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# List of Abbreviations

**CA-MRSA** Community-Associated MRSA

**DCM** Dichloromethane

**DMF** N,N-Dimethylformamide

**ESKAPE** *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp*

**FTsz** Filamentous temperature-sensitive protein Z inhibitors

**GTP** Guanosine triphosphate

**HA-MRSA** Hospitals-Associated MRSA

**ifMet** N-formylmethionine

**iMet** Initiator methionine

**LCMS** Liquid chromatography-mass spectrometry

**MDR** Multi-drug-resistance

**MetAP** Methionine aminopeptidase

**MIB** Sepharose beads

**MQO1** Malate:quinone oxidoreductase

**MRSA** Methicillin-resistant *Staphylococcus aureus*

**MS** Mass spectrometry

**NAG** N-acetylglucosamine

**NAM** N-acetylmuramic acid

**NBS** N-Bromosuccinimide

**OSA** OpenSourceAntibiotics **PBPs** Protein binding penicillin

**PBD** protein data bank **PGN** Peptidoglycan

**PurD** Phosphoribosylamine-glycine ligase

**SAR** Structure-activity relationship

**SCC** Staphylococcal cassette chromosome

**SodA** Superoxide dismutase

**TDR** Totally-drug-resistance

**UNC** University of North Carolina

**VISA** Vancomycin-Intermediate *S. aureus*

**VRs** Vancomycin-resistant organisms

**VRSA** Vancomycin-Resistant *S. aureus*

## Abstract

Antibiotic-resistant infections have been a global problem for decades, highlighting the necessity to develop new classes of antibiotics with new mechanisms of action, ideally limited by the development of resistance.

The series-2-Diarylimidazole project is part of an open-source *consortium* called “OpenSourceAntibiotics”, characterised by periodic meetings where people are free to join and exchange their ideas, contributing to the development and discovery of inexpensive drugs.

This project started with a small group of compounds tested against ESKAPE pathogens activity, with the result that some of them were found to be active against MRSA (Methicillin-resistant *Staphylococcus aureus*).

Initially, these compounds were characterised by a 2-(pyridin-2-yl)-6,7-dihydro-5H-pyrrolo[1,2-a]imidazole scaffold with limited SAR and target knowledge; then, by pursuing the traditional way of drug discovery, further improvements and studies increased their selectivity and cytotoxicity knowledge.

Following this, this thesis’ project aimed to find the molecular target, increase the SAR’s knowledge and clarify the mechanism of action of these molecules to gain potency, biological activity and selectivity. With these goals, computational tools were utilised for finding some targets candidate, and the synthesis of two new compounds is providing some interesting data regarding SAR learning.

Indeed, the computational studies helped select some candidate targets as MetAP1 and TGF- $\beta$ 1, studying which kind of interactions these compounds can make with these proteins, and which binding can be recognised from the docking software (especially coordination bonding with metals).

On the flip side, two different synthetic ways (Route I and II) were compared for synthesising a small library of new compounds.

The most successful route was Route II which involves a Suzuki reaction first, followed by a Hartwig-Buchwald amination. From this, two desired products were synthesised: compounds 9 and 10 with 18.2% and 37% yield, respectively. These molecules are being biologically tested with the goal of increasing the target active site’s and SAR’s learning.

## Acknowledgements and Declaration of Contributions

The author of this report made all the experiments and data collection with the help and the supervision of Dana Klug and Matthew Todd's research group (School of Pharmacy). I would like to thank Jr, J.L. Kiappes, Hugh Britton and all the demonstrators (Chemistry Department), who helped in the performance of the experiments, data collection and interpretation.

The University of North Carolina shared data regarding the mechanism of action of the tested compounds and PharmAlliance for funding underpinning this overall project.

## Declaration of Authenticity

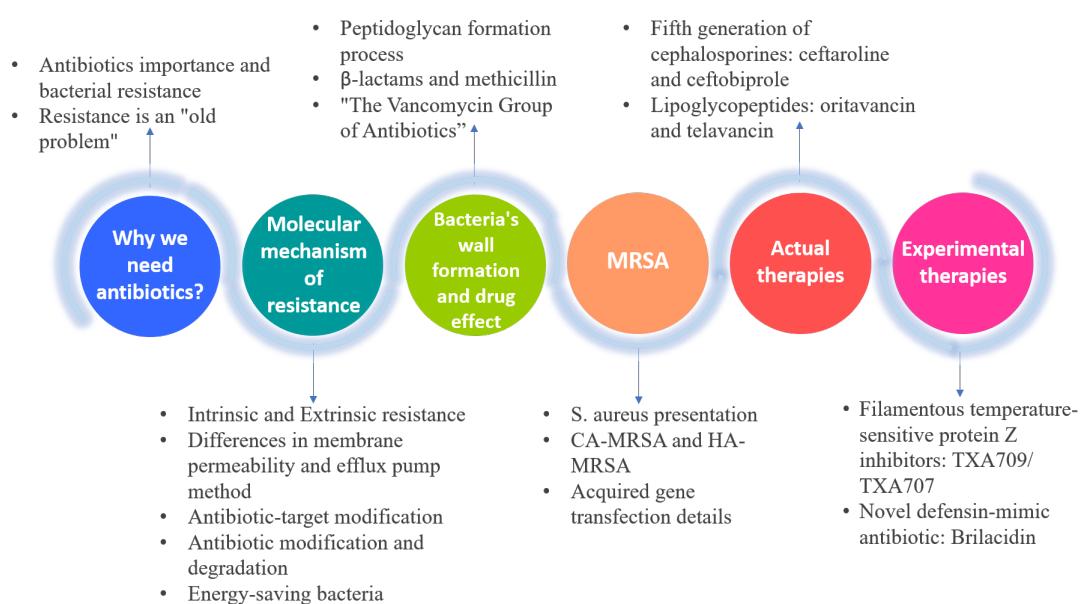
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# Introduction

In these introductory chapters, the various aspects of the situation regarding the problem of antibiotic resistance are shown. This provides an overview of why antibiotics are needed, which strategies bacteria use to achieve resistance, with a particular focus on methicillin-resistant *Staphylococcus aureus*, the main object of this research. The framework of the therapies in use and in development is then presented, in order to show and clarify the aim of this research thesis.



**Figure 1** *Introduction overview:* main points and key concepts of the introductory chapters are outlined here.

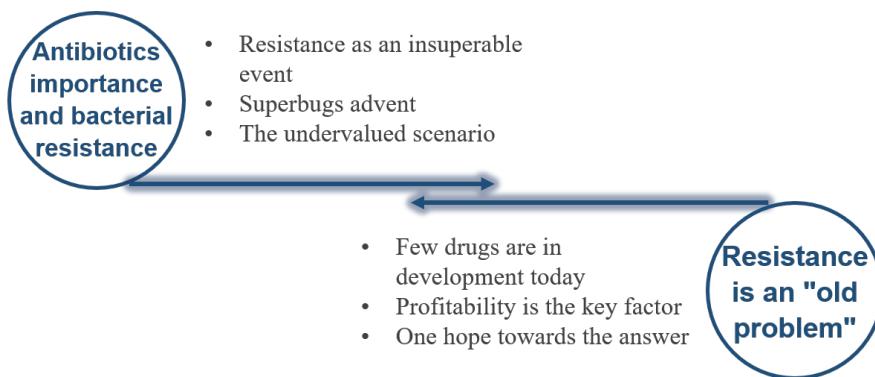
## 1 Why we need antibiotics?

### The importance of antibiotics and bacterial resistance

Since the discovery of the first antibiotic (1930 Sulfonamides) resistance phenomena have been observed and were first documented in 1940, with the end of the First World War [1].

This phenomenon happened because antibiotic resistance is a natural process which is expressed by genetic mutation occurring during genome replication or acquired by plasmid transmissions (circular DNA fragments) between bacteria. The result is the reduction in efficiency as a response of the

treatment-adaptability of the pathogenic organism and its continuous variability . The mutation can be specific against a particular class of drug or include chemical compounds characterized by different mechanism of action (cross-resistance). With the spread of this adaptation capacity, some organisms are becoming multiply drug resistant, giving rise to the so-called multi-drug-resistance (MDR) or, even worse, totally-drug-resistance (TDR) [2]. In this way "superbugs" (super-resistant bacteria) have been labelled such as MRSA (Methicillin-Resistant Staphylococcus Aureus) or VRs (Vancomycin-Resistant organisms) which are becoming a worldwide problem [3]. Within this trend, the resistance is constantly increasing, but the seriousness of the situation is still underestimated as few drugs are currently in development and even fewer of them act on new or possibly new targets. [4], [5] This underlines the lack of progress and funds for this topic of global interest.



**Figure 2 Why we need antibiotics? overview:** main points and key concepts about the need of antibiotics are outlined here.

### Resistance is an “old problem”

One of the biggest issues is that infections are considered an outdated problem, as a large number of drug classes were discovered after the Second World War and those same drugs are still used today, without any or with slight updates, used only for emergencies [3]. After the "golden era" of antibiotics discovery (1940-1970) [3], only few classes and few compounds were developed [4]; therefore, global healthcare needs innovation and progresses in this sector: there is a huge need to discover new mechanisms of action together with the necessity of MDR regression.

Notably, the pathogen's survival over our defences is spread due to the misuse and overuse of those old antibiotics that are still available: excessive demand and waste

of cares in agriculture, animals, food companies and in the society [6] contribute together to enhance the MDR. From this, 700000 people are dying every year for resistant infections [3], increasing the healthcare costs each year. [4] [2]

The slowdown is caused by economical interests [2] and the challenge in meeting the principles of drug development does not help (selective towards microbial processes, not interfering with human's intestinal flora and biochemistry, achieve growth inhibitory concentration, being immune to organisms' DNA variations etc...) [7].

At this stage, big companies do not invest their resources in this market (as it does not increase their profitability) and then, only small/ medium-sized industries or universities take on the issue [6], and these are struggling for funding.

From that emerges the need of an immediate global action [6] for supplying new technologies (no change for 140 years now) [3] and novel drugs, in order to decrease, control and prevent the bacterial multi-resistance.

This phenomenon is impossible to eradicate, but it is something to live with on a daily basis [2]. The discovery and development of new antibiotic is very challenging [5] and this project's goal is facing the threat.

## 2 Molecular mechanism of resistance

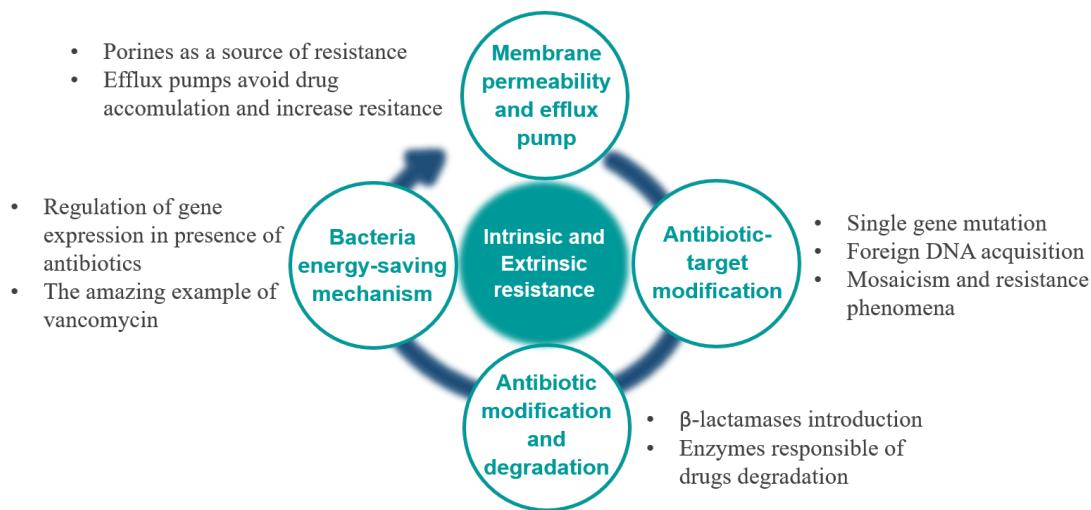
Bacterial resistance towards antibiotics is a complex and ancient phenomenon, which is based on the ability of these organisms to survive and mutate in presence of substances (antibacterial drugs) created for their elimination. [8] Furthermore, antibiotic resistance can be a natural event (intrinsic resistance) or can be acquired as a result of mutations or conjugations with other bacteria (extrinsic resistance): indeed, this ability is the result of a intrinsically developed structural and functional characteristics [9].

### Intrinsic and Extrinsic resistance

The intrinsic resistance is mainly represented by the different structure and constitution of bacterial protective wall (especially regarding gram-negatives) and constitutes a barrier for drug accessibility [10].

On the flip side, there are many mechanisms that bacteria can exploit to acquire

or develop resistance. These are the result of the altered expression of certain genes and give birth to the so-called extrinsic resistance.



**Figure 3 Intrinsic and Extrinsic resistance overview:** main aspects and key concepts about intrinsic and extrinsic resistance are outlined here.

### Differences in membrane permeability and efflux pump method

Charged proteins have the ability to cross bacterial wall and membrane, via protein channels called "porins". These structures allow proteins to enter selectively the cell and are presented both in gram-negative and some gram-positive. The relative mechanism of resistance is linked to the modification and down-regulation of these Porines (mainly in *Enterobacteriaceae* and in *E. coli*). [11] [12]

Compared to gram-positive bacteria, gram-negatives are more frequently characterized by these efflux systems that allow the outflow of toxic molecules outside the membrane [13]. The activity of these pumps not only avoids drug accumulation within bacterial cytoplasm but also represents the ability to increase resistance of unrelated drug classes, cause "species- or genus-specific intrinsic resistance to antibiotics" [13] and additionally, their over-expression is responsible of cross resistance in many organisms [14].

Pseudomonas strains are peculiar examples of intrinsic structural resistance, Porines and efflux pump systems. [15]

### Antibiotic-target modification

Drugs are created to be specifically selective for the binding with bacterial targets, therefore, the mutation of a single gene encoding for that specific target may induce a lack of effectiveness in drug-target bonds.

This can happen for a target that presents the genes in multiple copies: if the mutation occurs in only one of these genetic replicate, the formation of a new drug-resistant allele occurs.

Subsequently, selective pressure and gene-recombinations induce the prevalence of this allele over the others (e.g. linezolid resistance, given by a single 23S rRNA gene mutation). On the other hand, there is the acquisition of foreign DNA (transformation) which can give the mosaicism process (the union of different genetic heritages in a single organism, which expresses them simultaneously) or the acquisition (through plasmids or mobile elements) of a gene homologous to the original target that gives resistance. This occurs in MRSA strains, where the acquisition of SCCmec (staphylococcal cassette chromosome mec) leads to the development of PBPs (protein binding penicillin) insensitive to methicillin, as PBP2a. [9]

### **Antibiotic modification and degradation**

Another strategy that bacteria evolved to fight the drugs effect is identified as the direct modification on these molecules. In this way the drug loses its effectiveness and the target-bonding.

The drug-substrate changing by enzymatic catalysis is the oldest and the most common mechanism since the discovery of penicillases ( $\beta$ -lactamase) in 1940 [16].  $\beta$ -lactamase induces an hydrolysis in  $\beta$ -lactam ring, making it ineffective against their pharmaceutical target (PBPs): these enzymes are grouped in different subclasses, able to hydrolyze drugs of the same group as penicillin, cephalosporines, carbapenemes, and monobactams , which are part of  $\beta$ -lactam antibiotics.

Over time, the discovery and implementation of new drugs on the market, together with improvements of mechanisms of action, have led to fast-evolution hydrolytic enzymes activity, giving rise to broad-spectrum  $\beta$ -lactamases with increased activity and rate of action against most  $\beta$ -lactam antibiotics [17], [18].

Other enzymes are then identified, able to modify and degrade different drugs:

bacteria often carry one or more genes which encode for antibiotic covalent-modification catalysis. (e.g. aminoglycosides with acetyltransferases, phosphotransferase, nucleotidyltransferases, which attach functional groups on the antibiotic, limiting their activities [19]).

### Bacteria energy-saving mechanism

The approaches listed above are the demonstration of the mutability and adaptability of the bacteria, which after natural selection, has learned to control gene-expression.

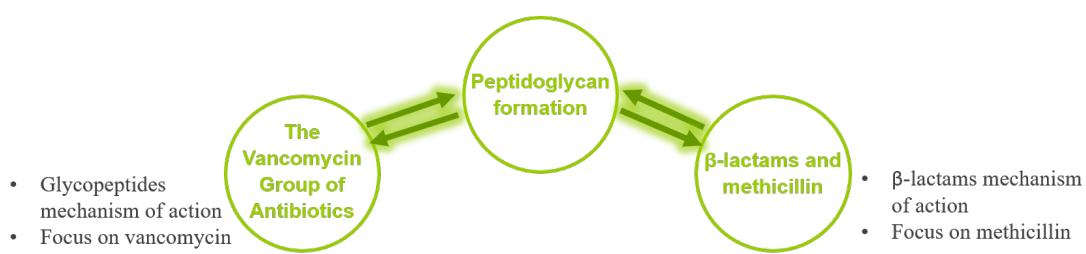
Since the transcription of these genes is not essential for the life of the bacterium, but represents only a defense mechanism, the bacteria exploit the so-called “regulation of gene expression”, inducing the expression of the modified gene only in the presence of the antibiotic for which the mutation occurred, gaining in energy and safety. [20]

Examples are *E. faecalis* with  $\beta$ -lactamase gene-transcription (activated only in the presence of inhibitors of peptidoglycan synthesis [21]), erm-gene methylase which confers macrolides and lincosamides resistance [22] and Vancomycin.

As Courvalin says, the most interesting case is that of vancomycin (antibiotic glycopeptide) where the mechanism of resistance consists on the changing between the dimer Dala (peptidoglycan precursor) and Dala-Dlac, thus escaping the pharmacological action. This change costs a lot of bacterial energy and therefore, this occurs only when it confers a selective advantage to the host [20].

## 3 Bacteria's wall formation and drug effect

The bacterial wall is of crucial importance for life, growth and survival of the bacterium organism and it is therefore widely used as a target for antibacterial drugs. In this chapter, the details of wall formation and the related drug targets of  $\beta$ -lactams and glycopeptides are considered, together with their mechanism of action. This description is relevant because this project's molecules can interfere with the cell wall formation process.



**Figure 4 Bacteria's wall formation and drug effect overview:** main aspects and key points about bacterial cell wall as a pharmaceutical target are outlined here.

### Peptidoglycan formation process

The bacterial cell wall consists of a rigid structure protecting the cytoplasmic membrane, which has a critical role in bacterial survival and development. This structure is made up of diaminopimelic, muramic and theicoic acids, which are bound together with carbohydrates, lipids and amino acids to form peptidoglycan unit (mucopeptide or murein). Murein is composed of glycan chains, which in turn are made up of alternating linear bands of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM), aminoglycosides that have cross-linked links to peptide chains. [23]

Peptidoglycan (PGN) biosynthesis involves three main steps:

1. Park nucleotide formation in cytoplasm
2. Lipid II assemblage and transglycosidase effect
3. Cross-links creation between peptidoglycan chains (transpeptidase catalysis)

**The first step** takes place in the cytoplasm and is characterised by the formation of the Park nucleotide (UDP-NAM-pentapeptide), where the last reaction involves the insertion of the D-Ala-DAla dipeptide obtained by the L-Ala racemization (to D-Ala) and its condensation. The catalyst is the enzyme D-alanyl-D-alanine synthase.

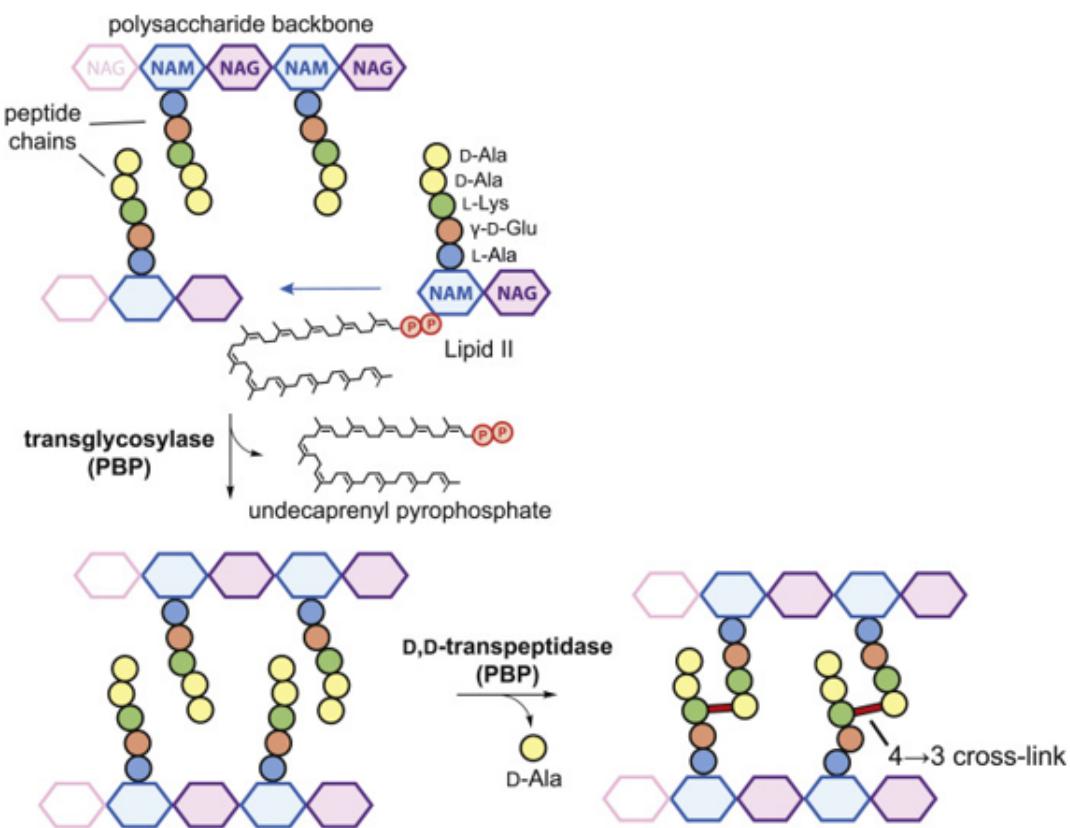
**The second step** is marked by the union of UDP-NAM-pentapeptide and UDP-acetylglucosamine (UDP-NAG), which form a long heteropolymer: first, the pentapeptide sugar is linked to a phospholipid transporter, then a second UDP-NAG molecule is added with the formation of a b-1,4 glycosidic bond

between the two sugars and this is followed by a further addition of five glycine residues (on the third amino acid). This cross-linked complex is transported through the membrane, from the cytoplasmic environment to the periplasm. Thus, this “complete unit” is called lipid II and contains the basic peptidoglycan component (NAG-NAM-pentapeptide). [23]

Once the lipid II is transported to the periplasmic side of the cell membrane, the transglycosylase activity occurs (Figure 5): it is represented by the linkage between the novel NAG-NAM-pentapeptide unit and the other structural components of the murein, leading to new  $\beta$ -1,4 NAM-NAG-glycosidic bonds creation. [24]

In **the third step**, the cross-linking of two adjacent chains is completed by transpeptidation between the newly created and a previously-created adjacent units. This interesting reaction is catalysed by transpeptidases (characterized by a nucleophilic serine residue in the active site) and results in peptide bond creations (Figure 5). Indeed, formation of cross-links occurs between the carboxyl of the fourth D-Ala residue (of the newly formed unit) and the last glycine amino-terminal residue of the adjacent chain’s pentaglycine unit (on the third amino acid).

In this way, the nucleophilic serine attacks the amide linkage between D-Ala4 and D-Ala5 of the pentapeptide, forming a transient ester-based intermediate with the enzyme side chain (cleavage of amide linkage and D-Ala released), and subsequently, the amino group of the glycine residue nucleophilically attacks the ester linkage, replacing the enzymatic serine (regenerating the catalyst) and forming the novel peptide bond (Figure 6). [25]



**Figure 5 Peptidoglycan formation:** Transpeptidase and transglycosidase actions within new peptidoglycan chain formation.

Image adapted from [25] with permission from Elsevier.

### $\beta$ -lactams and methicillin

As previously said, transpeptidases are essential enzymes for the bacteria's wall formation and they are also called PBPs (Penicillin-Binding Protein) because they are the  $\beta$ -lactams' characteristic target.  $\beta$ -lactams' activity against PBP is an exciting mechanism: a valuable source for synthesising new drugs and understanding their resistance mechanism.

Within bacteria, various number of PBPs are diffused with both

glycosyltransferase and transpeptidase domains or only characterized by the transpeptidase activity. [25]

The  $\beta$ -lactams' PBP inhibition is based on these drugs' structural similarity to D-Ala-D-Ala residue (the original target of these drugs). Indeed, the transpeptidase's nucleophilic serine attacks the carbonal group of the strained  $\beta$ -lactams ring with a covalent linkage (serine acylation) as a result. In this way these enzymes are blocked and not able to fulfil the new PGN's cross-links

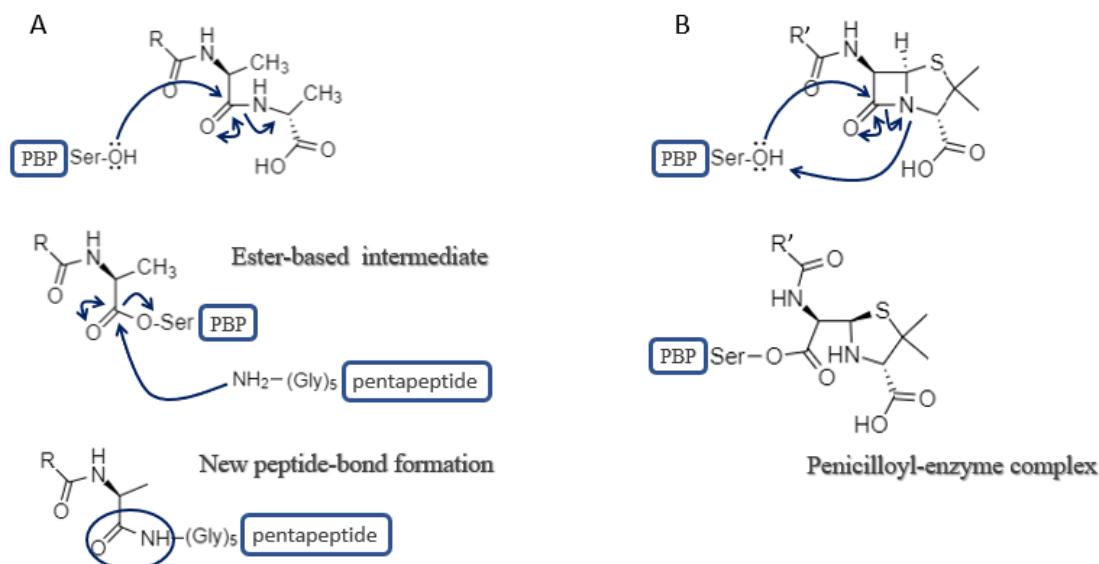
catalysation. [25]

Moreover, the stable unit formed between PBPs and penicillin ( $\beta$ -lactams) is called “penicilloyl-enzyme complex” which for steric reasons, prevent the pentaglycine’s amino-terminal attack in order to revert the acetylation-reaction as penicillin linkage result. This makes the enzyme bond irreversible and therefore weak PGN (peptidoglycan) cross-links are formed, which lead to cell lysis and pathogen’s death. [23]

Notably, the high electrophilic-character of the  $\beta$ -lactam system’s carbonyl and the steric-tension of the 4-members ring, make it more reactive than the carbamate at the side-chain of the general  $\beta$ -lactam antibiotics structure. Therefore, this ring characterises the  $\beta$ -lactam drugs and represents the main pharmacophoric group of all the classes of these antibiotics, as penicillins (methicillin, ampicillin), cephalosporins (carbenicillin, piperacillin), monobactams, carbapenems and penems. [23]

Methicillin in particular, presents two methoxy groups the 2, 6 positions of the phenyl ring consequently conferring steric-hindrance “ortho effect”, making therefore this antibiotic initially resistant to the  $\beta$ -lactamases effect (see above). [23]

As said before the mechanism of resistance of this antibiotics consists on the  $\beta$ -lactamases production and PBPs modification which together make the interaction target-drug less probable or unfeasible.



**Figure 6 Transpeptidase's mechanism of action and inhibition:** in Figure A, nucleophilic attack by enzymatic-serine to the amide bond between the two DAla amino acids (DAla4 DAla5 dimer of the pentapeptide unit), by forming the ester intermediate; then, the last glycine's amino group of the adjacent chain establishes the new peptide bond and regenerates the enzyme. In figure B, transpeptidase's serine attacks the carbonal group of the strained  $\beta$ -lactams ring with a covalent linkage, acylating the serine residue and forming the “penicilloyl-enzyme complex”. R= remaining elements of the new peptidoglycan’s chain, R'= generic penicillin’s substituent.

### The vancomycin group of antibiotics

Vancomycin was first isolated in the mid-1950s by the pharmaceutical company Eli Lilli; it is produced by *Amycolatopsis orientalis* and has been used clinically since 1959. [26]

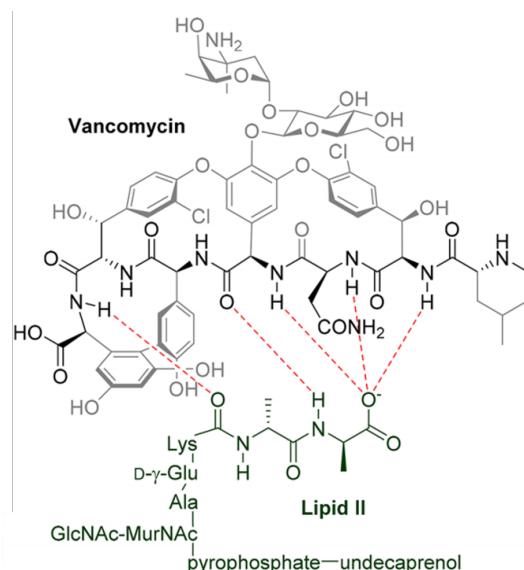
Vancomycin together with teicoplanin were the first commercially available glycopeptides, while telavancin, dalbavancina and oritavancin represent an improved version.

The conventional mechanism of action of this group of molecules involves the glycotransferases enzymes which catalyze glycosidic-bond formation within murein chains’ growth: in detail, their aglycone moiety establishes five-hydrogens interactions with the DAla-DAla dimer of the lipid II unit, therefore, these H-bounds sterically block the transglycosilation-activity of the glycotransferases enzymes, leading to weaker PGN cell wall bridges, especially in fast-growing bacteria. [27] [28]

This process is also increased by cooperative dimerization (homodimer formation

between glycopeptide molecules) which enhance binding affinity to the ligand, confers structural rigidity and displays the correct complex conformation for its own effect. [27] [29]

As said before, vancomycin's resistance concerns target-modification reactions which includes the replacement of the DAla-DAla dimer with DAla-DLactate (acquired genes: vanA, vanB, vanD) or DAla-DSerine (acquired-genes vanA, vanB, vanD), reducing vancomycin-binding affinity. The DAla-DLac (DLactate) exchange removes one of the five hydrogen-bonds from the drug-interaction and “it introduces a repulsive lone pair-lone pair interaction between the two oxygen atoms that is believed to be mainly responsible for the 1000-fold loss in affinity of vancomycin for Lipid II” [29].



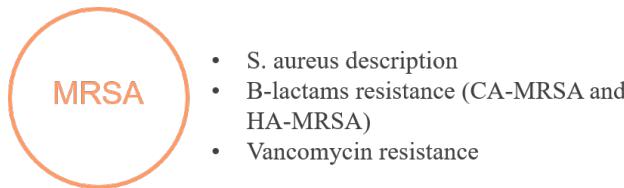
**Figure 7 Vancomycin bounds interaction with Lipid II:** Hydrogen bond interactions are illustrated between vancomycin backbone and Lipid II's D-Ala-D-Ala moiety.

*Image adapted from [29] and used here in accordance with the Creative Commons CC-BY licence.*

After a detailed illustration of the bacterial wall formation and the mechanisms of action of drugs that exploit cell wall relative construction, Methicillin-Resistant Staphylococcus Aureus (MRSA) is described: the evolution of its strains and the mechanisms by which resistance to vancomycin and methicillin has been acquired.

## 4 MRSA

Methicillin-resistant *S. aureus* (MRSA) represents the main focus of this master's thesis due to its ability to adapt and acquire resistance to the commonly used antibiotics ( $\beta$ -lactam, vancomycin). The aim is to develop new drugs able to fight it with a new mechanism which is not subject to resistance processes. In this section hospitals-associated and community-associated MRSA together with VRSA (vancomycin-resistant *S. aureus*) strains are described, which led to the  $\beta$ -lactam and vancomycin resistance that is still in expansion today.



**Figure 8 MRSA overview:** main aspects and key points about methicillin-resistant *S. aureus* chapter are outlined here.

*S. aureus* is a gram positive bacterium belonging to the *Micrococcaceae* family.  $\beta$ -lactams antibiotics would be the best choice in severe staphylococcal infections treatments, but their use was soon limited by the emergence of resistance. Indeed, penicillin resistance was immediately noticed after its introduction on the market, by the  $\beta$ -lactamases production. After that, although the antibiotics structure was improved with methicillin, oxacillin and other " $\beta$ -lactamase-insensitive penicillin derivatives" development, the *mecSSC* staphylococcal cassette acquisition led to modified PBPs production. This mobile genetic element encodes PBP2a, which have low affinity for  $\beta$ -lactam antibiotics and has been responsible for the development of methicillin-resistance *S. aureus* strains. [30]

This is because this type of PBP has developed an allosteric domain that allows interaction with the substrate only after the binding with PGN (allosteric substrate), otherwise, the active site is maintained in its closed conformation within a narrow channel inaccessible to  $\beta$ -lactams. [31]

Thus, different strains of MRSA emerged as hospitals-associated MRSA (HA-MRSA) and community-associated MRSA (CA-MRSA). The first strain is highly-resistant to oxacillin and causes invasive infections in immune-compromised patients in hospitals, while the latter is low-oxacillin

resistant and causes serious skin infections in previous-healthy people . [30] [32]

Moreover,  $\beta$ -lactams limitations lead to an increased use of other antibiotics, especially vancomycin and thus, cases of MRSA with reduced susceptibility to vancomycin, labelled as VISA (vancomycin-intermediate S. aureus) strains, were discovered in 1997. [32]

Consequently, with vanA gene transfections from Enterococcus faecalis, new high-level resistance types labelled VRSA (vancomycin-resistant S. aureus) have been isolated, demonstrating continued bacterial adaptability. [29]

VanA encodes a dehydrogenase that catalyses the reduction of pyruvate to D-lactate and a ligase, which synthesises the DAla-DLac moiety by forming an ester bridge between the two monomers. [28]

This fragments exchange leads to a loss in glycopeptides affinity for these peptidoglycan's modified chains and therefore, a drop in the effect of the drug. [33]

From this, what therapies are in use today? Are new drugs with new mechanisms of action available? How can these resistance problems be faced? In the following chapters, current and experimental molecules are discussed.

## 5 Actual therapies

This section outlines the molecules in use today against MRSA, with their mechanisms of action and biological targets. In addition, molecules representing improvements in  $\beta$ -lactams and glycopeptides are mainly described, together with a table showing a general overview of other current therapies (Table 1 within Appendix section).



- Fifth generation of cephalosporines: ceftaroline and ceftobiprole
- Lipoglycopeptides: oritavancin and telavancin

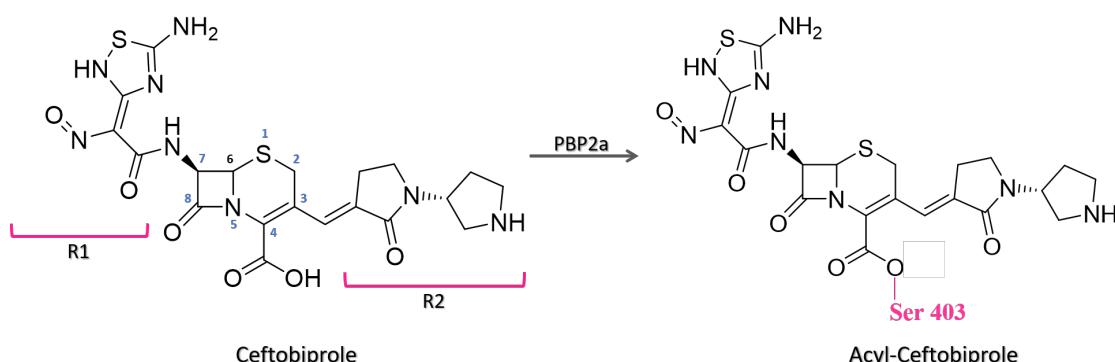
**Figure 9 Actual therapies overview:** main aspects and key points about current therapies chapter are outlined here.

In order to overcome these mechanisms of resistance in MRSA, drug-combination

and new antibiotic generations have been introduced. Important examples are ceftaroline and ceftobiprole (fifth cephalosporins generation [31]) that interact with the PBP2a-allosteric site, making the active site available and accessible to a second molecule of ceftaroline/ceftobiprole. This covalently-inhibits the transpeptidase catalytic-activity and leads to murein degradation. [31]

Ceftobiprole is a potent inhibitor of transpeptidases 2a, thanks to an increased binding-affinity with the target-enzymes: “The 1,3'-bipyrrolidin-2-one moiety (R2 group) located at the 3-position of the cephem nucleus sits deep in the narrow cavity of the PBP2a binding site making it closer to Tyr446, Met-641, and Thr-600 and favoring the acylation of PBP2a”. [31]

Moreover, the rigid structure of this substituent mimics the pentaglycine structure (same planarity) and therefore, the peptidoglycan natural cross-links. [31]



**Figure 10 PBP2a ceftobiprole binding:** serine acylation (Ser403) at PBPs' active site. R1: oxyimino aminothiadiazolyl substituent, which protects from  $\beta$ -lactamases hydrolysis; R2: 1,3'-bipyrrolidin-2-one moiety, is an hydrophobic residue that interacts with PBP2a active site and mimic pentaglycine planarity.

*Image adapted from [31].*

On the other side, new antimicrobial peptides have been developed with the role of small molecule antibiotics, as oritavancin and televancin. They are called Lipoglycopeptides and they are semi-synthetic vancomycin-derivatives with the presence of an additional lipophilic side-chain (hence the class-name) which confers them an innovative mechanism of action. [27]

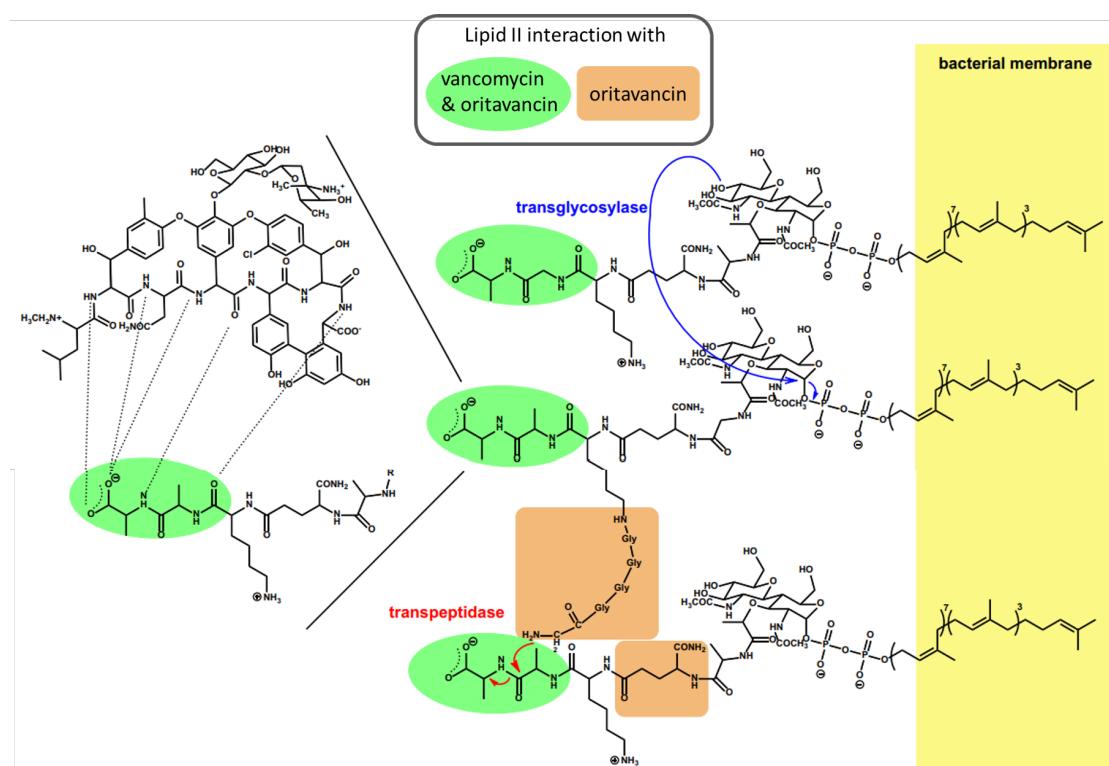
Notably, oritavancin is a lipoglycopeptide chloroeremomycin's derivative, presenting slight differences from vancomycin's structure (as an additional aminated sugar on the cyclic heptapeptide and the replacement of the 4-vancosamine by 4-epi-vancosamine) but, more important is the introduction of

a chlorobiphenylmethyl residue on the aglycone's side chain, which provides the amphipathic characteristic to the molecule.

In contrast, telavancin holds a decylaminoethyl hydrophobic-substituent on the aglycone's disaccharide side chain, which is responsible of the drug's liphophilicity. [27]

Owing to their increased hydrophobicity, phenomenons like homodimers-formation (mainly oritavancin with its additional sugar) and the anchorage to the bacterial membrane have been improved, giving improvements in terms of drug-target binding affinity. These new lipoglycosides have the ability to make selective and high-affinity interactions with lipid II during the cell wall's synthesis: leading to membrane degradation, depolarization and lysis, affecting its permeability. [34]

Oritavancin, in particular, is responsible for a dual mechanism of action: it makes specific non-covalent bonds with the pentaglycine segment and the pentapeptide moiety of lipid II (via its improved side-chain), while it blocks transpeptidation using its aglycone-structure, being able to bind the modified peptidoglycan dipeptide (DAla-DLac) of VISA strains. [35]



**Figure 11 Vancomycin and oritavancin in comparison:** vancomycin and oritavancin interactions with lipid II. In green, five hydrogen bounds are illustrated between the drugs and the D-Ala-DAla extremity of the lipid II. In red, oritavancin interactions with lipid II's structure and pentaglycyl bridge.

*Image adapted from [27] with licence from Springer Nature.*

In addition, the transglycosylase activity is also blocked, affecting the process of novel murein-chains formation; and the target-modification responsible of vancomycin resistance is solved. (See Table 1 within Appendix section)

## 6 Experimental therapies

This section aims to show examples of innovative molecules (TXA709, TXA707 and brilacidin) in trials, with new modes of action, demonstrating that unexplored processes/targets still need to be discovered; for this reason, research must continue (PEW website, Figure 40, Appendix section).

Furthermore, the idea behind these new compounds represents the future in this field and a guideline for this project, which aims to defeat and overcome resistance mechanisms.



- Filamentous temperature-sensitive protein Z inhibitors: TXA709/ TXA707
- Novel defensin-mimic antibiotic: Brilacidin

**Figure 12 Experimental therapies overview:** main aspects and key points about therapies in development are outlined here.

In recent years, many efforts have been made to combat MRSA and gram-positive bacteria resistance. Within a global scenario of certainty of the unstoppable development of resistance, the discovery of novel target and new mechanisms of action is becoming crucial and of high importance [36].

Nevertheless, from this kind of situation the most challenging and interesting findings emerge, arising among all the other molecules in development [37]. In addition, this project aims to make a contribution in the fight against antibiotic resistance by exploring promising new compounds.

With this purpose, two small experimental molecules, active against MRSA, are described as an example of a newly discovered innovative mechanism of action:

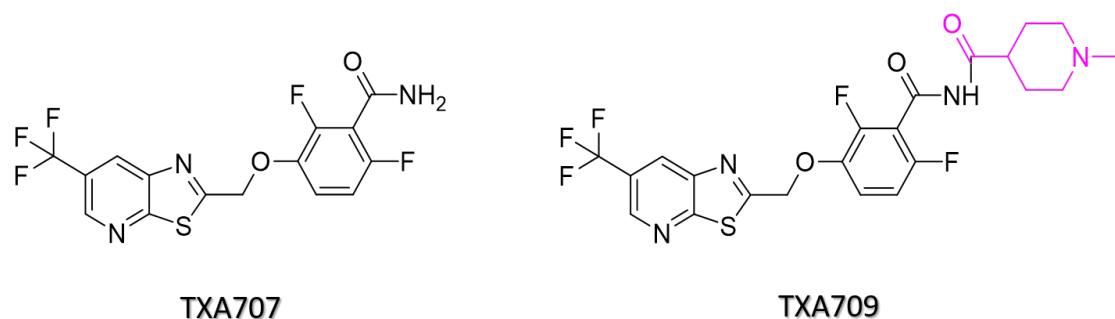
-TXA709 and TXA707 are “filamentous temperature-sensitive protein Z inhibitors” (FTsz)

-Brilacidin is a novel defensin-mimic antibiotic.

FTsz protein is a tubulin analogue has an essential role in cell division. It is involved in the cell-division’s septum formation via a process GTP dependent.

TXA709/ TXA707 (Figure 13) are benzamide-derivatives with a CF<sub>3</sub> (electron-withdrawing group), which help resist metabolic attacks (dehalogenation). [38]. These compounds act as FTsz inhibitors and interfere with the tubulin-homologues’ polymerization and its GTPase activity, resulting in the inability to form the cell-division septum [39]. This leads to the block of cell division and consequent cell death. [40]

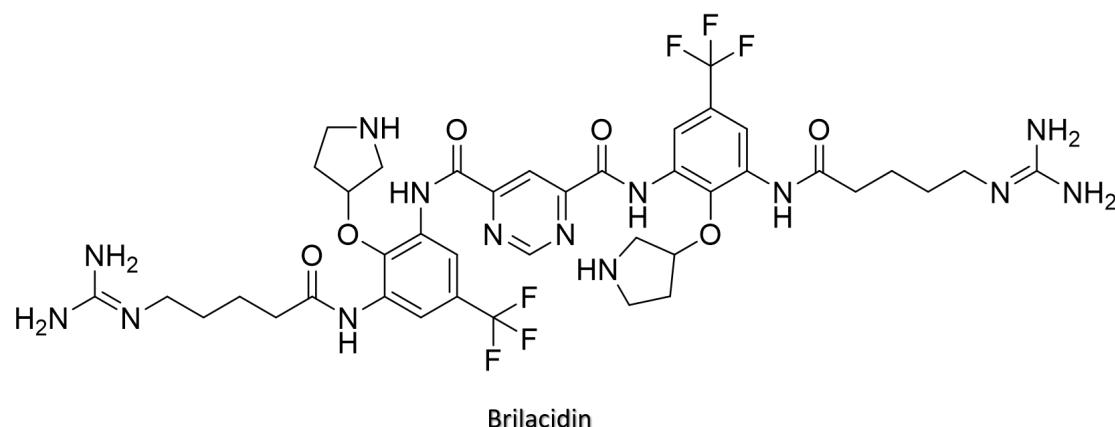
These drugs are actually in trials and are examples of an innovative mechanism of action developed by an accurate study and selection of a new target.



**Figure 13 FTsz antibiotics:** TXA707 and TXA709, the thiazolopyridine core attests the benzamide origin.

Brilacidin has a similar structure of the natural human defensin, which acts as immune-system defender against the pathogenic microorganisms within the human body. In addition, brilacidin causes cell membrane depolarization as main mechanism, recalling the daptomycin mode of action. [41]

Indeed, it is responsible of the induction of several systems and regulons (NsaSR, GraSR, and Dap) involved in cell wall/membrane stress, indicating its strong membrane disregulation effect. In addition, it emerged also the upregulation of pathways involved in bacterial membrane precursors' biosynthesis (VraSR and WalKR regulons) and those related to cytoplasmic proteases and chaperones, as a proof of cell wall disregulation and misfolded proteins accumulation. [42]



**Figure 14 Brilacidin structure:** this compound, also known as PMX30063, is a novel defensin-mimic antibiotic that causes membrane depolarisation and several systems and regulons induction.

Brilacidin (also known as PMX30063) is actually in trials, and it is described here due to its innovative mechanism against *S. aureus*.

It represents an improved version of small-molecule antimicrobial peptides due to intramolecular hydrogen-interactions and substituents of cationic or hydrophobic

nature, which generate a new stabilised, amphiphilic molecule. [42]. Indeed, it is characterised by a planar and conformationally restrained central structure with four positively charged guanidine and pyridine functionalities and two hydrophobic trifluoromethane substituents. [42]

Therapies in development are always up-to-date and visible on PEW website, “Antibiotics Currently in Global Clinical Development”. (Figure 40 in Appendix section)

## 7 Present and future reflections

In the preceding chapters, a general overview of the current state of antibiotic research and the mechanisms underlying the bacterial threat is provided, in order to understand them and evaluate the available options.

Bacterial resistance was discussed, with particular interest in MRSA, the focus of this project: the mechanisms of action of drugs (such as methicillin and vancomycin), which are the main subjects of resistance and the main contributors to these problems, were described. Inspiration can certainly be drawn from these old molecules, as demonstrated by the therapies currently in use, which represent the new generations of  $\beta$ -lactams and glycopeptides, molecules that overcome the resistance problems that arose in previous generations, but are still tied to the same redundant target and the similar approach.

As already mentioned, these improvements have not been extensive; in fact, drugs discovered more than 50 years ago are still used today because they are cheaper and have a broad spectrum. This phenomenon led to the development of superbugs such as MRSA or VRSA, organisms that are still an underestimated problem. But how can this world problem be overcome and solved?

Following this question, what represents a glimpse into the future and proposes a way out of the resistance phenomenon is provided by certain compounds under development, such as brilacidin and FTsz inhibitors, which embody new and promising modes of action, acting on unexplored targets about which little is yet known.

These innovative compounds are the proof that there are still unexplored fields,

new targets, maybe still hidden or unknown, but representing fertile ground just waiting to bear fruit.

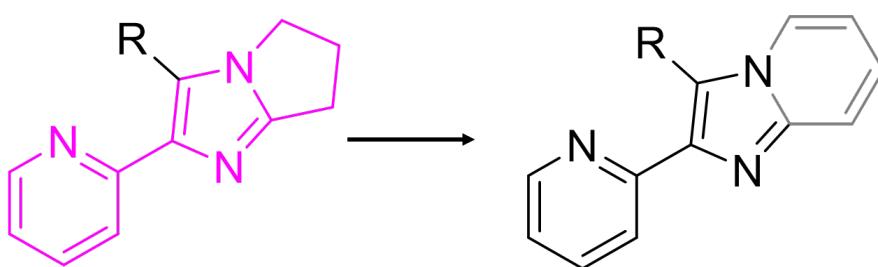
This is the beginning of a new line of research, far removed from the resistance processes of previous molecules and able to give a boost to overcoming them, in a new and effective way. With this objective, this project aims to make a contribution to the defeat of superbugs, such as MRSA, through the discovery of new compounds which do not involve old targets but symbolises a new era of antibiotic drugs.

## 8 Details of the project

This project is part of an open-source consortium called “OpenSourceAntibiotics” (called OSA [github.com/opensourceantibiotics](https://github.com/opensourceantibiotics)), characterised by periodic meetings where people are free to join and exchange their ideas, contributing to the development and discovery of inexpensive drugs.

For this reason this is an open source project, result of collaborations of different partners all over the world (Spain, USA etc.) and focuses on molecular structures active against methicillin-resistant *S. aureus*, referred to as “Series-2-Diarylimidazole” (Series-1 refers to “murligase”, another project within OSA [github.com/opensourceantibiotics/murligase](https://github.com/opensourceantibiotics/murligase)). This Series-2 idea started with a small group of compounds tested against ESKAPE pathogens activity, with the result that some of them were found to be active against MRSA.

These compounds were characterized initially by 2-(pyridin-2-yl)-6,7-dihydro-5H-pyrrolo[1,2-a]imidazole scaffold (Figure 15) with limited SAR and target knowledge. Later, by pursuing the traditional way of finding drugs, the molecular core was improved by exchanging the dihydro-pyrrolo for a benzene ring, gaining selectivity and limiting the cytotoxicity; this is one of the most significant enhancements in the molecular structure.

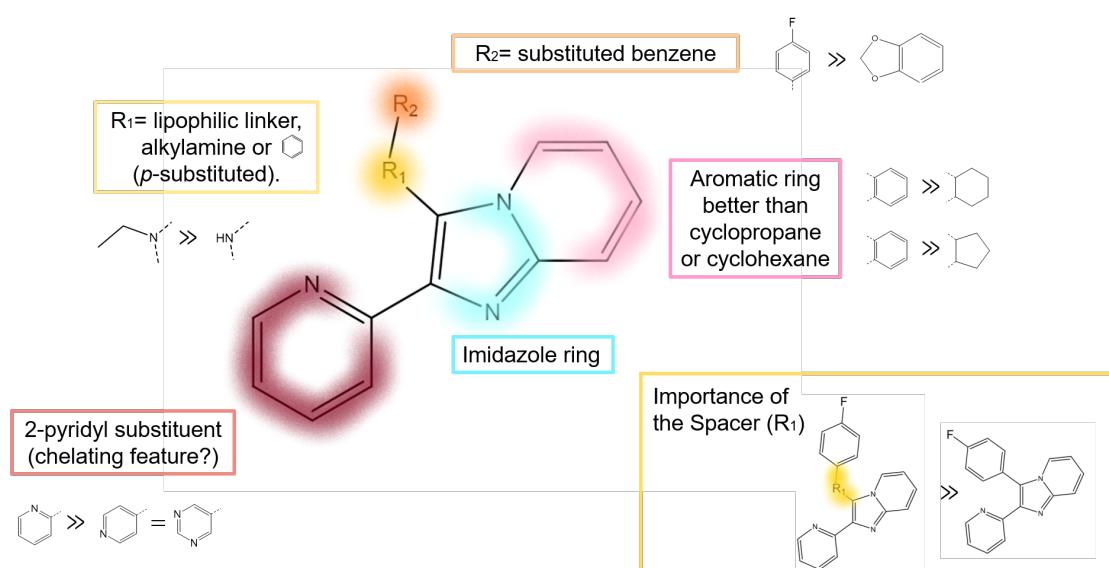


**Figure 15 Initial core structure of Series-2-Diarylimidazoles :** on the left, 2-(pyridin-2-yl)-6,7-dihydro-5H-pyrrolo[1,2-a]imidazole (violet) is the original molecular scaffold that gave birth to this project. On the right, the improved version with a benzene ring (grey) instead of the dihydro-pyrrolo. R= heteroaromatic cycles, as benzo tiazolo and benzofuran.

Moreover, as a result of the drug discovery traditional research, interesting SAR's (structure-activity relationship) aspects of Series-2-Diarylimidazoles molecules have been understood.

These were explored to gain potency, biological activity and selectivity (to limit human cells' toxicity); therefore, the results of these studies are summarised below (Figure 16):

- the 2-pyridyl substituent (dark red) is better than 3/4 pyridyl moieties or other nitrogens' rings, suggesting the chelating feature with the imidazole nitrogen.
- attached to the imidazole ring (light blue), a non-substituted aromatic ring, compared to cyclopropane or cyclohexane, is better for the potency and the cytotoxicity.
- in terms of activity and toxicity, the imidazole's 5-position substituent ( $R_1$ ) (yellow) should be lipophylic as a *p*-substituted aromatic ring or an alkylamine (as propylamine, 2-methylpropan-amine)).
- ( $R_2$ ) (orange) moiety gives promising results as a *p*-substituted benzene ring (as a 4-fluorobenzene, for example), instead of heteroaromatic rings (like a benzo[*d*][1,3]dioxole).
- it is crucial to have a spacer ( $R_1$ ) (yellow) between the benzimidazole ring and ( $R_2$ ); the length and space expansion should be critical factors for molecular potency and selectivity (increasing the interactions with the target pocket).



**Figure 16 Structure-activity relationship of Series-2-Diarylimidazoles molecules:** the crucial aspects for Series-2-Diarylimidazoles' SAR are summarised here; the imidazole ring is a key factor (light blue) and, as said before, the benzene cycle (pink) replaces non-aromatic 5/6 member-rings as the dihydro-pyrrolo. Furthermore, the 2-pyridyl substituent (dark red) gives good results in activity, suggesting the chelating activity's importance, compared to other nitrogens' moieties. In addition, (R<sub>2</sub>) (orange) provides positive outcomes as p-substituted benzene, while (R<sub>1</sub>) (yellow) seems to be help improve the activity as a lipophilic linker (an alkylamine or p-substituted benzene).

## 8.1 First data on the mechanism of action

One goal of this project is to understand and clarify the mechanism of action of these series' compounds; then, some studies have been done by the University of North Carolina (UNC Proteomics Core) by using proteomics techniques.

In particular, considering kinases as a possible target, competition assays have been done between our compounds (presenting the original scaffold, Figure 15) and other kinase inhibitors bound to immobilized bead, following the MIB-MS technology. [43] More in details, inhibitors of multiple kinase enzymes bound to Sepharose beads (MIB) constitute the stable phase, while *S. aureus* cell lysate represents the mobile phase. The binding degree of the enzymes to the beads in the presence of the compound of interest, which competes for the binding site of the kinase inhibitor, leads to the molecule-kinase binding capacity being detected and quantified using mass spectrometry (MS).

Decreased binding to Sepharose beads in the presence of the tested compound indicates that it is competing for the kinase inhibitor binding site.

Hence, after filtering the results and considering only those kinases that demonstrated a dose-dependent change, 16 kinases have been deemed significant. Among them, only 5 showed a notable dose-dependent decrease in MIB-binding: SodA (Superoxide dismutase), MQO1 (Malate:quinone oxidoreductase), PurD (Phosphoribosylamine-glycine ligase), MW0361 and SAR1965 (uncharacterised proteins) genes.

Then, the attention has focused on MQO1 and PurD genes: this is due to the lack of data of the uncharacterised proteins (MW036, SAR1965) and because SodA is involved in many inflammatory events meaning that it is unlikely to be a drug target. These proteins are candidate targets and led to further findings in the Result and Discussion chapter (Section 10).

## Materials and Methods

### 9 Computationl tools:

- Pymol used as 3D viewer for target-ligand molecules visualization.
- Databases as Zinc, Pubmed and chEMBL: useful for compound research; PDB (Protein Data Bank) utilised for 3D crystallographic structures of the proteins/targets.
- DataWarrior: used for screening the different conformations generated by “smina UCL” .
- Discovery Studio 2021 employed for 2D view of docking results.
- “Smina UCL” (software developed in UCL) was utilised for the docking part: it creates different conformations of the tested compounds and tries to dock them with the target, finding the best results.

### 10 Synthetic tools:

- All chemicals and compounds were purchased from Sigma Aldrich, Merck and Fischer, unless otherwise stated.
- $^1\text{H}$  NMR spectra were recorded on Bruker Avance with 300, 400 or 500 MHz, while for  $^{13}\text{C}$  NMR were used 126 or 75 MHz; MNOva NMR software assigned the peaks. The FT-IR was Bruker ALPHA 1 IR machine with an ART attachment; the software used was OPUS7.2.
- All the reactions were monitored by LCMS and thin-layer chromatography (TLC). Flash column chromatography was carried out with pre-loaded GraceResolvTM flash cartridges on a Biotage® Isolera Spektra One flash chromatography system.

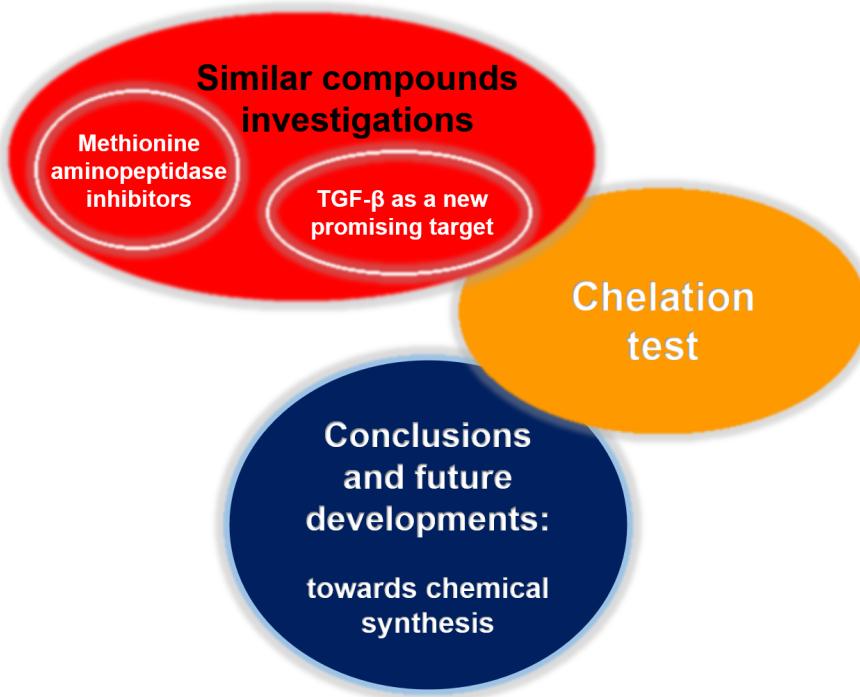
## Results and Discussion

This section contains two parts, the first concerning literature and computational search for possible drug targets identified after the interpretation of data from the UNC proteomics experiment (described in the introduction). The second part focuses mainly on synthetic work, which is valid for SAR enhancements, pursuing the traditional way of finding drug molecules.

### 11 Computational part's goals and descriptions

This chapter describes several *in silico* docking tests and searches carried out to identify and select the appropriate target for the Series-2-Diarylimidazoles molecules. The focus was on finding small molecules that resembled the structures of interest to study their specific targets and find valuable correlations with the tested compounds.

Moreover, the search of a metal ion (a chelation target by the core of this project molecules) was the main filter for the searches. Eventually, an experiment was carried out to test the software's ability to recognise metal coordination bonds between target and ligand.



**Figure 17 Computational part key points:** main concepts and critical details of the computational research chapters are outlined here.

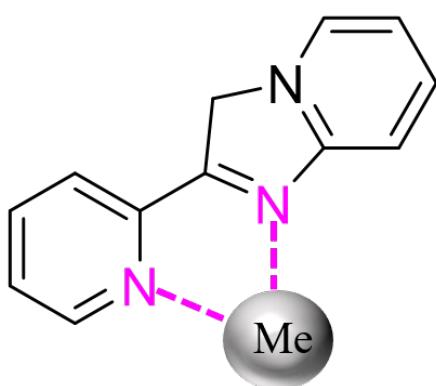
As said previously, following the the data interpretation process of the proteomic experiments performed by the University of North Carolina, some literature researches have been performed in order to investigate better the role of MQO1 (Malate:quinone oxidoreductase) and PurD (Phosphoribosylamine-glycine ligase) genes. These result as the most interesting candidates: the first one (MQO1) is implicated in the TCA cycle (tricarboxylic acid cycle) [44], while the last one (PurD) is involved in purine biosynthesis pathways [45].

In addition, it has been searched if these enzymes are known antimicrobial drug targets and it is found that MQO1 is a virulence factor [46] and a potential drug target [47], of which the crystallographic structure is not available [48]; on the flip side, PurD is indirectly involved in several pharmaceutical mechanisms but, currently, there are no targeted drugs for this kinase [49], [50].

Moreover, although these identified targets have fascinating and innovative profiles, it is crucial to also approach the research from the other side, identifying what is known about small molecules similar to those in Series-2-Diarylimidazoles and whether there is anything relevant about their mechanism of action.

## 12 Similar compounds investigations

Research has been performed about small molecules similar to the series scaffold aiming to learn more about the Series-2-Diarylimidazoles' features and mechanism of action. This structural scaffold is identified as 2-(pyridin-2 yl)H-imidazo [1,2-a]pyridine (Figure 18), and it is interesting because of its pyridine and imidazole's nitrogen atoms which may chelate metal ions. This chelation characteristic may be relevant to the series' mechanism of action if a metalloenzyme is the target. In this way, related motifs in complexation with metals in protein crystal structures have been explored.



**Figure 18 Series-2-Diarylimidazoles' chelating core :** 2-(pyridin-2-yl)H-imidazo[1,2-a]pyridine structure, chelating elements are highlighted in violet. Me = metal ion.

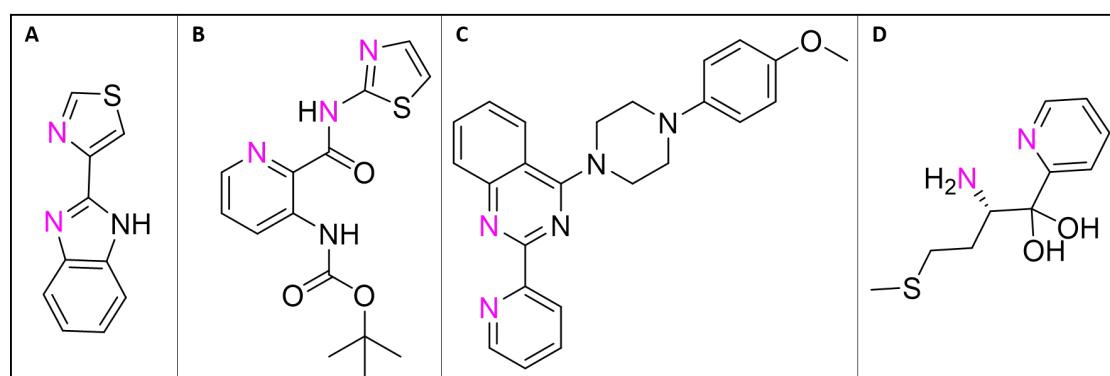
By researching in online databases such as PDB, Zinc and Pubchem, one of the first potential targets to be examined was Methionine aminopeptidase's inhibitors (MetAP): compounds A, B and C (Figure 19). These inhibitors' structures exhibit the previously mentioned chelating feature, which characterises the molecular scaffold studied in this project; therefore, similarly to this-project-compounds, nitrogenous elements such as pyridines and imidazole are presented.

### Methionine aminopeptidase inhibitors

Methionine aminopeptidase (MetAP) is a metalloprotease-enzyme present in two types (I and II); only type I is observed in bacteria, while in humans, both are present. It catalyses the amide-bond cleavage of N-formylmethionine (fMet) or initiator methionine (iMet) from newly synthesised proteins. [51]

The active site of this target is conserved among humans and bacteria: it has two cobalt ions (held together by a hydroxide ion), bound to active-site conserved amino acids such as two aspartates, two glutamates, and a histidine residue.

In addition, an auxiliary metal ion is usually present in silico prospects (usually cobalt, a metal routinely used for obtaining *in vitro* assays and crystallographic structures). However, this additional cobalt ion is rarely present *in vivo* within this enzyme. [51]



**Figure 19 Methionine aminopeptidase 1 chelators :** compound A: E coli MetAP1 inhibitor; B and C: human MetAP1 inhibitors; compound D: S. aureus MetAP1 inhibitor. Nitrogen atoms involved in compounds interactions are highlighted in violet. Compound A : 4-(1H-1,3-benzodiazol-2-yl)-1,3-thiazole, also known as thibendazole; compound B: t-butyl 2-(thiazol-2-ylcarbamoyl)pyridin-3-ylcarbamate; compound C: 4-[4-(4 Methoxyphenyl)piperazin-1-yl]-2-(pyridin-2-yl)quinazoline; compound D: (2S)-2-amino-4-(methylsulfanyl)-1-pyridin-2-ylbutane-1,1-diol.

Therefore, considering these structures similarities and the enzyme's active site, it was noteworthy performing computational docking investigations between Series-2-Diarylimidazoles' molecule and MetAP1 enzyme. These studies, unfortunately, yield poor results partly due to the limitation of the "smina UCL" software in detecting coordination bonds with metals (Section 13).

Following this, it was interesting to investigate the mechanism of action of the structures showed above by finding similarities with this project's molecules to understand whether MetAP1 may be a possible target.

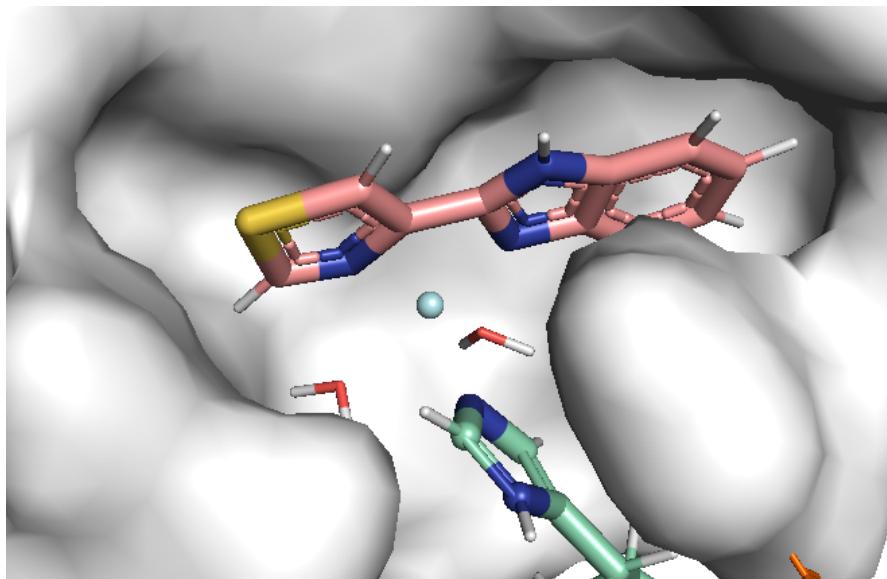
#### • What makes thiabendazole (compound A) an inhibitor?

The primary interaction of compound A (thiabendazole) with human MetAP1 occurs with an additional cobalt ion (Co III, called third cobalt) that also mediates binding to the conserved His79 residue in the active site (Figure 20). The catalytic metal ions (Co I, called first ion and Co II, referred to as second cobalt) are not involved in the interactions of compound A, while the "auxiliary" one is complexed by the nitrogen atoms of benzimidazole and thiazole, the histidine nitrogen, along with three molecules of water. [52]

Thus, active site entry is blocked, and inhibitory activity is shown.

In living cells, it is rare to find this auxiliary cobalt ion in the active site, but other metals characterized by higher blood concentrations *in vivo* (Zn, Mn) may

be present; this explains the lack of efficacy of this compound in living organisms. [51]



**Figure 20 Compound A interaction within the active site :** compound A (thiabendazole) binding geometries, resulted from docking runs within MetAP1 in 1YVM (PDB file), are illustrated. The ligand interactions with the auxiliary metal ion (in the centre, turquoise) are shown within the active site surface; the His 79 residue (in green, bottom part) is visualised in stick and binds the third cobalt ion, as well as the water molecules in the background (only two of them are visible). The non-catalytic metal ions are not illustrated in this picture.

*Image adapted from [52] with licence from American Chemical Society.*

- **Why compounds B and C are inhibitors?**

As thiabendazole, compound B exhibits chelation properties against the auxiliary ion cobalt and the “regular” metals are not involved, leading to decreased drug activity *in vivo*. Furthermore, these interactions are cobalt-dependent, so *in vitro* activity is decreased if the auxiliary ion is not cobalt. [51]

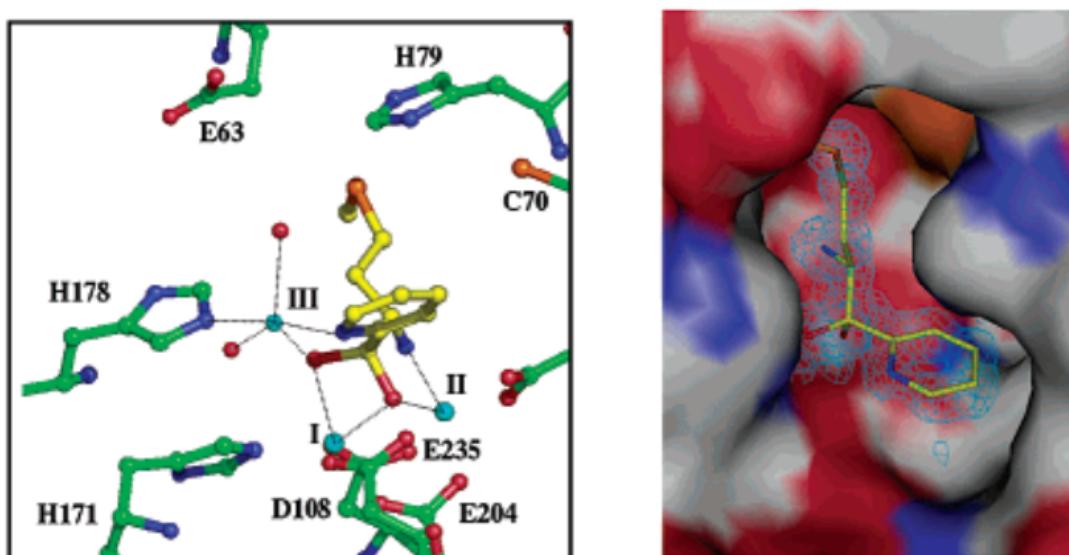
Compound C is created to overcome this problem and be active also in living cells: it is characterized by a “relaxed metal selectivity” that makes it active also with Zn(II) and Mn(II) *in vitro*.

Although the coordination with the extra atom is verified, other interactions are revealed. The nitrogen atoms of the 2-(pyridin-2-yl)quinazoline core chelate Co III, the pyridinyl moiety, makes hydrophobic interactions within a lipophilic pocket near the active site and simultaneously stacking with a histidine residue (His310 conserved) is shown. [51]

It is essential to mention that these details help understand and study the mechanism of action of these compounds, which show similar characteristics to the core of the project series and may relate to it.

- **What makes compound D an inhibitor?**

Compound D is a transition-state analogue: the diol mimics the tetrahedron that occurs naturally during hydrolysis of the peptide bond. [53] The pyridine nitrogen coordinates the additional cobalt ion (Co III) while the amine-group chelates Co II; the two hydroxyl groups also make other interactions with the metal ions, resulting in six different bonds in total (Figure 21). [53]



**Figure 21 Compound D interaction within the active site :** compound D (ligand), in yellow, within MetAP1 in 1QXY (PDB file) is illustrated. On the left the ligand is shown in ball-and-stick form; coordination interactions with metal ions (blue spheres) are represented by dashed-lines. On the right, the molecular surface of the inhibitors binding site with compound D is represented.

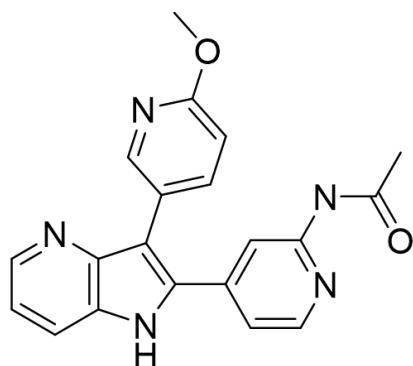
*Image adapted from [53] with licence from American Chemical Society.*

Considering these drugs' structure, their active site interactions (especially coordination with metals) and their mechanisms of action, it emerged that MetAP1 is a suitable target for Series-2-Diarylimidazoles molecules, and further researches are needed to explore its potential as a target.

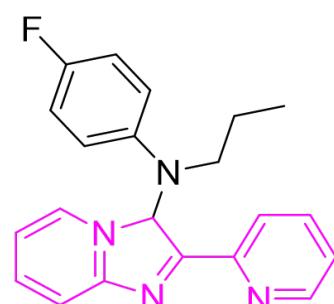
In addition, other studies have been conducted along the same lines as MetAP1, and attractive similitudes have also been found by not focusing only on the chelating aspect.

## TGF- $\beta$ as a new promising target

Among the researches involved in this “Similar compounds investigations” project side, TGF- $\beta$  Receptor 1 (human receptor) emerged as an attractive target candidate due to its binding-site and inhibitors’ features: its crystallographic structure (5QIL) includes N-4-[3-(6-methoxypyridin-3-yl)-1H-pyrrolo[3,2-b]pyridin-2-yl]pyridin-2-ylacetamide as an inhibitor (Figure 22). Interestingly, this molecule shows similar structure and elements to Series-2-Diarylimidazoles tested compounds (Figures 22).



TGF- $\beta$  Receptor 1 Inhibitor



OSA 982

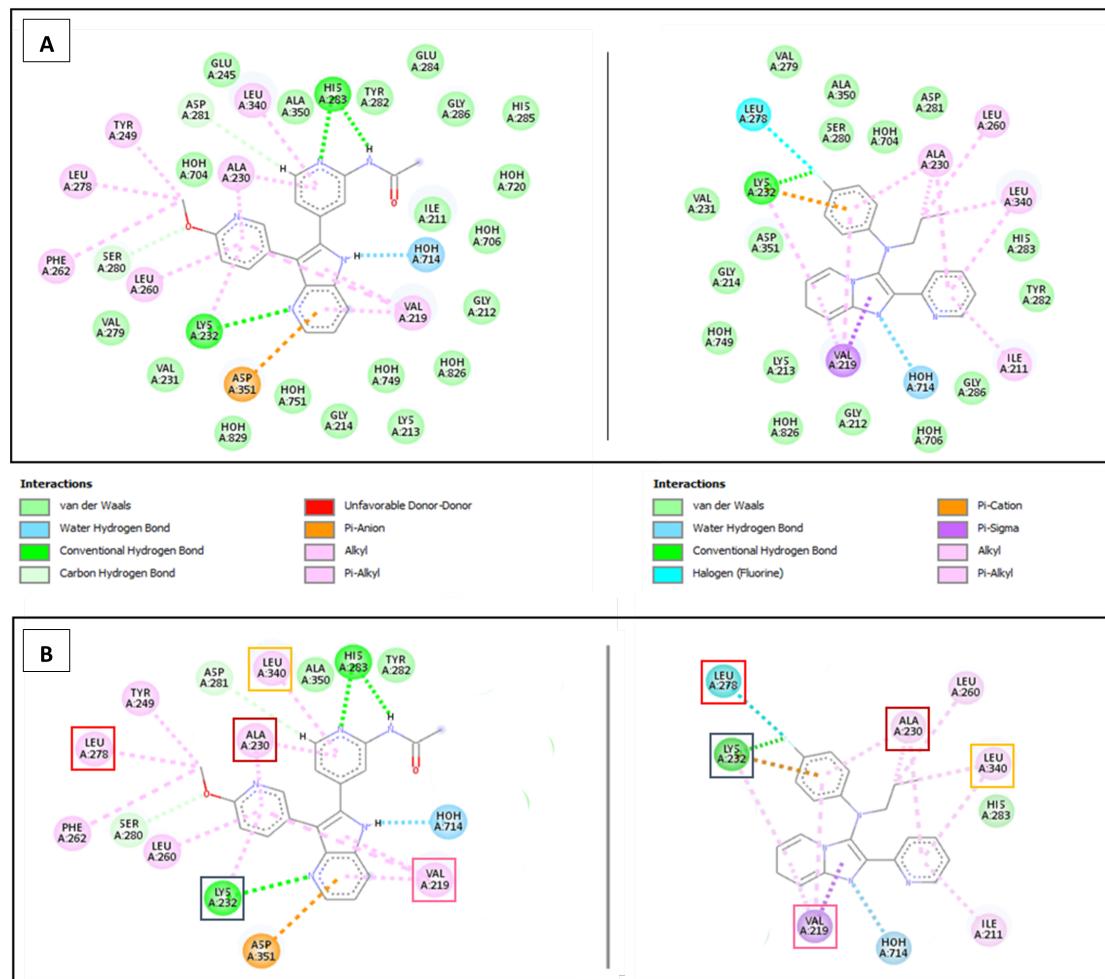
**Figure 22 Tested inhibitors for TGF- $\beta$  Receptor 1 :** on the left 5QIL-inhibitor’s structure (N-4-[3-(6-methoxypyridin-3-yl)-1H-pyrrolo[3,2-b]pyridin-2-yl]pyridin-2-ylacetamide) is illustrated while on the right, OSA982 compound is presented. The main core which characterises this project’s compounds is coloured in violet within 000982 structure.

Although these molecules’ core presents some similarities, as the pyridyl and pyrrolo-pyridin ring resembling the OSA982 scaffold, there are also some differences. These are listed below:

- the literature compound does not present a 2-pyridyl chelating motif. Indeed, compared to OSA982’s core, the nitrogen atom of the pyridine is in a different position around the five-member ring nearby (para position instead of the ortho for OSA982).

Computational studies have been performed to better investigate the similarities between these structures and understand if the lead compounds of this research (represented by OSA982) can inhibit TGF- $\beta$  Receptor 1. Indeed, these compounds

display the same binding site, and their 2D views diagrams show that they have in common a lot of critical interactions with the TGF- $\beta$  target (Figure 23).



**Figure 23 Comparison between TGF- $\beta$  Receptor 1 inhibitor (in 5QIL structure) and OSA982.** Figure A: on the left, 2D view of TGF- $\beta$  Receptor 1 and its inhibitor, from 5QIL PDB file. On the right, docking interactions of this project compound OSA982 with TGF- $\beta$  Receptor 1, 2D view.

Figure B: common and relevant interactions within the binding site are highlighted; Leu 278 (red square), Lys 232 (blue square), Val 219, Leu 340 and Ala 230 (mainly hydrophobic interactions).

These compounds have common interactions with Leu 278 (halogen or hydrophobic interactions), Lys 232 (hydrogen bonds), Val 219, Leu 340 and Ala 230 (mainly hydrophobic interactions).

Furthermore, the PDB inhibitor makes other lipophilic links with Tyr 249, Phe 626, Ser280, Leu 260, Asp 351, while it forms hydrogen bonds with a water molecule and a histidine (His 283), (Figure 23 left side). On the other hand, the OSA982 molecule makes additional lipophilic interactions with Leu 260 and Ile 211, together with an H bond with a water molecule (Figure 23 right side).

In conclusion, TGF- $\beta$  Receptor 1 is a possible target for Series-2-Diarylimidazoles molecules; however, further enzymatic or protein binding essays need to confirm its role.

## 13 Chelation test

This test aims to recapitulate the coordination bonding of metals, known interactions, which were the main focus of the experiments performed in the previous chapters. In addition, this proof of concepts has been conducted to verify the software functionality and usefulness in recognising these interactions. Following this, some experiments have been performed with the PDB 1QXY structure as a test subject to demonstrate the “smina UCL” software (used to perform all computational-docking tests in this project) efficiency about known ligand-target metal coordinations.

This software works by creating different conformations of the tested compounds at the beginning, and then it tries to dock them with the protein target, finding the best results.

The following procedure was the one utilised for this test’s purpose: firstly, the ligand (compound D, Figure 19, Section 12) was removed from the PDB file using Pymol, subsequently becoming the docking molecule. Then, the PDB protein alone (MetAP) acted as the target, while compound D (previous PDB ligand) represented the docking molecule as a proof of concept.

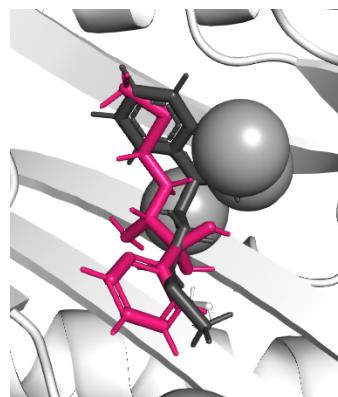
This procedure is helpful in not preset the active site in the docking program before experimenting and determine if the software can recognise the correct binding site with the appropriate interactions.

### 13.1 Result of the docking test

The first step was to ensure that at least one of the conformations (of compound D) created by “smina UCL” matched with the PBD one, and so as the interactions with the metals and other elements of the active site shown in the crystallographic structure and important for binding.

From the docking process emerged that the software generated 45 conformations,

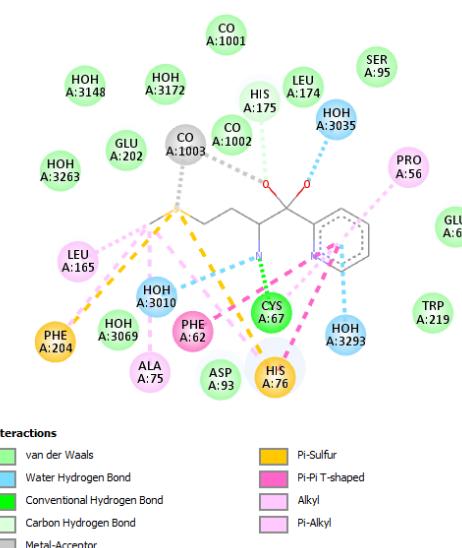
of which only one is related to the active site displayed in the 1QXY structure. Furthermore, this unique conformation does not resemble the compound D ligand in the crystallographic model, nor do the bonds identified with the enzyme (Figure 24).



**Figure 24 Comparison between “smina UCL” docking-result and 1QXY crystallography conformations :** the proper ligand conformation (in 1QXY) in grey with the conformation obtained from the proof-test (in pink) are shown, within the active site. The spheres behind the ligands are the metal ions (Co I, Co II and Co III).

Moreover, contrary to expectations, only interactions with one metal ion (third cobalt ion, Co III) occur, differently from the coordination bonds shown by the two nitrogen atoms with Co I and Co II in the crystallographic structure (Figure 21 left side within Section 12).

In addition, the chelating elements are represented by the sulfur atom and one hydroxyl element, while the nitrogen atoms (responsible for metal- coordination in PDB structure) are involved in hydrogen bonds with other amino acids within the active site (Figure 25).



**Figure 25 2D view of docking test results :** methionine amino peptidase docked with its inhibitor (2S)-2-amino-4-(methylsulfanyl)-1-pyridin-2-ylbutane-1,1-diol (compound D) in 2D view. Co I is called CO 1001, Co II is named CO 1002 and Co III is identified as CO 1003.

In this way, it is evident that the docking software, in this context, can not be helpful for metal coordination recognition and detection. The following suggestion is to try other procedures or not use the “smina UCL” program for new target finding (especially if a metal-coordination is involved), but rather, exploit this program to study and improve the connections between targets and ligands that are already well-established.

This observation helps gain confidence and awareness about the means and technologies used; it is challenging to know and explore all the computational tools available in this emerging sector. A great deal of experience is needed to deepen all the analyses and assumptions performed, hence the methodologies.

## 14 Computational part conclusions and future developments

From this chapter, the knowledge acquired is manifold: from the proteomic data analysis and the series’s core features, many experiments have been performed. In this way, some interesting candidate targets were discovered (MetAP1 and TGF- $\beta$ 1), studying their active site, drug interactions and conformations (thiabendazole, compounds A, B, C and D as MetAP1 inhibitors; TGF- $\beta$ 1 inhibitor) compared to

OSA982.

Even if there is no target X-ray structural information, there is a wide range in which the molecular structure can be iteratively optimised to improve its biological activity and potency. By generating hypotheses and synthesising the compounds' modifications that test these, the target pocket can be built, the mechanism of action explored, and the series's structure improved.

Following this, future calculations and modelling should be performed on these or other targets: first, by testing on bacteria synthesised compounds modification and at the same time, trying to built and visualize the binding pocket, maybe related to the enzymes/receptors studied in this chapter (Section 10).

In addition, the synthetic part is described in the following section.

## 15 Synthetic chemistry part

As already discussed, the initial SAR's data were not exhaustive, suggesting the synthesis of new compounds with different substituents in order to acquire info regarding the target's active site and improving the Series-2-Diarylimidazoles' structure. Therefore, one goal of this thesis project was to improve the SAR's knowledge to gain potency, biological activity and selectivity.

The SAR's previous information are listed here:

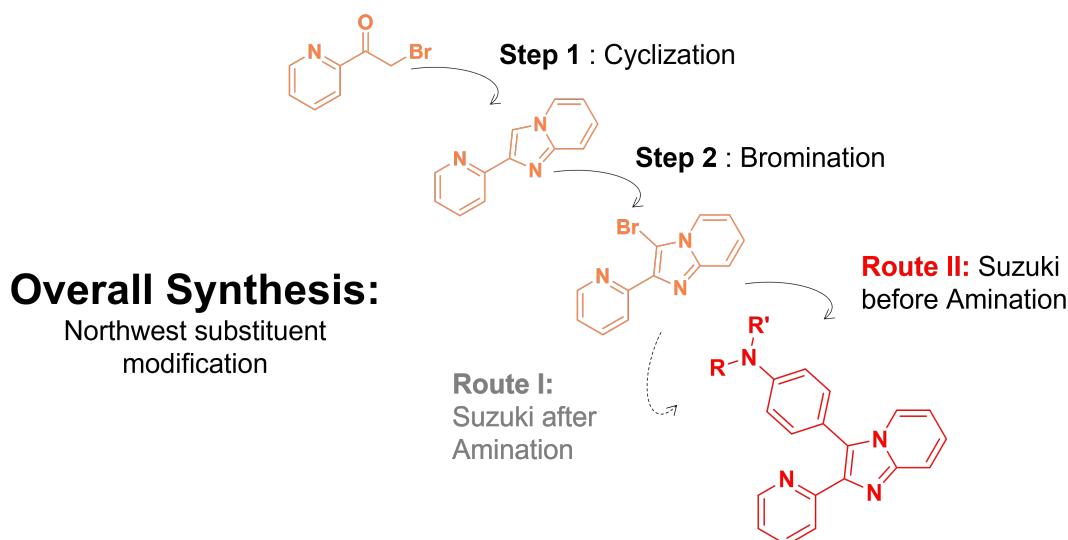
- the 2-pyridyl substituent (of the main core) is essential for the activity
- non-substituted aromatic ring attached to the imidazole improve the potency
- the imidazole 5-position substituent gives good results if lipophylic
- it is crucial having a spacer (R1) between the benzimidazole and another substituent.

Following this, it is interesting learning more about the imidazole's substituents to add details to identify the target's active site. Therefore, this section's main goal was to find a synthetic route for a small library of molecules with different northwest substituents: a benzene ring with various amines substituted.

With this aim, after the main core cyclization and bromination (steps 1 and 2,

already explored procedures within the project [54], Figure 26), two main routes were pursued, labelled as Routes I and II for obtaining the desired compounds. It is worthy of mentioning that the final compounds were produced following Route II (Figures 26 and 27, after the adjustment and modification of Route I (that was the initial plan). In this way, thanks to several improvements and careful researches, Route II successfully produced the two target molecules: compounds 9 and 10 with 18.2% and 37% yield, respectively.

These products are being biologically evaluated (to acquire SAR's data): all the information can be found on the OSA webpage, issue #84 for the most recent evaluations and #87 for the report of the last meeting ([github.com/opensourceantibiotics/Series-2-Diarylimidazoles/issues](https://github.com/opensourceantibiotics/Series-2-Diarylimidazoles/issues)). The image below summarises the overall synthetic pathway:



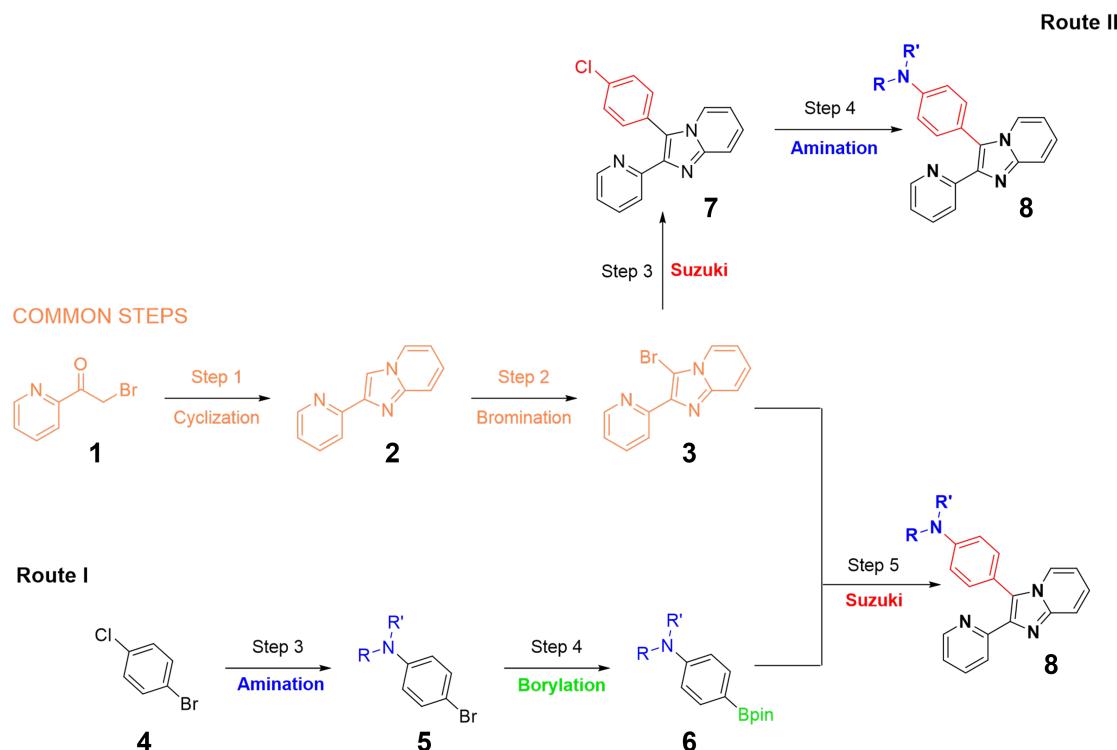
**Figure 26 Overall synthetic route diagram :** the main steps for the product's synthesis are illustrated here. Steps 1 and 2 represent the core formation and bromination (in orange), while the final compounds (in red) were produced exploring two different routes; the successful Route II was finalised and adopted.

The compounds resulted from Step 1 (cyclisation) and Step 2 (bromination) were synthesised adapting well-known protocols [54] while, the other molecules were results of research beneath this project at UCL. These researches will be the object of the discussion in the next section.

## 15.1 Discussion about the synthesis

The overall experiments in detail are illustrated in the picture below: together with the common steps (1 and 2), two different ways were pursued, Route I and

Route II. As already said, Route I was the initial pathway planned for the final compound synthesis; this underwent modification and improvements that led to the successful Route II. These two pathways are discussed in detail in this chapter.



**Figure 27 Synthetic scheme of the experiments :** the different steps of the routes used for the product's optimised synthesis are detailed here. Steps 1 and 2, which are common for both the routes, are in orange, while steps 3 and 4 are different for each way. Route I is characterised by an amination (blue) of a commercial starting material (4), the borylation (green) of the product (5) and a Suzuki (red) in the end, between the borylated molecule (6) and the brominated core (3). On the other hand, Route II is characterised by a Suzuki reaction of the brominated core (3) first, followed by the last amination, giving the product (8). Compound 8 represents the general formulae of the desired molecules 9 and 10, shown the picture below 33

Compound 1 and 2 were synthesised following previously reported protocols: the mechanism of action of the first two steps is illustrated in the Appendix section (Figure 41 and Figure 42), while the general procedures for Route II and the synthesis of the 1 and 2 compounds are shown in the 16 section.

### Route I Suzuki after amination

Various experiments have been done to reach the desired products; the initial plan was Route I, which begins with compound 4's amination. With this aim, three reactions were performed, and these are listed below.



**Figure 28 Amination attempts :** this image shows the three reactions for the amination of chloro-bromobenzene (compound 4). First is the initial SNAr tentative, followed by a Buchwald-Hartwig reaction and the last attempt to modify the starting material to obtain a Br-substituted product. This scheme follows the Route I pathway..

- **SNAr attempt:**

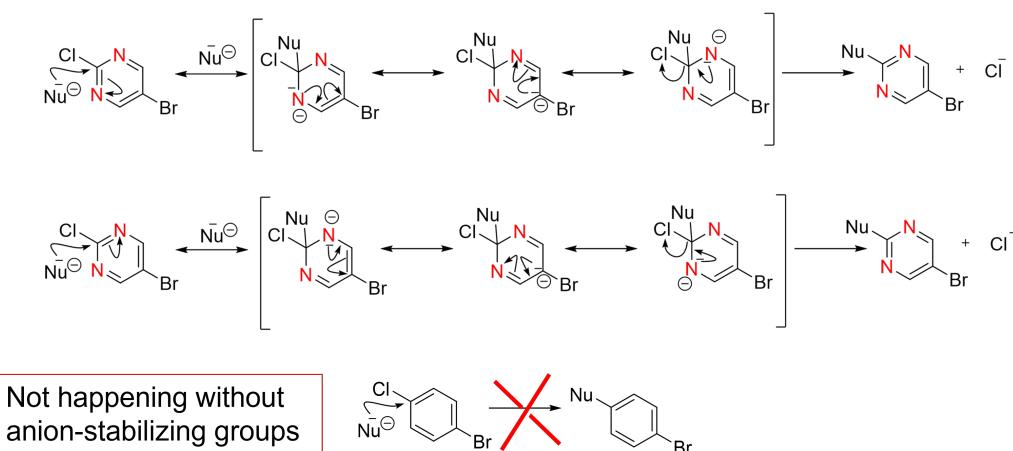
As shown in the scheme above, the initial plan was creating multiple borylated compounds with different amine substituents, ready for the Suzuki reaction (Step 5) with compound 3 (displacing the Br); this would give the desired result compound libraries.

With this idea in mind, starting with the commercial compound 4, an SNAr as an amination reaction was attempted by modifying an already known procedure ([55] Scheme 1/General Procedure A). Unfortunately, the product (compound 5) was not obtained following this procedure: but the result of this experiment was the starting material with reagents; they did not react with each other.

In the original scheme, there was a pyrimidine instead of benzene that undergoes an SNAr.

The two pyrimidine's nitrogens could handle the negative charge created during the reaction for the aromatic substitution since they are electron-withdrawing elements and have the function of anion-stabilizing groups [56] therefore, the benzene ring could not do the reaction because there are no heteroatoms that can have this role. Moreover, the presence of two nitrogens helps in the selective substitution of chlorine instead of bromine, valid for the borylation reaction (see below).

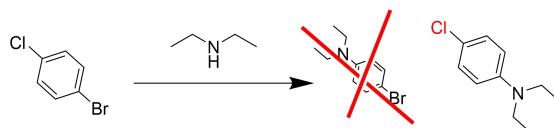
# SNAr



**Figure 29 Why was the product not obtained? Pyrimidine SNAr explanation :** Why was the product not obtained? Pyrimidine SNAr explanation: nucleophilic aromatic substitution of pyrimidine is illustrated here. The reaction does not occur without an electron-negative group (as the pyrimidine's nitrogens) which has the function of an anion-stabilizing group: this is the case of the chloro-bromobenzene shown in the bottom line. The pyrimidine's nitrogens are coloured in red within the SNAr mechanism (first two lines).

- **Buchwald-Hartwig amination:**

Following this, the amination of 1-Bromo-4-chlorobenzene was obtained by performing a Buchwald-Hartwig reaction: the used amine discarded the bromine substituent giving the chlorinated phenylamine; therefore, the product was not appropriate for the borylation reaction. The Buchwald-Hartwig mechanism is a metal catalysed (Pd) reaction, sensible to different substituents steric hindrance and electronic structure properties, such as bond length [57]. Therefore, it is selective for Br-substituents instead of chlorine ones.



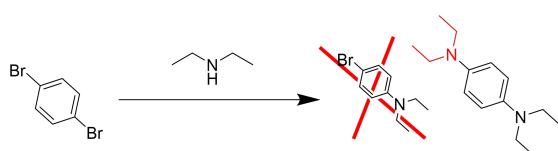
**Conditions:** Pd(OAc)<sub>2</sub>, RuPhos, NaOtBu, dioxane, 100°C overnight.

**Figure 30 Butchwal-Hartwig amination of 1-Bromo-4-chlorobenzene :** the occurred reaction is illustrated here; 4-chloro-N, N-diethylaniline was the result, rather than 4-bromo-N, N-diethylaniline (compound 5). The chlorine of the product obtained (rather than the Br-one) is coloured in red.

Indeed, the borylation of 4-chloro-N, N-diethylaniline did not give the desired product: only starting material was detected in the reaction mixture.

- **Amination of 1,4-dibromobenzene:**

Then, the last attempt was changing the starting material and do the amination using 1,4-dibromobenzene. The goal was to obtain a brominated phenylamine for the following borylation. With this aim, two experiments were performed with 1.5 and 3 equivalents of amine to avoid the amination of both the bromine and ensure enough amine quantity: dimethylamine was used, which has a low boiling point; therefore, the risk of evaporation is high. NMR analysis showed that both reactions resulted in the amination of both the Bromine substituents with some starting material.



**Conditions:** Pd(OAc)<sub>2</sub>, RuPhos, NaOtBu, dioxane, 100°C overnight.

**Figure 31 Change of the starting material within Route I :** this is shown the Butchwald-Hartwig amination of 1,4-dibromobenzene. Two reactions were performed using 1.5 and 3 equivalent on diethylamine. The result was the amination of both the Br substituents with some starting material.

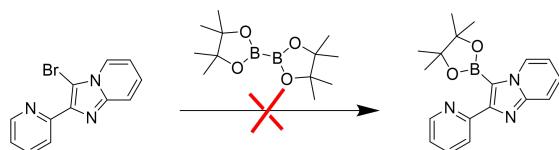
After all these experiments and without the desired products (5 and 6), the last attempt was directly borylate the molecular core (3) and then attaching the phenylamine with a Suzuki reaction. This last tentative is described in the next chapter.

### Borylation reaction

The borylation reaction was planned to be a metal catalysed (Pd) mechanism with the boronic acid as a reagent. However, according to the previous reactions, as it is a Pd-catalysed mechanism, it is selective for Br-substituents instead of chlorine ones. With this idea, there was the aim to directly borylate the compound 3, performing later the Suzuki with the phenylamine.

Unexpectedly, the resulted molecule was converted back to the cyclization product: hydrogen may have replaced the Bromine of the central core, turning the molecule to compound 2. The procedure was adapted from a previously

reported protocol [55] (Scheme 1/General Procedure C).



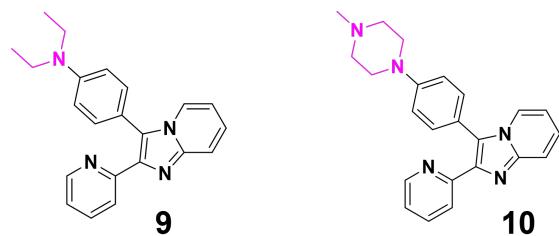
**Conditions:** KOAc, PdCl<sub>2</sub>(dpdpf)·CH<sub>2</sub>Cl<sub>2</sub>, Dioxane, MW 145°C, 30 min.

**Figure 32 Borylation of the core:** the last tentative for pursuing Route I is illustrated here. The desired product 2-(pyridin-2-yl)-3-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)imidazo[1,2-a]pyridine was not obtained but the reaction converted the starting material (compound 3) back to the compound 2.

Due to the lack of starting material, Route I (Suzuki reaction after the borylation) was not successful; therefore, for achieving the same goal (compounds 8), Route II was planned, deciding to switch the order of the reactions. The new scheme (Figure 27 shows the Suzuki before the amination: this procedure was successful, and two compounds were synthesised. This is described in the next chapter.

### Route II: Suzuki before amination

This route (Route II, Figure 27) involves a Suzuki reaction between compound 3 and (4-chlorophenyl)boronic acid, following by a Hartwig-Buchwald amination for the synthesis of the desired compounds 9 and 10 (Figure 33) with 18.2% and 37% yield, respectively. The detailed protocol is shown in Section 16.



**Figure 33 Desired compounds :** the two synthesised molecules are illustrated here. As northwest substituent (in violet), compound 9 has diethylamine, while compound 10 has methylpiperazine.

Regarding Step 3 (amination reaction of Route II), three different procedures were compared to find the one with the best yield: these protocols were performed by varying some conditions that are critical points of the reactions (the solvent, the catalyst and the temperature), and they are summarised in the table below; ethylamine was the reagent of these aminations. The crude LCMS

ratio between the product obtained and the starting material not consumed in the reaction mixture was used as a parameter for comparing the reaction yield.

	<b>Solvent</b>	<b>Base</b>	<b>Catalyst</b>	<b>Ligand</b>	<b>Crude LCMS Ratio Pdt/SM</b>
<b>A</b>	Dioxane (100°C reflux)	NaOtBu	Pd(OAc)2	RuPhos	1.18
<b>B</b>	Dioxane (100°C reflux)	NaOtBu	<b>RuPhos Pd G4</b>	RuPhos	0 (no Pdt)
<b>C</b>	<b>Toluene (110°C reflux)</b>	NaOtBu	Pd(OAc)2	RuPhos	0.05

**Figure 34 Comparison of amination's conditions :** three different procedures conditions for Butchwal-Hartwig amination reactions are illustrated here. Entries A, B and C relate various solvents and catalysts by maintaining the same base and ligand; the diverse conditions (compared to scheme A) are highlighted in red. The last column shows the crude LCMS ratio between product (Pdt) and starting material (SM) according to LCMS analysis of the crude reaction mixture. Ethylamine was used as a reagent for the three aminations.

Although none of the reactions did go to completion, comparing them reveals that scheme A (Pd(OAc)<sub>2</sub> as catalyst and dioxane as solvent) had the best yield. Following that, the successful procedure (scheme A) was also performed microwave (instead of refluxing overnight) to reduce the timings, but the product was not obtained. This change in scheme A was not successful, probably because the reaction mixture was not warmed up enough or for enough time.

It is noteworthy mentioning that the boiling point of the amine used as a reagent is an important condition for these experiments, because it influences their yield: the lower this is, the greater the likelihood that the amine will evaporate before the reaction is complete, thus limiting it. Indeed, this explains why the yield of compound 9 (ethylamine, boiling point 16.6°C, 18.2% yield) is lower than the one of compound 10 (methylpiperazine, boiling point 138°C, 37% yield). Probably this explains why all the reactions in the table (Figure 34) did not go to completion. Moreover, Route II represents the best way to create a drug library because, until the last step, the reactions can be done on a big scale, changing only the amine at the end (Step 4).

## Conclusions

### 16 General and Synthetic part conclusions and future developments

Antibiotic-resistant infections have been a global problem for decades, highlighting the necessity to develop new classes of antibiotics with new mechanisms of action, ideally limited by the development of resistance. The project started with some compounds active against *S. aureus* with limited knowledge about the target, mechanism of action and the SAR. However, after conducting researches pursuing the traditional way of drug discovery, the SAR's information acquired, and the ones regarding the target binding pocket were increased. Following this, this master thesis aimed to improve this knowledge with computational and synthetic tools.

Computational researches have been performed for studying the interactions of the Series-2-Diarylimidazoles' molecules with some potential targets. These proteins emerged from proteomic researches (about the mechanism of action) and studies about small molecules similar to those in Series-2-Diarylimidazoles (searching relevant information about their target). These computational studies helped select some candidate targets (MetAP1 and TGF- $\beta$ 1), understand which interactions these compounds can make with the selected proteins, and which binding can be recognised from the docking software (coordination bonding with metals).

On the other hand, the best way to synthesise a small library of compounds (with different northwest substituents) was selected, by comparing two different ways (Route I and II, Figure 26) in terms of yield and timing.

The most successful route was Route II (Figure 27): it involves a Suzuki reaction first, followed by a Hartwig-Buchwald amination.

Following this way, two desired products were synthesised: compounds 9 and 10 (Figures 38 and 39) with 18.2% and 37% yield, respectively; these molecules are being biologically tested for increasing the target active site's and SAR's learning.

The next step is about performing other proteomic assays for identifying the target and understanding the mechanism of action. Moreover, it is necessary to continue exploring and finding new valuable details regarding the SAR and facing the toxicity issues related to human cells: the selectivity index of the tested molecules should be increased since the molecule would be more specific for bacterial targets over the mammalian.

For this, new groups of compounds with different substituents should be produced and tested within this research, following the traditional way of drug discovery and analysing the obtained data, with the primary goal of creating a new class of antibiotics active against MRSA's infections.

## Experimental section

### 17 Procedure for Compound 2

Procedure for compound 2 synthesis (cyclization), following the scheme above (Figure 27): 2-Bromo-1-(pyridin-2-yl)ethan-1-one hydrobromide, 2-aminopyridine (1.5 equiv) and Na<sub>2</sub>CO<sub>3</sub> (4 equiv) were stirred in DMF (0.15 M) at 85 °C overnight. The reaction was cooled to room temperature, diluted with DCM, and poured over water. The aqueous layer was extracted 4 times with DCM (until organic layer was no longer orange), then the combined organic layers were dried with sodium sulfate and concentrated. The crude material was purified by flash chromatography (1-10% MeOH:DCM).

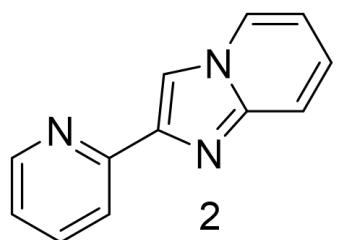


Figure 35 Compound 2.

The crude material was purified by flash chromatography (1-10% MeOH:DCM). Product 2: *2-(pyridin-2-yl)imidazo[1,2-a]pyridine*; orange solid; 24% yield.

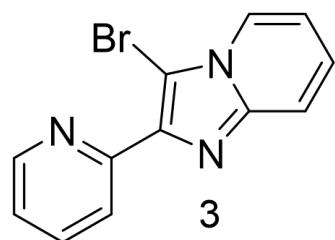
LCMS: m/z calcd 196.0 [M+H]<sup>+</sup> for C<sub>12</sub>H<sub>9</sub>N<sub>3</sub> + H<sup>+</sup> (196.0);  
1H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.62 (d, J = 5.4 Hz, 1H), 8.43 – 7.95 (m, 3H), 7.78 (td, J = 7.4, 1.6 Hz, 1H), 7.64 (d, J = 9.0 Hz, 1H), 7.20 (ddt, J = 9.1, 6.7, 1.3 Hz, 1H), 6.86 – 6.70 (m, 1H).

13C NMR (126 MHz, CDCl<sub>3</sub>) δ 153.04 (s, 1C), 149.49 (s, 1C), 145.73 (s, 1C), 145.69 (s, 1C), 136.92 (s, 1C), 126.10 (s, 1C), 125.09 (s, 1C), 122.77 (s, 1C), 120.59 (s, 1C), 117.88 (s, 1C), 112.85 (s, 1C), 111.01 (s, 1C).

The characterizations match with the literature ([58]).

## 18 Procedure for Compound 3

Procedure for compound 3 synthesis (bromination), following the scheme above (Figure 27): 2-(Pyridin-2-yl)imidazo[1,2-a]pyridine was dissolved in DCM (0.2 M) and NBS (1.1 equiv) was added. The reaction mixture was stirred at room temperature for 3 h and then concentrated. The resulting orange residue was dissolved in EtOAc. The organic layer was washed once with sat. aq. NaHCO<sub>3</sub> and once with brine, then dried with sodium sulfate and concentrated. The resulting residue was used in the next reaction without further purification.



**Figure 36 Compound 3.**

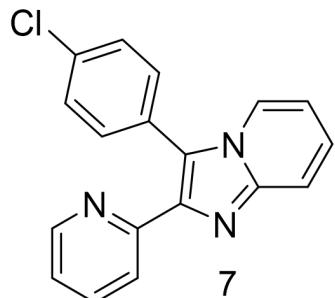
Product 3: *3-bromo-2-(pyridin-2-yl)imidazo(1,2-a)pyridine*; orange solid; 102% yield.

LCMS: m/z calcd 273.99 [M+H]<sup>+</sup> for C<sub>12</sub>H<sub>8</sub>BrN<sub>3</sub> + H<sup>+</sup> (273.99);  
1H NMR (300 MHz, CDCl<sub>3</sub>) δ 8.77 (ddd, J = 4.9, 1.9, 1.1 Hz, 1H), 8.25 (dt, J = 2.5, 1.1 Hz, 1H), 8.23 (dt, J = 3.8, 1.1 Hz, 1H), 7.81 (td, J = 7.8, 1.8 Hz, 1H), 7.71 (dt, J = 9.1, 1.1 Hz, 1H), 7.35 – 7.29 (m, 1H), 7.29 – 7.23 (m, 1H), 6.97 (td, J = 6.9, 1.2 Hz, 1H).  
13C NMR (75 MHz, CDCl<sub>3</sub>) δ 151.86 (s, 1C), 149.54 (s, 1C), 145.35 (s, 1C), 141.09 (s, 1C), 136.84 (s, 1C), 126.12 (s, 1C), 124.43 (s, 1C), 123.06 (s, 1C), 122.78 (s, 1C), 118.01 (s, 1C), 113.84 (s, 1C), 94.00 (s, 1C).

## 19 Procedure for Compound 7

Procedure for compound 7 (Suzuki cross-coupling reaction), following the scheme above (Figure 27): 3-Bromo-2-(pyridin-2-yl)imidazo[1,2-a]pyridine, (4-chlorophenyl)boronic acid (1.3 equiv), and Pd(PPh<sub>3</sub>)<sub>4</sub> (0.1 equiv) were

combined in a microwave vial that was filled with argon and evacuated three times. A 2M aqueous Na<sub>2</sub>CO<sub>3</sub> solution (5 equiv) was added, followed by the addition of 3:1 PhMe:MeOH (0.2 M). The reaction was run at 120 °C overnight. The reaction was cooled to room temperature, diluted with MeOH, filtered through celite, and concentrated. The crude material was purified by flash chromatography.



**Figure 37 Compound 7.**

The crude material was purified by reverse phase chromatography (5-100Product 7: *3-(4-chlorophenyl)-2-(pyridin-2-yl)imidazo[1,2-a]pyridine* ; white powder; 40% yield.

LCMS: m/z calcd 305.9 [M+H]<sup>+</sup> for C<sub>18</sub>H<sub>12</sub>ClN<sub>3</sub> + H<sup>+</sup> (305.9);  
1H NMR (300 MHz, DMSO) δ 8.36 (ddd, J = 4.7, 1.8, 0.9 Hz, 1H), 8.10 (ddt, J = 8.0, 6.9, 1.2 Hz, 2H), 7.84 (td, J = 7.7, 1.8 Hz, 1H), 7.70 (dt, J = 9.1, 1.2 Hz, 1H), 7.63 – 7.51 (m, 5H), 7.36 (ddd, J = 9.1, 6.7, 1.2 Hz, 1H), 7.26 (ddd, J = 7.5, 4.8, 1.3 Hz, 1H), 6.93 (td, J = 6.8, 1.3 Hz, 1H).  
13C NMR (75 MHz, DMSO) δ 153.38 (s, 1C), 148.80 (s, 2C), 144.12 (s, 1C), 136.69 (s, 1C), 133.07 (s, 1C), 132.82 (s, 2C), 128.74 (s, 2C), 128.55 (s, 1C), 125.85 (s, 1C), 124.14 (s, 1C), 122.61 (s, 1C), 122.18 (s, 1C), 121.46 (s, 1C), 117.26 (s, 1C), 113.20 (s, 1C).

## 20 Procedure for Compounds 9 and 10

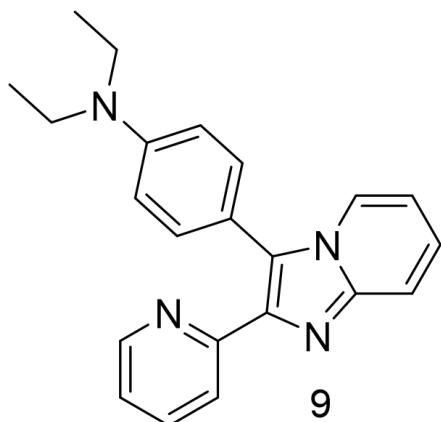
General route for compound 8 (Buchwald-Hartwig amination reaction), following the scheme above (Figure 27): the compounds generated are molecules 9 and 10 33.

3-(4-chlorophenyl)-2-phenylimidazo[1,2-a]pyridine, the proper amine\* (3 equiv)

and sodium 2-methylpropan-2-olate (1.2 equiv), were combined in dioxane (0.25 M). The reaction mixture was degassed for 2 mins. Pd(OAc)<sub>2</sub> (0.04 equiv) and Ruphos (dicyclohexyl(2',6'-diisopropoxy-[1,1'-biphenyl]-2-yl)phosphane) (0.02 equiv) were added to the reaction mixture which was again degassed. The reaction mixture was heated to 100 °C and stirred on a stir plate overnight. The reaction was stopped and cooled to room temperature, diluted with EtOAc and filtered through celite. The compound was purified by flash chromatography.

\* different amines were used to obtain compounds 9 and 10:

- ethylamine for compound 9
- methylpiperazine for compound 10

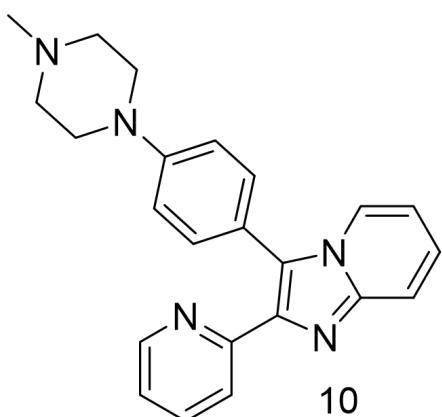


**Figure 38 Compound 9.**

The crude material was purified by reverse phase (50:100 MeOH: water).

Product 9: *N,N-diethyl-4-(2-(pyridin-2-yl)imidazo[1,2-a]pyridin-3-yl)aniline*; white powder; 18.2 %yield.

HRMS: m/z calcd 343.19 [M+H]<sup>+</sup> for C<sub>22</sub>H<sub>22</sub>N<sub>4</sub> + H<sup>+</sup> (343.19);  
1H NMR (400 MHz, CDCl<sub>3</sub>) δ 8.71 – 8.65 (m, 1H), 8.03 (d, J = 6.9 Hz, 1H), 7.71 (d, J = 9.1 Hz, 1H), 7.65 – 7.56 (m, 2H), 7.59 – 7.52 (m, 1H), 7.31 (d, J = 8.4 Hz, 2H), 7.23 – 7.14 (m, 1H), 7.17 – 7.09 (m, 1H), 6.80 (d, J = 8.5 Hz, 2H), 6.73 (t, J = 6.8 Hz, 1H), 3.45 (q, J = 7.1 Hz, 4H), 1.25 (t, J = 7.1 Hz, 6H).  
13C NMR (126 MHz, CDCl<sub>3</sub>) δ 152.85 (s, 1C), 149.83 (s, 2C), 148.07 (s, 1C), 144.17 (s, 1C), 140.17 (s, 1C), 135.94 (s, 1C), 131.76 (s, 2C), 125.27 (s, 1C), 124.01 (s, 1C), 122.78 (s, 1C), 121.97 (s, 1C), 117.78 (s, 1C), 114.60 (s, 1C), 112.59 (s, 1C), 111.85 (s, 2C), 44.38 (s, 2C), 12.63 (s, 2C).



**Figure 39 Compound 10.**

The crude material was purified by reverse phase (50:100 MeOH: water).

Product 10: *3-(4-(4-methylpiperazin-1-yl)phenyl)-2-(pyridin-2-yl)imidazo[1,2-a]pyridine*; yellow-orange powder; 10: 37% yield.

HRMS: m/z calcd 370.20 [M+H]<sup>+</sup> for C<sub>23</sub>H<sub>23</sub>N<sub>5</sub> + H<sup>+</sup> (370.20);

<sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>) δ 8.64 (ddd, J = 4.9, 1.8, 1.0 Hz, 1H), 7.99 (dt, J = 6.9, 1.2 Hz, 1H), 7.71 (dt, J = 9.1, 1.1 Hz, 1H), 7.62 – 7.58 (m, 1H), 7.58 – 7.54 (m, 1H), 7.38 (d, J = 8.7 Hz, 2H), 7.20 (ddd, J = 9.0, 6.7, 1.3 Hz, 1H), 7.15 – 7.12 (m, 1H), 7.06 (d, J = 8.8 Hz, 2H), 6.73 (td, J = 6.8, 1.2 Hz, 1H), 3.38 – 3.30 (m, 4H), 2.67 – 2.60 (m, 4H), 2.41 (s, 4H).

<sup>13</sup>C NMR (126 MHz, CDCl<sub>3</sub>) δ 153.40 (s, 1C), 151.20 (s, 1C), 149.76 (s, 2C), 144.75 (s, 1C), 141.41 (s, 1C), 135.89 (s, 2C), 131.64 (s, 2C), 124.74 (s, 1C), 123.71 (s, 1C), 122.70 (s, 1C), 121.89 (s, 1C), 119.76 (s, 1C), 118.11 (s, 1C), 115.95 (s, 2C), 112.35 (s, 1C), 55.04 (s, 2C), 48.31 (s, 2C), 46.14 (s, 1C).

## Appendix

### 1 PEW website

The screenshot shows a table from the PEW website. The columns are:

- Drug name
- Development phase
- Company
- Expected activity against ESKAPE pathogens?
- Expected activity against CDC urgent or WHO critical threat pathogens?
- Potential indication(s)

**Fetroja (cefiderocol)**

- Approved Nov. 14, 2019 (U.S. FDA)
- Shionogi & Co. Ltd.
- Yes: *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp.
- Yes: CRE, CRAB, CRPA
- 12

**Lasvic (las氟floxacin)**

- Approved Sept. 20, 2019 (Japan PMDA)
- Kyorin Pharmaceutical Co. Ltd.
- Yes: *S. aureus*, *K. pneumoniae*, *Enterobacter* spp.  
Possibly: *A. baumannii*
- No
- 8

Figure 40 *PEW website, "Antibiotics Currently in Global Clinical Development"*: [www.pewtrusts.org/en/research-and-analysis/data-visualizations/2014/antibiotics-currently-in-clinical-development](http://www.pewtrusts.org/en/research-and-analysis/data-visualizations/2014/antibiotics-currently-in-clinical-development)

## 2 Synthetic part images

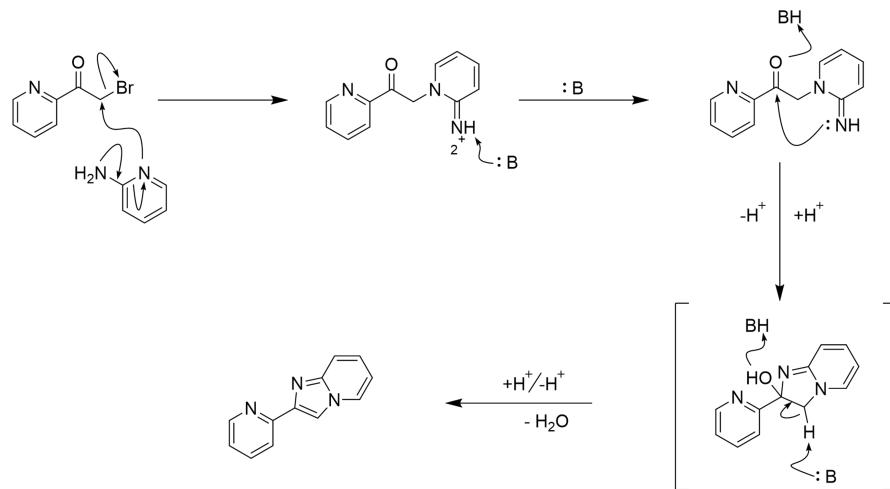


Figure 41 *Mechanism of Step 1 reaction* : synthesis of compound 2.

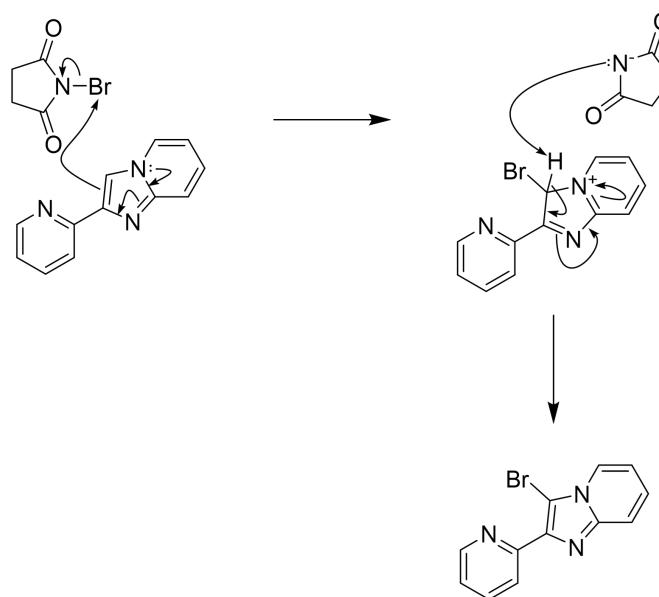


Figure 42 *Mechanism of Step 2 reaction* : synthesis of compound 3.

## Compound 2 characterizations

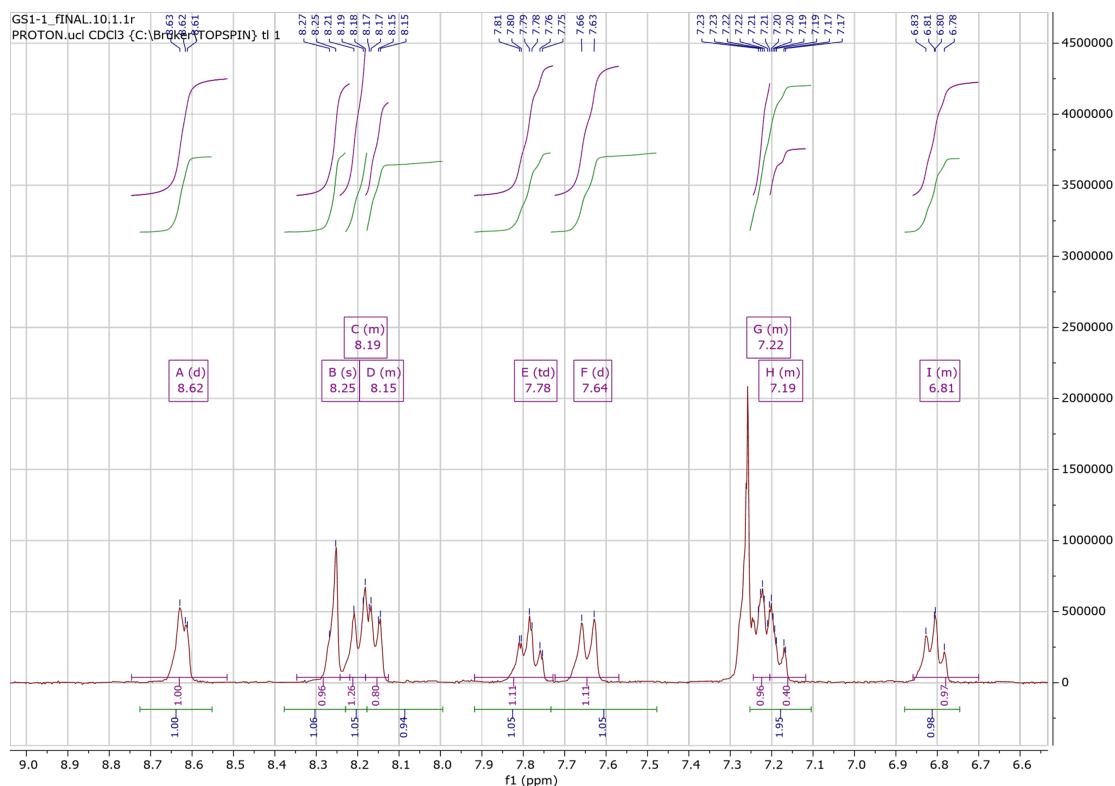


Figure 43 <sup>1</sup>H NMR spectrum compound 2.

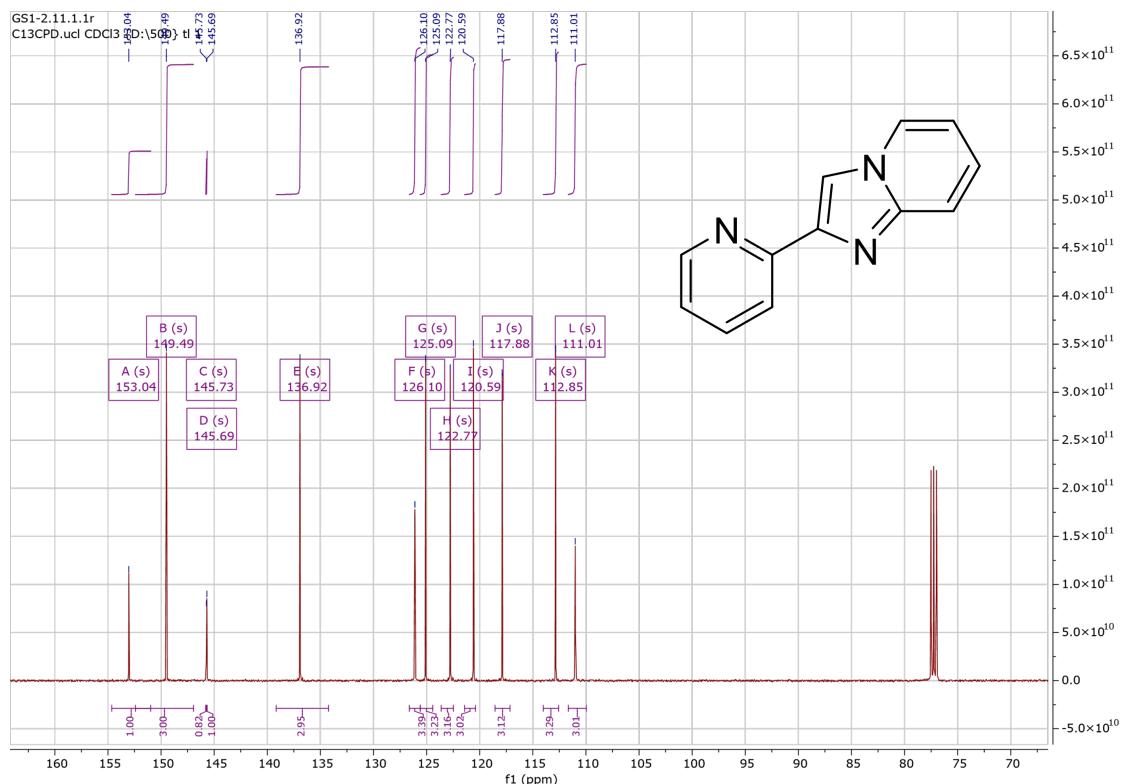


Figure 44 <sup>13</sup>C NMR spectrum compound 2.

### Compound 3 characterizations

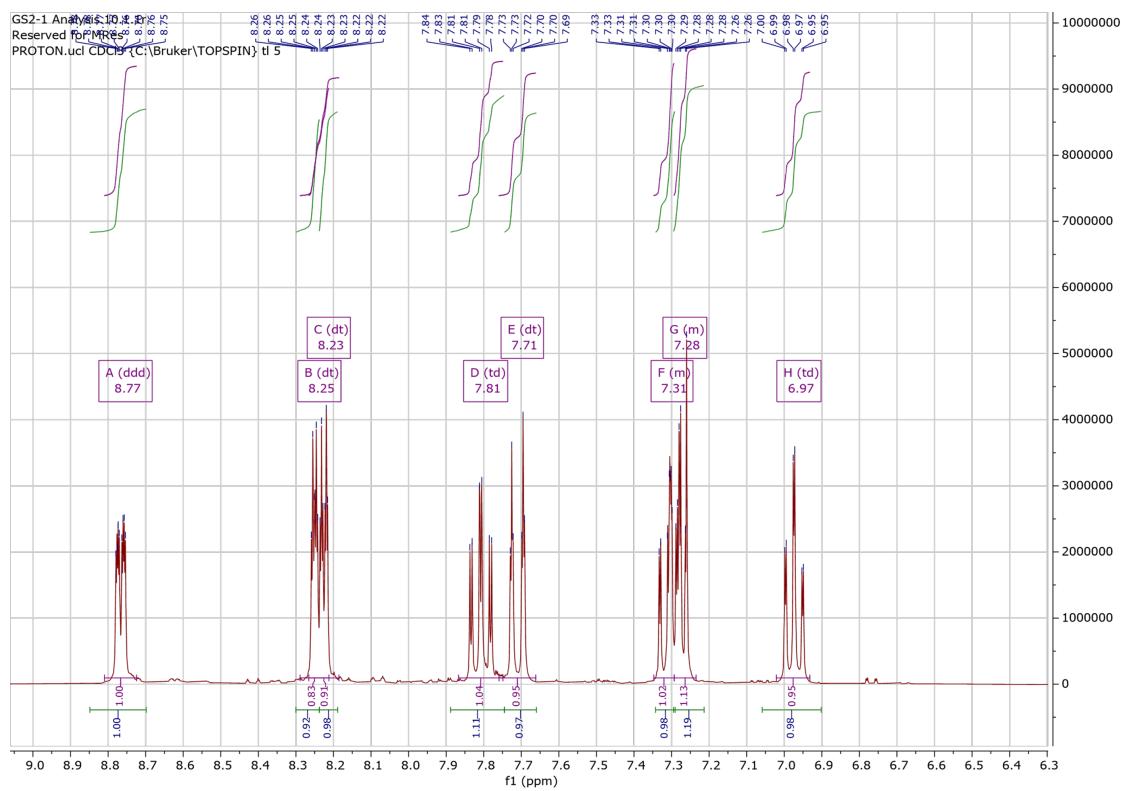


Figure 45  $^1\text{H}$  NMR spectrum compound 3.

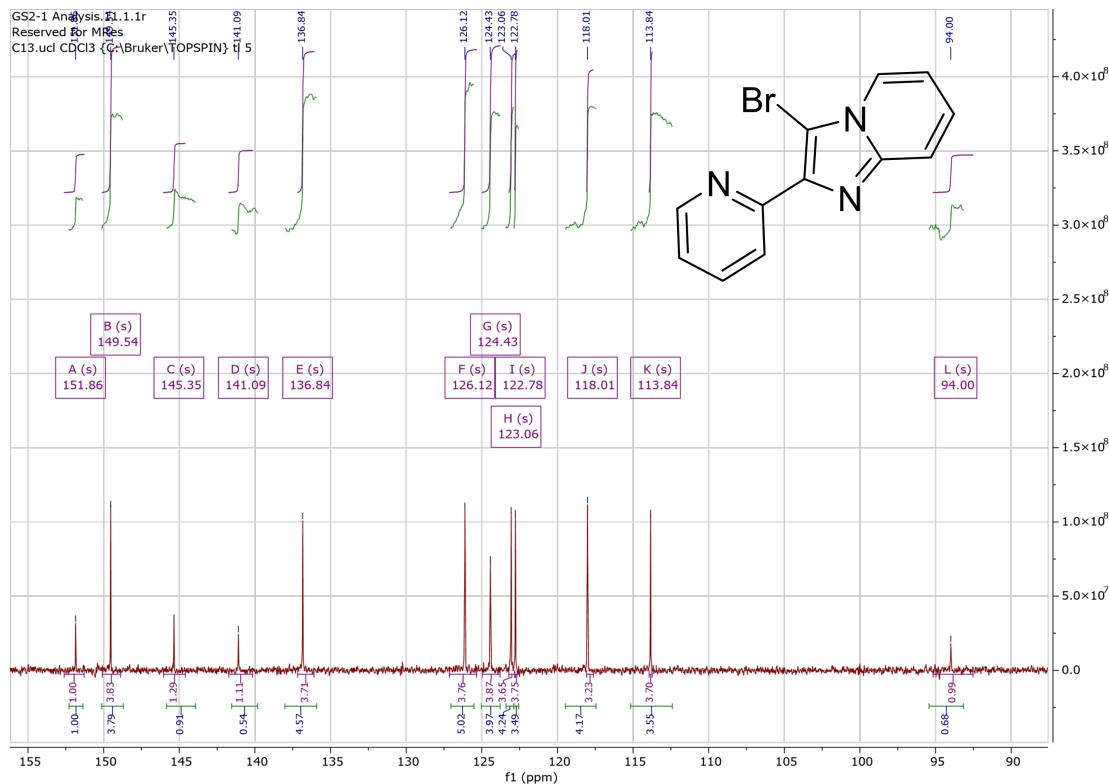


Figure 46  $^{13}\text{C}$  NMR spectrum compound 3.

## Compound 7 characterizations

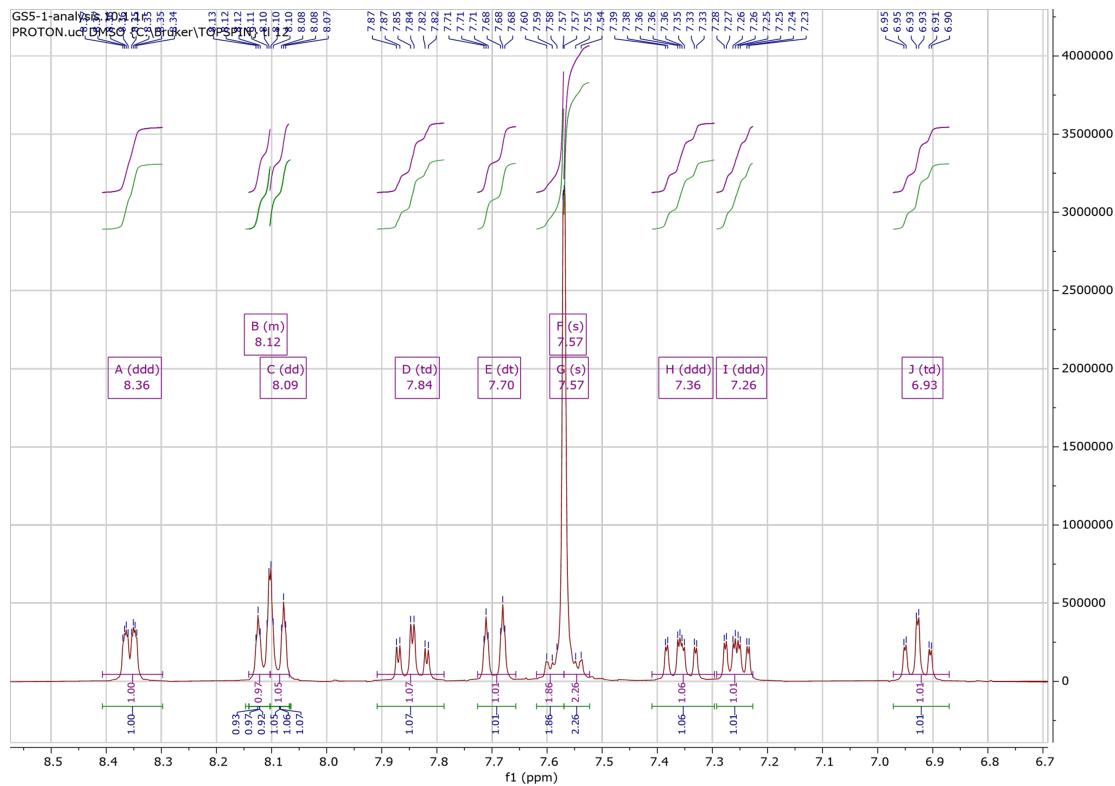


Figure 47 <sup>1</sup>H NMR spectrum compound 7.

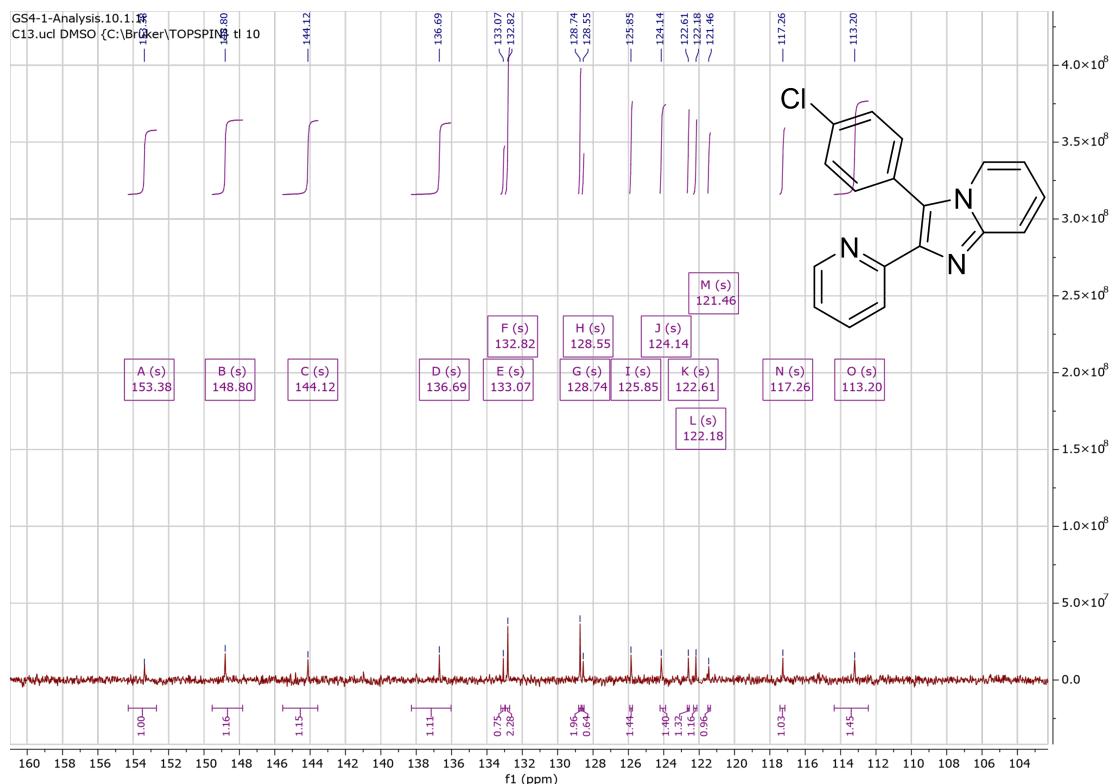


Figure 48 <sup>13</sup>C NMR spectrum compound 7.

### Compound 9 characterizations

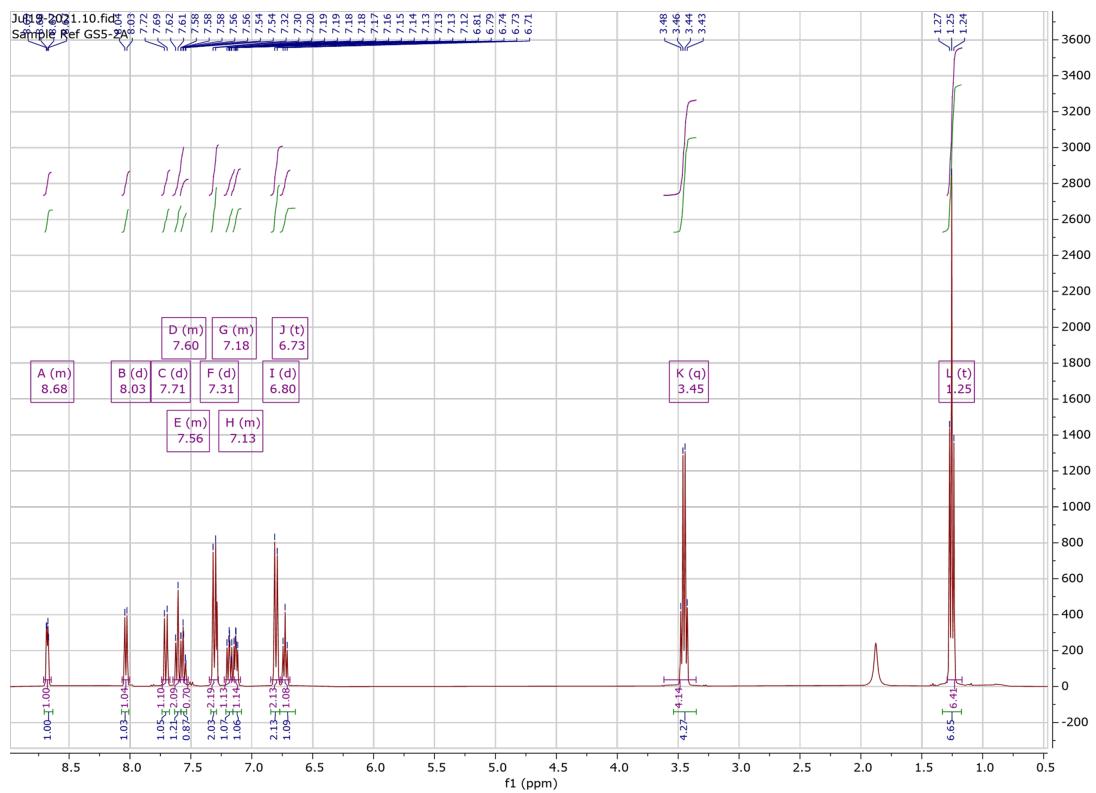


Figure 49  $^1\text{H}$  NMR spectrum compound 9.

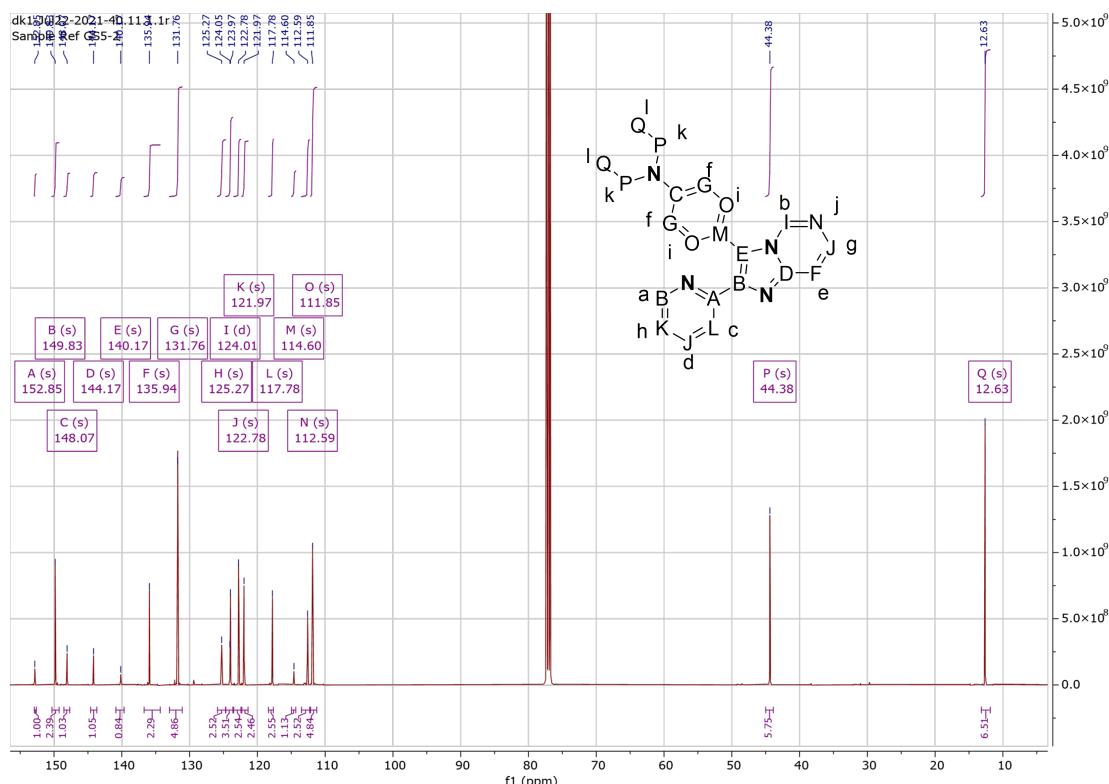


Figure 50  $^{13}\text{C}$  NMR spectrum compound 9.

## Compound 10 characterizations

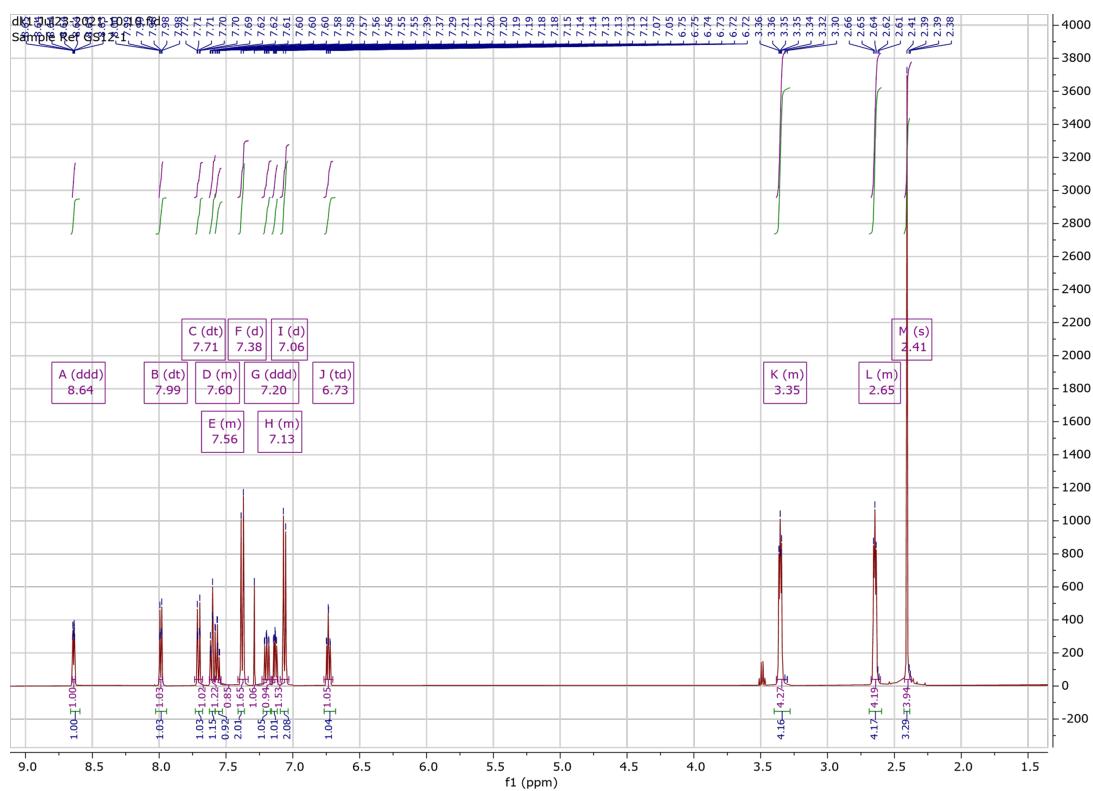


Figure 51  $^1\text{H}$  NMR spectrum compound 10.

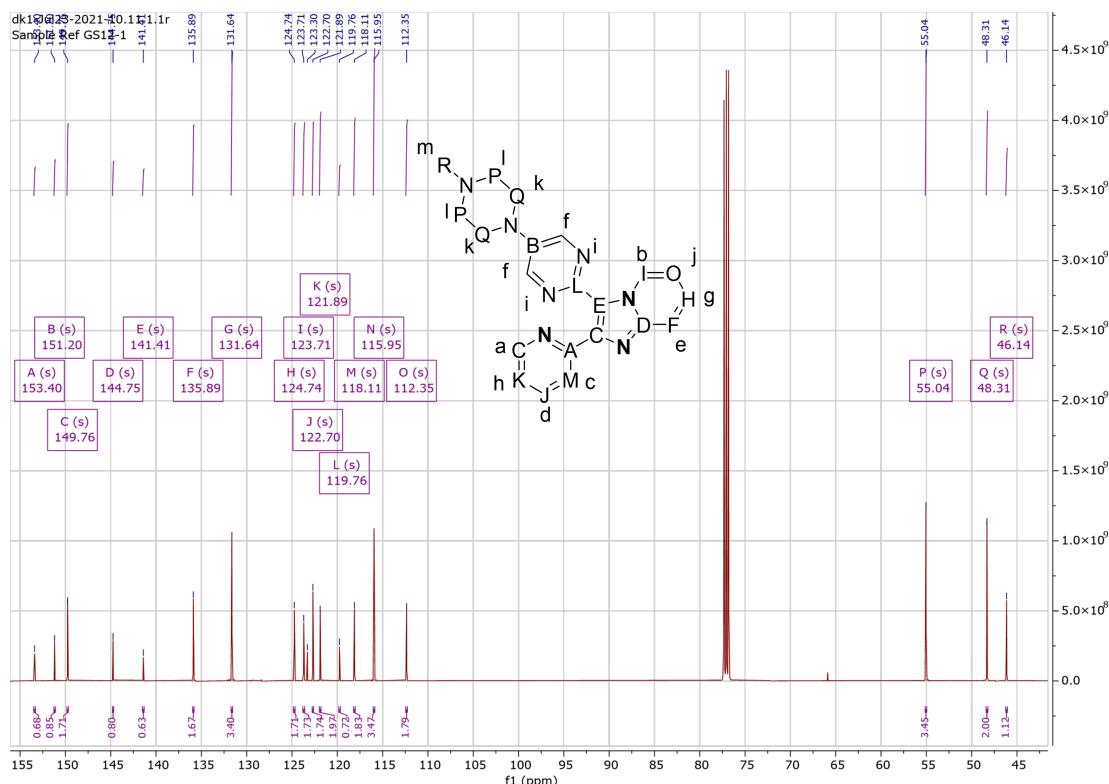


Figure 52  $^{13}\text{C}$  NMR spectrum compound 10.

### 3 Tables

**Table 1: *Actual therapies:*** List of antibiotic actually in use against S. aureus [36] [35]

TARGET	NAME	CLASS	MECHANISM OF ACTION
Bacterial membrane	<b>ampicillin,</b> <b>amoxicillin,</b> <b>oxacillin</b>	b-lactams	transpeptidases (PBPs) inhibition
	<b>ceftaroline,</b> <b>ceftrobiprole</b>	cephalosporines	transpeptidase 2a inhibition
	<b>iminepen</b>	carbapenems	penicillin-binding proteins (PBPs) inhibition
	<b>vancomycin,</b> <b>teicoplanin</b>	glycopeptides	transglycosidase activity inhibition
	<b>dalbavancin,</b> <b>telavancin,</b> <b>oritavancin</b>	lipoglycopeptides	transglycosidase and transpeptidase inhibition.
	<b>fosfomycin</b>	phosphonic acid	Enoylpyruvate transferase (MurA) inhibition

continued on next page

Table 1 – continued from previous page

TARGET	NAME	CLASS	MECHANISM OF ACTION
	<b>daptomycin</b>	lipopeptide	membrane-binding in calcium dependant manner, causing leakage of solutes through the lipid bilayer [59]
Protein synthesis	<b>linezolid,</b> <b>tedizolid</b>	oxazolidinones	the binding at the 23S rRNA prevents aminoacyl-tRNA proper placement (50S ribosomal subunit)
	<b>erithromycin</b>	macrolides	peptide chain elongation blocked by the binding with the peptidyl transferase center(50S ribosomal subunit) [60]
	<b>quinupristin,</b> <b>dalfopristin</b>	streptogramines	peptide chain elongation blocked by the binding with the peptidyl transferase center (50S ribosomal subunit) [60]

continued on next page

Table 1 – continued from previous page

TARGET	NAME	CLASS	MECHANISM OF ACTION
	<b>clindamycin</b>	lincosamides	tRNA placement within the peptidyl transferase center is hindered (50S ribosomal subunit) [60]
	<b>chloramphenicol</b>	chloramphenicols	interposition between the A- and P-site of the 50S subunit, blocking peptidyl-transpeptidase activity [60]
	<b>retapamulin</b>	pleuromutilins	peptidyl transferase activity and P-site interactions are blocked, due to ribosomal P site and peptidyl transferase centre bindings (50S ribosomal subunit) [61]

continued on next page

Table 1 – continued from previous page

TARGET	NAME	CLASS	MECHANISM OF ACTION
	<b>gentamicin</b>	aminoglycosides	incorrect protein synthesis by the A-site bounds (16S rRNA of the 30S ribosomal subunit), affecting membrane permeability
	<b>tetracycline,</b> <b>doxycycline</b>	tetracyclines	peptide chain elongation denied by binding with the ribosomal 30S subunit (prevented tRNA aminoacyl access to the acceptor site)
	<b>tigercyclin</b>	glycylcyclines	peptide chain elongation denied by binding with the ribosomal 30S subunit (prevented tRNA aminoacyl access to the acceptor site)
	<b>mupirocin</b>	pseudomonic acid	isoleucyl-tRNA synthetase inhibition

continued on next page

Table 1 – continued from previous page

TARGET	NAME	CLASS	MECHANISM OF ACTION
	<b>fusidic acid</b>		elongation factor G is inhibited, so binding to ribosomes and GTP is blocked, resulting in a lack of energy supply for protein synthesis
DNA replication	<b>fluoroquinolones</b>	fluoroquinolones	topoisomerase IV and DNA gyrase inhibition
RNA synthesis	<b>rifampicin</b>	rifamycins	DNA-transcription inhibition by prokaryotic- RNA polymerases binding
Metabolism of folic acid	<b>trimethoprim</b>		dihydrofolate reductase inhibition
	<b>sulphametoxazole</b>	sulfonamides	dihydropteroate synthase inhibition

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