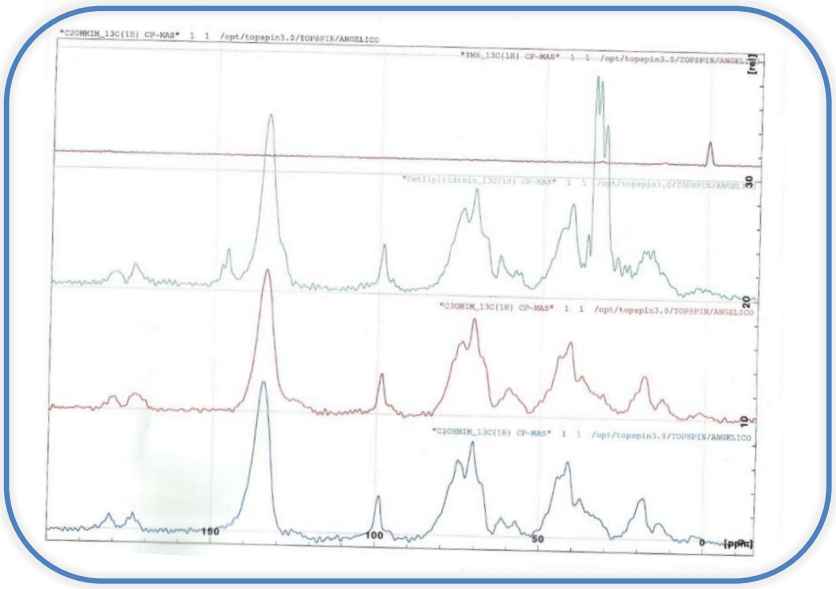


Figure 3.81. ^{13}CP-MAS NMR spectrum: a) TMS, b) [C_{16}Pyr][AmphB], c) [C_3OMIM][AmphB], d) [C_2OHMIM][AmphB].

3.2 Antimicrobial Activity Studies

3.2.1 Microorganisms and Growth Conditions

All the bacteria cells studied were stored at -80 ^{o}C by Vibakstore cryopreservation systems. A total of 8 bacterial strains were used in this study. The following reference

strains were obtained from the American Culture Collection (ATCC): *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. *Klebsiella pneumonia, Staphylococcus epidermidis* and *Enterococcus fecalis* and the resistant bacteria, *E coli* Tem CTX M9, *E. coli* CTX M2, *E. coli* AmpC Mox2 were clinical isolates from previous works[27-29,50-52]. These resistant strains were identified by Vitek®2 systems from bioMérieux . Bacteria were grown on Trypticase soy agar (TSA) for 24 h at 37 °C prior to each test. The bacteria assays were made in Tryptic Soy Broth (TSB).

The eukaryotic model used was *Saccharomyces cerevisiae* PYCC 4072 (from the Portuguese Yeast Culture Collection). This strain was stored at -80 °C in Yeast Extract Peptone Dextrose (YEPD) medium with 15% glycerol. The yeast cells were grown on YEPD with agar at 37 °C for 24 h prior to each test. The yeast assays were made in liquid YEPD.

3.2.2 Culture Medium

The composition and preparation of the different culture medium used in this work are described in this section.

Trypticase Soy Agar (TSA) – TSA (CULTIMED) is a dehydrated culture medium quite common for growth and isolation of bacteria[160]. It is a solid medium and was used for the growth and isolation of Gram-positive and Gram-negative bacteria. This medium was prepared by suspending 40 g of dehydrated medium in 1 L of distilled water. Then it was heated to boiling point while constantly stirring and boiled for 1 minute more. Then it was sterilized at 121 °C for 15 minutes and distributed into sterile Petri dishes. The final composition per litre of the medium was: Soy Peptone (5.0 g), Casein Peptone (15.0 g), Sodium Chloride (5.0 g) and Agar (15.0 g).

Tryptone Soy Broth (TSB) – TSA (CULTIMED) is a dehydrated culture medium. It is a liquid medium used for the micro dilution susceptibility testing[160]. This medium was prepared by suspending 30 g of dehydrated medium in 1 L of distilled water. It was heated to boiling point while constantly stirring and boiled for 1 minute more. Then it was sterilized at 121 °C for 15 minutes and distributed into test tubes (± 5 mL/tube) for

growth or distributed in 96-well microtiter plate for the micro dilutions susceptibility tests. The final composition per litre of the medium was: Soy Peptone (3.0 g), Casein Peptone (17.0 g), Sodium Chloride (5.0 g), di-Potassium Hydrogen Phosphate (2.5 g).

Yeast Extract Peptone Dextrose (YEPD) – This medium is used for maintaining and developing yeast. This medium could be liquid or solid (with agar). It was prepared by dissolving the following components: yeast extract (10.0 g, (SIGMA)), peptone (20.0 g, (CULTIMED)), glucose (20.0 g, (MERCK)) in one litter. In the case of a solid medium, agar was added (15.0 g, LIOFILCHEM) to the liquid medium. After medium preparation it was autoclaved at 121 °C for 15 minutes and distributed into test tubes (± 5 mL/tube) for growth or distributed in 96-well microtiter plate for the micro dilutions susceptibility tests.

3.2.3 Minimum Inhibitory Concentration (MIC)

The MIC values were determined in triplicate by the broth micro dilution method in a 96-well microtiter plate using TSB, according to the methodology from Clinical Laboratory Standard Institute (CLSI)[160]. Prior to MIC determination, each inoculum density was adjusted in TSB to 0.5 McFarland standard by a photometric device[160]. This resulted in a suspension containing approximately 1×10^8 to 2×10^8 colony forming units $CFUmL^{-1}$ for *E.* coli ATCC25922®[160]. A similar approach was used for the other strains. Following this step, 0.5 µL of the suspension was added to each well in order to obtain 0.5 – 2.5 x 10^4 $CFUmL^{-1}$ as final concentration. Bacteria were exposed to an IL concentration of 5.0 mM, 2.5 mM, 0.5 mM, 0.05 mM, 0.005 mM, 0.0005 mM and 0.00005 mM. With the exception of $[P_{6,6,6,14}]$ cations that were diluted in 1 % dimethylsulphoxide all others were dissolved in water and these results were compared with bacteria that had grown in TSB broth in the presence of 1% DMSO as a control. The MIC for each Amp-IL was recorded as the lowest concentration that showed no turbidity after 24 h of incubation at 37 ^oC[19,160]. The presence of turbidity is an indication of microbial growth and the corresponding concentration is considered ineffective.

In the case of *S. cerevisiae,* the method used to determine the MICs was the exact same procedure replacing the TSB medium by the YEPD liquid medium.

3.2.4 Minimum Bactericidal Concentration (MBC)

To study if the antimicrobial activity of the synthesized ILs was bacteriostatic (inhibited growth) or bactericidal, the colorimetric assay (XTT method) was used[161]. 5 μL of sodium salt of (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner salt) (XTT) was added to the non-turbid wells of the MIC assay plate and incubated for 3 h at 37 °C for the bactericidal status determination[161,162]. In the case of viable cells with inhibited growth, mitochondrial dehydrogenases of viable cells cleave the tretrazolium ring of XTT yielding dark orange aqueous soluble formazan crystals. However, a solution containing non-viable cells would remain with the same colour[162]. In this case, MICs values were equal to MBC for each of the ILs used, so the antibacterial activities of these compounds were considered to be bactericidal.

3.2.5 Relative Decrease of Inhibitory Concentration (RDIC)

To better understand and compare the results obtained from MIC values studies, we calculated the relative decrease of inhibitory concentration (RDIC). This is the ratio between the MIC values of the API and the ILs synthesised [Cat][API]. In the case of resistant bacteria, RDIC values were calculated, assuming that it is 5 mM in all cases (the highest value that was measured) providing us with the possibility to estimate the minimum positive effect of [Cat][API].

3.2.6 Growth Rate Studies

In order to complete the characterization of anti-bacterial activity, the Growth Rate (GR) when exposed to the MIC values was evaluated. Additionally, GR values in the presence of higher and lower concentration of the MIC previously found for each compound was also determined.

The GR parameter was determined in triplicate by the broth micro dilution method in a 96-well microtiter plate using Tryptic Soy Broth (TSB)[160,163,164]. Approximately 5000-

25000 CFUmL^{-1} of bacteria was exposed to an IL concentration superior, equal or inferior value of the determined MIC, making periodical readings of the optical density (OD) at 620 nm to establish the bacterial population growth curve and then determining the curve's slope. The GR was determined by fitting a linear function to the Exponential (log) phase curve.

3.3 Cell Line Culture Studies

All human cell lines (MG63, RKO, PC3 and T47D) were purchased from the American Type Culture Collection (ATCC). Skin and gingival fibroblasts were obtained from explants collected from healthy 25 to 35 year-old donors with, after informed consent.

Cells were maintained in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, 100 IUmL^{-1} penicillin, 2.5 μgmL^{-1} streptomycin, 2.5 μgmL^{-1} amphotericin B and 50 μgmL^{-1} ascorbic acid. At about 70-80 % confluence, cells were enzymatically detached with 0.05 % trypsin and 0.5 mM EDTA and seeded at 10^4 cellscm^{-2}. After an attachment period of 24 h, the culture medium was renewed, and supplemented with different concentrations (0.005-500 mM) of the ampicillin-derived ILs. Cell cultures were maintained in a 5 % CO_2 humidified atmosphere at 37 ^{o}C. Cellular viability/proliferation was assessed by the 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay at days 1, 3 and 5 of culture, as described before[165,166]. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. Shortly, cultured cells were incubated at 37 ^{o}C with 0.5 mgmL^{-1} of MTT during 4 h. After that the culture medium was removed. Then the stained product was dissolved with dimethylsulfoxide. Finally the absorbance determination was conducted at 550 nm in an ELISA plate reader. Results were expressed as absorbance per square centimetre (Acm^{-2})[165,166].

The values of the half maximal inhibitory concentration (IC50) and the median lethal dose (LD50) were obtained from nonlinear regression analysis of concentration-effect curves using the GraphPad Prism software[167,168]. The definition of the IC50 is given by

Sebaugh[169] as "the response corresponding to the 50% control (the mean of the 0% and 100% assay controls)" and is used to measure the efficacy of a compound in inhibiting any biochemical or biological function. The term LD50 means the median lethal dose and is the amount of material which causes the death of 50% of a population[170].

3.4 Statistical Analysis

In order to evaluate the significance of the growth curves of the selected microorganisms with and without the ILs, and the LD50 and IC50 of the ILs on the selected human cell lines, it was used the statistical software SPSS 15.0 (SPSS Inc., EUA). The sample distribution followed a non-normal distribution and therefore, verified by the Kolmorogov-Smirnov test and the variance analysis was studied by the Levene's test.

The influence of ILs of both microorganisms and human cells lines was evaluated by Mann-Whitney U with a significance level of 0.005.

IC50 and LD50 were calculated using Graphpad software.

Chapter 4.
Development of Novel Ionic Liquids Based on Ampicillin

This chapter is a reproduction of a published refereed article:

Development of novel ionic liquids based on ampicillin

Ricardo Ferraz, Luís C. Branco, Isabel M. Marrucho, João M. M. Araújo, Luís Paulo N. Rebelo, Manuel Nunes da Ponte, Cristina Prudêncio, João Paulo Noronha and Željko Petrovski

Med. Chem. Commun., 2012, **3**, 494-497.

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4 Development of Novel Ionic Liquids Based on Ampicillin

4.1 Abstract

Novel ionic liquids containing ampicillin as active pharmaceutical ingredient anion were prepared with good yields by using a new, efficient synthetic procedure based on the neutralization of a moderately basic ammonia solution of ampicillin with different organic cation hydroxides. The relevant physical and thermal properties of these novel ionic liquids based on ampicillin were also evaluated.

4.2 Introduction

Ionic liquids (ILs) are generally defined as organic salts with melting points below 100 ^{o}C (some of them are liquid at room temperature) and composed entirely of ions[9,95]. The large number of possible cation/anion combinations allows for great variety of tuneable interactions and subsequent applications[9,137,171]. Ionic liquids were initially used as greener alternatives to conventional, toxic and volatile organic solvents[3,9]. Some properties generally attributed to ILs, such as high thermal and electrochemical stability, negligible vapour pressure[171,172], high ionic conductivity[171], non-flammability and a tuneable solvation capacity, encouraged their application across a wide range of areas, including organic chemistry[9,137], chemical engineering[9,171], material science[171,173], physical chemistry[171,174,175], analytical chemistry[171,176], and biotechnology[2,9,107,171].

Recently, ILs have been combined with active pharmaceutical ingredients (APIs), and a so-called third generation of ILs has emerged[8]. These ILs-APIs compounds offer new and improved properties, such as stability, solubility, permeability and drug delivery, as compared to the corresponding solid pharmaceutical forms. The use of an active drug in the liquid form (at room temperature) can avoid some of the issues of polymorphism associated with crystalline solids and, thus, dramatically influence the

drug's solubility and dosages[9,71,150]. However, the entrance of ILs into the biosciences has been delayed mainly because of the toxicity of the counterions[9]. Most recent communications and reviews refer to the toxicity and activity of ILs against microorganism and cell cultures, especially their antimicrobial activity as well as drug delivery performance[9,71,133-135]. ILs have recently been tested in the fight against multi-drug resistance[21,135] and even against microbial biofilms, showing a broad and powerful spectrum of activity against several microbial pathogens, including Methicillin-resistant *Staphylococcus aureus*. The recent outbreak of *E. coli* O104[53,54] in Germany as well as the appearance of multi-drug-resistant organisms such as Gram-negative Enterobacteriaceae given by the New Delhi metallo β-lactamase[55-57] are becoming increasingly serious public health problems worldwide. Thus, the discovery of alternative and efficient pathways for the treatment of infections is one of the most urgent challenges of this century. Novel therapies using ILs as a drug delivery device[9] offer interesting avenues for exploration.

From the pharmaceutical point of view, the possibility of eliminating the negative side effects of a given active compound by delivering it as an ILs-APIs is extremely attractive. Taking advantage of the ILs building up platform, the counter-ion can be meticulously selected in order to minimize those undesirable side effects or to open up novel treatment therapies in which two active ions are paired.

4.3 Results and Discussion

This work intended to develop an efficient, synthetic methodology for preparing several ILs-APIs based on ampicillin [Amp]. Ampicillin has always been used as anion combined with the following organic cations 1-ethyl-3-methylimidazolium, [EMIM], 1-hydroxy-ethyl-3-methylimidazolium, [C_2OHMIM], choline, [Cholin], tetraethylammonium, [TEA], cetylpyridinium, [C_{16}pyr] and trihexyltetradecyl-phosphonium [$P_{6,6,6,14}$]. These anions were chosen due to their low toxicity, except in the case of [$P_{6,6,6,14}$] which was chosen to ensure that a ILs-APIs in the liquid form could be obtained. The combination of the adequate anion or cation with a specific drug can

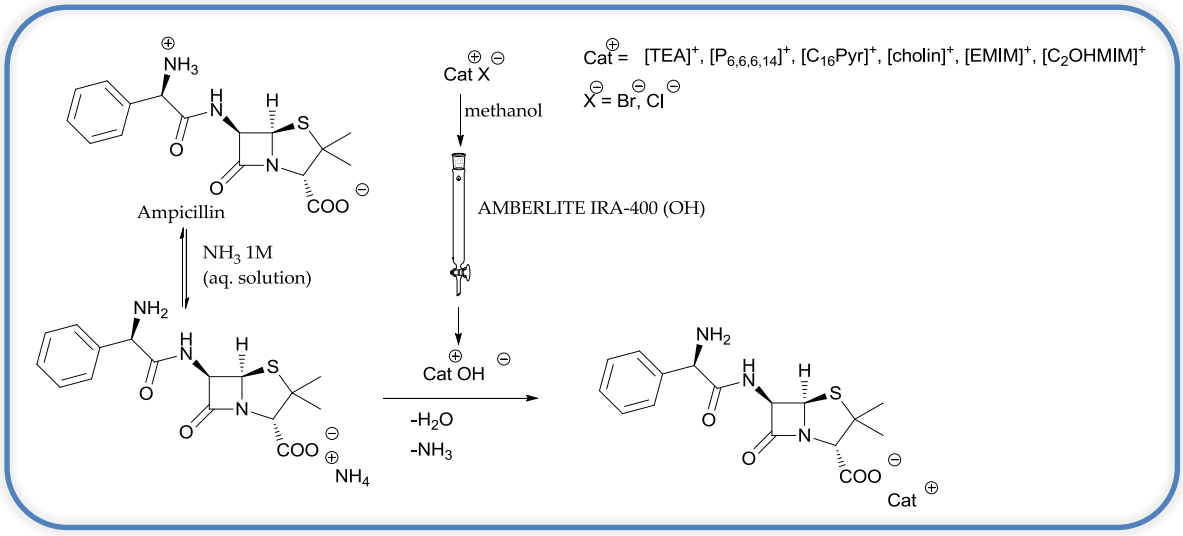
lead to an alteration in the compound's biopharmaceutical drug classification[32] as well as their drug formulation process.

The most conventional synthetic preparation of ILs involves a metathesis reaction of anion halide with an adequate anion and it was also used in the preparation of ILs containing ampicillin anion[19]. The pure IL can be obtained by eliminating undesirable inorganic salts (mainly sodium, potassium or lithium chloride or bromide) using precipitation followed by filtration[177]. The need to obtain pure ILs, especially halide-free ones, has been one of the central concerns within the IL community. The use of inorganic acids instead of salts is a potential approach to reducing these inorganic contaminations[178]. In the case of a large number of inorganic or organic anions, alternatives need to be considered, due to the fact that an anion exchange by weaker acids than hydrohalic acids[91] cannot be efficiently performed. In the case of imidazolium cations, Earle and Seddon proposed the use of imidazole carbenes[84] as strong bases. Nevertheless, this process is restricted to imidazolium cations, due to the high reactivity and low stability of the carbene intermediate[178].

Ion exchange resins methods recently developed by Ohno et al.[26] are being successfully used as alternative anion exchange processes and they have been extended to other reactions. Amberlite resin (in the OH form) has been used in order to exchange halides (bromide or chloride) to the hydroxide form and then this basic solution is neutralized by the addition of an adequate acid solution. The acid-base reaction yields the desired salt or IL[26,91,178]. Our first attempt to use this anion exchange method failed due to ampicillin´s poor solubility in most common organic solvents (with the exception of DMSO), as well as the instability of the β-lactam ring in the presence of strong bases. The decomposition of ampicillin was always detected by NMR analysis after several attempts.

The initial synthetic procedure was modified by dissolving ampicillin in a moderately basic ammonia solution and then neutralizing it with different hydroxides prepared with the Ohno method. Using an ammonia solution buffer (pH = 11.6), the hydrolysis of the sensitive β-lactam ring from a possible hydroxide attack was avoided. Pure ILs-

APIs based on ampicillin structure were obtained after eliminating the excess ammonia and/or ampicillin by evaporation and crystallization respectively. The organic cations were selected from substituted ammonium, phosphonium, pyridinium and methylimidazolium salts which were first transformed into hydroxides by the use of an ionic exchange column (Amberlite IRA-400 OH) in methanol[26]. The prepared hydroxides were then neutralized with the beta-lactam antibiotic (Scheme 4.1).



Scheme 4.1 Schematic synthetic procedure for the preparation of ampicillin-based ILs.

The structure of the cations synthesised and studied in this work appears in Figure 4.1.

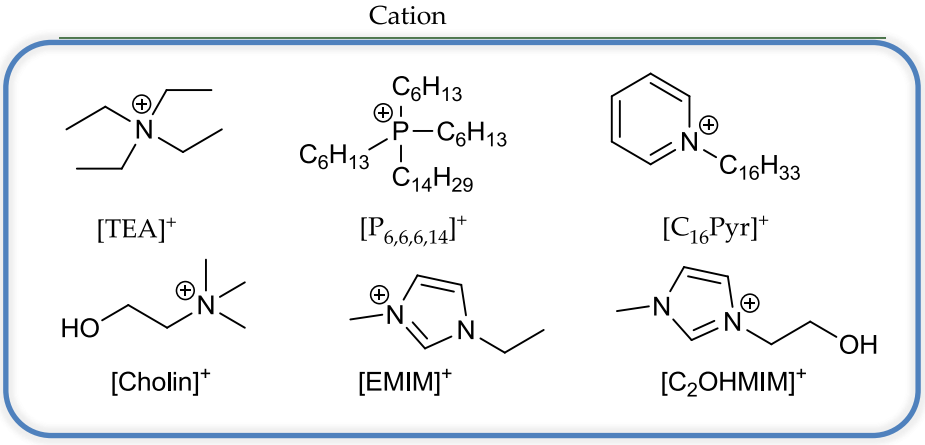


Figure 4.1 Structure of cations used.

All isolated products were completely characterized by ^{1}H and ^{13}C-NMR, FTIR and ESI mass spectra in order to check their expected structures and final purities. NMR

studies also elucidate about the expected cation/anion correlations by a quantitative integration of their characteristic ^{1}H resonance peaks.

4.3.1 Physical Properties

Table 4.1 summarizes some properties of the synthesized ILs-APIs based on Ampicillin. Our optimized synthetic procedure allowed us to obtain pure compounds in high yields (71 to 95%). All compounds are soluble in methanol, ethanol and water (except [$P_{6,6,6,14}$][Amp]). It is important to emphasize that the use of appropriate ammonium and imidazolium cations can effectively change the initial trihydrate ampicillin water solubility (6 $mgmL^{-1}$) and consequently their potential membrane permeability.

Table 4.1 Physical Properties of ILs-APIs based on ampicillin.

Compound	**Yield [%][a]**	**$[\alpha]_D^{27}$ [°] [b]**	**Solubility**	
			Miscible[c]	Immiscible[c]
[TEA][Amp]	76.0	+48.7±2.5	MeOH, EtOH, iPrOH, H_2O	Ac, AcOEt (pm)
[$P_{6,6,6,14}$][Amp] [d]	80.0	+23.3±1.5	MeOH, EtOH, *i*PrOH, Ac, AcOEt	H_2O
[C_{16}Pyr][Amp]	76.4	+51.7±0.9	MeOH, EtOH, *i*PrOH	Ac, AcOEt, H_2O (pm)
[Cholin][Amp]	70.7	+52.3±0.8	MeOH, EtOH, H_2O	*i*PrOH, Ac, AcOEt
[EMIM][Amp]	94.6	+89.3±5.5	MeOH, EtOH, *i*PrOH, H_2O	Ac, AcOEt (pm)
[C_2OHMIM][Amp]	86.8	+86.3±4.5	MeOH, EtOH, H_2O	*i*PrOH (pm), Ac, AcOEt (pm)

[a] Isolated yields. [b] Optical rotation values measured in methanol (2 $mgmL^{-1}$) by polarimetry at 27 °C. [c] Observed complete solubilisation (miscible), partial miscible (pm) or non-solubilisation (immiscible) by adding solvent to a small amount of ionic liquid, MeOH (methanol), EtOH (ethanol), *i*PrOH (*iso*-propanol), Ac (acetone) and AcOEt (ethyl acetate). [d] The water content of [$P_{6,6,6,14}$][Amp] was 14.7 ppm (determined by Karl Fisher titration).

[Cholin][Amp] and [C_2OHMIM][Amp] are immiscible in isopropanol contrarily to other ILs-APIs. [$P_{6,6,6,14}$][Amp] is the only IL-API completely miscible in acetone and ethyl acetate.

Optical rotations values of the prepared ILs-APIs based on ampicillin (+23.3º ± 1.5 to +89.3º ± 4.5 in methanol, Table 4.1) are significantly lower compared with the initial trihydrate ampicillin (+163.0 º ± 2.0 in water) but are of the same order of magnitude as commercial sodium ampicillin (+40.0º ± 4.0). The highest optical rotations of the prepared compounds were observed when imidazolium cation structures were used.

4.3.2 Thermal Properties

The thermal properties of synthesized ILs-APIs based on ampicillin are summarized in Table 4.2.

All the ILs-APIs were obtained as pale yellow solids (melting temperature, ™, between 58 ºC and 86 ºC) except in the case of [$P_{6,6,6,14}$][Amp] (viscous yellow liquid). [C_2OHMIM][Amp] was obtained as a yellow molten salt (T_m = 117 ºC). Particularly relevant is the successful reduction of the initial melting point of commercial ampicillin or sodium ampicillin (higher than 300 ºC) by the appropriate selection of organic cations. The glass transition temperature (Tg) was determined at a heating/cooling rate of 1 ºCmin^{-1} for all compounds. Similar values of glass transition temperatures were detected for all ILs-APIs (-17.86 ºC to -20.84 ºC) except in the case of [$P_{6,6,6,14}$][Amp].

In the case of [$P_{6,6,6,14}$][Amp], DSC curves showed two transition peaks at low temperature which can be attributed to crystallization (-80.07 ºC) and melting (-23.01 ºC) processes, respectively.

Decomposition studies were performed by TGA analysis for all the synthesized compounds. As expected, these studies indicated that the selection of the organic cation influences the thermal stability of ILs-APIs based on ampicillin. [$P_{6,6,6,14}$][Amp] and [C_{16}Pyr][Amp] presented higher thermal stability than those ILs-APIs based on imidazolium and ammonium cations.

Table 4.2 Thermal Properties (T_m, T_g and T_{dec}) of ILs-APIs based on ampicillin.

Compound	Physical State	T_m[a] [°C]	T_g[b] [°C]	T_{dec}[c] [°C]
[TEA][Amp]	Pale yellow Solid	79.0	-18.64	214.75
[$P_{6,6,6,14}$][Amp]	Yellow Viscous Liquid	-	-	297.65
[C_{16}Pyr][Amp]	Pale yellow Solid	86.0	-19.64	269.39
[Cholin][Amp]	Pale yellow Solid	58.0	-20.12	221.29
[EMIM][Amp]	Pale yellow Solid	72.0	-17.86	239.64
[C_2OHMIM][Amp]	Pale yellow Solid	117.0	-20.84	246.40

[a] Melting temperature ™ was determined by a melting point apparatus (Stuart Scientific). [b] Glass transition temperature (Tg) was determined by DSC measurements at a heating/cooling rate of 1 °C.min^{-1} for all ILs. [c] Decomposition temperature (Tdec) was determined by TGA studies.

4.3.3 NMR Studies

A preliminary ^{1}H-NMR study was performed in the case of IL [Cholin][Amp] for different temperatures (25 °C to 85 °C). Figure 4.2 illustrates a comparative ^{1}H-NMR study of [Cholin][Amp] at four temperatures (25, 45, 65 and 85 °C) in d-DMSO. Two different regions of the ^{1}H-NMR spectra between 1 to 2 ppm and 6.5 to 8.8 ppm were particularly selected to check the aliphatic (methyl peaks) and aromatic (phenyl peaks) signals of ampicillin anion, respectively.

In the case of two methyl peaks (1.10 and 1.41 ppm) of ampicillin, a chemical shift to the left was observed between 25 °C and 85 °C. This variation of the chemical shift (at least 0.4 ppm of difference between 25 °C to 85 °C) can be explained by the possible interaction of the carboxylate group of the ampicillin anion with the choline cation.

In the case of the aromatic peaks from ampicillin (7.20 to 7.50 ppm), no significant variation of chemical shift was observed, indicating that the phenyl ring of ampicillin does not interact with the choline cation.

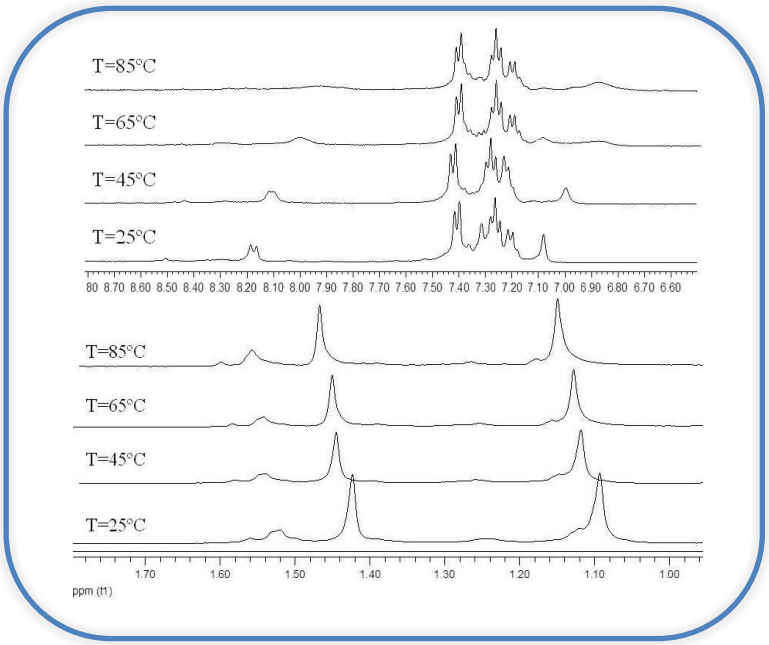


Figure 4.2 Comparative ^{1}H-NMR study of [Cholin][Amp] for four temperatures (25, 45, 65 and 85 °C) and two NMR regions (1 to 2 ppm and 6.5 to 8.8 ppm) in DMSO.

4.4 Conclusions

The present work reports a new and efficient method for the synthesis of beta-lactam antibiotics like ampicillin, which may prove useful for the development of new bioactive materials [1] (antiseptics and anti-biofilm, for example) or to reduce drug resistance in microorganisms. With the careful selection of the organic cation, it is possible to provoke important physical and thermal properties of ILs-APIs based on ampicillin, such as water solubility, melting point and thermal stability. [Cholin][Amp] is the most interesting example of a prepared API-IL in what concerns their particular IL properties (low melting point, high water solubility) as well as the biocompatibility and low toxicity of the choline cation.

Chapter 5.
Antibacterial Activity of Ionic Liquids Based on Ampicillin Against Resistant Bacteria

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Antibacterial activity of Ionic Liquids based on ampicillin against resistant bacteria

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5 Antibacterial Activity of Ionic Liquids Based on Ampicillin Against Resistant Bacteria

5.1 Abstract

Antibacterial activity of novel Active Pharmaceutical Ingredient Ionic Liquids (API-ILs) based on ampicillin anion [Amp] have been evaluated. They showed growth inhibition and bactericidal properties on some sensitive bacteria and especially some Gram-negative resistant bacteria when compared to the [Na][Amp] and the initial bromide and chloride salts. For these studies, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBIC) were analysed against sensitive Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* (clinically isolated). Sensitive Gram-positive *Staphylococcus. Aureus* ATCC 25923, *Staphylococcus epidermidis* and *Enterococcus faecalis* were also analysed. This was completed with MIC and MBIC evaluation of clinically isolated resistant strains such as *E. coli* TEM CTX M9, *E. coli* CTX M2 and *E. coli* AmpC Mox. From the obtained MIC values of studied APIs-ILs and standard [Na][Amp], RDIC (relative decrease of inhibitory concentration) values were calculated. High RDIC values of [C_{16}Pyr][Amp] especially against two resistant Gram-negative strains *E. coli* TEM CTX M9 (RDIC > 1000) and E. coli CTX M2 (RDIC > 100) point clearly to a potential promising role of APIs-ILs as antimicrobial drugs, particularly against resistant bacterial strains.

5.2 Introduction

Ionic Liquids (ILs) are usually defined as organic salts with melting points lower than 100 ^{o}C (several of them are liquid at room temperature)[3]. They became popular due to large number of possible cation/anion combinations allowing different tuneable interactions and potential applications. Some ILs properties, such as their high thermal and chemical stability, negligible vapour pressure[172,179], high ionic conductivity, lack of

inflammability and adjustable solubility have attracted numerous applications across an extensive variety of research areas[2,107,174,176], in particular related to organic chemistry, chemical engineering, material science, physical chemistry, analytical chemistry and biotechnology, among others.

One of the most promising applications of ILs seems to be the so-called third generation of ILs (their arrangement with active pharmaceutical ingredients, APIs) or APIs-ILs[9]. It is suggested that these compounds can solve problems associated to the pharmaceutical industry related with polymorphism and drug solubility[8,9,30,71,151]. At the beginning, the uses of ILs in biosciences was difficult because of ILs' toxicity[136,180]. However, more recently lower inherent toxicity was shown and thus a lower impact on human health and the environment from some hydrophobic ILs derived from toxic herbicides[136,180]. Presently, biocidal properties of large cations, such as benzalkonium and imidazolium species, have also been largely used as an advantage to kill or inhibit bacteria[18,97] or yeast[181,182] growth. In respect to this area, different publications have reported antimicrobial activity studies using microorganisms or cell culture for long alkyl chain quaternary ammonium[133,183]. Recently, Cole and co-workers[19] reported the use of metathesis reaction to produce ILs with long alkyl chain quaternary ammonium and ampicillin anion. Some ILs have been efficiently tested with clinically significant microbial pathogens, including Methicillin-resistant Staphylococcus aureus (MRSA)[135].

Bacterial resistance to different antibiotics, commercially available, is one of the major public health problems[27,50,51]. Recent outbreaks of *E. coli* O104[53,54] in Germany as well as the emergence of multi-drug resistant organisms such as Gram-negative Enterobacteriaceae associated to the New Delhi metallo β-lactamase[55,80] confirm that this is a serious problem for public health, but also from an economic and social point of view. Besides the fact that bacterial resistance increases mortality and morbidity, some recent publications have reported the financial burden of health care-associated infections (HAIs) in the USA[60,61].

Innovative therapies involving the use of ILs as a drug delivery platform suggest other attractive opportunities for exploration. The possibility to eliminate or reduce the

negative side effects of a given active compound by delivering it as an API-IL is extremely attractive for pharmaceutical, environmental technologies and other medical applications. It is suggested that the appropriate combination of counter ion and a specific drug as API-IL can influence the final biopharmaceutical drug classification (BCS), toxicity and biodegradability behavior[32,181,182], water solubility, permeability as well as the drug formulation process. Besides, it can changes some biological properties.

As mentioned before, the bacterial resistance to antibiotics is a serious public health threat and needs novel and urgent action. In this context, we studied the bacterial activity of different API-ILs based on Ampicillin[30] against a panel of sensitive Gram-negative and Gram-Positive bacteria as well as a panel of resistant bacteria. In particular, we selected sensitive Gram-negative bacteria *Escherichia coli* ATCC 25922 and *Klebsiella pneumonia* (clinically isolated); sensitive Gram-positive *S. Aureus* ATCC 25923, *Staphylococcus epidermidis* (clinically isolated) and *Enterococcus faecalis* (clinically isolated) and the resistant bacteria (clinically isolated strains) *E. coli* TEM CTX M9, *E. coli CTX M2*, *E. coli* AmpC Mox2[27,29,50-52].

5.3 Results and Discussion

In this work, API-ILs or pharmaceutical organic salts were produced by the neutralization method[30] from ampicillin. The toxic effect of cationic counter ion of API-ILs should not be neglected[156]. All prepared Ampicillin based ILs are considered non-toxic, except in the case of $[P_{6,6,6,14}]$ cation, which is toxic according to standard toxicity test against human colon carcinoma cell line (CaCo-2)[25]. Contrarily, Choline cation ($[Cholin]^+$) is used as an essential nutrient; low molecular substituted ammonium or imidazolium cations ($[TEA]^+$ or $[C_nMIM]^+$) as alternative low-toxic cations and cetylpyridinium cation ($[C_{16}Pyr]^+$) have already been applied in pharmaceutical applications[184]. For comparative studies, the activity tests were also performed using starting materials (bromide and chloride salts) while ampicillin sodium salt was always used as the standard API. The purity of compounds was checked by ^1H and

^{13}C-NMR and mass spectra analysis. Figure 5.1 summarizes all prepared API-ILs based on Ampicillin anion ([Amp]).

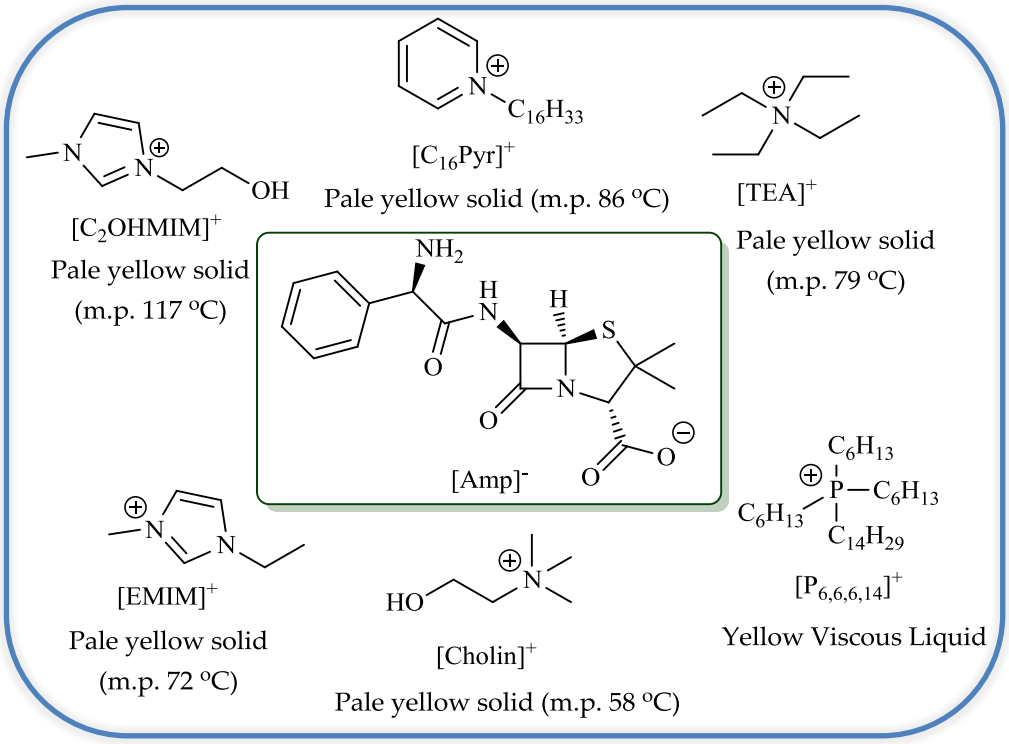

Figure 5.1 Representation of the prepared API-ILs based on ampicillin anion.

Table 5.1 shows the MICs for the API-ILs based on ampicillin sensitive bacteria and Table 5.2 presents the MICs for resistant strains. The analysis from Table 5.1 suggests that in the case of sensitive bacteria, the [C$_{16}$Pyr][Amp] exhibits the highest activity and significantly lower MIC values when compared to [Na][Amp]. In the case of *K. pneumonia*, *S. epidermidis* and *E. fecalis* suggest that this could be a promising antibacterial compound for this kind of bacteria.

Some toxicity studies indicated that the least toxic anions are Cl^- and BF_4^-[185] and a there is a possible relation between toxicity and the length of alkyl chains in the case of imidazolium, pyridinium and quaternary ammonium cations for several microorganisms such as rods, cocci and fungi[157].

In our work, the combination effect of ampicillin anion (API) with selected organic cation has been evaluated. Also, the cation's own contribution or synergetic effect from

cation/anion were checked by measuring MIC values of [Cat][Cl] and [Cat][Br] (shown in Table 5.1 and Table 5.2) that were used in the synthesis of [Cat][Amp][30].

Table 5.1 Minimum inhibitory concentrations (mM) on the ampicillin sensitive bacterial strains tested.

Strains / Comp.	Gram-negative		Gram-positive		
	E. coli ATCC 25922	*K. pneumoniae*	*S. aureus* ATCC 25923	*E. fecalis*	*S. epidermis*
[Na][Amp][a)]	0.05	2.5	0.005	0.05	0.05
[TEA][Amp]	>5	>5	>5	>5	>5
[TEA][Br]	>5	>5	2.5	>5	>5
[$P_{6,6,6,14}$][Amp]	2.5	5	0.05	0.05	0.05
[$P_{6,6,6,14}$][Cl]	2.5	2.5	2.5	>5	2.5
[C_{16}Pyr][Amp]	0.5	**0.05**	0.005	**0.005**	**0.005**
[C16Pyr][Cl]	0.5	2.5	0.5	0.5	2.5
[Cholin][Amp]	>5	>5	>5	>5	>5
[Cholin][Cl]	>5	>5	2.5	>5	>5
[EMIM][Amp]	>5	>5	>5	>5	>5
[EMIM][Br]	>5	>5	0.05	>5	5
[C_2OHMIM][Amp]	5	>5	>5	5	2.5
[C_2OHMIM][Cl]	5	>5	>5	5	5

[a)] The [Na][Amp] was used as control.

The results showed that the toxic effect of the chloride and bromide is almost negligible in diluted solutions as already described in the literature[156,185]. With the exception of [C_2OHMIM][Cl] providing lower MIC value against resistant *E. coli* CTX M2 than [C_2OHMIM][Amp].

MIC values of ionic liquids and salts containing highly polar cations in particular [Cholin][Amp] or [C_2OHMIM][Amp] are always higher than [Na][Amp]. Contrarily, the ampicillin ILs containing apolar cations such as [$P_{6,6,6,14}$][Amp] and [C_{16}Pyr][Amp] presented lower MIC values than [Na][Amp].These observations can be explained with the fact that the highly polar cations are more prone to stay in aqueous solution instead

of hydrophobic cell membranes and therefore anchor themselves to ampicillin anions (ion trapping effect)[186]. The correspondent ampicillin salts thus stay trapped to aqueous solution which eventually becomes hydrolysed.

Table 5.2 Minimum inhibitory concentrations (mM) on the ampicillin resistant bacterial strains tested.

Strains / **Comp.**	*E. coli* TEM CTX M9	*E. coli* CTX M2	*E. coli* AmpC MOX2
[Na][Amp][a)]	>5	>5	>5
[TEA][Amp]	>5	>5	>5
[TEA][Br]	>5	2.5	>5
[$P_{6,6,6,14}$][Amp]	**0.5**	**0.5**	>5
[$P_{6,6,6,14}$][Cl]	**2.5**	**5**	>5
[C_{16}Pyr][Amp]	**0.005**	**0.05**	**0.05**
[C16Pyr][Cl]	**0.5**	>5	>5
[Cholin][Amp]	>5	>5	>5
[Cholin][Cl]	>5	>5	>5
[EMIM][Amp]	>5	>5	>5
[EMIM][Br]	**5**	>5	>5
[C_2OHMIM][Amp]	>5	>5	5
[C_2OHMIM][Cl]	>5	**2.5**	**5**

[a)] The [Na][Amp] was used as control.

Unlike penicillin G, which is active only against Gram-positive bacteria, ampicillin possesses an additional amino group and is active against both Gram-positive and some Gram-negative bacteria. Bacteria may be resistant (or develop) resistance on β-lactam antibiotics by developing very efficient β-lactamase enzymes ("penicillinases"), by developing change (resistance gene) in penicillin-binding proteins (PBPs) and therefore providing poor affinity for antibiotic or development of porin channel blockage (in case of Gram-negative bacteria)[187-189].

As observed from Table 5.3 and Table 5.4, [C_{16}Pyr][Amp] exhibits the most noticeable Relative Decrease of Inhibitory Concentration (RDIC). Among sensitive Gram-positive

bacteria [C_{16}Pyr][Amp] shows no effect on *S. aureus* ATCC 25923 (RDIC = 1) or mild positive effect against *E. fecalis* and *S. Epidermis* (RDIC=10 for each). Among sensitive and resistant Gram-negative strains (Table 5.3 and Table 5.4) [C_{16}Pyr][Amp] IL presents a slight increase of the MIC value on *E. coli* ATCC 25922 (RDIC = 0.1). MIC decreases in the case of *K. Pneumonia* (RDIC = 50) and regarding the resistant strains *E. coli* TEM CTX M9 (RDIC > 1000) and *E. coli* CTX M2 (RDIC > 100) the decrease was remarkable.

Table 5.3 Relative Decrease of Inhibitory Concentration (RDIC) of ampicillin anion in [Cat][Amp] (RDIC) comparing with sodium ampicillin for ampicillin sensitive bacteria[a)].

Strains / Comp.	Gram-negative		Gram-positive		
	E. coli ATCC 25922	*K. pneumoniae*	*S. aureus* ATCC 25923	*E. fecalis*	*S. epidermis*
[Na][Amp][a)]	1	1	1	1	1
[$P_{6,6,6,14}$][Amp]	0.02	0.5	0.1	1	1
[C_{16}Pyr][Amp]	0.1	50	1	10	10
[C_2OHMIM][Amp]	0.01	-	-	0.01	0.02

[a)] for explanation of RDIC values see Experimental section.

Lehn *et al*. demonstrated that transport across biological membranes of highly polar anionic compounds can be facilitated if they are paired with lipophilic ammonium ions, which act as phase transfer[190]. We and others have recently suggested the application of the same principle for penetration and drug delivery in APIs-ILs with lipophilic counterions[8,9,30,135,151]. The results of [C_{16}Pyr][Amp] on ampicillin sensitive Gram-positive and both sensitive and resistant Gram-negative strains are according to this principle. [C_{16}Pyr][Amp] usually does not influence the RDIC values of sensitive strains as peptidoglycan of Gram-positive bacteria is already directly exposed to an aqueous solution while outer membrane of sensitive Gram-negative bacteria does not represent a physical barrier for [Na][Amp]. Regarding small fluctuations in the case of *E. coli* ATCC 25922 (RDIC = 0.1), *K. pneumonia* (RDIC = 50), *E. fecalis* and *S. Epidermis* (each RDIC = 10) all can be explained in morphological differences of the cell surface of these bacteria, peptidoglycans, outer lipid layers among others and their interaction with API-IL. However, the most remarkable effect of [C_{16}Pyr][Amp] is observed

against resistant Gram-negative strains. For *E. coli* CTX M2 RDIC value is higher than 100 and for *E. coli* TEM CTX M9 is even higher (RDIC > 1000). No activity (and consequently no RDIC value) has been found for *E. coli* AmpC Mox2. In the case of [$P_{6,6,6,14}$][Amp] the MIC values decrease in resistant bacteria *E. coli* TEM CTX M9 and *E. coli* CTX M2. In this case the RDIC values are lower than for [C_{16}Pyr][Amp]. However, the RDICs are in order of tens. These results clearly suggest that antibiotic APIs-ILs help in the deliver of antibacterial agent [Amp] to some Gram-negative bacteria that developed resistance against beta-lactam antibiotics[187,189], most probably acting as lipophilic phase transfer agent across the outer membrane of the bacteria[190]. If this transportation mechanism is true, APIs-ILs obviously may solve some resistance problems – e.g. in the case of porin channel blockage, they should be fully effective against bacterial resistance but if mutation on Penicillin-binding proteins (PBPs) occurred, they would show no effect, while in the case of resistance based on the development of beta-lactamases, their success may depend on several factors. However, as bacteria frequently developed more than one resistance mechanism, the full effect of ILs-APIs on them should be further studied. Regarding sensitive strains, the MIC values of [$P_{6,6,6,14}$][Amp] present an opposite effect. In this group of bacteria RDIC values were equal or lower than 1, indicating a more complex interaction.

Table 5.4 Relative Decrease of Inhibitory Concentration (RDIC)[a)] of ampicillin anion in [Cat][AMP] comparing with Sodium Ampicillin for ampicillin resistant bacteria[a.]

Strains / **Comp.**	*E. coli* TEM CTX M9	*E. coli* CTX M2	*E. coli* AmpC Mox2
[Na][Amp]	1	1	1
[$P_{6,6,6,14}$][Amp]	>10	>10	-
[C_{16}Pyr][Amp]	>1000	>100	>100
[a)] for explanation of RDIC values see Experimental section.			

The Growth Rate (GR) studies were performed by exposing approximately 0.5 to 2.5 x 10^4 $CFUmL^{-1}$ of bacteria to an ampicillin based IL concentration. The selected concentrations were of superior, equal or inferior value of the determined MIC.

As expected, in the presence of concentrations equal and superior to MIC, no growth was observed. In the presence of inferior MIC values, a decrease was observed on GR in most of the cases (Table 5.5, Figure 5.2). These observations indicate that in the presence of an IL with an inferior concentration of MIC, a possible growth inhibition occurs in a similar way, especially in the case of pathogenic strains. These results point to a potential use of these compounds as antibacterial drugs mainly against resistance strains at very low concentrations.

Table 5.5 Growing rates for the different organisms in the presence and absence of $[P_{6,6,6,14}][Amp]$.

Organism	**MIC/ mM**	**Conc. Tested/ mM**	**Growing rate.min^{-1}**		**Decreasing rate**
			With $[P_{6,6,6,14}][Amp]$	no compound	
E. coli ATCC 25922	2.5	0.5	0.0007 ± 0 .0001	0.0020 ± 0.0001	0.035
S. aureus ATCC 25923	0.005	0.0005	0.0004 ± 0 .0003	0.0052 ±0 .0007	0.077
K. pneumonia	5.0	0.5	0.00045 ± 0 .00007	0.0025 ± 0.0005	0.18
E. fecalis	0.05	0.005	0.0009 ± 0.0004	0.0033 ± 0.0001	0.28
S. epidermis	0.05	0.005	0.0006 ± 0.0000	0.0047 ±0 .0002	0.13
E. coli TEM CTX M9	0.5	0.05	0.00067 ± 0.00006	0.0033 ± 0.0007	0.20
E. coli AmpC Mox2	0.5	0.05	0.00077 ±0 .00006	0.0032 ± 0.0004	0.24

Figure 5.2 illustrates resistant Gram-negative strains bacteria *E. coli* AmpC Mox2 (Figure5.2 a); *E. coli* TEM CTX M9 (Figure 5.2 b); *E. coli* CTX M2 (Figure 5.2 c) growth curves in the case of $[C_{16}Pyr][Amp]$ for different concentrations (0.5, 0.05 and 0.005 mM).

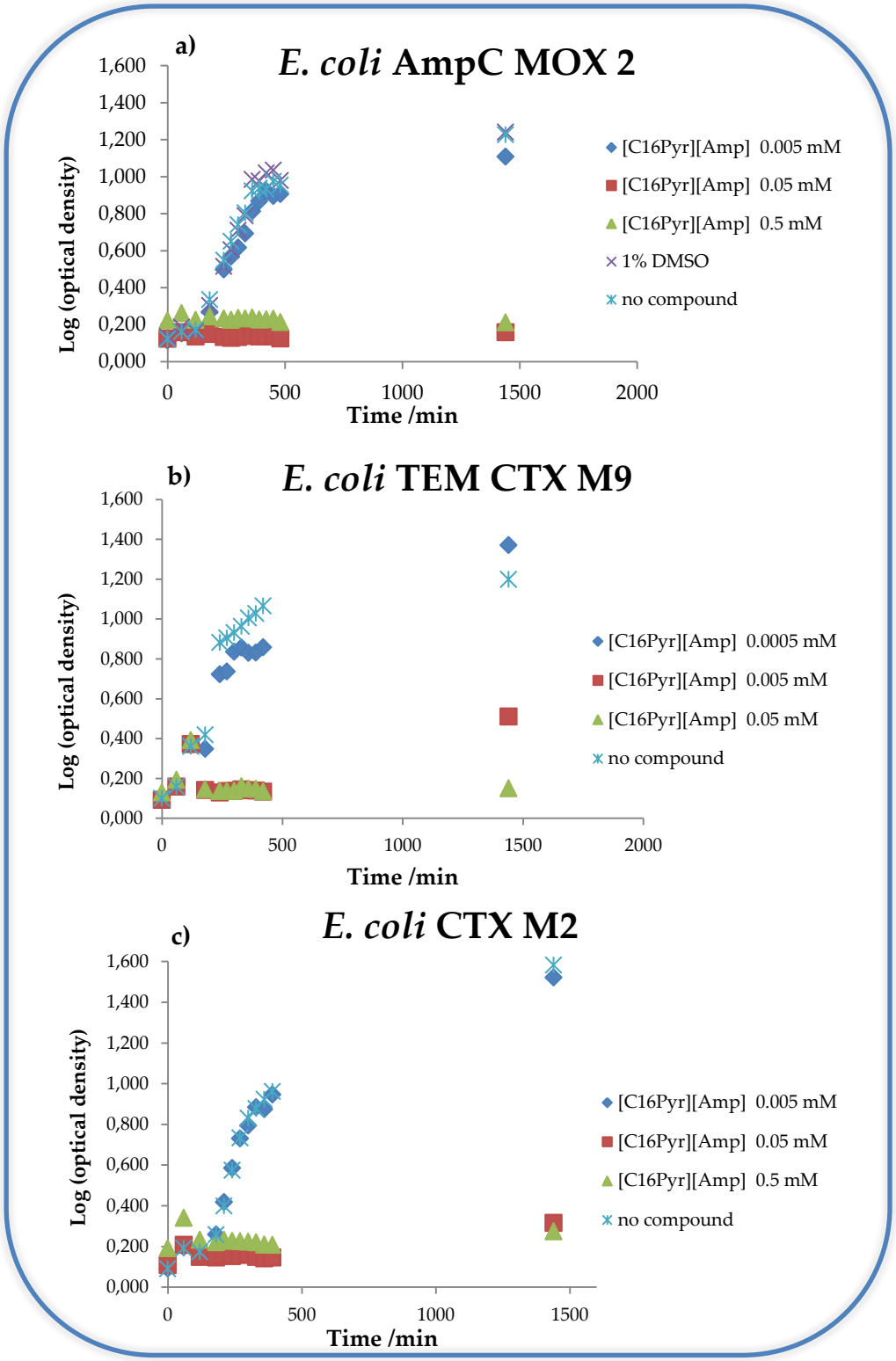

Figure 5.2 Representation of growth curves (Log(optical density) vs Time) from resistant Gram-negative strains bacteria *E. coli* AmpC Mox2 (a); *E. coli* TEM CTX M9 (b); *E. coli* CTX M2 (c) in the case of API-IL [C_{16}Pyr][Amp] for different concentrations. For comparison no compound addition experiments were performed.

5.4 Conclusion

The present work suggested that ILs based on ampicillin can reverse the resistance in some clinical strains previously isolated and tested as resistant. With the appropriate selection of the organic cation, it is possible to provoke important biological variations in their antibacterial properties. This report also showed that the ion-pair effect is crucial in the ampicillin mechanism of action, being required a selection of hydrophobic ampicillin counterions.

[C_{16}Pyr][Amp] demonstrated the highest potential in reversion of resistance. [C_{16}Pyr][Cl] is already used in some types of mouthwashes and toothpastes[14] although it is irritant in higher concentrations[15]. The highest improvement of activity is obtained against Gram-negative resistant bacteria (*E. coli* TEM CTX M9 and *E. coli* CTX M2, RDIC >1000 and 100 respectively). These results are clearly promising and appoint to a beneficial effect of drug delivery assisted by API-IL through outer membrane of Gram-negative bacteria, opening possibilities for new similar applications and explorations especially in reversing drug resistance. Drug delivery processes have never seemed to be a problem for Gram-positive and Gram-negative sensitive strains and therefore they showed more moderate oscillation in RDIC values (0.1-50), which are obviously connected to their morphology. RDIC values proved to be a good indicator for the measurement of the efficiency effectiveness of the process. In the future they can be used to quantify interaction of ILs-APIs and resistant bacteria in order to determine the influence of ILs-APIs on various bacterial resistance mechanisms.

Our results also showed that future developments of novel APIs-ILs must been taken into consideration not only in the toxicity and hydrophobicity of the counter ion but also to be more focused on it. Example of [$P_{6,6,6,14}$][Amp] which generally follows the trend of [C_{16}Pyr][Amp], but with very low RDIC values (demonstrate that there may be more factors at stake to be considered).

The reversion mechanism of the ampicillin based ILs should be further studied in order to improve infection control within the hospital environment and in the

community. Nevertheless, the use of ILs based antimicrobials will contribute to the decrease of nosocomial infections (in terms of morbidity and mortality) and costs associated with them.

5.5 Experimental Section

5.5.1 Reagents and Materials

[Amp] has been used as anion combined with the following organic cations: 1-ethyl-3-methylimidazolium, [EMIM]; 1-hydroxy-ethyl-3-methylimidazolium, [C_2OHMIM]; choline, [Cholin]; tetraethylammonium, [TEA]; cetylpyridinium, [C_{16}Pyr] and trihexyltetradecylphosphonium [$P_{6,6,6,14}$]. In addition, in order to be accurate and make comparisons, activity tests were performed on starting materials (cation bromide and chloride salts). Sodium salt of ampicillin is always used as standard for comparison.

5.5.2 Bacterial Strain

A total of 10 bacterial strains were used in this study. The following reference strains were obtained from the American Culture Collection (ATCC): *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. All other strains, including several resistant bacteria are clinically isolated from previous work: *K. pneumonia*, *S. epidermidis* and *E. fecalis* and the resistant bacteria, *E coli* Tem CTX M9, *E. coli* CTX M2, *E. coli* AmpC Mox2. These resistant strains were tested in Vitek®2 systems from bioMérieux.

5.5.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

The MIC values were determined in triplicate by the broth micro dilution method in a 96-well microtiter plate, using Tryptic Soy Broth (TSB), which is an adapted methodology from the Clinical Laboratory Standard Institute (CLSI)[160] (Table 5.1 and Table 5.2). The organisms tested were grown individually on Tryptic Soy agar for 24 h at 37 ^{o}C prior to each antibacterial test. Prior to MIC determination, each inoculum density was adjusted in TSB to 0.5 McFarland standard by photometric device[160]. This resulted in a suspension containing approximately 1×10^8 to 2×10^8 colony forming

units $CFUmL^{-1}$ for E. coli ATCC25922®. A similar approach was used for the other strains. Then 0.5 μL of the suspension was added to each well to have 0.5-2.5x10^4 $CFUmL^{-1}$ as final concentration. Bacteria were exposed to an Ampicillin-IL salt using the following concentration: 5 mM, 2.5 mM, 0.5 mM, 0.05 mM, 0.005 mM, 0.0005 mM and 0.00005 mM. With the exception of $[P_{6,6,6,14}]$[Amp] that was diluted in 1% dimethylsulfoxide (DMSO), all others were dissolved in water and these results were compared with bacteria that had grown in TSB broth in the presence of 1% DMSO as a control. The MIC for each Amp-IL was recorded as the lowest concentration that showed no turbidity after 24 h of incubation at 37 ^oC[160]. The presence of turbidity is an indication of microbial growth and the corresponding concentration of an antibacterial agent is considered ineffective. To determine whether the Amp-ILs inhibited growth or had bactericidal activity, 5 μL XTT sodium salt of (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner salt) was added to the non-turbid wells of the MIC assay plate and incubated for 3 h at 37 ^oC for bactericidal status determination[161,162]. In the case of viable cells with inhibited growth, mitochondrial dehydrogenases of viable 100 cells cleave the tetrazolium ring of XTT yielding dark orange aqueous soluble formazan crystals. However, a solution containing non-viable cells would remain with the same colour[162]. In this case, MIC values were equal to MBC for each Ampicillin based ILs, so the antibacterial activities of these compounds were considered to be bactericidal.

In order to obtain the ratio of MIC values for various sensitive bacteria they were grouped as ratio of MIC values of [Na][Amp] and [Cat][Amp] or so-called RDIC values (relative decrease of inhibitory concentration).

As in the case of resistant bacteria, the MIC values of [Na][Amp] could not be found experimentally. RDIC values of resistant bacteria are calculated assuming that the MIC value of [Na][Amp] is at least 5 mM (>5 mM) in all cases (the highest value that can be measured experimentally), providing the numerical RDIC values for all [Cat][Amp] with MIC values (Table 5.4).

5.5.4 Growth Rate Studies

In order to complete the characterization of anti-bacterial activity, the Growth Rate (GR) when exposed to the MIC value was evaluated. Additionally, GR values in the presence of higher and lower concentration of the MICs, previously found for each compound, was also determined (Table 5.5 and Figure 5.2).

The GR parameter was determined in triplicate by the broth micro dilution method in a 96-well microtiter plate using Tryptic Soy Broth (TSB)[160,163,164]. Approximately 5000-25000 $CFUmL^{-1}$ of bacteria is exposed to an ampicillin based IL of concentration superior, equal or inferior value of the determined MIC, performing periodical readings of the optical density (OD) at 620 nm. The growing rate was determined by fitting a linear function to the Exponential (log) phase curve.

Chapter 6.
Anti-tumoral Activity of Ampicillin Ionic Liquids and Their Salts

This chapter is a reproduction of a submitted article to the Chemical Science (SC-EDG-08-2013-052438):

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6 Anti-tumoral Activity of Ampicillin Ionic Liquids and Their Salts

6.1 Abstract

Ionic Liquids (ILs) are a peculiar class of organic salts with melting points below 100 °C. They are finding increased interest from the scientific community and industry due to the large number of potential cation/anion combinations, which allows a variety of tuneable interactions and applications. These type of combinations can be an innovative solution to the polymorphism behaviour of several drugs as well as to their reduced bioavailability. The focus of this work is to study the anti-cancer activity of ILs based on the ampicillin antibiotic, using cell viability tests. We evaluated anti-proliferative activity against some human cancer cell lines and found an anti-proliferative effect against diverse tumour cell lines using innovative active pharmaceutical ingredients (APIs) as molten salts or ionic liquids.

6.2 Introduction

As said before, Ionic Liquids (ILs) are organic salts, generally with melting points below 100°C (in many cases they are liquid at room temperature)[9,171] and which have been applied in several research areas. One of the most promising applications of ILs seems to be the so-called third generation of ILs (their arrangement with active pharmaceutical ingredients, APIs) or APIs-ILs[8,9]. Recently, we described the successful combination of API ampicillin as anion with six different organic cations.[30] This type of combination can be an innovative solution in order to eliminate polymorphism, characteristic of a large number of drugs, as well as, to improve their solubility and permeability behaviour.

The compound [C2OHMIM][Amp] possess relevant discriminative and strong anti-proliferative activity against five different human cancer cell lines, in particular T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon).

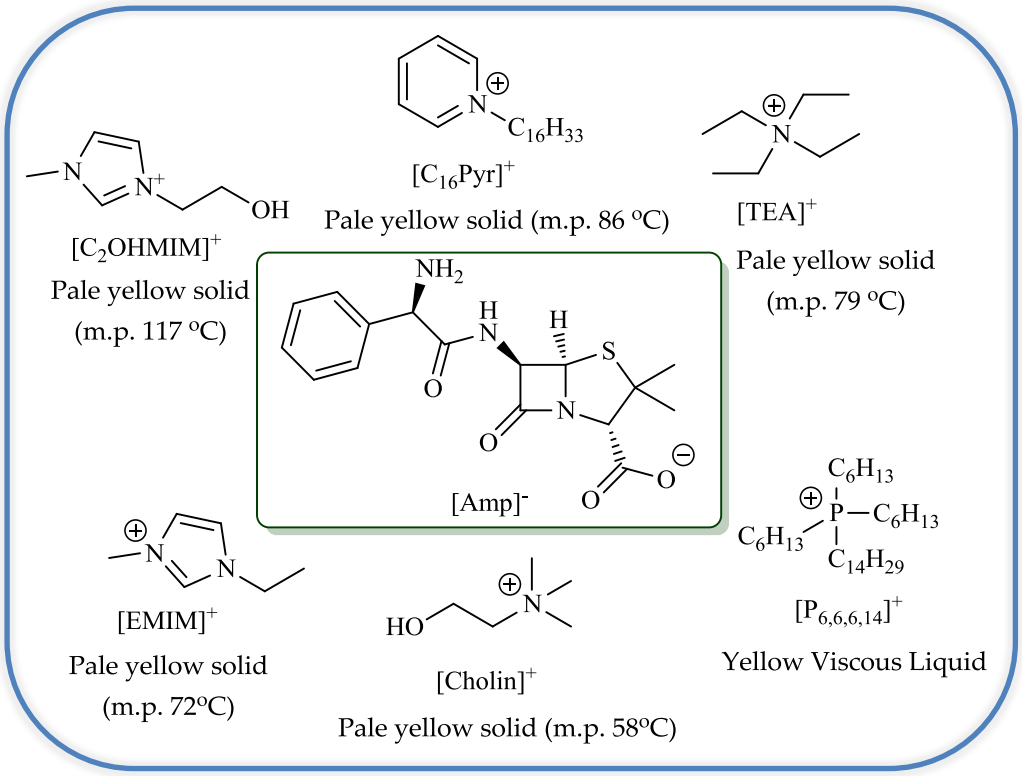


Figure 6.1 Structure of compounds prepared based on ampicillin.

The relevant question of the toxicity of the active pharmaceutical ingredient counter-ion in ILs has been the major cause for the delay of the entrance of ILs in biosciences.[9] The toxicities towards microorganisms and eukaryotic cell cultures cover the whole range of biocidal potencies, from rather inactive molecular solvents that are biocompatible up to high concentrations in aqueous solutions. This leads to the successful proposal of ionic liquids as wood preservatives as well as different pharmaceutical applications,[9,142] and even as potential anticancer therapeutic agents[23,31,71] (*in vitro*). Consequently, the development of novel anti-cancer drugs that decrease the toxicity associated with active chemotherapies[4,5] can also be pursued within the ionic liquid framework. The focus of this work is to further explore the outstanding properties of the previously synthetized ampicillin compounds, in particular their anti-proliferative effect against diverse tumour cell lines and compare

to results obtained of the primary human cell lines. Figure 6.1 illustrates the ILs based APIs (or molten salts-APIs) used in this study.

6.3 Experimental section

6.3.1 Biological studies

All compounds were prepared using an optimized and sustainable method described in a previous publication[30].

The synthesized compounds were then used in order to evaluate their anti-proliferative activity against some human cancer cell lines, such as T47D (ductal breast epithelial tumour cell line), PC3 (prostate cancer cell line), HepG2 (hepatocellular liver carcinoma cell line), MG63 (osteosarcoma cell line) and RKO (colon cancer cell line). In addition, two primary human cell types, namely, skin and gingival fibroblasts (SF and GF, respectively) were also tested.

6.3.1.1 Cell viability/proliferation

Cells were maintained in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, 100 IumL^{-1} penicillin, 2.5 μgml^{-1} streptomycin, 2.5 μgmL^{-1} amphotericin B and 50 μgmL^{-1} ascorbic acid. At about 70-80% confluence, cells were enzymatically detached with 0.05% trypsin and 0.5mM EDTA and seeded at 10^4 cellscm^{-2}. After an attachment period of 24 h, the culture medium was renewed, and supplemented with different concentrations (0.005-500 mM) of the ampicillin-derived ILs. Cell cultures were maintained in a 5% CO_2 humidified atmosphere at 37 ^{o}C. Cellular viability/proliferation was assessed by the MTT assay at days 1, 3 and 5 of the culture, as described before[165,166]. This assay is based on the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to a purple formazan product by viable cells. Shortly, cultured cells were incubated at 37 ^{o}C with 0.5 mg mlL^{-1} of MTT for 4 h. The culture medium was then removed; the stained product dissolved with dimethylsulfoxide and absorbance determination was conducted at 550 nm in an ELISA plate reader. Results were expressed as absorbance per square centimetre

(A cm^{-2}).[165,166] The values of the half maximal inhibitory concentration (IC50) and the median lethal dose (LD50) were obtained from nonlinear regression analysis of concentration-effect curves using the GraphPad Prism software[167,168]. The definition of the IC50 is given by Sebaugh[169] as "the response corresponding to the 50% control (the mean of the 0% and 100% assay controls)" and is used to measure the efficacy of a compound in inhibiting any biochemical or biological function. The term LD50 means the median lethal dose and is the amount of material which causes the death of 50% of a population[170].

6.4 Results and discussion

Table 6.1 summarizes the anti-tumoral (IC50 and LD50) activities of the prepared ILs and molten salts based on the ampicillin anion against two primary human cell types, namely, skin (SF) and gingival fibroblasts (GF).

Table 6.1 IC50 and LD50 in μMof the ILs based on ampicillin against primary human cell lines.

Compounds		**Cell lines**	
		SF	**GF**
[TEA][Amp]	IC50/ μM	nd	nd
	LD50/ μM	nd	58.040
[$P_{6,6,6,14}$][Amp]	IC50/ μM	0.249	0.173
	LD50/ μM	>0.249	0.176
[C_{16}Pyr][Amp]	IC50/ μM	0.032	0.012
	LD50/ μM	0,815	45.510
[Cholin][Amp]	IC50/ μM	48.480	nd
	LD50/ μM	49.790	nd
[EMIM][Amp]	IC50/ μM	6.366	0.853
	LD50/ μM	>6.366	9.357
[C_2OHMIM][Amp]	IC50/ μM	5.084	0.462
	LD50/ μM	22.600	30.470
[Na][Amp]*	IC50/ μM	nd	109.100
	LD50/ μM	8.104	>109.100

nd – IC50 and LD 50 not detected in the concentration range used. SF (skin fibroblasts), gingival fibroblasts (GF). [Amp] Ampicillin anion, [TEA] tetraethylammonium, [$P_{6,6,6,14}$] trihexyltetradecylphosphonium, [C_{16}pyr] cetylpyridinium, [Cholin] choline, [EMIM] 1-ethyl-3-methylimidazolium, [C_2OHMIM] 1-hydroxy-ethyl-3-methylimidazolium. * [Na][Amp] was used as a control.

The [Na][Amp] was used as a control and, as can be seen in Table 6.1, it is one of the least toxic. The IC50 was not detected in the concentration range used against SF and the value of the IC50 for the GF was 109.1 μM. Analysing the other synthesised compounds and comparing the values with [Na][Amp], we can say that the most toxic to the human cell lines are [$P_{6,6,6,14}$][Amp] and [C_{16}Pyr][Amp] (the lowest values of the IC50). [TEA][Amp] and [Cholin][Amp] are the least toxic. The ILs based on imidazolium [EMIM][Amp] and [C_2OHMIM][Amp] have values in the middle. These results are according to the literature which says that the increase of hydrophobicity and the increase of the alkyl chain leads to the increase of the toxicity[25,155,157,191,192].

Table 6.2 IC50 and LD50 in μM of the ILs based on ampicillin against cancer cell lines.

Compounds		**Cell lines**				
		MG63	**HepG2**	**T47D**	**PC3**	**RKO**
[TEA][Amp]	IC50/ μM	0.030	nd	0.042	35.650	56.700
	LD50/ μM	1.368	0.0951	43.990	58.920	68.370
[$P_{6,6,6,14}$][Amp]	IC50/ μM	0.312	0.322	0.264	0.354	0.180
	LD50/ μM	>0.312	15.770	4.970	9.335	11.860
[C_{16}Pyr][Amp]	IC50/ μM	0.011	nd	0.005	132.700	0.226
	LD50/ μM	183.200	nd	nd	>132.700	59.170
[Cholin][Amp]	IC50/ μM	0.017	1.619	110.900	0.982	nd
	LD50/ μM	738.000	15.030	>110.900	17.160	0.707
[EMIM][Amp]	IC50/ μM	1.122	24.270	nd	29.990	0.269
	LD50/ μM	>1.122	>24.270	32.370	31.020	>209.700
[C_2OHMIM][Amp]	IC50/ μM	0.738	0.319	0.146	0.297	0.359
	LD50/ μM	4.197	0.4040	0.498	>0.297	0.362
[Na][Amp]*	IC50/ μM	nd	nd	nd	0.597	2.406
	LD50/ μM	506.600	0.720	47.790	560.900	920.800

nd – IC50 and LD 50 not detected in the concentration range used. MG63 (osteosarcoma cell line), HepG2 (hepatocellular liver carcinoma cell), T47D (ductal breast epithelial tumour cell line), PC3 (prostate cancer cell line), line) and RKO (colon cancer cell line). [Amp] Ampicillin anion, [TEA] tetraethylammonium, [$P_{6,6,6,14}$] trihexyltetradecylphosphonium, [C_{16}pyr] cetylpyridinium, [Cholin] choline, [EMIM] 1-ethyl-3-methylimidazolium, [C_2OHMIM] 1-hydroxy-ethyl-3-methylimidazolium. * [Na][Amp] was used as a control.

Table 6.2 shows the results of Ampicillin-ILs on five human cancer cell lines: osteosarcoma cell line (MG63), hepatocellular liver carcinoma cell (HepG2), ductal breast epithelial tumour cell line (T47D), prostate cancer cell line (PC3), line) and colon cancer cell line (RKO). Table 6.2 shows that each IL based on Ampicillin have very low

values of IC50 and LD50 for at least one of the cancer cell lines, which demonstrate the potential of these organic salts as selective anti-tumour agents. However, [C_2OHMIM][Amp] has low values of LD50 and IC50, in particular T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon), when compared to the results of IC50 and LD50 on primary human cell lines, SF and GF, (more than 5 times lower). So, these results demonstrate that [C_2OHMIM][Amp] possess relevant discriminative and strong anti-proliferative activity against five different human cancer cell lines. Additionally, the values of LD50 ([C_2OHMIM][Amp]) for this salt are much lower than those of the Ampicillin Sodium salt [Na][Amp], with the exception of the HepG2 cell line. Considering these results and analysing the only variant that had been changed, the structure of the cation, [C_2OHMIM] is a cation that could have 3 points of interactions with ampicillin (Figure 6.2). [C_2OHMIM] has 2 protons that could make a hydrogen bond and a π system that could interact with the π system of the ampicillin that provides more ways of interaction between the cation and the anion. This could help the salt entering the cell.

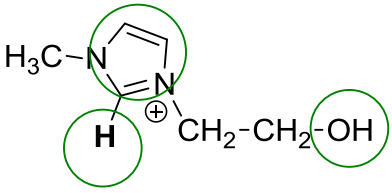


Figure 6.2. Struture of [C_2OHMIM] cation. The circles is show the possible point of interactions with ampicillin.

The results from Table 6.1 and Table 6.2 indicate that [TEA][Amp] is more toxic (LD50) for MG63 (osteosarcoma cell line) and T47D (human ductal breast epithelial tumor cell line). [$P_{6,6,6,14}$][Amp] also reveals a higher toxicity for the cancer cell lines than for the two normal cell types, a very low value of IC50 when comparing Ampicillin Sodium salt [Na][Amp]. [C_{16}Pyr][Amp] is only significantly toxic against skin fibroblasts and [Cholin][Amp] against RKO (colon cancer cell line). The lowest value of LD50 obtained for [EMIM][Amp] was for MG63 cell line. The high cytotoxic effect, but low selectivity of compounds containing the $[P_{6,6,6,14}]^+$ cation, is not surprising as the very high cytotoxicity of compounds with this cation towards other cell lines has already been

described.[25] Regarding the compounds containing $[C_{16}Pyr]^+$ cation, although frequently applied in pharmaceutical formulations, are also proven as very potent skin irritants.[15] This fact is in line with the results obtained here for the skin fibroblasts, where a small LD50 was obtained.

Regarding the IC50 values of Ampicillin-ILs, which is related to the anti-tumoral activity, $[P_{6,6,6,14}]$[Amp] seems to significantly inhibit the growth of all tested cells (low values of IC50 for all the cancer cell lines. The compound [TEA][Amp] displays a high activity against MG63 (osteosarcoma cell line) and HepG2 (hepatocellular liver carcinoma cell), while no effect was found in the remaining cell lines. $[C_{16}Pyr]$[Amp] has anti-proliferative activity against all of the tested cells, except for the cases of HepG2 and PC3 (prostate cancer cell line).

For [Cholin][Amp], a significant anti-proliferative behaviour was only observed in the case of MG63 (osteosarcoma cell line). Contrarily to the other bulky cations, the high cytotoxicity of [Cholin][Amp] towards MG63 cancer cell lines and high selectivity towards non-cancerogeneous cells can only be attributed to the xenobiotic (ampicillin) and its facilitated transport to the cytosol of cancer cell (effect of the ionic liquid) as the cation $[Cholin]^+$ is an essentially nutrient[9,184].

On the other hand, [EMIM][Amp] only shows activity against RKO (colon cancer cell line). The IC50 values for the molten salt $[C_2OHMIM]$[Amp] are quite satisfactory, with very low values of IC50 for 4 of the 5 tumour cell lines tested. The only exception was MG63 (IC50 = 0.7384 μM), which revealed an inhibition of proliferation lower than the gingival fibroblasts (IC50 = 0.4616 μM). For [Na][Amp], the lowest value of IC50 was obtained for PC3 cell line (IC50 = 0.5973 μM).

6.5 Conclusions

In conclusion, $[C_2OHMIM]$[Amp] presented low values of IC50 and LD50 against five different cell lines (T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon)) showing that it could be used against cancer cell lines. A very low

cytotoxicity against two primary cell lines (skin (SG) and gingival fibroblasts (GF)) shows that these compounds are not toxic to human cell lines. The IC50 values also indicated that [C_2OHMIM][Amp] presents an anti-proliferative activity against several tumour cell lines and a lower activity against primary fibroblasts. The other ampicillin ILs also show anti-tumoral activity against specific cell lines. It is important to emphasize that the appropriate selection of the cations and anions could render salts (or the ILs) with specific activity against different tumour cell lines, and therefore allow the modulation of the final biological properties of the pharmaceutical salts.

Chapter 7.
Synthesis of ILs based on Penicillin G, Amoxicillin and Amphotericin B

7 Synthesis of ILs based on Penicillin G, Amoxicillin and Amphotericin B

7.1 Introduction

The use of ILs in pharmaceutical sciences has increased in recent years[193]. They have been used to solve the problems related to the solubilisation of some drugs[30,193-197]. They are being exploited as solvents and/or (co)solvents and/or reagents to synthetize active pharmaceutical ingredients (APIs)[7-9,30,150,193,198]. Besides these applications, many ILs show antimicrobial activity as presented in Chapter 5. This leads to the use of ILs as active pharmaceutical ingredients (APIs) or formulation preservatives[9,30,193].

One of the many challenges that the pharmaceutical industry is facing is the solid form administration of many drugs. The problems with solid forms could be there low solubility, polymorphic conversion and low bioavailability[9,30,198]. The properties inherent to ILs could be of extreme importance to overcome the difficulties of solid form drugs[9,30]. Recent works have shown that ILs-APIs prove to have many attractive properties when compared to conventional drugs[69]. Our group has recently evaluate their relevant pharmacological properties, such as water solubility, octanol–water partition coefficient, hexadecylphosphocholine (HDPC) micelle–water partition coefficient and critical micelle concentration of the ampicillin-based pharmaceutical active ionic liquids synthesized by us[30,199]. In the work "Evaluation of solubility and partition properties of ampicillin-based ionic liquids"[199] the result clearly confirms the great potential of the API-IL methodology, since ampicillin-based ILs enhances properties regarding solubility in water, as well as more adequate properties regarding membrane affinity and permeation. This work[199] also confirms that an accurate selection of the organic cation allows the fine-tuning of some important physical and thermal properties, such as water solubility, membrane permeation, melting point and thermal stability[199].

In this chapter we present a sustainable methodology to synthesize ILs from APIs such as the one used to synthesize ampicillin ILs. The IL-APIs synthesised here are not only beta-lactamics like amoxicillin and penicillin, but also an antifungal containing amphotericin B. Although these molecules are completely different, the same preparative methodology could be applied to all of them with minor adaptations.

As said before, the classic methodology for the synthesis of ILs is the metathesis reaction. This is a reaction where cations and anions exchange partners. The purification of ILs is made by the elimination of undesirable inorganic salts (mainly sodium, potassium or lithium chloride or bromide) using precipitation followed by filtration[177]. This reaction has two major problems. One is the difficulty to obtain halide-free ILs. The other problem relies in the ion exchange. When there is an anion exchange with weaker acids than hydrohalic acids[91], it cannot be efficiently done[30].

The methodology used here for the preparation of the ILs-APIs is the same as used in Chapter 4 to obtain pure ILs from ampicillin. It is an adaptation based in the ion exchange resins developed by Ohno *et al.*[26] The Amberlite resin is in the OH form and is used to obtain the hydroxide form from the halide salts (bromide or chloride), then this basic solution is neutralized with the buffered API solution.

7.1.1 Amoxicillin and Penicilin

Amoxicillin [Amx] and Penicillin [Pen] are two antibiotics that belong to the beta-lactamic group like Ampicillin [Amp] (Figure 7.1).

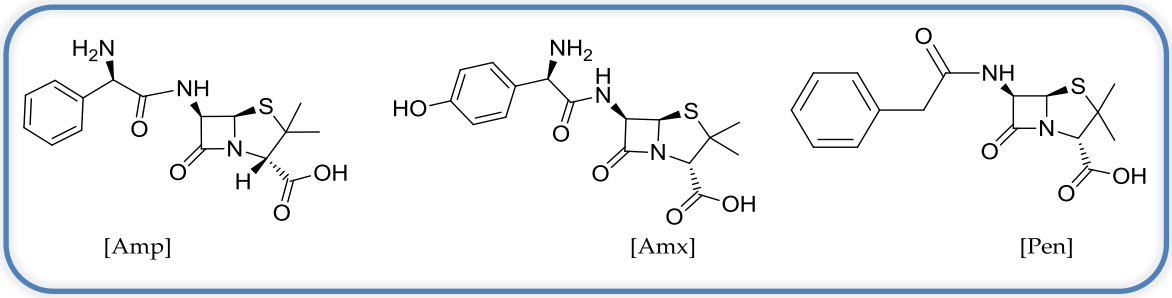


Figure 7.1. Struture of Ampicillin [Amp], Amoxicillin [Amx] and Penicillin [Pen].

From the point of view of microbiological synthesis, antibiotics are the most important bioactive and chemotherapeutic compounds[200]. Penicillin was the first product from an

organism to allow the distinction between the toxicity to the bacterial cell and toxicity to the mammalian host. This allows its use in the general treatment of infections caused by Gram-positive and Gram-negative organisms in humans and animals. The structure of beta-lactamics antibiotics consists in a nucleus that includes a beta-lactam ring fused through nitrogen and adjacent tetrahedral carbon to a second heterocycle (five-membered thiazolidine ring).[200]

In this work, as well as in Chapter 4, we pretend to obtain ILs from amoxicillin and penicillin. For the synthesis of most of these compounds we used the recently developed method of *Ohno et al.*[26] for the preparation of quaternary ammonium, phosphonium, imidazolium and pyridinium hydroxides, which were later neutralized by appropriate acids (amoxicillin and penicillin) in a buffer solution to avoid antibiotic decomposition.

7.1.2 Amphotericin B

Amphotericin B is also an antimicrobial agent. In this case it is a polyene used mainly against fungal infections and parasitic disease. The main problems with amphotericin B are its low solubility and the capacity to self-aggregate and therefore cause severe nephrotoxicity, which may lead to kidney failure[43]. This drug has different formulations, most of them have recently been discovered (*AmBisome*®, *Abelcet*®, and *Amphotec*®). Due to the low solubility drug delivery systems, such as liposome, developments on nanosolubility and microsphere are being done. These drug delivery systems have the advantage to increase the concentration in the liver and spleen, but decrease the concentration in the kidney resulting in low toxicity. An alternative to this drug delivery systems is ILs. The transformation of amphotericin B as ILs could lead to the increase of the solubility or even the decrease of the toxicity.

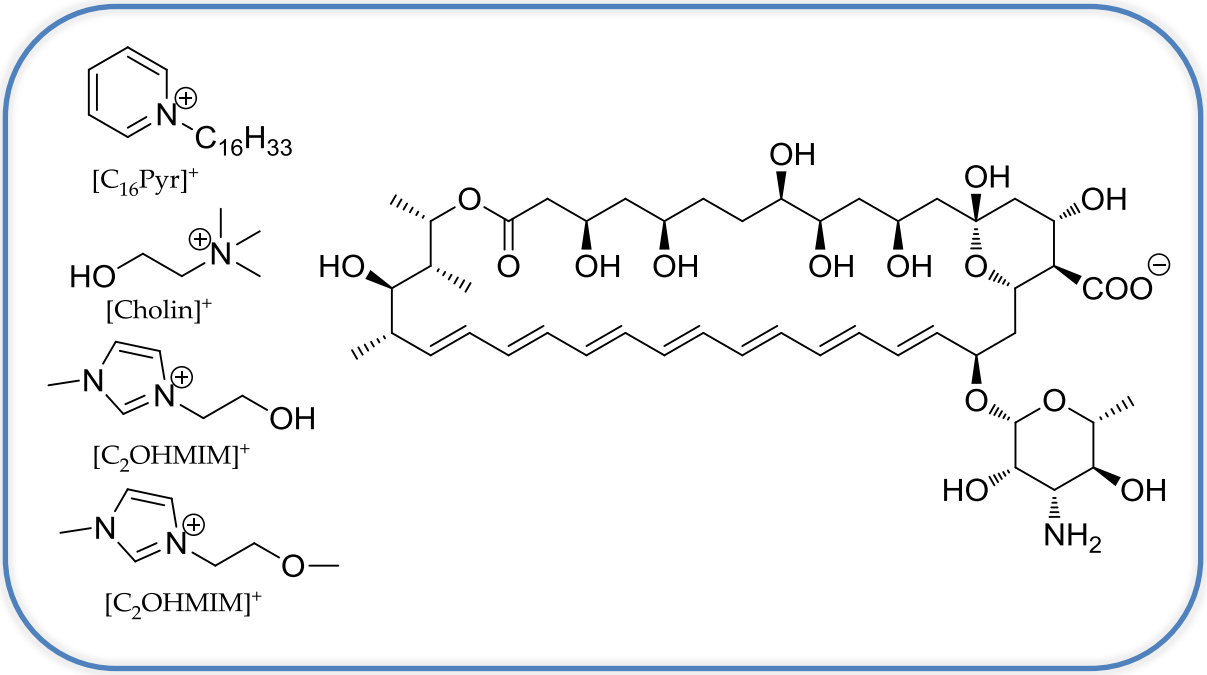


Figure 7.2 Ionic Liquids based on amphotericin B.

7.2 Material and Methods

7.2.1 General remarks

Commercially available reagents were purchased from Aldrich, BDH – laboratory reagents and Solchemar and were used as received. The solvents were from Valente & Ribeiro and distilled before used. The basic anion-exchange resin Amberlite IRA-400-OH (ion-exchange capacity 1.4 $Eq.mL^{-1}$) was purchased from Supelco. ^{1}H and ^{13}C-NMR spectra in $(CD_3)_2SO$ or CD_3OD (from Euriso-Top) were recorded on a Bruker AMX400 spectrometer at room temperature unless specified otherwise. Chemical shifts are reported downfield in parts per million (ppm). IR spectra were measured on a Perkin Elmer 683. Optical rotations were recorded on a Perkin Elmer 241MC.

7.3 Results and discussion

7.3.1 Chemistry

This work proposed to show that it is possible to prepare ILs from several types of APIs and develop an efficient and sustainable synthetic methodology for that. The

APIs used in this work have been used as an anion combined with the several organic cations 1-ethyl-3-methylimidazolium, [EMIM], 1-hydroxy-ethyl-3-methylimidazolium, [C_2OHMIM], choline, [Cholin], tetraethylammonium, [TEA], cetylpyridinium, [C_{16}pyr], trihexyltetradecylphosphonium [$P_{6,6,6,14}$], 1-methoxyethyl-3-methylimidazolium [C_3OMIM], 1-(2-hydroxyethyl)-2,3-dimethyl-1*H*-imidazol-3-ium chloride [C_2ODMIM]. The anions used in this work were chosen due to their low toxicity, except in the case of [$P_{6,6,6,14}$] which was chosen to ensure that at least one of the ILs-APIs could be obtained in the liquid form. The several combinations made between the cations and the anion as a specific drug can lead to an alteration in the compound's biopharmaceutical drug classification[32] as well as their drug formulation process.

The Table 7.1 presents the results for the synthesis of the ILs.

Table 7.1 Yield for the synthesis of ILs based on APIs.

Anion	Cation	Yield
[Amx]	[EMIM]	80
	[C_2OHMIM]	62%
	[$P_{6,6,6,14}$]	94%
	[C_{16}Pyr]	55%
[Pen]	[EMIM]	84%
	[C_2OHMIM]	86%
	[Cholin]	98%
	[$P_{6,6,6,14}$]	99%
	[C_{16}Pyr]	91%
	[TEA]	92%
[AmphB]	[C_3OMIM]	53%
	[Cholin]	51%
	[C_{16}Pyr]	71%
	[C_2OHMIM]	90%

7.3.2 Isolation of the Products

The isolation of the ILs based on amoxicillin and penicillin was made in the exact same way as for ampicillin. The excess of the antibiotics was removed by filtration after the crystallization with acetonitrile/methanol (9:1).

Amphotericin B was the most problematic compound to work. This compound is very unstable and is insoluble in water and in most common solvents. Besides, the fragmentation starts easily from hemiacetal ring. As in the case of beta-lactam antibiotics it was also decided that buffered neutralization method (using 1.0 M ammonium solution) should be used. However this solution did not managed to protect amphotericin B from decomposition. After the reaction with the desired cation hydroxide we tried to analyse the final mixture by ^{1}H-NMR. This spectrum was inconclusive and therefore it was analysed by MALDI-TOF-MS the desired peak of the amphotericin B was not found. One of the peaks found was 420.7 u.m.a (**Figure 7.3**). Trying to understand this result, we analysed the structure of amphotericin B and it looked as if amphotericin B could suffer Grob fragmention[158,159].

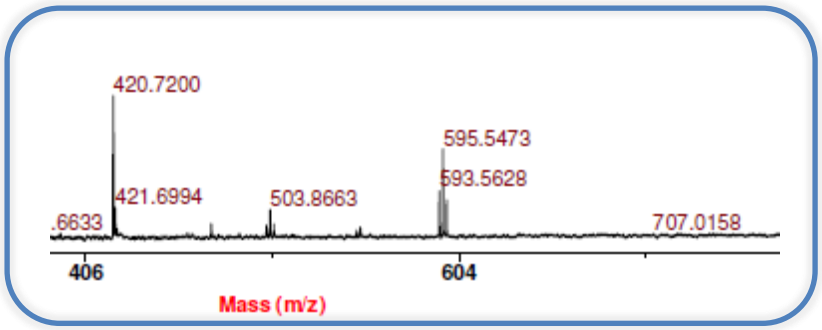
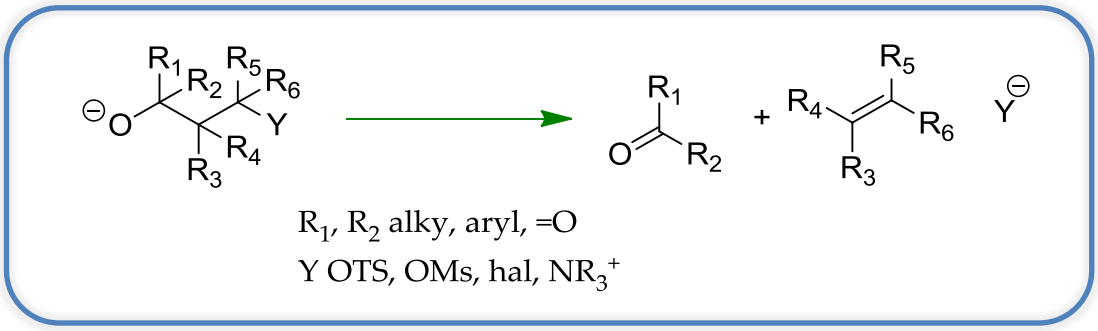


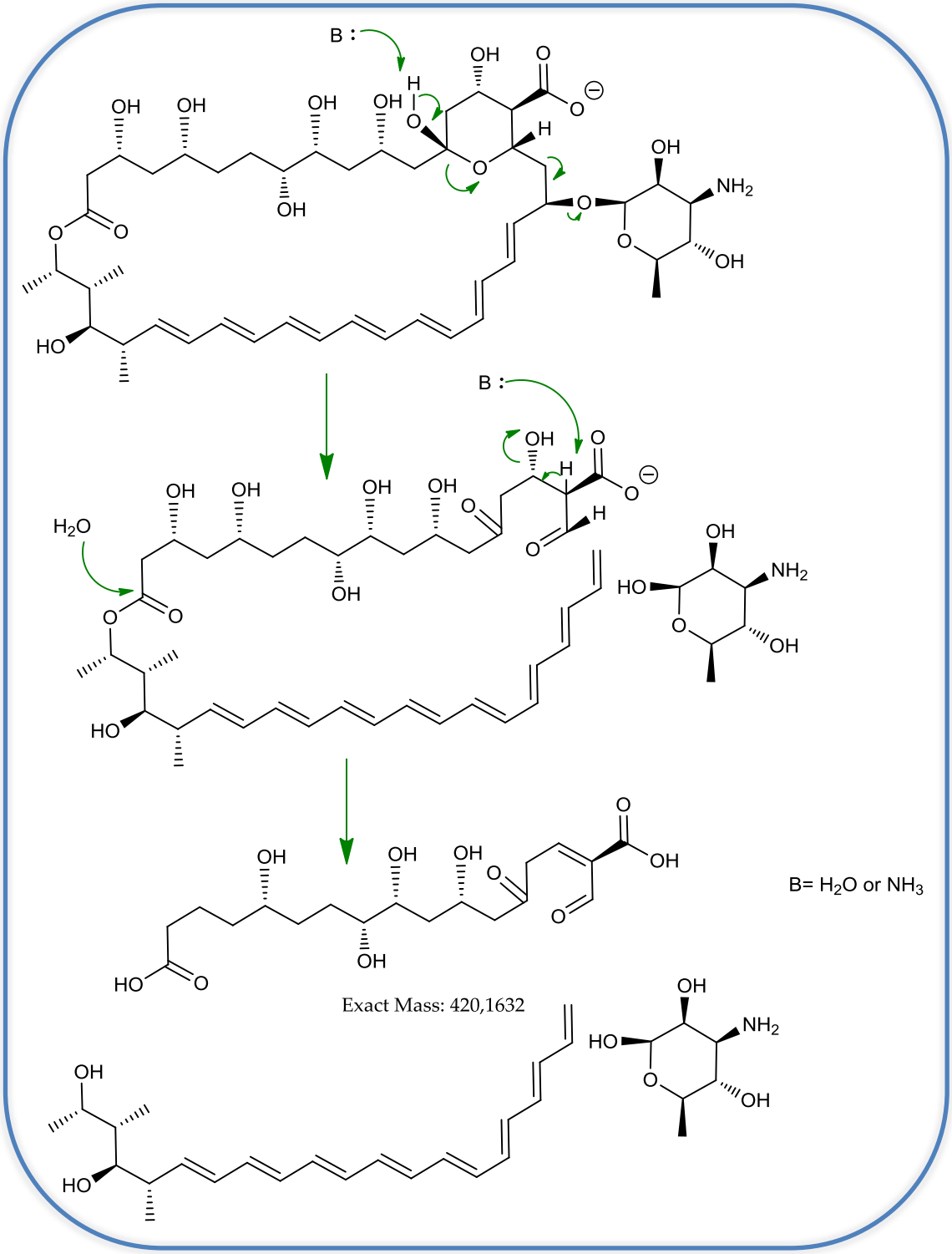
Figure 7.3 MALDI-TOF-MS mass spectrum of the reaction mixture after the first attemtp to synthesise ILs based.on AmphB.

Grob fragmention is a heterolytic fragmentation reaction where a molecule with a certain carbon-heteroatom (N, O, S, P, Si, B, or halogen) combination is cleaved under specific mechanistic principles[158] (Scheme 7.1).



Scheme 7.1 Grob fragmentation of a general 1,3-diheterosubstituted substrate (from Prantz *et al.*[158]).

In the case of AmphB, the degradation could start in the labile hemiacetal point, obviously promoted by attack of an aqueous base. Attack of a base on hemiacetal will promote the opening of acetal ring and further Grob fragmentation with the separation of amino sugar. In addition elimination of beta-hydroxy group of the ester functionality will occur, a reaction similarly catalysed by aqueous base and finally proper ester hydrolysis is plausible in aqueous basic condition (Scheme 7.2)). So, in order to minimize impact from Grob fragmentation and ester hydrolysis, new preparative conditions had to be explored. We decided then to test conditions in the absence of large quantities of water in reagents at the beginning of the reaction (so-called water-free condition).Then we decided to use 1.0 M triethylamine methanolic solution as the buffer solution. This buffer solution managed to dissolve amphotericin B and did not helped its degradation The isolation of pure ILs based on AmphB was made by recrystallization from methanol in order to remove the amphotericin crystalline impurities, insoluble in methanol and then it was treated with calcium carbonate to remove acid impurities.



Scheme 7.2 AmphB degradation by Grob fragmention – a possible mechanism.

7.3.3 Biological Activity

In the present study, a series of ILs-APIs based on amoxicillin and penicillin (Table 7.2)[30] was tested against sensitive Gram-negative bacteria *Escherichia coli* ATCC 25922 and Gram-positive *Staphylococcus aureus* ATCC 25923, as well as against the yeast *Saccharomyces cerevisiae,* an eukaryotic model organism.

The Minimum Inhibitory Concentration (MIC) values were determined in triplicate by the broth micro dilution method, in a 96-well microtiter plate, using Tryptic Soy Broth (TSB) and adapted methodology from the Clinical Laboratory Standard Institute (CLSI)[160]. The *E. coli* and *the S. aureus* strains were grown individually on *Tryptic Soy* agar for 24 h at 37 ^{o}C prior to each antibacterial test and the *S. cerevisiae* was grown in YEPD solid medium. Prior to MIC determination, each inoculum density was adjusted in TSB to 0.5 McFarland standard by photometric device[160]. This resulted in a suspension containing approximately 1×10^8 to 2×10^8 colony forming units $CFUmL^{-1}$ for *E.* coli ATCC25922®[160]. A similar approach was used for the other strains. Then 0.5 μL of the suspension was added to each well to have 5000-25000 $CFUmL^{-1}$. Bacteria were exposed to an IL-API concentration of 5 mM, 2.5 mM, 0.5 mM, 0.05 mM, 0.005 mM, 0.0005 mM and 0.00005 mM. With the exception of ILs with [$P_{6,6,6,14}$], which were diluted in 1% dimethylsulphoxide, all others were dissolved in water and these results were compared with bacteria that had grown in TSB broth in the presence of 1% DMSO. The MIC for each IL was recorded as having lowest concentration, showing no turbidity after 24 h of incubation at 37 ^{o}C[19,160]. The presence of turbidity is an indication of microbial growth and the corresponding concentration of antibacterial agent is considered ineffective. To study whether the antimicrobial activity of the synthesized ILs was bacteriostatic (inhibited growth) or bactericidal (fungicidal for the *S. cerevisiae)*, the colorimetric assay (XTT method) was used[161]. 5 μL of XTT sodium salt of (2,3-bis[2-Methoxy-4-nitro-5-sulfophenyl]-2*H*-tetrazolium-5-carboxyanilide inner salt) was added to the non-turbid wells of the MIC assay plate and incubated for 3 h at 37 ^{o}C for the bactericidal status determination[161,162]. In the case of viable cells with inhibited growth, mitochondrial dehydrogenases of viable cells cleave the tretrazolium ring of XTT, yielding dark orange aqueous soluble formazan crystals; however, a solution

containing dead bacterial cells would remain with the same colour[162]. In this case, MIC values were equal to MBC for each amoxicillin and penicillin based ILs. Therefore, the antibacterial activities of these compounds were considered to be bactericidal. For *Sacharamyces cerevisiae* it was considered fungicidal.

Table 7.2 shows that for ILs based on Amoxicillin have high or equal values of MIC for the bacteria and for the yeast used with the exception of [Amx][C_2OHMIM], which has a Relative Decrease of Inhibitory Concentration (RDIC) of 10.

Table 7.2 Minimum inhibitory concentrations (mM) of the new compounds produced on the microbial stains.

Compounds	*Escherichia Coli*	*Staphylococcus Aureus*	*Saccharomyces cerevisiae*
[$P_{6,6,6,14}$][Amx]	2.5 mM	5.0 mM	>5.0 mM
[C_{16}Pyr][Amx]	5.0 mM	0.050 mM	>5.0 mM
[EMIM][Amx]	0.5 mM	>5.0 mM	0.50 mM
[C_2OHMIM][Amx]	0.050 mM	>5.0 mM	**0.050 mM**
[Amx]*	0.005 mM	0.050 mM	0.500 mM
[TEA][Pen]	>5.0 mM	>5.0 mM	2.5 mM
[$P_{6,6,6,14}$][Pen]	>5.0 mM	**0.005 mM**	**0.050 mM**
[C_{16}Pyr][Pen]	5.0 Mm	>5.0 mM	>5.0 mM
[Cholin][Pen]	>5.0mM	>5.0mM	>5.0 mM
[EMIM][Pen]	>5.0mM	>5.0mM	>5.0 mM
[C_2OHMIM][Pen]	**0.050mM**	>5.0mM	>5.0 mM
[Pen][K]*	0.500mM	0.500mM	>5.0 mM

*[Amx] and [Pen][K] were used as controls.

For the case of the ILs based on Penicillin, Table 7.2 shows that for each microorganism tested there is only one ILs that has decreased the MIC values of Penicillin. In the case of *E. coli* [C_2OHMIM][Pen] has a RDIC value of 10. For *S. aureus* and *S. cerevisiae* the IL that lead to the decrease of MIC value was the one that has [$P_{6,6,6,14}$], a cation whose toxicity is well known[25]. In the case of *S. aureus* [$P_{6,6,6,14}$][Pen] lead to the decrease of the MIC value related to Penicillin (RDIC = 10). For the yeast *S. cerevisiae*, RDIC is bigger than 100 and shows that, when conjugated with right cation, penicillin anion has the potential to be used as an anti-fungal drug.

7.4 Conclusions

This chapter shows that the methodology developed to synthesise ampicillin ILs could be used as an efficient methodology for the synthesis of ILs based on APIs like other beta-lactamics (amoxicillin and penicillin G). It can also be used to synthesise more complex antimicrobial agents like amphotericin B. This could be of extreme importance for the development of new bioactive materials[1] (antiseptics and anti-biofilm, for example), to fight drug resistance in microorganisms or to improve the biopharmaceutical classification system (BCS)[32,146] and, just like ampicillin, they may have the potential to be used as anti-cancer agents. The right selection of the organic cation can provoke important physical and thermal properties of ILs-APIs, such as water solubility, melting point and thermal stability.

The MIC results show us that [C_2OHMIM][Amx], [C_2OHMIM][Pen] and [$P_{6,6,6,14}$] [Pen] are the compounds that could lead to further studies. These results show that [$P_{6,6,6,14}$] [Pen] could be more efficient in fighting *S. cerevisiae* contaminations than traditional antimicrobial agents. Due to the fact that MIC values of [Pen][$P_{6,6,6,14}$] for *S. aureus* are lower than MIC values of amoxicillin and penicillin G, we could say that [Pen][$P_{6,6,6,14}$] in the *S. aureus* tested, could be an alternative to amoxicillin and penicillin G antibiotics and they can also be useful in other applications like surface decontamination and disinfectant/antiseptic in a diverse range of antimicrobial infections.

Chapter 8.
Discussion and Conclusion

8 Discussion and Conclusion

8.1 Discussion

The following discussion focuses essentially on the data presented in the previous research chapters and on future perspectives. In this work, each chapter of results is presented as an accepted or submitted paper and already includes a comprehensive discussion and a conclusion of the work presented.

8.1.1 Synthesis

One of the first references to the Ionic Liquids as Active Pharmaceutical Ingredients (ILs-APIs) was in 2007 ("The third evolution of ionic liquids: active pharmaceutical ingredients")[8], where Hough-Troutman *et a*l.[8] describe the first generation of ILs. The first generation of ILs is characterized by the use of ILs as solvents. The second generation uses ILs as energetic materials and lubricants. In this work Hough-Troutman *et a*l[8] produced ranitidine docusate, lidocaine docusate and didecyldimethylammonium ibuprofen and studied the physical properties and biological efficacy. Since then, many publications have appeared describing the applications of ILs in pharmaceutical sciences. Also in 2007, Rogers and co-workers patented "Preparation of ionic liquid composition, e.g. pesticide composition, involves combining cations and anions having bioactive property or cation precursors and anion precursors having bioactive property when ionized"[10]. In this patent[10], they show the use of preparing an ionic liquid composition (e.g. pesticide, herbicide, cosmetic, food additive, or explosive) with the advantage to overcome "polymorphism, solubility and delivery problems to control release rates, add functionality, enhance efficacy and improve ease of use and manufacture"[10].

The study of ILs toxicity has led to the use of ILs as antimicrobial agents. There are some publications showing the antimicrobial activity of ILs[18,21,60,134,135]. Most of the ILs in

these publications are imidazolium and/or phosphonium halides. In spite of this, there is another work with ILs as antimicrobial agents, like the use of ammonium based salts with artificial sweetener anions, from Hough-Troutman[133]. More recently, in 2011 Cole produced biocidal ILs consisting of cationic imidazolium or pyridinium and an anionic beta-lactam antibiotic[19]. Pernak has developed, between 2011 and 2013, ILs based in herbicides[136,154,201-203]. Nevertheless, besides Hough-Troutman's, Cole's and Pernak 's works, there are not many study cases about ILs based on API and most ILs produced are by metathesis reaction. As a matter of fact, in "Ionic Liquids - New Aspects for the Future"[149], as referred by Clarissa P. Frizzo and co-workers those alternative methods to metathesis reaction were used by us in the synthesis of beta-lactamic antibiotics and by Bica and co-workers in the synthesis in solvent-free conditions of salicylic acid ILs[149]. As referred before, metathesis reaction has some inconvenients[24,85]: contamination with a small amount of halide ions, which may react with solute materials, and the presence of water or silver residues as impurities.

There is another problem with the metathesis reaction related to the synthesis of pharmaceutical compounds: the use of halide solvents (like chloroform) for the majority of the purification process[204-206]. The pharmaceutical industry avoids the use of halide solvents[205,206].The advantage of the method presented in this work is that it uses methanol as an organic solvent, avoiding the use of halide solvents in the synthesis and in the purification.

The method used to synthesize the ILs in this work is the neutralization method. As said before, this method solved the problem of contamination[90]. The only difficulty with this method is when we have weaker acids than hydrohalic acids[91]. This problem was solved using hydroxide quaternary cations[30]. In this work we adapted ion exchange resin methods developed by Ohno and co-workers[26,91-93]. Amberlite resin (in the OH form) has was used in order to exchange halides (bromide or chloride) into hydroxide form and then this basic solution was neutralized by the adding an adequate buffer solution of the API. Because the amberlite resin used is regenerated, it brings about another advantage, since we could use the same resin for several syntheses without spending further resin.

When this method was applied to the synthesis of the ILs and salts based on beta-lactamic antibiotics and amphotericin B, some were detected:

1) the beta-lactam ring strain, which promotes the opening of the ring,

2) the low solubility and the low stability of the amphotericin B;

3) the purification of the final compounds.

1) the beta-lactam ring strain, which promotes the opening of the ring

To avoid the opening of the ring and to give more stability to the antibiotics, we decided to dissolve the antibiotic in a buffer. This process worked very well and the beta-lactamic ring did not open as shown in the analysis.

2) the low solubility and the low stability of the Amphotericin B;

To solve this issue, we applied the same methodology as used in the antibiotics, where a buffer was needed. The first buffer we tried was ammonium solution in water, but it did not work. Amphotericin was degraded. It seemed that Amphotericin B suffered Grob fragmentation[158] Then it was decided that dried triethylammonium should be used to avoid degradation. The ^{1}H-NMR shows that the Amphotericin B anion was presented in the final product. Also the triethylammonium solution in methanol helped to dissolve the Amphotericin B.

3) the purification of the final compounds

These syntheses did not have any side product. Nevertheless, all the syntheses used a slight excess of the acid (antibiotics and Amphotericin B), so it was necessary to remove the excess in the end.

To remove the excess of beta-lactamic antibiotics (Ampicillin, Penicillin G and Amoxicillin) it was used a similar method to the one Ohno and co-workers had used: a mixture of f acetonitrile and methanol (9:1) to induce the crystallization of the antibiotic excess and then, the crystals were removed by filtration.

In the case of Amphotericin B, it was necessary to modify the process. In this case, after 2 h of reaction, in solution, it was observed that the solution had some undissolved powder. To remove these impurities, first the solution was filtered. After filtration in order to remove any residues of triethylammonium, dry calcium carbonate was added. Then we filtered the solution and evaporated the solvent. The analysis confirmed that ILs had been obtained ILs based on amphotericin B.

8.1.2 Biological Studies

In this work, the new synthesized ILs based on Ampicillin were the most studied in terms of biological studies. The studies of the antibacterial activity against resistant strains and also their anti-tumoral activity were made for the first time with these ampicillin-ILs.

The analysis of the results for the antibacterial activity demonstrated that the lowest MIC values of Ampicillin ILs used are for Ampicillin ILs containing apolar cations such as [$P_{6,6,6,14}$][Amp] and [C_{16}Pyr][Amp]. Probably because highly polar cations like [Cholin] or [C_2OHMIM] are more prone to stay in aqueous solution instead of in hydrophobic cell membranes and therefore anchor themselves to ampicillin anions (ion trapping effect)[186]. This could lead the correspondent ampicillin salts to stay in aqueous solution where they could eventually could be hydrolysed. These results are according to the literature which says that the increase of hydrophobicity and the increase of the alkyl chain increase the toxicity[25,155,157,191]. These studies describe the toxicity of imidazolium, pyridinium, quaternary ammonium and phosphonium cations in several microorganisms such as rods, cocci and fungi[155,157,191] and on human colon carcinoma cell line[25,192].

These results suggest that antibiotic APIs-ILs help in the delivery of an antibacterial agent [Amp] to some Gram-negative bacteria that developed resistance against β-lactam antibiotics[187,189], most probably acting as a lipophilic phase transfer agent across the outer membrane of the bacteria as referred by Lehn *et al*.[190] Many more studies are needed, in particular, to study the mechanism of action of the ILs. The understanding of this mechanism could be of extreme importance to fight bacterial resistance, because

it could lead to the finding of the right cation needed to conjugate with the antibiotic that could fight resistant bacteria.

The study of the anti-tumoral activity was based on Kumar work[23,31]. Kumar had studied the anti-tumoral activity of imidazolium[23], phosphonium[31] and ammonium[31] ILs and because the ILs based on ampicillin have all kinds of these cations (imidazolium, phosphonium and ammonium), we decided to study these ILs in several cancer cell lines. The results obtained demonstrated the potential of these organic salts as selective anti-tumour agents. For example, [C_2OHMIM][Amp] possesses relevant discriminative and anti-proliferative activity against five different human cancer cell lines, in particular T47D (breast), PC3 (prostate), HepG2 (liver), MG63 (osteosarcoma) and RKO (colon) and low or none anti-proliferative activity against two primary human cell types: skin fibroblasts (SF) and gingival fibroblasts (GF). As expected, Ampicillin ILs with the $[P_{6,6,6,14}]^+$ cation had very high cytotoxicity in all cell lines studied (cancer and non-cancer cell lines). Frade *et al.* had already studied several compounds based in this cation towards other cell lines, as has already been described showing its toxicity[25].

The biological characterization of the ILs based on Penicillin G, Amoxicillin, and Amphotoricin B are still being studied. In this work, we already presented some preliminary studies of the anti-microbial activity of Penicillin G and Amoxicillin ILs against *E. coli*, *S. aureus* and *S. cerevisiae*. The microorganisms were chosen because they are the classic study models of Gram-negative bacteria (*E. coli*), Gram-positive bacteria (*S. aureus*) and *S. cerevisiaes*, being the most common eukaryotic model organisms. These preliminary studies once again show that the presence of the cation $[P_{6,6,6,14}]^+$ (an apolar cation with large alkyl chain) led to a bigger antimicrobial activity.

In the case of Amphotericin B ILs, studies are being prepared to study the activity against several strains of fungals *Aspergillus fumigatus, Candida albicans* and *Sacharomyces cerevisiae*. Because Amphotericin B is also the most important drug for the treatment of visceral and cutaneous leishmaniasis, studies on *Leishmania* (visceral and

cutaneous) will also be made in the future. Besides the solubility studies of ILs based on Amphotericin B are being prepared.

8.2 Conclusion

This Thesis is mainly focused on the synthesis of ILs based on APIs (Chapters 4 and 8) and their biological analysis (Chapter 5, 6 and 7). The use of ILs as solvents for synthesis is the major application of ILs. There is no doubt that ionic liquids are promising alternative solvents in synthesis, often improving the efficiency and sustainability of the synthesis. This work both anticipates and contributes to the interest of the pharmaceutical industry in ILs-APIS. It also demonstrates some new methods of preparing ILs-APIs and shows some of their potential applications.

After the synthesis one of the focuses was on anti-bacterial activity on resistant strains. As said previously, the emergence of multi-drug resistant organisms has limited clinicians to the number of effective anti-infective agents available for treatment. Therefore, there is a need to reuse and find new applications for some of the drugs, not only for economic reasons but also to find new ways of therapy and treatment which will allow some drugs to be reconsidered. Based on Kumar work[23,31], it was evaluated the anti-proliferative activity of this compounds on human cell line (cancer and non-cancer) to find new alternative to chemotherapy.

This work brings some new perspectives for the use of ILs. The main achievements are summarized in the following topics:

a) Synthesis – A new sustainable strategic methodology was developed for the synthesis of ILs based APIs. This method is more useful when the exchange by acids weaker than hydrohalic acids[91] cannot be efficiently performed. It is halogenated solvents free and the resin used can be recovered. In this work we have successfully synthesised the following ILs based on APIs with good yields, completely characterized (^{1}H-NMR, ^{13}C-NMR, IR and mass spectra):

- **6** ILs based on ampicillin: [TEA][Amp], [$P_{6,6,6,14}$][Amp], [C_{16}Pyr][Amp], [Cholin][Amp], [EMIM][Amp], [C_2OHMIM][Amp].
- **4** ILs based on amoxicillin: [$P_{6,6,6,14}$][Amx], [C_{16}Pyr][Amx], [EMIM][Amx], [C_2OHMIM][Amx].
- **6** ILs based on penicillin G: [TEA][Pen], [$P_{6,6,6,14}$][Pen], [C_{16}Pyr][Pen], [Cholin][Pen], [EMIM][Amp], [C_2OHMIM][Pen].
- **4** ILs based on amphotericin B: [C_{16}Pyr][AmphB], [Cholin][AmphB], [C_2OHMIM][AmphB], [C_3OMIM][Amp].

The method of purification developed for each compound was efficient and proved to be appropriate. It showed to be efficient not only with antibiotics but also with anti-fungal drugs, like amphotericin B.

b) Antimicrobial activity – The biological activity for the ILs based on antibiotics showed us that ILs based on antibiotics could lead to alternative ways of fighting bacteria and even resistant bacteria. This proves that with a careful selection of the organic cation, it is possible to provoke important biological alterations in their antibacterial properties. In summary:

- [C_{16}Pyr][Amp] demonstrated the highest potential in reversion of resistance against Gram-negative resistant bacteria (*E. coli* TEM CTX M9 and *E. coli* CTX M2, RDIC > 1000 and 100 respectively);
- [$P_{6,6,6,14}$][Amp] with RDIC values > 10 against *E. coli* TEM CTX M9 and *E. coli* CTX M2 was also able to fight the resistant bacteria presented.
- The use of RDIC values allows a better understanding of the MIC values, as well as the evaluation of the real effect of ILs-APIs activity.

The application of ILs based on antimicrobial agents in fighting infections may help to decrease the nosocomial infections (in terms of morbidity and mortality) and the costs associated to them.

a) **Human cell line studies** – besides the antimicrobial activity studies which were made for the ampicillin ILs, we also studied the toxicity effect of these compounds on two primary human cell types, namely, skin and gingival fibroblasts (SF and GF, respectively) and five cancer cell lines such as T47D (ductal breast epithelial tumour cell line), PC3 (prostate cancer cell line), HepG2 (hepatocellular liver carcinoma cell line), MG63 (osteosarcoma cell line) and RKO (colon cancer cell line). In summary:

- [C_2OHMIM][Amp] showed the highest cytotoxicity against five different cell lines (T47D, PC3, HepG2, MG63 and RKO) and very low cytotoxicity against two primary cell lines (skin (SG) and gingival fibroblasts (GF);
- [Na][Amp] mostly did not show activity;
- [$P_{6,6,6,14}$][Amp] was the most toxic;
- [TEA][Amp] and [Cholin][Amp] were the least toxic.

It is important to note that the correct choice of the cations and anions could render the salts or the ILs with specific activity against different tumour cell lines, and, in a broader way, modulate the final biological properties of the ILs[9,23,31,142,155]. This is in accordance with the recently proposed idea that neutral ionic liquids can modulate the biopharmaceutical drug classification system (BCS)[1] of an active pharmaceutical ingredient and therefore enhance its membrane transport, as recently shown with protic ionic liquids, by other researchers[15].

This research also offers the groundwork for the application of this technology in other fields of the pharmaceutical industry. In addition to targeting drug-resistant or pathogenic bacteria, the consideration of ILs based on ampicillin as anti-tumour agents can be useful from this research. Likewise, this may be beneficial in designing new agents as anti-epileptic or anti-fungal drugs. The simple and rapid synthesis of ILs from active pharmaceuticals allows this flexible, pragmatic approach to lead to the next generation of combination drug therapy.

8.3 Future Work

These methods of synthesis developed and studied in the present work seem to be suitable on what concerns the practical application for synthesis of ILs with other APIs, for example, for the synthesis of other anti-epileptics like valproic acid, anti-cancer drugs or any other kind of drugs.

Bacteria resistance is a well-known problem. Therefore, these promising results could lead to further studies in order to better understand the resistance mechanism and how ILs based on antibiotics could act to eliminate or fight that resistance. First of all, it is necessary to study the bacteria activity of ILs based in amoxicillin and penicillin G against resistant bacteria. Then, it is necessary to study the mechanism of action of this ILs based on antibiotics in bacteria and resistant bacteria to better understand how they act.

The biological activity of ILs based on Amphotericin B is already in progress, so there is a need to further understand their behaviour. ILs based on Amphotericin B are being analysed to study their mechanism of action in *Aspergillus fumigatus*, which could help to understand the fungicidal activity of these compounds.

Chapter 9.
References

10 References

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