Charles University

Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

Design, synthesis, and evaluation of heterocyclic compounds with potential antimicrobial activity III

Master's Thesis

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Hradec Králové, 2024

„I declare that this thesis is my original author's work, which has been composed solely by myself (under the guidance of my consultant). All the literature and other resources from which I drew information are cited in the list of used literature and are quoted in the paper. The work has not been used to get another or the same title.“

Fahim Joe Rahi
Hradec Králové, May 15th 2024

2 Table of Contents

Table of Contents *3*

Acknowledgements *4*

2 Abbreviations *5*

3 Abstract *7*

4 Czech Abstract *8*

5 Theory *9*

5.1 Introduction **9**

5.2 Bacterial resistance **9**

5.3 Classification of Acquisition of Bacterial Resistance **12**

5.3.1 Gene Mutation 12

5.3.2 Horizontal Gene Transfer 13

5.3.3 Inactivation by bacterial enzyme 15

5.4 Novel drugs in the treatment of resistant bacterial infections **18**

5.5 Mycobacterial diseases **21**

5.6 Drugs used to treat Mtb **23**

6 Aim of the Study *26*

7 Design Rationale *27*

8 Experimental *29*

8.1 Materials and instruments **29**

8.2 Synthesis **30**

8.2.1 2-Acetoxyphenyl acetic acid 30

8.2.2 Synthesis of final compounds 31

8.2.3 Acetylation of final compounds (final compounds 8–10) 34

8.3 Title Compounds **35**

8.4 In silico prediction of protonation and 3D structure of final compounds **45**

8.5 Biological Activity Assay **46**

9 Discussion *51*

9.1 Intended compounds **51**

9.2 Synthesis **51**

9.3 Pharmacokinetic Analysis **53**

9.4 Biological Activity Assays **54**

10 Conclusion *56*

11 References *57*

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3 Abbreviations

AME – Aminoglycoside modifying enzymes

ATP – Adenosine Triphosphate

CDI – 1,1'-Carbonyldiimidazole

CIP – Ciprofloxacin

CNS – Central Nervous System

DCM – Dichloromethane

DMF – Dimethyl Formamide

DMSO – Dimethyl Sulfoxide

DIPEA – *N, N*-Diisopropylethylamine

EMA – European Medicines Agency

FDA – Federal Drug Administration

HCl – Hydrochloric Acid

HGT – Horizontal Gene Transfer

IGRA – Interferon-Gamma Release Assay

INH – Isoniazid

KP – *Klebsiella pneumoniae*

KPC – Klebsiella pneumoniae Carbapenemase positive

MDR – Multiple Drug- Resistance

MDR-TB – Multiple Drug- Resistant *Mycobacterium tuberculosis*

MGE – Mobile Genetic Elements

MIC – Minimum Inhibitory Concentration

MP – Melting point

MRSA –Methicillin-Resistant *Staphylococcus Aureus*

MS-ASAP – Mass Spectrometry – Assisted Screening for Accelerated Process

Mtb – *Mycobacterium tuberculosis*

MTN NTM – Non-tuberculous mycobacteria

NAAT – Nucleic Acid Amplification Test

NMR – Nuclear Magnetic Resonance

PBP – Penicillin Binding Protein

PCR – Polymerase Chain Reaction

PGP – P-glycoprotein

SAR – Structure- Activity Relationship

THF – Tetrahydro furane

TLC – Thin Layer Chromatography

TLC-MS – Thin Layer Chromatography – Mass Spectrometry

UV – Ultraviolet

VRE – Vancomycin-Resistant *Enterobacter*

WHO – World Health Organisation

4 Abstract

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Department of Pharmaceutical Chemistry and Pharmaceutical Analysis

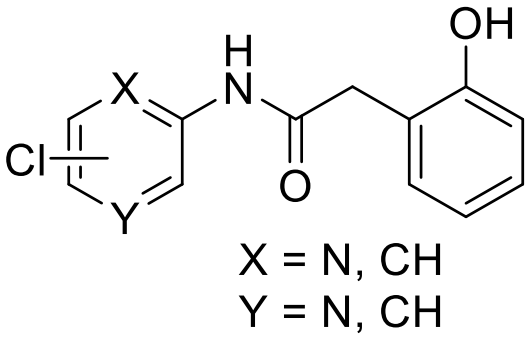
Candidate: Fahim Joe Rahi

Supervisor: Assoc. Prof. PharmDr. Jan Zitko, Ph.D.

Title of diploma thesis: Design, synthesis, and evaluation of heterocyclic compounds with potential antimicrobial activity III

The overuse of antibiotics has led to an alarming increase in resistance. The acute need for antibiotics with novel mechanism of action has driven our research to find promising new compounds through a HIT-SAR study.

The scaffold was inspired by a recent publication. Our compounds have an additional methylene bridge between the heterocycle and the benzene ring. This methylene bridge increases the flexibility of the structure, which might improve the interaction potential of the compounds.



In general, the methylene bridge did not improve the antimicrobial properties of the intended compounds. In some cases, the antimicrobial activity was significantly reduced in comparison to published compounds. MIC was insufficient to perform additional microbiological testing.

5 Czech Abstract

Univerzita Karlova

Farmaceutická fakulta v Hradci Králové

Katedra farmaceutické chemie a farmaceutické analýzy

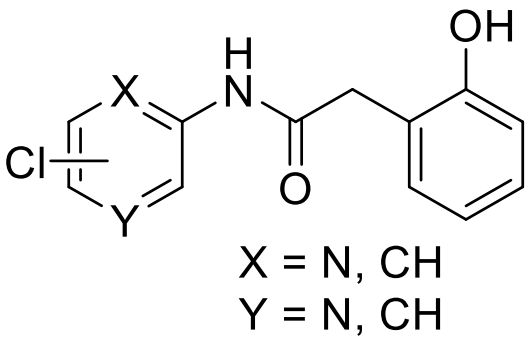
Kandidát: Fahim Joe Rahi

Vedoucí: doc. PharmDr. Jan Zitko, Ph.D.

Název diplomové práce: Návrh, syntéza a hodnocení heterocyklických sloučenin s potenciální antimikrobní aktivitou III

Nadužívání antibiotik vedlo k alarmujícímu nárůstu rezistence. Akutní potřeba antibiotik s novým mechanismem účinku vedla náš výzkum k nalezení slibných nových sloučenin prostřednictvím studie HIT-SAR.

Cílená struktura byla inspirována nedávnou publikací. Naše sloučeniny mají navíc methylenový můstek mezi heterocyklem a benzenovým kruhem. Tento methylenový můstek zvyšuje flexibilitu struktury, což může zlepšit interakční potenciál sloučenin.



Obecně lze říci, že methylenový můstek nezlepšil antimikrobiální vlastnosti zamýšlených sloučenin. V některých případech byla antimikrobiální aktivita významně snížena ve srovnání s publikovanými sloučeninami. MIC nebyla dostatečná k provedení dalšího mikrobiologického testování.

6 Theory

6.1 Introduction

Bacteria have always coexisted with humans. Many bacterial species are beneficial to humans. However, many pose a significant threat to humanity. They can field many ways to infect. They can use virulence factors such as toxins, enzymes, and adhesions molecules to propagate. Through these mechanisms, the bacteria can form colonies, invade, and evade the defenses of the body. Bacteria can manifest on any organ, most commonly on the respiratory tract, gastrointestinal tract, and the urinary tract. In severe cases, untreated bacterial infections can lead to sepsis, necrosis, and death.

The pathogenicity of bacteria extends much further than just on the individual level. Public health is under threat from the ability of bacteria to adapt and form in multiple environments. This adaptability can cause outbreaks of disease and pandemics.

Bacteria possess versatility which is capable of exerting detrimental effects on human health. Even more so with the bacteria's ability to develop resistance. The increase in multidrug-resistant bacteria have crippled treatment options available and has increased morbidity and mortality as well as healthcare costs. Dealing with bacterial resistance demands a multifaceted approach ranging from careful antibiotic use to finding new antibacterial agents.

6.2 Bacterial resistance

Bacterial resistance is a problem that we cannot look away from. The father of Antibiotics Sir Alexander Flemings raised his concerns about the over-use of antibiotics[1]. The World Health Organization (WHO) published a priority list of bacteria, which pose a major threat in terms of resistance, this includes *Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumonia, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter*[2]. All together these bacterial strains are known as ESKAPE[3]. In 2019 alone, 4.95million people died

from illnesses where drugs resistant bacteria contributed and were co-present. Of those, 1.27 million deaths were attributed to drug resistant bacteria directly[4] (Figure 1).

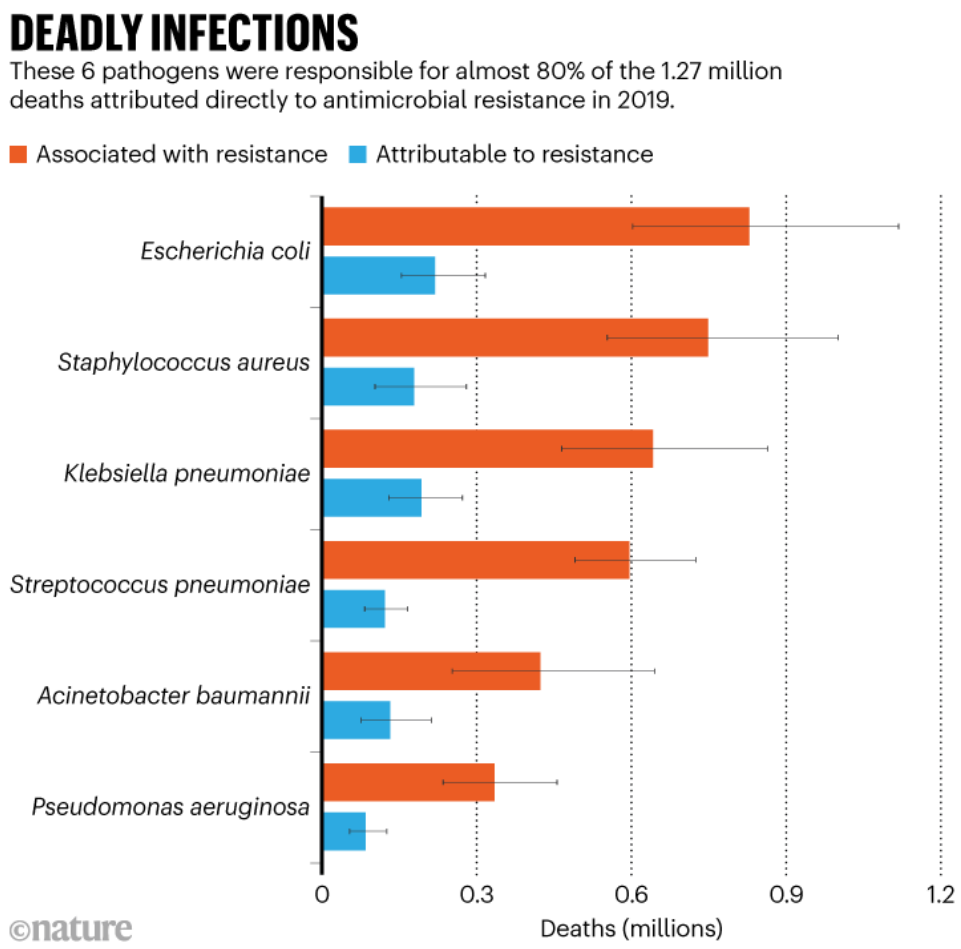


Figure 1 – Distribution of deaths from each strain -taken from: Nature[5]

It is possible for one bacterial cell to use a vast arsenal of weapons against antibacterial chemicals. For example, a fluoroquinolone resistant bacterium such as *Streptococcus pneumonia* can form resistance by mutating only one or two genes[6]. Thus, fluoroquinolone resistant bacteria can use 3 distinct resistant mechanisms to defend itself. These mechanisms can be present at the same time and are:

i) mutations in genes encoding the target site of fluoroquinolones (DNA gyrase and topoisomerase IV[7])

ii) increase in the amount of efflux transports capable of pumping out the drug from the bacterium

iii) protection of the target site of the fluoroquinolone.

Gram negative bacteria resistant to beta-lactams are capable of fielding enzymes capable of breaking down beta-lactam and inactivate the antibiotic. This enzyme is well known as beta-lactamase. Gram positive bacteria on the other hand could modify their Penicillin Binding Protein (PBP)[8]. The mutation on the PBP occurs as a shift in the position of the amino acids on the PBP also known as a point mutation. Specifically, the decreased affinity of PBP1a is reported to play a very important role in the resistance against ß-lactams[9].

A difference is noted between the two defense mechanisms due to the difference in the cell envelope. In gram negatives, the presence of an outer membrane gives the cell control on what enters – specifically into the periplasmic space. This demonstrates how most beta-lactams need specific porins to reach the PBPs on the inner membrane. Thus, the cells allow access into the periplasmic space at a pace that allows it to release enough Beta-lactamase to counter the threat posed by the antibacterial molecule. This phenomenon can be labeled as a sort of "compartmentalization" which gives the cell control of the field[10].

Some bacteria have acquired such strong resistance that they pose a genuine threat to human life. Bacteria such as **Methicillin-resistant *Staphylococcus aureus*** (MRSA), **Vancomycin-resistant *Enterococcus*** (VRE), and **Multidrug-resistant *Mycobacterium tuberculosis*** (MDR-TB) continue to be the biggest threat faced today.

MRSA was first observed among isolates from patients hospitalized in 1960 but has since spread like wildfire[11]. MRSA colonization increases the risk for infection. The infecting strains of MRSA match colonizing strains as much as 50-80 %[12]. The difficulty with MRSA is its survivability. Colonization can maintain longevity in domestic setting because MRSA persists on many surfaces which complicates attempts at eradication[13].

In the case of VRE, the first isolated resistant cases reported were *Enterococcus faecalis* and *Enterococcus faecium* in England in 1988[14]. They have since rapidly spread and are routinely encountered in hospitals in most countries[15]. Its vancomycin resistance is expressed in 5

different recognized phenotypes VanA, VanB, VanC, VanD and VanE[16]. In case of VanA, for example, the resistance is expressed by the synthesis of abnormal peptidoglycan precursors. This changes the binding sites to lower affinity binding site and thus vancomycin binds with lower affinity compared to when it binds to receptors with normal dipeptide[17].

6.3 Classification of Acquisition of Bacterial Resistance

It is important to classify the mechanisms by which the cells acquire resistance. There is a genetic element involved in the basis of the formation of the resistance. Evolutionally, bacteria rely on two major genetic strategies to adapt to the threat posed by antibiotics. These can be described as:

1- Gene mutation
2- Acquisition of foreign DNA

6.3.1 Gene Mutation

A group of susceptible bacterial cells can develop a mutation to a threat present in their surrounds. The threat eradicates the weak organisms that lack the mutation and leave only the cells which possess the new defense mechanism. This is a kind of bacterial Darwinism where only the strong (bacteria with mutation) survive and the weak (the ones without the mutation) do not[18].

Therefore, the mutations change the antibiotic action in one of four ways: modification of the target of the antibiotic (which leads to the decrease in the affinity of the drug to the cells); a decrease in uptake; an activation of a new efflux mechanism to throw out the threat; or a change in the metabolic pathways through the alteration of regulatory networks.

However, this method of acquisition is very costly on the cell's fitness. As this requires a lot of energy to maintain the mutation. Thus, this defense mechanism is only active while the threat is present[19].

Not only is it hard to maintain this resistance, but it is also usually only a phenotypic expression of the gene. The phenotypic expression does not always reflect the genotypes in mutated cells because a mutation in different genes is more than capable of producing the same resistance. Thus, pinpointing the reason for the resistance becomes more complex. When we take into consideration the same phenotypic expression that arise, we calculate this expression from different genotypical mutations. One example can be derived from quinolone resistance. When the resistance is being determined, a combination of different genes encode the synthesis of GyrA, GyrB, ParA, ParC and a plethora of other multidrug resistance systems[20; 21].

6.3.2 Horizontal Gene Transfer

One of the most important drivers for evolutionary change in bacteria is the acquisition of foreign genetic material through horizontal gene transfer (HGT). It is the most frequent reason for the development of resistance. Normally, bacteria receive genetic material on three different axes (Figure 2).

1- Transformation – incorporation of genetic material from naked DNA.
2- Phage mediated transduction.
3- Conjugation.

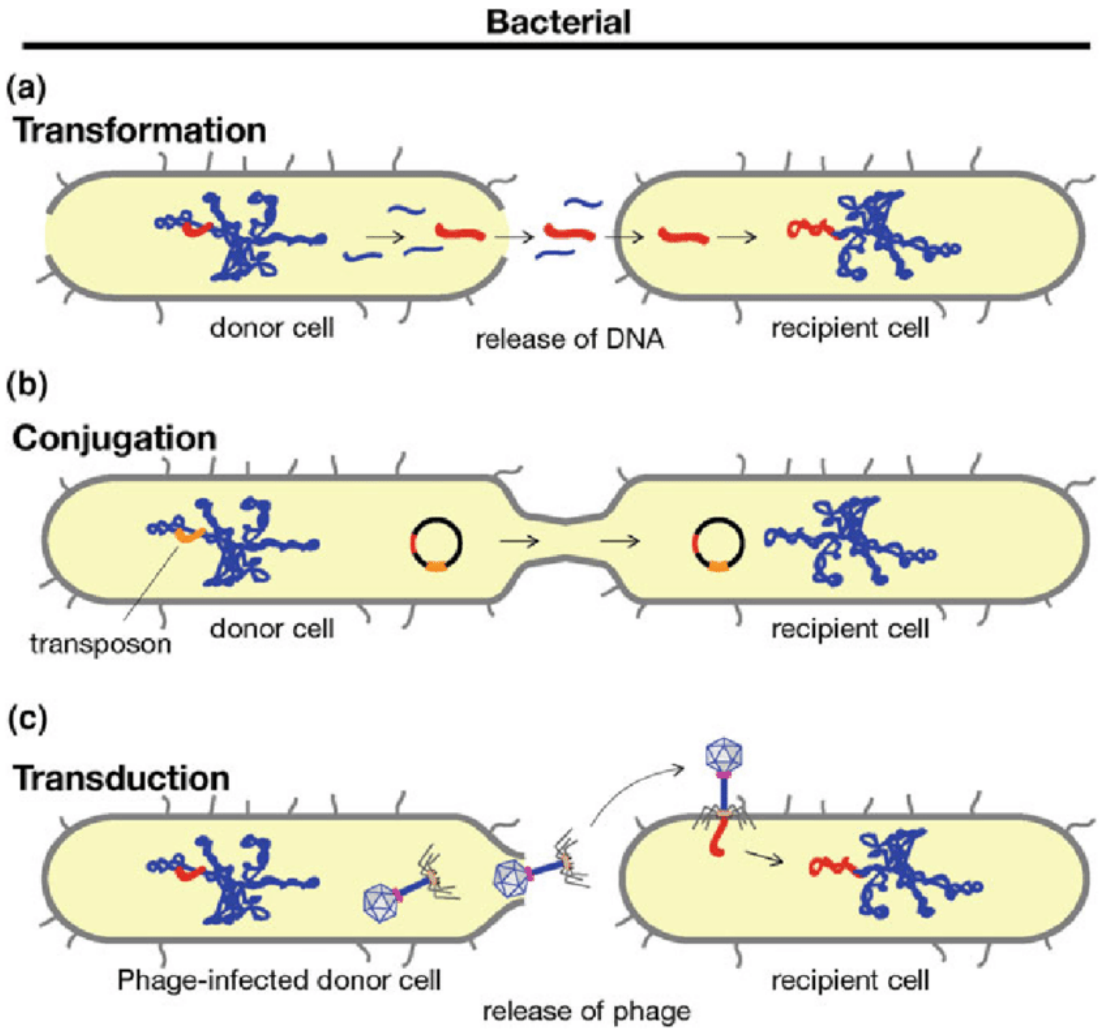


Figure 2 – Visualized description of Horizontal Genetic Transfer. Taken from[22]

Transformation is the simplest form of gene transfer. It enables the bacteria to acquire new genetic material from many sources and integrating it to its own genome. These sources can range from other bacteria, viruses and even decaying organisms in its surroundings. This leads to the acquisition of new genetic potential and increasing its genetic arsenal. This method helps the bacteria to adapt to changing environments and, in this case, acquire bacterial resistance.

However, only a few clinically significant bacterial species can use this method due to the availability of essential processes. One such example is *Streptococcus pneumoniae* – a pathogen notorious for its ability to cause respiratory tract infections and pneumonia in

humans. Despite its limitation to a few significant strains of bacteria, this method cannot be left out or ignored as its significance is in the adaptability of the bacteria.

Transduction is another mechanism of HGT observed, it is facilitated by bacteriophages or viruses that infect the bacteria. During the process, a bacteriophage infects the bacteria and hijacks its cellular machinery to replicate. This mechanism was first discovered in Salmonella Phage P22[23].

Conjugation is the reason behind the emergence of bacterial resistance in hospital settings. This method is incredibly efficient as it only requires only contact with other bacteria to transfer the biological data. This occurs at very high rates in the gastrointestinal tract of humans during antibiotic treatment. This method uses Mobile Genetic Elements (MGE) to share valuable genetic data. The most crucial MGEs are plasmids and transposons[24].

6.3.3 Inactivation by bacterial enzyme

the bacteria has multiple ways of defending itself and can vary between gram positive and gram negative (Figure 3). The most successful way the bacteria can defend itself is through creating enzymes that inactivate the antibiotic. This is achieved either by:

I) Chemical alteration of the Antibiotic

II) Destruction of the antibiotic molecule

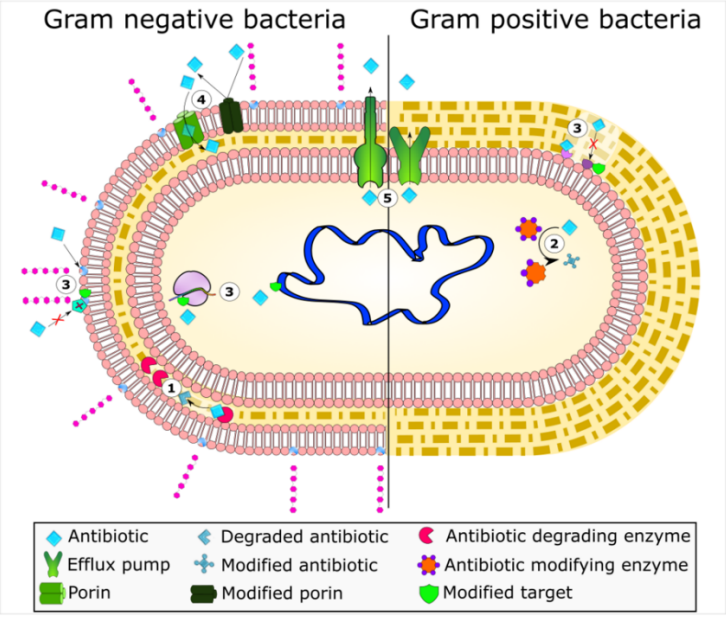


Figure 3- Depiction of Mechanisms of antibiotic resistance – taken from[27]

I- <u>Chemical alteration of the Antibiotic</u>:

This method is a well-known mechanism of acquired resistance in both gram-positive and gram-negative bacteria. This method involves the production of enzymes capable of altering the chemical structure of antibiotic rendering inactive. Most antibiotics which are affected by this defense mechanism are the ones that usually affect protein synthesis at the ribosomal level[25]. Many types of modifying enzymes have been identified and described. These enzymes usually employ a multitude of biochemical reactions to defend the bacteria including **acetylation**, **phosphorylation**, and **adenylation**.

Regardless of the method used, the effect is often a steric hindrance which leads to a decreased affinity to the target, followed by increased Minimum Inhibitory Concentration (MIC). It is also important to note that some of these enzymes can employ more than one kind biochemical activity.

For example, aminoglycoside 6'-acetyltransferase/2"-phosphotransferase AAC (6') APH (2'') (UniprotKB Accession Number: P0A0C2) mainly found in gram-positive bacteria has two functions – Acetylation and phosphotransferase activities that probably came from a fusion of two aminoglycoside modifying enzymes (AME) encoding genes. This protein allows for high

level resistance from almost all aminoglycosides with the exception of streptomycin. This protein is encoded on a Tn4001-like transposon which has been distributed widely to enterococci and staphylococci. Moreover, this enzyme's bifunctionality is the reason why high levels of gentamicin resistance was detected in enterococci and MRSA worldwide[26].

II- Destruction of the antibiotic molecule:

As stated earlier, this method works by breaking or destroying the molecule. A good example of this mechanism would be beta-lactamase. Its main function is to destroy the molecule by attacking the amide (lactam) bond in the beta-lactam ring (Figure 4).

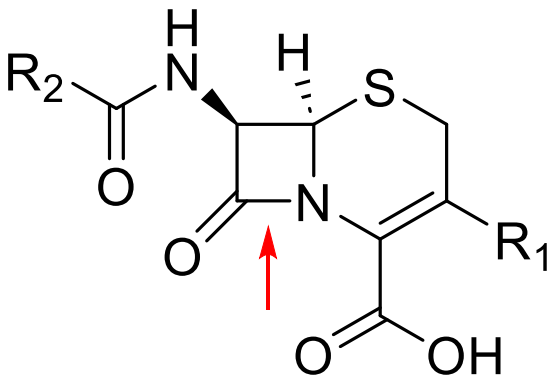


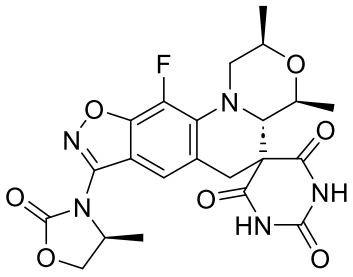
Figure 4 - The general structure of cephalosporines as an example of beta-Lactamase target

The beta-lactamase enzyme has evidence of its existence from millions of years ago[28]. This resistance was detected as early as 1940, one year before penicillin would hit markets. However, its existence only became relevant after the resistance started to spread. This spread can be attributed to the plasmids which resulted in the rapid genetic sharing of the enzyme[29]. After the discovery of the resistance, a new push to create new beta-lactams was underway. After they hit the market in the 1960s, a new plasmid encoding beta-lactamase capable of hydrolyzing ampicillin was found[30]. This led to a vicious cycle which today is increasing in severity.

6.4 Novel drugs in the treatment of resistant bacterial infections

New drugs for resistance bacterial infections are critical to fight off the growing threat of bacterial resistance. Since 2017, the Federal Drug administration (FDA) has approved 8 new antibiotics such as cefiderocol, and meropenem/vaborbactam. Most of these drugs were developed from existing traditional molecules and target *Enterobacteriaceae* resistant to carbapenems. Promising new drugs currently in clinical trials such as zoliflodacin and ridinilazole show increasing potential.

Zoliflodacin (Figure 5) is a new potential antibiotic in phase III of clinical trials (NCT0395952) for MDR *Neisseria gonorrhea*[31]. It is the first synthesized antibiotic to belong to the spiropyrimidintrione group. It was developed by Entasis Therapeutics in collaboration with Global Antibiotic Research Development Program. It targets bacterial topoisomerase type II by binding to a distinct site from fluoroquinolones, inhibiting DNA gyrase (in gram-negative bacteria) and topoisomerase IV (in gram-positive bacteria) – crucial enzymes for DNA replication[31]. Its chemical structure consists of a benzisoxazole scaffold with piperimidinetrione spirocyclic pharmacophore.



zoliflodacin

Figure 5 -Zoliflodacin structure

Another potential antibacterial chemotherapeutic is **ridinilazole** (Figure 6). It is a synthetic antibiotic made up of bis-benzimidazoles. It was discovered by Summit therapeutics (Menlo Park, CA, USA) to treat *Clostridium difficile*. It showed rapid bactericidal activity[32]. It is composed of double benzimidazoles with each substituted with pyridin-4-yl. Its mechanism of action is theorized to be the inhibition of cell division of the bacteria by binding to the minor groove of the DNA strand. Transcriptome analyses has altered expression of the genes

responsible for the cell division. And during testing there were no evident resistant strains detected[32].

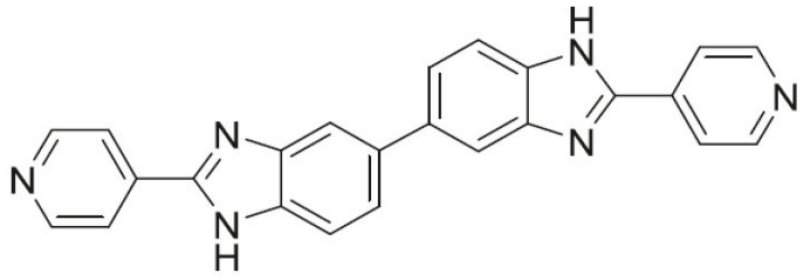


Figure 6 -Structure of ridinilazole

Another new combination with great potential is **meropenem and vaborbactam** (Figure 7) (Vaborbactam being a new beta-lactamase inhibitor). It was found to be very potent in vitro activity against *Klebsiella pneumoniae* carbapenemase (KPC)[33]. It is marketed as Vabomere after it received market authorization in 2018 for treatment of complicated urinary tract infection including acute pyelonephritis, complicated intra-abdominal infection, and hospital acquired pneumonia.

Meropenem is a 1ß-methyl carbapenem with greater stability compared to imipenem due to the ß-methyl in position 1 and a unique substitution at position 2[33]. **Vaborbactam** is based on the boronic acid formula that demonstrated potent inhibition of ß-lactamase including KPC. In vitro experimentation has demonstrated that its ability to enter the outer membrane of KP which was facilitated by porins OmpK35 and OmpK36[34]. The addition of thienyl-acetyl in position 2 of vaborbactam ring indicated promising results when in combination with meropenem.

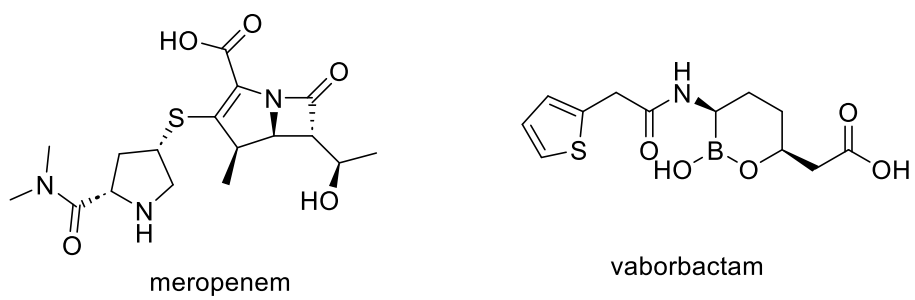
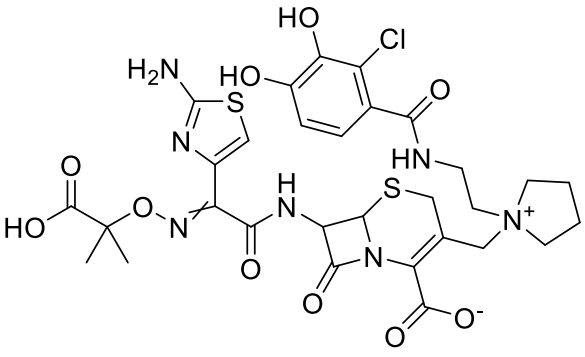


Figure 7 – Meropenem and vaborbactam structures

One last addition to this list would be **cefiderocol** (Figure 8). Developed by Shionoigi & Co (Osaka, Osaka, Japan). In the early 2000s, it is a siderophoric cephalosporin with enhanced properties[35]. It has a similar structure to Cefepime, with a pyrrolidine group at C3 and additional chains for increased antibiotic activity. It also contains chloro-catechol moiety facilitating increased plasma concentration by chelating iron ions and exploiting the iron transport system[36].

Cefiderocol is stable against ß-lactamases, including cephalosporinases. It can penetrate bacterial cell through the iron transport system. Cefiderocol is effective against ß-lactamase resistance, ß-lactamase production, efflux pump up-regulation as well as porin modification. So far, no cross-resistance has been detected with other cephalosporins[37]. However, there is a potential for resistance to form on the iron transport system. Therefore, deeper research into this mechanism is underway[38].

The Interesting aspect of cefiderocol is its ability to target clinically relevant gram-negative strains – Enterobacteriaceae such as *Klebsiella spp.*, *Salmonella spp.*, *Acinetobacter*, *Pseudomonas spp.*, and *Burkholderia spp.*, it also has activity on *Haemophilus*[39]. It also holds promise for treating carbapenem-resistant strains. In fact, a global surveillance study indicated a very high sensitivity of 96.2% of *Enterobacteriaceae, p A. baumannii*, *P. aeruginosa*, and *Sterotrophomonas maltophila* to ≤ 4mg/ml of cefiderocol. This indicates efficacy against resistant strains[40].



cefiderocol

Figure 8 – Structure of cefiderocol

6.5 Mycobacterial diseases

Mycobacterium comprises a diverse genus of bacteria boasting 190 different species. These bacteria are characterized by their acid-fast staining properties due to their waxy cell wall structures. These cell walls are composed of mycolic acids. Among the most clinically significant is ***Mycobacterium tuberculosis*** (Mtb) and ***Mycobacterium leprae*** (*M. leprae*) and ***non-tuberculous mycobacteria*** (NTM); these three mycobacteria are pathogenic. NTM manifest usually as skin and soft tissue infection, and superficial lymphaditis[41].

The terrifying aspect of tuberculosis is its ability to stay dormant long after its initial infection. It is estimated that due to this property, one third of the world's population is infected with the latent form of Mtb[42].

Mtb has many virulent factors (cellular structure molecules that allow colony formation that evade immune response), which makes it a very versatile bacterium. This includes **lipid and fatty acid metabolism, cell envelope protein, macrophage inhibitors, kinase proteins, proteases, and gene expression regulators**[42]. This arsenal of weapons makes Mtb very potent and dangerous. Its intrinsic property of its mould-like cell wall structure makes penicillin totally ineffective which requires stronger antimycobacterial drugs such as pyrazinamide to inhibit coenzyme A synthesis[43].

Tuberculosis is diagnosed with a handful of methods. Which include a Chest X-ray, **Nucleic Acid Amplification Test (NAAT)**, and **Interferon-Gamma Release Assay (IGRA)**.

Nucleic Acid Amplification Test (Figure 9)is a molecular test used to detect DNA of Mtb in sputum or other respiratory tissue. This Assay is commercially available and were developed by Cepheid Inc (Sunnyvalle, CA, USA). – among other assays available from other companies – called Xpert MTB/RIF assay. This automated PCR based test can identify Mtb DNA and Rifampicin resistance[44]. Figure 8 depicts the stages of the NAAT process.

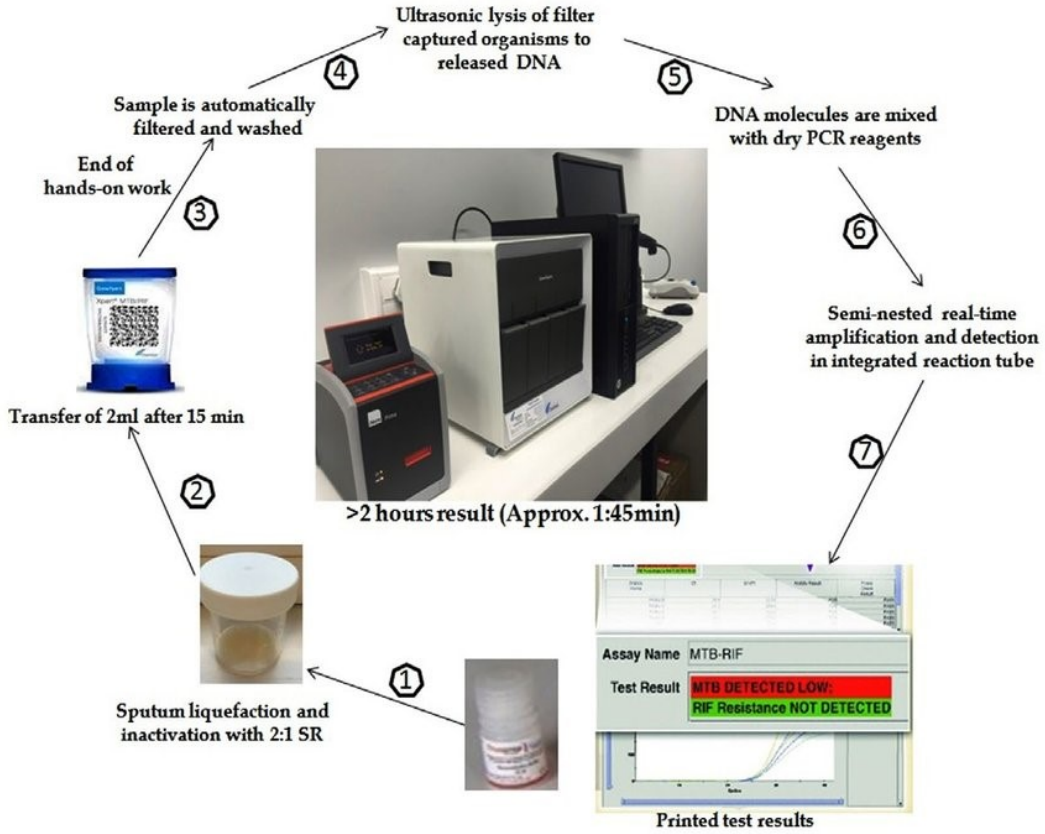


Figure 9 - Nucleic Acid Amplification Test steps -taken from10 [45]

Interferon-Gamma Release Assay (Figure 10) measures a person's immune reactivity to Mtb. This method revolutionized tuberculosis detection: this method was the first standardized accurate commercial for Mtb detection. This genomic analysis allows to find Mtb-specific antigen on RD-1 region which induces strong interferon-gamma activity from sensitized T-cells which signals ongoing infection[46]. The test is conducted with a fresh sample of blood which is then mixed with Mtb specific antigen. A specific response is detected through IFN-gamma release. This test is commercially available as QuantiFERON-TB and T-SPOT TB[46].

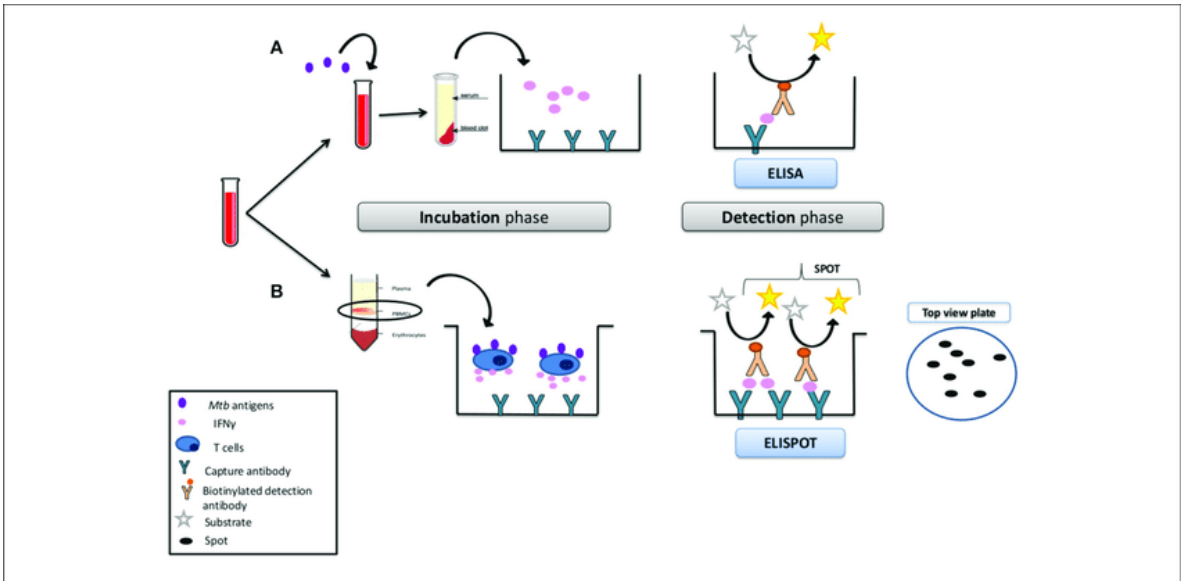


Figure 10-- IGRA Interferon-Gamma Release Assay visualization taken from[46]

6.6 Drugs used to treat Mtb

Currently, the treatment regimen of Mtb comprises a cocktail of four first-line drugs. These include pyrazinamide, isoniazid, rifampicin and ethambutol (Figure 11). These four drugs are used together in the first two months of the treatment, which is called the initial phase. Then, the continuation phase is focused on isoniazid and rifampicin for an additional four months to ensure eradication.

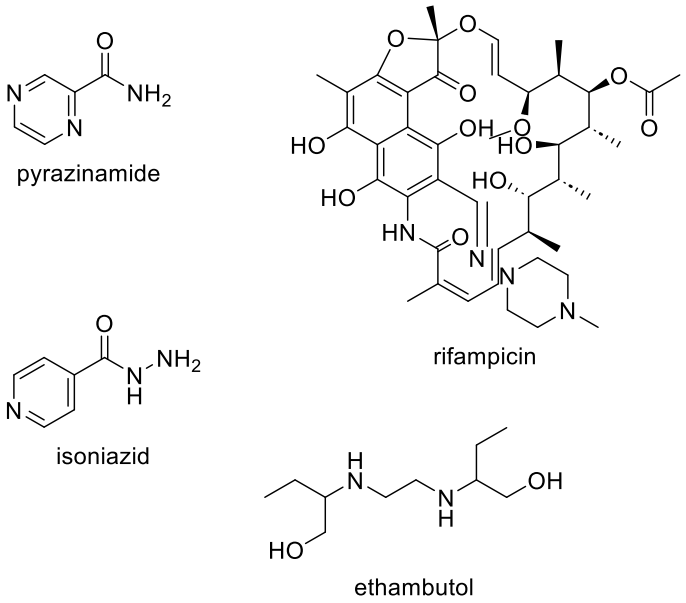


Figure 11 - The structures of 1st line Mtb treatment – taken from[47]

In the case of *Mycobacterium tuberculosis*, recent new drugs in development have gained significant attention. One of which is **bedaquiline** (). This drug belongs to the diarylquinolines family and is in – at the time of writing – phase III of clinical trials. Its mechanism of action is that it selectively targets the proton pump of ATP synthesis which leads to a decrease of ATP available[48]. Basically, it starves the Mtb from its energy source. It has shown significant bactericidal activity greater than isoniazid and rifampicin. It has also shown significant activity against both sensitive and resistant strains of Mtb[49].

The result of the studies conducted on that structure has indicated a possible 2-month treatment regimen that has high culture conversion rate, rapid sputum conversion and lower acquired resistance in MDR Mtb[50]. It is currently being dosed at 400 mg daily for two weeks and then 200 mg three times a week for 22 weeks added to a background of optimized treatment for MDR Mtb[51].

Another potential drug – at the time of writing – in phase II of clinical trials is **delamanid** (). It belongs to the piperidine family of structures derived from nitro-dihydro-imidazoxazole. It's mechanism of action is that it inhibits the synthesis of mycotic cell wall[52]. In deeper detail, Delamanid is activated by the F420 coenzyme system to form a reactive intermediate metabolite that inhibits the synthesis of cell wall components which depletes the mycobacterium from cell wall components effectively killing the mycobacterium[47]. Delamanid is active against both replicating and anaerobic non-replicating Mtb persisters. It has potential to improve treatment outcomes for MDR Mtb[52]. It is marketed under the brand name Deltyba by Otsuka Pharmaceutical Co. Ltd in the EU. The market authorization was granted by the EMA.

It Is dosed currently at 100 mg twice daily for 6 months with a background of optimized drugs[53]. A spontaneous resistance was observed due to F420 mutation. One of the enzymes responsible for Delamanid activation mutated and blocked the activation.

Another contribution to the list of new potential antimycobacterial drugs is **sutezolid** (Figure 12)**.** This drug currently – at the time of writing – in phase IIB of clinical trials has potent

antimycobacterial activity. It belongs to the oxazolidinone group of structures. Its mechanism of action is binding to 23S RNA, and 50S subunit ribosome prevents protein synthesis[54]. Clinical trial testing has shown that it is safe and well tolerated by patients[55].

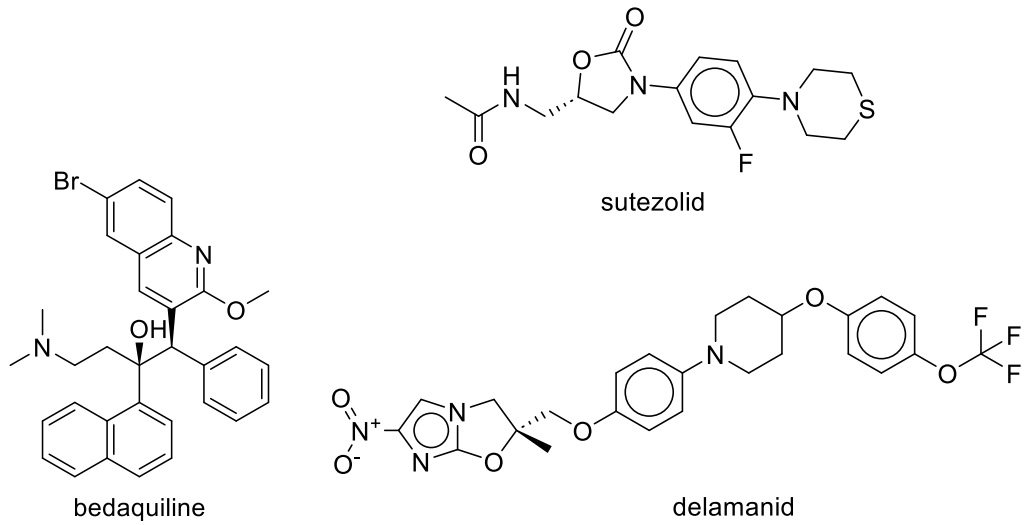


Figure 12 -Structures of sutezolid, delamanid, and bedaquiline

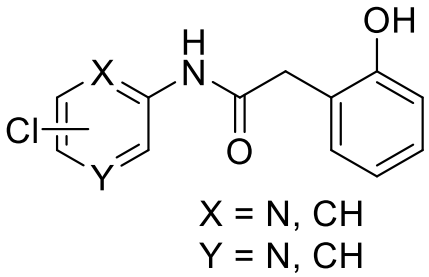
It is important to mention **salicylanilides** as a potential antimycobacterial. It has shown substantial antibiotic activity compared to isoniazid and benzylpenicillin. They also showed mild inhibition of mycobacterial isocitrate lyase. Salycilanilides studied in M. Krátký's publications were tested against different strains of *Mycobacterium tuberculosis.* The tested compounds showed MIC of maximum of 32 µmol/L[56].

This development influenced the scaffold of the compounds in this thesis.

7 Aim of the Study

The work to be presented in this thesis will be aimed at synthesizing multiple different derivatives of *N*-pyrazinyl- and *N*-pyridylhydroxybenzamides that were derived from previously published compounds. These previously studied compounds underwent a HIT expansion study to study the Structure Activity Relationships (SAR) with potential activity[57].

We will prepare a series of **methylene homologues** (extension of the linker, red arrow) and isosteres (pyrazine vs pyridine) to provide additional knowledge on structure activity relationships and biological activities.



In addition to the chemical synthesis, this thesis will dive deeper into the biological activities of the synthesized compounds and their potential against an array of bacterial and mycobacterial strains. These activities will be compared to previously reported compounds without the methylene bridge in order to examine the idea that the added flexibility of the methylene-containing linker would lead to better interaction with a hypothetical bacterial and mycobacterial receptor. The new compounds will also undergo pharmacokinetic evaluations.

8 Design Rationale

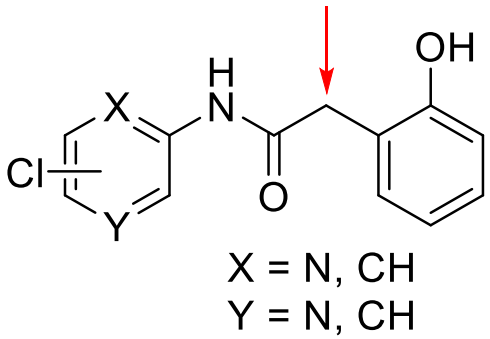


Figure 13 - General structural formula of the current study

The structure from the study was modified with a methylene bridge located between the phenyl group and the carbonyl group which allows for greater flexibility. This modification could help to obtain a new interaction with the hypothetic receptor or strengthen one which might be present in compounds without a methylene bridge (Figure 13

This change in the compound was done with the intention of increasing the freedom of rotation and thus increasing the conformational flexibility. Thus, potentially establishing new interactions with biological receptors found on bacteria or even strengthening existing ones. That leads to improved biological activity which is the aim of this study.

Additionally, the presence of the bridge increases the lipophilicity of the structure. Although a slight improvement this could mean a lot on the molecular level as the log P characteristics of the structure with and without the bridge differs when other constituents of the structure are taken into consideration.

We used the 2-OH substitution modification on the compound due this substitution being present on salicylanilides which have interesting antistaphylococcal acitivity[58]. Thus, my compounds can be considered homologues of salicylanilides due to the methylene bridge addition and can be considered isosteres as well because we exchanged the benzene ring of the salicyllanilides with heterocycles – pyridine and pyrazine.

Overall, these modifications represent an optimized approach to fine-tuning the molecular architecture of the compound, with the potential to optimize its pharmacological and physico-chemical properties and therapeutic efficacy.

9 Experimental

9.1.1 Materials and instruments

All experimental work was completed at the Department of Pharmaceutical Chemistry and Pharmaceutical Analysis at the Faculty of Pharmacy in Hradec Králové, Charles University. The Chemicals used for synthesis were from Merck (Darmstadt, Germany). All chemicals fit the parameters set by the manufacturer.

Monitoring of the reaction process and initial evaluation of the purity of products was done on TLC plates Merck Silica 60 F_{254} (Merck, Darmstadt, Germany). The TLC chromatography detection was conducted using UV light at a wavelength of 254 nm. The retention factor Rf was measured through thin layer chromatography method and the mobile phase used was ethyl acetate and hexane used in a ratio of 2:1 The purification of the synthesized compounds was carried out on an automated flash chromatography PuriFlash XS420+ (Interchim, Montluçon, France) using original columns (spherical silica 30 µm, F0040). The mixture of ethyl acetate and hexane was used as a mobile phase with elution and detection performed by ultraviolet-visible detector at a wavelength of 254 and 280 nm.

The mass spectra in both positive and negative modes were measured using the Expression® Compact Mass Spectrometer (Advion, NY, USA) with a single-quad detector.

NMR spectra was measured on Varian VNMR S500 (Varian, Palo Alto, CA, USA) at frequency 500 MHz for ^{1}H spectrum, and at the frequency 126 MHz for ^{13}C spectrum; and on Jeol ECG600 NMR (JEOL, Tokyo, Japan) at 600MHz for ^{1}H and at the frequency 151 MHz for ^{13}C. The spectra were measured in deuterated dimethyl sulfoxide (DMSO-d_6) if not stated otherwise. Chemical shifts were measured in ppm (δ). Reference signals were residual signals of DMSO (2.49 ppm for ^{1}H and 39.7 ppm for ^{13}C).

Melting points were measured using the open capillary method on a Stuart SMP30 spot meter (Bibby Scientific Limited, Staffordshire, UK). Measured values were not corrected.

Reaction yields are shown as a percentage of the theoretical yield of reactions of the isolated product after all purification processes and refer to the last synthetic step.

The theoretical lipophilicity parameters log P were determined using ChemDraw Professional, Version 23.0.1 (PerkinElmer Informatics, Inc., Waltham, MA, USA).

All structures were put through Molecular modelling software MOE (Molecular Operating Environment) version 2022.02 (Chemical Computing Group, Montreal, Canada).

All physico-chemical and pharmacokinetic predictions were conducted on SwissADME http://www.swissadme.ch (Lausane, Switzerland) [59].

9.2 Synthesis

9.2.1 2-Acetoxyphenyl acetic acid

9.2.1.1 Method 1

The first acetylation attempt was performed under biphasic conditions according to Schotten-Baumann. 10 g of 2-hydroxyphenyl acetic acid (65.7 mmol) was dissolved in 200 ml mixture (1:1 v/v) of chloroform and sodium hydroxide water solution (7.8 mmol, 0.31 g of NaOH in water solution). The reaction mixture was stirred for 5 minutes and 2 ml of acetic anhydride (7.5 ml excess) was added. The reaction was stirred vigorously for 12 hours to form 2-acetoxyphenylacetic acid. After the reaction was completed (TLC showed no starting material, both phases were checked). The reaction was acidified with 2M HCl to pH 4. The organic layer was separated, washed with brine and dried with Na_2SO_4. The solvent was evaporated resulting in an oil product. The MS analysis showed no trace of the product.

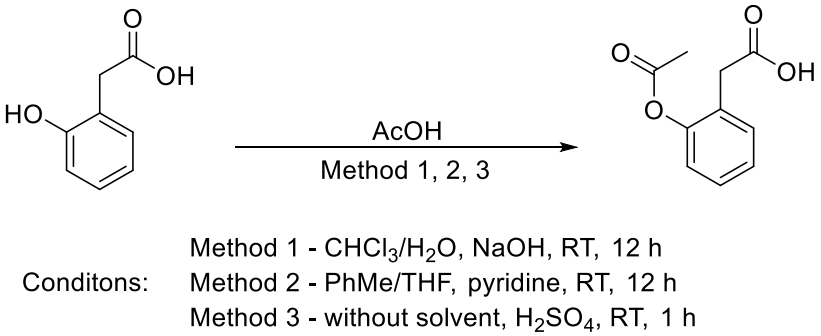
9.2.1.2 Method 2

The second acetylation attempt was performed under monophasic conditions, using a mixture of two miscible organic solvents. 10 g 2-hydroxyphenylacetic acid (65.7mmol) was dissolved in a mixture of toluene and THF (4:1). Then, acetic anhydride (1.1 eq, 72.3 mmol) and 1.5 ml of pyridine was added to the mixture. The mixture was then stirred for 12 hours. The solvent was evaporated then the crude was diluted in ethyl acetate and washed with 50 ml of diluted

HCl, brine and dried with Na_2SO_4. The solvent was then evaporated resulting in an oil. The TLC-MS analysis showed no trace of the compound.

9.2.1.3 Method 3

The third acetylation approach was attempted directly in acetic anhydride without any additional solvent. 10 g of 2-hydroxyphenylacetic acid (65.7 mmol) was dissolved in 20 ml of acetic anhydride. Then 10 drops of concentrated sulfuric acid were added to the mixture and then mixed at room temperature for 1 hour. The reaction was then poured on ice and left to mix until the ice had melted. The product extracted with ethyl acetate was separated, washed with brine and dried with Na_2SO_4. The solvent was then evaporated and produced an oil. TLC of the oil revealed no trace of the compound.

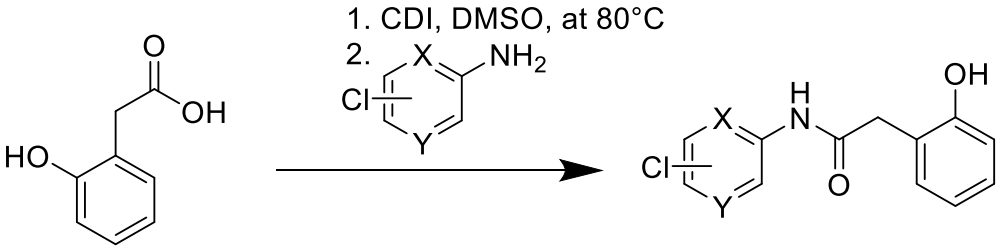


Scheme 1 - Acetylation of free hydroxy group by different methods

9.2.2 Synthesis of final compounds

9.2.2.1 Method 4

0.608 g (4 mmol) of 2-hydroxyphenyl acetic acid was put in a test tube with 713.5 mg of CDI (4.4 mmol, 1.1 eq.) and a few drops of DMSO. The reaction was heated to 80 °C for 20 minutes, until the activation was completed (liquified from solid to liquid and bubbles have subsided). Then, selected substituted aminopyridine or aminopyrazine (4 mmol, 1 eq.) and 10 ml of DMSO were added to the mixture and left to stir overnight at 80°C. The reaction was cooled to room temperature and put to 100 ml of H_2O. The precipitated product was filtered and purified with Flash chromatography. The resulting product was not pure and in low yield, so the method was abandoned.

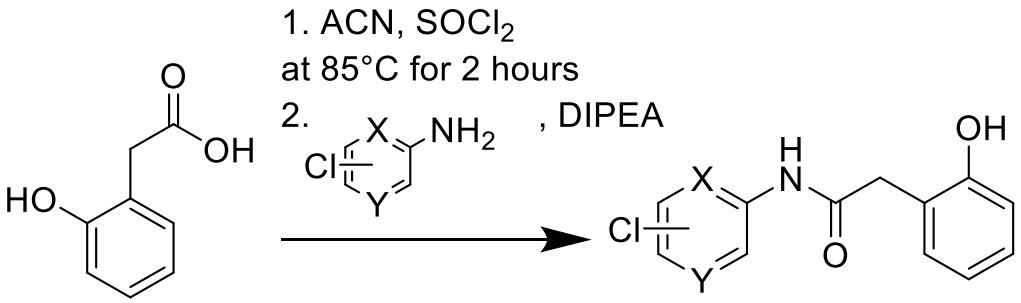


Scheme 2 - General reaction scheme of method 4

9.2.2.2 Method 5

0.608 g of 2-hydroxyphenyl acetic acid (4 mmol) was dispersed in 20 ml of acetonitrile. 4.4 mmol of $SOCl_2$ (thionyl chloride) was added to the mixture and stirred for 2 hours at 85 °C. The solvent was evaporated under a high vacuum to dispose of all traces of leftover thionyl chloride. The crude was dissolved in 20 ml of acetonitrile and selected aminopyrazine or aminopyridine (4 mmol, 1 eq.) and 12 mmol of *N, N*-Diisopropylethylamine (DIPEA) were added to the reaction. The reaction was heated at 80°C overnight. The reaction was then evaporated and purified using flash chromatography and then analysed with TLC-MS.

The reaction yielded the product, but in a low amount and insufficient purity; thus, the method was abandoned.

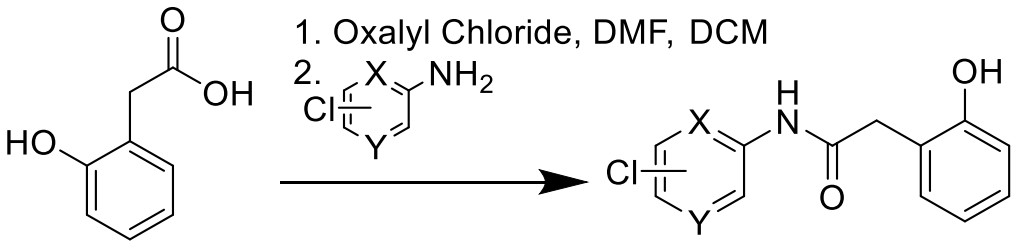


Scheme 3 - General reaction scheme of method 5

9.2.2.3 Method 6

0.684 g (4.5 mmol) of 2-hydroxyphenyl acetic acid was dispersed in 5 ml of dichloromethane (DCM). 5 mmol of oxalyl chloride and 2 drops of *N*, *N*-dimethylformamide (DMF) as a catalyst were added. The reaction was stirred at room temperature for approximately 45 minutes. After the activation was completed – checked visually after bubbling had subsided and confirmed by TLC – the activated acid was then added dropwise to an ice-cooled solution of 3.5 mmol of the corresponding aminopyridine or aminopyrazine which were beforehand dispersed in 5 ml of DCM and 3 equivalents of pyridine (725 µl). The reaction was then left to heat to room temperature and stirred for 24 hours. The reaction was checked with TLC. The reaction mixture was washed with acidic water (50 ml of distilled water with a 10 ml of 2M HCl) and extracted to ethyl acetate. The mixture was evaporated, adsorbed on silica and purified with Flash chromatography, resulting in a pure compound.

This method yielded the most product and resulted in the purest form.



Scheme 4 - General reaction Scheme of method 6

Table I. Weights for final products prepared by Method 6

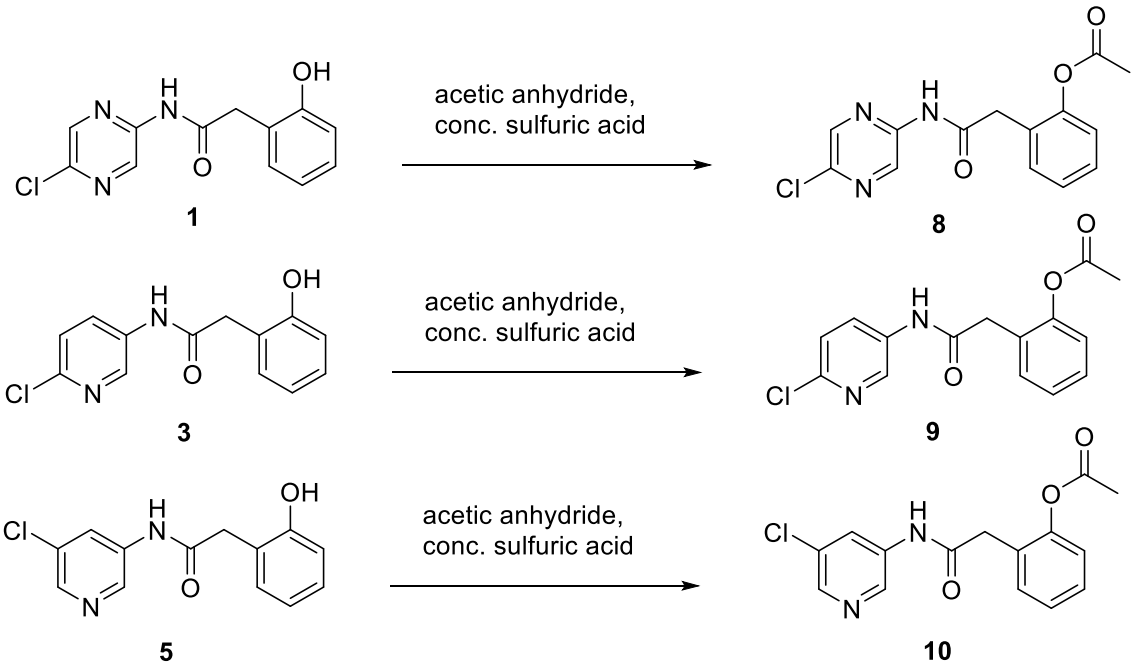
Code	Acid		Amino compound			Product – Isolated yield	
	n (mmol)	m (mg)	R	n (mmol)	m (mg)	m (mg)	% to theoretical
1	4.5	685	2-NH_2-5-Cl-Pz	3.5	453	27	6
2	4.5	685	2-NH_2-5-Cl-Py	3.5	450	18	4
3	4.5	685	3-NH_2-6-Cl-Py	3.5	450	130	29
4	4.5	685	2-NH_2-6-Cl-Py	3.5	450	27	6
5	4.5	685	3-NH_2-5-Cl-Py	3.5	450	72	16
6	4.5	685	3-NH_2-2-Cl-Py	3.5	450	45	10
7	4.5	685	2-NH_2-Pz	3.5	333	17	5

Pz – pyrazine, Py - pyridine

9.2.3 Acetylation of final compounds (final compounds 8–10)

9.2.3.1 Method 7

A total of 0.5 mmol of the respective hydroxyderivatives (compounds 1, 3, 5) was dissolved in 2 ml of acetic acid anhydride. Two drops of concentrated sulfuric acid were added. The solution was stirred continuously at room temperature for one hour. The reaction mixture was then poured on ice and stirred until the ice melted. A white precipitate was formed and then filtered, washed with cold water several times and dried in an oven at 90°C. The solid compound was triturated with hexane and resulted in pure compound. Due to insufficient yields, the reactions for compounds 1, 3 ,5 were repeated.

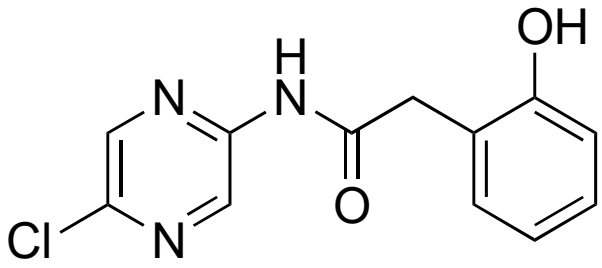


Scheme 5 – Acetylation of final compounds

9.3 Title Compounds

PzH Pyrazine hydrogen
PyH Pyridine hydrogen
ArH Aromatic hydrogen

***N*-(5-chloropyrazin-2-yl)-2-(2-hydroxyphenyl) acetamide**



Code (Lab code): 1 (AZV-JZ-4)

CAS#: 1986789-38-1

Chemical Formula: $C_{12}H_{10}ClN_3O_2$

Molecular weight: 263.68 g/mol

Yield: 6%

Appearance: dark yellow powder

m.p.: 158.8 – 162.2°C

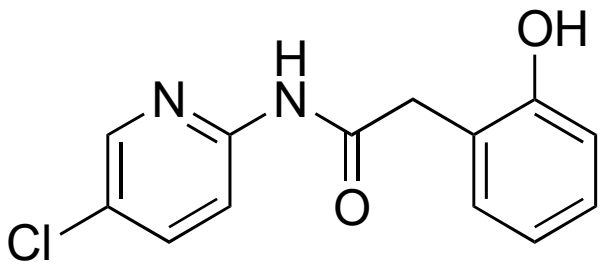
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 11.00 (s, 1H, CONH), 9.50 (s, 1H, OH), 9.13 (d, J = 1.4 Hz, 1H, PzH), 8.54 (d, J = 1.4 Hz, 1H, PzH), 7.16 – 7.12 (m, 1H, ArH), 7.05 – 7.09 (m, 1H, ArH), 6.82 – 6.78 (m, 1H, ArH), 6.73 – 6.77 (m, 1H, ArH), 3.72 (s, 2H, CH_2).

^{13}C NMR: ^{13}C NMR (151 MHz, DMSO-D_6) δ 171.22, 155.87, 148.41, 142.68, 141.75, 135.44, 131.75, 128.48, 122.34, 119.37, 115.37, 38.04.

Elemental Composition (%): C, 54.66; H, 3.82; Cl, 13.44; N, 15.94; O, 12.14

IR: 3308 ν(N-H, CONH), 3095 ν(O-H, OH), 1675 ν(C=O, CONH), 1545, 1515, 1503, 1451, 1340, 1127, 1036, 752.

***N*-(5-chloropyridin-2-yl)-2-(2-hydroxyphenyl)acetamide**



Code (Lab code): 2 (AZV-JZ-16)

CAS#: 1522773-71-2

Chemical Formula: $C_{13}H_{11}ClN_2O_2$

Molecular weight: 262.69 g/mol

Yield: 4%

Appearance: light yellow powder

m.p.: n/a

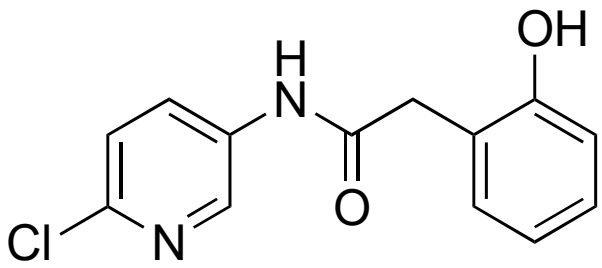
^{1}H NMR: ^{1}H NMR (500 MHz, DMSO-D_6) δ 10.59 (s, 1H, CONH), 9.53 (s, 1H, OH), 8.35 (dd, *J* = 2.7, 0.7 Hz, 1H, PyH), 8.09 (dd, *J* = 8.9, 0.7 Hz, 1H, PyH), 7.87 (dd, *J* = 8.9, 2.7 Hz, 1H, PyH), 7.15 – 7.11 (m, 1H, ArH), 7.05 – 7.09 (m, 1H, ArH), 6.82 – 6.78 (m, 1H, ArH), 6.72 – 6.76 (m, 1H, ArH), 3.68 (s, 2H, CH_2).

^{13}C NMR: n/a

Elemental Composition (%): C, 59.44; H, 4.22; Cl, 13.49; N, 10.66; O, 12.18

IR: n/a

***N*-(6-chloropyridin-3-yl)-2-(2-hydroxyphenyl)acetamide**



Code (Lab code): 3 (AZV-JZ-18)

CAS#: 1920554-27-3

Chemical Formula: $C_{13}H_{11}ClN_2O_2$

Molecular weight: 262.69 g/mol

Yield: 28%

Appearance: off-white powder

m.p.: 184.2 – 185.0 °C

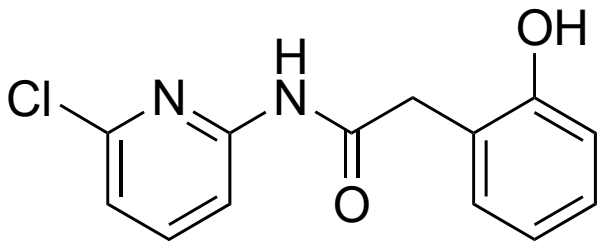
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 10.41 (s, 1H, CONH), 9.48 (s, 1H, OH), 8.62 (dd, *J* = 2.8, 0.7 Hz, 1H, PyH), 8.09 (dd, *J* = 8.7, 2.8 Hz, 1H, PyH), 7.45 (dd, *J* = 8.7, 0.6 Hz, 1H, PyH), 7.16 – 7.12 (m, 1H, ArH), 7.09 – 7.05 (m, 1H, ArH), 6.82 – 6.78 (m, 1H, ArH), 6.77 – 6.72 (m, 1H, ArH), 3.63 (s, 2H, CH_2).

^{13}C NMR: ^{13}C NMR (151 MHz, DMSO-D_6) δ 170.72, 155.87, 143.83, 140.73, 136.19, 131.59, 130.07, 128.39, 124.71, 122.53, 119.34, 115.36, 38.24.

Elemental Composition (%): C, 59.44; H, 4.22; Cl, 13.49; N, 10.66; O, 12.18

IR: 3239 ν(N-H, CONH), 3107 ν(O-H, OH), 1660 ν(C=O, CONH), 1536, 1467, 1371, 1274, 838, 751, 733.

***N*-(6-chloropyridin-2-yl)-2-(2-hydroxyphenyl)acetamide**



Code (Lab code): 4 (AZV-JZ-20)

CAS#: 1913392-54-7

Chemical Formula: $C_{13}H_{11}ClN_2O_2$

Molecular weight: 262.69 g/mol

Yield: 6%

Appearance: white powder

m.p.: 159.1 – 162.0 °C

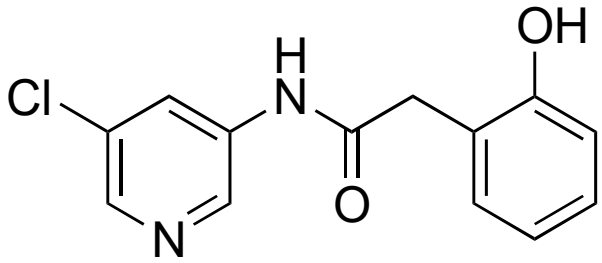
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 9.76 (s, 1H, CONH), 9.46 (s, 1H, OH), 8.28 (dd, *J* = 8.1, 1.8 Hz, 1H, PyH), 8.11 (dd, *J* = 4.6, 1.8 Hz, 1H, PyH), 7.37 (dd, *J* = 8.1, 4.6 Hz, 1H, PyH), 7.17 – 7.13 (m, 1H, ArH), 7.09 – 7.03 (m, 1H), 6.84 – 6.80 (m, 1H, ArH), 6.77 – 6.71 (m, 1H, ArH), 3.67 (s, 2H, CH_2).

^{13}C NMR: ^{13}C NMR (151 MHz, DMSO-D_6) δ 170.82, 155.66, 145.13, 142.10, 132.66, 132.59, 131.51, 128.70, 123.99, 122.25, 119.65, 115.52, 38.72.

Elemental Composition (%): C, 59.44; H, 4.22; Cl, 13.49; N, 10.66; O, 12.18

IR: 3330 v (N-H, CONH), 3098 v (O-H, OH), 1671 v (C=O, CONH), 1572, 1538, 1436, 1396, 1139, 797, 754.

***N*-(5-chloropyridin-3-yl)-2-(2-hydroxyphenyl)acetamide**



Code (Lab code): 5 (AZV-JZ-22)

CAS#: N/A

Chemical Formula: $C_{13}H_{11}ClN_2O_2$

Molecular weight: 262.69 g/mol

Yield: 16%

Appearance: red powder

m.p.: 158.7 – 163.5 °C

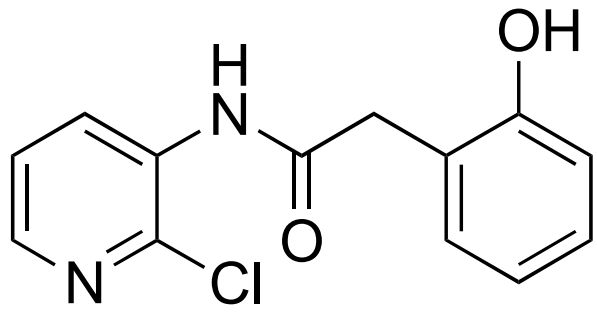
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 10.49 (s, 1H, CONH), 9.49 (s, 1H, OH), 8.65 (d, J = 2.1 Hz, 1H, PzH), 8.29 (d, J = 2.2 Hz, 1H, PzH), 8.26 (t, J = 2.2 Hz, 1H, PzH), 7.16 – 7.12 (m, 1H, ArH), 7.10 – 7.05 (m, 1H, ArH), 6.82 – 6.78 (m, 1H, ArH), 6.77 – 6.73 (m, 1H, ArH), 3.64 (s, 2H, CH_2).

^{13}C NMR: ^{13}C NMR (151 MHz, DMSO-D_6) δ 171.10, 155.87, 142.57, 139.28, 137.44, 131.59, 131.13, 128.43, 125.51, 122.42, 119.35, 115.35, 38.31.

Elemental Composition (%): C, 59.44; H, 4.22; Cl, 13.49; N, 10.66; O, 12.18

IR: 3312 ν(N-H, CONH), 3034 ν(O-H, OH), 1694 ν(C=O, CONH), 1606, 1581, 1459, 1340, 1120, 749, 665.

***N*-(2-chloropyridin-3-yl)-2-(2-hydroxyphenyl)acetamide**



Code (Lab code): 6 (AZV-JZ-34)

CAS#: 1911317-22-0

Chemical Formula: $C_{13}H_{11}ClN_2O_2$

Molecular weight: 262.69 g/mol

Yield: 10%

Appearance: dark yellow powder

m.p.: 199.0 – 201.2 °C

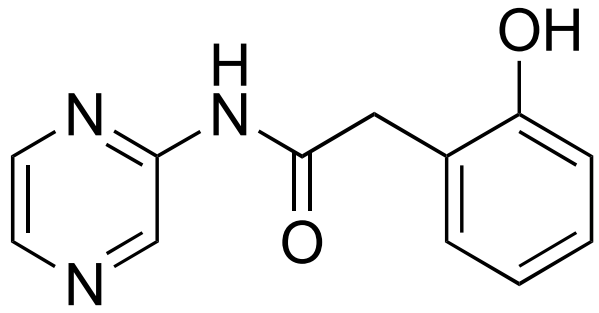
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 9.76 (s, 1H, CONH), 9.46 (s, 1H, OH), 8.28 (dd, *J* = 8.1, 1.8 Hz, 1H, PyH), 8.11 (dd, *J* = 4.6, 1.8 Hz, 1H, PyH), 7.37 (dd, *J* = 8.1, 4.6 Hz, 1H, PyH), 7.17 – 7.13 (m, 1H, ArH), 7.09 – 7.03 (m, 1H, ArH), 6.84 – 6.80 (m, 1H, ArH), 6.77 – 6.71 (m, 1H, ArH), 3.67 (s, 2H, CH_2).

^{13}C NMR: 13C NMR (151 MHz, DMSO-D6) δ 170.82, 155.66, 145.13, 142.10, 132.66, 132.59, 131.51, 128.70, 123.99, 122.25, 119.65, 115.52, 38.72.

Elemental Composition (%): C, 59.44; H, 4.22; Cl, 13.49; N, 10.66; O, 12.18

IR: 3326 ν(N-H, CONH), 3098 ν(O-H, OH), 1711 ν(C=O, CONH), 1558, 1516, 1504, 1394, 1297, 756.

2-(2-hydroxyphenyl)-*N*-(pyrazin-2-yl)acetamide



Code (Lab code): 7 (AZV-JZ-42)

CAS#: 1513476-22-6

Chemical Formula: $C_{12}H_{11}N_3O_2$

Molecular weight: 229.24 g/mol

Yield: 5%

Appearance: yellow powder

m.p.: 147.8 – 153.1 °C

^{1}H NMR: N/A

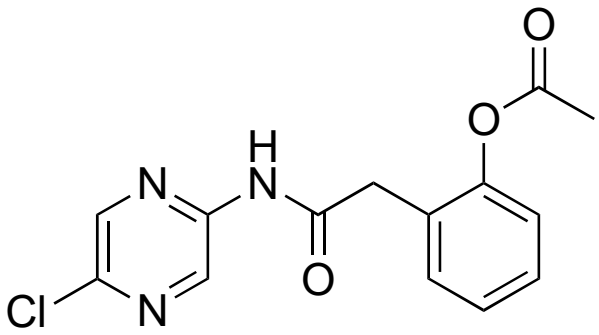
^{13}C NMR: N/A

Elemental Composition (%): C, 62.87; H, 4.84; N, 18.33; O, 13.96

IR: N/A

NB: this compound lacked sufficient purity and is kept only for comparison.

2-(2-((5-chloropyrazin-2-yl)amino)-2-oxoethyl)phenyl acetate



Code (Lab code): 8 (AZV-JZ-A4)

CAS#: N/A

Chemical Formula: $C_{14}H_{12}ClN_3O_3$

Molecular weight: 305.72 g/mol

Yield: 62%

Appearance: yellow powder

m.p.: 100.0 – 104.0 °C

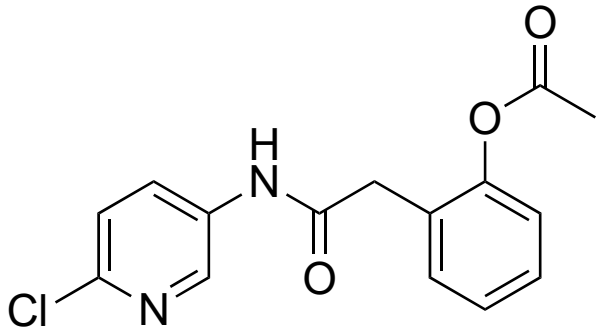
^{1}H NMR: ^{1}H NMR (600 MHz, ACETONE-D_6) δ 9.91 (s, 1H, CONH), 9.22 (d, J = 1.4 Hz, 1H, PzH), 8.37 (d, J = 1.4 Hz, 1H, PzH), 7.51 – 7.45 (m, 1H, ArH), 7.37 – 7.33 (m, 1H, ArH), 7.28 – 7.24 (m, 1H, ArH), 7.17 – 7.13 (m, 1H, ArH), 3.85 (s, 2H, CH_2), 2.27 (s, 3H, CH_3).

^{13}C NMR: N/A

Elemental Composition (%): C, 55.00; H, 3.96; Cl, 11.60; N, 13.75; O, 15.70

IR: N/A

2-(2-((6-chloropyridin-3-yl)amino)-2-oxoethyl)phenyl acetate



Code (Lab code): 9 (AZV-JZ-A18)

CAS#: N/A

Chemical Formula: $C_{15}H_{13}ClN_2O_3$

Molecular weight: 304.73 g/mol

Yield: 74%

Appearance: white powder

m.p.: 184.2 – 185.0 °C

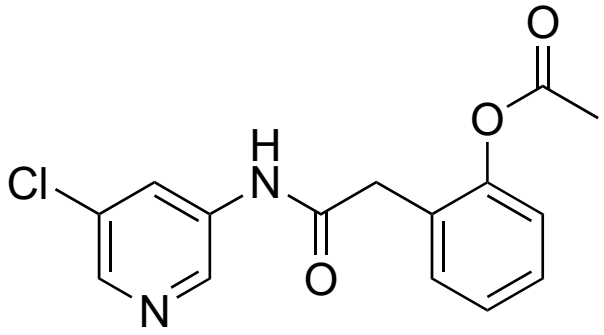
^{1}H NMR: ^{1}H NMR (600 MHz, DMSO-D_6) δ 10.50 (s, 1H, CONH), 8.59 (d, J = 2.8 Hz, 1H, PyH), 8.07 (dd, J = 8.7, 2.8 Hz, 1H, PyH), 7.47 (d, J = 8.6 Hz, 1H, PyH), 7.42 – 7.38 (m, 1H, ArH), 7.35 – 7.29 (m, 1H, ArH), 7.26 – 7.21 (m, 1H, ArH), 7.13 – 7.09 (m, 1H, ArH), 3.64 (s, 2H, CH_2), 2.23 (s, 3H, CH_3).

^{13}C NMR: ^{13}C NMR (151 MHz, DMSO-D_6) δ 169.48, 149.57, 144.14, 140.84, 135.91, 132.14, 130.25, 128.59, 128.47, 126.36, 124.80, 123.10, 38.42, 21.22.

Elemental Composition (%): C, 59.12; H, 4.30; Cl, 11.63; N, 9.19; O, 15.75

IR: 3348 ν(N-H, CONH), 1742 ν(C=O, CONH), 1683 ν(C=O, OAc), 1529, 1458, 1216, 1180, 750, 716, 628

2-(2-((5-chloropyridin-3-yl)amino)-2-oxoethyl)phenyl acetate



Code (Lab code): 10 (AZV-JZ-A22)

CAS#: N/A

Chemical Formula: $C_{15}H_{13}ClN_2O_3$

Molecular weight: 304.73 g/mol

Yield: 87%

Appearance: white powder

m.p.: 121.0 - 122.5 °C

^{1}H NMR: ^{1}H NMR (500 MHz, DMSO-D_6) δ 10.60 (s, 1H, CONH), 8.62 (d, *J* = 2.2 Hz, 1H, PyH), 8.32 (d, *J* = 2.2 Hz, 1H, PyH), 8.22 (t, *J* = 2.2 Hz, 1H, PyH), 7.43 – 7.37 (m, 1H, ArH), 7.35 – 7.28 (m, 1H, ArH), 7.27 – 7.20 (m, 1H, ArH), 7.14 – 7.08 (m, 1H, ArH), 3.65 (s, 2H, CH_2), 2.23 (s, 3H, CH_3).

^{13}C NMR: n/a

Elemental Composition (%): C, 59.12; H, 4.30; Cl, 11.63; N, 9.19; O, 15.75

IR: 3349 ν(N-H, CONH), 1737 ν(C=O, CONH), 1671 ν(C=O, OAc), 1520, 1455, 1219, 1180, 748.

9.4 In silico prediction of protonation and 3D structure of final compounds

All structures underwent molecular modelling testing to test their conformational properties. The compounds were imported to the MOE software from the respective SMILEs codes. The compounds were analyzed for the most probable protomers at pH 7.4 and subsequently minimized using the mmff94x force field. The operations were performed using the built-in utility called Wash with default settings (Figure 14).

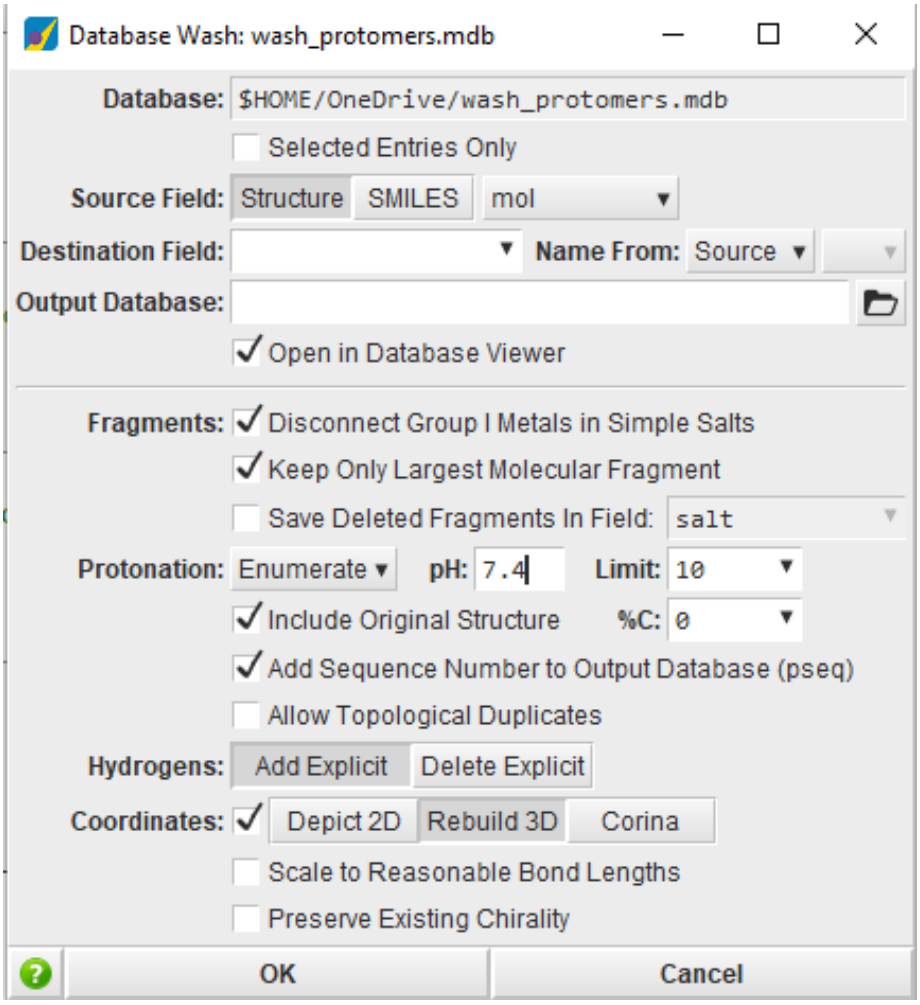


Figure 14- Wash settings and Protomers settings

The result of the protonation test at pH 7.4 indicated a similar result for all compounds – the uncharged protomer (phenolic OH not deprotonated, optionally pyridine nitrogen not protonated) had an abundance > 98%. The LowMD protocol as implemented in MOE was used to perform a search of the conformational space of the compounds. Within the default + 7 kcal/mol energy window, the conformers did not exert significant intramolecular H-bonds.

9.5 Biological Activity Assay

The biological activity tests were run in the Department of Biological and Medical Sciences by Dr. Konečná, Dr. Janďourek and Dr. Paterová to determine the compounds' potential antimycobacterial, antibacterial or antifungal activity.

In the **antimycobacterial** assay, five trains of mycobacteria were tested and are *Mycolicibacterium smegmatis* DSM 43465 (ATCC 607), *Mycolicibacterium aurum* DSM 43999 (ATCC 23366), *Mycobacterium avium* DSM 44156 (ATCC 25291), *Mycobacterium kansasii* DSM 44162 (ATCC 12478) from German collection of Microorganisms and Cell Cultures (Braunshweig, Germany), and avirulent strain of *Mycobacterium tuberculosis* H37Ra ITM-M006710 (ATCC 9431) from Belgian coordinated Collections of Microoragnisms (Antwerp, Belgium). The technique used was binary microdilution using 96-well microtitration plates in a culture medium of Middlebrook 7H9 broth (Merck, Darmstadt, Germany) enriched with 0.5% glycerol and 10% Middlebrook OADC growth supplement (Merck, Darmstadt, Germany). The mycobacterial strains were cultivated on the supplemented Middlebrook 7H9 agar (Himedia, Mumbai, India).

The suspensions prepared in the supplemented broth were adjusted to 1.0 on the McFarland scale. They were diluted in the ratio of either 1:20 – for rapid growth mycobacteria – or 1:10 – for slow growth mycobacteria – with broth.

The tested compounds were dissolved in DMSO (Merck, Darmstadt, Germany), and then Middlebrook broth was added to obtain a concentration of 2000 µg/ml. the standard used for activity determination was isoniazid (INH) (Merck, Damstadt, Germany). Final concentrations were reached by binary dilution and addition of mycobacterial suspension and were set as 500, 250, 125, 62.5, 31.25, 15.625, 7.81, and 3.91 µg/ml. isoniazid was diluted to the range 500-3.81 µg/mL for screening against rapidly growing mycobacteria, 2000-15.625 µg/mL for *M. avium*, 50-0.39 µg/mL for *M. kansasii*, and 1-0.0078 µg/mL for Mtb. The final concentration of DMSO did not exceed 2.5% (v/v) and did not affect the growth of all strains. Positive (broth, DMSO, bacteria) and negative (broth, DMSO) growth controls were included.

The antimycobacterial activity was expressed as minimum inhibition (MIC) determined based on the stain colour change (blue colour – no growth, active compound/concentration; pink

colour – positive growth inactive compound/concentration) all experiments were conducted in duplicates.

For the **antibacterial** activity, the assessment was done using microdilution broth method following the guidelines provided by EUCAST (The European Committee on Antimicrobial Susceptibility Testing). Eight strains were acquired from either the Czech Collection of Microorganisms (CCM, Brno, Czechia) or the German Collection of Microorganisms and Cell Culture (DSM, Braunschweig, Germany). The included strains are *Staphylococcus aureus* subsp. *aureus* CCM 4223 (ATCC 29213), *Staphylococcus aureus* subsp. *aureus* methicillin-resistant (MRSA) CCM 4750 (ATCC 43300), Staphylococcus epidermidis CCM 4418 (ATCC 12228), *Enterococcus faecalis* CCM 4224 (ATCC 29212), *Escherichia coli* CCM 3954 (ATCC 25922), *Klebsiella pneumoniae* CCM 4415 (ATCC 10031), *Acinetobacter baumannii* DSM 30007, ATCC 19606, *Pseudomonas aeruginosa* CCM 3955 (ATCC 27853).

They were prepared in a Cation-adjusted Mueller-Hinton broth (CAMHB, M-H2, Merck) at 35°±2°C. They were dissolved in DMSO (Merck, USA) for stock solutions. The final concentration did not exceed 1% (v/v) and did not affect the growth of the bacteria.

Positive controls were conducted on the test microbes alone while negative controls were focused on the cultivating medium and DMSO.

Antibacterial activity was assessed as the Minimum Inhibitory Concentration (MIC) after 24 and 48 hours of static incubation in a dark and humidified environment at 35°±2°C. Endpoint evaluations were done using visual methods and spectrophotometry. Internal control quality standards were also included in the assay for MIC determination and included gentamicin and ciprofloxacin.

For the Antifungal assays, they were conducted using the microdilution method according to EUCAST recommendation with slight alteration. 4 yeast and 4 mold strains were selected for the evaluation and were *Candida albicans* CCM 8320 (ATCC 24433), *Candida krusei* CCM 8271 (ATCC 6258), *Candida parapsilosis* CCM 8260 (ATCC 22019), *Candida tropicalis* CCM 8264 (ATCC 750), *Aspergillus fumigatus* ATCC 204305, *Aspergillus flavus* CCM 8363, *Lichtheimia corymbifera* CCM 8077, and *Trichophyton interdigitale* CCM 8377 (ATCC 9533). They were acquired from either the Czech Collection of Microorganisms (CCM, Brno, Czechia) or from

the American Type collection cultures (ATCC, Manassas, VA, USA). The selected strains were dissolved in DMSO and subjected to two-fold Dilution with RPMI 1640 medium which was supplemented with glutamine and 2% glucose. They were buffered to pH 7.0 with MOPS (3-morpholinopropane-1-sulfonic acid). The DMSO concentration in the testing medium did not exceed 1% (v/v) of total composition. The incubation was set statically in the dark under humid condition at 35°±2°C for 24 and 48 hours (72 or 120 hours for *Trichophyton Interdigitale* respectively).

Positive control consisted of microbe evaluation alone and negative controls consisted for cultivation medium and DMSO. The MIC end point evaluation was done through visual methods and spectrophotometry. Additionally, internal quality standards, amphotericin B (Merck), and voriconazole (Toronto Research Chemicals, Inc., Toronto, ON, Canada) were included in the assays for comparative purpose.

Antifungal assays showed no activity from any of the tested compounds at all tested concentrations. The compounds have no activity on tested fungal strains.

Table II – Results of Antibacterial activity assay expressed as MIC in µM.*

Antibacterial activity expressed as MIC in µM.																
Code	*S. aureus subs. aureus*		*S. aureus subsp. aureus* methicillin-resistant		*S. epidermidis*		*E. faecalis*		*E. coli*		*K. pneumoniae*		*A. baumannii*		*P. aeruginosa*	
	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge
1	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
2	250	≥ 500	500	≥ 500	500	≥ 500	500	≥ 500	>500	≥ 500	>500	≥ 500	>500	≥ 500	>500	≥ 500
3	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
4	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
5	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
8	≥500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
9	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
10	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500	≥ 500
CIP	1.252		0.625		1.252		10.008		0.039		0.039		2.502		2.502	
Gentamicin	2.094		16.94		0.131		16.94		2.094		1.046		16.751		1.046	

* NB: compounds 6 and 7 are still undergoing testing. Values for lower homologues without the methylen bridge were taken from[57]

Table III – Results of Antimycobacterial activity assay expressed as MIC in µg/ml.

Antimycobacterial activity expressed as MIC in µg/ml										
Code	*Mycobacterium tuberculosis* H37Ra		*Mycobacterium kansasii*		*M. avium*		*Mycobacterium aurum*		*M. smegmatis*	
	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge	without bridge	with bridge
1	≥500	**125**	**15.625**	**15.625**	**15.625**	**31.25**	**125**	**125**	≥500	**250**
2	**31.25**	≥ 500	**15.625**	**7.81**	**15.625**	**31.25**	**31.25**	≥ 500	**31.25**	≥ 500
3	**62.5**	n/a	**15.625**	n/a	**31.25**	n/a	125	n/a	≥500	n/a
4	≥500	≥ 500	**31.25**	**15.625**	**31.25**	**250**	≥500	**500**	**125**	≥ 500
5	**62.5**	**125**	**15.625**	**62.5**	**15.625**	**62.5**	**125**	**250**	**125**	**250**
6	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
7	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a
8	250	250	**62.5**	**15.625**	125	**62.5**	500	250	≥500	250
9	**31.25**	125	**15.625**	**31.25**	**31.25**	250	**62.5**	500	**62.5**	≥ 500
10	**62.5**	250	**15.625**	125	**125**	125	**125**	500	**125**	≥ 500
INH	15.625-31.25		1.95-3.91		500-1000		3.125-6.25		0.125-0.25	

NB: compounds 6 and 7 are still undergoing testing. Values for lower homologues without the methylen bridge were taken from[57]

10 Discussion

10.1 Intended compounds

In the previous study of the lower homologues related to our current series, the acetoxy derivatives tended to be of higher antimicrobial activity compared to hydroxy derivatives[57]. We intended at first to synthesize acetoxy compounds, the synthesis of compounds with acetoxy group was needed and three final compound (compounds 1,3,5) were acetylated to produce acetoxy derivatives (compounds 8, 9, 10, respectively). Other compounds will be prepared in the future. Surprisingly, we did not observed problems with steric hindrance and instability of the final product after the acetylation step.

10.2 Synthesis

Methods 1-3 focused on preparing the 2-acetoxyphenyl acetic acid intermediate by acetylating the hydroxy group on the ortho position. Such acetylation is a bit challenging because of steric hindrance in the structure. Even though we were successful to acquire the product with some of the methods, we were not able isolate it in decent purity. The issues could be with the instability of the final product on the silica gel and tailing of the product. There is possibility to order this protected starting material from some of the vendors, but the cheapest of them offers 1g for 739 USD. This also represents the difficulty of such protection (standard price for positional isomers is only fraction of ours).

Method 1, used 2-hydroxyphenyl acetic acid in a biphasic mixture of NaOH water solution, chloroform, and acetic anhydride, and was unsuccessful. Acetylation did take place, but the compound was lost in purification.

In **Method 2**, solvents were changed to a monophasic mixture of toluene and THF (4:1), base was changed for pyridine in a ratio of 4:1, This was done with the intention of optimizing the reacting conditions. However, it also did not work, and the method was abandoned.

Method 3, the acetylation was performed directly in acetic anhydride without any additional solvent and catalysed by concentrated sulfuric acid. The product was isolated but in low yields and purity. Since there was a strong catalyst multiple side reactions could have occurred. Concentrated sulfuric acid could have caused these side reactions and threw the reaction into a different direction – starting material was seen on the TLC plates. It could have ended in either incomplete reaction or inside reactions producing impurities. Therefore, we decided to change the reacting conditions once more.

We changed the scheme and work up of the reaction and went in a different direction. We decided to ditch the *O*-protected intermediate (2-acetoxyphenylacetic acid) – even though using it would mean higher yields – in comparison with unprotected 2-hydroxyphenylacetic acid. Instead, the final compounds were prepared directly by the reaction of unprotected 2-hydroxyphenylacetic acid and the aminopyrazine/aminopyridine together in one step.

Methods 4–6 were the reaction that would lead to the final compound.

Method 4, which used CDI as an activator for the starting 2-hydroxyphenylacetic acid. The reaction performed in DMSO proved successful. The product was observed on the TLC-MS. However, the work-up and purification failed to provide pure product. It could be due to some tracks of DMSO in the crude even after several washings with water. When this reaction is attempted with other solvents, the yield was even lower.

Method 5, which used activation of 2-hydroxyphenylacetic acid by thionyl chloride dissolved in acetonitrile, followed by coupling with the corresponding aminopyridine or aminopyrazine with DIPEA as a base. The method provided the intended final compounds but again in lower yields. We had initially used DCM as solvent but DCM was ultimately changed to acetonitrile because it has a higher boiling point and is more convenient for nucleophilic reactions especially for aminopyrazines (and is less toxic).

Method 6 was the method that provided enough yield, had better consistency and repeatability and purity. The low yields could be caused by side reactions of the activated acid and non-protected hydroxy group. We used DCM even though it is not the most optimal for

nucleophilic reactions. We also tried the reaction in acetonitrile (Method 5) due better suitability for nucleophilic reactions and higher boiling point. However, with acetonitrile, more side reactions appeared resulting in more difficult purification. Therefore, in method 6, we switched back to DCM.

10.3 Pharmacokinetic Analysis

We conducted a pharmacokinetic prediction analysis through SwissADME in order to predict what are the potential pharmacokinetic parameters of each compound. Seven compounds can cross the Blood Brain Barrier (BBB) which are compounds 2, 3, 4, 5, 6, 9 and 10 (). It is important to note that the presence and the position of the chloro substituent on the heterocycle seems to be one of the deciding factors on whether the compound can cross the BBB as evidenced by the SwissADME analysis. Another factor seems to be the number of nitrogen atoms on the heterocycle. Compound 8 has no chlorine atoms but has two nitrogen atoms on the heterocycle. Meanwhile, compound 1 has a chlorine atom and two nitrogen atoms. The lipophilicity is greatly decreased by the presence of one extra nitrogen and the lack of a chlorine atom. The acetoxy group also added lipophilicity and made the compounds much more likely to cross the BBB. However, the power of the nitrogen substituent can still change whether it crossed. Ass seen in molecule 8, it is not predicted to cross the BBB.

The analysis also predicted that none of the compounds capable of crossing the BBB will be effluxed out of the CNS by PGP. All compounds fit the Lipniski, Ghose, Veber, Egan, Mueggue drug-likeness criteria. All compounds have high gastrointestinal absorption and have a bioavailability score of 0.55, which indicates that all the compounds are viable for per oral administration.

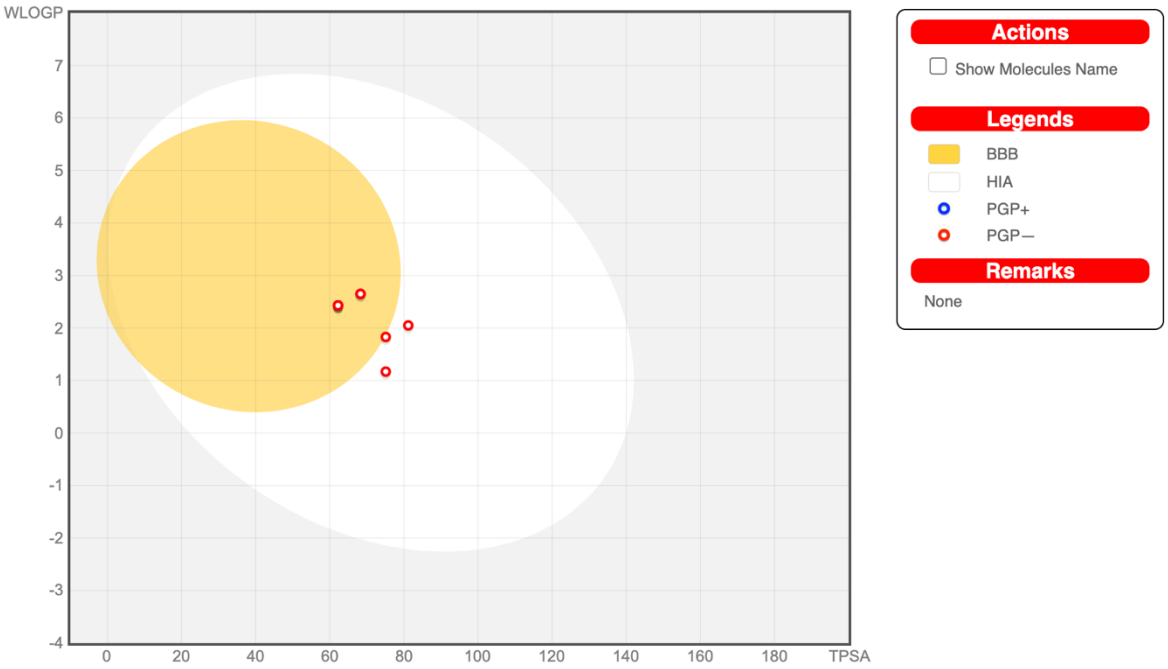


Figure 15- -BOILED-egg analysis from SwissADME - taken from: swissadme.ch. TPSA – Topological Polar Surface Area, WLOGP – Water and LogP, BBB – Blood Brain Barrier, HIA – Human Intestinal Absorption, PGP+ - compounds effluxed by P-glycoprotein, PGP- - compounds not effluxed by P-glycoprotein. NB - there are seven compounds overlap under the same point because they have the same WLOGP and TPSA value.

All tested compounds indicated no alerts when tested for Pan Assay Interference Structures (PAINS). PAINS are substructures capable of interfering with various biological assays which lead to false positives. Therefore, no alerts are a positive sign that the compounds tested active by their own design and not from substructures.

A more important note from the Pharmaceutical Chemistry analysis on SwissADME is the synthetic accessibility score. The range does not go above 2.43, indicating that all tested compounds can be synthesized.

Moreover, the pharmacokinetic analysis also predicted that all compounds have the potential to inhibit CYP1A2 while molecules 8, 9 and 10 have potential to inhibit isoenzyme CYP2C19 as well.

10.4 Biological Activity Assays

We theorized that adding a methylene bridge would increase conformational flexibility of the compound and provide increased activity. This increased activity could have been characterized as either a new avenue of activity on the receptor or the strengthening of

already existing activity. However, as the biological activity results showed, the reality did not match the expectation.

The biological activity of compounds with methylene bridges did not provide any improvement in the bacterial biological activity assay, seen in Table II. There was not any activity present on all tested strains. Compared to the control group using ciprofloxacin and gentamicin which had expected high results, the tested compounds did not show any activity at all. The antibacterial MIC for each tested compound > 500 µM, so even at the highest tested concentration there was no activity. Compounds 6 and 7 are in the process of testing. Overall, these results mean that the tested compounds do not possess a suitable mechanism of action to handle bacterial infections.

In the mycobacterial biological activity assay seen in Table III, both structures with and without the methylene bridge had activity on tested mycobacterial strains. In the case of Compound 1, it had higher biological activity – MIC 15.625 µg/ml – compared to its homologue without the methylene bridge on *M. kansasii* and Mtb H37Ra. Furthermore, Compound 2 also had higher biological activity on *M. kansasii* compared to its homologue without the bridge, having an MIC of 7.81 µg/ml. In general, however, the methylene bridge decreased the activity in total but there were 3 exceptions (compounds 1, and 2).

The mycobacterial strains most affected by the synthesized compounds were *M. kansasii* and *M. avium*. Specifically, *M. kansasii*, compound 2 had the most biological activity and had an MIC of 7.81 µg/ml. compounds 1 and 3 also had significant biological activity at MIC 15.625 µg/ml. For *M. avium* compounds 1 and 2 had good biological activity of MIC 31.25 µg/ml.

11 Conclusion

We prepared total of 10 derivatives of *N*-pyrazinyl- and *N*-pyridylhydroxybenzamides (6 with CAS number, 4 completely new). Even though some of the compounds have their CAS number, they are only listed in some vendor catalogues as possible to be prepared on request. None of the compounds have previously reported biological activity in the time of writing this thesis. The new compounds were properly reported with the analytical data. We performed prediction of pharmacokinetic properties and prediction of 3D structure The idea of the design and attempt to improve antimicrobial activity of the previously published compounds failed.. This development proved that the methylene bridge linker does not warrant further research.

Thankfully, as previously stated in this thesis, a strive is still being made to find a solution to antibiotic resistance. Many companies, research institutes and universities are pooling their resources to solve this problem. From the new drugs approved by the FDA and EMA to the new potential drugs currently in clinical trials, all the way down to discovery studies of potential new antimicrobials like this thesis aimed to do; a new horizon is dawning on antibiotic resistance research.

12 References

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