Nitrothiazole-thiazolidinone hybrids: Synthesis and *in vitro* antimicrobial evaluation

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PREFACE

This thesis is presented in article format in compliance with the North-West University's General Academic Rules (A.13.7.3).

Chapter 1: Introduction and problem statement

Chapter 2: Literature review

Chapter 3: Article

Nitrothiazole-thiazolidinone hybrids: Synthesis and *in vitro* antimicrobial evaluation

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Chapter 4: Summary and conclusion

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In honour of my grandmother, **Susan Hart**, I will continue to strive to make you proud.

ABSTRACT

Tuberculosis (TB), caused by the bacterium *Mycobacterium tuberculosis* (*Mtb*), is one of the world's most fatal infectious diseases. Annually, 10 million people fall ill with TB and 1.5 million people die from it globally. TB control and management solely rely on the use of drugs, most of which were developed 40–60 years ago. The emergence and spread of drug-resistant *Mtb* strains, as well as toxicity associated with these drugs create an irrefutable demand for novel drugs with potent activity against active and latent TB infections. Treatment can take up to 4–9 or 18–24 months with a cocktail of first- and second-line antitubercular drugs, depending on *Mtb* drug susceptibility. This often results in patients not complying with their antitubercular treatments, which is the major cause of treatment failure or relapse. Novel oral drugs that may shorten treatment regimens while still achieving complete *Mtb* clearance would have a positive influence on patient compliance, slowing the onset of disease as well as the evolution of drug-resistant *Mtb* strains.

In addition to *Mtb*, other pathogenic bacteria commonly referred to as ESKAPE pathogens are also currently seen as a global public health threat. In the last decade, the prevalence of antimicrobial-resistant (AMR) bacteria in hospitals has increased, as has the challenge of hospital-acquired infections (HAIs). The majority of HAIs include central line-associated bloodstream infections, catheter-associated urinary tract infections, surgical site infections, and ventilator-associated pneumonia, all of which are linked to a high prevalence of multidrug-resistant pathogens known as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa,* and *Enterobacter* spp.). In addition, ESKAPE pathogens are also wreaking havoc on many areas of individual patient healthcare, including increased direct health care expenses, increased length of hospitalisation, and higher fatality rates. The identification of new antimicrobial drugs with activity against drug-resistant ESKAPE pathogens is vital and urgent since treatment

options for them are rapidly becoming limited. Indeed, if no new antimicrobial is discovered promptly, it is predicted that no drug will be available to treat AMR bacteria by 2050.

Nitazoxanide (NTZ), an antimicrobial agent harbouring a 5-nitrothiazole moiety, has been demonstrated to have activity against both replicating and non-replicating *Mtb* through a novel mechanism of action. Thiazolidin-4-one is another well-known privilege scaffold with possible substitution sites at the second, third, and fifth positions of its heterocyclic ring system. With this in mind, the molecular hybridisation strategy has been employed in this study as a rational design strategy for generating novel ligands or prototypes in which the pharmacophoric subunits in the molecular structure of NTZ and the biologically active thiazolidin-4-ones were merged to produce novel hybrid compounds with a possible poly-pharmacological mechanism of action.

In this study, 17 novel nitrothiazole-thiazolidinone hybrids were synthesised and evaluated *in vitro* against *Mtb* and certain ESKAPE pathogens. Compounds were also screened for antifungal activity against *Candida albicans*. The hybrids were synthesised using a multi-step synthetic protocol that includes *N*-acylation, dehydrative cyclization (*S*-alkylation and intermolecular cyclization), and Knoevenagel condensation. The condensation of different electron withdrawing and electron donating benzylidene moieties in the fifth position (C5) of the thiazolidin-4-one scaffold was used to investigate the structure-activity relationship of the hybrids. After purification, the hybrid-target compounds were synthesised in yields ranging from 26% to 69%. However, the poor solubility of the hybrid-target compounds in standard solvents such as dichloromethane, ethanol, or methanol was recognised as a limitation in this work, emphasising the need for optimisation in future research. Nuclear magnetic resonance (^{1}H and ^{13}C), high resolution mass spectrometry, and Fourier-transform infrared spectroscopy were used to characterise all synthesised hybrids, and the purity of the compounds was assessed using high performance liquid chromatography. Finally, the target compounds were

tested for overt cell toxicity against human embryonic kidney cells (HEK-293) and haemolysis against whole human red blood cells (RBCs).

All hybrid compounds displayed remarkable antitubercular activity of < 0.24 – 2.00 µM. Most compounds had low micromolar activity against *S. aureus* and *C. albicans*. Compound **3b**, in particular, demonstrated sub-micromolar activity against *Mtb* and *C. albicans* while also having superior activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (< 0.25 µg/ml) as compared to the reference drug vancomycin (1 µg/ml). Compound **3g** had activity against *C. albicans* (≤ 0.25 µg/ml) comparable to that of the drug fluconazole (0.12 µg/ml). The antimicrobial SAR reveals that *ortho* substitution of the C5-benzylidene moiety favours activity against *S. aureus*.

Keywords: Antimicrobial; 5-nitrothiazoles; Thiazolidin-4-ones; Molecular hybridization; ESKAPE pathogens

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LIST OF ABBREVIATIONS

µM	Micromolar
^{1}H NMR	Proton nuclear magnetic resonance
^{13}C NMR	Carbon nuclear magnetic resonance
ACP	Acyl carrier protein
AFB	Acid-fast bacilli
AM	Amikacin
AMB	Antimicrobial-resistant bacteria
APCI	Atmospheric pressure chemical ionization
ATP	Adenosine 5′-triphosphate
BBB	Blood-brain barrier
BCG	Bacillus Calmette-Guérin
BDQ	Bedaquiline
BSIs	Bloodstream infections
CAS	Casitone
CAMHB	Cation-Adjusted Mueller Hinton Broth
CAUTIs	Catheter-associated urinary tract infections
CC_{50}	Cytotoxic concentration (50%)
CDC	Centre for Disease Prevention and Control
CFU	Colony-forming units
CFZ	Clofazimine

CLABSIs	Central line-associated bloodstream infections
CNS	Central nervous system
Coa	Coagulase
CS	Cycloserine
CSF	Cerebrospinal fluid
CYP450	Cytochrome P450
(D_6)DMSO	Deuterated dimethyl sulfoxide
Da	Daltons
Ddn	Deazaflavin-dependent nitroreductase
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate synthase
DHPS	Dihydropteroate synthase
DLM	Delamanid
DLS	Damped least-squares
DMEM	Dulbecco's Modified Eagle Medium
DMF	Dimethylformamide
DNA	Deoxyribonucleic acid
DR-TB	Drug-resistant tuberculosis
DS-TB	Drug-susceptible tuberculosis
EDG	Electron donating group
ESBLs	Extended spectrum β-lactamases

ESKAPE	*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacte*r spp.
ESX	Type VII secretion system
ETH	Ethambutol
ETO	Ethionamide
EWG	Electron withdrawing group
FAS II	Fatty acid synthase II
FBP	Folate biosynthesis pathway
FBS	Foetal bovine serum
FDA	Food and drug administration
FLDs	First-line drugs
FQNs	Fluoroquinolones
FTIR	Fourier-transform infrared spectroscopy
GI	Gastrointestinal
HAIs	Hospital-acquired infections
HBA	Hydrogen bond acceptors
HBD	Hydrogen bond donors
HBHA	Heparin-binding hemagglutinin
HC_{10}	Haemolysis concentration (10%)
HEK-293	Human embryonic kidney cells
HIV	Human immunodeficiency virus

HPLC	High-performance liquid chromatography
HRMS	High-resolution mass spectrometry
Hz	Hertz
ICU	Intensive care unit
IFN-γ	Interferon gamma
IGRAs	Interferon-γ-release assay
INH	Isoniazid
LAM	Lipoarabinomannan
LFX	Levofloxacin
LPAs	Line probe assays
LZD	Linezolid
mAGP	Mycolyl arabinogalactan–peptidoglycan
MDR-TB	Multidrug-resistant tuberculosis
MFX	Moxifloxacin
MH	Molecular hybridisation
MIC	Minimum inhibitory concentration
MOA	Mechanism of action
m.p.	Melting point
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
Mtb	*Mycobacterium tuberculosis*

MTZ	Metronidazole
MW	Molecular weight
NA	Nalidixic acid
NAATs	Nucleic acid amplification tests
NADH	Nicotinamide adenine dinucleotide
NO	Nitric oxide
NTZ	Nitazoxanide
PA	Pyrazinoic acid
PABA	*para*-Aminobenzoic acid
PAS	*para*-Aminosalicylic acid
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PFOR	Pyruvate-ferredoxin oxidoreductase
PPD	Purified protein derivative
ppm	Parts per million
PTM	Pretomanid
PTO	Prothionamide
PZA	Pyrazinamide
RBCs	Red blood cells
RIF	Rifampicin
RNA	Ribonucleic acid

RoB	Rotatable bonds
rRNA	Ribosomal ribonucleic acid
r.t.	Room temperature
SAR	Structure-activity relationship
SLDs	Second-line drugs
SM	Streptomycin
SSIs	Surgical site infections
$T_{1/2}$	Half-life
TB	Tuberculosis
TDR-TB	Total drug-resistant tuberculosis
TEA	Triethylamine
TIZ	Tizoxanide
TLC	Thin-layer chromatography
TNF-α	Tumour necrotic factor
*t*PSA	Topological polar surface area
TRD	Terizidone
tRNA	Transfer ribonucleic acid
TST	Tuberculin skin test
UTIs	Urinary tract infections
VAP	Ventilator-associated pneumonia
VISA	Vancomycin-intermediate *Staphylococcus aureus*

VRE	Vancomycin-resistant enterococci
VRSA	Vancomycin-resistant *Staphylococcus aureus*
vWbp	von Willebrand factor-binding protein
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis
YEPD	Yeast Extract Peptone Dextrose
ZN	Ziehl-Neelsen

CHAPTER 1: INTRODUCTION AND PROBLEM STATEMENT

1.1. Background

Before COVID-19, tuberculosis (TB) was the world's foremost infectious disease caused by a single pathogen. The COVID-19 pandemic seems to be erasing the advances made in the past 20 years toward eradicating TB (Togun *et al.*, 2020).

The COVID-19 pandemic has also increased awareness regarding the threat posed by infectious diseases, highlighting the silent epidemic of antimicrobial-resistant (AMR) bacteria. Antibiotics' diminishing efficacy against bacteria is jeopardising the practice of modern medicine. This is especially evident in infections caused by a group of pathogens collectively referred to as ESKAPE. ESKAPE pathogens are the major cause of hospital-acquired infections (HAIs) (infections acquired while receiving treatment in a healthcare facility) globally. In fact, antimicrobial resistance among these pathogens is wreaking havoc on global public health (Ayobami *et al*., 2022).

Therefore, there is a continuous demand for effective antimicrobial drugs for the prevention and treatment of infections such as TB and HAIs. This project intends to address this need by discovering and evaluating a novel antimicrobial drug having activity against ESKAPE pathogens as well as Mtb, as described in this report.

First, this chapter will provide introductory information on TB and HAIs, followed by a hypothesis, the rationale and strategy for the study, as well as its aims and objectives.

Chapter two provides a literature review of current research relevant to the study topic, while chapter three presents the results of the study in article format. The fourth chapter is a summary and conclusion.

1.2. Tuberculosis

The second leading cause of mortality worldwide from a single infectious pathogen is tuberculosis (TB), a clinically progressive chronic disease. *Mycobacterium tuberculosis* (Mtb), a pathogenic mycobacterium of the *Mycobacteriaceae* family that is thought to have originated in the ancient African human ancestors some three million years ago, is the culprit behind TB (Ernst *et al.*, 2007:1738). Despite the fact that TB can be prevented and treated for many of the most vulnerable populations, such as children, pregnant women, people living with human immunodeficiency virus (HIV), miners and medical professionals, the year 2020 witnessed little progress toward the elimination of TB. Public health services – notably TB prevention and control - were severely impacted by the COVID-19 pandemic. The worldwide decline in the number of TB diagnoses is the most obvious consequence. Evidence of such a significant decline includes limited access to healthcare, decreased desire and availability to seek treatment during lockdowns and activity restrictions, and the stigma associated with symptoms shared by TB and COVID-19 (WHO, 2021).

Latent TB and active TB need to be distinguished from one another in order to contextualise the transmission of TB. Latent TB is an asymptomatic stage of immune sensitisation to Mtb. Conversely, Mtb infections that progress to various life-threatening pulmonary or extrapulmonary clinical symptoms are what are known as "active TB" (CDC, 2014; Laycock *et al.*, 2021:2). One-third of the world's population, or over 2.3 billion people, have latent TB and act as a reservoir for reactivation into active TB at any given time (Houben & Dodd, 2016:3). It is unlikely that people with latent TB who do not progress to active TB would transmit Mtb to others. However, their lifetime chance of acquiring active TB is 10% (CDC, 2014). When people with active TB cough, sneeze or engage in other strong expiratory behaviours, they create Mtb-containing droplet nuclei (airborne particles) which can be transmitted and subsequently infect healthy people (Churchyard *et al.*, 2017:S630-S631). TB is the top risk factor for mortality among people who are HIV-positive. It is responsible for an estimated 26% of all HIV-related deaths, 99% of which occur in developing nations. Furthermore, in high-

burden countries like those in Africa, co-infection with HIV multiplies the chance of latent TB reactivation by 20. In fact, the two conditions amplify one another in the host, accelerating the loss of host immune functionalities and, if untreated, bringing about untimely death (Bruchfeld *et al.*, 2015:2; Pawlowski *et al.*, 2012:1).

1.3. The burden of treating tuberculosis

The first-line drugs (FLDs) used to treat drug-susceptible TB (DS-TB) include isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PZA) (see **Fig. 1.1**). Drug-resistant TB (DR-TB) is more challenging to treat despite the availability of these treatments due to Mtb's immense ability to develop resistance to them (Mehari *et al.*, 2019:2). DR-TB prevalence has exponentially increased and reached epidemic levels in recent years, killing more people than malaria and HIV combined (Moreira *et al.*, 2020:1). TB that is resistant to or does not react to at least two of the most potent antitubercular drugs, INH and RIF, is known as multidrug-resistant TB (MDR-TB). Extensively drug-resistant TB (XDR-TB), a subtype of MDR-TB, is sporadic and responds to even fewer treatment regimens. XDR-TB has evolved resistance to other antitubercular drugs known as second-line drugs (SLDs) in addition to FLDs (WHO, 2018).

A two-month intensive phase of INH, RIF, PZA, and ETH, followed by a four-month continuation phase of INH and RIF, continues to be the recommended treatment plan for TB infections. However, even following culture conversion, treatment for those with DR-TB on prolonged regimens may take up to 20 months with additional SLDs (Mabhula & Singh, 2019:1345-1346; Migliori *et al.*, 2020:S20). Patient compliance to antitubercular drugs is crucial for achieving successful treatment outcomes, limiting the spread and minimizing the emergence of DR-TB. Several variables are known to influence compliance with TB drugs, including socioeconomic considerations, complexity of regimens, and extended treatment duration (Bea *et al.*, 2021:2). Poor compliance with TB drugs is generally recognized as a source of increased morbidity, mortality, and financial burden. According to a worldwide meta-

analysis, patient non-compliance with antitubercular drugs is shown to be a major risk factor for MDR-TB. Moreover, MDR-TB patients usually have worse treatment outcomes than drug-susceptible patients (Pradipta *et al.*, 2020:1).

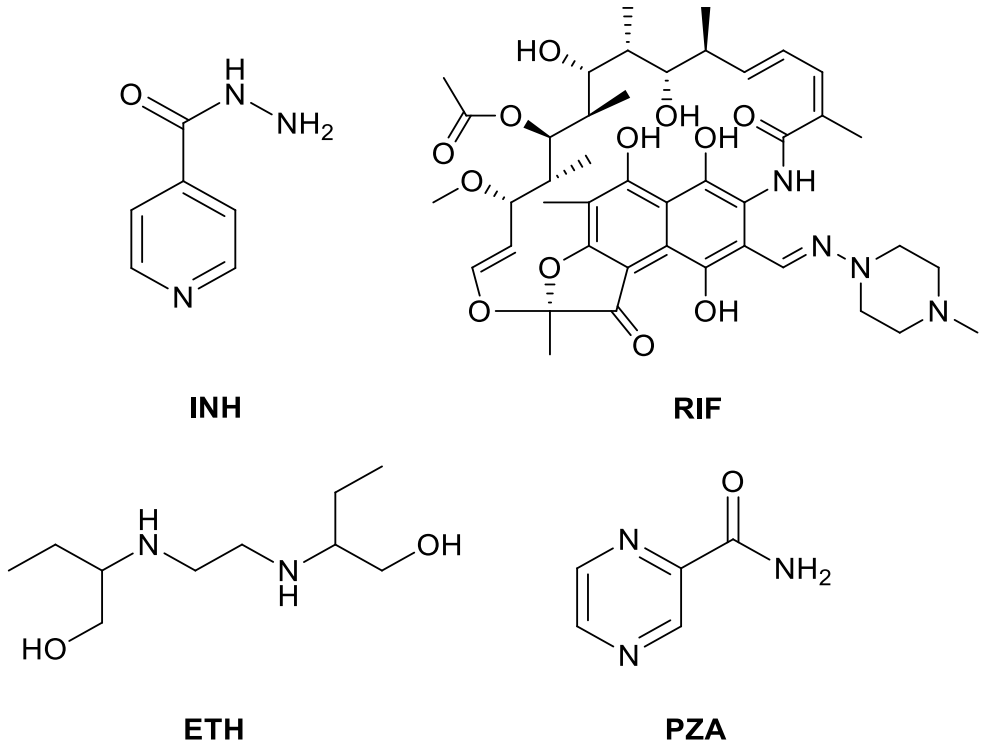


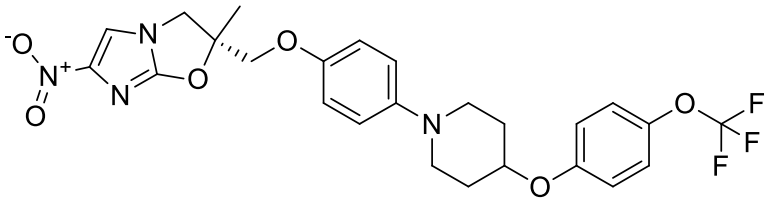
Figure 1.1 First-line antitubercular drugs

1.4. Advances in antitubercular treatment

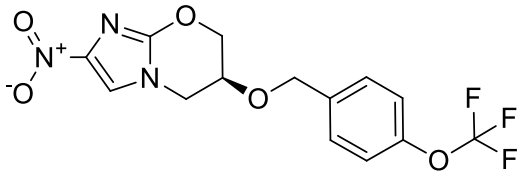
After more than four decades of rigorous study, bedaquiline (BDQ) and delamanid (DLM) have received accelerated or conditional regulatory approval based on the outcomes of their Phase II clinical trials against Mtb strains. The first innovative antitubercular drug with a novel mechanism of action (MOA) approved in 2012 to treat DR-TB was BDQ (Rodriguez *et al.*, 2019:32). Additionally, two novel nitroazole families of antibiotics have been approved for the treatment of DR-TB, including DLM and its structural analogue, pretomanid (PTM) (see **Fig. 1.2**). Recently, these two 5-nitroimidazole-based compounds have shown potent *in vivo* and *in vitro* activity against Mtb, particularly MDR- and XDR-TB (Wen *et al.*, 2019:1293). Furthermore, they have the potential to considerably shorten the course of extended MDR-

and XDR-TB regimens while also promoting patient treatment outcomes. However, mutations related to Mtb resistance to both DLM and PTM were soon discovered (Kadura *et al.*, 2020:2031).

Linezolid (LZD), an oxazolidinone that has been repurposed owing to its antimycobacterial properties, has also earned its position in the World Health Organization's (WHO) consolidated guidelines as a group A antitubercular drug for the treatment of MDR-TB and XDR-TB. However, a major drawback of adopting LZD as a treatment is the frequency of treatment discontinuation and serious adverse effects that study participants often experience (Padayatchi *et al.*, 2020:1024).



DLM



PTM

Figure 1.2 Delamanid (DLM) and pretomanid (PTM)

1.5. Antimicrobial resistance

The emergence and spread of AMR bacteria are a serious global public health problem. AMR bacteria, sometimes known as "superbugs," can thrive in the presence of antimicrobials, creating countless treatment problems as well as elevated morbidity and mortality rates in both hospitals and the community (Allcock *et al.*, 2017). AMR bacteria cause over two million infections and at least 23,000 deaths in the United States each year. This statistic is expected to rise by a factor of ten every year, with mortality estimates by 2050 varying by region (Ma *et*

al., 2020:1). Globally, antimicrobial resistance is now expected to cause over 700,000 deaths each year. Unless the resistance trend is reversed, this number may rise to 10 million by 2050, with more than 4 million deaths occurring each year in Africa and Asia alone (Xu *et al.*, 2019:3209).

The relationship between antibiotic use and antimicrobial resistance establishment complicates antimicrobial resistance even more and has long been linked to its development in individual patients. Antimicrobial resistance is primarily driven by selective pressures exerted on these pathogens due to long-term exposure to antimicrobials such as *β*-lactams, aminoglycosides, fluoroquinolones (FQNs) and macrolides. Bacteria can, however, adapt and become resistant to antimicrobials through a variety of other mechanisms, such as enzyme production and drug target site modification. Thus, resistant bacteria may possess one or more of these resistance mechanisms, resulting in antimicrobial resistance to more than one class of antibiotics (Allcock *et al.*, 2017; Cristino, 1999:199-202). Today, existing antibiotics are rendered ineffective as a result of these resistant infections.

1.6. Hospital-acquired infections (HAIs)

Infections that are not present or incubating at the time of hospitalisation but manifest at least 48 hours later in addition to the main reason for hospitalisation are referred to as HAIs (Viderman *et al.*, 2019:35). HAIs include respiratory tract infections, urinary tract infections (UTIs), infections related to the use of surgical equipment, as well as intravascular cannulas and catheters (Spelman, 2002:286-288). ***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa and* ***E****nterobacter spp*. are among a group of potentially fatal hospital-acquired bacteria known as ESKAPE pathogens that have the ability to "escape" the biocidal activity of currently used antimicrobial drugs. The greatest clinical threat currently comes from drug-resistant ESKAPE pathogens like methicillin-resistant *S. aureus* (MRSA), vancomycin-resistant *enterococci* (VRE), and other *β*-lactam resistant pathogens. These bacteria usually show novel

pathogenesis, transmission and resistance mechanisms, resulting in difficult-to-treat diseases (Navidinia *et al.*, 2017:779; Pandey *et al.*, 2021:2202). Treatment options for infections caused by these bacteria may become more problematic to manage in the future as long as the resistance patterns for these pathogens persist. As a result, it is critical to discover novel antimicrobials with activity against ESKAPE pathogens (De Oliveira *et al.*, 2020:2).

1.7. Hypothesis

HAIs are increasingly being associated with ESKAPE pathogens, which have developed several mechanisms to nullify the activity of all antimicrobials now in clinical use and are therefore resistant or not susceptible to routinely prescribed antibiotics such as FQNs and *β*-lactams. Furthermore, despite progress in reducing the global incidence of TB via DOTS (Directly Observed Treatment Shortcourse) (WHO, 1999) or antitubercular prevalence-and-surveillance studies, the rise of MDR- and XDR-TB during the past decade threatens to undo these gains (Zignol *et al.*, 2016:1086-1087). As a result, and considering the poor success rates of DR-TB, novel and innovative TB drugs to treat both drug-susceptible TB (DS-TB) and drug-resistant TB (DR-TB) are urgently required.

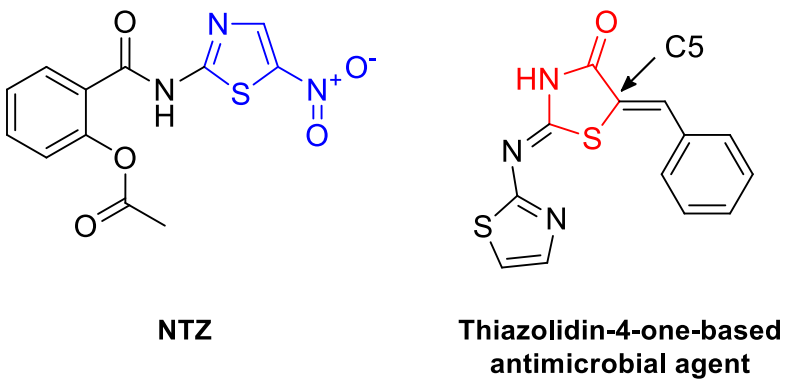
Herein, it is hypothesized that synthesizing poly-pharmacophoric ligands as potential drug leads for the treatment of both drug-susceptible and drug-resistant pathogens, such as Mtb and ESKAPE pathogens, will slow the global onset of drug resistance development. On the other hand, poly-pharmacophoric ligands act simultaneously on several microbiological targets, which should limit resistance development since concurrent mutation of multiple sites in bacteria is associated with a fitness cost.

1.8. Rationale and strategy

The 5-nitroimidazole-based drugs DLM and PTM, both of which are used to treat MDR-TB, were discovered and given the green light in recent years. The availability of these drugs has rekindled interest in nitroazole scaffolds as a source of potent antimicrobials. While the focus

is still primarily on nitrofurans and nitroimidazoles, such as metronidazole (MTZ), which are well-known as bio-reductive pro-drugs, other 5-membered nitroazole compounds with a 5-nitrothiazole moiety, such as nitazoxanide (NTZ) (see **Fig. 1.3**), have also been reported (Rice *et al*., 2021:2). NTZ exhibits promising antitubercular and antimicrobial activity in addition to having a MOA that differs from the conventional reductive activation of the 5-nitro group (like for MTZ), while simultaneously avoiding resistance from bacteria (De Carvalho et al., 2009:5789–5792). In addition, NTZ has the potential to shorten current TB regimens (Iacobino *et al.*, 2019:1-4).

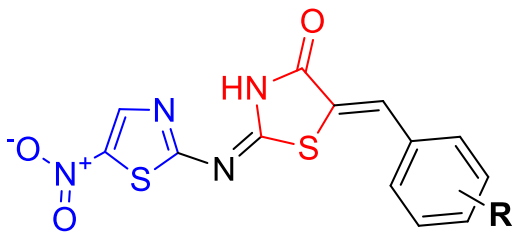
Another well-known class of heterocycles that are thought to offer promising structures as preferred scaffolds for the development of novel drugs are the thiazolidin-4-ones. Thiazolidin-4-one-based compounds exhibit a wide range of biological activities, and this class contains many known lead compounds, potential drug candidates, and commercially available drugs (Kaminskyy *et al.*, 2017:543; Mech *et al.*, 2021:1; Skóra *et al.*, 2022:1-2). Due to their immense chemical significance at their potential substitution sites, thiazolidin-4-ones are widely and successfully used in many drug development methodologies. For instance, among all thiazolidin-4-one subtypes, C5 substituted thiazolidin-4-ones, specifically C5-benzylidene derivatives (see **Fig. 1.3**), are of particular interest in terms of their chemical properties and antimicrobial activities (Kaminskyy *et al.*, 2017:554; Vicini *et al.*, 2006:3859; Vicini *et al.*, 2008:3714).



NTZ **Thiazolidin-4-one-based antimicrobial agent**

Figure 1.3 Nitazoxanide (NTZ) and thiazolidin-4-one antimicrobial agent.

The molecular hybridisation (MH) approach has produced chemical libraries based on privileged structures, with heterocyclic moieties receiving special attention due to their proven utility in medicinal chemistry. It is clear, for example, that nitrogen-containing heterocyclic moieties are present in more than 75% of drugs approved by the American Food and Drug Administration (FDA) and now available on the market. The presence of non-carbon atoms in heterocycles is responsible for the unique properties of this scaffold (Kerru *et al.*, 2020:1). As a result, we believe that drugs containing the 2-amino-5-nitrothiazole (5-nitrothiazole) head group from NTZ and the thiazolidin-4-one scaffold will be active against microorganisms via a poly-pharmacophoric mechanism. Given these considerations, and especially in light of the therapeutic potential of 5-nitrothiazoles and thiazolidin-4-ones, an attempt was made to integrate these moieties to generate novel hybrid compounds (see **Fig 1.4)** that are potentially active against Mtb as well as a number of Gram-positive and Gram-negative ESKAPE pathogens.



Hybrid compound

R = F, Cl, Br, NO_2, OH, Me, MeO

Figure 1.4 Design of hybrid-target compounds

1.9. Aims and objectives

This study aims to synthesise a series of a novel (*2E,5Z*)-5-benzylidene-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one derivatives and investigate their antimicrobial potential against some ESKAPE pathogens, *Mycobacterium tuberculosis* and the fungus *Candida albicans*.

Objectives:

- Synthesis of conceptualised target compounds.
- Characterization of intermediates and target compounds using analytical methods such as Fourier-transform infrared spectroscopy (FTIR), proton nuclear magnetic resonance (^{1}H NMR) spectroscopy, carbon nuclear magnetic resonance (^{13}C NMR) spectroscopy, high-resolution mass spectrometry (HRMS), and high-performance liquid chromatography (HPLC) analysis.
- *In vitro* antimicrobial activity evaluation of hybrid-target compounds.
- *In vitro* cytotoxic activity evaluation of hybrid-target compounds.
- Analyse the structure-activity relationship (SAR) of antimicrobial inhibition by various derivatives synthesized in this study.
- *In silico* drug-like and ADME predictions of hybrid-target compounds.

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CHAPTER 2: LITERATURE REVIEW

The literature review will be divided into two parts, with the first concentrating on tuberculosis (TB) and the second on hospital-acquired infections (HAIs), in order to gain a more in-depth understanding of current research relevant to the study topic.

PART ONE: TUBERCULOSIS (TB)

2.1. Background

The bacterium *Mycobacterium tuberculosis*, or Mtb, causes tuberculosis (TB), one of the oldest known human diseases that has co-evolved alongside human populations for many centuries. For example, evidence of skeletal TB (Pott's lesions) was discovered in ancient Egyptian mummy's spinal cord segments dated to 2400 B.C., indicating a definite pathological sign of TB deterioration up to this point (Barberis *et al.*, 2017:E9).

In 1679, Sylvius de la Boe, a Dutch researcher from Amsterdam, published the earliest anatomical and pathological descriptions of TB by recognising tubercles as a typical clinical manifestation in the lungs of Mtb-infected individuals. The first to identify TB as an infectious disease was Benjamin Martin. He also postulated that prolonged contact with a Mtb-infected individual was needed to acquire TB (Herzog, 1998:6-7). However, a French military physician named Jean-Antoine Villemin physically demonstrated the infectious nature of Mtb for the first time in 1865 and learned that it could be transmitted from animals to humans. At the time, the mode of transmission was unknown (Schluger, 2005:251). Later, in 1882, Robert Koch presented a unique staining method for visualizing and isolating the Mycobacterium responsible for TB. Subsequently, he also established the airborne method of transmission of Mtb. These key developments paved the way for scientists to conduct extensive research on Mtb in an attempt to eradicate the bacterium responsible for TB (Cambau & Drancourt, 2014:197-199).

Selman Waksman's discovery of streptomycin (SM) in the early 1940s set the groundwork for effective TB treatment, leading many healthcare experts to assume that TB would soon be eradicated (Zumla & Grange, 1999:104). However, its usage in extended treatment regimens proved challenging owing to the difficulties of administering SM orally and its toxicity profile. Additionally, it was also discovered that TB could not be treated with a single drug, due to the emergence of Mtb resistance as a result of ribosomal target site mutations (Prabhu & Singh, 2019:936).

TB has been known by multiple names throughout the years. Hippocrates, the father of medicine, who was fully aware of TB and its clinical manifestations, introduced the term "phthisis", which means "consumption" in Greek. The phrase "consumption," points to how TB seems to "devour or consume" the host through significant weight loss and "wasting away", primarily striking adults between the ages of 18 and 35 (Daniel, 2006:1863; Moonan, 2018:592). In the late 18th and early 19th centuries, TB spread over Europe and the United States, labelled as "The Great White Plague." It was dubbed "white" because of the extreme anaemia-related paleness of those affected. The World Health Organization (WHO) declared TB a global emergency because every year during this time, the mortality rate fluctuated from 800 to 1,000 per 100,000 Mtb infected individuals (Frith, 2014:32; Zumla *et al.*, 2009:197).

TB remains a public health concern even today, despite the production of newer antitubercular drugs, making it one of the leading causes of death among people in developing countries, especially those co-infected with the human immunodeficiency virus (HIV) (WHO, 2017a).

2.2. Epidemiology of TB

According to the WHO (2021a), approximately 10 million people worldwide contracted TB in 2020. Males accounted for 56% (5.6 million) of all TB infections, women bore 33% (3.3 million) of the TB burden, and children 11% (1.1 million). Africa, Southeast Asia and the Western Pacific had the most TB cases, followed by the Eastern Mediterranean, Europe and the

Americas. High-prevalence countries accounted for 86% of all TB cases. Eight countries accounted for two-thirds of the global TB burden: the Philippines (6%), India (26%), Pakistan (5.8%), China (8.5%), Bangladesh (3.6%), Indonesia (8.4%), Nigeria (4.6%) and South Africa (3.3%).

The COVID-19 pandemic has resulted in the first annual increase in TB deaths since 2005, mainly due to the limited access to TB diagnosis and treatment. The number of fatalities has increased from 1.2 million to 1.3 million, including 240,000 HIV-related deaths in 2020. The prevalence of multidrug-resistant TB (MDR-TB) is steadily increasing with the number of people treated for MDR-TB declining from 177,100 to 150,359 in 2020. It seems that just one in three people in need of MDR-TB therapy sought medical care. This 2020 results render TB a public health emergency and a global threat, making it the second most infectious killer after COVID-19 (WHO, 2021a).

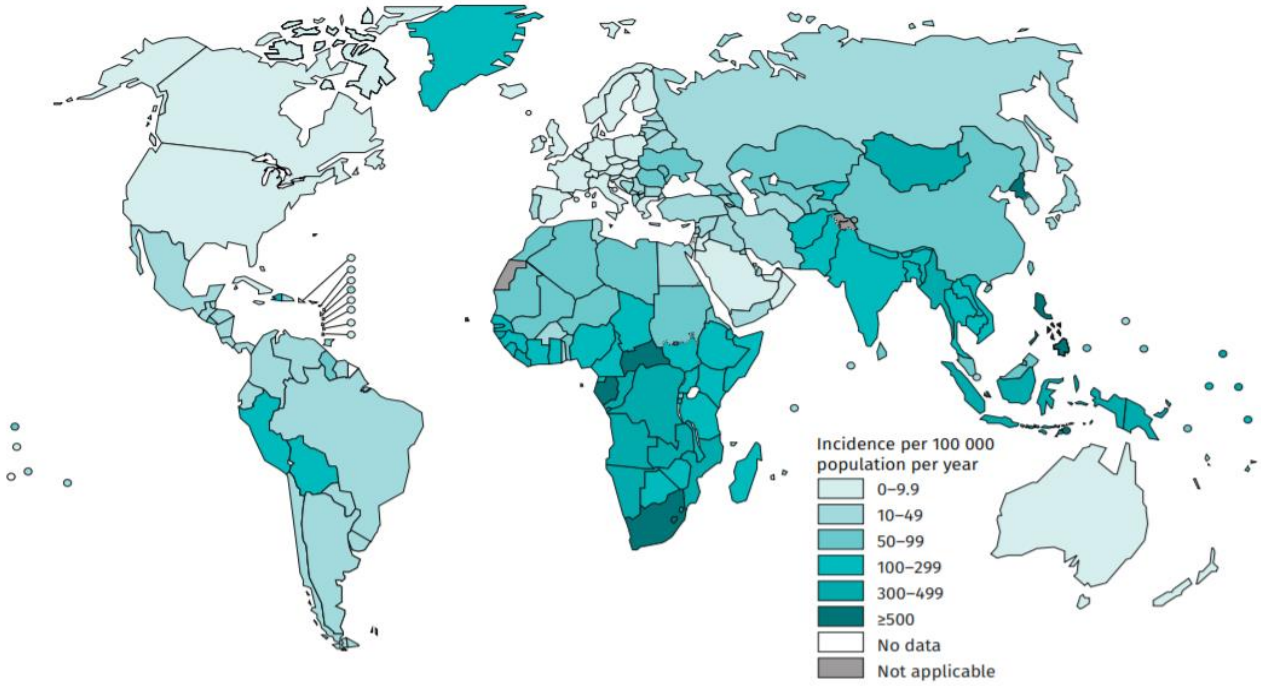

Figure 2.1 Tuberculosis incidence rates in 2020 (WHO, 2021a).

2.3. Mtb cellular morphology

Depending on the environmental circumstances, Mtb develops as long, straight or slightly thin, curved rods (1.0–10 µm long and 0.2–0.7 µm wide). Mtb does not produce spores and is normally aerobic to microaerophilic. Mtb grows slowly and create observable colonies in 2–60 days at ideal conditions (Ahamad *et al.*, 2022:3). Furthermore, it replicates at a far slower rate (16–20 hours) than other bacteria, which often divide in less than an hour (Sharma *et al.*, 2011:244).

Microscopy reveals that the cell wall of Mtb consists of an outer membrane similar to Gram-negative bacteria, packed in an asymmetrical lipid bilayer composed of glycolipids and waxy materials on the outside and long fatty acid chains on the inside (mycolic acids). The periplasmic space is made up of the inner and outer membranes. It is formed by peptidoglycan that is covalently bound to arabinogalactan and lipoarabinomannan (LAM), which are subsequently covalently bonded to mycolic acids. This forms the mycolyl arabinogalactan-peptidoglycan (mAGP) complex, or commonly termed, the cell wall nuclei (Alderwick *et al.*, 2015:1; Delogu *et al.*, 2013). The cell wall of Mtb is additionally surrounded by a mycobacterial capsule. The capsule contains a high concentration of neutral polysaccharides, lipids and proteins, which, along with glycolipids and inert waxes, form an impenetrable barrier against noxious TB drugs and compounds (Shaku *et al.*, 2020:2). Mtb is resistant to Gram staining due to its overall lipid-rich cell wall composition. This is because, when stained with Gram stain dyes, the cell wall is resistant to the hydrophilic acid alcohol decolorizing agent, hence they are classified as "acid-fast" bacilli (Vilchèze & Kremer, 2017:1).

2.4. Pathophysiology of Mtb

The risk of contracting TB is solely dependent on the probability of inhaling an infectious dosage or quantity of Mtb and the development thereof into an established active infection (assuming total susceptibility). Individuals with pulmonary TB produce Mtb-contaminated dehydrated residua droplet nuclei, or larger aerosols generated through sneezing, coughing,

laughing or shouting (Nardell, 2015:1-2). These physical acts pave the way for Mtb to enter the respiratory system and be caught by goblet cells, which release mucus. However, after slipping past the body's first mucociliary defensive barrier, the bacilli infiltrate the oxygenated alveoli of the lungs, triggering the host's innate immunological response. After activation of the latter, alveolar macrophages engulf Mtb by primary phagocytosis (Chai *et al.*, 2020:1860; Luies & Du Preez, 2020:2). Phago-lysosomal fusion is responsible for the early elimination of Mtb. However, while in the phagosome, Mtb may produce the phosphatase *SapM* and the serine/threonine kinase *PknG*, which prevents such fusion, and subsequently promote the survival and multiplication of Mtb within macrophages (Pieters, 2008:402).

During this phase, which lasts approximately two to three weeks, the human body mounts a complex cell-mediated immunity to combat Mtb. Monocytes, dendritic cells and neutrophils begin to surround the site of the infection. The infected dendritic cells become activated and migrate to a nearby draining lymph node, where they present antigens to T lymphocytes, allowing for a host T cell response. The infected macrophages, on the other hand, release pro-inflammatory cytokines (Silva Miranda *et al.*, 2012:2; Welin, 2011:11). The excreted cytokines activate natural killer cells, which then produce interferon gamma (IFN-*γ*) and subsequently activate macrophages to release tumour necrosis factor (TNF-*α*) as well as bactericidal chemicals in an attempt to destroy Mtb. Additional immune cells and macrophages are recruited from the blood circulation through cytokine and chemokine signaling, which aggregate into cell clusters at the primary site of infection called the granuloma (Welin, 2011:11) (see **Fig. 2.2**).

The formation of such granulomas, which contain both uninfected and infected cells covered by a lymphocytic cuff is the hallmark of TB infection. Granulomas are the main site of the host-pathogen interaction and functions by limiting Mtb dissemination from the lungs to other places in the body (Kotze *et al.*, 2021:2; Sia & Rengarajan, 2019:1). However, research using the zebrafish TB model has recently indicated that granulomas may potentially offer a niche for

the survival of Mtb and may also facilitate the dissemination of the bacilli inside the host (Ravimohan *et al.*, 2018:10; Volkman *et al.*, 2010:466). Some infected granulomas may heal entirely (sterilising cure) over time or stay in a balanced, compact form, restricting Mtb at their centres as a result of the host's reaction to infection (Kotze *et al.,* 2021:2; Russell *et al.*, 2009:3).

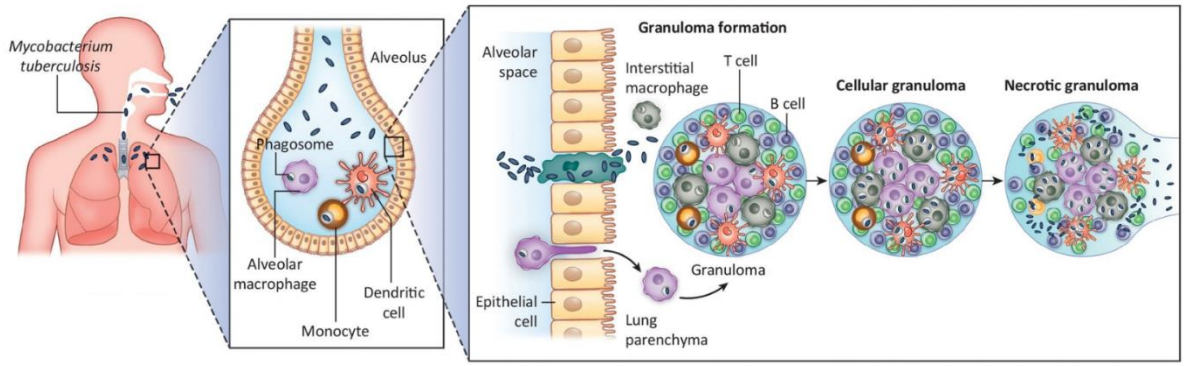

Figure 2.2 The formation and progression of granulomas (Koch & Mizrahi, 2018).

The healing process requires the macrophages within the granulomas to undergo necrotic destruction through a process called caseous necrosis. The necrotic tissue is now known as a ghon focus (Ani Fatonah *et al.*, 2019). Mtb may additionally spread via lymphatic vessels to regional lymph nodes as a direct extension of the ghon focus. At this point, the ghon focus and its accompanying lymph nodes are referred to as the ghon complex (Pillay *et al.*, 2020:66). The tissue contained by the ghon complex gradually undergoes fibrosis and calcification, generating scar tissue or lesions that may be seen on a chest X-ray. The radiological appearance of the calcified ghon complex on a chest X-ray is referred to as a ranke complex (Hicks *et al.*, 2014:129). At this stage, the progression of the infection has been halted. However, some Mtb bacilli that are capable of surviving under the stressful conditions executed by the host escape death by avoiding elimination by the immune response and entering a state of dormancy (Ahmad, 2011:4).

During the dormancy period, the disease is usually asymptomatic and not contagious because the Mtb bacilli cannot enter the airways to be exhaled and transmitted, leaving the patient with a latent TB infection (Sharma *et al.*, 2021:4362). It is believed that viable Mtb bacilli may stay dormant and reactivate days, months or even years later (Ai *et al.*, 2016:1). Of all people latently infected with TB, 5–15% will develop active TB over their lifetime (Dutta & Karakousis, 2014:344).

Reactivation of latent TB occurs when the patient's immune system weakens, as with HIV co-infection which is the most significant single risk factor for disease escalation to active TB in adults (Ahmad, 2011:9). The immune system is incapable of controlling the infection, enabling the dormant Mtb bacilli to reactivate by replicating at a high density and increasing their concentration within granulomas (Fattorini *et al.*, 2013:2). As a result, the caseous necrotic core of the granuloma liquefies and cavitates, spewing hundreds of reactivated Mtb into the airways (as seen in **Fig. 2.2**) (Ndlovu & Marakalala, 2016:3). The reactivated bacilli may rapidly disseminate from the initial infection, and Mtb can be found in lymph nodes near the lungs and subsequently in other organs within two weeks, known as extrapulmonary TB (Moule & Cirillo, 2020:5).

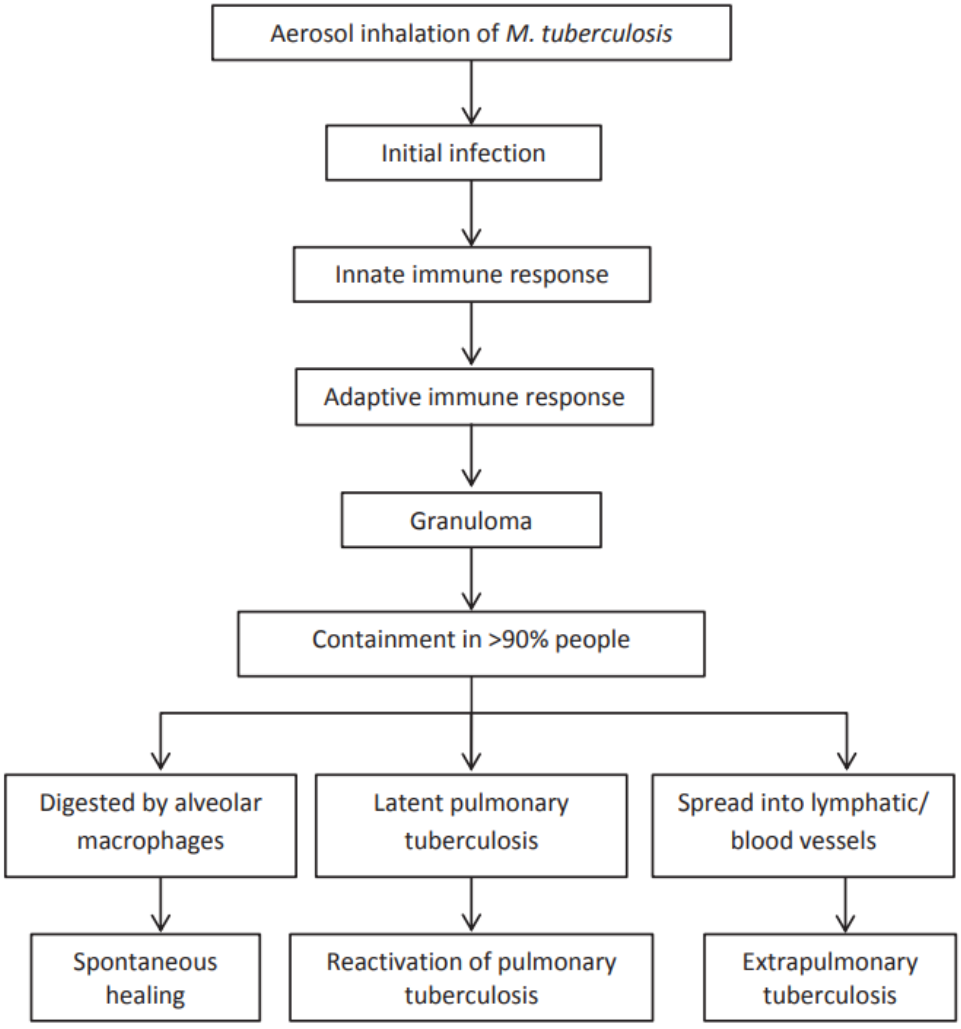

Figure 2.3 Pathophysiology of tuberculosis (Susilawati & Larasati, 2019).

2.5. Virulence factors of Mtb

The pathophysiology of Mtb clearly shows that mycobacteria can withstand host immunity. Mtb lacks the typical virulence factors that are generated by the majority of other bacteria, such as endotoxins to poison host cells, capsules to avoid phagocytosis, flagella for mobility, and other adhesins for attachment to host tissues (Cambier *et al.*, 2014:1497; Smith, 2003:468). Mtb, on the other hand, employs a number of additional characteristics that are critical for the progression of TB in order to subvert and/or evade the host's immune system (Sundararajan & Muniyan, 2021:6182) and subsequently promote mycobacterial replication and survival within the host:

- **Mycolic acids** are *β*-hydroxy fatty acids with a long *α*-alkyl side chain. They appear as a homologous sequence of C_{54} to C_{63} fatty acids with C_{22} to C_{24} *α* side chains. Mtb specifically contains *α*-, methoxy-, and keto-mycolic acids (Takayama *et al.*, 2005:82) that create a lipid shell surrounding Mtb and alter cell permeability. They are also known to protect mycobacteria against lysozyme, oxygen radicals and cationic proteins (Rao & Meena, 2011a:2).
- **Trehalose 6,6′-dimycolate** (cord factor) is the most abundant extractable lipid on the surface of Mtb (Harland *et al.*, 2008:4718). The cord factor is toxic to mammalian cells and is known to inhibit neutrophil migration during host cell-mediated immune response. It also promotes the Mtb infected macrophages to form granulomas by inducing cytokine (INF-*γ*) production (Rao & Meena, 2011b: 3-4). In addition, the cord factor inhibits phago-lysosomal fusion as well as the migration of Mtb to acidic compartments in macrophages (Harland *et al.*, 2008:4718).
- **Lipoarabinomannan (LAM)** is a complicated cell wall glycolipid comprised of phosphatidylinositol and a mannose nucleus, branched with arabinofuranosyl side chains (Maiti *et al.*, 2001:329). LAM inhibits INF-*γ* mediated macrophage activation. In addition, LAM may scavenge toxic oxygen free radicals, inhibit protein kinase C activity, and prevent the transcriptional activation of INF-*γ* inducible genes in macrophages (Joshi *et al.*, 2020:252).
- **Type VII secretion system (T7SS; ESX):** The ESX protein secretion system exports intracellular virulence-promoting proteins over Mtb's cell envelope. Mtb encodes five unique type VII secretion proteins, ESX–1 through ESX–5 (Bunduc *et al.*, 2020:316). ESX–1 enables Mtb to escape phagosome vesicles to avoid destruction (Rao & Meena, 2011a:3) and secretes antigens that cause the membrane of phagosomes to rupture. ESX–2 ensures survival of Mtb, while ESX–3 regulates iron and zinc influx and hinders phagosome maturation. ESX–4 is needed for conjugation, whereas ESX–5 releases immunomodulatory proteins (Roy *et al.*, 2020:203).

2.6. Clinical manifestations of TB

2.6.1. Post-primary pulmonary TB (reactivation TB)

Reactivation of primary Mtb infection or a new infection from an external source, for example an infected individual, can result in post-primary TB. Post-primary TB may be misdiagnosed and possibly be contagious for up to three years or more, with symptoms only manifesting late in the disease's progression (Pozniak, 2019). It normally affects just the uppermost lobes of the lungs, with no involvement of lymph nodes or other organs. It accounts for about 80% of all confirmed TB cases and almost 100% of infection transmission (Hunter, 2011:498). Patients may manifest symptoms such as chest discomfort, shortness of breath, low grade fever and a slight, non-productive cough. As the infection worsens, cough may become more productive with bloody-greenish sputum. Anorexia, weight loss, muscular atrophy and wasting ("consumption") may be seen in extreme cases (Alzayer & Al Nasser, 2021:4; Pozniak, 2019).

2.6.2. Extrapulmonary TB

TB can affect every organ system in the human body, despite the fact that TB in the pulmonary system is the most common form. Extrapulmonary TB is a kind of TB disease that involves organs other than the lungs and represents 10–50% of confirmed TB cases (Shivakumar *et al.*, 2022:341). Extrapulmonary TB is more prevalent in individuals with weaker immune systems, particularly those who are also HIV-infected. The most likely affected extrapulmonary TB sites are the lymphatic system, the bones and joints, the genito-urinary system and the central nervous system (CNS), followed by peritoneal and other abdominal organs (De Backer *et al.*, 2006:248-249).

Extrapulmonary TB clinical manifestations include typical post-primary pulmonary TB symptoms such as fever, weight loss, night sweats or malaise, as well as various systemic symptoms related to the affected organ (Gopalaswamy *et al.*, 2021:141). The most prevalent manifestation of extrapulmonary TB, for example, is lymphadenitis, or inflammation of the

cervical lymph nodes. Lymphadenitis may cause significant swelling in the neck that mimics a growth or tumour in extreme instances (Moule & Cirillo, 2020:2). The renal system is commonly affected, It is, however, typically asymptomatic. Mtb may enter the glomerular system and get caught in the Henle loop, causing new focal points of infection to develop in the renal pyramids. Peritoneal TB is often associated with other types of abdominal TB. Necrotic lymph node rupture induces peritoneal TB, with ascites being the most common manifestation (Rodriguez-Takeuchi *et al.*, 2019:2028-2032).

The most severe form of extrapulmonary TB with CNS involvement is TB meningitis (5–10% of all TB cases). Fatigue, nausea, fever, headaches and disorientation are all symptoms of TB meningitis. Convulsions, comas and stupor may also occur (Azad & Chowdhury, 2022:132). Miliary TB develops via hematogenous spread of the primary infection. If not treated properly, this is the most lethal type of extrapulmonary TB, accounting for 15–20% of teenage and 25–30% of adult TB fatalities (Vohra & Dhaliwal, 2022:9). Miliary TB most often affects the lymph nodes, liver, meninges, adrenal glands, spleen, lung and bone marrow. Septicaemia with fever, lethargy, anorexia, weight loss, and coughing are all common manifestations of miliary TB (Azad & Chowdhury, 2022).

2.7. Detection and diagnosis of TB

Rapid and accurate diagnostic tests for TB, with the management of latent TB are vital to Mtb eradication attempts. The diagnosis of primary pulmonary TB and latent TB incorporates culture development, immunological tests and molecular assays to identify the presence of Mtb. Extrapulmonary TB is detected similarly to post-primary and latent TB, albeit invasive biopsies are typically used. The diagnostic tests are less effective in HIV-positive patients, those with latent TB, and adolescents unable to produce Mtb-containing sputum. Furthermore, they require expensive, sophisticated laboratory equipment and highly qualified staff, both of which are uncommon or inaccessible in low- and middle-income countries (MacGregor-Fairlie *et al.*, 2020:436).

2.7.1. Microbiological assays

A. TB Culture

The ultimate gold standard diagnostic test for TB is incubating a sputum sample for Mtb culture growth, which may also provide evidence of drug susceptibility or resistance, including emerging mutations (Oommen & Banaji, 2017:325). Culture has a 99% specificity for diagnosing TB. However, owing to Mtb's slow growth, it may take a long time to develop a suitable culture. This, in turn, delays treatment initiation in the absence of a confirmed diagnosis (Acharya *et al.*, 2020:4069).

There are three types of culture media: Löwenstein-Jensen (egg-based), Middlebrook 7H10 or 7H11 (agar-based), and Middlebrook 7H12 (liquid-based). Mycobacterial growth is more conducive to egg-based media than agar-based media, and growth in liquid media is often more rapid than growth in solid media. A further benefit of using agar medium is that it enables researchers to examine the morphology of Mtb colonies and distinguish between diversified mycobacterial cultures (Sia & Wieland, 2011:354). To diagnose TB, sputum isolates are inoculated onto various media containing increasing doses of antibiotics to determine the lowest concentration at which 99% of growth is inhibited. To determine resistance and susceptibility, two media are prepared, one with antibiotics and one without, and the percentage of Mtb colonies growing in the presence of antibiotics is compared to those growing in the absence of antibiotics. The isolate is classified as sensitive or resistant based on predetermined ranges (Cudahy & Shenoi, 2016:191).

Traditional solid culture growth is time-consuming, difficult, and have little therapeutic benefit. Automated liquid culture methods have essentially supplanted solid culture, with around 10% greater yields and a shorter time to diagnosis. However, automated liquid cultures are costly, prone to contamination, and need a significant amount of laboratory equipment and experience (Peter *et al.*, 2012:128). Growth requires 4 to 8 weeks on a typical egg-based

medium, with an additional 4 weeks for drug sensitivity testing. As a result, diagnosing MDR-TB takes an average of 70 days (Oommen & Banaji, 2017:325).

B. Acid-Fast Bacilli (AFB) smear microscopy

Mtb can be detected through AFB smear microscopy using the Ziehl-Neelsen (ZN) staining technique. Staining for AFB has been the foundation of TB diagnosis since its discovery by Koch in 1882. The effectiveness of AFB smear microscopy lies in its ability to detect the most infectious patients. However, there are a number of variables that might alter the yield of a smear, including collection time and sputum processing (Cudahy & Shenoi, 2016:190).

The procedure includes carbolfuchsin staining of the sputum-smeared slides by submerging the smear in carbolfuchsin and subsequently heating the slide until steam rises without boiling the dye. This allows the stain to be fixed into the cell wall of Mtb and the whole smear will become red at this stage. The second step after stain fixation is to cover the slide with acid alcohol for 2–3 minutes. Acid alcohol decolourises all non-acid-fast organisms, leaving behind only the red stained acid-fast Mtb. Finally, the slides are then counterstained with methylene blue, which enhances the visual contrast of the red acid-fast Mtb against a blue background during microscopy (Bayot *et al.*, 2019). The benefits of the ZN include low-cost rapid testing for Mtb. However, results are often hampered due to low sensitivity, which may range from 20–80%. The smear needs high bacilli count from sputum to be successful and its sensitivity is further significantly reduced in HIV-coinfected individuals (Ojha *et al.*, 2020:1431–1432). Other limitations of smear microscopy include the test's inability to differentiate between viable and non-viable organisms, Mtb and non-tuberculous mycobacteria, and drug-susceptible and drug-resistant strains of Mtb (Harries & Kumar, 2018:3-4).

2.7.2. Immunoassays

A. Mantoux Tuberculin Skin Test (TST)

A TST is an intradermal injection of tuberculin purified protein derivative (PPD) extracted from Mtb cultures. The test detects a type IV delayed-type hypersensitivity response in Mtb-exposed people based on immunological recognition of the PPD antigen (Pahal & Sharma, 2021). This reaction is delayed since it takes time to recruit immune cells and activated macrophages to the injection site. Intradermal injection of 0.1 ml of five test unit PPD stimulates antigen-specific lymphocyte influx and the release of inflammatory cytokines. The subsequent inflammatory reaction causes an indurated zone at the injection site, which is needed for diagnosis. After 48–72 hours, a healthcare professional palpates the injection site for induration and measures it. Depending on a patient's comorbidities or history of TB exposure, 5–15 mm of induration indicates a positive result (Kestler & Tyler, 2022:L415).

The TST is not always reliable – it often delivers false-positive and false-negative results. False-positive results are due to its inability to differentiate between Mtb and either non-tuberculosis mycobacteria or the Bacille Calmett-Guérin (BCG) vaccine (*Mycobacterium bovis*). These false-positive results are often ascribed to an immunological response generated by homologous antigens from environmental mycobacteria or *M. bovis* (Yang *et al.*, 2012:276). In addition, HIV infection, severe acute diseases, cancer, ageing and immunosuppressive drugs might decrease the immune system's capacity to react to the TST, causing false-negative results (Almeida Santos *et al.*, 2020:542). Finally, a positive TST solely indicates Mtb infection. It does not tell whether someone has latent TB or active TB. A chest X-ray and sputum analysis are, therefore, required for a complete diagnosis after a TST (CDC, 2022).

B. Interferon-γ Release Assays (IGRAs)

IGRAs are Mtb antigen-specific IFN-*γ* release assays designed to measure *in vitro* IFN-*γ* concentration after incubation of whole blood or peripheral blood mononuclear cells with antigens (ESAT-6 and CFP10), encoded within the region of difference–1 locus of Mtb. These antigens are absent in the vast majority of non-TB mycobacteria and BCG strains, making the IGTAs more selective than the TST (Kestler & Tyler, 2022:L416; Pourakbari *et al.*, 2019:437). Two IGRAs are available: the QuantiFERON-TB Gold In-Tube Plus test, which measures the amount of IFN-*γ* in culture supernatant using an enzyme-linked immunosorbent assay (ELISA), and the T-SPOT.TB assay which recognises the amount of IFN-*γ* producing T cells using an enzyme-linked immunospot (ELISPOT) assay (Zhou *et al.*, 2015:2).

For the QuantiFERON-TB Gold In-Tube Plus test, blood samples are incubated with Mtb-specific antigens and controlled at 37°C ± 1°C for 16 to 24 hours. After incubation, tubes are centrifuged, plasma is extracted, and ELISA measures the amount of IFN-*γ* produced by T cells (Lee *et al.*, 2021:1695). However, for the T-SPOT.TB, the procedure is more complex and carried out in microtiter wells pre-coated with Mtb-specific capture antigens (IFN-*γ*-specific). Sample preparation involves separating, quantifying and diluting peripheral blood mononuclear cells from whole blood, after which it is incubated at 37°C with 5% CO_2 for 16 to 20 hours. The pre-coated antibodies capture the released IFN-*γ* at the bottom of each well. After adding a second antigen and a soluble substrate, an insoluble precipitate forms on the membrane at the bottom of each well. Finally, using an ELISPOT plate reader, spots are counted. Each spot represents an individual cytokine-secreting T cell, and the number of spots observed indicates the abundance of Mtb-sensitive effector T cells in peripheral blood compared to the control (ECDP, 2018).

The sensitivity of IGRAs is lowered in immunocompromised individuals, similar to the TST. In addition, it cannot discriminate between latent and active TB (Sotgiu *et al.*, 2019:444). Recent evidence indicates, however, that the combination of IGRAs and mycobacterial heparin-

binding hemagglutinin (HBHA) is a potential test for differentiating between latent and active TB. This is because HBHA is a typical Mtb antigen linked with latency (Tang *et al.*, 2021:1-2). The specificity of IGRAs is believed to exceed 95%, whereas the sensitivity ranges between 80% and 90%. IGRAs are substantially more expensive than TSTs and require phlebotomy, scientific equipment, and technical competence for sample collection, processing, and assessment (Kestler & Tyler, 2022:L416).

2.7.3. Nucleic Acid Amplification Tests (NAATs)

A. Molecular Line Probe Assays (LPAs)

Numerous molecular strategies and technologies have been developed to enable a rapid turnaround time of one day for NAATs, as opposed to two weeks or more for culture development (Teo *et al.*, 2011:3659). The GenoType® MTBDR*plus* v1.0 and GenoType® MTBDR*sl* v2.0 tests are both LPAs based on genotype rather than phenotype. They rely on gene mutations associated with antitubercular drug resistance to detect Mtb complex (Stephen *et al.*, 2019:84). GenoType® MTBDR*plus* v1.0 detects resistance to rifampicin (RIF) and isoniazid (INH), while GenoType® MTBDR*sl* v2.0 detects resistance to fluoroquinolones (FQNs) and other second-line injectables (Chen *et al.*, 2019:2). Although LPAs aid in the rapid detection of MDR-TB, deoxyribonucleic acid (DNA) extraction, amplification, hybridization and the result interpretation are manual, laborious operations that necessitate error-prone multistep processes and at least three different rooms to avoid contamination, which makes them labour-intensive (David *et al.*, 2020:3302).

The procedure for LPAs includes the extraction of DNA, the preparation and addition of master samples, multiplexed nucleic acid amplification using biotinylated primers through polymerase chain reaction (PCR), detection of the amplicons by reverse hybridization with oligonucleotide probes attached on a nitrocellulose strip in parallel lines, and finally colorimetric development that enables lines to be seen where the probes are situated on the strip (Eddabra & Ait Benhassou, 2018:5; Javed *et al.*, 2018:2; Meaza *et al.*, 2017:2). The GenoType® MTBDR*plus*

v1.0 test has a sensitivity of 95.8% and a specificity of 98.4% for detecting RIF resistance, and 94.5% sensitivity and 99.3% specificity for detecting INH resistance. The GenoType® MTBDR*sl* v2.0 test, on the other hand, has a sensitivity of 86.2% and a specificity of 98.6% for detecting FQN resistance, and 87.0% sensitivity and 99.5% specificity for detecting second-line injectable drug resistance (WHO, 2022a).

B. Xpert MTB/RIF assay

The Xpert MTB/RIF assay (a novel NAAT) was authorized by the WHO in 2010 as a primary diagnostic test for MDR-TB or HIV-associated TB, and it may be used as a follow-up test to AFB smear microscopy in countries where MDR-TB or HIV are less of a problem (WHO, 2011). The Xpert MTB/RIF assay has revolutionised TB control by further improving the turnaround time for NAATs. Results can now be delivered in less than two hours, either directly from a sputum sample or after decontamination and concentration of the sputum (CDC, 2016a; Steingart *et al.*, 2013:6-7). It helps TB patients commence treatment much sooner (as opposed to have them wait for other drug susceptibility tests). A retrospective cohort study in South Africa, where MDR-TB and HIV rates are extremely high, confirms this. The Xpert MTB/RIF test reduced treatment delays by 50% between 2011 (after deployment of Xpert MTB/RIF) and 2013 (Cox *et al.*, 2017:3).

Xpert MTB/RIF uses hemi-nested real-time PCR and molecular beacon technology to simultaneously detect Mtb and RIF resistance by recognising mutations in the normal, RIF-susceptible "wild-type" sequence of Mtb's ribonucleic acid (RNA) polymerase gene, *rpoB*. Five distinct coloured beacons are applied, each covering a specific nucleic acid sequence in the amplified *rpoB* gene (Rufai *et al.*, 2014:1846; Steingart *et al.*, 2013:6-7). The Xpert MTB/RIF assay, unlike other standard NAATs (like LPAs), integrates sputum processing, DNA extraction, PCR amplification and Mtb detection into a single self-contained cartridge. After inserting the sample, the assay is completely automated and confined inside the cartridge, which reduces the possibility of errors related to other NAATs (WHO, 2013). The cartridge-

based device benefits from convenience of use and a sealed amplification system that prevents cross-contamination between specimens (Caulfield & Wengenack, 2016:40).

In an attempt to boost the sensitivity of the Xpert MTB/RIF, the WHO (2017b) suggested and employed the use of Xpert MTB/RIF Ultra as an alternative to Xpert MTB/RIF in 2017. The same analyser as in the Xpert MTB/RIF assay is used, but with an upgraded specimen cartridge and operating system. This implies that the Xpert MTB/RIF Ultra test detects Mtb and RIF resistance in the same way as the Xpert MTB/RIF assay. However, it seems to be substantially more sensitive than Xpert MTB/RIF for detecting Mtb in smear-negative, culture-positive, paediatric, extrapulmonary and HIV-infected samples (WHO, 2017b). Unlike LPAs, the Xpert MTB/RIF and Xpert MTB/RIF Ultra cannot detect INH resistance. Hence, RIF resistance coexists with INH resistance in 85–90% of TB patients and the two assays combined may thus be used to predict MDR-TB cases (Liu *et al.*, 2020:6).

2.8. Vaccination

The only WHO-approved TB vaccine is a live-attenuated Bacille Calmett-Guérin vaccine derived from *M. bovis,* which shares > 90% of its genetic makeup with Mtb. Over four billion people worldwide have received the BCG vaccine, making it the most common and oldest vaccine in use (Li *et al.*, 2021:1). The BCG vaccine has a 90-year safety record. Its effectiveness, though, is disputed and only lasts for 10 to 20 years after inoculation (Escobar *et al.*, 2020:7720; Fatima *et al.*, 2020:1-2). The controversial aspect of BCG is how inconsistently well it works to prevent TB. It provides adequate protection for children against meningitis TB and other extrapulmonary TB, such as miliary TB, however, protection against adult pulmonary TB fluctuates between 0% and 80%. Hence, some countries, like the USA, do not routinely administer the BCG vaccine due to the vaccine's questionable effectiveness against pulmonary TB and the need to preserve the TST as a diagnostic tool. Furthermore, the BCG vaccine does not prevent or protect against reactivation of latent TB (Teo & Shingadia, 2006:530; WHO, 2008).

A single dose of BCG is administered to new-borns in TB-prone areas where BCG is included in the national childhood immunisation program, within hours or days of birth. Only children at risk of contracting TB and healthcare workers at risk of exposure to drug-resistant TB (DR-TB) receive BCG in countries with low TB incidence (WHO, 2008). BCG is contraindicated for people with compromised immune systems (such as those who are HIV-positive or have had organ transplantation), pregnant woman, and those who have already had TB (CDC, 2016b). According to the last scenario, the host has already acquired some sort of immunity to TB as a result of prior mycobacterial exposure. The "blocking hypothesis" describes how this immunity causes a response to antigens that are cross-reactive with BCG antigens, preventing the dissemination and replication of BCG within the host, and subsequently depleting its effectiveness. Thus, receiving the vaccine in such case could result in unwanted side effects (Andersen & Doherty, 2005:659).

2.9. Overview of TB Treatment

The standard treatment for drug-susceptible TB (DS-TB) consists of giving four antitubercular drugs over a minimum period of six months (two months for the intensive phase, followed by four or seven months for the continuation phase) (CDC, 2016c). This regimen has an overall success rate of 85%. First-line drugs (FLDs) are a feasible therapeutic option, but they are not always successful in treating TB. One of the reasons is that the vast majority of Mtb strains are resistant to two or more of the most potent FLDs which can only be cured by using an intricate cocktail of additional drugs referred to as second-line drugs (SLDs) (CDC, 2017). Increased drug resistance has been brought on by a variety of factors, including inadequate healthcare facilities, the wrong treatment being prescribed (either the incorrect dosage or the incorrect duration), poor quality drugs, drug shortages or Mtb re-infection, among others (Allué-Guardia *et al.*, 2021:2).

The primary reasons for TB treatment failure are patient negligence and non-compliance with antitubercular drug regimens, making TB treatment seemingly virtually impossible. Patient

non-compliance compromises treatment outcomes, increases the risk of TB transmission and reactivation, causes the rapid emergence of DR-TB, and elevates TB mortality rates (Fang *et al.*, 2019:1929). One of the main reasons patients do not complete taking their prescribed antitubercular drugs is the prolonged period of treatment that must continue even after clinical improvement (Woimo *et al.*, 2017:2). Patients do not follow suggested treatments for a number of additional reasons, such as apprehension about side effects, stigma, a lack of information, the price of medicines and a lack of awareness on the part of patients and their families about the possible repercussions of TB (AlSahafi *et al.*, 2019:2). As a result, the End TB Strategy's goal of lowering TB infections by 90% by 2035 is under threat due to the rapid rise of DR-TB, especially in places where TB is endemic (WHO, 2015a).

2.10. First-line drugs for the treatment of drug-susceptible TB (DS-TB)

2.10.1. Isoniazid (INH)

INH (**1**), also known as iso-nicotinic hydrazide, is a small, freely soluble molecule used to treat DS-TB since 1952 and continues to serve as the cornerstone of TB treatment together with RIF. INH is effective against extracellular and intracellular metabolically active Mtb bacilli owing to its ability to penetrate macrophages (Deck & Winston, 2012a:840; Palomino & Martin, 2014:318-319). The structure of INH is simply a hydrazine-and-carbonyl ($CONHNH_2$) group joined to the *para* position of a pyridine ring containing nitrogen (Fernandes *et al.*, 2017:299). The structure-activity relationship (SAR) of INH demonstrates that the pyridine heterocycle is optimum, but substitution with alternative heterocycles results in a significant potency decrease. The $CONHNH_2$ moiety is, however, required for activity. The activity is furthermore diminished by even seemingly minor alterations, including adding a methyl group to the pyridine ring or isomerising the nitrogen atom to a different position on the pyridine ring (Hegde *et al.*, 2021:2).

INH is a prodrug that enters Mtb by passive diffusion and must first be activated by *KatG*, the catalase-peroxidase produced by mycobacteria, in order to have any effect. Mycolic acid

production depends on the Mtb nicotinamide adenine dinucleotide (NADH)-dependent enoyl-acyl carrier protein (ACP) reductase of fatty acid synthase II (FAS II), which is encoded by *inhA* and targeted by INH (Marrakchi *et al.*, 2014:77). INH inhibits mycolic acid biosynthesis by forming a covalent complex with *AcpM*, an ACP, and *KasA*, a *β*-keto-ACP synthase, which ends up killing Mtb cells. INH resistance has been linked to mutations that increase the expression of *inhA*, which encodes NADH-dependent enoyl-ACP, *KatG* mutations or deletions that prevent INH prodrug activation, promoter mutations that cause overexpression of *ahpC* (which shields Mtb from oxidative stress), and *KasA* mutations (Deck & Winston, 2012a:840). *KasA* mutations are uncommon but have been noted in isolates that are INH susceptible. Low levels of INH resistance are caused by *InhA* mutations, but high levels of resistance by *KatG* mutations (Jaiswal *et al.*, 2017:144).

INH is readily absorbed from the gastro-intestinal (GI) tract after a single 300 mg oral dose, and it diffuses rapidly throughout body tissues, including the cerebrospinal fluid (CSF) (Deck & Winston, 2012a:840). However, food and antacids impede INH from being absorbed. It is therefore recommended to consume INH on an empty stomach (Rossiter, 2020:331). The peak plasma concentration reaches 3–5 µg/ml in one to two hours after administration of the drug (Deck & Winston, 2012a:840). Metabolism of INH occurs in the liver through acetylators, including *N*-acetylation by *N*-acetyltransferase–2 to form *N*-acetyl-INH and hydrolysis by amidase to produce iso-nicotinic acid and hydrazine. Metabolites are mainly excreted in urine (Wang *et al.*, 2016:385).

INH causes unfavourable side effects. Rashes and fever are two occasional, dose-dependent side effects. INH-induced hepatitis (hepatotoxicity) occurs in 10–20% of individuals receiving INH treatment. Clinical signs of INH-induced hepatotoxicity include lack of appetite, nausea, jaundice, vomiting and right upper quadrant pain (Deck & Winston, 2012a:841; Rossiter, 2020:332). INH-induced neurotoxicity is dose-dependent and most typically presents as seizures in pyridoxine (Vitamin B6) deficient people due to first-pass metabolism (Badrinath &

John, 2018). Less common CNS complications include seizures, memory loss and psychosis. Other adverse effects include haematological abnormalities, anaemia (from pyridoxine deficiency), hearing impairment and GI irritation. Drug-induced systemic lupus erythematosus has been reported, but it is rare (Deck & Winston, 2012a:841).

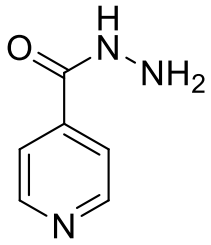

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Figure 2.4 Isoniazid (**1**)

2.10.2. Rifamycins

Rifamycins are macrocyclic antibiotics that belong to the ansamycin family. They are made up of a naphthoquinone core with two ends connected by a poly-hydroxylated ansa-chain (long-chain) (Aristoff *et al.*, 2010:96). Rifamycins were discovered as natural products from the soil actinomycete *Amycolatopsis mediterranei*, with the first compound of rifamycin B being found to have relatively weak antimicrobial activity (Qi *et al.*, 2016:3803). However, researchers have improved their activities and developed new effective commercial rifamycins through hemi-synthesis (Dey & Chatterji, 2012:88).

The most well-known member of the rifamycin family, RIF (**2**), is a semi-synthetic derivative of rifamycin that was first made available in 1968 (Qi *et al.*, 2016:3803). It is quite effective against both replicating and non-replicating Mtb bacilli. Its activity against the latter is believed to be responsible for shortening the length of TB treatment regimens from 12 or 18 months to 9 months when RIF is included. One of RIF's most salient characteristics is that it is the most effective "sterilising" antitubercular drug. This means that the bacillary population can be

adequately reduced to avoid infection relapse (Peloquin & Davies, 2021:1459). Additional rifamycin derivatives have been developed over time, such as rifapentine (**3**), which has a longer half-life ($T_{1/2}$) and greater activity than RIF but is inactive against RIF-resistant Mtb strains (Zhang, 2005:537). Rifabutin (**4**) is used for the treatment of *Mycobacterium avium* complex in HIV patients and rifaximin (**5**), a non-oral rifamycin, is used for the treatment of enteropathogenic *E. coli*, which causes traveller's diarrhoea (Aristoff *et al.*, 2010:94).

RIF inhibits the Mtb RNA transcription process by restricting elongation of the RNA chain by binding to the DNA-dependent RNA polymerase of Mtb in its *β*-subunit (encoded by *rpoB*) (Khawbung *et al.*, 2021:2). When RIF is bound, it prevents the synthesis of RNAs larger than dinucleotides, sterically blocking the route of nascent RNA at the beginning of the transcription process (Dey & Chatterji, 2012:88). When the RNA reaches a length of 3–4 nucleotides, the RNA and RIF collide, releasing the RNA from the promoter complex as an interrupted transcript and eventually causing Mtb cell death (Mosaei *et al.*, 2018:263). Resistance to RIF may develop as a consequence of any *rpoB* gene mutation. Over 95% of Mtb clinical isolates seem to have a mutation in its RIF resistance-determining region, an 81-base pair hotspot region of the *rpoB* gene (Rando-Segura *et al.*, 2021:2; Xu *et al.*, 2018:2).

Following oral administration, RIF is well absorbed and widely disseminated throughout body fluids and tissues (Deck & Winston, 2012a:841). However, RIF is highly protein bound (80–90%) making CSF penetration (10–20%) limited (Cresswell *et al.*, 2018:3; Rossiter, 2020:323). RIF is eliminated *via* the liver into the biliary system. It then undergoes enterohepatic recirculation followed by deacetylation by Cytochrome P450 (CYP450) iso-enzymes to generate the metabolites 25-O-desacetyl-RIF and 3-formyl-RIF SV. Deacetylated metabolites are mostly excreted in the faeces and a fraction is also excreted in the urine (Deck & Winston, 2012a:841; Pacifici, 2019:1).

RIF turns tears, urine and body fluids orange to reddish brown. Patients on RIF should know that it may permanently stain contact lenses. GI problems include nausea, vomiting, diarrhoea and mild abdominal pain, which may be reduced by taking RIF with meals. However, an empty stomach is best for optimum absorption (Rossiter, 2020:333). Hypersensitivity symptoms include fever, chills, myalgias, anaemia and thrombocytopenia. Hepatotoxicity is uncommon, yet fatal. The risk of hepatotoxicity is increased when RIF is co-administrated with INH, PZA or other drugs metabolised in the same manner, such as contraceptives, protease inhibitors, anticoagulants, methadone, cyclosporine, anticonvulsants and other non-nucleoside reverse transcriptase inhibitors. This is because RIF induces most CYP450 iso-enzymes, which cause overexpression of multiple drug metabolising enzymes and transporters, accelerating the elimination of these drugs. This ultimately results in decreased serum concentrations, which necessitates larger drug dosages and in effect promote hepatotoxicity (Deck & Winston, 2012a:842; Rossiter, 2020:333).

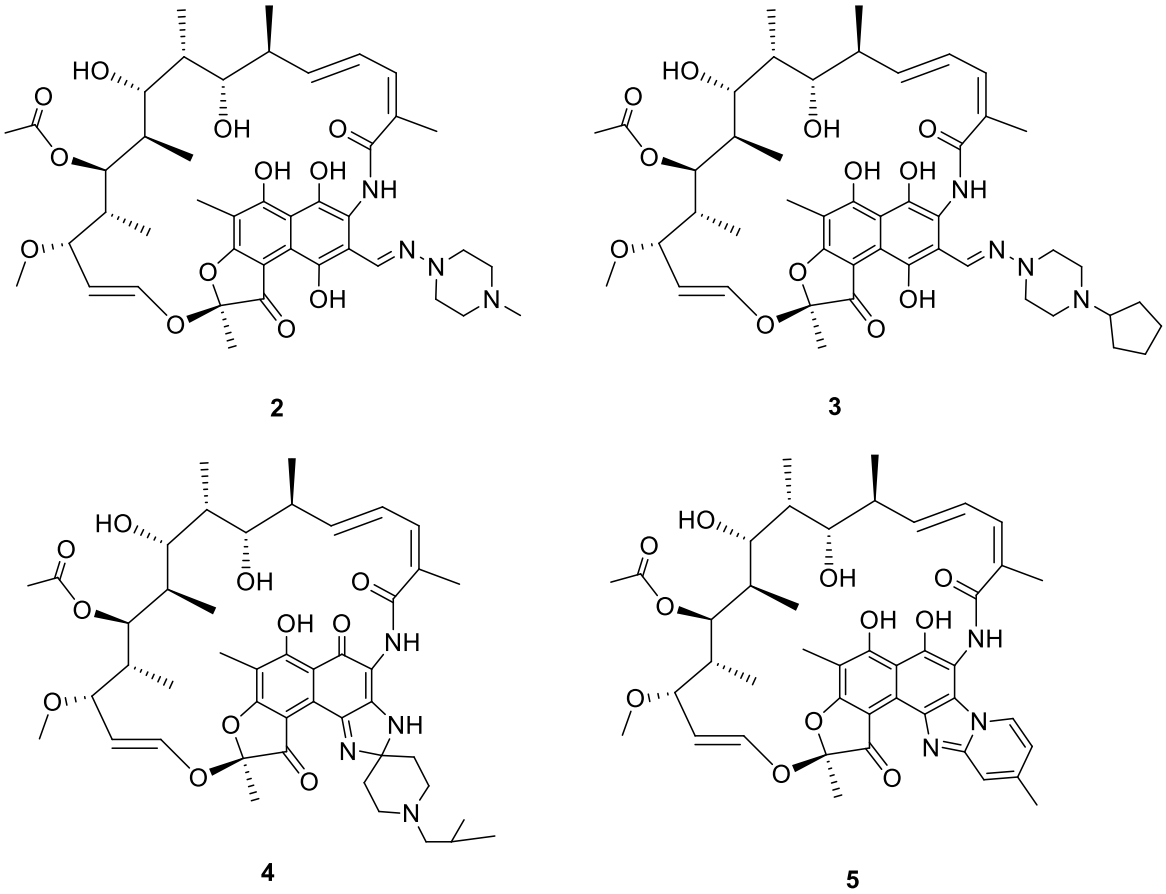

Figure 2.5 Rifampicin (**2**), rifapentine (**3**), rifabutin (**4**), and rifaximin (**5**)

2.10.3. Ethambutol (ETH)

ETH (**6**) is a synthetic, heat-stable, water-soluble molecule that is administered as a dihydrochloric salt. It is bacteriostatic, although higher dosages of ETH are bactericidal (Deck & Winston, 2012a:842; Rossiter, 2020:331). ETH acts on rapidly replicating Mtb bacilli that are both intracellular and extracellular in nature (Arbex *et al.*, 2010:635). It was recently discovered that *EtbR*, a *TetR* transcriptional regulator in Mtb, is a novel ETH-binding protein involved in a synergistic effect between INH and ETH. As a repressor, *EtbR* inhibits the expression of *inhA*, the target gene of INH. Thus, when both drugs are taken at the same time, a non-lethal dose of ETH may increase Mtb's susceptibility to INH, which in turn promotes INH's activity (Lee & Nguyen, 2021; Zhu *et al.*, 2018:16741).

As its primary MOA, ETH disrupts the synthesis and integrity of Mtb's cell wall. It is believed that it does this by inhibiting the mycobacterial arabinosyl transferases, which are encoded by Mtb's *embCAB* operon. Thus, it inhibits the polymerisation phase of arabinogalactan (encoded by the *embB* gene) and the formation of LAM, which are required for the integrity of the Mtb cell wall (Deck & Winston, 2012a:842). Three genes with a 65% similarity make up the *emb* operon: *embA*, *embB*, and *embC*. Mutations in the *embA* and *embC* genes are rarely observed and may be a factor in ETH resistance. However, the majority of the time, mutations in the *embB* gene, particularly at codon 306, are associated with ETH resistance. Mutations in *embB306* are present in between 50–70% of Mtb strains that are resistant to ETH (Mohammadi *et al.*, 2020:1-2). It is interesting to note that *embB306* mutations were only found in ETH-resistant strains by Plinke *et al.* (2006:1900). However, Hazbón *et al.* (2005:3796-3798) studied 1,020 clinical isolates of Mtb from over the globe and found that *embB306* mutations also confer resistance to other drugs, not only ETH.

Similar to RIF, ETH is rapidly absorbed via the GI tract after oral administration (plasma concentration of 2–5 µg/ml after 2–4 hours), diffuses widely across body fluids and tissues (Deck & Winston, 2012a:842), and has little CSF penetration (10–50% penetrates only

inflamed meninges) (Rossiter, 2020:331). Alcohol dehydrogenase initially oxidises ETH to an aldehyde in the liver before aldehyde dehydrogenase converts it into a dicarboxylic acid (Arbex *et al.*, 2010:635-636). About 20% of unaltered ETH is eliminated in faeces and 50% is excreted in urine (Deck & Winston, 2012a:842).

Chronic ETH usage often causes severe toxicity to appear at doses of 25 mg/kg/day. Dose-dependent retrobulbar optic neuritis (Deck & Winston 2012a:842), which may result in red-green colour blindness and varying degrees of vision impairment, is the most serious side effect of ETH. Blue-yellow visual abnormalities can also exist. Additionally, it has been noted that contrast sensitivity can be reduced (Saxena *et al.*, 2021). Due to this adverse effect on the eyes, ETH should not be used in children who cannot yet complete visual acuity and red-green colour discrimination tests (WHO, 2006). ETH ocular toxicity may typically be reversed if identified and managed early (Saxena *et al.*, 2021:3734). However, nerve damage to the eye is irreversible (Rossiter, 2020:331).

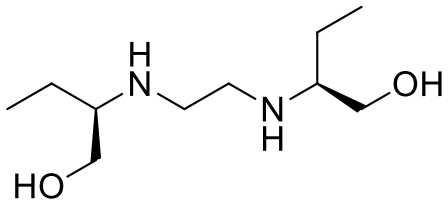

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Figure 2.6 Ethambutol (**6**)

2.10.4. Pyrazinamide (PZA)

PZA (**7**) is a nicotinamide relative that is stable but only moderately water soluble, having a chemical structure similar to INH (Deck & Winston, 2012a:842). Under typical *in vitro* conditions at neutral pH, PZA is not active against Mtb. However, it was discovered that it is only active at an acidic pH of 5.5. It has an exceptional *in vivo* sterilising effect, and when included in regimens containing RIF, it is mostly responsible for the eradication of persistent

Mtb bacilli during the intensive phase of treatment, enabling the duration of treatment to be shortened from 12 months to 6 months (Zhang & Mitchison, 2003:6).

The MOA of PZA is not widely understood and the precise pharmacological target is unclear (Deck & Winston, 2012a:843). However, a pH-dependent model is still regarded as an accepted mechanism. PZA is a prodrug like INH, and for it to have an effect, it needs to be in its active form. The *pncA* gene, which encodes mycobacterial pyrazinamidase, mediates PZA activation. Mycobacterial pyrazinamidase converts PZA into pyrazinoic acid (PA), which is released extracellularly after administration of the drug. PA becomes protonated in an acidic environment and permeates Mtb where it accumulates. Following that, the protonated PA reduces inter-mycobacterial pH and subsequently de-energises the membrane potential by compressing the proton gradient and interfering with membrane transport, which eventually results in Mtb cell death. However, the activity of PZA has also been shown to be pH-independent, and additional MOAs have been proposed. For instance, PZA inhibits the aspartate decarboxylase *PanD* from functioning and promotes its degradation via the *Clp* protease system, thus preventing the synthesis of coenzyme-A (Santucci *et al.*, 2021:2; Zhang & Mitchison, 2003:10). Resistance to PZA has been documented and is attributed to mutations in the *pncA* gene, which prevents PZA from being converted to its active form. Additional resistance mechanisms are known but have not been adequately investigated. Among these is the lack of absorption of PZA and mutations in *PanD*, preventing coenzyme-A depletion (Gopal *et al.*, 2016:623; Raynaud *et al.*, 1999:1359). Mtb develops resistance to PZA rapidly but does not demonstrate cross-resistance to INH or other antitubercular drugs (Deck & Winston, 2012a:843).

PZA is well absorbed and widely distributed throughout body tissues, with a maximum plasma concentration of 30–50 µg/ml achieved after 2 hours of administration (Arbex *et al.*, 2010:633). It is extensively metabolised in the liver by CYP2E1 and CYP3A4 to PA and 5-hydroxy-PA, which may cause hepatotoxicity – the most significant adverse effect of PZA. The mechanism

of PZA hepatotoxicity is unknown. However, it might be caused by either PA (the drug itself) or its metabolites (Rawat *et al.*, 2018:374). Hepatotoxicity may range from a reversible increase in blood transaminases to an overt toxic hepatitis (Rossiter, 2020:332). Hyperuricemia with non-gouty joint pain is a common side effect of PZA owing to decreased uric acid clearance. However, it is often self-limiting and may only rarely necessitate the discontinuation of PZA (Deck & Winston, 2012a:843; Rossiter, 2020:332).

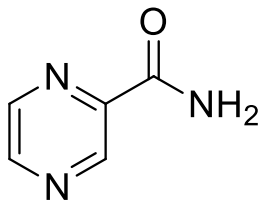

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Figure 2.7 Pyrazinamide (**7**)

2.11. Drug-resistant tuberculosis

Drug-resistant Mtb isolates are emerging despite the effectiveness of antitubercular drugs. It further complicates TB treatment and threatens global public health. Several mechanisms, including compensated evolution, epistasis, clonal interference, cellular membrane permeability, efflux pumps, drug modification and degradation, drug target site mimicking, and phenotypic drug tolerance, among others, facilitate antitubercular drug resistance in Mtb isolates. The main culprit, however, is that prolonged TB treatment regimens often result in patient non-compliance, resulting in the rapid evolution of Mtb from mono-to-multi-to extensively drug-resistant (Singh *et al.*, 2019:1554).

Charles Darwin's theory of evolution (survival of the fittest) and the emergence of drug-resistant Mtb isolates are closely related because, like any unique characteristic that arises during the process of selective evolution, Mtb-resistant strains can only propagate among Mtb populations if their resistance phenotypes (like virulence factors) offer the mutants a greater

chance of survival than their susceptible counterparts. As a result of ongoing drug use in response to an increase in DR-TB cases, Mtb strains that are more resistant to available antitubercular drugs are constantly evolving (Khawbung *et al.*, 2021:1; Smith *et al.*, 2013:54).

Multi-drug resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) are the two main subtypes of DR-TB. MDR-TB occurs when Mtb strains are resistant to the FLDs INH and RIF, two of the most potent antitubercular drugs. MDR-TB progresses to XDR-TB as Mtb strains become resistant to additional SLDs (WHO, 2020). The WHO recently amended the definition of XDR-TB, which should have gone into effect globally in January 2021: "XDR-TB is a kind of DR-TB in which Mtb strains are resistant to RIF, INH, at least one FQN, and either bedaquiline (BDQ) or linezolid (LZD) (or both)" (WHO, 2019b). Furthermore, some studies have identified Mtb strains that are completely resistant to all currently available antitubercular drugs, resulting in a TB condition known as total drug-resistant TB (TDR-TB) (Migliori *et al.*, 2013:169-179; Velayati *et al.*, 2009:420-425). However, due to a scarcity of drug susceptibility testing and the difficulties in defining such a highly resistant pathogen, the WHO has yet to recognise TDR-TB and continues to refer to it as XDR-TB (Cegielski *et al.*, 2012; WHO, 2015b; WHO, 2021a).

2.12. Second-line drugs for the treatment of drug-resistant TB (DR-TB)

The WHO published consolidated guidelines in 2019 on SLDs to be used in the treatment of DR-TB, and the MDR-/XDR-TB treatment paradigm has significantly changed since then. The recommendations include both longer and shorter treatment regimens and divide the drugs used in the longer regimens into three categories – groups A, B and C (**Table 2.1**). Additionally, it emphasises that for the great majority of DR-TB patients, oral treatment should be their first option (WHO, 2019b). This is a significant improvement since it minimises the need for injectable drugs (group C aminoglycosides) when developing a longer regimen for treating DR-TB patients (Mase & Chorba, 2019:776). However, a personalised "rescue regimen" including injectable drugs is still advised for treating TB cases resistant to RIF who are

suspected or confirmed to be resistant to BDQ or LZD from group A, and/or clofazimine (CFZ) from group B, in TB-endemic areas like South Africa (DOH, 2019).

SLDs are less effective than FLDs, more costly, and have a greater toxicity profile, making tolerance and treatment adherence a problem. Furthermore, since SLDs have limited sterilising activity, DR-TB treatment often takes 18–24 months (Seung *et al.*, 2015:5). The cost of treatment with SLDs alone is anticipated to be 50–200 times higher for DR-TB patients when compared to treating individuals with DS-TB (Pinto & Menzies, 2011:130).

Table 2.1 Second-line drugs for the treatment of drug-resistant tuberculosis

GROUP A	GROUP B	GROUP C
Levofloxacin (LFX) OR Moxifloxacin (MFX)	Clofazime (CFZ)	Ethambutol (ETH)
Bedaquiline (BDQ)	Cycloserine (CS) OR Terizidone (TRD)	Delamanid (DLM)
Linezolid (LZD)		Pyrazinamide (PZA)
		Imipenem-cilastatin (IPM-CLN) OR Meropenem (MPM)
		Amikacin (AM) OR Stretomycin (SM)
		Ethionamide (ETO) OR Prothionamide (PTO)
		para-Aminosalicylic acid (PAS)

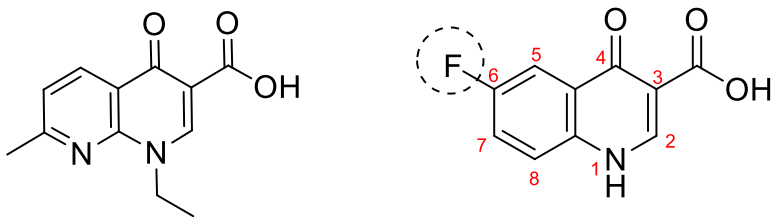
2.12.1. Quinolones

2.12.1.1. Discovery of fluoroquinolones (FQNs)

Nalidixic acid (NA) (**8**) was discovered in the 1960s during the analogue synthesis of a lead structure, which was found as an impurity in the synthesis of the antimalarial drug chloroquine. NA stood out for its moderate antimicrobial activity against Gram-negative pathogens, including *E. coli*, with the exception of *Pseudomonas aeruginosa* (Bush *et al.*, 2020:2). NA has a rapid renal elimination rate and a significant risk of neurological, dermatological and GI

adverse effects. As a result, one may conclude that NA was never an appropriate drug for treating systemic infections. However, because of its urinary antiseptic properties, NA was eventually approved for the treatment of uncomplicated urinary tract infections (UTIs). Notwithstanding, NA provided the chemical groundwork for the synthesis of NA analogues with the aim of maximising its activity (Sharma *et al.*, 2017:2). However, in terms of activity and plasma concentration levels, these analogues demonstrated minimal improvement over NA. The NA analogues, together with NA (which, according to its structure, is a 1,8-naphthyridone and not a genuine quinolone) were dubbed first-generation quinolones (Bush *et al.*, 2020:2).

It was not until the 1980s that the structure of NA was modified to increase absorption and bioavailability and hence broaden its activity against Gram-positive pathogens. Fluorination of the quinolone scaffold at position C6 was one of these modifications, which resulted in the discovery of FQNs (**9**) (Pallo-Zimmerman *et al.*, 2010:E1). Norfloxacin, the first novel clinically accessible second-generation quinolone with broad antimicrobial activity, was far more used than NA. Following the success of this drug, numerous other FQNs were synthesised over the following three years, several of which are still routinely used to treat infections (Telfer, 2011:14).



8 **9**

Figure 2.8 Nalidixic acid (**8**) and the fluoroquinolone scaffold (**9**)

2.12.1.2. Classification and structural modifications of fluoroquinolones

The different generations of FQNs have been classified based on their increasing antimicrobial activity against Gram-negative and Gram-positive strains of bacteria, with the first-generation (NA and its analogues) having the narrowest pharmacological spectrum and the later generations having greater structural complexity and increased antimicrobial activity. The addition of carefully chosen substituents to critical sites of the quinolone nucleus (N1, C6, C7, and C8) (see **Fig. 2.8**) enables the quinolone to target certain groups of pathogens. **Table 2.2** summarizes the classification and structural modifications associated with the common antimicrobial activities of major FLQs (information adapted and tabulated from Ball, 2000; Mohammed *et al.*, 2019; Pham *et al.*, 2019; Rusu *et al.*, 2021).

Table 2.2 Classification and structural modification-associated activities of FQNs.

Classification of quinolones	Drug	Structural modifications	Activity
1st generation	Nalidixic acid	- Nitrogen atom (C8)	Limited to Gram-negative *Enterobacteriaceae.*
		- Methyl group (C7)	No activity for Gram-negative *pseudomonas spp.*
		- Ethyl group (N1)	No Gram-positive pathogen activity.
2nd generation (See **Fig. 2.9**)	Norfloxacin (**10**) Ciprofloxacin (**11**) Ofloxacin	- Fluorine atom (C6)	Predominant Gram-negative activity (including *pseudomonas spp.)*
		- Piperazine ring (C7)	Improved Gram-positive activity to *S. aureus*.
		- Cyclopropyl (**11**) and ethyl groups (**10**) (N1)	Atypical pathogens (*Mycoplasma pneumoniae* and *Chlamydia pneumoniae*).
3rd generation (See **Fig. 2.9**)	Levofloxacin (**12**) (L-isomer of ofloxacin)	- Fluorine atom (C6)	Increased predominant Gram-negative activity.
		- Alkylated piperazine ring (C7)	4-fold increased Gram-positive activity.

Classification of quinolones	Drug	Structural modifications	Activity
		- Cyclic $–OCH_2$ substituent (C8)	Increased atypical pathogen activity. Mycobacterial activity (Mtb).
4th generation (See **Fig. 2.9**)	Moxifloxacin (**13**)	- Fluorine atom (C6) - Azabicyclonyl side chain on pyrrolidine (C7) - Cyclopropyl group (N1) - $–OCH_3$ (MeO) substituent (C8)	Superior potency against Gram-positive pathogens. Atypical pathogens. Anaerobic activity (nitrogen atom). Mycobacterial activity (Mtb).

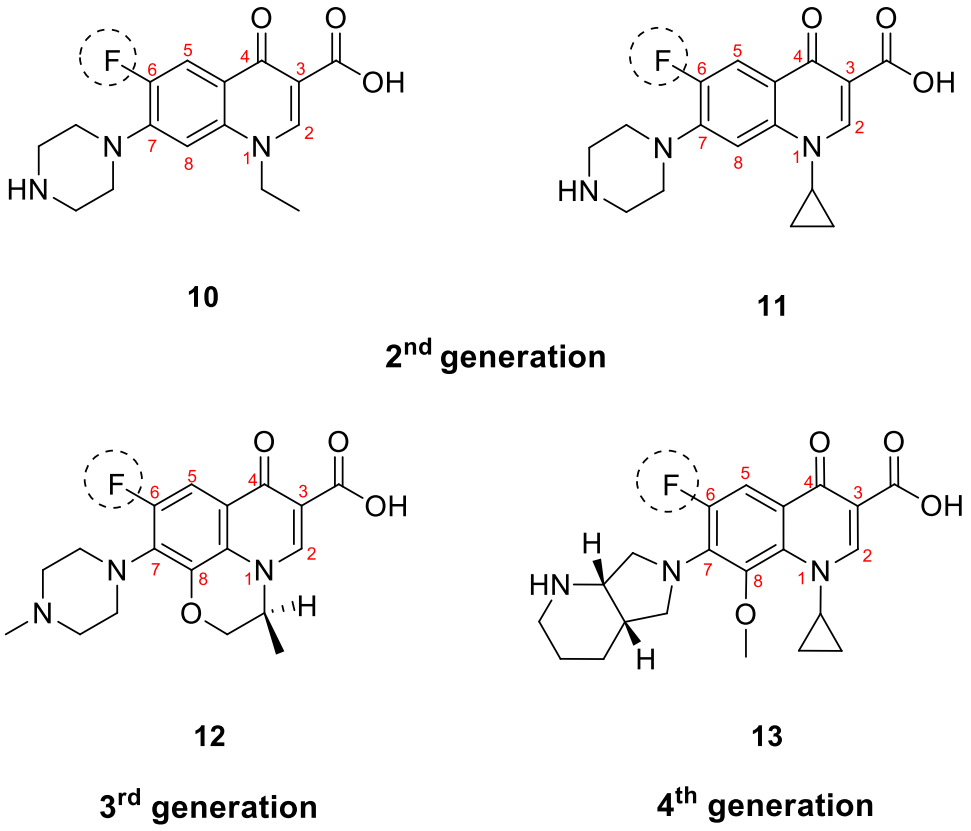


Figure 2.9 Norfloxacin (**10**), ciprofloxacin (**11**), levofloxacin (**12**), and moxifloxacin (**13**)

2.12.1.3. Mechanism of action of fluoroquinolones

FQNs target DNA topoisomerase IV and DNA gyrase, two vital type II bacterial topoisomerase enzymes involved in DNA synthesis during bacterial replication and transcription (Hooper & Jacoby, 2016:2). Helicase enzymes uncoil the DNA double helix to initiate DNA replication, which results in excessive supercoiling of the remaining DNA double helix. In order for the process to continue, the tension building up in this remaining DNA double helix must be released (Ezelarab *et al.*, 2018:15). The only enzyme capable of inducing negative supercoiling at the remaining supercoiled DNA double helix is DNA gyrase. DNA gyrase is made up of two A subunits and two B subunits, which are encoded by the genes *gyrA* and *gyrB*, respectively. The FQNs bind to the DNA gyrase subunits and inhibit negative supercoiling, preventing the supercoiled DNA double helix from relaxing and ultimately resulting in cell death. On the other hand, DNA topoisomerase IV acts to separate the daughter chromosomes following bacterial DNA replication, enabling segregation into daughter cells. FQNs inhibit DNA topoisomerase IV, which prevents Mtb cells from dividing and ultimately results in cell death (Hooper, 2000:S24).

DNA gyrase is situated ahead of the DNA replication complex of enzyme-binding sites on chromosomal DNA, while topoisomerase IV is positioned behind it. As a result, FQN interactions with DNA gyrase appear to lead to a more rapid inhibition of DNA replication than FQN interactions with topoisomerase IV (Hooper & Jacoby, 2016:2). In Gram-negative pathogens such as *E. coli*, FQNs interact with DNA gyrase as their main target and topoisomerase IV as their secondary target. The opposite is true for Gram-positive pathogens such as *S. aureus*. However, exceptions to this rule do occur. For example, it was revealed that DNA gyrase in *Streptococcus pneumonia* (a Gram-positive bacterium) is more susceptible to FQNs than topoisomerase IV. In addition, Mtb lacks the topoisomerase IV enzyme. Thus, it was revealed that DNA gyrase is FQN's only target in its antitubercular activity against Mtb (Somasundaram & Manivannan, 2013:300).

2.12.1.4. Fluoroquinolones for the treatment of drug-resistant TB

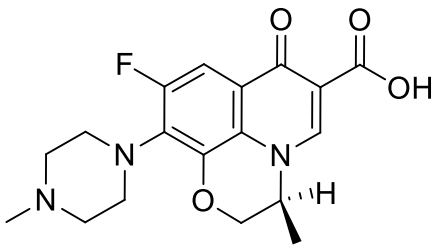
According to current MDR-TB treatment guidelines, all patients should be treated with later-generation FQNs. The two most commonly prescribed second-line FQNs for the treatment of MDR-TB are Levofloxacin (LFX (**12**)) and Moxifloxacin (MFX (**13**)) (Kang *et al.*, 2016:364; WHO, 2019b). FQN resistance in Mtb emerges rapidly when an FQN is added to other SLDs or added alone to a failed regimen and basically serves as the only agent in a multidrug regimen. Since Mtb lacks topoisomerase IV, mutations in the quinolone resistance-determining region of *gyrA* (the target of FQNs in Mtb) are the most common source of FQN resistance. The most prevalent mutation in FQN-resistant Mtb isolates is a codon 94 substitution in the *gyrA* gene, which reduces the drug's affinity to the gyrase DNA complex (Ginsburg *et al.*, 2003). The widespread use of FQNs (for example, for lower respiratory tract infections) further complicates Mtb FQN resistance and may result in resistance in undetected TB patients. In light of this, especially in areas with a high incidence of TB, such as South Africa, newer FQNs (e.g., LFX and MFX) should not be used for respiratory tract infections unless the patient has an allergy to *β*-lactam antimicrobial drugs (Rossiter, 2020:309; Ziganshina *et al.*, 2005:4).

After oral treatment, FQNs are well absorbed, with peak plasma concentrations reached 1–3 hours later. FQN concentrations surpass blood levels in urine, kidney, lung and prostate tissue, as well as in faeces, bile, macrophages and neutrophils (MacDougall, 2017a:1016). However, the presence of multivalent cations in milk and antacids containing aluminium and magnesium reduces oral bioavailability. This reaction is the consequence of chelation between the metallic cations and the 4-oxo and adjacent carboxyl groups present on all FQNs, which leads to the formation of insoluble, non-absorbable complexes in the GI tract (Pitman *et al.*, 2019:2). Hence, it is advised to take FQNs two hours before and at least four hours after consuming products containing these cations. The majority of FQNs are eliminated via renal routes by tubular secretion or glomerular filtration. MFX is, however, eliminated via nonrenal

routes, negating the need for dosage adjustments in patients with renal impairment (Deck & Winston, 2012b:836).

In general, FQNs are well tolerated. However, GI problems are the side effects of newer generation FQNs like LFX and MFX that are often reported. Four to six percent of participants in preregistration clinical trials reported experiencing diarrhoea, while eight percent reported nausea as the most frequent side effect. Milder neurotoxic side effects, including fatigue, headaches and insomnia, are followed by more severe forms like psychosis, depression and hallucination (Stahlmann, 2002:271-272). The FQNs' ability to reduce the seizure threshold and increase the risk of an epileptic episode in individuals using non-steroidal anti-inflammatory drugs or those with a history of epilepsy, is a serious neurological symptom. There is debate regarding the precise mechanism through which FQNs cause seizures. However, a substantial correlation between the chemical structures of several substituents at position C7 of the quinolone nucleus and gamma-aminobutyric acid (the principal inhibitory neurotransmitter in the cerebral cortex) seems to exist (Owens & Ambrose, 2005:S146-S147).

Because MFX inhibits the human cardiac K^+ channel hERG, it has been widely associated with electrocardiographic QT prolongation, and the risk rises significantly when used in conjunction with other SLDs like BDQ and DLM (Alexandrou *et al.*, 2006:904; Angula *et al.*, 2021:4). FQNs have also been associated with arthropathy (joint disease) and cartilage degeneration (tendonitis). As a result, these drugs are typically not advised for individuals under the age of 18. Contrarily, tendonitis is a very uncommon adult complication despite risk factors such as advanced age and concomitant steroid usage (Deck & Winston, 2012b:836).



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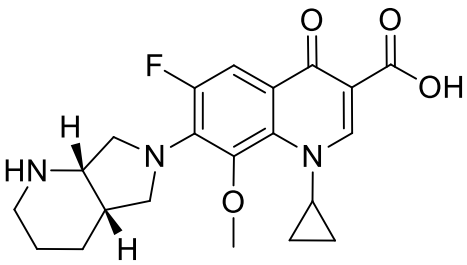

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Figure 2.10 Levofloxacin (**12**) and moxifloxacin (**13**)

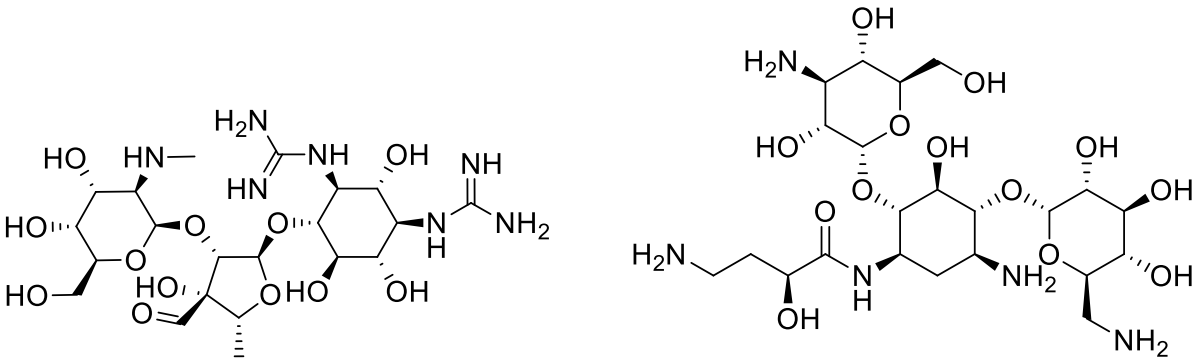
2.12.2. Aminoglycosides: streptomycin (SM) and amikacin (AM)

The structure of aminoglycosides consists of a hexose ring to which different amino sugars are joined through glycosidic bonds. Based on the aminocylitol nucleus, aminoglycosides are divided into two major structural classes: streptidine (for SM) and deoxystrepatamine (for AM and others) (Childs-Kean *et al.*, 2019:2). The first antimicrobial drug with demonstrated activity against Mtb was SM (**14**). Its current therapeutic value is limited due to the early onset of SM resistance brought on by its initial widespread usage in the treatment of numerous diseases (Cohen *et al.*, 2020:1-2). Therefore, SM is often exclusively used to treat endocarditis when combined with a *β*-lactam as well as DR-TB. SM is also active against other nontuberculous diseases such as brucellosis, tularaemia and plague (*Y. pestis*) (Deck & Winston, 2012c:824). In contrast to SM, AM (**15**) exhibits activity against highly resistant Gram-negative pathogens such as *A. baumanii* and *P. aeruginosa*. It is also highly effective against the majority of aerobic Gram-negative pathogens belonging to the *Enterobacteriaceae* family, as well as *Nocardia* spp. and other mycobacteria (Sizar *et al.*, 2021).

All aminoglycosides exhibit concentration-dependent bactericidal activity via protein synthesis inhibition regardless of structural differences (Childs-Kean *et al.*, 2019:2). The 30S ribosomal subunit of mycobacterial pathogens is the major intracellular site of action of aminoglycosides. Aminoglycosides disrupt the protein synthesis initiation complex by binding to polysomes in

the 30S ribosomal subunit and causing mRNA (messenger ribonucleic acid) misinterpretation and premature termination. As a result, incorrect amino acids are added to growing polypeptide chains, creating a protein that is not functional. The resultant aberrant proteins may be introduced into the cell membrane of Mtb, altering its permeability and enhancing the transport of aminoglycosides into the pathogenic cell. The interference with the protein synthesis of Mtb caused by these events eventually results in cell death (MacDougall, 2017b:1039-1040). The 16S mRNA, which is encoded by the *rpsL* gene, makes up the 30S ribosomal subunit. To optimise the effectiveness of tRNA (transfer ribonucleic acid) binding and mRNA interpretation, the 16S mRNA binds to the ribosomal protein S12, which is encoded by the *rrs* gene. Mutations in the *rpsL* and *rrs* genes are often seen in mycobacteria that have developed high levels of aminoglycoside resistance. Additionally, low-level SM resistance is linked to *gidB* gene mutations. *GidB* functions as an rRNA (ribosomal ribonucleic acid) methyltransferase for 16S rRNA. In addition, clinical isolates of Mtb with low levels of SM resistance were shown to have efflux pump-mediated resistance (Gumbo, 2017:1075).

The oral pharmacokinetic properties of aminoglycosides are unsatisfactory because of their polarity. For example, none of them are absorbed well enough after oral administration, their concentrations in the CSF are low, and almost the entire oral dosage is eliminated in the faeces (MacDougall, 2017b:1039). Aminoglycoside has some potentially dangerous side effects, the severity of which is dose- and drug-dependent. Ototoxicity and nephrotoxicity are the characteristic features of aminoglycoside toxicity. These side effects typically manifest themselves within five days of treatment initiation, at high dosages, in patients who have renal insufficiency and in elderly patients (Deck & Winston, 2012c:824). In the most severe cases, ototoxicity can cause irreversible vestibular impairment in addition to deafness. Aminoglycosides can cause nephrotoxicity due to proximal tubule necrosis, which can range from small to large lesions. Manifestations of nephrotoxicity include mild proteinuria, an increase in cellular excretion and elevations in blood urea (Waters & Tadi, 2022).



14 **15**

Figure 2.11 Streptomycin (**14**) and amikacin (**15**)

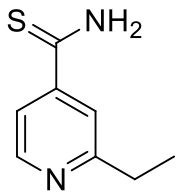
2.12.3. Thioamides: ethionamide (ETO) and prothionamide (PTO)

The nicotinamide derivatives ETO (**16**) and PTO (**17**), which is its propyl analogue, are structurally related to INH. The antitubercular activity of these drugs, like that of INH, requires enzyme activation, but thioamides are activated in a different manner by flavin-containing monooxygenase enzymes (encoded by *EthA*), leading to the oxygenation of thioamides to the corresponding sulfenic acid, sulfoxide metabolite (*S*-oxide) (**18**), and then to a highly reactive sulfonic acid that subsequently spontaneously decomposes to 2-ethyl-4-amidopyridine (Palmer *et al.*, 2012:1148).

The NADH-dependent-enoyl-ACP reductase of FAS II, which is the same target enzyme for INH, is inhibited by the *S*-oxide metabolite (active drug of thioamides). This inhibits the production of mycolic acid, which affects mycobacterial cell wall synthesis (Gumbo, 2017:1077; Nishida & Ortiz de Montellano, 2011:2). Thioamide resistance is brought on by mutations in the *inhA* gene (its target site), which is linked to INH resistance. Mutations in the genes encoding its activation (*ethA*) or the *ethA* regulator, *ethR*, may also contribute to Mtb resistance to ETO. Compared to *katG* mutants, which may be found in up to 94% of Mtb clinical isolates that are INH-resistant, no dominant *ethA* mutation is found in clinical isolates that are ETO-resistant (Vilchèze & Jacobs, 2014:13). Surprisingly, the absence of cross-

resistance is the main distinction between ETO and INH. INH is effective against the majority of ETO-resistant pathogens. Some INH-resistant pathogens do, however, only show slight susceptibility to ETO (Baulard *et al.*, 2000:28326-28327).

Food has no effect on the absorption of ETO from the GI tract, and the drug is freely distributed throughout the body, reaching the same concentration in both the plasma and the CSF. ETO can only be consumed orally. However, it is poorly tolerated due to severe neurologic side effects (e.g., seizures, anxiety, acute psychosis, depression and optic neuritis) and GI irritation (such as nausea, vomiting, metallic taste, abdominal pain, diarrhoea and weight loss). Thioamides should not be used in combination with other antitubercular drugs like INH because they may cause additive neurological adverse effects, or with cycloserine or terizidone because they raise the probability of neurological side effects. Pyridoxine may be used to treat ETO-related neurological symptoms (Deck & Winston, 2012a:843; Rossiter, 2020:337).



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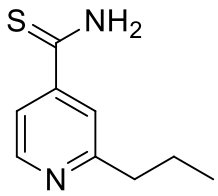

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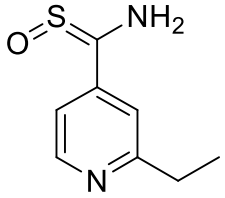


18

Figure 2.12 Ethionamide (**16**), prothionamide (**17**), and ethionamide *S-oxide* (**18**)

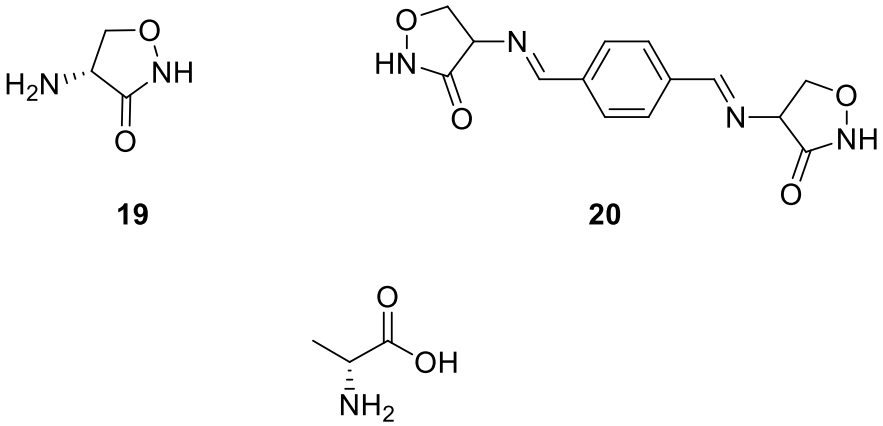
2.12.4. Cycloserine (CS) or terizidone (TRD)

Both CS (D-4-amino-3-isoxazolidone) (**19**) and TRD (**20**) are D-alanine analogues (**21**). It was found that *Streptococcus orchidaceous* produced the antimicrobial drug CS. TRD, a dimer of CS, is often more tolerated than CS, although its effectiveness is debatable. While these D-alanine analogues are almost exclusively used to treat DR-TB, they are also effective against a wide variety of Gram-positive and Gram-negative pathogens, including *Enterococci*, *E. coli*, *S. aureus*, *Nocardia spp*. and *chlamydia* (Deck & Winston, 2012d:805; Gumbo, 2017:1078; Hwang *et al.*, 2013:1257).

CS and TRD both inhibit alanine racemase, an enzyme that converts L-alanine to D-alanine used for the synthesis of the cell wall of Mtb, as well as D-alanine-D-alanine ligase, which is necessary for the adenosine 5′-triphosphate (ATP)-dependent synthesis of the D-alanine-D-alanine dipeptide bond. These two vital enzymes are also necessary for the synthesis of the cell wall of Mtb (Prosser & De Carvalho, 2013:7145). CS resistance has been seen in 10–80% of clinical isolates of Mtb and has been associated with mutations in alanine racemase and D-alanine-D-alanine ligase, despite the fact that the molecular basis of CS resistance among clinical isolates of Mtb is poorly known (Desjardins *et al.*, 2016:2-3; Gumbo, 2017:1078).

Compared to other SLDs, CS is very toxic and has a significant risk of neuropsychiatric disorders, hence the moniker of CS, psych-serine. Nevertheless, a meta-analysis revealed that CS was more effective than some of the most widely used antitubercular drugs, and the WHO has included CS or TZD as a group B drug for extended MDR-TB treatment regimens despite its known toxicity (Court *et al.*, 2021:688). One gram of CS per day causes psychiatric side effects in 50% of consumers, ranging from headaches and sleep disturbances to severe psychosis, convulsions and suicidal tendencies (Gumbo, 2017:1078). Thus, according to WHO recommendations, CS should not be used if a patient has a history of seizures or epilepsy or a serious current mental illness such as depression or psychosis (Hwang *et al.*, 2013:1257). Although CS is not registered in South Africa, it may be accessed with the South

African Health Products Regulatory Authority approval on a named-patient basis. However, TZD is accessible and pyridoxine supplementation is advised to address neuropsychiatric side effects when used to treat DR-TB (Rossiter, 2020:337-338).



19 **20**

21

Figure 2.13 Cycloserine (**19**), terizidone (**20**), and D-alanine (**21**)

2.12.5. *para*-Aminosalicylic acid (PAS)

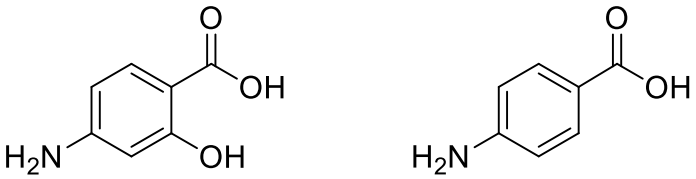
PAS (**22**) is an antagonist of folate synthesis that was rediscovered as an antitubercular drug in the 1940s. It is structurally related to *para*-Aminobenzoic acid (PABA) (**23**) and is virtually active only against Mtb (Deck & Winston, 2012a:844). PAS is often reserved for the treatment of XDR-TB and the management of complicated MDR-TB cases (De Kock *et al.*, 2014:6242). Zhang *et al.* (2019:827) reported on *in vitro* data revealing that an INH–PAS drug combination had synergistic activity against the majority of Mtb isolates in their study. In other words, the presence of PAS significantly elevates the Mtb isolates' susceptibility to INH. This discovery may be beneficial in shortening the extensive duration of MDR-TB treatment regimens that are currently required.

PABA participates in the Mtb *de novo* folate biosynthesis pathway (FBP) of Mtb. In the upper stream region of the FBP, dihydropteroate synthase (DHPS), which is encoded by *FolC*,

catalyses the conversion of PABA to dihydropteroate. Meanwhile, dihydrofolate synthase (DHFS) catalyses the ATP-dependent addition of L-glutamate to dihydropteroate (converted PABA) to produce dihydrofolic acid (Minato *et al.*, 2015:5099).

The precise sites of PAS activity on the Mtb FBP are yet unknown. However, it is thought that when PAS is administered, its structural resemblance to PABA enables it to act as a deceptive substrate for DHPS via its conversion through *FolC*. As a result, the incorporation of PAS instead of PABA culminates in the formation of hydroxyl (OH)-containing dihydrofolate antimetabolites (the active form of PAS), which further inhibits the dihydrofolate reductase (DHFR) enzyme down the line of the FBP. This ultimately results in Mtb's growth being inhibited since it is unable to synthesize purines and DNA (Chakraborty *et al.*, 2013:1; Heo *et al.*, 2022). *ThyA*, *FolC*, *DfrA*, and *RibD* gene mutations may be responsible for PAS resistance. Cells receive DHFR activity from *DfrA* and *RibD*, which is necessary for the conversion of dihydrofolate to tetrahydrofolate downstream of the FPB. Therefore, mutations that result in enhanced *DfrA* or *RibD* gene expression would shield Mtb against PAS inhibition. Decreased *FolC* expression or DHFS enzymatic activity, result in PAS not being converted to its active form. Regarding *ThyA*, it is not quite known why mutations in this gene promote PAS resistance (Nguyen, 2016:1589).

PAS is easily absorbed in the GI tract and reaches a plasma concentration of at least 50 µg/ml (Deck & Winston, 2012a:844). It undergoes *N*-acylation in the liver, where it may generate hepatotoxins (*N*-acetyl PAS). The advantages of PAS are very constrained due to the predominance of GI problems, which often impair patient compliance. The likelihood of undesirable GI symptoms linked to PAS use ranges from 10% to 30% (Gumbo 2017:1077). In severe cases, peptic ulcers and haemorrhage might occur. Increased body temperature, joint pain, skin rashes, hepatitis, hepatosplenomegaly, adenopathy and granulocytopenia are all common signs of PAS hypersensitivity. Very high PAS levels discharged in the urine have also been linked to crystalluria (Deck & Winston, 2012a:844).



22 **23**

Figure 2.14 *para*-Aminosalicylic acid (**22**) and *para*-Aminobenzoic acid (**23**)

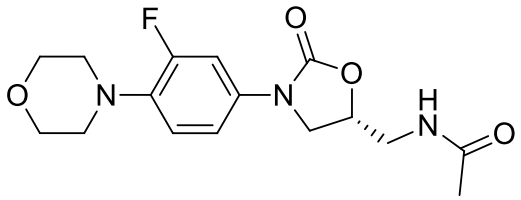
2.12.6. Linezolid (LZD)

LZD (**24**) is a member of the oxazolidinone class of drugs. Oxazolidinones are novel synthetic antimicrobial drugs used to treat pneumonia acquired in a hospital or community setting as well as complicated and minor skin and soft tissue infections caused by Gram-positive pathogens. *Nocardia* infections are one of the off-label indications of LZD (Deck & Winston, 2012e:817). The WHO recently repurposed LZD as a group A antitubercular drug and added it to the list of SLDs to treat MDR-TB due to its activity against resistant Mtb isolates (WHO, 2021b).

Unlike other protein synthesis inhibitors, LZD has a distinct MOA. The four primary stages of bacterial protein synthesis are initiation, elongation, termination and recycling. While LZD inhibits the initiation phase of protein synthesis, other protein synthesis inhibitors such as chloramphenicol, macrolides, lincosamides and tetracyclines interfere with the elongation (polypeptide extension) phase (Ament *et al.*, 2002:664-665). LZD targets the 50S ribosomal subunit, which is present on the 23S rRNA of bacterial pathogens, impeding the assembly of its complex with the 30S subunit and, therefore, the formation of the 70S initiation complex, a crucial step in the translation process for bacterial pathogens (De Vriese *et al.*, 2006:1115). The binding of LZD to the 50S subunit delays the formyl methionyl-tRNA recognition, ultimately slowing the initiation of translation (Angula *et al.*, 2021:12). As for its activity against Mtb, a team of researchers used commercial oligonucleotide microarrays to examine transcriptional mutations throughout the whole genome. They discovered that a number of vital genes,

including those involved in protein synthesis, the metabolism of sulphite, the cell envelope and virulence of Mtb, were significantly altered (Jadhavar *et al.*, 2015:4384).

The co-administration of antacids has no effect on the oral absorption of LZD and results in 100% bioavailability, requiring no dose adjustments when switching to intravenous administration. Approximately 30% of LZD is eliminated in the urine – the remaining is metabolised *via* renal and non-renal pathways, where it is oxidised to two major inactive metabolites (Ager & Gould, 2012:88-97). LZD tolerance and safety, on the other hand, are dose – and duration dependent. A metallic taste, headaches, moniliasis, cramps and GI symptoms such as nausea, vomiting, diarrhoea and cramps, are all common adverse effects of LZD (Rossiter, 2020:316). Long-term treatment has been associated with ocular and peripheral neuropathy, as well as lactic acidosis. The inhibition of mitochondrial protein synthesis by LZD is likely to be the source of these adverse effects. Serotonin syndrome may occur when LZD is combined with serotonergic drugs, most often antidepressants such as serotonin reuptake inhibitors (Deck & Winston, 2012e:817).



24

Figure 2.15 Linezolid (**24**)

2.12.7. Bedaquiline (BDQ)

Andries *et al.* (2005:223) discovered the diarylquinoline BDQ (**25**) as having a unique range of potent and selective *in vitro* activity against Mtb (0.030 to 0.120 µg/ml, compared to 0.500 µg/ml for RIF and 0.120 µg/ml for INH). The American Food and Drug Administration (FDA)

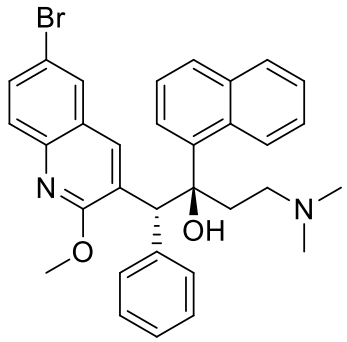
has authorized BDQ as the first drug approved for the treatment of TB in almost four decades. The approval was based on the findings of two Phase IIb trials that demonstrated improved efficacy in the clinical context of DR-TB, with cure rates of 62% and 44% in patients treated with BDQ and a placebo, respectively (Cholo *et al.*, 2017:334). This potent activity is reflected through *in vivo* reductions in treatment duration where it has been proven that the addition of BDQ for 6 months to an MDR-TB treatment regimen considerably enhanced short-term treatment results with sputum culture conversion to negative just after 2 months of treatment (Svensson *et al.*, 2015:1106). Importantly, BDQ has bactericidal activity against non-replicating Mtb at therapeutically achievable doses (Sarathy *et al.*, 2019:2). This is often accomplished by combining BDQ with other bactericidal drugs such as third-generation FQNs (like LFX), PTO or PZA (Li *et al.*, 2019:99).

BDQ is the first of a new class of antitubercular drugs with a novel MOA. It inhibits mycobacterial F_1F_o-ATP synthase, an important membrane-bound enzyme, interfering with energy synthesis and altering intracellular metabolism in both replicating and non-replicating Mtb (Field, 2015:173). BDQ exerts its MOA by binding to the membrane-bound c-ring rotor of the F_o component of ATP synthase. This binding inhibits c-subunit rotation (a required conformational change for the flow of protons) and proton transfer. Concurrently, ATP production is hindered, and intracellular ATP levels decrease, ultimately leading to Mtb cell death (Hards *et al.*, 2018:7326; Worley & Estrada, 2014:1189). Surprisingly, BDQ's bactericidal activity is delayed, i.e., cell death does not occur immediately after ATP depletion (Sarathy *et al.*, 2019:2).

Unlike in many other bacteria, ATP synthase is required for Mtb survival, and any mutation in the c-subunit may result in BDQ resistance (Chahine *et al.*, 2014:108). The *atpE* gene, which encodes the ATP synthase c-subunit, may contain a variety of point mutations that confer high-level resistance, all of which impact amino acid residues adjacent to the BDQ binding site (Worley & Estrada, 2014:1189). Mutations in *Rv0678*, a transcriptional regulator of the genes

encoding the *MmpS5–MmpL5* efflux pump, indicate non-target-based resistance to BDQ (Nguyen *et al.*, 2017:1626). According to Hartkoorn *et al.* (2014:2980), resistance to azoles and CFZ is caused by the active efflux of these compounds by these multi-substrate transporter pumps. Thus, even before BDQ was extensively used, cross-resistance between BDQ and CFZ had been established. In rare situations, *Rv0678* mutations co-occur with genetic polymorphism in other genes encoding the unknown transporter *Rv1979c* and the cytoplasmic peptidase *PepQ* (*Rv2535c*), both of which are linked to CFZ resistance (Degiacomi *et al.*, 2020:1).

There is a significant lag period between oral intake and BDQ absorption after which the drug is extensively distributed throughout the body tissues (Gumbo, 2017:1076). BDQ is further distinguished by a very long terminal $T_{1/2}$ of about 5.5 months, which is mostly due to the delayed redistribution from the tissues where it is stored (Gumbo, 2017:1076). In the liver, CYP450 iso-enzyme 3A4 predominantly converts BDQ to the *N*-mono-desmethyl metabolite M2. M2 is effective against Mtb, although about 5 times less effective than BDQ (Van Heeswijk *et al.*, 2014:2312). M2 has been observed to prolong the QT interval, creating safety concerns for torsades de pointes, particularly when taken with other QT-prolonging drugs such as FQNs, DLM and CFZ (Koele *et al.*, 2022:1-2). BDQ was also related to an unexplained increase in late mortality in one randomized Phase II study. Patients who were given BDQ died at a higher rate than those who were given a placebo (Diacon *et al.*, 2014:723). Other commonly reported BDQ side effects include joint pain, nausea, headaches, haemoptysis (coughing up blood), chest pain, skin rashes and elevated liver enzymes (Gumbo, 2017:1077; Rossiter, 2020:335).



25

Figure 2.16 Bedaquiline (**25**)

2.12.8. Clofazimine (CFZ)

CFZ (**26**) is a fat-soluble riminophenazine dye with antibacterial activity against a wide range of Gram-positive pathogens, including *S. aureus*, coagulase-negative *staphylococci*, *Streptococcus pyogenes* and *Listeria monocytogenes*. CFZ is also used to treat *Mycobacterium ulcerans*-caused chronic skin ulcers (Buruli ulcers) (Gumbo, 2017:1075-1076). In general, CFZ is thought to be unsuccessful in the treatment of pulmonary TB. This disagreement stems from early research that revealed inconsistencies in CFZ's therapeutic effect in diverse animal models (Cholo *et al.*, 2011:290). As a consequence, CFZ was abandoned as a treatment for TB in humans, most likely because INH and other TB drugs introduced at the same time were superior to CFZ (Deck & Winston, 2012a:846; Murray *et al.*, 2015:1755).

The antitubercular activity of CFZ was re-evaluated as the global incidence of DR-TB rose. A pivotal trial conducted in Bangladesh in 2010 found that a treatment regimen combining CFZ with additional antitubercular drugs was especially beneficial for reducing the treatment duration of MDR-TB, which may have aided the achievement of a 9-month TB treatment regimen (Gopal *et al.*, 2013:1006). As a result, the WHO now considers CFZ to be a key component of the new short-course MDR-TB regimen (WHO, 2019b).

The CFZ MOA is not well understood. However, it has been proposed that the principal site of action is Mtb's outer membrane. CFZ inhibits two potential targets: the mycobacterial respiratory chain and ion transporters. Phenazine dyes, such as CFZ, are auto-oxidizable compounds that generate reactive oxygen species when re-oxidized in air. As a result, CFZ serves as an artificial electron acceptor, and Mtb's respiratory system oxidizes CFZ rather than NADH, resulting in a decrease in the amount of ATP available for all cellular functions (Mirnejad *et al.*, 2018:1354; Yano *et al.*, 2011:10276). *Rv0678* modulates the expression of the *MmpS5-MmpL5* efflux pump, and its overexpression or mutation increases CFZ MIC by two- to four-fold. Mutations in *Rv1979c*, which encodes a possible amino acid membrane transporter with permease activity, have also been linked to CFZ resistance. Mutations in the proline aminopeptidase gene *pepQ* have additionally been demonstrated to confer low-level cross-resistance between CFZ and BDQ *in vitro* (Park *et al.*, 2022:2).

The oral bioavailability of CFZ is 45–60%. High-fat meals increase it two-fold while antacids reduce it by 30%. CFZ is extremely lipophilic and forms crystal-like deposits within the tissues of the mononuclear phagocyte system. The $T_{1/2}$ of these crystalline deposits is at least 2–3 months (Baik *et al.*, 2013:1218; Gumbo, 2017:1076). This might provide a rationale for the shortened CFZ-based antitubercular regimens. On the one hand, tissue accumulation and the resulting long $T_{1/2}$ provide a benefit in reducing treatment duration, while crystal deposition of the drug in tissues, on the other hand, is at the root of the most common undesired side effects of reddish-brown skin and conjunctival discoloration (Rossiter, 2020:336).

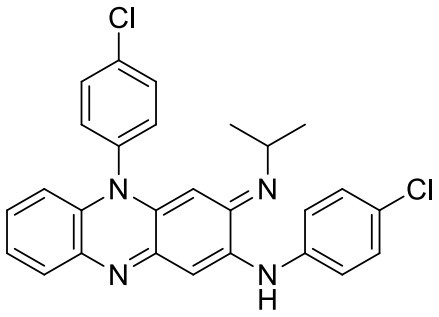


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Figure 2.17 Clofazimine (**26**)

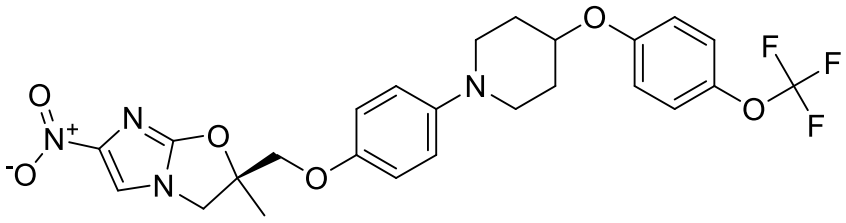
2.12.9. Nitroazoles: delamanid (DLM) and pretomanid (PTM)

Nitroazole-based drugs have long been used to treat bacterial and parasitic infections. Both DLM (a nitroimidazooxazole) (**27**) and PTM (a nitroimidazooxazine) (**28**) are two newer nitroimidazole-containing drugs that inhibit both replicating and non-replicating Mtb. Both DLM and PTM are lipophilic, which may aid penetration through the highly lipophilic cell wall of Mtb and account for their significant efficacy. They are, however, poorly water soluble and tend to bind to human plasma proteins (Showalter, 2020:2-3). The European Medicines Agency authorized DLM in 2014 as the second novel antitubercular drug after BDQ, based on a Phase 2 study that showed that a higher percentage of MDR-/XDR-TB patients taking DLM compared to a placebo achieved sputum culture conversion after just 8 weeks of therapy. The FDA authorised PTM in 2019 and the EMA approved it in 2020 as part of a six-month regimen (called BPaL) that also included LZD and BDQ. The Phase 3 Nix-TB study was the pivotal trial for PTM approval. Six months following therapy, 90% of patients who received the BPaL regimen had a favourable result. Thus, for the first time in history, there is evidence that effective chemotherapy for at least sub-categories of DR-TB may be accomplished in the same time period as DS-TB treatment utilising the nitroimidazole based drugs DLM and PTM (Black & Buchwald, 2021:7).

Following bio-activation through the denitrification process, these pro-drugs seem to have a multi-target MOA. In the redox cycling of cofactor F_{420}, the enzyme deazaflavin-dependent nitro-reductase (Ddn) converts DLM and PTM to their des-nitroimidazole metabolites, which yield reactive nitrogen species such as nitric oxide (NO). The release of NO during drug metabolism under anaerobic conditions is thought to facilitate Mtb respiratory poisoning. However, under aerobic conditions, the major MOA of these nitroazoles is the inhibition of mycolic acid biosynthesis, which is required for the synthesis of Mtb's outer lipid membrane (Grzelak *et al.*, 2019:721; Mudde *et al.*, 2022:882). Interestingly, human nitro-reductases were shown to be incapable of activating DLM, perhaps due to the fact that humans use nicotinamide adenine dinucleotide phosphate as an electron donor, which has a higher redox

potential than cofactor F_{420}. The fact that pro-drug activation of these drugs is specific to mycobacterial Ddn may represent some of the selective activity against Mtb while remaining non-mutagenic to humans (Mudde *et al.*, 2022:882). Mtb resistance to DLM and PTM is induced by mutations in the genes *fgd1*, *fbiA*, *fbiB* and *fbiC*, which are responsible for the production and reactivation of cofactor F_{420}, preventing DLM and PTM pro-drug activation by Ddn (Kadura *et al.*, 2020:2035-2040). Recently, a novel mechanism of resistance was revealed in Mtb strains with mutations in *Rv2983* (*fbiD*), a gene that had not previously been linked to nitroimidazole resistance but was shown to be a guanylyl-transferase necessary for the production of cofactor F_{420} (Rifat *et al.*, 2020:1).

The oral bioavailability of DLM varies between 35% and 60%. DLM is highly protein-bound (> 97%), and its metabolism is predominantly controlled by plasma albumin. DLM had no adverse effects on the CNS or respiratory systems in animal studies at concentrations 18.5 or 3.2 times greater than the highest blood concentrations predicted in humans at a dosage of 100 mg twice a day. However, QT interval prolongation is one of the adverse side effects of DLM usage (Lewis & Sloan, 2015:781). PTM has a more favourable pharmacokinetic profile than DLM. It is easily absorbed, well tolerated and has good bioavailability (Bahuguna & Rawat, 2019:271). Some of the most commonly reported side effects of PTM usage in various research include vomiting, acne, nausea, migraines, musculoskeletal discomfort and headaches. Optic problems, hepatic enzyme imbalances, dermatitis, GI upset and severe cutaneous side effects are more likely to be induced by PTM (as seen in patients taking the BPaL regimen in the Nix-TB trial) (Fekadu *et al.*, 2022:179).



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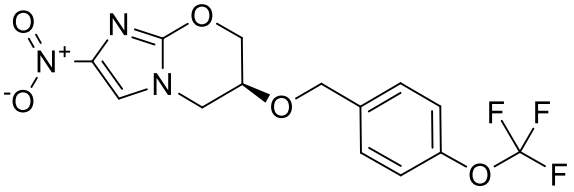

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Figure 2.18 Delamanid (**27**) and pretomanid (**28**)

PART TWO: HOSPITAL-ACQUIRED INFECTIONS (HAIs)

2.13. Background

An HAI is defined as an infection that is neither present nor incubating when a patient is admitted to a healthcare facility. The time for diagnosing an HAI will therefore be reliant on the incubation period of the infection, which is commonly suspected to be 48 to 72 hours after admission. Although these infections are mainly associated with hospitalisation (hence the phrase "hospital-acquired infections"), they may develop at any given time after admission to any healthcare facility (Vincent, 2003:2068). HAIs are widespread in the intensive care unit (ICU), where acute life-threatening conditions are assessed and treated (WHO, 2022b). ICU patients have a five to ten times higher risk of acquiring HAIs owing to intrinsic factors such as their clinical state, weakened immune systems and external factors such as the requirement for advanced surgical procedures or the administration of medical devices (Alves *et al.*, 2021:1744; Dadi, 2021:1).

The most prevalent HAIs include central line-associated bloodstream infections (CLABSIs), catheter-associated urinary tract infections (CAUTIs), surgical site infections (SSIs) and ventilator-associated pneumonia (VAP) (CDC, 2014). Hospitalised patients acquire such infections through pathogenic organisms that organise themselves into biofilms and colonize on medical equipment. The type of pathogen causing an HAI differs based on the type of medical intervention and medical device used as well as where and how a procedure was given to a patient (Dadi *et al.*, 2021:2).

The ESKAPE pathogens are responsible for the majority of HAIs. The acronym ESKAPE refers to six hypervirulent and antibiotic resistant pathogens, including ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and other ***E****nterobacter* spp. These pathogens display significant degrees of antimicrobial resistance because they frequently exhibit novel pathophysiology, modes of

transmission and resistance patterns that makes HAIs caused by them nearly untreatable (Navidinia *et al.*, 2017:779; Orosz *et al.*, 2022:27).

Currently, the ESKAPE pathogens are resistant to the majority of existing antimicrobial drugs, including FQNs, tetracyclines, lipopeptides, *β*-lactams, *β*-lactam-*β*-lactamase inhibitor drug combinations, macrolides, oxazolidinones and last-line antibiotics such as carbapenems, glycopeptides and polymyxins (De Oliveira *et al.*, 2020:2). These pathogens are very effective in up-regulating or adopting genes that code for antimicrobial resistance mechanisms, particularly in the presence of antibiotic drug selection pressure, as observed in most healthcare settings. With a plethora of antimicrobial resistance mechanisms at their disposal, they have the capacity to deploy several of these mechanisms against the same antimicrobial drug or a single mechanism against multiple drugs, representing a new paradigm in contemporary medicine (De Oliveira *et al.*, 2020:2; Peleg & Hooper, 2010:1).

The morbidity and mortality rates associated with HAIs are quite high, and as a consequence, the healthcare system loses billions of dollars. The expenditures associated with the abovementioned HAIs total around $9.8 billion per year, with SSIs being the most expensive of the bunch. In addition to this, an increase in the number of days spent in the hospital is almost always connected to the presence of these infections (Monegro *et al.*, 2020). Marturano and Lowery (2019:1) investigated whether ESKAPE pathogens were associated with worse hospital outcomes than non-ESKAPE pathogens in a data set of more than 1.1 million patient encounters in the United States and reported that ESKAPE pathogens accounted for 42.2% of species isolated from bloodstream infections (BSIs) and were additionally related to a 3.3–day increase in hospital stay, a $5,500 increase in healthcare expenditure per case, and a 2.1% increase in mortality.

2.14. Call for antimicrobial drug development

According to the WHO 2022 global report on infection prevention and control, seven patients in high-income countries and 15 patients in low-and middle-income countries are expected to acquire at least one HAI for every 100 hospitalised patients (WHO, 2022b). It is also predicted that if new drugs and means to address this global menace are not discovered by 2050, drug-resistant pathogens will kill 10 million individuals globally each year. In fact, if left unchecked, the rapid spread of antimicrobial resistance might make many pathogens much more fatal than they are now (Murray *et al.*, 2022:630).

The WHO (2017c) published a global priority pathogen list (GPPL) in 2017 to identify pathogens for which new antimicrobial development is urgently needed. The GPPL prioritises bacterial pathogens into three categories: critical, high and medium, with the ESKAPE pathogens receiving critical and high priority rank. The critical priority category includes carbapenem-resistant *A. baumannii*, *P. aeruginosa*, and *Enterobacteriaceae* spp., including *K. pneumonia*. This category of multidrug-resistant organisms poses a significant threat in hospitals, especially among patients who need ventilators and catheters for treatment and management. Methicillin-resistant and vancomycin-intermediate/resistant *S. aureus*, as well as *E. faecium*, are classified as high priority (WHO, 2017c). This clearly illustrates the global need for antimicrobial drugs with novel molecular targets against ESKAPE pathogens, as well as to alleviate the burden of HAIs.

Table 2.3 WHO priority pathogens list (tabulated from WHO, 2017c).

PRIORITY	RESISTANT PATHOGENS	DRUGS TO WHICH IT IS RESISTANT
Priority 1: CRITICAL*	**- *Acinetobacter baumannii***	Carbapenem-resistant
	- *Pseudomonas aeruginosa*	Carbapenem-resistant
	- *Enterobacteriaceae*[#]	Carbapenem-resistant, ESBL-producing
Priority 2:	**- *Enterococcus faecium***	Vancomycin-resistant

PRIORITY	RESISTANT PATHOGENS	DRUGS TO WHICH IT IS RESISTANT
HIGH	**- *Staphylococcus aureus***	Methicillin-resistant, vancomycin-intermediate and resistant
	- *Helicobacter pylori*	Clarithromycin-resistant
	- *Campylobacter spp.*	Fluoroquinolone-resistant
	- *Salmonellae*	Fluoroquinolone-resistant
	- *Neisseria gonorrhoeae*	Fluoroquinolone-resistant, cephalosporin-resistant
Priority 3: MEDIUM	- *Streptococcus pneumoniae*	Penicillin-non-susceptible
	- *Haemophilus influenzae*	Ampicillin-resistant
	- *Shigella spp.*	Fluoroquinolone-resistant
*****Mtb is not included in the priority list since the need for drug development is already established. [#]*Enterobacteriaceae* include *E. coli*, *K. pneumoniae*. *Enterobacter* spp. *Citrobacter* spp. *Morganella* spp. *Providencia* spp. *Proteus* spp. *Serratia* spp.		

2.15. The ESKAPE pathogens

2.15.1. *Enterococcus faecium*

Enterococcus spp. are Gram-positive, facultative anaerobes that generally colonise the intestines of healthy individuals. They are members of the lactic acid bacteria group, which produces bacteriocins (Fischer & Phillips, 2009:1749-1751). The majority of *enterococci* are employed in the manufacture of pharmaceutical probiotics and have an important role in the maintenance of human intestinal homeostasis. Furthermore, they serve as the initiating culture in the fermentation of meats and cheeses, and are commonly used during food preservation (Krawczyk *et al.*, 2021:1).

However, *enterococci* are opportunistic pathogens that, outside of their normal commensal habitats, propagate themselves as life-threatening pathogens. Among the *enterococci*, *E. faecalis* and *E. faecium* are the most common pathogens that cause infections in humans,

and they have aggressively expanded as global hospital-acquired pathogens by successfully adapting to circumstances in a healthcare facility (Zhou, 2020:1). They are often associated with HAIs such as BSIs, UTIs, endocarditis (endocardium inflammation) and sepsis. In addition, catheter-related infections that may cause meningitis have also been recorded in new-borns and babies (Krawczyk *et al.*, 2021:6).

Enterococci resistance to penicillin/ampicillin, aminoglycosides (high level resistance) and glycopeptides is emerging in a growing number of isolates, indicating a limited treatment spectrum in the future (Orsi & Ciorba, 2013:485). Ampicillin resistance is explained by penicillin binding protein (PBP) mutations and/or overexpression, or by mutations in the *β*-subunit of this protein. Ampicillin resistance is uncommon in *E. faecalis*, but it is found in 90% of *E. faecium* isolates (Gagetti *et al.*, 2019:180). Vancomycin (a glycopeptide) resistance is found in about 35.5% of *enterococci* and is caused by the production of a mutated cell wall precursor that is unable to attach to glycopeptides. *VanA*, *VanB*, *VanD*, *VanN*, and *VanM* mutations are often found in vancomycin-resistant *enterococci* (VRE) isolates, with *VanA* and *VanB* being the most prevalent and abundant (Darvishi *et al.*, 2020:165). As a consequence, the available antimicrobials for treating VRE infections are limited to bacteriostatic drugs such as LZD, tigecycline and quinupristin-dalfopristin (inactive against *E. faecalis*) (Orsi & Ciorba, 2013:489).

2.15.2. *Staphylococcus aureus*

S. aureus is a Gram-positive coccus that colonises the cutaneous microbiota of the nose and perineum of humans, and their cells are typically organised in unique grape-like clusters. Transmission of *S. aureus* may occur *via* direct contact or by airborne vectors (Santajit & Indrawattana, 2016:5). *Staphylococci* are classified as either coagulase (Coa)-positive or coagulase (Coa)-negative depending on their ability to induce plasma clotting (Pickering *et al.*, 2021:1). *S. aureus*, for example, stimulates the human coagulation cascade by secreting two coagulases, staphylo-Coa and von Willebrand factor-binding protein (vWbp), which binds to

prothrombin in the blood. The interaction of any of these enzymes with prothrombin causes a morphological shift in the thrombin's active site, converting plasma-soluble fibrinogen into insoluble fibrin segments – initiating clot formation (Vanassche *et al.*, 2013:93).

The propensity of *S. aureus* to clot host plasma has been used to identify it. In this dimension, staphylo-Coa is crucial for distinguishing the virulent pathogen from less virulent Coa-negative *S. aureus* spp., and may be identified using a tube test or a slide test. The former is often employed to detect free extracellular Coa, while the latter detects bound Coa (Kateete *et al.*, 2010:3). *S. aureus* infections are effectively treated with *β*-lactam antibiotics such as penicillin, cephalosporins and carbapenems. Overuse of these drugs, however, led to the emergence of *β*-lactamase-producing *S. aureus* spp., including the well-known MRSA strain, which is resistant to all *β*-lactam antimicrobials. This resulted in a more than 80% increase in *S. aureus* related infections in both communities and hospitals over the course of two decades (Santajit & Indrawattana, 2016:5).

MRSA is a major cause of life-threatening HAIs. Vancomycin, a glycopeptide cell wall synthesis inhibitor that has been in use for more than 50 years, remains the FLD for MRSA infections and the majority of *S. aureus* infections. However, there are substantial concerns since *S. aureus* susceptibility to this drug has decreased and total resistance has increased during the last 20 years (Choo & Chambers, 2016:267; McGuinness *et al.*, 2017:270). For example, MRSA exposure to high vancomycin concentrations resulted in a predicted rise in vancomycin-intermediate *S. aureus* (VISA) and vancomycin-resistant *S. aureus* (VRSA). VISA and VRSA originated as a response to modifications in bacterial cell wall thickness and composition, which restricted drug penetration through the cell wall to the site of action (Conly & Johnston, 2002:283).

S. aureus's key virulence factors include the capacity to form a biofilm on implanted medical devices or damaged host tissue, providing a niche for survival. This is particularly noticeable

in hospital settings where antibiotics are extensively used. This matrix encourages antimicrobial resistance, which contributes to the pathogen's role as a prevalent cause of chronic and recurrent infections (McCarthy *et al.*, 2015:1). Furthermore, staphylo-Coa and vWbp are well-known *S. aureus* pathogenesis attributes for catheter-related biofilm production and survival during BSIs (Pickering *et al.*, 2021:8-9).

2.15.3. *Klebsiella pneumoniae*

K. pneumoniae is a Gram-negative bacillus from the *Enterobacteriaceae* family. It colonises human mucosal surfaces such as the nasal cavity (up to 19%) and the GI tract (up to 77%). *K. pneumoniae* causes major HAIs such as pneumonia, UTIs (the most prevalent) and BSIs. However, *Klebsiella spp*. is the most common cause of VAP in ICU patients. Environmental strains of *K. pneumoniae* are much more sensitive to antimicrobial drugs than clinically isolated *K. pneumoniae*, indicating that drug resistance is often driven by selective antimicrobial drug pressure (Martin & Bachman, 2018:2-3). *K. pneumoniae* infections are often treated with *β*-lactam antibiotics such as penicillin, cephalosporins and carbapenems. However, they develop a range of *β*-lactamase enzymes that confer resistance to all *β*-lactams (Babic *et al.*, 2006:143-144). An example is the production of extended spectrum *β*-lactamases (ESBLs), which hydrolyse the oxyimino group contained in the structure of third-generation cephalosporins, rendering them ineffective before they reach the PBP target (Ashurst & Dawson, 2022).

The genes encoding ESBLs are located on the same plasmids as those encoding resistance to aminoglycosides and sulphonamides. This implies that ESBL-producing *Enterobacteriaceae* are often multidrug resistant, posing a significant challenge for the treatment of HAIs (Paterson, 2006:S21). As a result, carbapenems became one of the few drugs available to treat infections caused by ESBL *K. pneumoniae*. However, it has been shown that carbapenem-resistant *K. pneumoniae* causes over 80% of *Enterobacteriaceae*

related infections, thus limiting the therapeutic choices available for this family (Ashurst & Dawson, 2022).

2.15.4. *Acinetobacter baumannii*

A. baumannii is a Gram-negative opportunistic pathogen that spreads rapidly, causing HAIs such as septicaemia, UTIs, kidney infections, meningitis, bone infections and pneumoniae, particularly in ICU patients. Given their capacity to resist severe environmental conditions, *Acinetobacter* spp. are very adaptive and resilient. It can, for example, multiply at temperatures ranging from 150°C to 440°C (Hassani, 2014:80-81). Furthermore, *A. baumannii* seems to remain viable for relatively long periods on inanimate objects and surfaces, such as catheters, ventilators and tubes, and may withstand regular disinfecting attempts (Mohammed *et al.*, 2022:2).

The spread of *A. baumannii* in a healthcare setting is aided by both resistance to desiccation and the establishment of antimicrobial resistance by antimicrobial selection pressure (Navidinia, 2016:46). MDR *A. baumannii* is resistant to three or more antimicrobials, including *β*-lactams, aminoglycosides, FQNs and third-generation cephalosporins. As a consequence, colistin and tigecycline are the only active antimicrobial drugs available and have become the last lines of treatment against MDR *A. baumannii* (Cai *et al.*, 2012:1607).

2.15.5. *Pseudomonas aeruginosa*

P. aeruginosa is an anaerobic bacterium that can grow in the presence of oxygen. Human colonisation takes place in the GI tract and spreads to moist cutaneous areas, including the perineum and axilla (Pachori *et al.*, 2019:110). It can, however, be isolated from a variety of sources, including several life-threatening infections in cystic fibrosis patients, wounds, UTIs and pulmonary infections, as well as medical equipment such as inhalers, dialysis machinery, ventilators, anaesthetic equipment, toilets and sinks (Azam & Khan, 2019:350). *P. aeruginosa* is the fourth most prevalent pathogen identified in hospitals, accounting for 10% of all HAIs,

the second most common cause of VAP, and the third most common Gram-negative cause of BSIs. It can survive for 6 hours to 6 months due to its adaptability and excellent resilience (Pachori *et al.*, 2019:111).

P. aeruginosa can thrive at a wide range of temperatures. Their cells proliferate rapidly on water delivery systems' surfaces to produce a biofilm. Some investigations show that hospital plumbing reservoirs can be contaminated with *P. aeruginosa* and cause patient colonisation and infection. The use of tap water, that contaminates hospital staff hands have been identified as a transmission vehicle. Furthermore, the discharge of wash water and ventilator traps is thought to be a possible way for *P. aeruginosa* to be transported from patient to plumbing (Loveday *et al.*, 2014:8-9).

P. aeruginosa resists most antimicrobials by preventing them from penetrating its outer membrane. However, some FLQs (e.g., CFX and LFX), aminoglycosides (e.g., gentamicin, tobramycin and AM), *β*-lactams (e.g., piperacillin-tazobactam, ceftazidime, cefepime, ceftolozane-tazobactam, ceftazidime-avibactam, imipenem, meropenem and doripenem), and polymyxins remain active against a hand full of *P. aeruginosa* isolates. *P. aeruginosa* may furthermore generate resistance to these agents via modified pharmacological targets, active efflux, decreased permeability and degradation enzymes (Spagnolo *et al.*, 2021:171).

2.15.6. *Enterobacter* spp.

The *Enterobacter* genus comprises 22 species of opportunistic Gram-negative pathogens that colonize the normal commensals of the human GI microbiota. However, only a few pathogens within this species including *E. aerogenes*, *E. cloacae*, and *E. hormaechei* have been linked to HAIs (Davin-Regli *et al.*, 2019:2). Transmission occurs via hand carriage and fomites. It is also known that aspiration of colonised pharyngeal *Enterobacteriaceae* causes hospital-acquired pneumonia, while cutaneous organisms introduced into sterile areas may cause UITs, SSIs and CLABSIs (Mehrad *et al.*, 2015:1414).

An inducible, chromosomally encoded beta-lactamase known as *AmpC*, which confers resistance to penicillins, second- and third-generation cephalosporins, and aztreonam, is present in all *Enterobacter* species (Lim & Webb, 2005:895). In addition, *β*-lactamase inhibitors such as clavulanate have little effect on *Enterobacter* species (Lim & Webb, 2005:895). ESBLs and carbapenemases are used by a large number of *Enterobacter* organisms to confer resistance to almost all available antimicrobial drugs, except tigecycline and colistin. These include the Verona integron-borne metallo-*β*-lactamase, oxacillin hydrolysing enzymes, metallo-*β*-lactamase–1, and *K. pneumoniae* carbapenemase (Santajit & Indrawattana, 2016:6). However, with the discovery of the polymyxin-resistant gene *MCR-1*, which is responsible for plasmid-mediated colistin resistance in *Enterobacter* isolates, there have been a great number of studies pointing to the rapidly spreading nature of this transmissible mechanism conferring resistance to these last line drugs (Ma *et al.*, 2020:1).

2.16. Common ESKAPE-associated hospital-acquired infections

2.16.1. Central line-associated bloodstream infections (CLABSIs)

A central line is an intravenous catheter that originates at or near the heart or from one of the major arteries. It is used for intravenous drug infusion, the withdrawal of blood or hemodynamic monitoring. CLABSIs may occur within 48 hours of central line placement due to sterility breaches at the time of central line insertion or during the maintenance of the catheter, which is unrelated to any previous infection or condition (Clover *et al.*, 2022:15; Lobdell *et al.*, 2012:66). The rates of CLABSIs are mostly determined by two factors related to exposure risk: the (i) length and (ii) frequency of central line catheterisation. Other risk factors, in addition to this exposure, include the kind of catheter, the site at which it is inserted, and infection control measures (Blot *et al.*, 2022:1). The rate of CLABSIs was determined to be 5.05 cases per one thousand central line days, as stated in a study that was compiled by the International Nosocomial Infection Control Consortium and carried out in 45 different countries between 2012 and 2017 (Clover *et al.*, 2022:15).

Between 40% and 80% of all CLABSIs are caused by Gram-positive pathogens. These pathogens include *enterococci*, coagulase-negative *staphylococci*, and *S. aureus*. Gram-negative pathogens are responsible for 20% to 30% of CLABSIs and MRSA-related CLABSIs are rather common (Farrington & Allon, 2019:612). A meta-analysis of approximately 4000 publications indicated that the estimated additional healthcare costs associated with CLABSIs are $48,108 per annum, with excess mortality estimated at 0.15 per HAI case, implying that there are 150 excess fatalities for every 1,000 in-hospital CLABSI cases (AHRQ, 2017).

2.16.2. Catheter-associated urinary tract infections (CAUTIs)

A CAUTI is an infection associated with the use of indwelling urinary catheters. Approximately 1 million CAUTIs are diagnosed yearly, accounting for over 40% of all HAIs, thus making CAUTIs the most common type of HAI (Jacobsen *et al.*, 2008:26). Indwelling urinary catheters cause up to 80% of hospital-acquired UTIs. Fever is the most common indicator of a UTI in patients with indwelling catheters (Tenke *et al.*, 2017:138-140). 60–80% of hospitalised patients with a catheter receive antimicrobials, mainly for non-UTI indications. This extensive antimicrobial exposure implies that resistant pathogens are often detected in the urine of catheterised patients (Nicolle, 2014:1-2). *E. coli* (21.4%) and *Candida* spp. (21.0%) are the most prevalent pathogens associated with CAUTIs, followed by *Enterococcus* spp. (14.9%), *P. aeruginosa* (10.0%), *K. pneumoniae* (7.7%), and *Enterobacter* spp. (4.1%), amongst others less prevalent (CDC, 2009).

More than 100,000 colony-forming units (CFU)/ml of urine is generally regarded to be diagnostic of a UTI. However, lesser numbers of organisms might become troublesome in a catheterised patient if not adequately treated. Thus, having more than 1000 to 10,000 CFU/mL of urine are often characterised as a CAUTI (Lobdell *et al.*, 2012:71). CAUTIs pose a major morbidity and mortality risk to the general hospital inpatient population. This risk, however, is almost five to ten times higher among patients admitted to the ICU. Every year, over 13,000 people die as a result of CAUTIs, and it is estimated that each CAUTI case costs almost $600

to diagnose and treat, resulting in nearly $131 million in annual national medical costs. Younger people and females have been identified as risk factors for CAUTIs (Podkovik *et al.*, 2019:5).

2.16.3. Surgical Site Infections (SSIs)

SSIs are the third most prevalent HAI, accounting for 10 to 40% of all HAIs (Legesse Laloto *et al.*, 2017:2). About 1% to 3% of surgical operations result in SSIs. SSIs are defined as infections that develop within 30 days following operative procedures due to microbial pathogens infiltrating the wound at the time of surgery, or within a year if an implant is left in place and the infection is believed to be attributable to surgery. The frequency of SSIs after abdominal surgery is 15–25%, depending on the extent of contamination, which is much greater than other types of surgery (Alkaaki *et al.*, 2019:112). *S. aureus* is one of the most prevalent SSI causative pathogens in 2% to 5% of patients receiving extra-abdominal surgery and up to 20% of patients receiving intra-abdominal surgery. The global prevalence of SSIs ranges from 2.5% to 41.9%, with South Africa's incidence rate of 5.6 SSIs per 100 surgeries being much higher than published rates in developed countries (Legesse Laloto *et al.*, 2017:2). SSIs involving the subcutaneous tissues, organs or implanted material may be more severe than superficial skin infections around the epidermis (WHO, 2018).

SSIs continue to be a burden to post-operative patients despite the use of prophylactic antimicrobials before and after surgery, as well as other preventative measures like upgraded ventilation in the operating room, better disinfection techniques and surgical skills (Hope, 2019:1). Patients who acquire SSIs have a fivefold increase in death and a fivefold increase in readmissions to hospital (Nel, 2014:33). In addition, SSIs, on average, lengthen hospital stays by 7–10 days (Isik *et al.*, 2015:458). Furthermore, it has been stated that the extra healthcare expenses ascribed to SSIs vary from $11,778 to $42,177 per case, and the mortality rates of SSIs are estimated to be 26 excess deaths per 1,000 cases (AHRQ, 2017).

2.16.4. Ventilator-associated pneumonia (VAP)

VAP is the second most prevalent HAI, affecting 5–40% of patients receiving invasive mechanical ventilation for more than 2 days (Papazian *et al.*, 2020:888). Mechanical ventilation entails supplying oxygen *via* a nasopharyngeal tube placed in a patient's mouth or nose or through a tracheostomy (a hole in the front of the neck) (CDC, 2014). Mechanical ventilation is associated with 86% of hospital-acquired pneumonias, and VAP is often diagnosed when a patient acquires a new or progressive infiltrate on a chest radiograph, leucocytosis, and purulent tracheobronchial secretions (Koenig & Truwit, 2006:637).

Gram-negative bacilli such as *P. aeruginosa*, *E. coli*, *K pneumoniae*, and *Acinetobacter* are frequent pathogens of VAP, as are Gram-positive cocci such as *S. aureus*. The most prevalent pathogen of VAP is *P. aeruginosa* (Wu *et al.*, 2019:4). The length of mechanical ventilation prior to the onset of VAP is a significant predictor of the pathogen most likely to cause VAP. Within four days after endotracheal intubation, VAP infections caused by antimicrobial susceptible pathogens are more prevalent, but later VAP infections are more typically caused by multidrug resistant pathogens such as *P. aeruginosa*, *Acinetobacter spp*. and MRSA (Hunter, 2012:3). Fungi such as *Candida* spp. can also be a causative pathogen of VAP. A lung biopsy containing yeast or pseudo-hyphae, on the other hand, is diagnostic of *Candida*-associated VAP (Lobdell *et al.*, 2012:69).

When compared to patients without VAP, it is projected that VAP elongates mechanical ventilation by 7.6 to 11.5 days and hospital stay by 11.5 to 13.1 days (Klompas, 2018). In 2017, the average additional medical expenses associated with VAP were estimated to range from $19,325 to over $80,013 per case (AHRQ, 2017). The risk of mortality from VAP is one of the highest. The reported death rate for VAP varies from 24 to 50%, and in extreme circumstances, it may reach as high as 76% (Chastre & Fagon, 2002:867).

2.17. Compound classes of interest in this study

2.17.1. Nitroazole: Nitazoxanide (NTZ)

NTZ (**29**) is a thiazolyl-salicylamide containing a 5-nitrothiazole head group attached to a salicylamide via an amide bond. Nitrothiazole unit is structurally analogous to nitroimidazole – a moiety present in drugs such as metronidazole (MTZ) (**30**). Following oral administration, NTZ is rapidly metabolised to its desacetyl derivative tizoxanide (TIZ) (**31**), which is the active metabolite *in vivo* (Hemphill *et al.*, 2006:954).

NTZ was primarily used as an anthelmintic and parasiticide in 1975 (Rossignol & Cavier, 1976:1–8). Human trials conducted in the 1980s, however, discovered that it was beneficial in treating infections caused by *Hymenolepis nana* and *Taenia saginata* (Rossignol & Maisonneuve, 1984). Later, *in vitro* evaluations of NTZ demonstrated a very broad activity range, thus expanding its therapeutic use. Today, NTZ is often used as a single agent to treat mixed intestinal infections caused by protozoa and helminths, as an antibacterial agent primarily for *Clostridium difficile* and *Helicobacter pylori* infections, rotavirus diarrhoea in children, and chronic Hepatitis B and C infections (Singh & Narayan, 2011:67-68).

NTZ's antiviral properties were unintentionally discovered while treating cryptosporidium diarrhoea in HIV-positive patients co-infected with hepatitis B or C. Since then, scientists have investigated NTZ's potential as an antiviral agent. For instance, it has been claimed that NTZ can inhibit the most recent outbreak of SARS-CoV-2 at a low micromolar concentration of 2.12, following infection in Vero E6 cells (Mahmoud *et al.*, 2020:2-3).

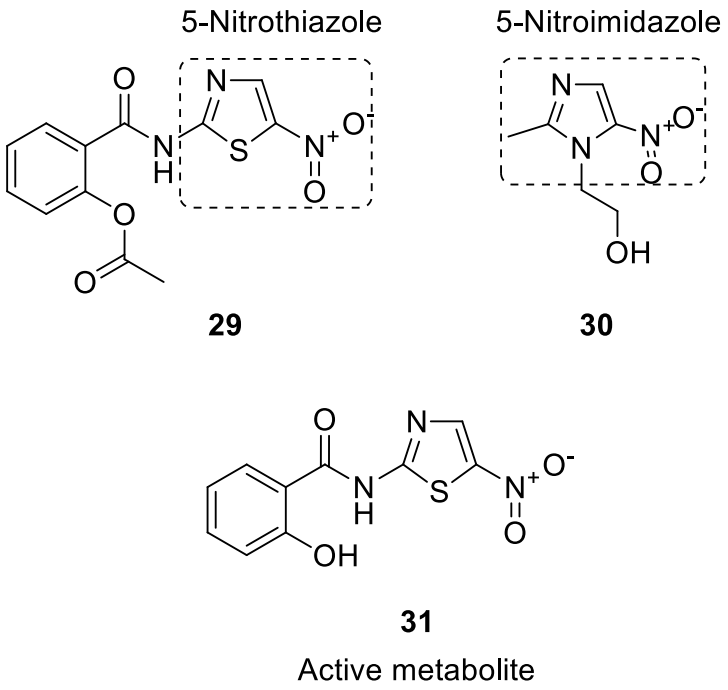


Figure 2.19 Nitroazoles: nitazoxanide (**29**), metronidazole (**30**), and tizoxanide (**31**)

2.17.1.1. Antimicrobial activity of nitazoxanide

The antimicrobial MOA of NTZ has been found to be selective inhibition of pyruvate-ferredoxin oxidoreductase (PFOR), which is required for protozoan and anaerobic bacterial energy metabolism (Singh & Narayan, 2011:67). In contrast to its anaerobic activity, NTZ is almost completely inactive against aerobically cultured *Staphylococcus*, *Enterococcus*, *Pseudomonas* and *Enterobacteriaceae* (De Carvalho *et al.*, 2009:5789). NTZ inhibition occurs independently of reduced ferredoxin and acetyl-coenzyme-A, unlike other drugs that target PFOR – such as MTZ. This is since inhibition of NTZ occurs earlier in the catalytic cycle (Hoffman *et al.*, 2007:869; Soria-Arteche *et al.*, 2013:6839). In other words, unlike MTZ, which interacts with the catalytic sites during PFOR metabolism, NTZ interferes with the binding of pyruvate to the thiamine pyrophosphate cofactor during the first phase of the PFOR cycle. This differential MOA may aid in avoiding cross resistance between NTZ and MTZ derivatives. Other possible NTZ MOAs remain unclear since some species lack the PFOR target (Anderson & Curran, 2007:1950).

2.17.1.2. Antitubercular activity of nitazoxanide

During a drug repurpose study, De Carvalho *et al.* (2009:5789) discovered that *in vitro* evaluation of NTZ and its active metabolite, TIZ, exhibited activity against replicating and non-replicating Mtb strains at low inhibitory concentrations, as well as that these compounds avoided resistance development. NTZ was shown to be effective against clinical isolates of drug-susceptible and drug-resistant Mtb strains, with a median MIC of 16 µg/ml. No resistance was identified when 10^{12} CFU of Mtb were exposed to NTZ (Walsh *et al.*, 2020:1). Furthermore, it was recently observed during a RIF–NTZ conjunctional study that RIF co-administered with NTZ eliminated latent Mtb cells in 28–35 days vs. the six months required therapy by other first-line TB combinatory regimens. This clearly indicates that NTZ has significant TB treatment shortening potential and could be useful in the development of novel antitubercular drugs (Iacobino *et al.*, 2019:1-4). In addition, NTZ exhibited antimycobacterial activity at 25 mg/kg when administered by gavage to *Mycobacterium leprae* infected mice, which implies that NTZ could also be considered for the treatment of leprosy. This was demonstrated in a study that was carried out by Bailey and colleagues (2017:1-9) in 2017.

NTZ's MOA against Mtb was unexpected since Mtb lacks a homologue for the anaerobic bacterial target, PFOR, which NTZ is known to target. Furthermore, Mtb-NTZ susceptibility is irrespective of whether the environment is microaerophilic or anaerobic. However, NTZ has a distinct MOA that may encompass numerous targets in Mtb. NTZ functions as an uncoupler, interfering with oxidative phosphorylation and thereby affecting membrane potential and intra-organism pH homeostasis, both of which are required for Mtb survival (De Carvalho *et al.*, 2011:850). Plasma protein interactions seem to limit the potential of NTZ for the treatment of TB in humans, despite the promising outcomes of NTZ studies against MDR Mtb (Umumararungu et al., 2020:550).

2.17.2. Thiazolidin-4-ones

Thiazolidinones are heterocyclic ring structures containing a sulphur atom (S) in the first position, a nitrogen atom (N) in the third position, and a carbonyl (C═O) functional group in the second, fourth or fifth position, yielding the derivatives thiazolidin-2-one, thiazolidin-4-one, and thiazolidin-5-one (Nirwan *et al.*, 2019:1239). The thiazolidin-4-one derivative (**32**) is regarded as a "wonder nucleus" due to its wide array of biological activities, including anti-inflammatory, antiproliferative, antiviral, anticonvulsant, antidiabetic, antihyperlipidemic, cardiovascular, anti-tubercular, antidiarrheal, antiarthritic, antiparasitic and antibacterial (Kulkarni *et al.*, 2022:2031). This extensive library of activity can be ascribed to its broad and non-specific inhibition of several pathogenic molecular targets, including peroxisome proliferator activated receptor, aldose reductase, cyclooxygenase, phosphoinositide 3-kinase or mitogen activated protein kinase, pim kinase, protein tyrosine phosphatase–1B, UDP-N-acetylmuramoylalanine D-glutamate ligase, amongst others (Kaur Manjal *et al.*, 2017:1; Trotsko, 2021:2-3).

The thiazolidin-4-one core is a well-known privileged scaffold in pharmaceutical chemistry and a valuable resource for designing novel drug-like compounds, particularly in rational privileged substructure-based diversity-oriented synthesis (Lesyk, 2020:33). For example, ralitoline, etozoline, pioglitazone and thiazolidomycin are just a few drugs based on this pharmacophore available on the market for the treatment of epilepsy, high blood pressure, hyperglycaemia and *streptomyces* spp. infections (Tripathi *et al.*, 2014:52). The physicochemical properties of thiazolidin-4-one are significantly affected by its numerous possible ring system modifications. However, it has been established that modifications to the C2 and C5 positions often result in compounds with optimised biological activity (Molina *et al.*, 2021:130).

Vicini *et al.* (2006:3863) demonstrated that C5-benzylidene substituted thiazolidin-4-one derivatives have significantly greater antibacterial activity compared to analogues wherein the C5 position is unsubstituted. Due to the potential Michael addition of nucleophilic protein

residues to the exocyclic double bond complex, the conjugation of the C5-benzylidene fragment to the C4 carbonyl functional group to form an α,β-unsaturated carbonyl group make such compounds electrophilic and possibly reactive (Kaminskyy *et al.*, 2017:552; Lesyk, 2020:34). Thus, these compounds may be regarded as Michael acceptors and covalent binders, which function similarly to *β*-lactams in their antimicrobial activity (Hammad *et al.*, 2020:2).

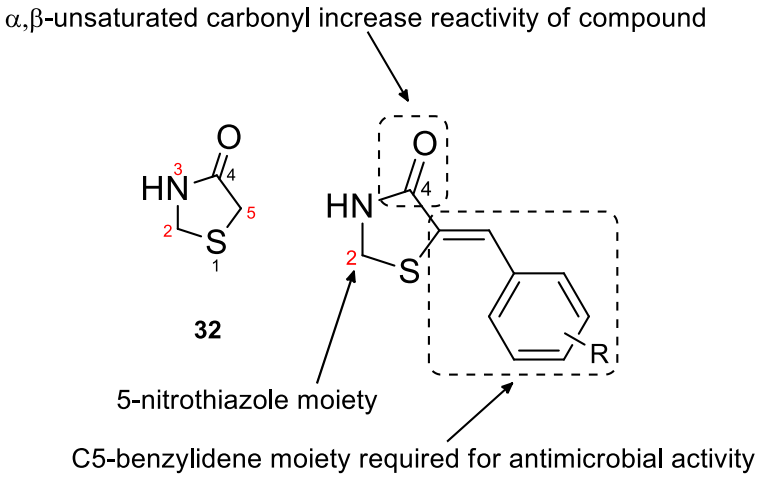
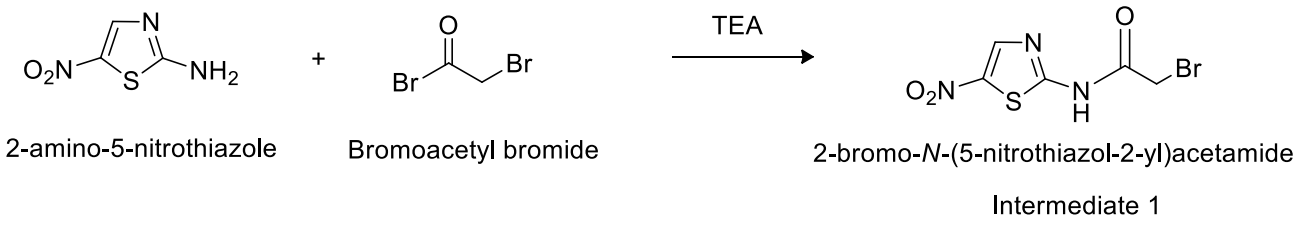


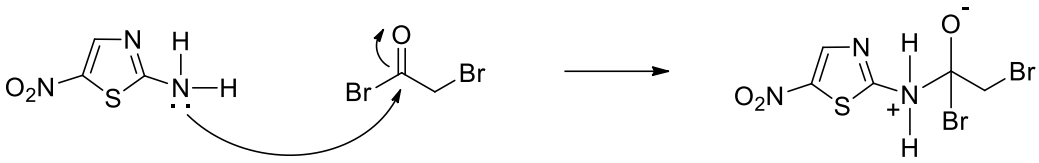
Figure 2.20 Thiazolidin-4-one (**32**) and structure-activity relationship

2.17.3. Mechanism for *N*-acylation reaction (intermediate 1)

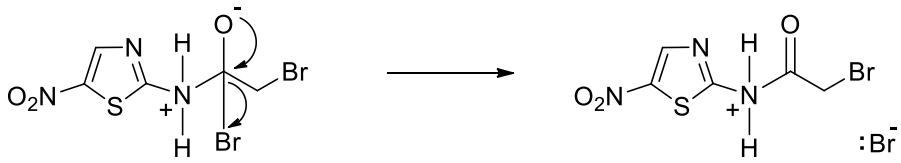


One of the most often utilised reactions in the synthesis of secondary and tertiary amides is the *N*-acylation reaction of amines (Taylor & Bull, 2014). It's interesting to note that varied regioselectivity for acylation agents may be seen in *N*-acylated products. However, the products are often *N*-acylation products with the reaction sites on the primary amine (NH_2) when it is treated with acylation reagents under alkaline conditions at room temperature or

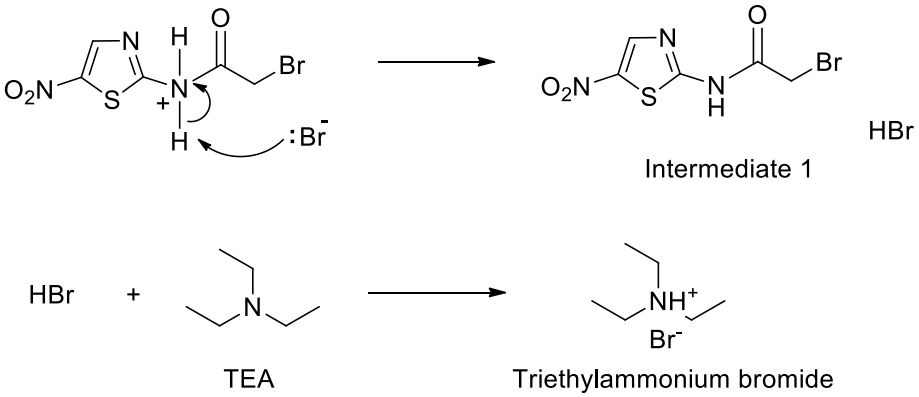
below (Cui *et al*., 2022:1). In the presence of triethylamine (TEA), the reaction between 2-amino-5-nitrothiazole and bromoacetyl bromide proceeds in two stages: an addition stage and an elimination stage. The amine's (NH_2) lone pair on the nitrogen atom engages in a nucleophilic attack on the carbonyl carbon (C═O) of bromoacetyl bromide during the addition stage. This mechanism totally repels the two electrons from one of the C═O double bonds onto the oxygen, leaving it negatively charged (Clark, 2000).



The elimination stage is divided into two stages. The C═O double bond reforms in the first stage, and a bromide ion is driven off.



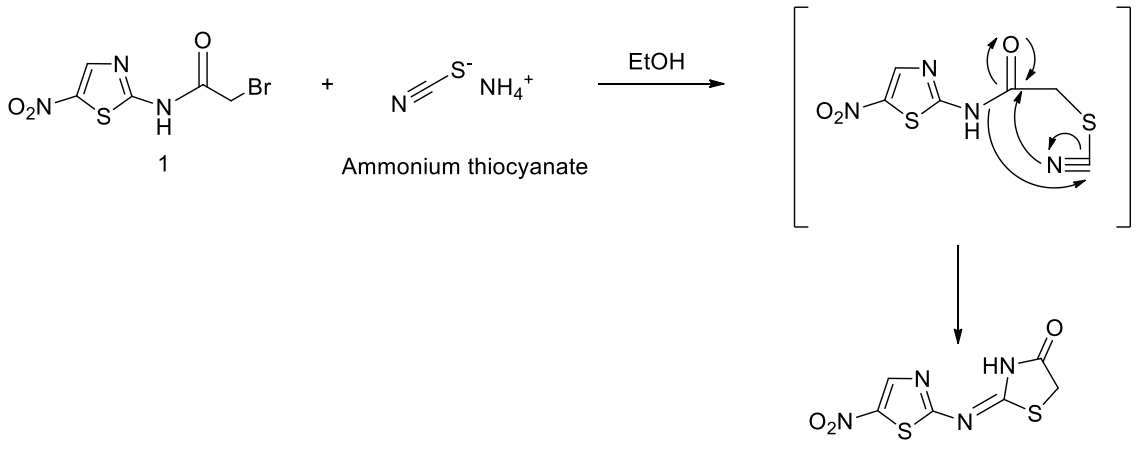
Finally, one of the hydrogen ions attached to the nitrogen is eliminated to afford the desired product (intermediate 1). The hydrogen is eliminated by the free bromide ion, generating HBr, which would instantly react with the TEA (an acid acceptor) in the process to produce insoluble triethylammonium bromide, the hydrobromide salt of triethylamine (Clark, 2000).



2.17.4. Mechanism for dehydrative cyclization reaction (intermediate 2)

Numerous approaches may be used to synthesize the 4-thiazolidinone ring system. One of these reactions is the cyclization of halogen-containing acetamide compounds in the presence of potassium thiocyanate (Baboo *et al*., 2017:247; Liu *et al*., 2000:1056) or ammonium thiocyanate (Çakır *et al*., 2015:3; Kulabaş *et al*., 2010:372; Küçükgüzel *et al*., 2013:9).

In this study, the product (intermediate 1) from the *N*-acylation reaction will be treated with ammonium thiocyanate in refluxing ethanol (EtOH) to attain the desired intermediate 2. It is well known that the reaction does not terminate at the nucleophilic substitution step. Following the removal of water, the reaction proceeds by spontaneous intramolecular cyclization of the α-thiocyanatoamide intermediate and Dimroth-like rearrangements (Abu-Melha, 2018:3–4; Gzella *et al*., 2014:813; Tripathi *et al*., 2014:53). The reaction's most probable mechanism is shown here.

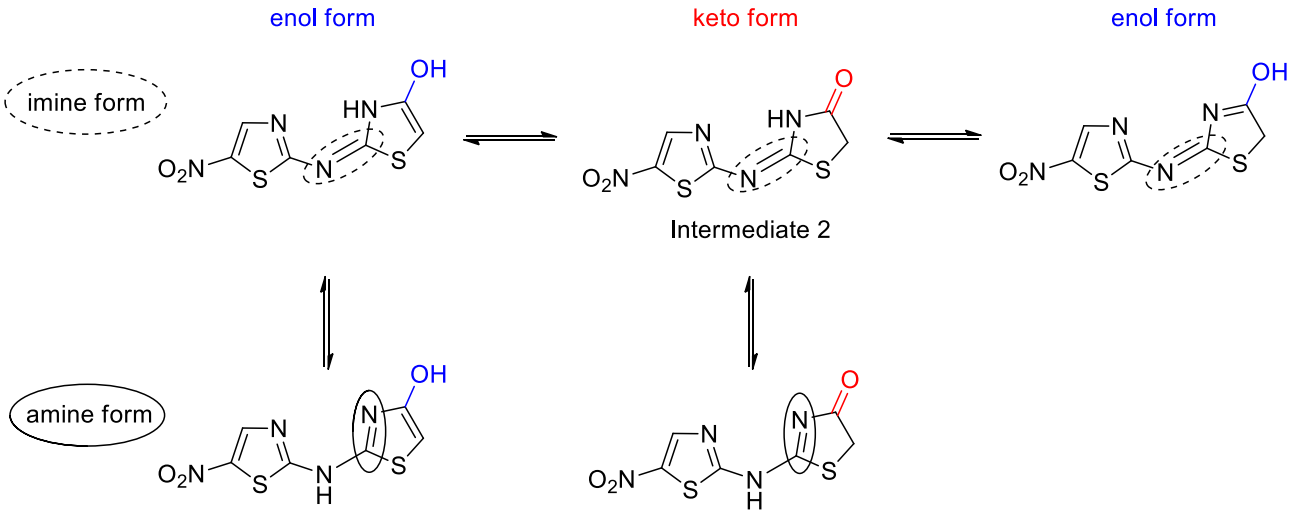


(*E*)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one

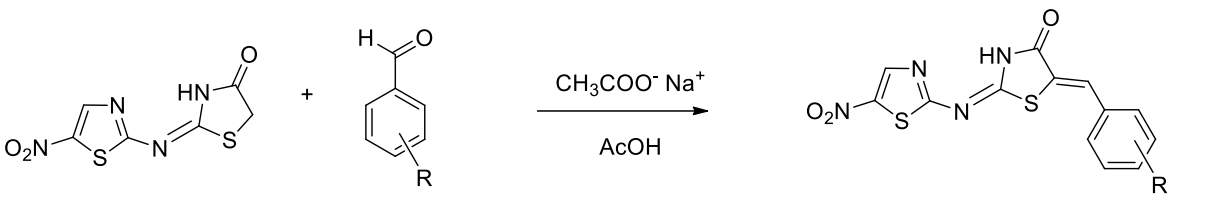
Intermediate 2

The synthesis of intermediate 2 comprises a tautomeric mixture of keto/enol and amino/imino forms. The structure below is an example of a probable tautomeric equilibrium for the synthesis of intermediate 2 (adapted from Türe *et al*., 2021:1029). The lack of hydroxylic (OH) functional groups in intermediate 2's FTIR and ^{1}H NMR spectra will rule out tautomerism involving an enol form (Chawla *et al*., 2012:3268; Nowaczyk *et al*., 2014:2). 2-imino-4-thiazolidinones (exocyclic C═N bond) and 2-amino-4-thiazolidinones (endocyclic C═N bond)

are two more tautomeric forms of intermediate 2. (Mishchenko *et al*., 2020:7). A ^{1}H NMR resonance of a –NH– proton peaking at roughly 12-13 ppm (downfield) indicates the presence of a –NH-lactam proton. These downfield –NH-lactam signals will confirm intermediate 2's keto-imine (exocyclic C=N) tautomeric structure. This is also significant evidence for ring closure (Vicini *et al*., 2008:3715; Geronikaki *et al*., 2008:5222).



2.17.5. Mechanism for Knoevenagel condensation reaction (target compounds)



Intermediate 2 | Aldehyde | (2*E*,5*Z*)-5-benzylidene-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one

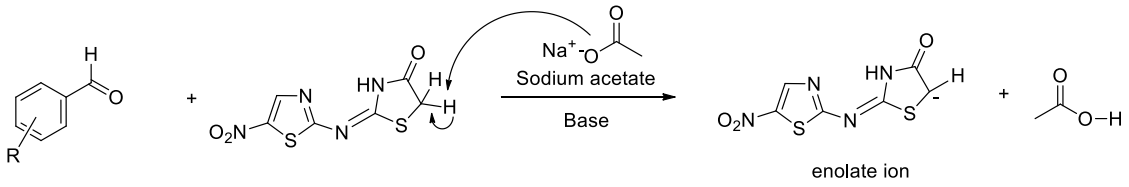
R = F, Cl, Br, NO_2, OH, Me, MeO

Target compound

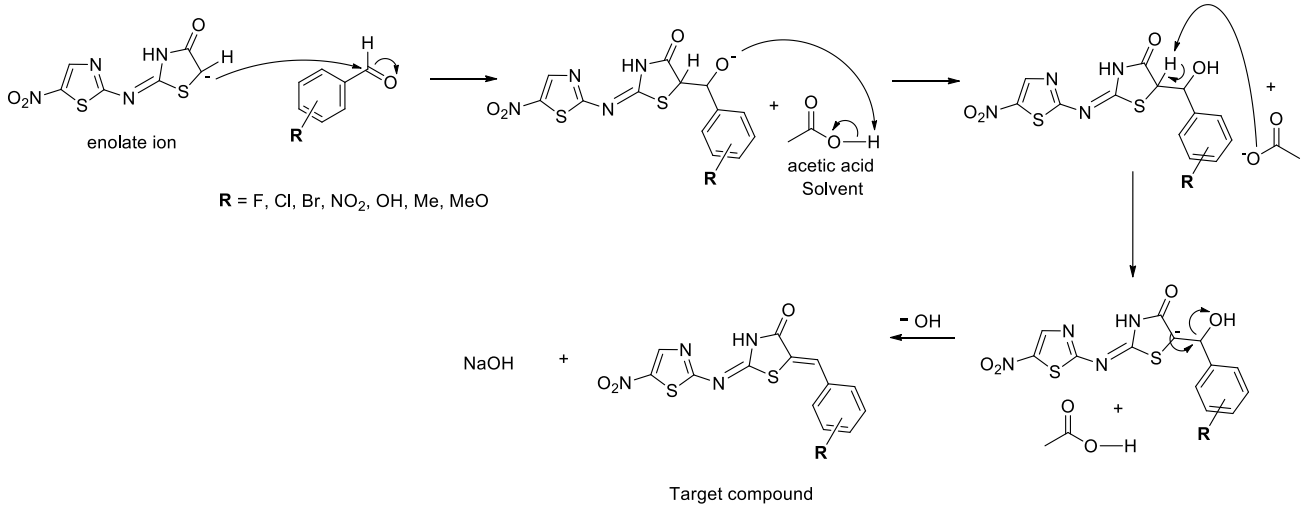
The Knoevenagel condensation reaction is a modified Aldol condensation in which an aldehyde or ketone is added to an active methylene (CH_2) compound in the presence of a basic catalyst, followed by dehydration to yield a conjugated enone product. The reaction is known as nucleophilic addition and the product is often referred to as an α, β-unsaturated ketone (Heravi *et al*., 2020:439; Lin *et al*., 2017:10).

In the instance of intermediate 2, the active methylene compounds are those flanked by two electron withdrawing groups, namely a carbonyl carbon (C═O) and a sulphur atom (S). These electron-withdrawing groups render the methylene group's hydrogen atoms acidic, which may be readily removed with a suitable base. As a result, these hydrogen atoms are known as "active hydrogen atoms," since they are ionisable owing to the electron withdrawing effect of the surrounding groups (Bakhshi *et al*., 2013).

Thus, the mechanism of Knoevenagel condensation necessitates the formation of a carbanion, which attacks the electrophilic center of the other molecule as a nucleophile. So, deprotonation of the activated methylene by the base (sodium acetate) generates a carbanion, which is stabilized by resonance with an enolate ion (Rupainwar & Pandey, 2019:424).

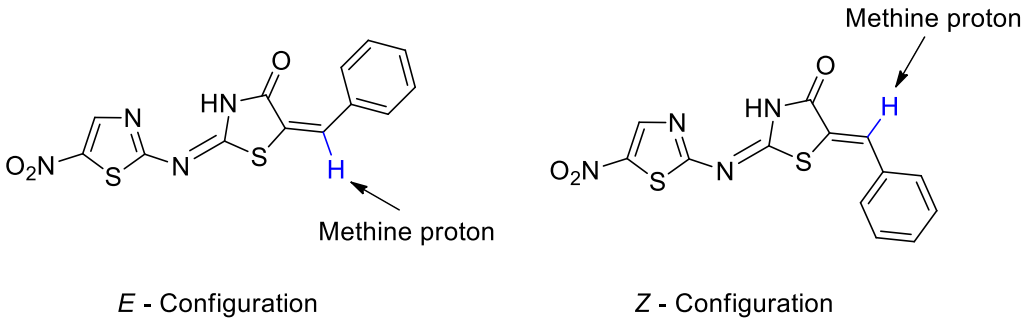


Furthermore, according to the mechanistic considerations, this enolate ion attacks as a nucleophile on the carbonyl carbon (C═O) of the aldehyde. The resulting intermediate further undergoes base-induced elimination to afford the target compounds (Bakhshi *et al*., 2013).



With regard to the exocyclic C═C bond, the target compounds may exist as possible *E* and *Z* geometrical isomers (see structure below). ^{1}H NMR spectral analysis will be used to validate the *Z* configuration of the exocyclic C═C bond in the target compounds. Because of the

deshielding effect of the adjacent C=O group, the methine proton resonates at higher chemical shift values (7.7-7.8 ppm as a single peak of one proton) in the *Z* isomer, whereas it resonates at lower chemical shifts in the *E* isomer due to the comparatively less deshielding effect of the sulphur atom (Abdellatif *et al*., 2015:153; Çakır *et al*., 2015:4; Chawla *et al*., 2012:3268).



E - Configuration *Z* - Configuration

2.18. Summary and rational design of target compounds

The literature review pertaining to this study focused on providing more insight into existing literature and information on infectious diseases caused by various bacteria, that are difficult to treat due to a global increase in antimicrobial drug resistance.

Part one focused on tuberculosis (TB), and amongst others, how it presents in terms of epidemiology, cellular morphology, pathophysiology, virulence factors and clinical manifestation thereof. The detection and diagnosis of TB were also discussed, followed by an overview of the available treatment options, and the effectiveness of these drugs for both drug-susceptible and drug-resistant TB cases.

Part two focused on hospital-acquired infections (HAIs) and the so-called ESKAPE pathogens which are responsible for most of these infections, secondary to the initial reason for hospital admission. As with the TB-section, different treatment options were also discussed. However, it has been established that most bacteria are resistant or not susceptible to routinely prescribed antibiotics now in clinical use.

Literature reveals that the MOA of NTZ largely depends on the reduction of the 5-nitrothiazole moiety through PFOR. However, it has been shown that NTZ exhibit a unique and interesting MOA against Mtb as well as a low frequency of resistance. Lastly, Thiazolidin-4-one derivatives have been reported to exhibit broad pharmacological activities. Their MOA, although not precisely known, is unlikely akin to that presented by 5-nitrothiazole derivatives in general. Based on this assumption, we do believe that generating molecules that contain a 5-nitrothiazole moiety appended to thiazolidin-4-one might lead to novel compounds with a novel MOA and/or poly-pharmacology against a variety of resistant microbial pathogens.

The next chapter contains the crux of the study in the form of the published research article on the topic, and will focus more on the synthesis of novel compounds screened for antimicrobial activity against the bacterium that causes TB as well as the ESKAPE pathogens.

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CHAPTER 3: ARTICLE FOR SUBMISSION

Chapter 3 contains a manuscript that was accepted for publication in Chemistry & Biodiversity. This article is divided into an introduction, materials and methods (including an experimental section), results and discussion, and a conclusion section. It also includes *in vitro* biological and cytotoxic assessments, as well as *in silico* drug-like prediction. Additionally, it has an abstract and a graphic abstract.

This paper was written in accordance with the author's guidelines, which are accessible on the journal's webpage:

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NITROTHIAZOLE-THIAZOLIDINONE HYBRIDS: SYNTHESIS AND *IN VITRO* ANTIMICROBIAL EVALUATION

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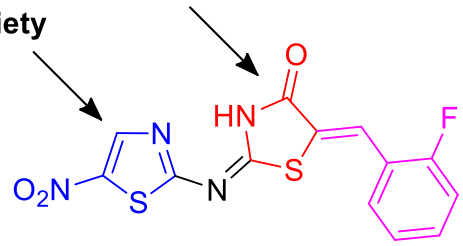
GRAPHICAL ABSTRACT

Thiazolidin-4-one scaffold

5-Nitrothiazole moiety



Hybrid compound 3b

(2*E*,5*Z*)-5-(2-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one

Mtb gfp MIC_{90} 0.45 µM
S. aureus MIC_{80} 2 µg/ml
MRSA MIC < 0.25 µg/ml
C. albicans MIC $\leq$ 0.25 µg/ml
EKAP MIC > 32 µg/ml
HEK-293 CC_{50} > 32 µg/ml
RBC HC_{10} > 32 µg/ml
cLogP 2.53

ABSTRACT

Herein we report the synthesis of novel compounds inspired by the antimicrobial activities of nitroazole and thiazolidin-4-one based compounds reported in the literature. Target compounds were evaluated *in vitro* for antitubercular, antibacterial, antifungal, and overt cell toxicity properties. All compounds exhibited potent antitubercular activity. Most compounds exhibited low micromolar activity against *S. aureus* and *C. albicans* with no overt cell toxicity against HEK-293 cells nor haemolysis against human red blood cells. Notably, compound **3b** exhibited low to sub-micromolar activities against *Mycobacterium tuberculosis* (Mtb), methicillin-resistant *Staphylococcus aureus* (MRSA), and *Candida albicans*. Compound **3b** showed superior activity (< 0.25 µg/ml) against MRSA compared to vancomycin (1 µg/ml).

Keywords: Antimicrobial; 5-nitrothiazoles; Thiazolidin-4-ones; Molecular hybridisation; ESKAPE pathogens.

3.1. Introduction

Before COVID-19, tuberculosis (TB) was the world's foremost infectious disease caused by a single pathogen. TB is caused by a bacterium called *Mycobacterium tuberculosis* (Mtb). The COVID-19 pandemic is erasing the laborious advances made in the past 20 years toward eradicating TB [1]. Without concrete actions, the global 10 million estimated TB cases (including 815, 000 HIV-positive individuals) and more than 1.4 million TB deaths (including 208, 000 HIV-positive individuals) that occur annually will increase, and low-to-middle income countries will be the most affected [2]. The currently available first-line antitubercular treatment regimen is a 6-month course of rifampicin (RIF), isoniazid (INH), ethambutol (ETH), and pyrazinamide (PZA). This regimen presents adverse side effects, a prolonged treatment period, and non-compliance from TB patients, which ultimately leads to the emergence of drug-resistant strains of Mtb [3]. There is apparently a need for the discovery of efficacious chemotherapeutics with minimal toxicity and a novel mechanism of action (MOA) to address Mtb, with particular relevance to multidrug-resistant TB (MDR-TB) and extensively drug-resistant TB (XDR-TB) [4,5]. After approximately forty years of no novel antitubercular drugs entering the market, bedaquiline (BDQ) and nitro-containing drugs (delamanid (DLM) and pretomanid (PTM)) achieved conditional regulatory approval as novel antitubercular drugs to treat MDR-TB [6-8]. The approval of these three drugs is a great achievement in the fight against TB but does not suffice to completely eradicate TB, hence the need to continuously search for novel antitubercular agents.

The COVID-19 pandemic has also increased awareness regarding the threat posed by infectious diseases, and it has served as a major catalyst for reviving efforts to tackle the silent epidemic of antimicrobial-resistant (AMR) bacteria [9]. Antibiotics' diminishing efficacy against bacteria is jeopardising the practice of modern medicine. This is especially evident in infections caused by *Enterococcus* spp.*, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp. (ESKAPE-pathogens), and *Escherichia coli*. ESKAPE pathogens are the major cause of hospital-acquired infections

(HAIs) (an infection acquired while receiving treatment in a healthcare facility) globally. In fact, antimicrobial resistance among these pathogens is wreaking havoc on global public health [10].

ESKAPE pathogens are responsible for approximately 80% of HAIs — which include pneumonia, bloodstream infections, urinary tract infections, or surgical site infections [11]. These pathogens represent the top bacterial isolates in a hospital setting, possessing significant intrinsic drug resistance. Data from Centre for Disease Control and Prevention (CDC), suggests that 1 in 31 hospitalised patients develops a HAI every day, and approximately 99 000 deaths due to HAIs occur each year in the United States alone [12, 13]. These infections are anticipated to increase the cost of patient treatment by 4.5 to 5.7 billion dollars annually [14]. The increased burden of drug-resistant ESKAPE pathogens has prompted the WHO to classify ESKAPE pathogens into critical and high priority groups, which indicates an urgent need for the identification and development of new antibiotics against these pathogens [15].

Newly approved antitubercular drugs, DLM and PTM (see **Fig. 3.1**), belong to the nitroazole class of compounds. They have been identified as a novel and potent chemical class, exhibiting a MOA not seen with currently available antitubercular drugs [8]. Other notable nitroazole compounds are nitazoxanide (NTZ) and its hydrolysed active metabolite, tizoxanide (TIZ) (see **Fig. 3.1**). NTZ and TIZ are antiprotozoal 5-nitrothiazole-based agents that have been repurposed owing to their efficacy against Mtb. Targets perturbed by NTZ include pyruvate-ferredoxin oxidoreductase (PFOR) in anaerobic bacteria and protozoans [16], pyruvate dehydrogenase in *E. coli* [17], membrane potential, and pH homeostasis of Mtb [18], bacteria's chaperone/usher (CU) pathway [19], macrophage autophagy and *mTORC1* signalling [20]. At low concentrations, NTZ and TIZ eradicate both replicating and non-replicating (slow growing) Mtb while also appearing to evade resistance formation [21]. NTZ

is therefore a suitable template to inspire the identification and development of novel broad-spectrum anti-infective agents.

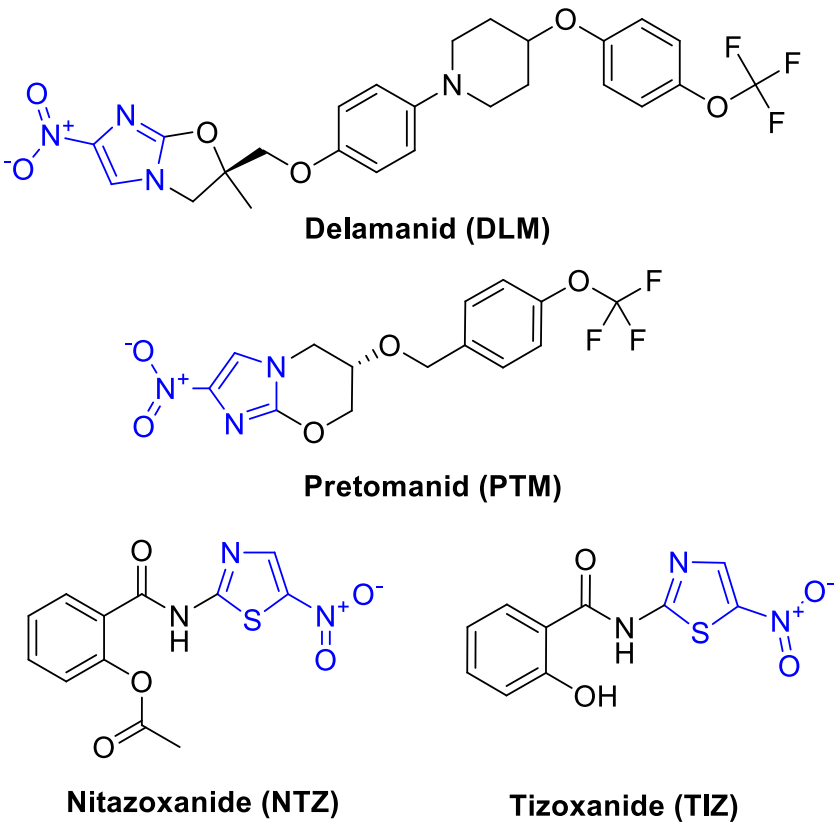


Figure 3.1 Structures of biological active nitroazole drugs

Thiazolidinone is a saturated heterocyclic thiazole bearing a carbonyl (C═O) functional group, a sulphur (S) atom and a nitrogen (N) atom [22]. There are predominantly three types of thiazolidinones: thiazolidin-2-one, thiazolidin-4-one, and thiazolidin-5-one [23]. The thiazolidin-4-one scaffold (see **Fig. 3.2**) has long been regarded as a magical structure ("wonder nucleus") capable of almost any biological activity [24], including antibacterial (against Gram-positive and Gram-negative bacteria) [25-27], antimycobacterial [28], antifungal [27,29], anti-HIV [30,31], anticancer [32] and anticonvulsant [33] properties. Although the MOA of thiazolidin-4-one is unknown, Vicini *et al.* (2006) demonstrated that substituting a benzylidene moiety in the fifth position (fifth carbon-C5) of the thiazolidin-4-one heterocyclic core enhances its antibacterial properties [34].

The molecular hybridisation (MH) approach has been widely employed to develop novel molecules with potential poly-pharmacophoric characteristics. The MH strategy commonly employed involves the use of linkers to join two or more compounds with well-characterized biological activities [35]. This practice often leads to the synthesis of new compounds with an excessive molecular weight (> 500 Da) beyond Lipinski's recommendation. In this study, we employ the concept of MH (see **Fig. 3.2**) to generate novel compounds incorporating the 2-amino-5-nitrothiazole moiety (present in NTZ) and thiazolidin-4-one. C5 of the thiazolidin-4-one was used to explore SARs for this library. The target compounds (**3a – 3j**) were evaluated *in vitro* against Mtb, some ESKAPE pathogens, MRSA, and the fungus *C. albicans*. Additionally, these compounds were evaluated against human embryonic kidney and red blood cells for overt cell toxicity and haemolysis, respectively.

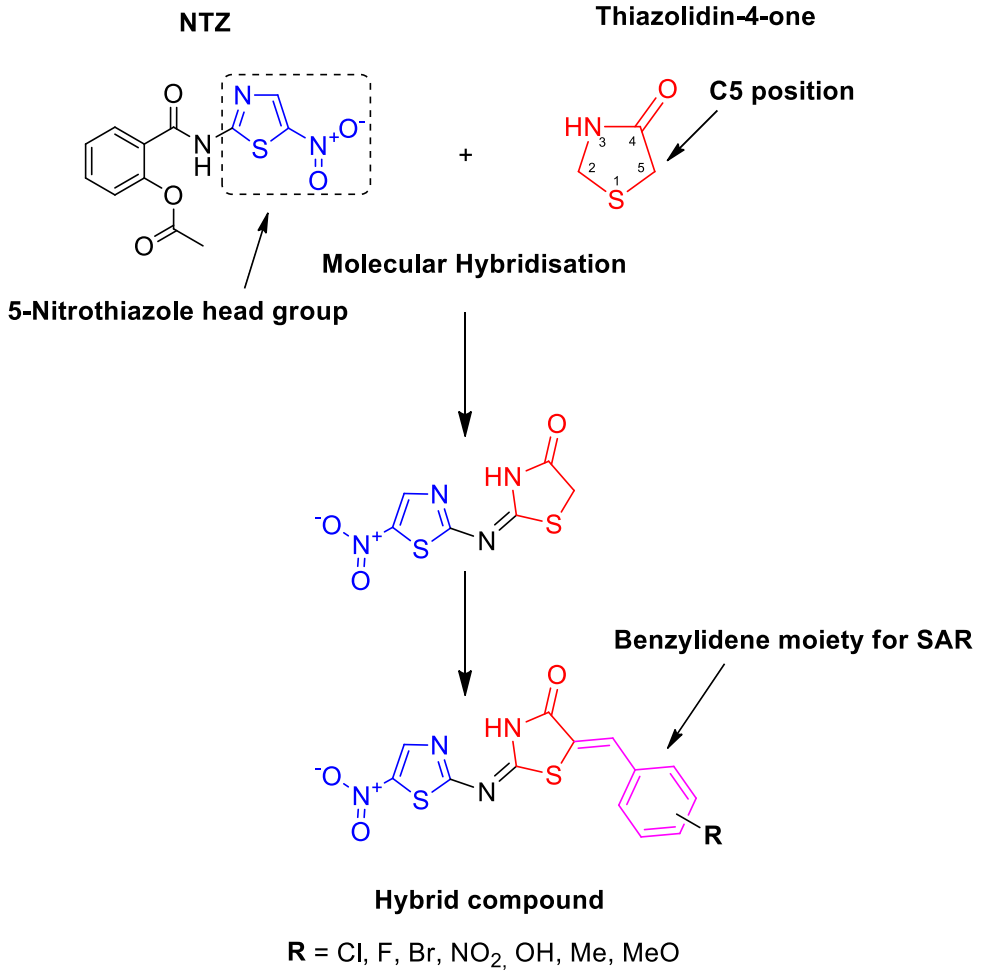
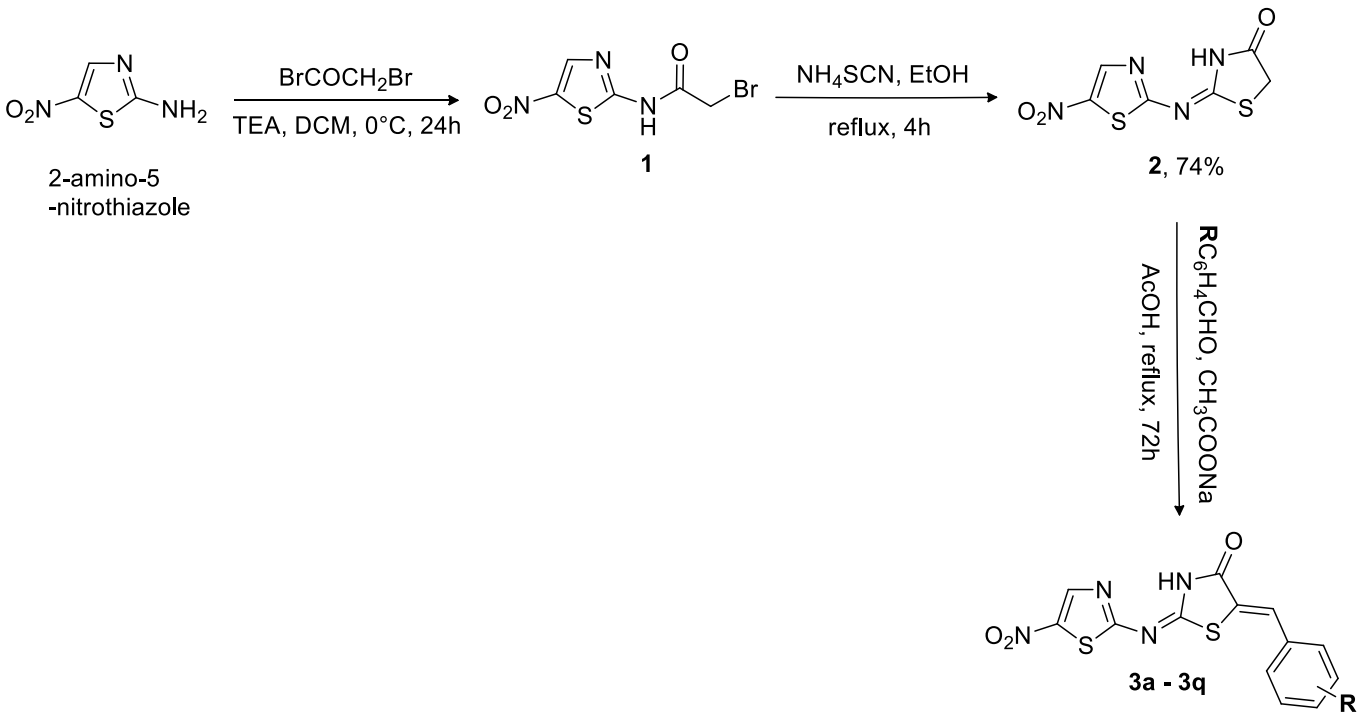


Figure 3.2 Molecular hybridization strategy

3.2. Results and discussion

3.2.1. Chemistry and structure characterisation

The target compounds were synthesized following a multi-step synthetic protocol presented in **Scheme 1**. *N*-acylation of the 2-amino-5-nitrothiazole with bromoacetyl bromide afforded intermediate **1** in 80% yield. Refluxing **1**, and ammonium thiocyanate in ethanol led to successive *S*-alkylation and intramolecular cyclization to provide the thiazolidine-4-one based heterocycle, intermediate **2** in 74% yield [26,34]. Target compounds (**3a – 3q**) were synthesized in 26–69% yield using the standard Knoevenagel condensation procedure by treating intermediate **2** with commercially available aldehydes.



* Percentage yield

R					
3a: H, 52%*	**3e**: 3-F, 45%	**3h**: 4-F, 42%	**3l**: 2-Me, 38%	**3n**: 2,4-MeO, 51%	**3q**: 3,4-Cl, 35%
3b: 2-F, 26%	**3f**: 3-Cl, 46%	**3i**: 4-Cl, 40%	**3m**: 4-Me, 43%	**3o**: 2,4,6-MeO, 42%	
3c: 2-Cl, 52%	**3g**: 3-Br, 69%	**3j**: 4-NO_2, 31%		**3p**: 3-MeO, 54%	
3d: 2-NO_2, 36%		**3k**: 4-OH, 32%			

Scheme 1 Synthesis of conceptualised target compounds

The structures of intermediate **2** and target compounds **3a – 3q** were determined using FTIR, ^{1}H and ^{13}C-NMR and HRMS. The observable diagnostic peaks are stated below:

The FTIR spectra of intermediate **2** and all the target compounds **3a – 3q** showed absorbance at different wavelengths indicative of the functional groups present. For example, the 5–NO_2 group present on the fifth position of the thiazole moiety can be observed in all compounds as an intense broad stretch between 1521.91–1582.33 cm^{-1} (N–O) and 1294.77–1351.42 cm^{-1} (N–O). The –NH– stretch appeared around 3067.70–3144.49 cm^{-1} and the stretch around 1676.96–1729.23 cm^{-1} can be attributed to the amide carbonyl (C═O) functional group. In the FTIR spectra of compound **3k**, the broad stretching at 3260.80 cm^{-1} is indicative of the presence of an OH group.

The broad singlet at *ca.* 12.56–13.26 ppm in the ^{1}H NMR spectra of intermediate **2** and target compounds indicates the presence of a lactam –NH– group; this signal is however absent in compounds **3i**, **3j**, **3o**, **3q**, probably due to exchange with H_2O in deuterated (D_6)DMSO. In the ^{1}H NMR of **2**, the active methylene protons appear as a singlet, integrating to 2 protons at *ca.* 4 ppm. The disappearance of this singlet in the ^{1}H NMR of compounds **3a – 3q** suggests that the Knoevenagel condensation step was successful. In the ^{1}H NMR spectra of all compounds, the singlet peak, integrating to one proton at approximately 8.66 ppm, is assignable to the thiazolyl proton. Compound **3o** can be identified as an outlier due to the thiazolyl proton appearing at 7.40 ppm. The substituted methyl (R–CH_3) or methoxy (R–OCH_3) groups at the benzylidene moiety of compounds **3l – 3p**, are observed in the ^{1}H NMR spectra in the range of 2.36–3.91 ppm as three or more protons, depending on the structure. This series of compounds suffers from poor solubility in deuterated (D_6)DMSO and, as a result, all carbon peaks could not be accounted for.

The molecular mass for each compound was confirmed using HRMS. All compounds (except **3e**, **3n**, and **3o**) were confirmed to be > 96% pure using HPLC. Compounds **3e**, **3n**, and **3o**

displayed a purity of 79.04%, 88.02%, and 85.47%, respectively. Trace solvent impurities (1,4-dioxane, DMF, toluene, and ethanol) are present in the NMR spectra of some of the target compounds. Prior to biological evaluation, the target compounds were vacuum-dried for 72 h at 150°C and 200 Pa to remove solvent impurities.

3.2.2. Antitubercular activity

All compounds, **2**, **3a – 3q**, were screened *in vitro* for their antitubercular activity in CAS medium versus the gfp strain of Mtb. The first-line antitubercular drug, RIF, was used as a reference. Following incubation, the minimum inhibitory concentration (MIC_{90}) needed to inhibit 90% of mycobacterial growth was determined on day 14. The antitubercular activity results for the tested compounds are summarized in **Table 3.1**. Astoundingly, compounds **3a – 3q** were presented with excellent antitubercular activity, ranging from < 0.24–2.00 µM. Compounds **3c**, **3g – 3i**, **3l**, **3n**, and **3q** are the most potent against Mtb, showing MIC_{90} values of < 0.24 µM. Compound **3k**, having a hydroxy (OH) group at the *para*-position of the benzylidene moiety, presented the least antitubercular activity of 2 µM. Comparing the MIC_{90} value of intermediate **2** (31.25 µM) against the values of compound **3a – 3q**, suggests that appending on to **2** a benzylidene moiety bearing electron withdrawing– (EWG) or electron donating groups (EDG) is essential for antitubercular activity.

Table 3.1 Antimicrobial and cytotoxic activities of compounds **2** and **3a – 3j**

ID	R	Mtb[a] (CAS) Day 14 MIC_{90} µM	Sa[b] MIC (µg/ml)	MRSA[c] MIC (µg/ml)	Ca[d] MIC (µg/ml)	EKAP[e] MIC (µg/ml)	HEK-293[f] CC_{50} (µg/ml)	RBCs[g] HC_{10} (µg/ml)
2	**-**	31.25	>32	>32	>32	>32	>32	>32
3a	**H**	0.48	8	>32	4	>32	>32	>32
3b	**2-F**	0.45	2	<0.25	4	>32	>32	>32
3c	**2-Cl**	<0.24	4	>32	>32	>32	>32	>32
3d	**2-NO_2**	0.72	2	>32	>32	>32	>32	>32
3e	**3-F**	0.52	4	>32	16	>32	>32	>32
3f	**3-Cl**	0.29	>32	>32	>32	>32	>32	>32
3g	**3-Br**	<0.24	2	>32	≤0.25	>32	>32	>32
3h	**4-F**	<0.24	>32	>32	32	>32	>32	>32
3i	**4-Cl**	<0.24	4	>32	>32	>32	>32	>32
3j	**4-NO_2**	0.92	>32	>32	>32	>32	>32	>32
3k	**4-OH**	2.00	>32	>32	>32	>32	>32	>32
3l	**2-Me**	<0.24	2	>32	>32	>32	>32	>32
3m	**4-Me**	0.48	2	>32	>32	>32	>32	>32
3n	**2,4-MeO**	<0.24	>32	>32	>32	>32	>32	>32
3o	**2,4,6-MeO**	0.48	>32	>32	>32	>32	>32	>32
3p	**3-MeO**	0.50	8	>32	>32	>32	>32	>32
3q	**3,4-Cl**	<0.24	>32	>32	32	>32	>32	>32
RIF		0.018	-	-	-	-	-	-
AMP		-	0.5	-	-	-	-	-
CPFX		-	0.5		-	-	-	-
VM		-	-	1	-	-	-	-
FZ		-	-	-	0.12	-	-	-
CL		-	-	-	-	0.25	-	-
TF		-	-	-	-	-	9	-
MT		-	-	-	-	-	-	2.7

[a]*M. tuberculosis* strain *(*Mtb pMSp12: gfp) in Middlebrook 7H9 CAS: 0.03% casitone, 0.4% glucose, and 0.05% tyloxapol; [b]*S. aureus;* [c]*Methicillin-resistant S. aureus;* [d]*C. albicans*; [e]*E. coli, K. pneumoniae, A. baumannii*, *P. aeruginosa*; [f]Human embryonic kidney cells; [g]Red blood cells; **Reference drugs:** Rifampicin (RIF); Ampicillin (AMP) Ciprofloxacin (CPFX), Colistin (CL), Vancomycin (VM), Fluconazole (FZ), Tamoxifen (TF), Mellitin (MT)

3.2.3. Antibacterial and antifungal activities

In addition to the antitubercular assay, the antibacterial activity of **2** and **3a – 3q** was determined against *Escherichia coli* (ATCC25922), *Klebsiella Pneumoniae* (ATCC700603), *Acinetobacter baumannii* (ATCC19606), *Pseudomonas aeruginosa* (ATCC27853), *Staphylococcus aureus* and *methicillin-resistant Staphylococcus aureus* (MRSA) (ATCC43300). Approved antibacterial drugs, ampicillin, colistin, vancomycin, and ciprofloxacin, were used as reference drugs, and the screening results are tabulated in **Table 3.1**. Hits were classified as any compound exhibiting an MIC value of ≤ 16 µg/ml. Generally, these compounds were not active against MRSA and Gram-negative bacteria (*E. coli*, *K. pneumoniae, A. baumannii, P. aeruginosa, Enterobacter* spp.), but exhibited varying degrees of activity (MIC: 2 – > 32 µg/ml) against *S. aureus*, a Gram-positive bacterium. This disparity in activity could be due to the cell wall of Gram-negative bacteria, which act as a barrier, preventing molecules from entering these bacteria.

Ten of the eighteen compounds tested were active against *S. aureus*. Compounds **3a**, **3b**, **3c**, **3d**, **3e**, **3g**, **3i**, **3l**, **3m**, and **3p** all exhibited potent antibacterial activity in the range of 2–8 µg/ml against *S. aureus*. Among the active compounds, the fluoro-, nitro-, bromo-, and methyl substituted compounds, **3b**, **3d**, **3g**, **3l**–**3m**, respectively, had the highest and equipotent antibacterial activity (MIC: 2 µg/ml), whereas chloro- (**3c** and **3i**) and 3-fluoro-substituted (**3e**) compounds showed a two-fold increase in MIC values from 2 to 4 µg/ml. Compound **3a**, having an unsubstituted benzylidene moiety, and **3p**, harbouring a methoxy group, exhibited the lowest activity (MIC: 8 µg/ml).

In general, activity against *S. aureus* increases with substitution of the benzylidene moiety with EWG and EDG. For example, compounds **3b** and **3l**, bearing EWG and EDG, respectively, have equipotent activity (MIC: 2 µg/ml), which is a fourfold increase in activity when compared to compound **3a** (MIC: 8 µg/ml) bearing an un-substituted benzylidene moiety. No change in MIC values could be found between compounds **3c** and **3i** (4 µg/ml) having a chloro

substituent in the *ortho*- and *para*-positions, suggesting that both positions are preferred. Having said that, it is worth noting that compound **3f**, with a chloro substituent in the *meta*-position, exhibited no antibacterial activity against *S. aureus* (MIC > 32 µg/ml). Compounds **3b** and **3e** with a fluoro atom in the *ortho* and *meta*-positions, respectively, exhibited activity, while compound **3h**, with a fluoro atom in the *para*-position, showed no activity. Furthermore, the findings demonstrated that compound **3j** with a nitro substituent in the *para*-position is inactive against *S. aureus*, but the activity was restored to 2 µg/ml when the nitro atom was present in the *ortho*-position (**3d**). All these suggest that substitution at the *ortho* position of the benzylidene moiety favours activity against *S. aureus*.

It is noteworthy to note that compounds **3n** and **3o** bearing multiple methoxy substituents on the benzylidene moiety are inactive against *S. aureus*, but compound **3p** bearing a single methoxy substituent in the *meta*-position is active (MIC: 8 µg/ml). Finally, unlike the compounds with a single chloro-substituted atom (**3c** and **3i**), compound **3q** with disubstituted chlorine atoms in the *meta*- and *para*-positions exhibits no activity. This suggest that poly-substitution of the benzylidene moiety does not favour activity. Compound **3k**, with a hydroxy substituent in the *para*-position, showed no activity.

Except for compound **3b**, all compounds showing activity against *S. aureus* were inactive against MRSA. This suggests penicillin-binding protein (PBP) as the likely target for these compounds. **3b**, however, exhibited sub-micromolar activity (< 0.25 µg/ml) against MRSA, suggesting a different target from PBP and/or binding to a different pocket within PBP.

Compounds were also evaluated for antifungal activity against *Candida albicans* (ATCC90028). Only six compounds showed activity against *C. albicans,* and they include **3a** (4 µg/ml), **3b** (4 µg/ml), **3e** (16 µg/ml), **3g** (≤ 0.25 µg/ml), **3h** (32 µg/ml) and **3q** (32 µg/ml). Notably, compound **3g** exhibited comparable activity to the reference drug fluconazole (MIC: 0.12 µg/ml).

3.2.4. Cytotoxicity and haemolytic studies

Overt cell toxicity was determined against human embryonic kidney cells (HEK-293) and human red blood cells (RBCs), which are reported in **Table 3.1**. These cells were treated with test compounds at 32 µg/ml and their viability was assessed. No compound in this study reduced cells viability to below 80%, and as such, these compounds are assigned a CC_{50} and HC_{10} value of > 32 µg/ml.

3.2.5. *In silico* drug-like prediction

In silico physiochemical prediction of novel compounds is an integral part of drug discovery since it provides insight into a molecule's drug-like properties and safety. Using the SwissADME online web tool (http://www.swissadme.ch), we calculated and tabulated the consensus logarithm of the partition coefficient (cLogP), the molecular weight (MW), the number of hydrogen bond acceptors (HBA), the number of hydrogen bond donors (HBD), the topological polar surface area (*t*PSA), and the number of rotatable bonds. The results in **Table 3.2** were compared to Lipinski's five-point rule, as described in his foundational work [36]. According to the Lipinski rule of five, a molecule with a MW of under 500 Da, an octanol–water partition coefficient (cLogP) of less than 5, an HBA of less than 10, and no more than 5 potential HBD is more likely to be an acceptable oral active drug [37]. In the results, it can be observed that compounds **2**, **3a – 3q** do not violate Lipinski's five-point rule.

While lipophilicity is a critical physicochemical criterion, it is not the only property that determines effective drug design, as shown from the findings of a GlaxoSmithKline-funded study of rat availability data for 1100 drug candidates, conducted by Veber *et al.* (2002). This finding suggests that additional physicochemical properties, for example, *t*PSA and molecular flexibility (RoB), should be investigated, as they affect the oral bioavailability of drugs [38]. To select drugs with expected oral bioavailability, two simple guidelines were confirmed by Veber *et al.* (2002). If a rat oral bioavailability of ≥ 20–40% is acceptable, then *t*PSA ≤ 140 $Å^2$ paired with RoB ≤ 10 appears to be an efficient and selective parameter [39]. Interestingly, the newly

synthesized target compounds showed one violation of Veber's parameters regarding their *t*PSA, which is above 140 Å^2 but presents with good molecular flexibility according to the RoB parameter.

Table 3.2 Physiochemical properties of compounds **2** and **3a – 3j**

ID	cLogP$_{o/w}$[a]	MW[b] (Da)	HBA[c]	HBD[d]	RoB[e]	*t*PSA[f] Â^2	LogS[g] mol/l	Lipinski Violations	Veber Violations
2	0.62	244.25	5	1	2	153.71	-2.38	0	1
3a	2.26	332.36	5	1	3	153.71	-4.32	0	1
3b	2.53	350.35	6	1	3	153.71	-4.47	0	1
3c	2.78	366.80	5	1	3	153.71	-4.91	0	1
3d	1.49	377.36	7	1	4	199.53	-4.38	0	1
3e	2.53	350.35	6	1	3	153.71	-4.47	0	1
3f	2.76	366.80	5	1	3	153.71	-4.91	0	1
3g	2.85	411.25	5	1	3	153.71	-5.22	0	1
3h	2.52	350.35	6	1	3	153.71	-4.47	0	1
3i	2.75	366.80	5	1	3	153.71	-4.91	0	1
3j	1.45	377.36	7	1	4	199.53	-4.38	0	1
3k	1.54	348.36	6	2	3	173.94	-4.18	0	1
3l	2.34	346.38	5	1	3	153.71	-4.61	0	1
3m	2.30	346.38	5	1	3	153.71	-4.61	0	1
3n	2.01	392.41	7	1	5	172.17	-4.46	0	1
3o	1.98	422.44	8	1	6	181.40	-4.54	0	1
3p	1.98	362.38	6	1	4	162.94	-4.39	0	1
3q	3.31	401.25	5	1	3	153.71	-5.51	0	1

[a]Consensus octanol–water partition coefficient indicating lipophilicity; [b]Molecular weight of compounds; [c]Number of hydrogen bond acceptors; [d]Number of hydrogen bond donors; [e]Number of rotatable bonds; [f]Topological polar surface area; [g]Logarithm of the solubility in water.

Furthermore, the cLogP for the target compounds correlate negatively with the water solubility potentials (LogS). Predictions of distribution and absorption properties (see **Annexure A – Table 1)** revealed that none of the examined compounds can penetrate the blood–brain barrier (BBB), and none of the compounds are estimated to be substrates of P-glycoprotein.

Additionally, the passive human gastrointestinal (GI) absorption predictions found that all generated compounds had a limited absorption potential and displayed reasonably high inhibition of many or all the important CYP450 iso-enzymes associated with drug metabolism: CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4

3.3. Materials and methods

3.3.1. General methods

Reagents and solvents were purchased from various chemical suppliers including, Ambeed, AK Scientific, Rochelle, Labchem and Merck. Reaction progress was monitored through thin-layer chromatography method using Merck $60F_{254}$ silica gel sheet supported on aluminium. UV light (254 and 366 nm) or iodine vapor staining was used to visualise the developed sheets. Melting points (m.p.) were determined with a Buchi B-545 apparatus. Fourier-transform infrared spectroscopy (FTIR) was done using a Bruker ALPHA FTIR Routine spectrometer. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were acquired on a Bruker Avance III 600 spectrophotometer at 600 MHz and 151 MHz, respectively, in deuterated DMSO ((D_6)DMSO). Chemical shifts are reported in parts per million (ppm) and were referenced to the residual solvent peaks ((D_6)DMSO: 2.50 and 39.52 ppm for ^{1}H and ^{13}C-NMR, respectively). Spin multiplicities are given as s (singlet), d (doublet), t (triplets), m (multiplet), dt (doublet of triplets), td (triplet of doublets), and dd (doublet of doublets). Coupling constants (*J*) are reported in Hz. A Bruker micrOTOF-Q II mass spectrometer was used to record high-resolution mass spectra (HRMS) utilising atmospheric pressure chemical ionisation (APCI) in positive ion mode. A full scan from 50 to 1600 *m/z* was carried out at a capillary voltage of 4500 V, an end plate offset of –500 V, 1.8 Bar nebulisers, and a collision cell RF voltage of 150 Vpp. Compound purity was determined by high performance liquid chromatography (HPLC) analysis using an Agilent 1200 series HPLC system equipped with a quaternary pump and an Agilent 1200 series diode array detector. A Venusil XBP C18 column (4.60 × 150 mm, 5 µm) was used for separation, and the mobile phase consisted initially of 10% methanol and

90% MilliQ water at a flow rate of 1 ml/min. The compounds were prepared in HPLC-grade acetonitrile and injected at a volume of 10 µl. The flow rate was set to 1 ml/min, and the samples were analysed at 280 nm and 400 nm wavelengths. The equilibration period between runs was 5 minutes, and each HPLC run was 13 minutes in length.

3.3.2. Synthesis (Experimental)

3.3.2.1. *N*-Acylation (1)

A round-bottom flask was charged with 35 ml of anhydrous CH_2Cl_2, 2.3 g of 2-amino-5-nitrothiazole (15.9 mmoles) and 2.7 ml (1.2 equiv., 18.7 mmoles) of TEA. The mixture was cooled to 0°C using an ice bath, and 3 ml (2.2 equiv., 34 mmoles) of bromoacetyl bromide was added dropwise, after which it was stirred for 24 h at room temperature (r.t.) The reaction was monitored with TLC, and upon completion, the insoluble triethylamine hydrobromide salt formed was filtered off and the product-containing filtrate was concentrated *in vacuo*, followed by the addition of deionised water (20 ml), and the mixture stirred vigorously at r.t for 15–20 min. The ensued precipitate was filtered, dried, and washed with hexane to furnish intermediate **1** in 80% yield.

3.3.2.2. Dehydrative cyclization (2)

In a round-bottom flask, 3 g (11.4 mmoles) of **1** (2-bromo-N-(5-nitrothiazol-2-yl)acetamide), 1.7 g (2 equiv., 22.7 mmoles) of ammonium thiocyanate (NH_4SCN) and 20 ml of ethanol were added and the mixture heated under reflux for 4 h. The reaction was monitored with TLC, and after completion, it was left overnight at r.t. to precipitate. The product was filtered, washed with a cold ethanol: water (50:50) mixture to afford parent intermediate **2** at a 74% yield.

(E)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**2**)

Black powder; 74% yield; m.p.: > 400°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 12.56 (s, 1H, NH), 8.65 (s, 1H, thiazole–CH), 4.11 (s, 2H, methylene–CH_2); ^{13}C NMR (151 MHz, (D_6)DMSO) δ

143.53, 143.08, 35.94; IR V_{max} cm^{-1}: 3083.04 (N–H lactam), 2988.08 (Ar–H), 1721.67 (C═O stretch), 1630.95 (C═N stretch), 1550.72 (N–O stretch), 1333.53 (N–O stretch). *m*/*z* HRMS (APCI) found 244.9790, calcd for $C_6H_5N_4O_3S_2$: 244.9798 $[M + H]^+$; Purity (HPLC): 100%.

3.3.2.3. Knoevenagel condensation (3a – 3q)

Into a round-bottom flask was added 0.3 g (1.2 mmoles) of **2**, 0.35 g (3.5 equiv., 4.2 mmoles) of anhydrous sodium acetate (CH_3COONa), 20 ml of glacial acetic acid (AcOH), and 1.2 equiv. of the appropriate aldehyde. The mixture was heated under reflux for 72 h. The reaction was monitored with TLC and, after completion, left to cool to r.t. to initiate precipitation of the target compounds. The resultant precipitate was filtered, washed with water, and recrystallised from 1.4-Dioxane (**3a**, **3d**, **3e**, **3g**, **3h**, **3i**, **3k**, **3m**, **3p**, **3q**) and DMF (**3j**, **3n**, **3o**). Compounds **3b**, **3c**, and **3f** were recrystallised from toluene through hot filtration. For **3l**, it was precipitated out of ice cooled water, and recrystallised from ethanol.

(2E,5Z)-5-benzylidene-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3a**)

Green powder; 52% yield; m.p.: 288–290°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.20 (s, 1H, NH), 8.73 (s, 1H, thiazole–CH), 7.80 (s, 1H, CH), 7.64 (d, J = 7.5 Hz, 2H, Ar-CH), 7.56 (t, J = 7.6 Hz, 2H, Ar–CH), 7.50 (t, J = 7.3 Hz, 1H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 144.06, 131.13, 130.66, 129.88; IR V_{max} cm^{-1}: 3082.67 (N–H lactam), 2987.76 (C–H arom), 1707.34 (C═O stretch), 1602.63 (C═N stretch), 1561.62 (N–O stretch), 1328.90 (N–O stretch). *m*/*z* HRMS (APCI) found 333.0136, calcd for $C_{13}H_9N_4O_3S_2$ 333.0111 $[M + H]^+$; Purity: 97.9%.

(2E,5Z)-5-(2-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3b**)

Dark-maroon crystals; 26% yield; m.p.: 262–263°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.21 (s, 1H, NH), 8.69 (s, 1H, thiazole–CH), 7.78 (s, 1H, CH), 7.57 (dt, J = 8.7, 6.8 Hz, 2H, Ar–CH), 7.40 (dt, J = 9.0, 8.0 Hz, 2H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 161.29, 159.62, 143.26, 132.83, 129.08, 125.35, 121,01, 120.94, 116.15; IR V_{max} cm^{-1}: 3144.49 (N–H lactam), 2987.84 (C–H arom), 1701.86 (C═O stretch), 1606.48 (C═N stretch), 1548.74 (N–O stretch),

1330.56 (N–O stretch). *m*/*z* HRMS (APCI) found 351.0039, calcd for $C_{13}H_8FN_4O_3S_2$: 351.0016 $[M + H]^+$; Purity (HPLC): 97%.

(2E,5Z)-5-(2-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3c**)

Brown powder; 52% yield; m.p.: 290–292°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.21 (s, 1H, NH), 8.68 (s, 1H, thiazole–CH), 7.92 (s, 1H, CH), 7.66 – 7.61 (m, 2H, Ar–CH), 7.56 (t, *J* = 7.2 Hz, 1H, Ar–CH), 7.51 (td, *J* = 7.7, 1.5 Hz, 1H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.26, 134.45, 131.89, 131.12, 130.33, 129.05, 128.07; IR V_{max} cm^{-1}: 3093.36 (N–H lactam), 2987.81 (C–H arom), 1723.31 (C═O stretch), 1641.63 (C═N stretch), 1582.33 (N–O stretch), 1351.42 (N–O stretch); *m*/*z* HRMS (APCI) : 366.9749, calcd for $C_{13}H_8ClN_4O_3S_2$: 366.9721 [M + H]$^+$; Purity (HPLC): 97.9%.

(2E,5Z)-5-(2-nitrobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3d**)

Black crystals; 36% yield; m.p.: 281–282°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.26 (s, 1H, NH), 8.66 (s, 1H, thiazole–CH), 8.23 (dd, *J* = 8.2, 0.8 Hz, 1H, Ar–CH), 8.05 (s, 1H, CH), 7.94 (td, *J* = 7.6, 0.8 Hz, 1H, Ar–CH), 7.79 – 7.73 (m, 2H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 147.71, 143.22, 134.55, 131.07, 129.54, 129.04, 125.38; IR V_{max} cm^{-1}: 3109.15 (N–H lactam), 2994.28 (C–H arom), 1702.15 (C═O stretch), 1618.43 (C═N stretch), 1521.91 (N–O stretch), 1338.19 (N–O stretch); *m*/*z* HRMS (APCI) found 377.9994, calcd for $C_{13}H_8N_5O_5S_2$ 377.9961 [M + H]$^+$; Purity (HPLC): 99%.

(2E,5Z)-5-(3-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3e**)

Light-maroon powder; 45%; m.p.: 281–283°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 12.98 (s, 1H, NH), 8.72 (s, 1H, thiazole–CH), 7.81 (s, 1H, CH), 7.64 – 7.58 (m, 1H, Ar–CH), 7.34 (t, *J* = 8.2 Hz, 1H, Ar–CH), 7.24 (t, *J* = 7.5 Hz, 1H, Ar–CH), 7.19 – 7.12 (m, 1H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 163.03, 143.39, 131.37, 128.76, 128.06, 125.62; IR V_{max} cm^{-1}: 3108.69 (N–H lactam), 2987.67 (C–H arom), 1708.91 (C═O stretch), 1607.92 (C═N stretch), 1562.45

(N–O stretch), 1304.24 (N–O stretch); *m/z* HRMS (APCI) found 351.0039, calcd for $C_{13}H_8FN_4O_3S_2$ 351.0016 $[M + H]^+$; Purity (HPLC): 76%.

(2E,5Z)-5-(3-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3f**)

Yellow-mustard powder; 46% yield; m.p.: 262–264°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.18 (s, 1H, NH), 8.69 (s, 1H, thiazole–CH), 7.76 (s, 1H, CH), 7.69 (s, 1H, Ar–CH), 7.61 – 7.50 (m, 3H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.27, 135.72, 133.93, 131.05, 130.01, 129.86, 128.78, 127.81; IR V_{max} cm^{-1}: 3091.14 (N–H lactam), 2987.72 (C–H arom), 1701.66 (C═O stretch), 1677.56 (C═N stretch), 1577.63 (N–O stretch), 1343.04 (N–O stretch); *m/z* HRMS (APCI) found 366.9746, calcd for $C_{13}H_8ClN_4O_3S_2$: 366.9721 $[M + H]^+$; Purity (HPLC): 99%.

(2E,5Z)-5-(3-bromobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3g**)

Green-mustard powder; 69% yield; m.p.: 220–282°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.21 (s, 1H, NH), 8.70 (s, 1H, thiazole–CH), 7.83 (d, *J* = 1.6 Hz, 1H, Ar–CH), 7.76 (s, 1H, CH), 7.67 (dd, *J* = 7.8, 1.6 Hz, 1H, Ar–CH), 7.60 (d, *J* = 7.8 Hz, 1H, Ar–CH), 7.52 (t, *J* = 7.9 Hz, 1H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.32, 135.50, 132.95, 132.87, 131.28, 128.11, 122.45; IR V_{max} cm^{-1}: 3069.42 (N–H lactam), 2988.11 (C–H arom), 1693.43 (C═O stretch), 1604.87 (C═N stretch), 1578.68 (N–O stretch), 1343.29 (N–O stretch); *m/z* HRMS (APCI) found 410.9241, calcd for $C_{13}H_8BrN_4O_3S_2$ 410.9216 $[M + H]^+$; Purity (HPLC): 97%.

(2E,5Z)-5-(4-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3h**)

Green off-white puffy powder; 42% yield; m.p.: 273–275°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.20 (s, 1H, NH), 8.72 (s, 1H, thiazole–CH), 7.82 (s, 1H, CH), 7.71 (dd, *J* = 8.7, 5.4 Hz, 2H, Ar–CH) 7.43 (t, *J* = 8.8 Hz, 2H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 163.04, 143.54, 132.71, 132.64, 129.76, 116.69, 116.55; IR V_{max} cm^{-1}: 3093.91 (N–H lactam), 2988.02 (C–H arom), 1721.96 (C═O stretch), 1610.31 (C═N stretch), 1580.26 (N–O stretch), 1343.87 (N–O

stretch); *m*/*z* HRMS (APCI) found 351.0039, calcd for $C_{13}H_8FN_4O_3S_2$ 351.0016 $[M + H]^+$; Purity (HPLC): 96%.

(2E,5Z)-5-(4-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3i**)

Yellow crystals; 40% yield; m.p.: 298–300°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 8.65 (s, 1H, thiazole–CH), 7.78 (s, 1H, CH), 7.64 (d, J = 8.6 Hz, 2H, Ar–CH), 7.61 (d, J = 8.6 Hz, 2H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 142.94, 135.00, 131.88, 131.42, 129.18; IR V_{max} cm^{-1}: 3093.37 (N–H lactam), 2987.93 (C–H arom), 1729.23 (C=O stretch), 1690.96 (C=N stretch), 1573.42 (N–O stretch), 1342.30 (N–O stretch); *m*/*z* HRMS (APCI) found 366.9744, calcd for $C_{13}H_8ClN_4O_3S_2$ 366.9721 $[M + H]^+$; Purity (HPLC): 96%.

(2E,5Z)-5-(4-nitrobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3j**)

Fine golden crystals; 31% yield; m.p.: 326–327°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 8.70 (s, 1H, thiazole–CH), 8.38 (s, 1H, CH), 8.36 (s, 1H, Ar–CH), 7.88 (d, *J* = 8.7 Hz, 3H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.44, 130.93, 124.37; IR V_{max} cm^{-1}: 3093.08 (N–H lactam), 2987.89 (C–H arom), 1720.21 (C=O stretch), 1596.96 (C=N stretch), 1571.73 (N–O stretch), 1332.92 (N–O stretch); *m*/*z* HRMS (APCI): found 377.9984, calcd for $C_{13}H_8N_5O_5S_2$ 377.9961 $[M + H]^+$; Purity (HPLC): 96%.

(2E,5Z)-5-(4-hydroxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3k**)

Mustard powder; 32%; m.p.: > 400°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.00 (s, 1H, NH), 10.35 (s, 1H, OH), 8.68 (s, 1H, thiazole–CH), 7.70 (s, 1H, CH), 7.52 – 7.47 (m, 2H, Ar–CH), 6.96 – 6.91 (m, 2H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 160.25, 143.46, 134.50, 132.67, 123.87, 116.38; IR V_{max} cm^{-1}: 3260.80 (O–H stretch), 3110.97 (N–H lactam), 2987.31 (C–H arom), 1676.96 (C=O stretch), 1590.67 (C=N stretch), 1555.18 (N–O stretch), 1294.77 (N–O stretch); *m*/*z* HRMS (APCI) found 349.0084, calcd for $C_{13}H_9N_4O_4S_2$ 349.0060 $[M + H]^+$; Purity (HPLC): 99%.

(2E,5Z)-5-(2-methylbenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3l**)

Golden-brown crystals; 38% yield; m.p.: 268–269°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.19 (s, 1H, NH), 8.70 (s, 1H, thiazole–CH), 7.91 (s, 1H, CH), 7.49–7.46 (m, 1H, Ar–CH), 7.41 – 7.34 (m, 3H, Ar–CH), 2.40 (s, 3H, CH_3); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.58, 138.84, 132.21, 131.05, 130.55, 127.46, 126.73, 19.42; IR V_{max} cm^{-1}: 3067.70 (N–H lactam), 2987.58 (C–H arom), 1689.15 (C═O stretch), 1605.42 (C═N stretch), 1562.61 (N–O stretch), 1341.91 (N–O stretch); *m/z* HRMS (APCI) found 347.0297, calcd for $C_{14}H_{11}N_4O_3S_2$ 347.0267 $[M + H]^+$; Purity (HPLC): 99%.

(2E,5Z)-5-(4-methylbenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3m**)

Light-green puffy powder; 43% yield; m.p.: 268–269°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.09 (s, 1H, NH), 8.70 (s, 1H, thiazole–CH), 7.75 (s, 1H, CH), 7.51 (d, *J* = 8.0 Hz, 2H, Ar–CH), 7.36 (d, *J* = 8.0 Hz, 2H, Ar–CH), 2.36 (s, 3H, CH_3); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.47, 141.02, 130.19, 129.93, 21.04; IR V_{max} cm^{-1}: 3080.21 (N–H lactam), 3007.85 (C–H arom), 2880.37 (C–H methyl), 2798.27 (C–H methyl) 1719.61 (C═O stretch), 1690.79 (C═N stretch), 1577.20 (N–O stretch), 1343.38 (N–O stretch); *m/z* HRMS (APCI) found 347.0288, calcd for $C_{14}H_{11}N_4O_3S_2$: 347.0267 $[M + H]^+$; Purity (HPLC): 98%.

(2E,5Z)-5-(2,4-dimethoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3n**)

Bright-red powder; 51% yield; m.p.: 281°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 12.98 (s, 1H, NH), 8.68 (s, 1H, thiazole–CH), 7.94 (s, 1H, CH), 7.42 (d, *J* = 8.7 Hz, 1H, Ar–CH), 6.75 (dd, *J* = 8.7, 2.4 Hz, 1H, Ar–CH), 6.69 (d, *J* = 2.4 Hz, 1H, Ar–CH), 3.91 (s, 3H, CH_3), 3.86 (s, 3H, CH_3); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 163.42, 160.04, 143.53, 130.44, 114.33, 106.77, 98.64, 55.95, 55.66; IR V_{max} cm^{-1}: 3101.73 (N–H lactam), 2988.13 (C–H arom), 2797.78 (O–CH_3 stretch), 1702.07 (C═O stretch), 1610.93 (C═N stretch), 1550.36 (N–O stretch), 1343.65 (N–O stretch), 1207.07 (C–O stretch); *m/z* HRMS (APCI) found 393.0343, calcd for $C_{15}H_{13}N_4O_5S_2$: 393.0322 $[M + H]^+$; Purity (HPLC): 88%.

(2E,5Z)-5-(2,4,6-trimethoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3o**)

Black flakes; 42% yield; m.p.: 292–293°C ; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 7.63 – 7.54 (m, 2H, Ar–CH), 7.49 (t, *J* = 7.5 Hz, 1H, Ar–CH), 7.40 (t, *J* = 7.2 Hz, 1H, Ar–CH), 3.30 (s, 9H, 3 × CH_3); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 180.27, 178.86, 156.66, 134.53, 129.80, 129.34, 129.05, 128.99, 127.79, 45.33, 25.24, 23.47; IR V_{max} cm^{-1}: 3081.72 (N–H lactam), 2836.20 (O–CH_3 stretch), 1705.11 (C═O stretch), 1608.07 (C═N stretch), 1542.99 (N–O stretch), 1345.12 (N–O stretch), 1293.02 (C–O stretch); *m*/*z* HRMS (APCI) found 423.0450, calcd for $C_{16}H_{15}N_4O_6S_2$ 423.0428 $[M + H]^+$; Purity (HPLC): 85%.

(2E,5Z)-5-(3-methoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3p**)

Green-yellow powder; 54%; m.p.: 252°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 13.14 (s, 1H, NH), 8.70 (s, 1H, thiazole–CH), 7.76 (s, 1H, CH), 7.48 (t, *J* = 8.2 Hz, 1H, Ar–CH), 7.19 (d, *J* = 7.4 Hz, 2H, Ar–CH), 7.10 – 7.04 (m, 1H, Ar–CH), 3.82 (s, 3H, CH_3); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 159.60, 143.36, 134.40, 130.38, 121.90, 116.06, 115.86, 55.25; IR V_{max} cm^{-1}: 3110.50 (N–H lactam), 2988.16 (C–H arom), 2797.54 (O–CH_3 stretch), 1720.46 (C═O stretch), 1603.05 (C═N stretch), 1569.55 (N–O stretch), 1345.01 (N–O stretch), 1232.32 (C–O stretch); *m*/*z* HRMS (APCI) found 363.0023, calcd for $C_{14}H_{11}N_4O_2S_3$ 363.0039 $[M + H]^+$; Purity (HPLC, PDA, 280nm): 99%.

(2E,5Z)-5-(3,4-dichlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3q**)

Green puffy powder; 35%; m.p.: 279–281°C; ^{1}H NMR (600 MHz, (D_6)DMSO) δ 8.70 (s, 1H, thiazole–CH), 7.92 (d, *J* = 1.8 Hz, 1H, Ar–CH), 7.84 (d, *J* = 8.4 Hz, 1H, Ar–CH), 7.77 (s, 1H, CH), 7.59 (dd, *J* = 8.4, 1.8 Hz, 1H, Ar–CH); ^{13}C NMR (151 MHz, (D_6)DMSO) δ 143.39, 137.96, 132.07, 132.01, 131.42, 128.90, 119.62; IR V_{max} cm^{-1}: 3089.46 (N–H lactam), 2989.50 (C–H arom), 1729.79 (C═O stretch), 1698.65 (C═N stretch), 1579.35 (N–O stretch), 1345.73 (N–O stretch); *m*/*z* HRMS (APCI) found 400.9355, calcd for $C_{13}H_7Cl_2N_4O_3S_2$ 400.9331 $[M + H]^+$; Purity (HPLC): 96%.

3.3.3. *In vitro* antitubercular evaluation

The antitubercular property of target compounds was established using the method reported by Beteck *et al.* (2019). In brief, a 10 ml culture of the gfp reporter Mtb strain was grown to an optical density (OD_{600}) of 0.6–0.7 in Middlebrook 7H9 medium supplemented with 0.03% casitone (CAS), 0.4% glucose, and 0.05% tyloxapol. Target compounds at 0.244–125 µM concentration range were added in 96-well plates and 50 ml of Mtb containing medium was introduced to each well. This was followed by sealing and incubation of the microplates at 37°C with 5% CO_2 and humidification. The antitubercular activity was determined with rifampicin (RIF) (2 x MIC_{90}) and 5% DMSO acting as the minimum, and maximum growth controls, respectively. On day 14, fluorescence readings at 485, and 520 nM were recorded for each well using a plate reader (FLUOstar OPTIMA, BMG LABTECH). These readings were standardised and used to generate dosage response curves with the help of the CDD Vault from Collaborative Drug Discovery. The minimal inhibitory concentration (MIC) was estimated using the Levenberg-Marquardt damped least-squares method and it is reported as the lowest concentration that inhibits the growth of more than 90% of the bacterial population [40].

3.3.4. *In vitro* antibacterial and antifungal evaluation

Starting from 32 µg/ml, eight-fold serial dilutions of each sample were prepared in 384-well, non-binding surface plates while keeping the final concentration of DMSO below 0.5%. Bacteria were cultured at 37°C overnight using Cation-Adjusted Mueller Hinton Broth (CAMHB) overnight and followed by 40-fold dilution, incubation at 37°C for 1.5–3 h.

Cultures at mid-log phase were diluted (CFU/ml measured by OD_{600}) and plated to each compound-containing well plate to attain a volume of 50 µl and a cell density of 5 x 10^5 CFU/ml. Plates were incubated at 37°C for 18 h without shaking. Using a Tecan M1000 Pro monochromator plate reader, absorbance at 600 nm (OD_{600}) were recorded for each well and used to determined inhibition of bacterial growth. The minimum inhibitory concentration (MIC), defined as the lowest concentration at which > 80% of bacteria growth is inhibited, was

determined following CLSI guidelines. Compounds exhibiting MIC ≤ 16 µg/mL in either replicate (n=2) were classified as hits. *C. albicans* was cultured using Yeast Extract-Peptone Dextrose (YEPD) agar at 30°C for 3 days. A suspension of culture was made and subsequently diluted and added to each well of the compound-containing plates to give a final cell density of 2.5 x 10^3 CFU/mL and a volume of 50 µl. All plates were sealed and incubated for 36 h at 35°C, followed by addition of resazurin (0.001% final concentration) and incubating for additional 2 h at 35°C. Absorbance at 630 nm (OD_{630}) for each well was measured using a Biotek Multiflo Synergy HTX plate reader to determine growth inhibition.

3.3.5. *In vitro* cytotoxicity evaluation: resazurin assay

HEK-293 cells were counted manually in a Neubauer haemocytometer and then plated in 384-well compound-containing plates to give a density of 5×10^3 cells and a volume of 50 µl per well. Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum was used as growth media. Compounds at 32 µg/ml were added and incubated for 20 h at 37°C in 5% CO_2, followed by the addition of resazurin (5 µl) and incubation for 3 more hours at 37°C in 5% CO_2. The fluorescence of each well was recorded at 590 nm using a Tecan M1000 Pro monochromator plate reader and these data along with concentrations of compounds was used to generate the CC_{50} (concentration required to inhibit 50% cell growth) values.

3.3.6. *In vitro* haemolytic evaluation

Saline solution (NaCl, 0.9%) was used to wash human whole blood, after which the cells were re-suspended at concentration of 0.5×10^8 cells/ml and then added to the 384-well compound-containing plates to a final volume of 50 µl. The plates were shaken for ten minutes, they were then incubated at 37°C for 1 h. Plates were subsequently centrifuged at 1000 g for 10 min, after which the supernatant (25 µl) was transferred to a 384-well plate. Absorbance of the supernatant at 405 nm was recorded using a Tecan M1000 Pro monochromator plate reader, these data together with compounds concentrations were used to generate HC_{10} (concentration at which 10% of RBCs are haemolysed) values.

3.4. Conclusion

In this study, we successfully synthesised low molecular weight (< 500 Da), novel thiazolidin-4-one appended 5-nitrothiazole derivatives. The compounds were evaluated *in vitro* for antibacterial, antifungal, and overt cell toxicity. All compounds exhibited potent antitubercular activity, less than 5 µM. Most of the compounds showed potent activity against *S. aureus*, wherein SAR analyses suggest that the *ortho* substituted benzylidene moiety promotes activity against *S. aureus.* Notably, compound **3b** exhibited superior activity (< 0.25 µg/mL) against *methicillin-resistant S. aureus* compared to the standard (vancomycin; 1 µg/mL). With regards to antifungal activity, a few compounds showed low micromolar activity against *C. albicans*, with **3g** exhibiting comparable activity (≤ 0.25 µg/mL) to the standard fluconazole. Compounds in this study were not cytotoxic towards HEK-293 and human red blood cells. These compounds were generally inactive against Gram-negative bacteria and presented with insoluble problems. Future work will be aimed at overcoming these challenges.

3.5. Acknowledgements

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3.6. Conflict of interest

The authors have no competing interest to declare.

3.7. Data availability statement

The data that support the findings of this study are available in the supplementary material (**Annexure A**).

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CHAPTER 4: SUMMARY AND CONCLUSION

Tuberculosis (TB), a disease caused by *Mycobacterium tuberculosis* (*Mtb*), kills thousands of people each year despite the fact that it is preventable and curable with proper treatment. In 2020, 10 million people contracted TB, and it was responsible for 1.3 million deaths globally (WHO, 2021). The primary concerns about TB are as follows: (i) HIV co-infection; (ii) the predominance of drug-resistant *Mtb* strains leading to multidrug-resistant TB (MDR-TB) cases; (iii) the length of treatment and chemotherapy, which increases in the case of resistant TB; (iv) the toxicity of antitubercular drugs (Fang et al., 2019:1929; Woimo et al., 2017:2); and (v) latent-TB infection, which is thought to affect one-third of the world's population and serves as a reservoir for future TB infections (active TB) (Houben & Dodd, 2016:3).

Developing effective chemotherapeutic drugs with minimal toxicity and novel mechanisms of action to treat TB, particularly MDR-TB, has been an ongoing struggle since the discovery of the first antitubercular agent almost half a century ago. With the advent of MDR-TB, the utility of first-line drugs has been substantially reduced, rendering the majority of patient TB treatment outcomes unsatisfactory. Improving these outcomes in MDR-TB is further hampered in part by inadequate second-line drugs (WHO, 2019). MDR-TB treatment includes drugs with many side effects, which promotes patient noncompliance and treatment discontinuation. Patients who fail or default on TB treatment have a higher risk of death and develop additional complications. Furthermore, these patients accelerate the spread of drug-resistant *Mtb* within communities (Podewils et al., 2013:1).

Antimicrobial resistance is of high prevalence in hospital settings and is frequently related to ESKAPE pathogens. ***E****nterococcus faecium*, ***S****taphylococcus aureus*, ***K****lebsiella pneumoniae*, ***A****cinetobacter baumannii*, ***P****seudomonas aeruginosa*, and ***E****nterobacter* spp. are the six hypervirulent, antimicrobial resistant bacteria represented by the abbreviation ESKAPE (Orosz et al., 2022:27). These pathogens are associated with potentially fatal hospital-

acquired infections (HAIs), such as catheter-associated urinary tract infections, central line-associated bloodstream infections, ventilator-associated pneumonia, and surgical site infections in critically ill and immunocompromised patients (CDC, 2014). The prevalence of HAIs is rising at an alarming rate for a number of reasons, including the emergence of new infectious diseases and the reappearance of previously eradicated infectious diseases; the rise in antimicrobial resistance; and the lengthening of patients' duration of stay in hospitals while they receive specialised care, *i.e*., ICU patients (Nouri et al., 2020:2365).

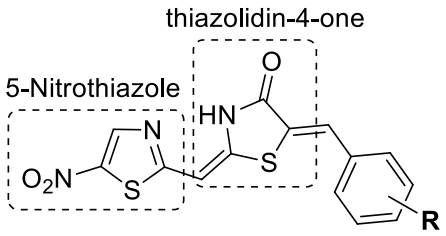
When compared to their susceptible counterparts, ESKAPE pathogens result in much worse clinical outcomes (Kamurai et al., 2020:1). Higher mortality and morbidity rates, as well as increased healthcare costs, are some of the major consequences of HAIs caused by ESKAPE pathogens (Ma et al., 2020:3). Furthermore, despite the plethora of antibiotics accessible for clinical use, the principles of evolution and natural selection, as well as antibiotic misuse, have all contributed to the development and spread of drug-resistance among these pathogens (Wang et al., 2020:1). This jeopardises modern medicine's accomplishments, and in the absence of effective antibiotics for the prevention and treatment of HAIs, medical procedures such as organ transplants may become considerably riskier and more futile in the future (WHO, 2020). In fact, if no new antibiotics are developed or discovered by 2050, it is anticipated that there will be no effective antibiotics available to treat infections caused by resistant bacteria like ESKAPE pathogens (Vivas et al., 2019). To avert this eventuality, novel antimicrobial drugs to combat the rise in antimicrobial resistance are urgently required.

It has been shown that the drug nitazoxanide (a 5-nitrothiazole based drug) has potent inhibitory activity against both replicating and non-replicating *Mtb* via a unique mechanism of action (MOA), which seems to prevent resistance (De Carvalho et al., 2009:5789–5792). Recent studies also demonstrated that a RIF–NTZ combination was successful in eradicating latent *Mtb* strains in as little as 28 to 35 days, in contrast to the approximately 6 month time frame required by other antitubercular drug combinations employed in human treatment.

Nitazoxanide is therefore indicated to be useful as a suitable lead compound in designing novel antitubercular drugs with a short development time (Iacobino et al., 2019:1-4; Umumararungu et al., 2020:550).

The thiazolidin-4-one scaffold is referred to as a "wonder nucleus" because it demonstrates a wide variety of biological activities, including those that are anti-tuberculous, antibacterial, anti-inflammatory, anticarcinogenic, and antiprotozoal, amongst others (Mech et al., 2021:1). In the field of medicinal chemistry, thiazolidin-4-ones are well-known examples of privileged scaffolds, and they have become the focus of many studies as potential scaffolds for the synthesis of novel drugs. For example, it is a well-known fact that modifying the C2 and C5 positions of the thiazolidin-4-one scaffold often results in the generation of compounds with optimised biological activity (Molina et al., 2021:130). Also, Vicini et al. (2006:3863) reported that C5-benzylidene substituted thiazolidin-4-one analogues had considerably increased antibacterial activity when compared to counterparts in which the C5 position is unsubstituted.

In this study, target compounds (see **Fig. 4.1)** containing a 5-nitrothiazole moiety appended to thiazolidin-4-one, were conceptualised, synthesised, characterised (through ^{1}H NMR, ^{13}C NMR, FTIR, HRMS, and HPLC), and biologically evaluated *in vitro*.



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3a: H	**3e**: 3-F	**3h**: 4-F	**3l**: 2-Me	**3n**: 2,4-MeO	**3q**: 3,4-Cl
3b: 2-F	**3f**: 3-Cl	**3i**: 4-Cl	**3m**: 4-Me	**3o**: 2,4,6-MeO	
3c: 2-Cl	**3g**: 3-Br	**3j**: 4-NO_2		**3p**: 3-MeO	
3d: 2-NO_2		**3k**: 4-OH			

Figure 4.1 General structure of target compounds (**3a – 3j**)

The compounds in this study were generally active against the gfp reporter strain of *Mtb* (cultured in Middlebrook 7H9 medium), with potent MIC_{90} values (the lowest concentration of compound needed to inhibit the growth of more than 90% of *Mtb*) in the range of < 0.244–2.004 µM. Comparing the MIC_{90} value of parent intermediate **2** (31.25 µM) against the values of target compounds **3a – 3q**, suggests that appending on to **2** a benzylidene moiety bearing electron withdrawing– (EWG) or electron donating groups (EDG) is essential for antitubercular activity.

Target compounds were also evaluated for antimicrobial activities against some ESKAPE pathogens and compounds **3a**, **3b**, **3c**, **3d**, **3e**, **3g**, **3i**, **3l**, **3m**, and **3p** demonstrated significant activity against *S. aureus*, with MIC_{80} values (the lowest concentration at which > 80% of bacterial growth was inhibited) in the range of 2–8 µg/ml. However, the compounds were generally inactive against Gram-negative ESKAPE pathogens (*Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*), probably owing to the lack of penetration through the highly lipophilic cell walls of these bacteria. Furthermore, the compounds were inactive against methicillin-resistant *Staphylococcus aureus* (MRSA) (except for **3b**), suggesting that penicillin binding protein might be a probable target for this series. Compound **3b**, demonstrated sub-micromolar activity against MSRA (MIC < 0.25 µg/ml), indicating an alternative target from penicillin binding protein. It is also worth noting that **3b** exhibited superior activity against the reference drug vancomycin (1 µg/ml) for MSRA activity. Furthermore, the SAR of the target compounds (**3a – 3q)** demonstrated that substitution in the *ortho* positions of the C5-benzylidene moiety favoured *S. aureus* activity.

In addition to antitubercular and antimicrobial screening, target compounds were evaluated for antifungal activity against *C. albicans*. Six compounds displayed varying inhibitory concentrations, including **3a** (4 µg/ml), **3b** (4 µg/ml), **3e** (16 µg/ml), **3g** (≤ 0.25 µg/ml), **3h** (32 µg/ml), and **3q** (32 µg/ml). It is noteworthy to mention that **3g** exhibit comparable activity to the reference drug fluconazole (0.12 µg/ml).

Cell toxicity against HEK-293 cells (cultured in Dulbecco's Modified Eagle Medium) and haemolysis against human red blood cells were also investigated. The target compounds herein reported displayed no cytotoxicity nor haemolytic activity (HC_{10} / HC_{50} > 32 µg/ml).

Collectively, the study's aims and objectives were met, and the results indicate the successful synthesis (utilising a molecular hybridization approach) and antimicrobial evaluation of 17 novel nitrothiazole-thiazolidinone hybrids (**3a – 3q**) with a molecular weight (MW) of less than 500 Da. Moreover, these hybrids may be considered as possible oral drug candidates according to Lipinski's Rule of 5 (based on *in silico* evaluation). However, lack of solubility in commercially available solvents has been identified as a limitation for this series of compounds, which will be addressed in future work. The findings of this study warrant further investigation and optimisation as potential lead compounds for overcoming and treating resistant pathogens associated with TB and HAIs.

Table 4.1 Target compounds with the best MIC values against evaluated pathogens

Pathogen	Target compound	MIC
Mycobacterium tuberculosis	**3c, 3g, 3h, 3i, 3l, 3n, 3q**	< 0.24 µM
Staphylococcus aureus	**3b**, **3d**, **3g**, **3l**, **3m**	2 µg/ml
Methicillin-resistant *S. aureus*	**3b**	< 0.25 µg/ml
Candida albicans	**3g**	≤ 0.25 µg/ml

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ANNEXURE A: SUPPLEMENTARY MATERIAL FOR CHAPTER 3

Nitrothiazole-thiazolidinone hybrids: Synthesis and *in vitro* antimicrobial evaluation

SUPPORTING DATA FOR *IN SILICO* PREDICTIONS

Table 1: Prediction of distribution and absorption properties of **2** and **3a – 3j**

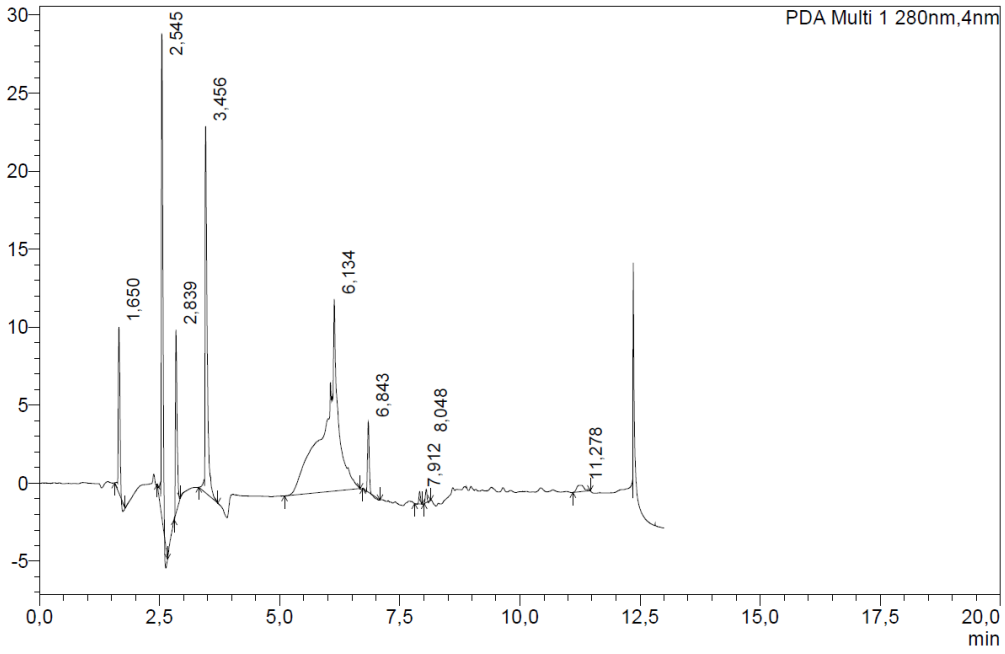
ID	GI Absorption	BBB permeant	P-gp substrate	CYPP450				
				CYP1A2	CYP2C19	CYP2C9	CYP2D6	CYP3A4
2	Low	No	No	✓	X	X	X	X
3a	Low	No	No	✓	✓	✓	X	X
3b	Low	No	No	✓	✓	X	X	X
3c	Low	No	No	✓	✓	X	X	X
3d	Low	No	No	✓	✓	X	X	X
3e	Low	No	No	✓	✓	X	X	X
3f	Low	No	No	✓	✓	✓	X	X
3g	Low	No	No	✓	✓	✓	X	X
3h	Low	No	No	✓	✓	X	X	X
3i	Low	No	No	✓	✓	✓	X	X
3j	Low	No	No	✓	✓	X	X	X
3k	Low	No	No	✓	✓	✓	X	✓
3l	Low	No	No	✓	✓	✓	X	✓
3m	Low	No	No	✓	✓	✓	X	✓
3n	Low	No	No	✓	✓	✓	X	✓
3o	Low	No	No	✓	✓	✓	✓	✓
3p	Low	No	No	✓	✓	✓	X	✓
3q	Low	No	No	✓	✓	✓	X	✓

SUPPORTING DATA FOR CHEMISTRY, CHARACTERIZATION AND SYNTHESIS

Blank HPLC test

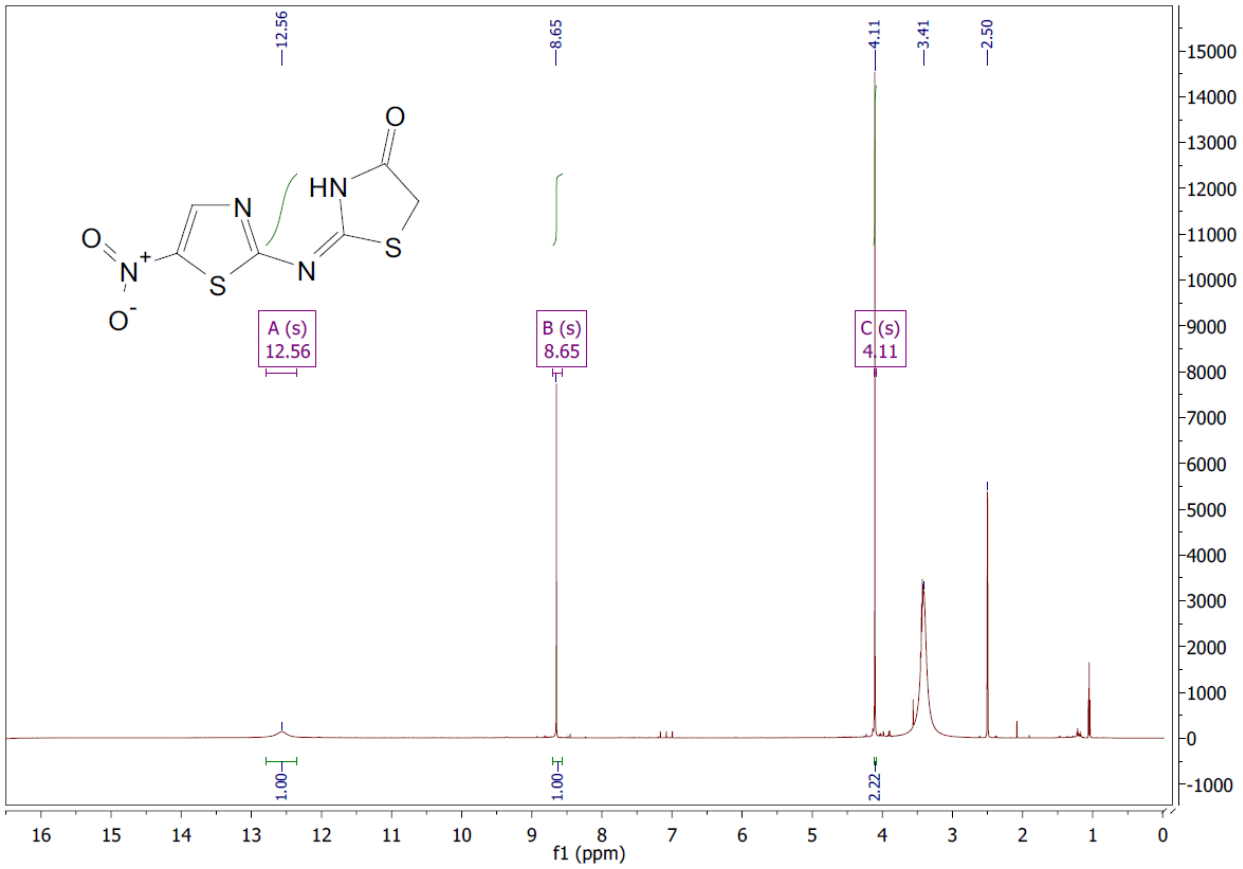
A blank experimental HPLC control analysis using just acetonitrile (solvent) was conducted to rule out any visibly undesirable peaks that may be present in the HPLC data of the test compounds owing to solvent or column impurities. The HPLC data of parent intermediate **2** and hybrid-target compounds **3a – 3j** with identical peaks to the blank were excluded from the calculation (AUC) of purity for each individual compound.

Blank HPLC result (Solvent: Acetonitrile)

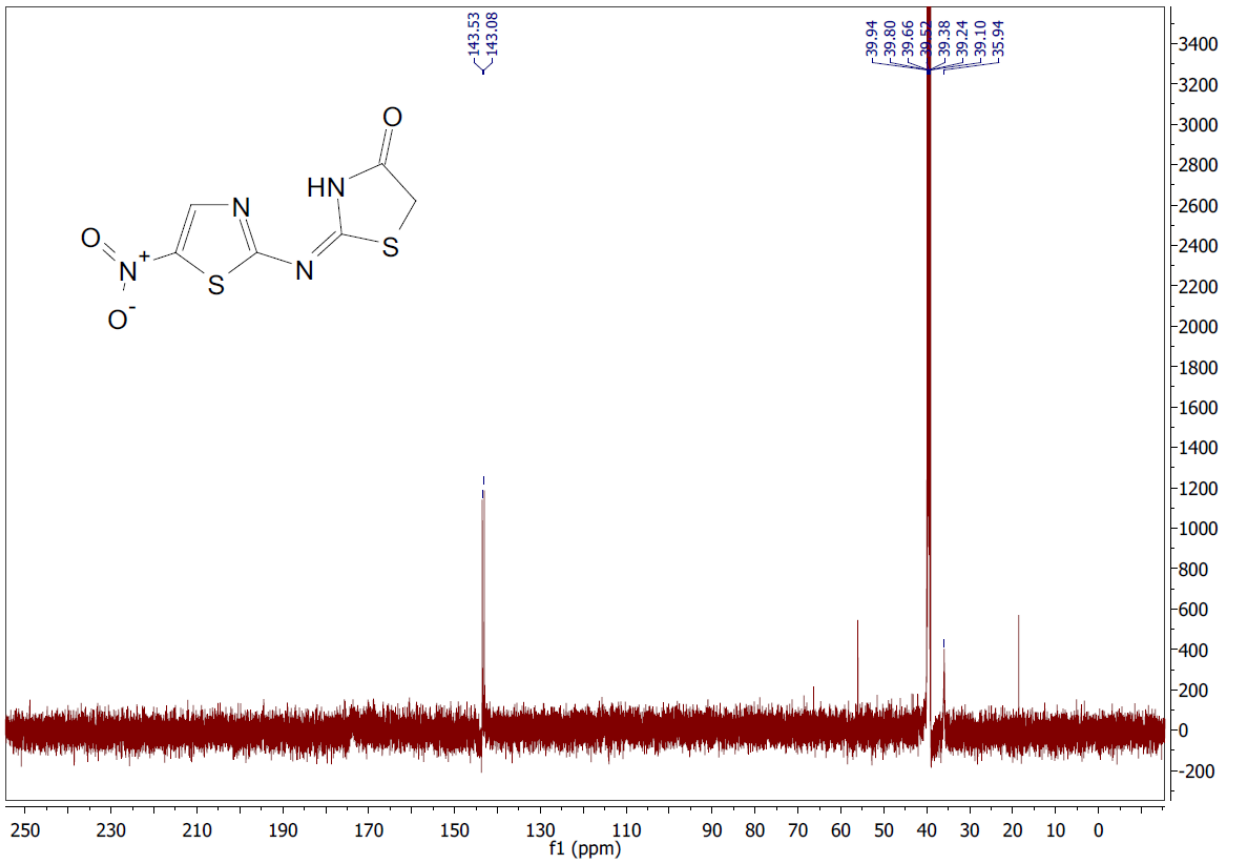


(E)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**2**)

^{1}H NMR



^{13}C NMR



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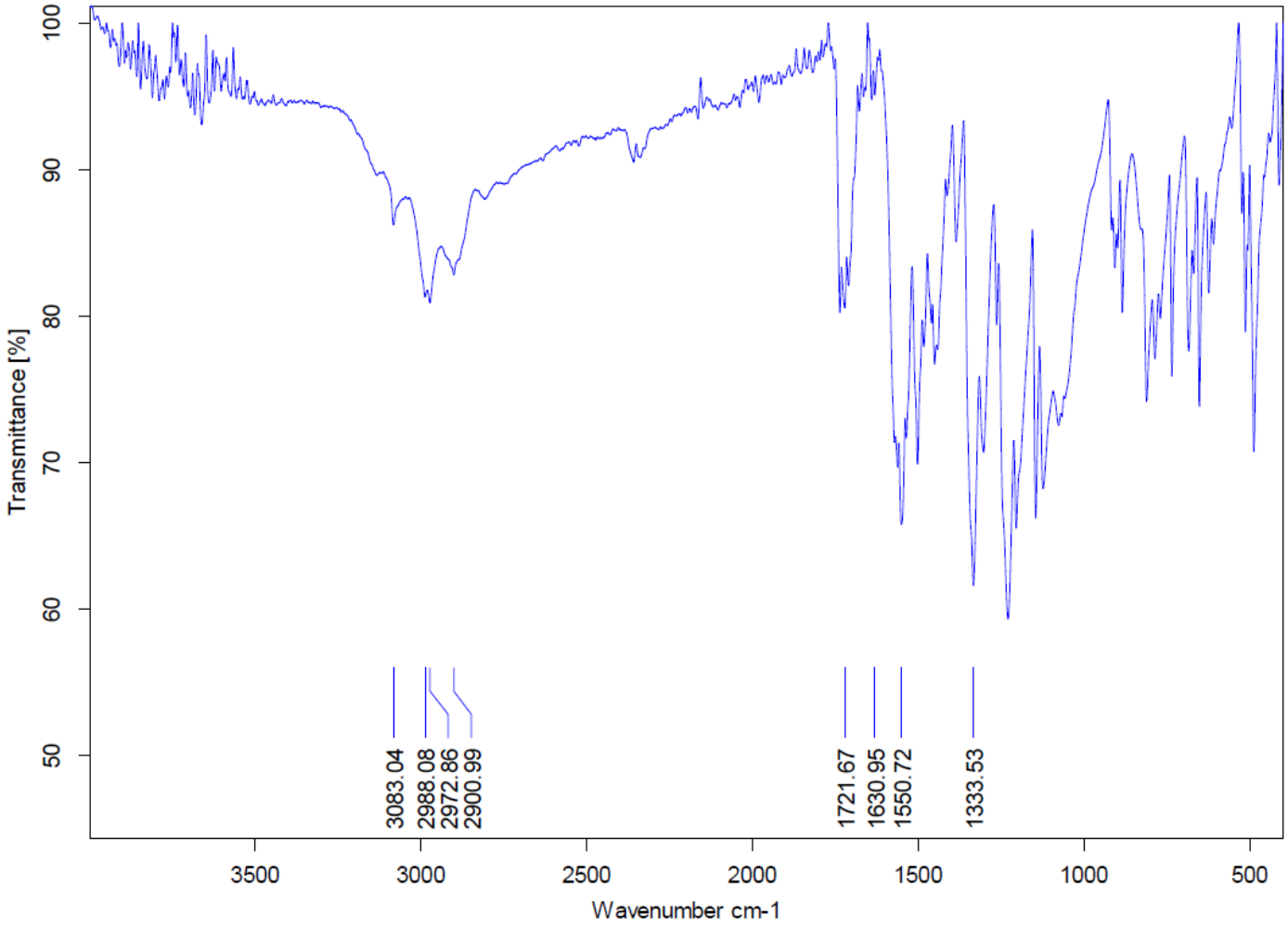

HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
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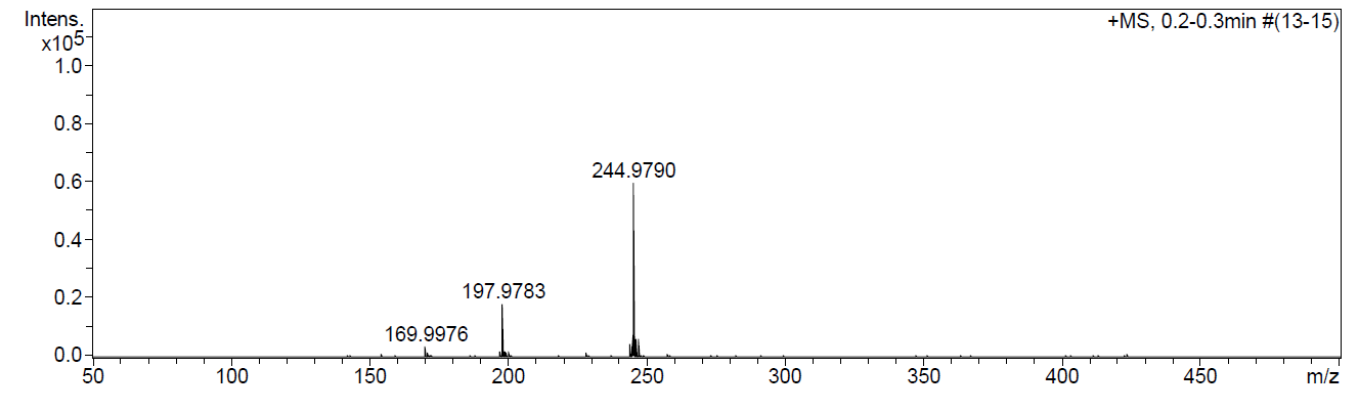

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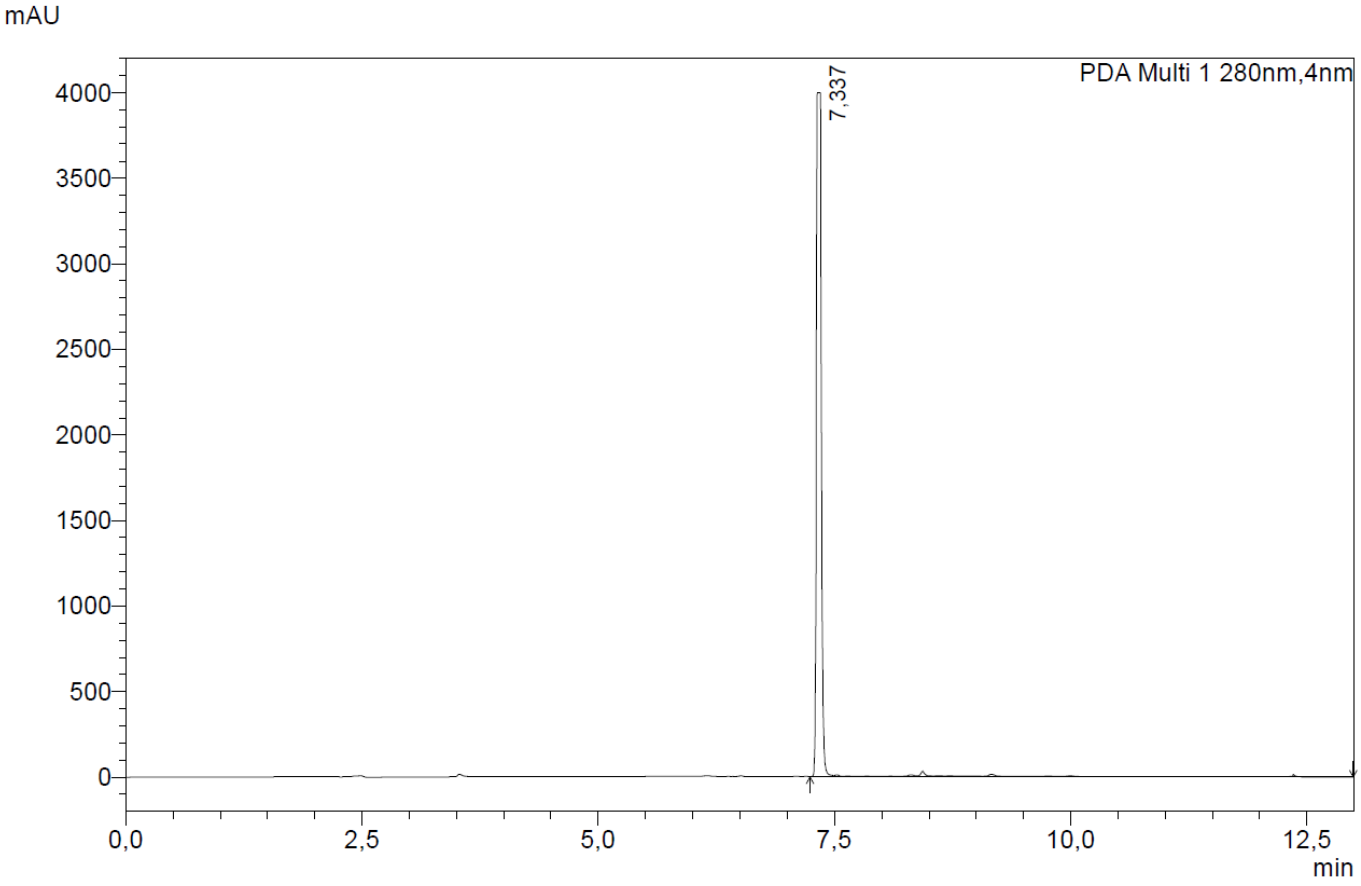
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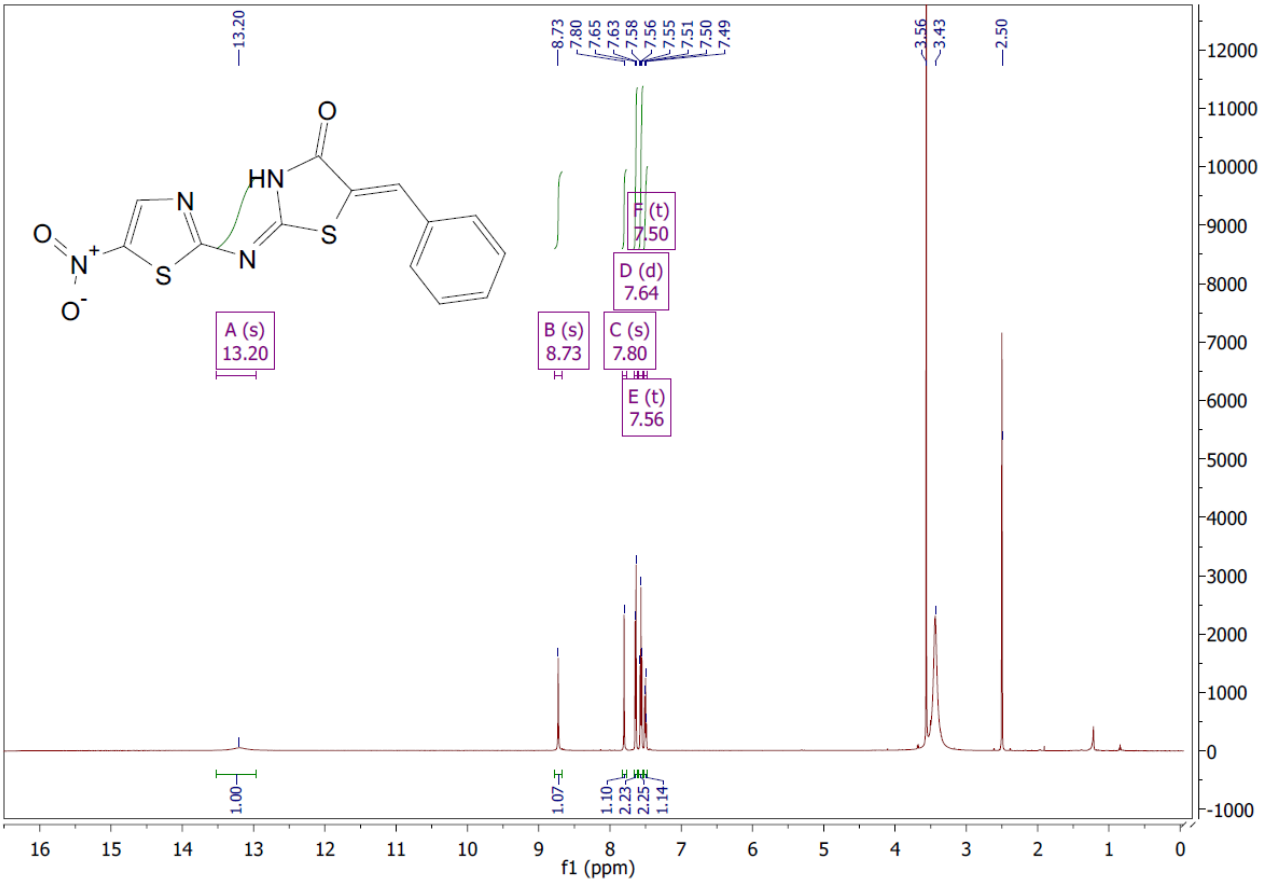
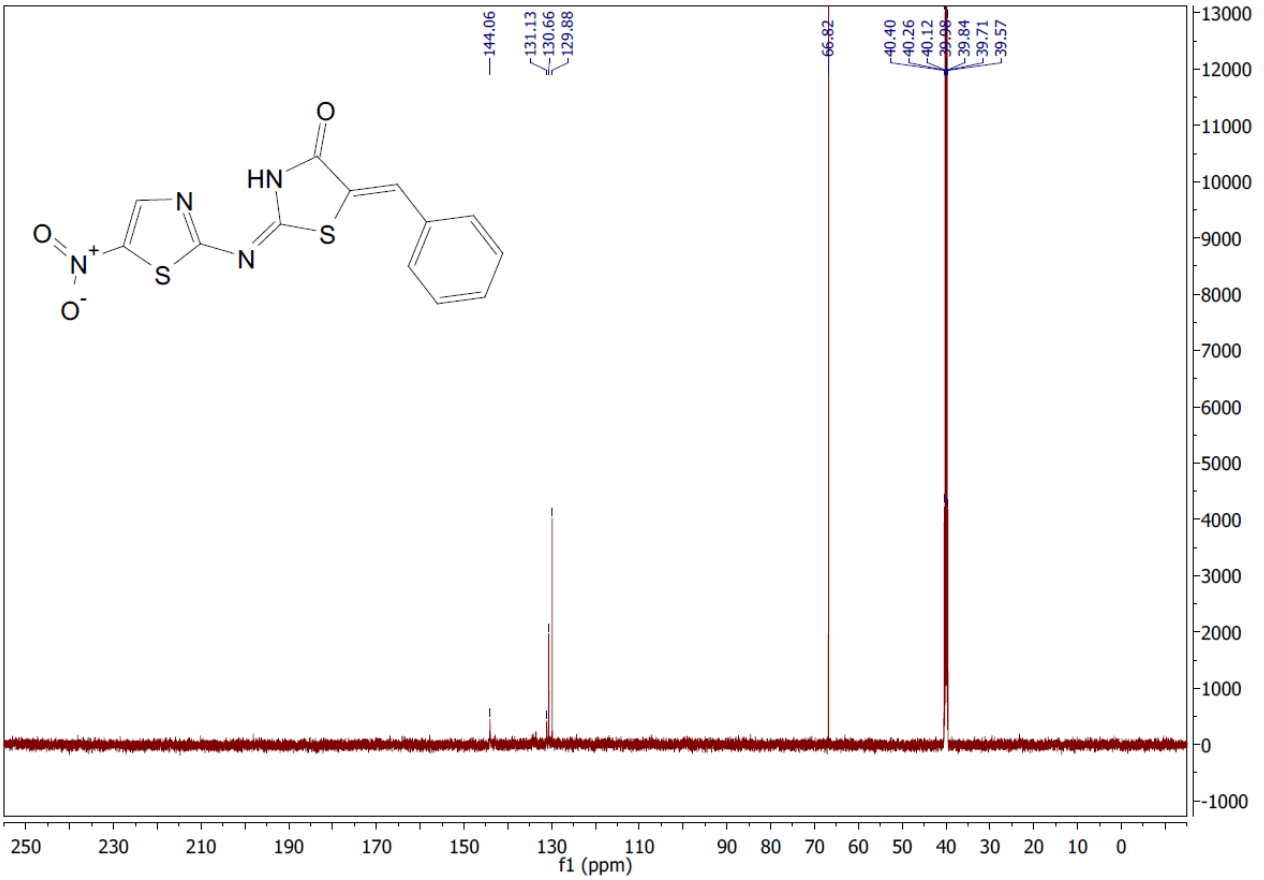
3000
2500
2000
1500
1000
500
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-benzylidene-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3a**)

^{1}H NMR



^{13}C NMR



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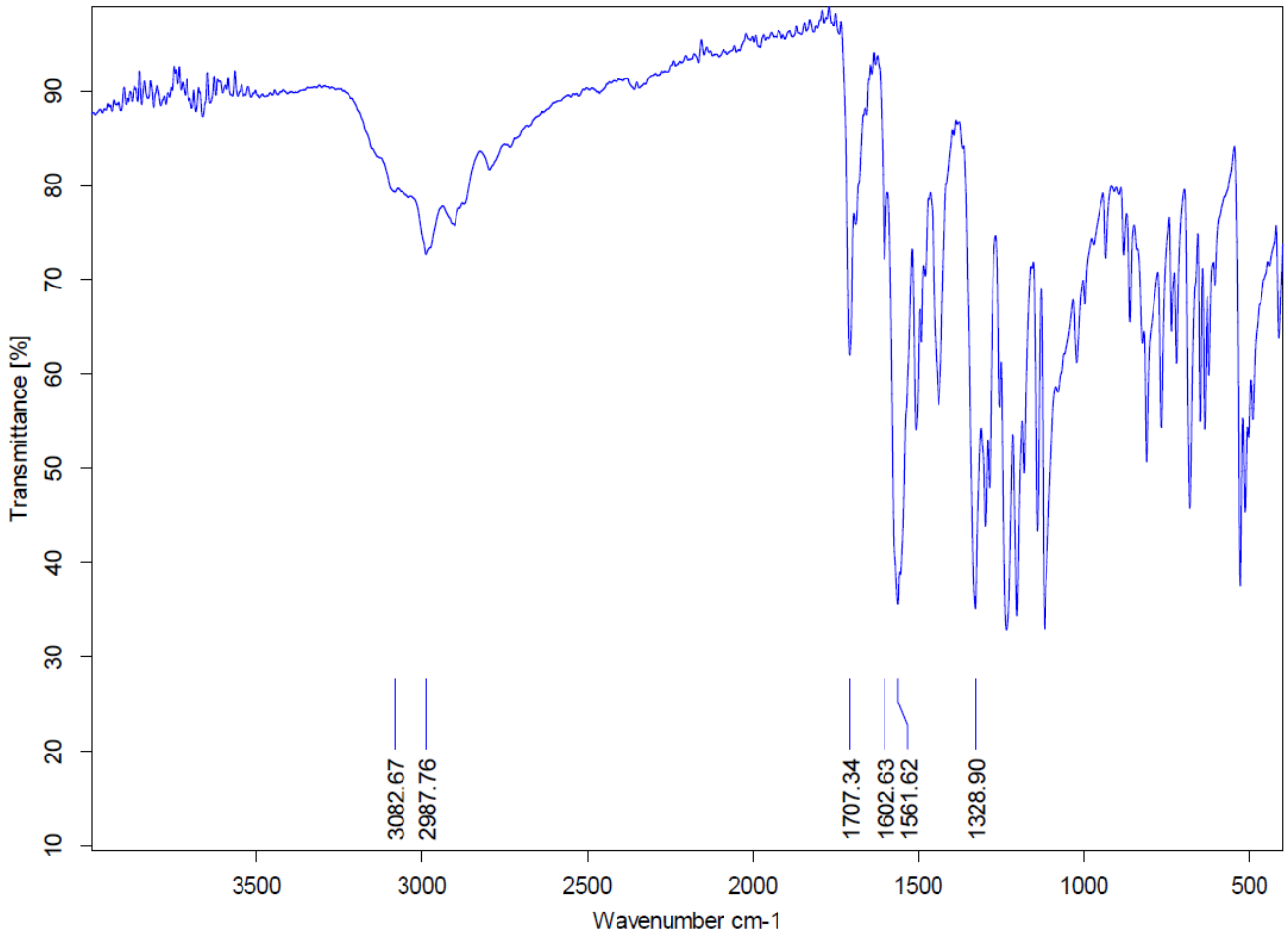

HRMS

Acquisition Parameter

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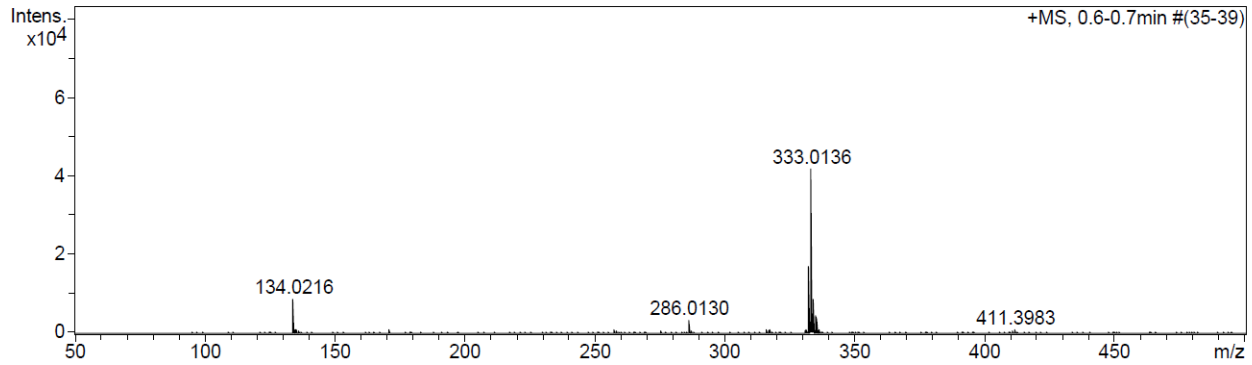

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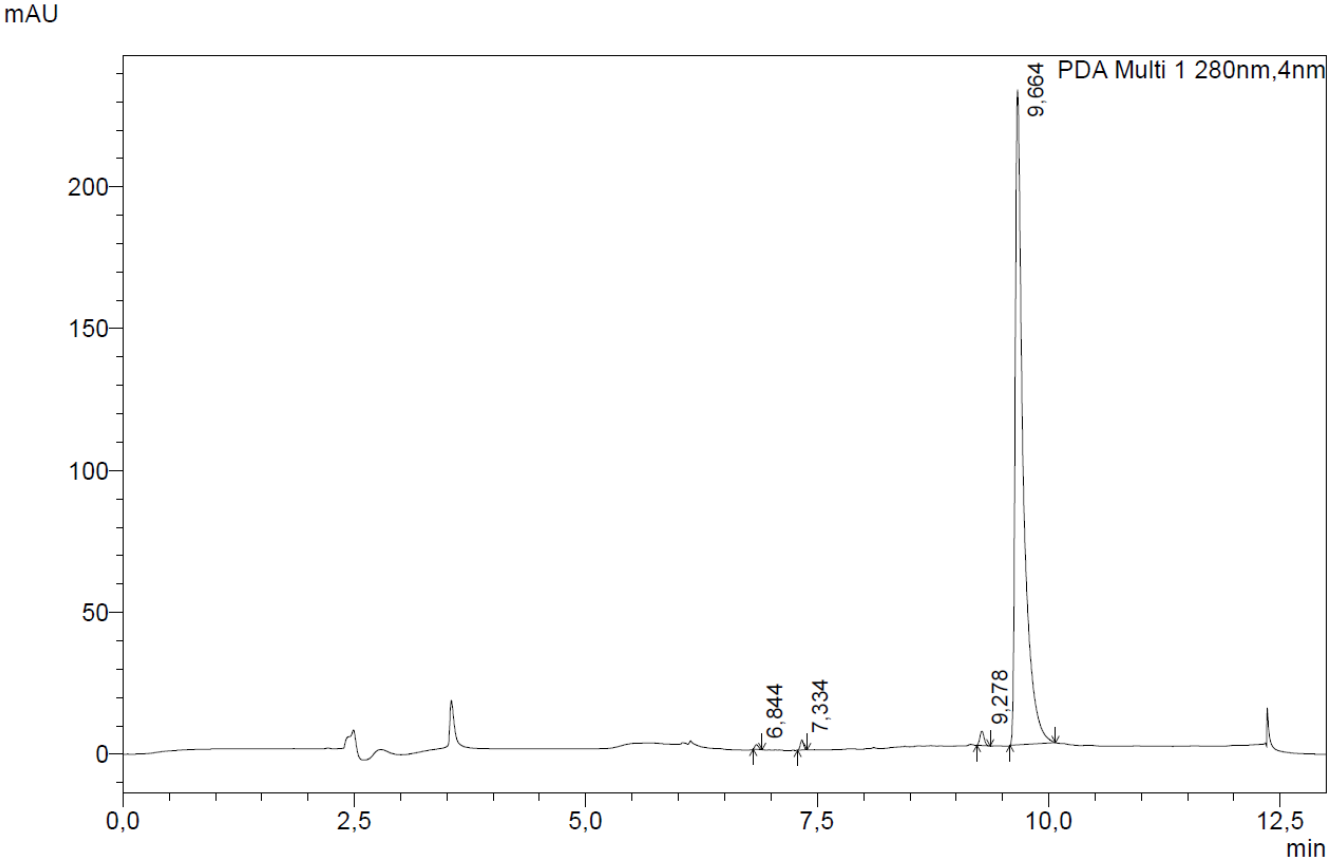
	2	C 5 H 10 S 2	100.00	134.0218	0.2	1.4	25.2	1.0	odd	ok
	3	C 7 H 4 N O 2	32.73	134.0237	2.0	15.0	27.1	6.5	even	ok
	4	C 5 H 2 N 4 O	68.15	134.0223	0.7	4.9	32.0	7.0	odd	ok
333.0136	1	C 13 H 9 N 4 O 3 S 2	100.00	333.0111	-2.5	-7.5	16.7	11.5	even	ok

HPLC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150
100
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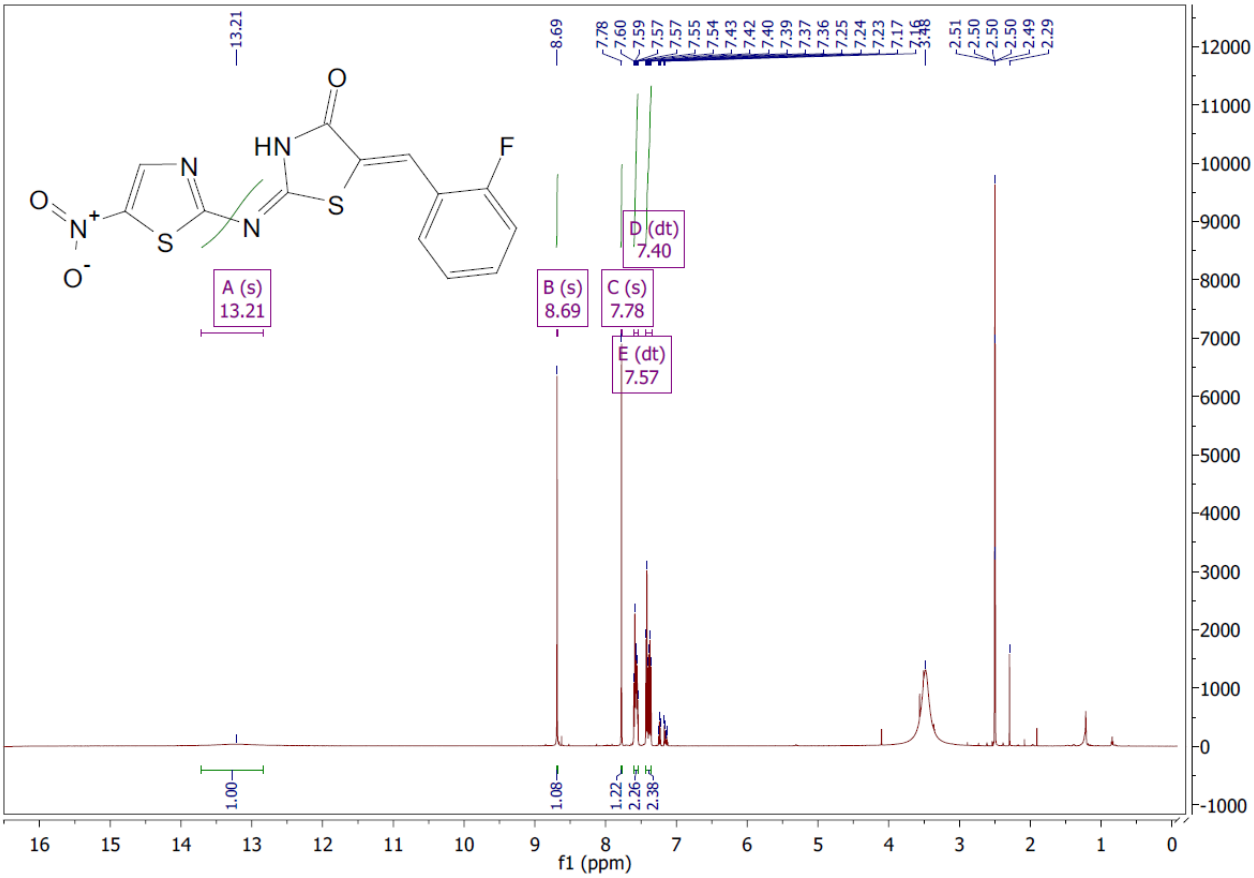
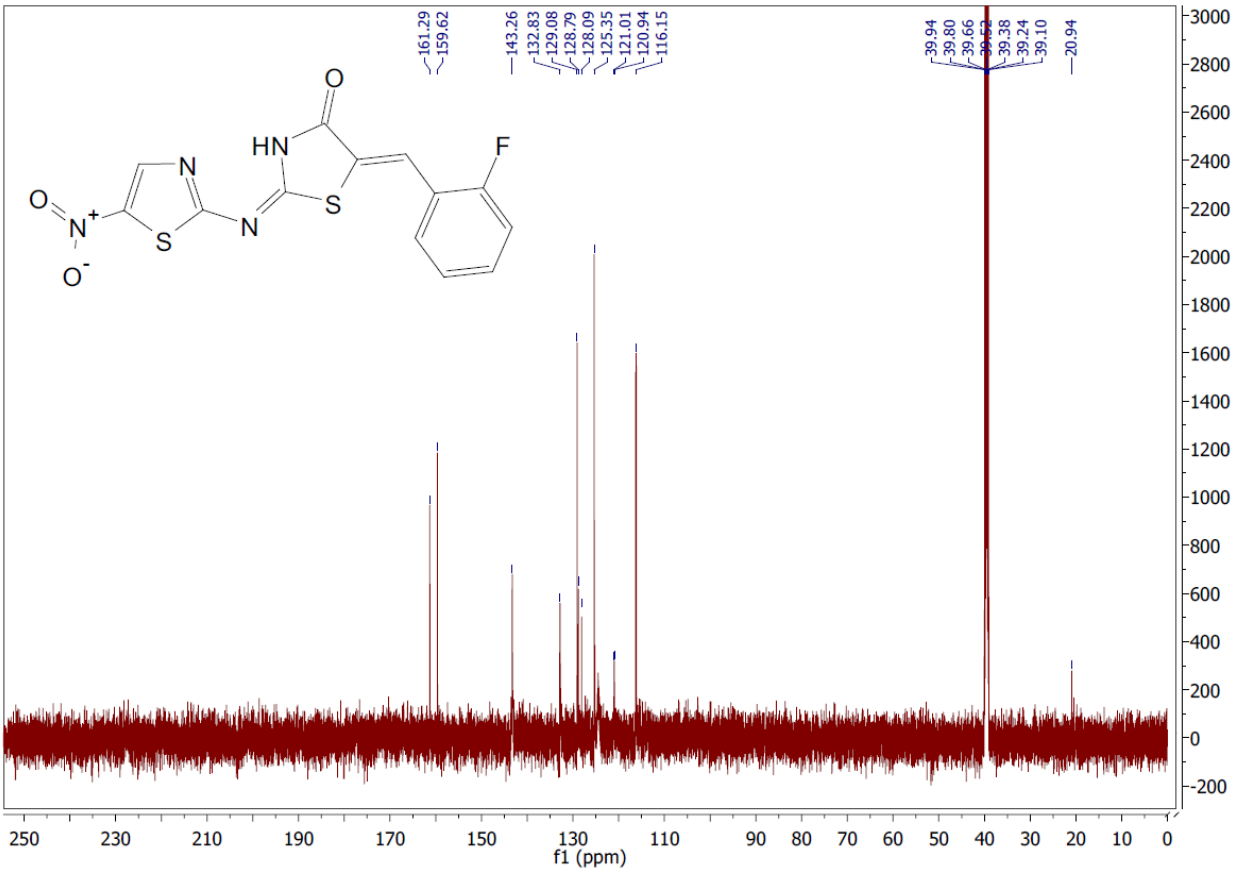
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(2-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3b**)

^{1}H NMR



^{13}C NMR



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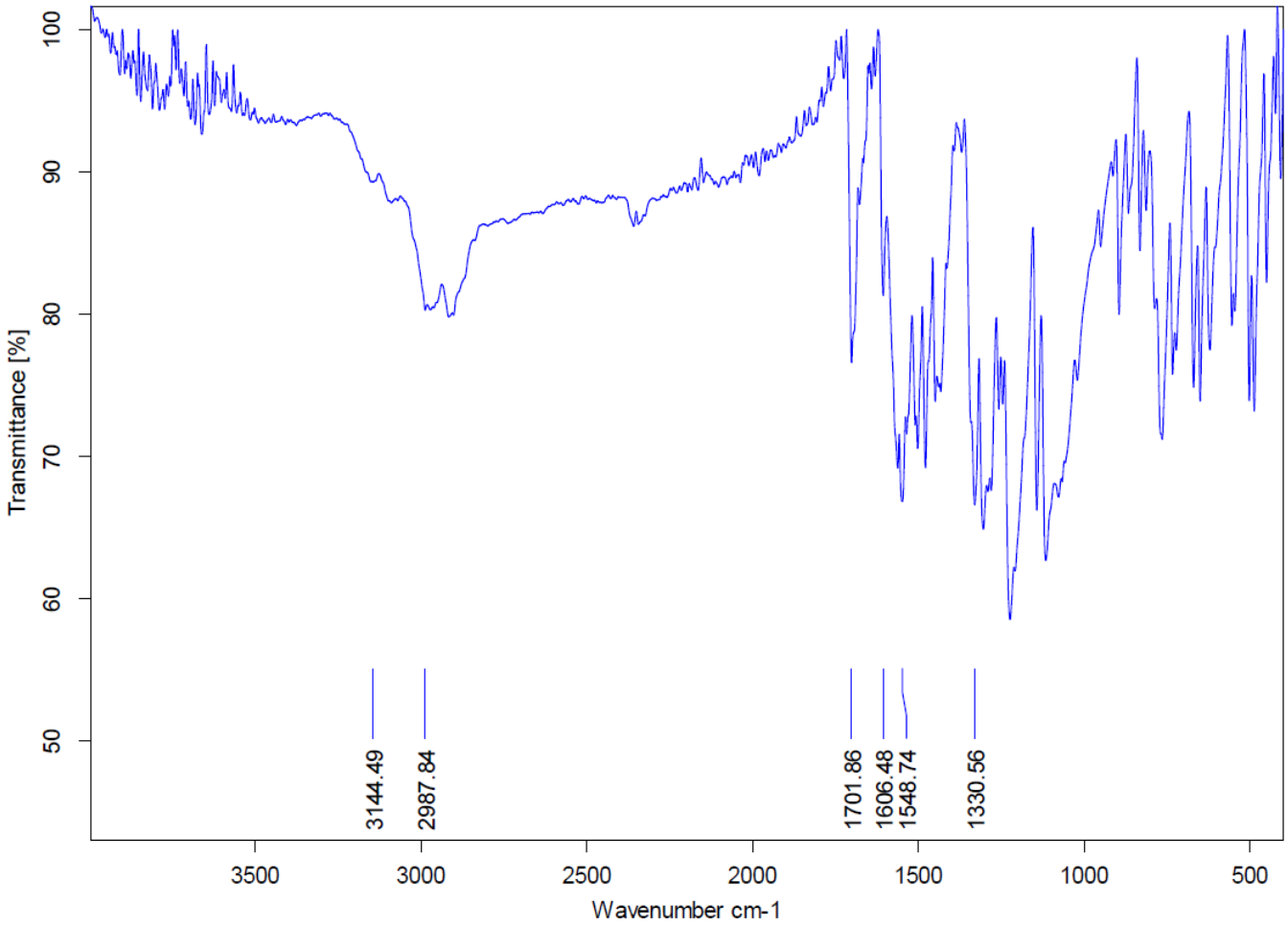

HRMS

Acquisition Parameter

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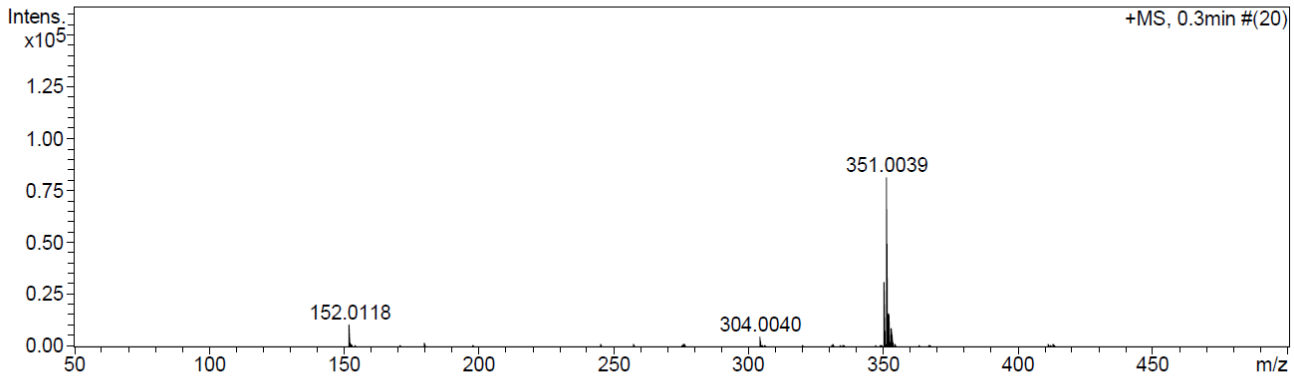
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



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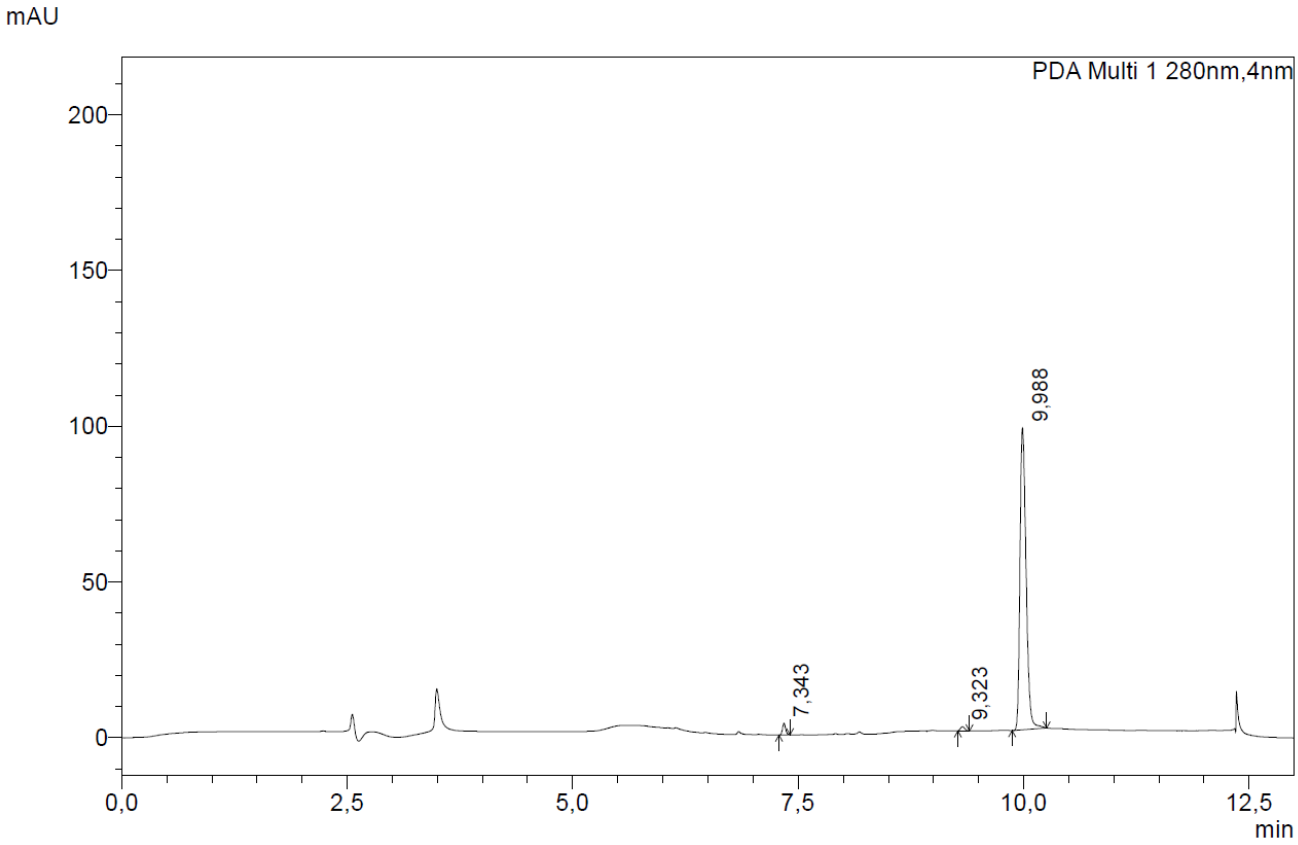
HPLC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100
50
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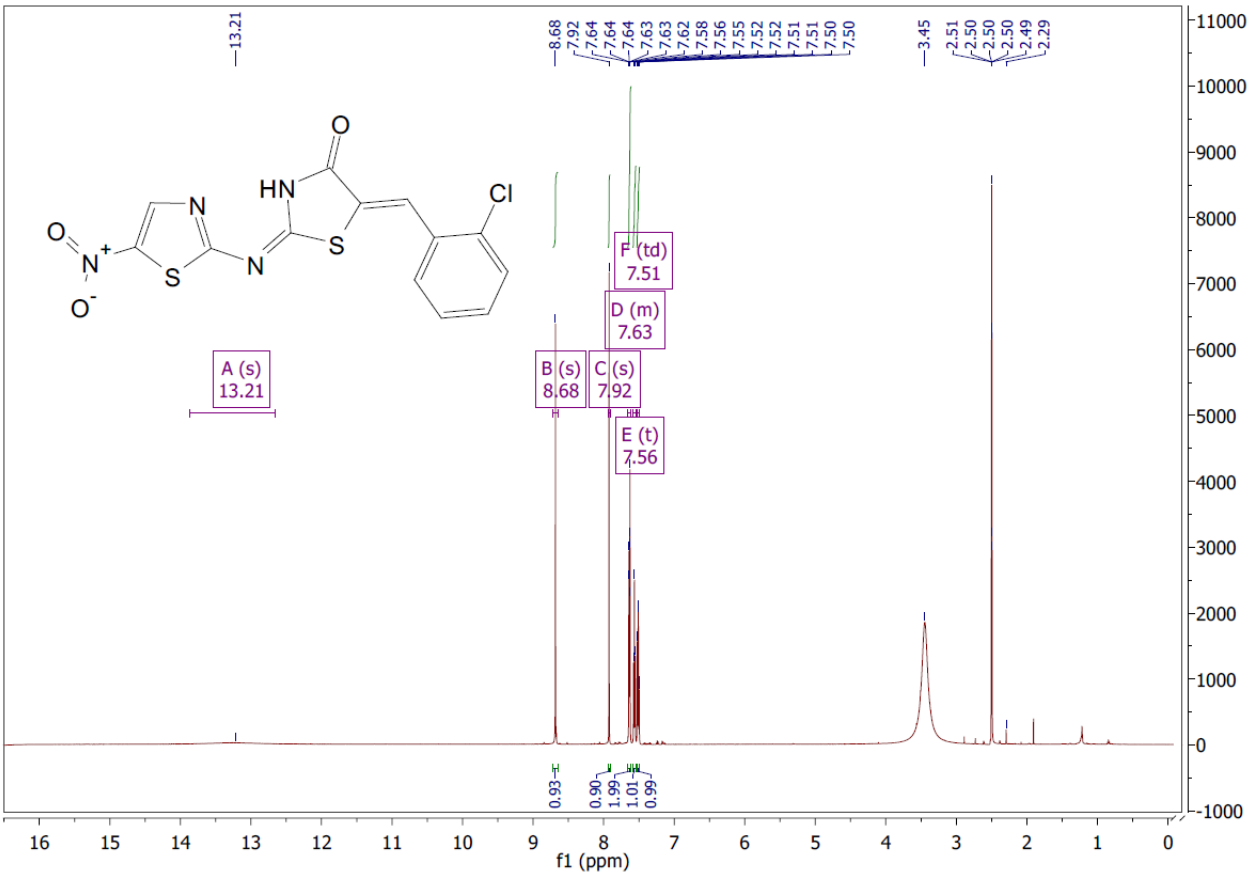
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(2-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3c**)

^{1}H NMR



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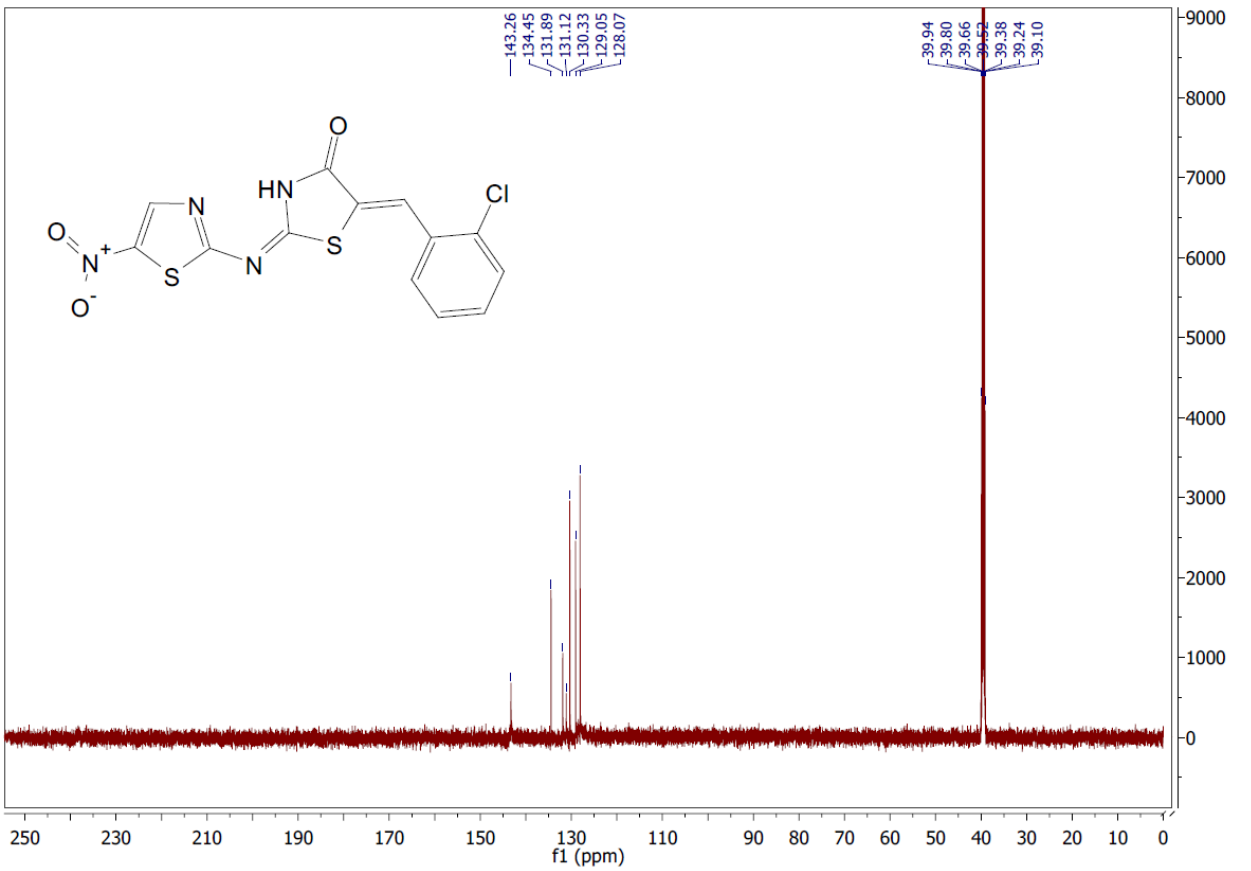
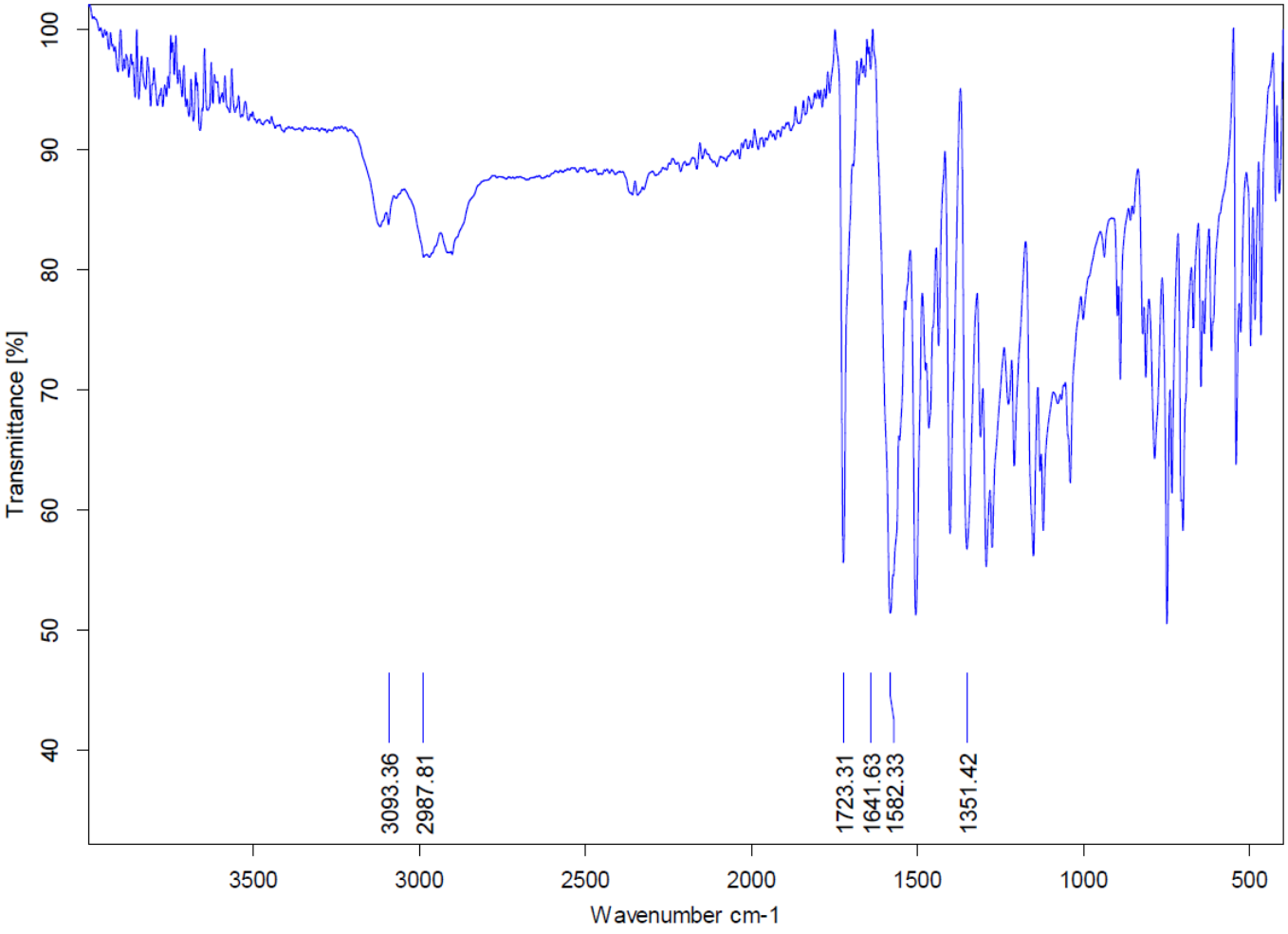


FTIR



HRMS

Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
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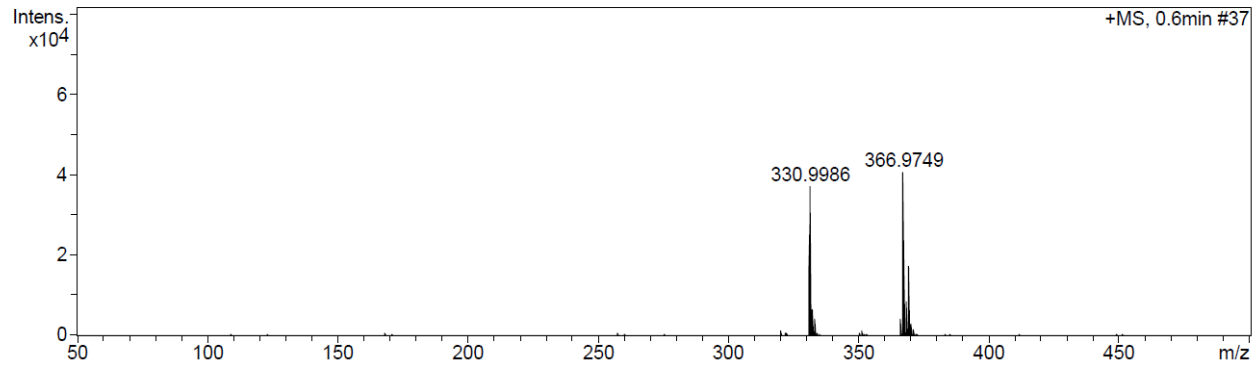
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



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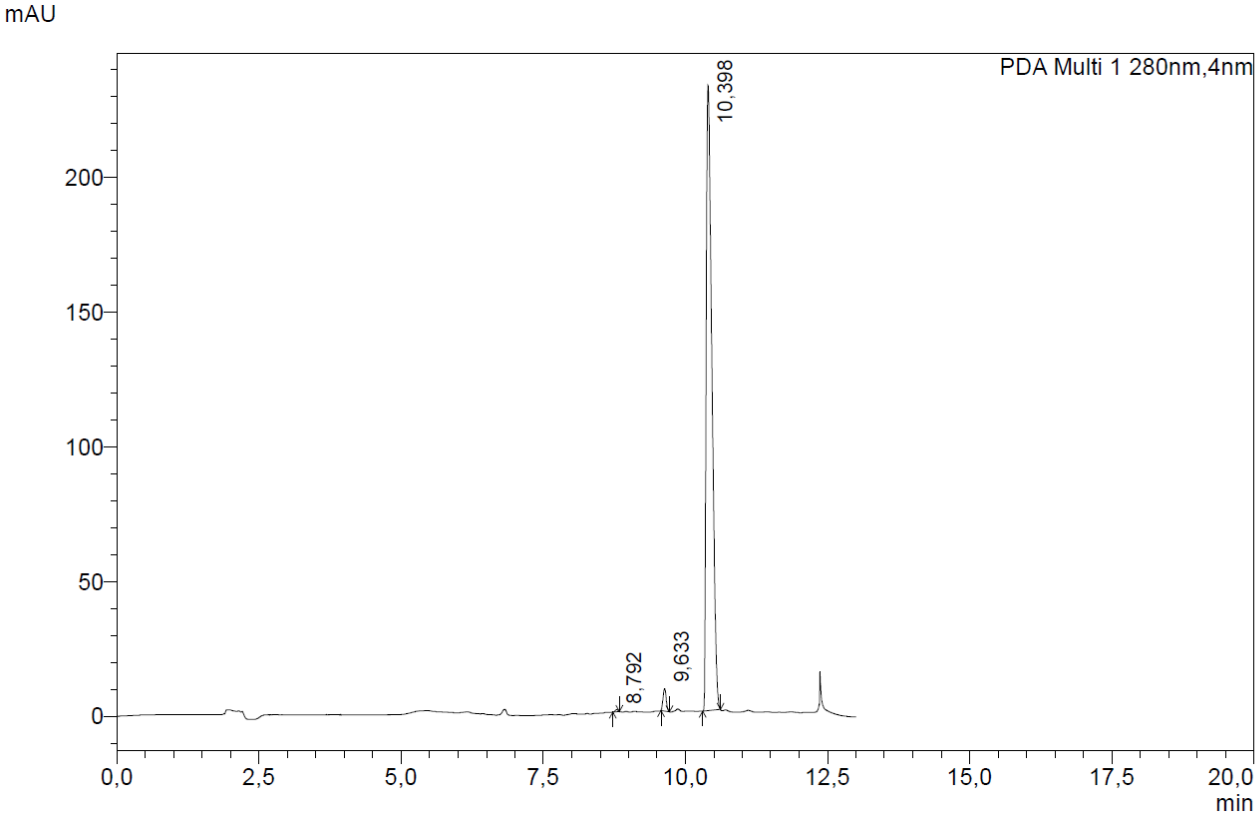
	2	C 10 H 11 N 4 O 3 S 3	100.00	330.9988	0.2	0.5	22.0	7.5	even	ok
	3	C 12 H 12 Cl N 2 O 3 S 2	0.70	330.9972	-1.4	-4.1	131.2	7.5	even	ok
366.9749	1	C 13 H 8 Cl N 4 O 3 S 2	100.00	366.9721	-2.8	-7.6	16.0	11.5	even	ok

HPLC


mAU
PDA Multi 1 280nm,4nm
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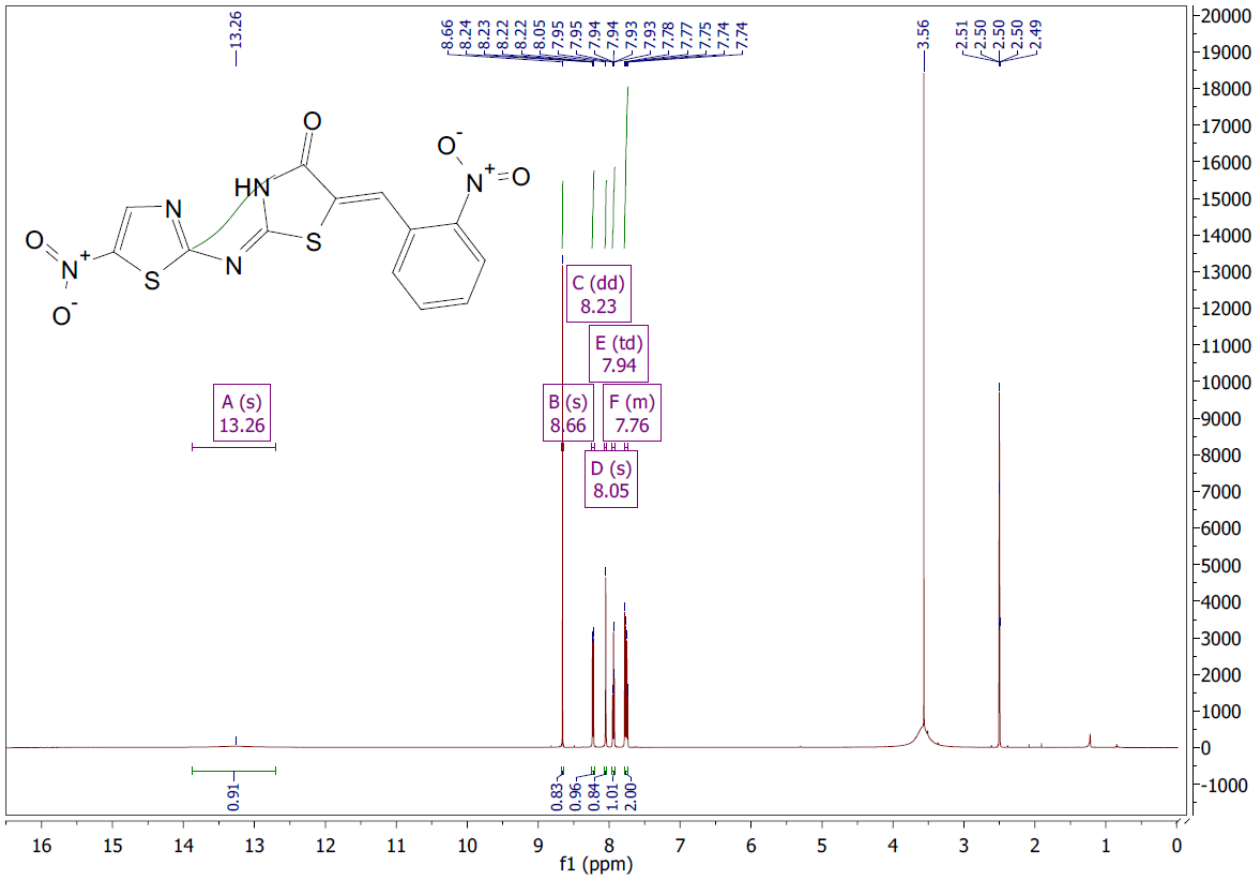
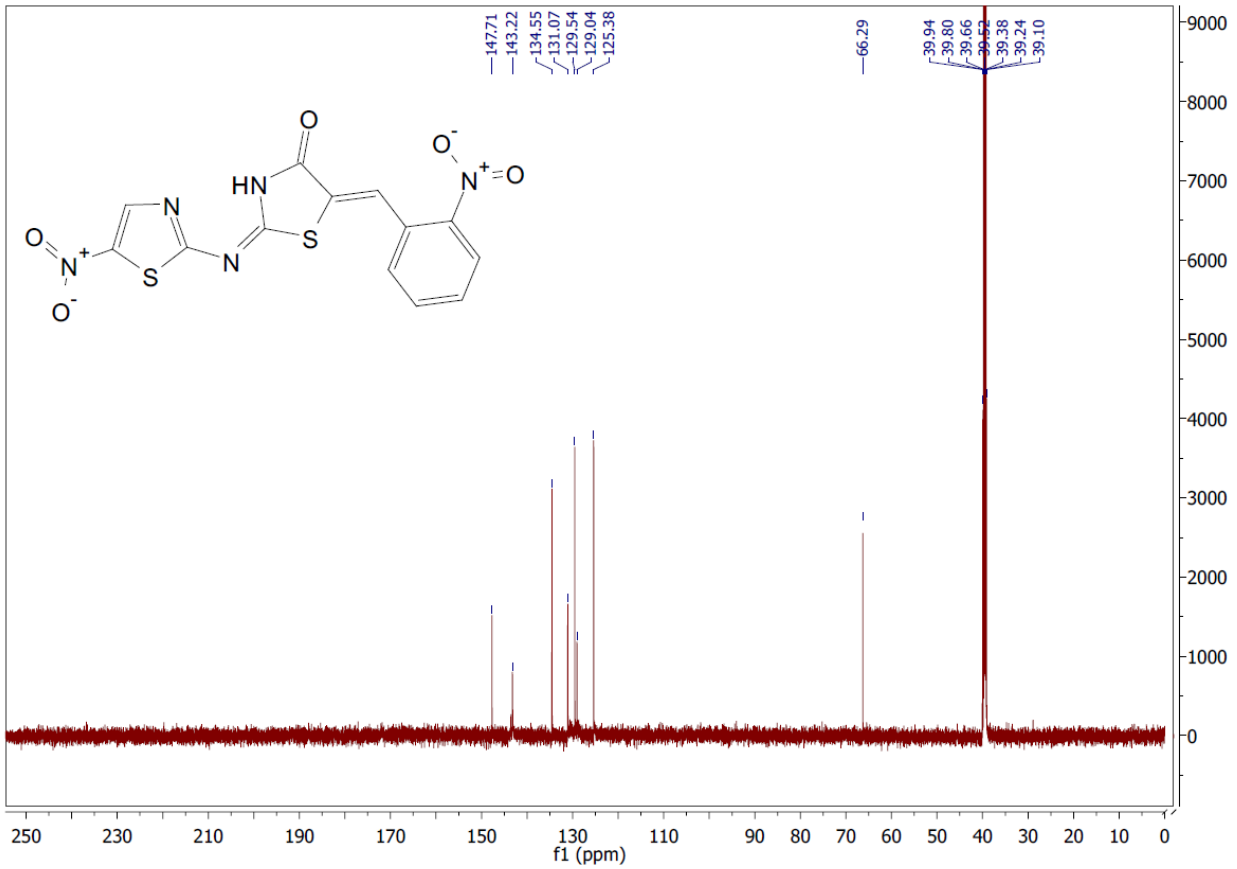
8,792
9,633
200
150
100
50
0
0,0
2,5
5,0
7,5
10,0
12,5
15,0
17,5
20,0
min

(2E,5Z)-5-(2-nitrobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3d**)

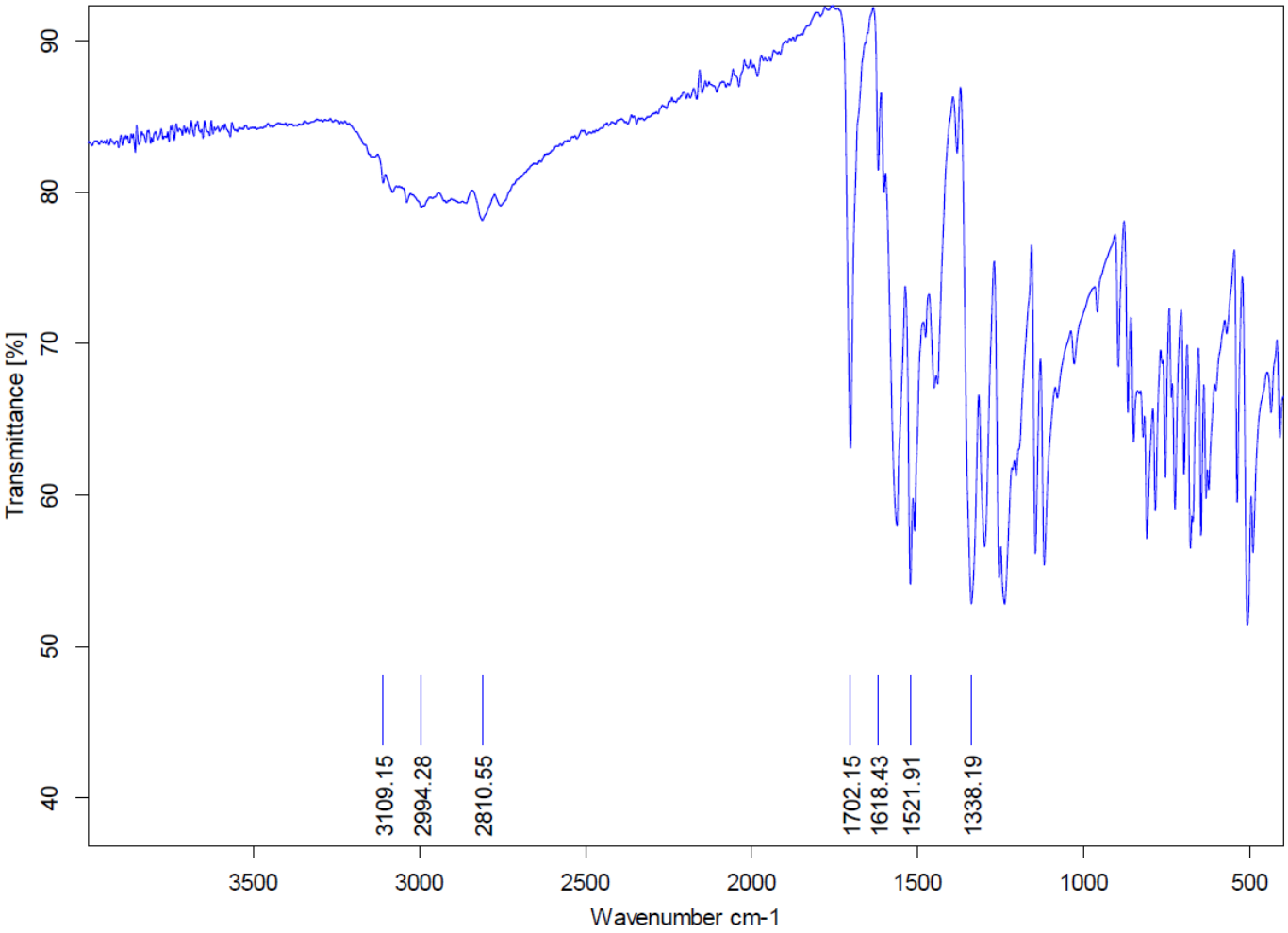
^{1}H NMR



^{13}C NMR



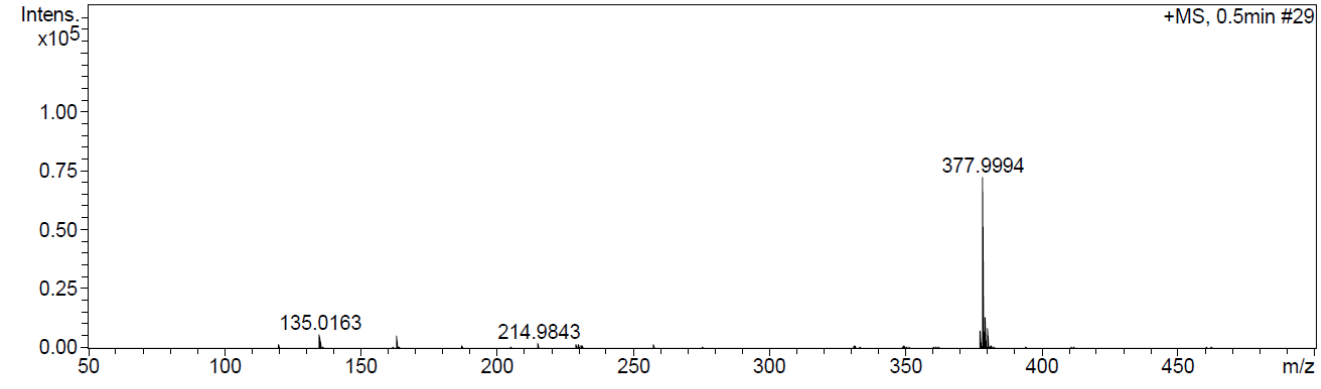
FTIR



HRMS

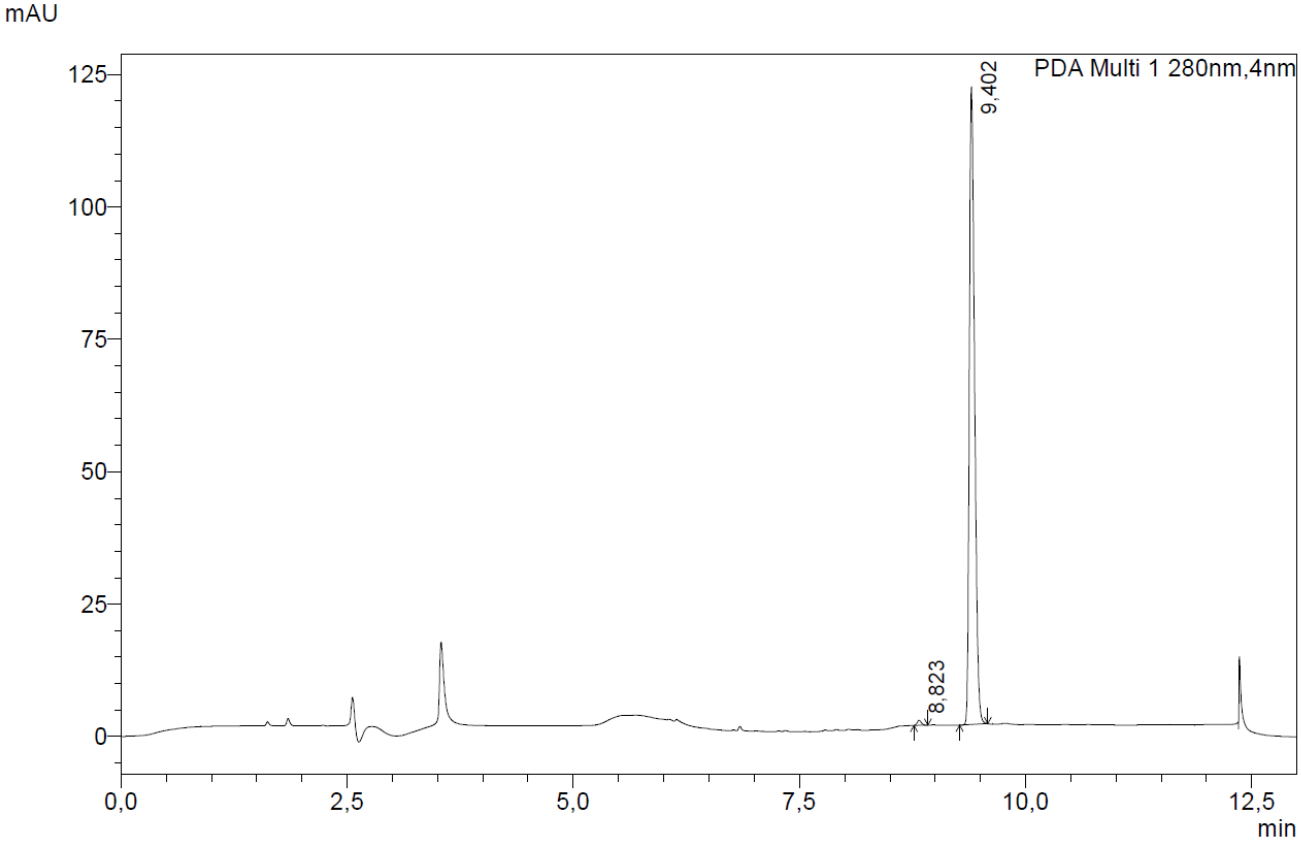
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



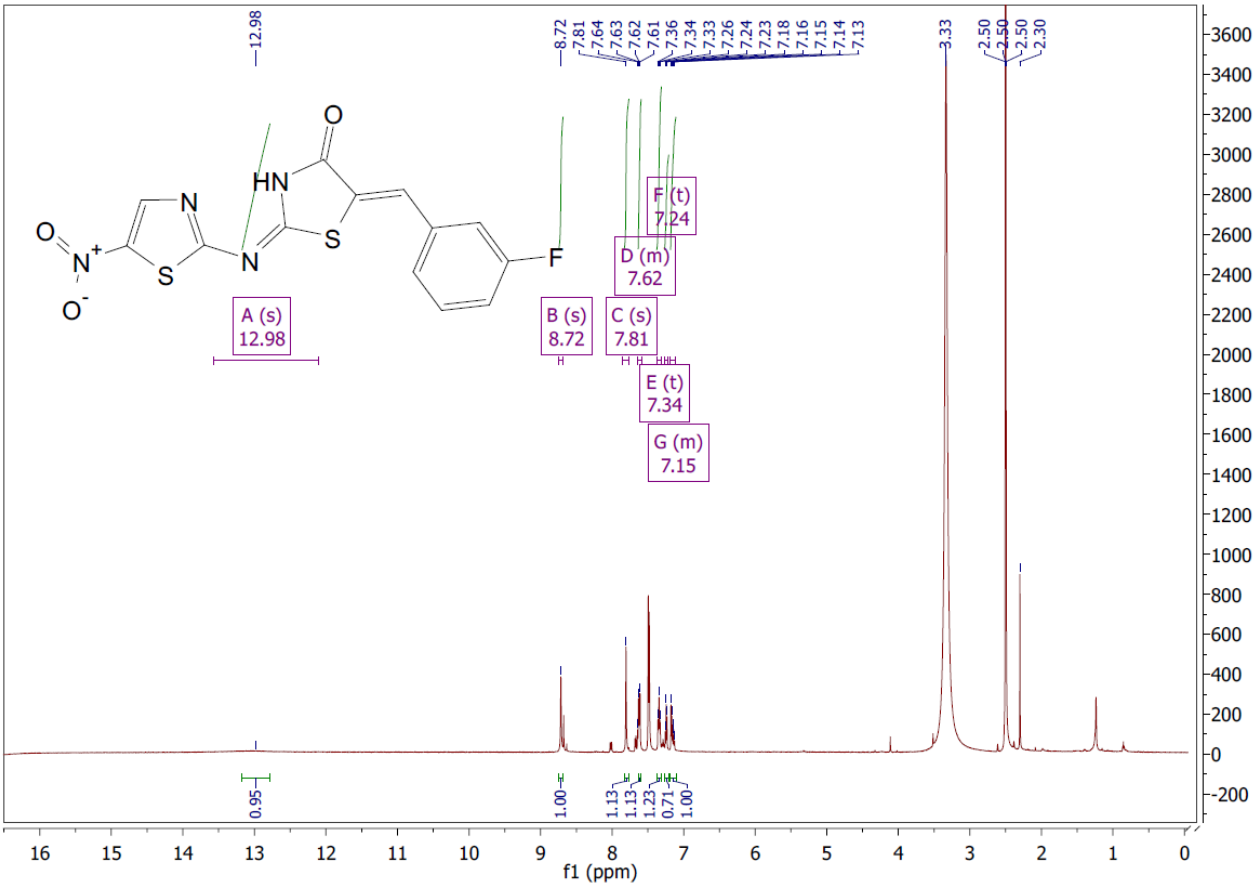
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
377.9994	1	C 13 H 8 N 5 O 5 S 2	100.00	377.9961	-3.3	-8.7	3.8	12.5	even	ok

HPLC

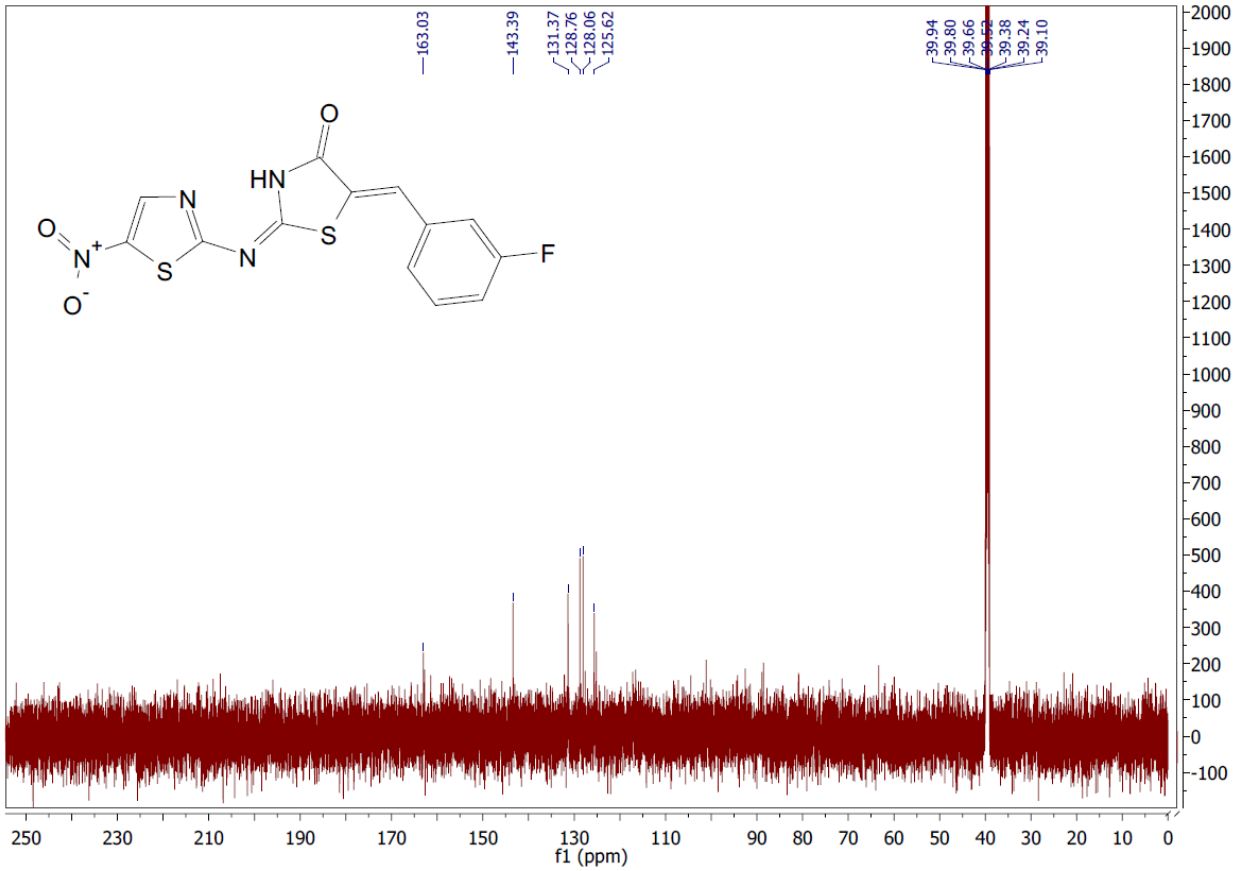

mAU
125
100
75
50
25
0
PDA Multi 1 280nm,4nm
9,402
8,823
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(3-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3e**)

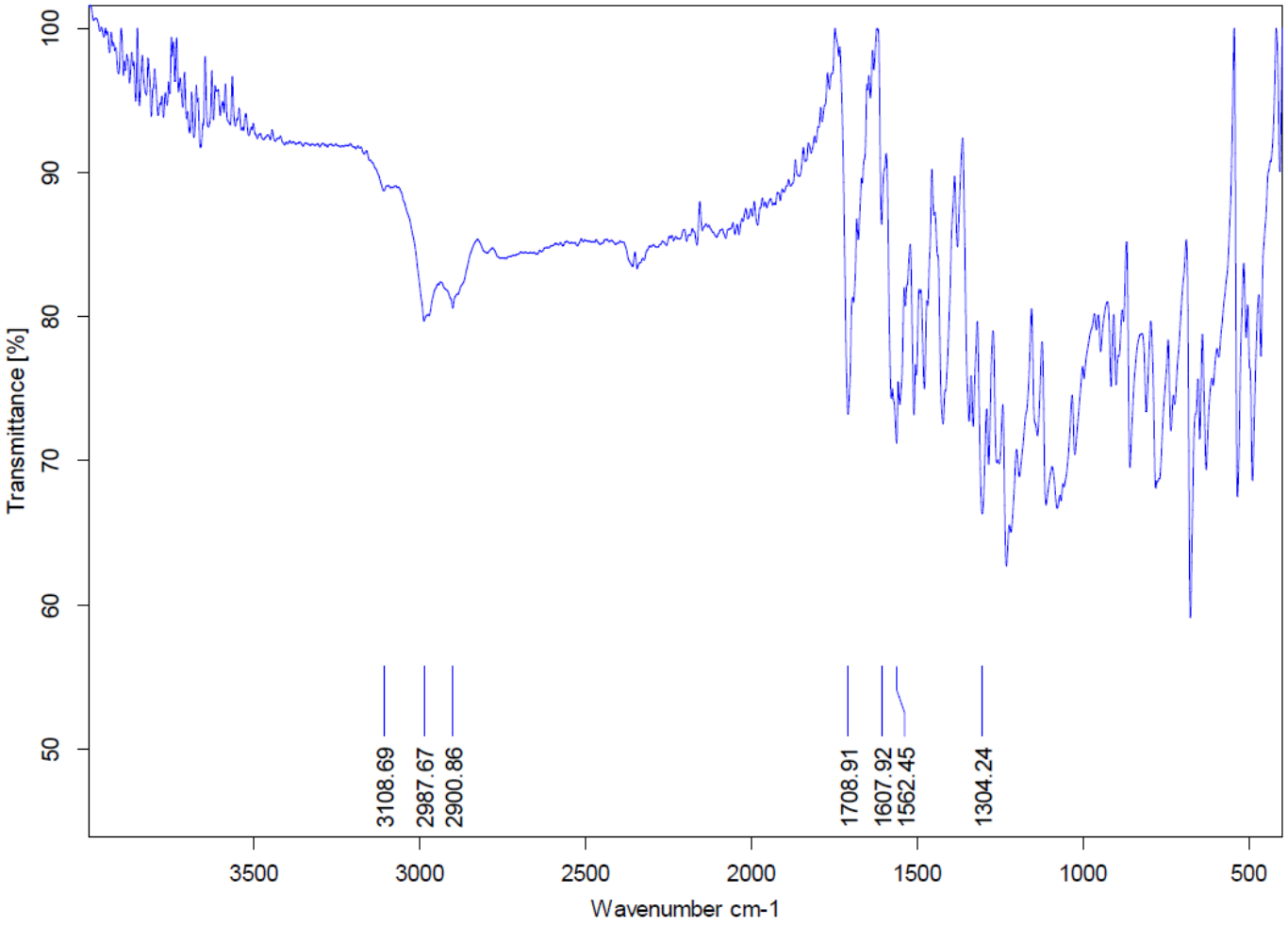
^{1}H NMR



^{13}C NMR



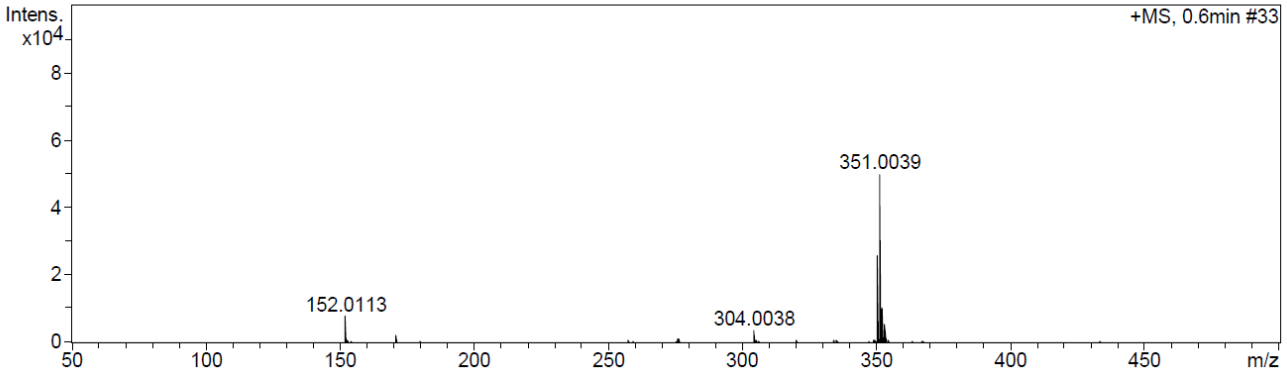
FTIR



HRMS

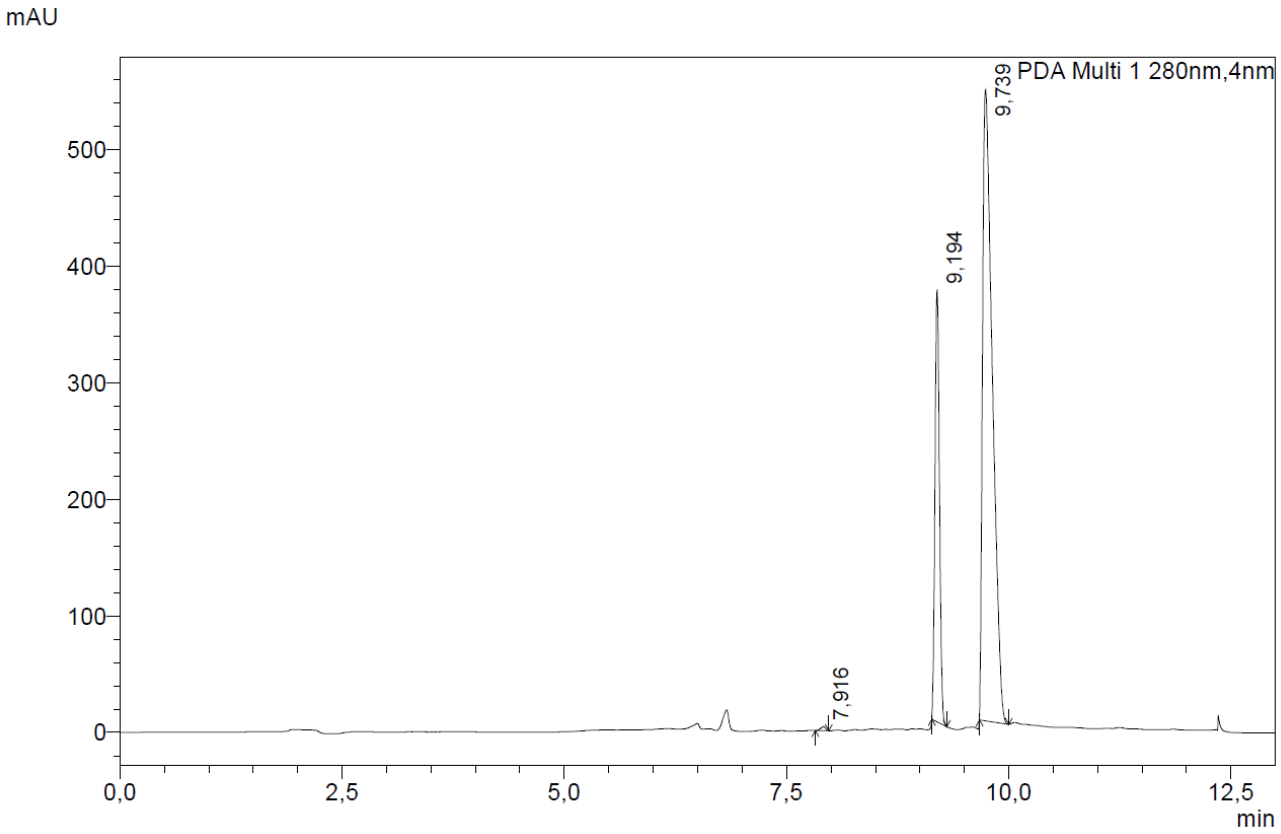
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



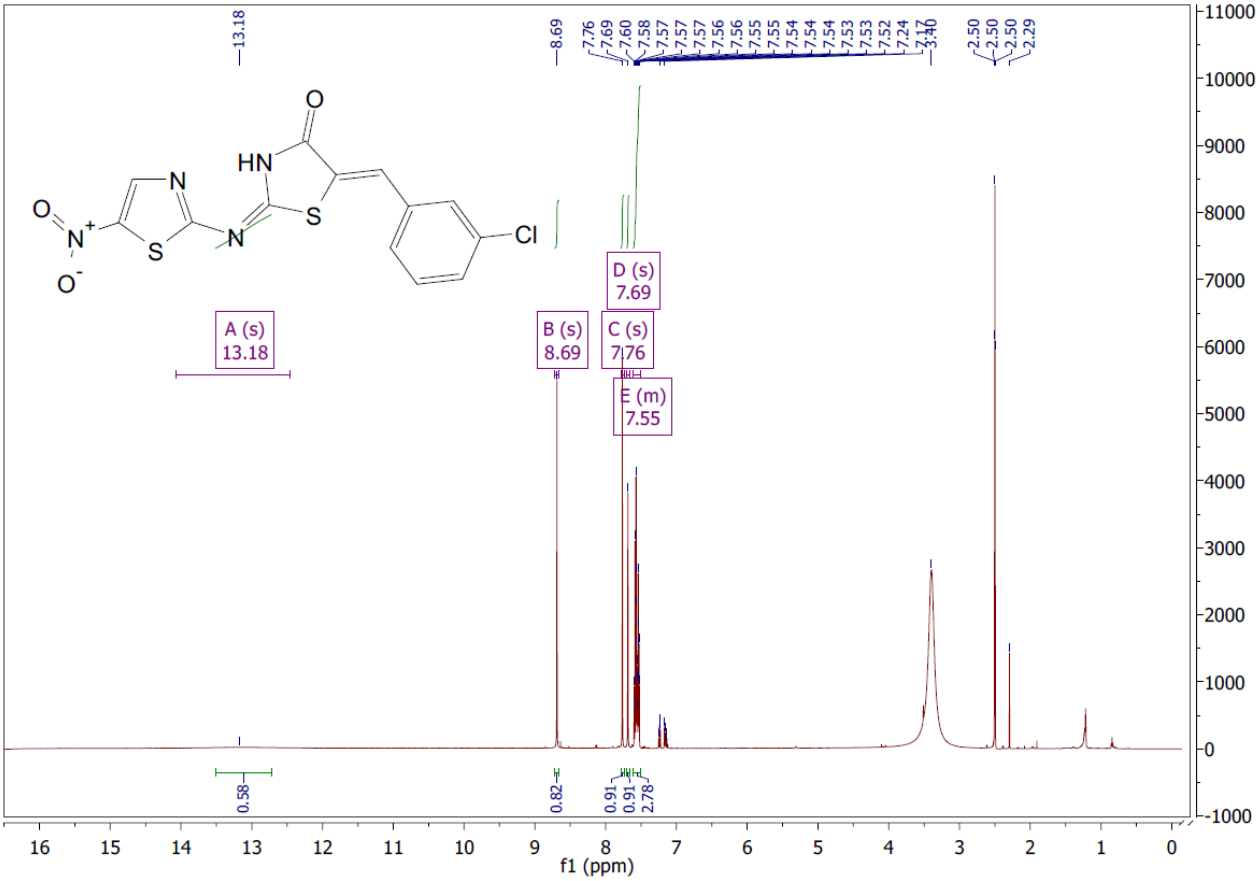
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e¯ Conf	N-Rule
351.0039	1	C 13 H 8 F N 4 O 3 S 2	29.12	351.0016	-2.3	-6.6	22.3	11.5	even	ok
	2	C 13 H 11 N 4 O 2 S 3	100.00	351.0039	-0.1	-0.2	27.8	10.5	even	ok

HPLC

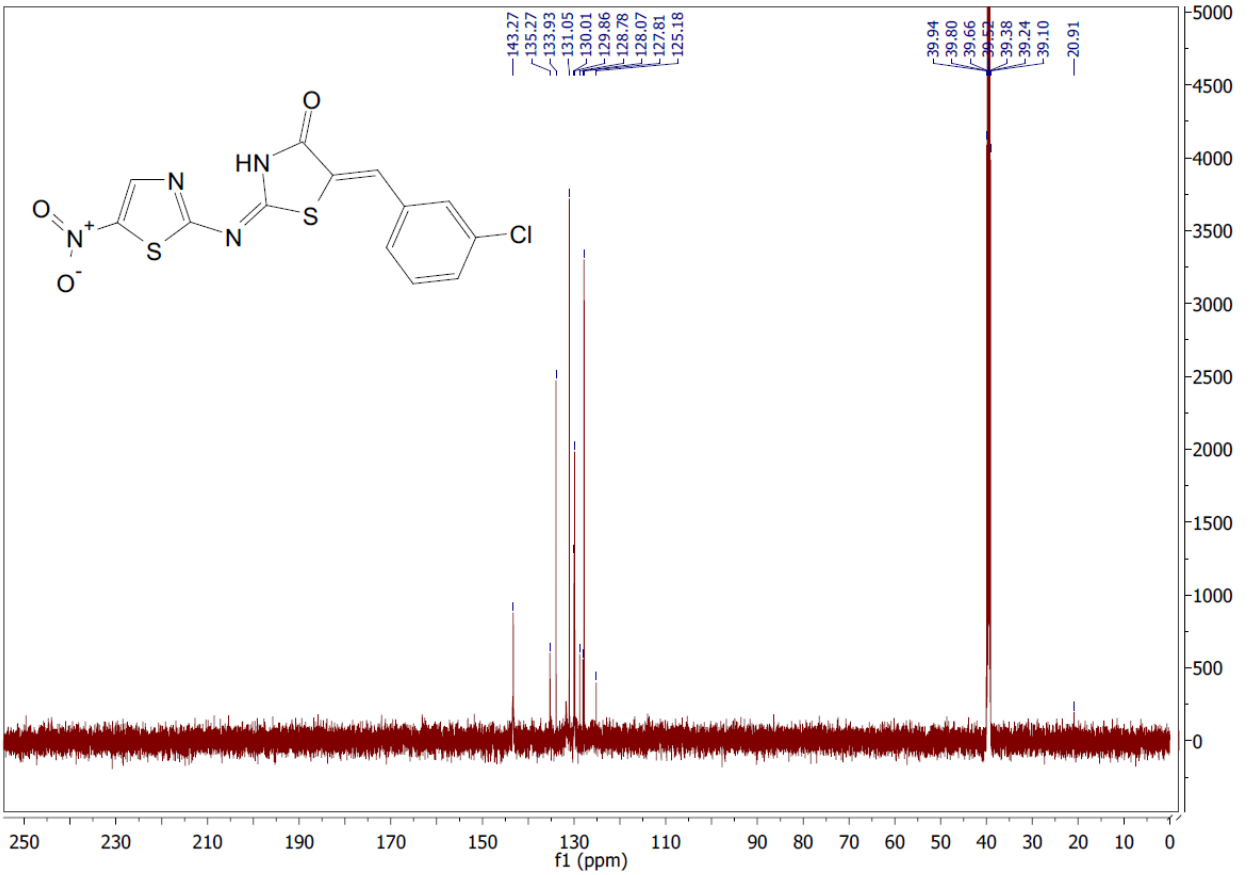

mAU
PDA Multi 1 280nm,4nm
9,739
9,194
7,916
500
400
300
200
100
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(3-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3f**)

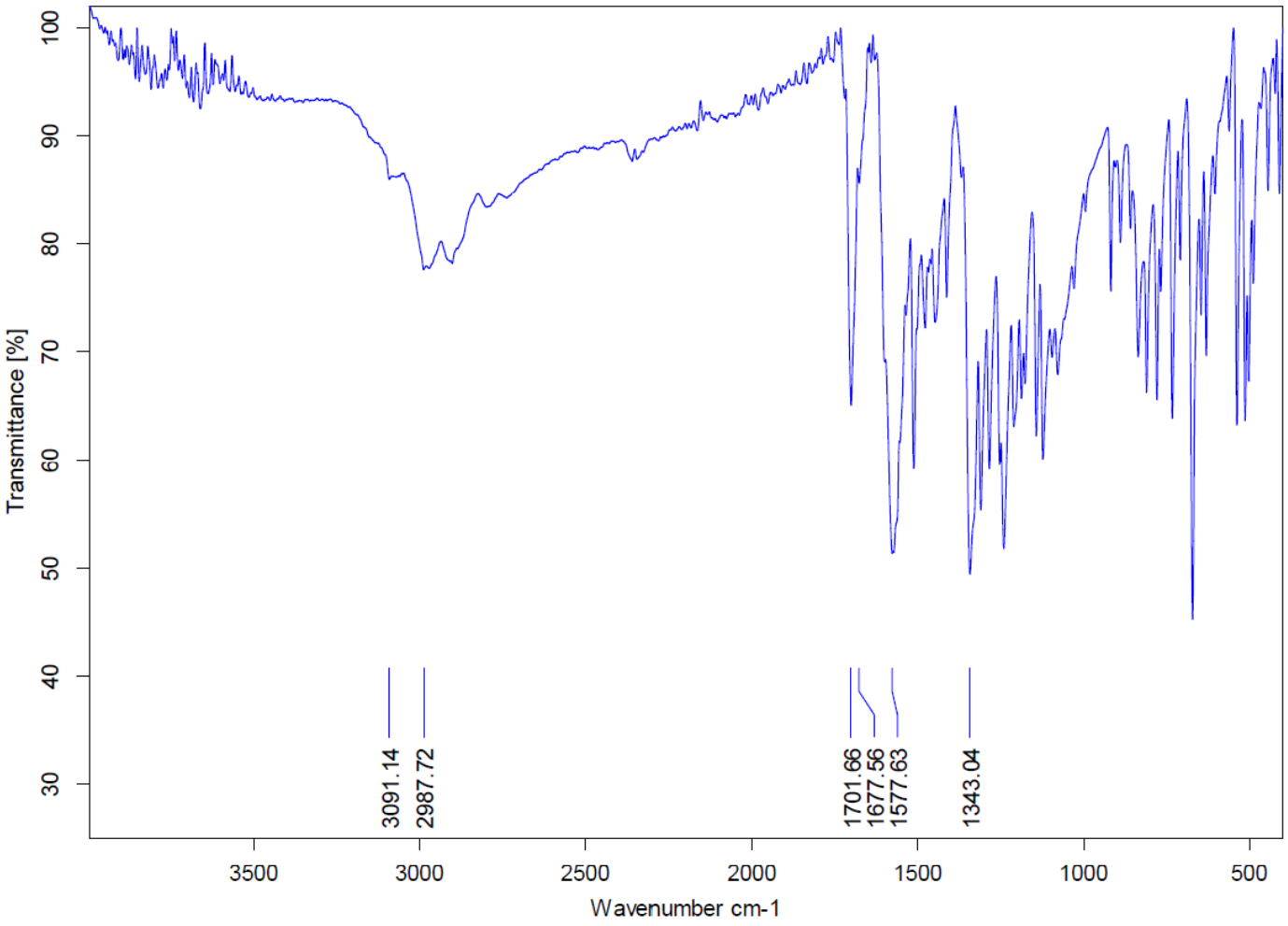
^{1}H NMR



^{13}C NMR



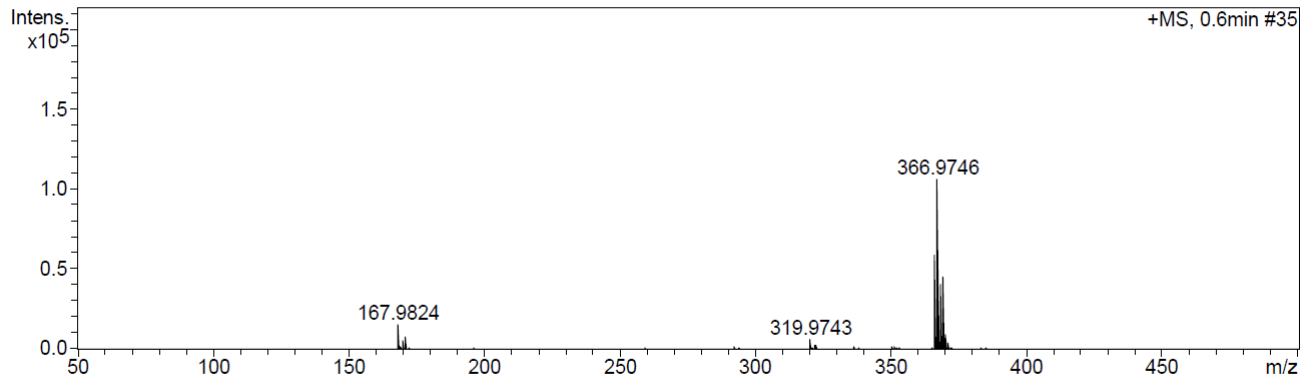
FTIR



HRMS

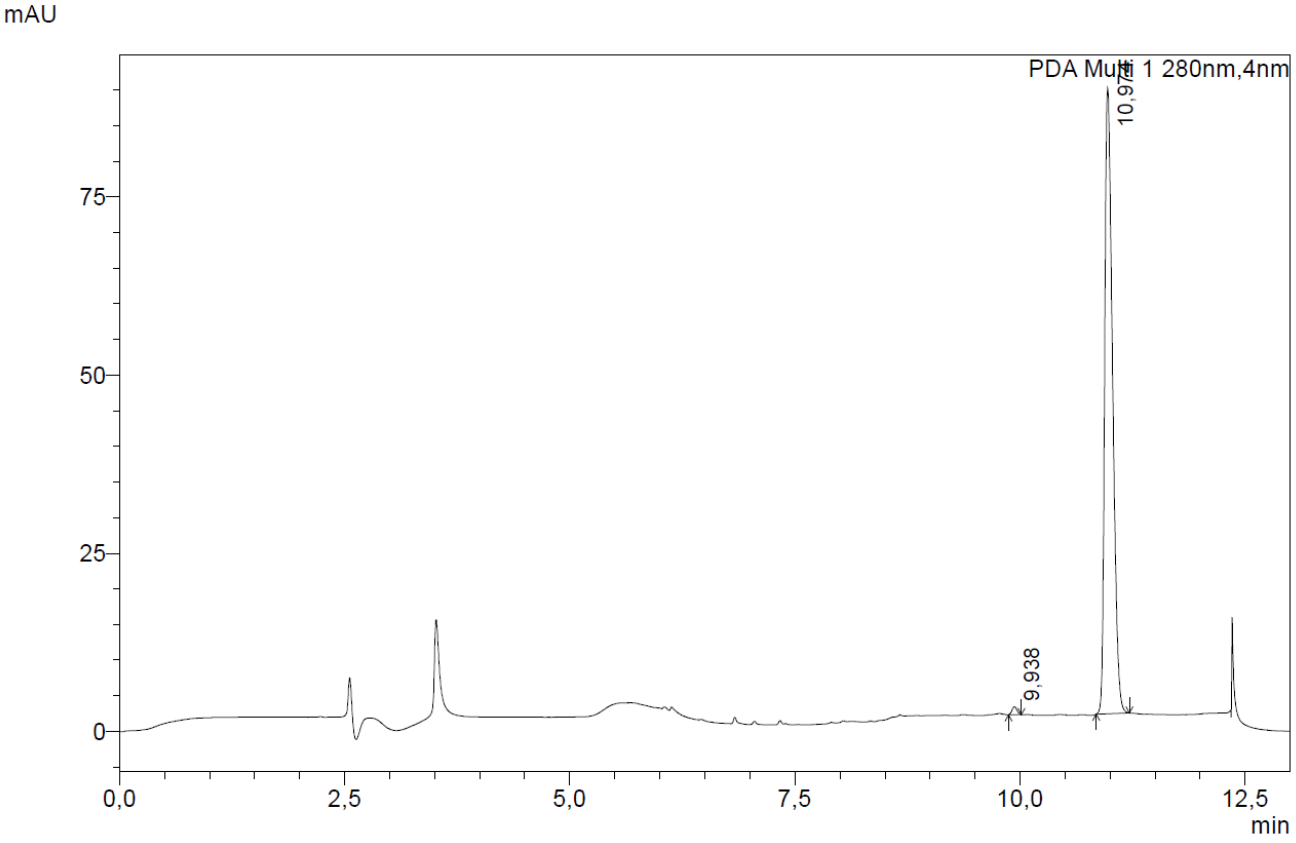
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



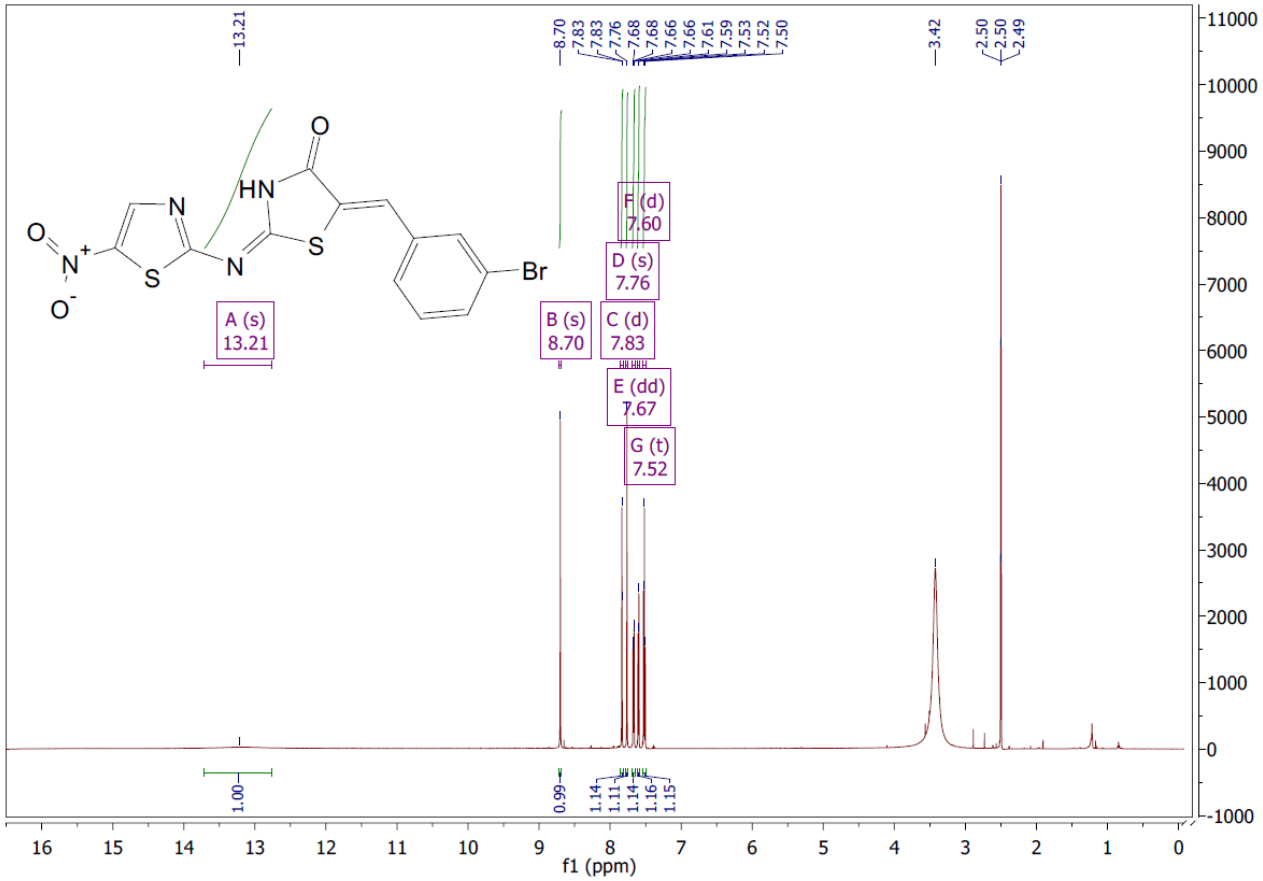
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
366.9746	1	C 13 H 8 Cl N 4 O 3 S 2	100.00	366.9721	-2.5	-6.7	85.2	11.5	even	ok

HPLC

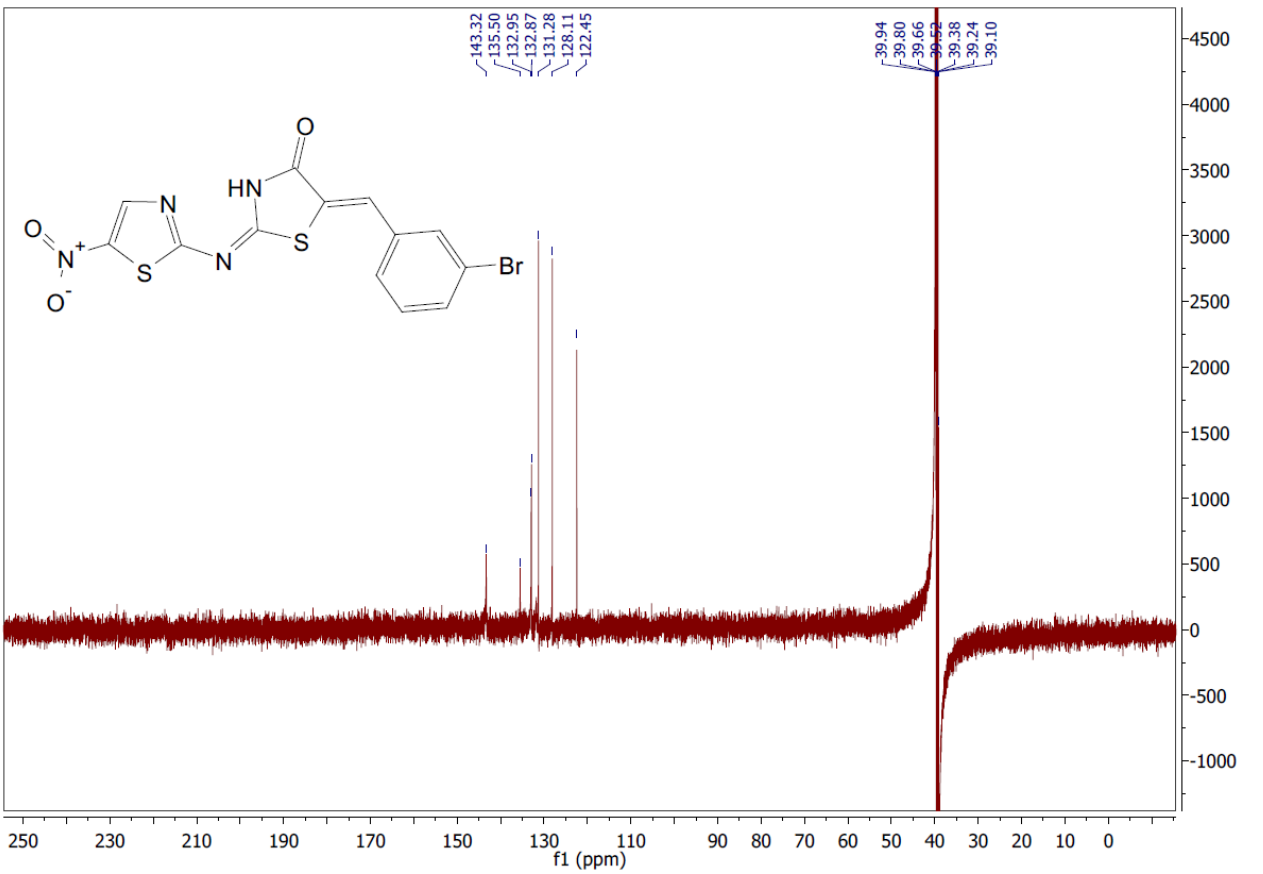

mAU
PDA Multi 1 280nm,4nm
10,974
9,938
75
50
25
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(3-bromobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3g**)

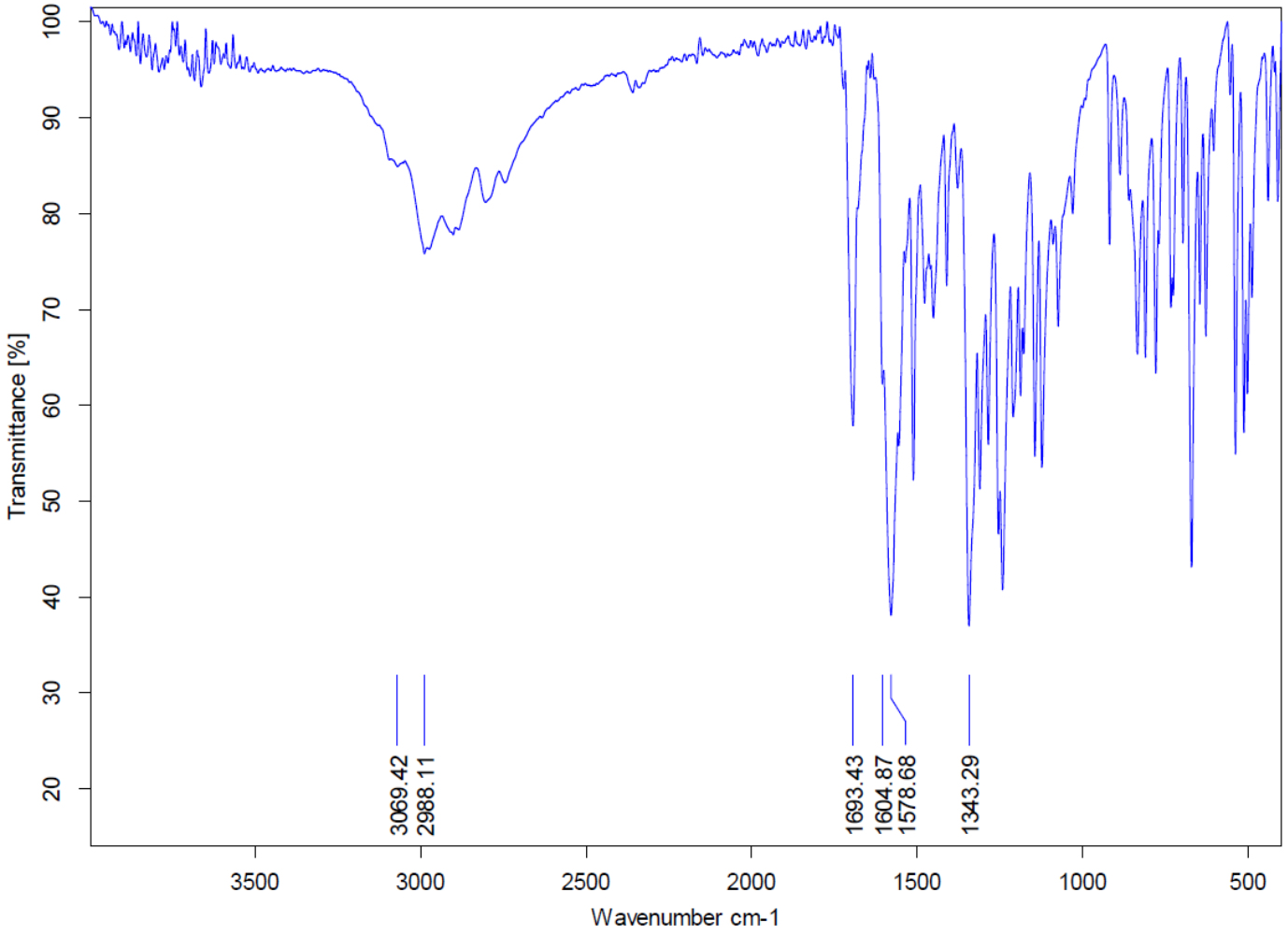
^{1}H NMR



^{13}C NMR



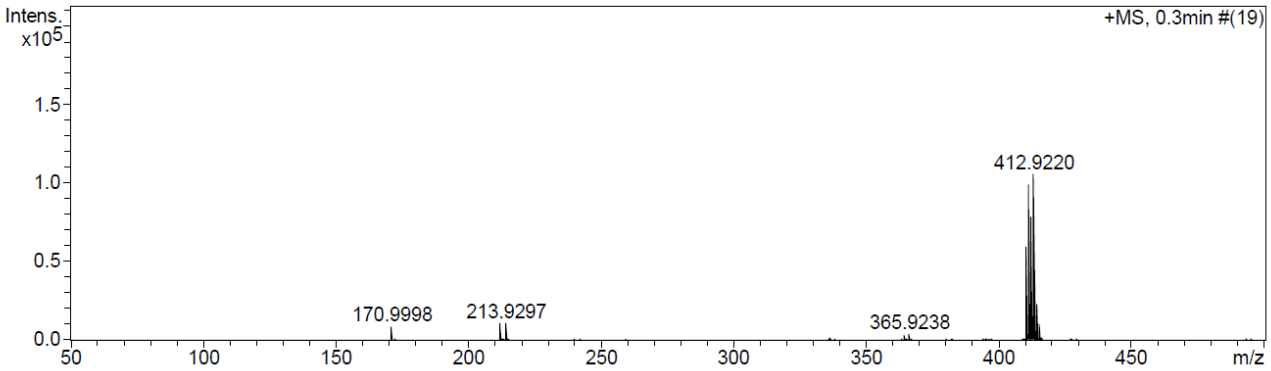
FTIR



HRMS

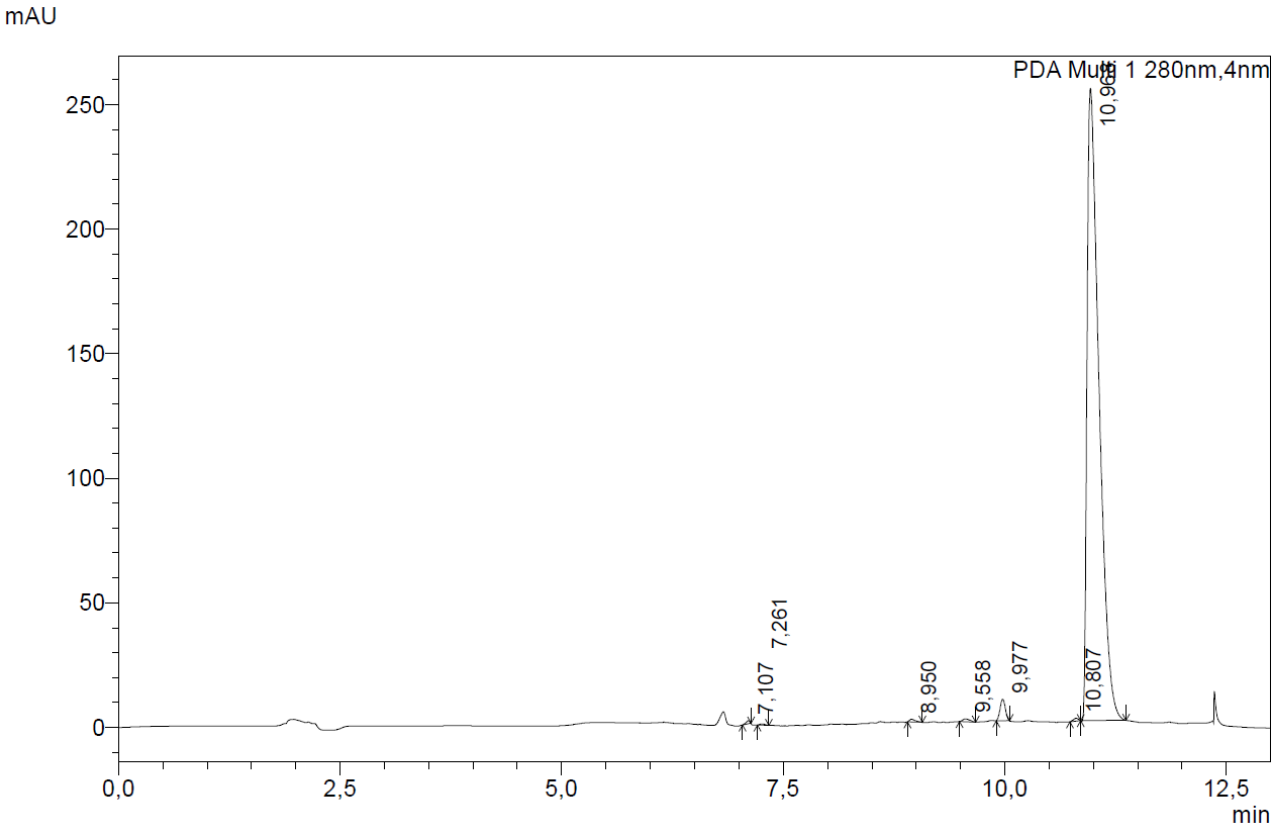
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



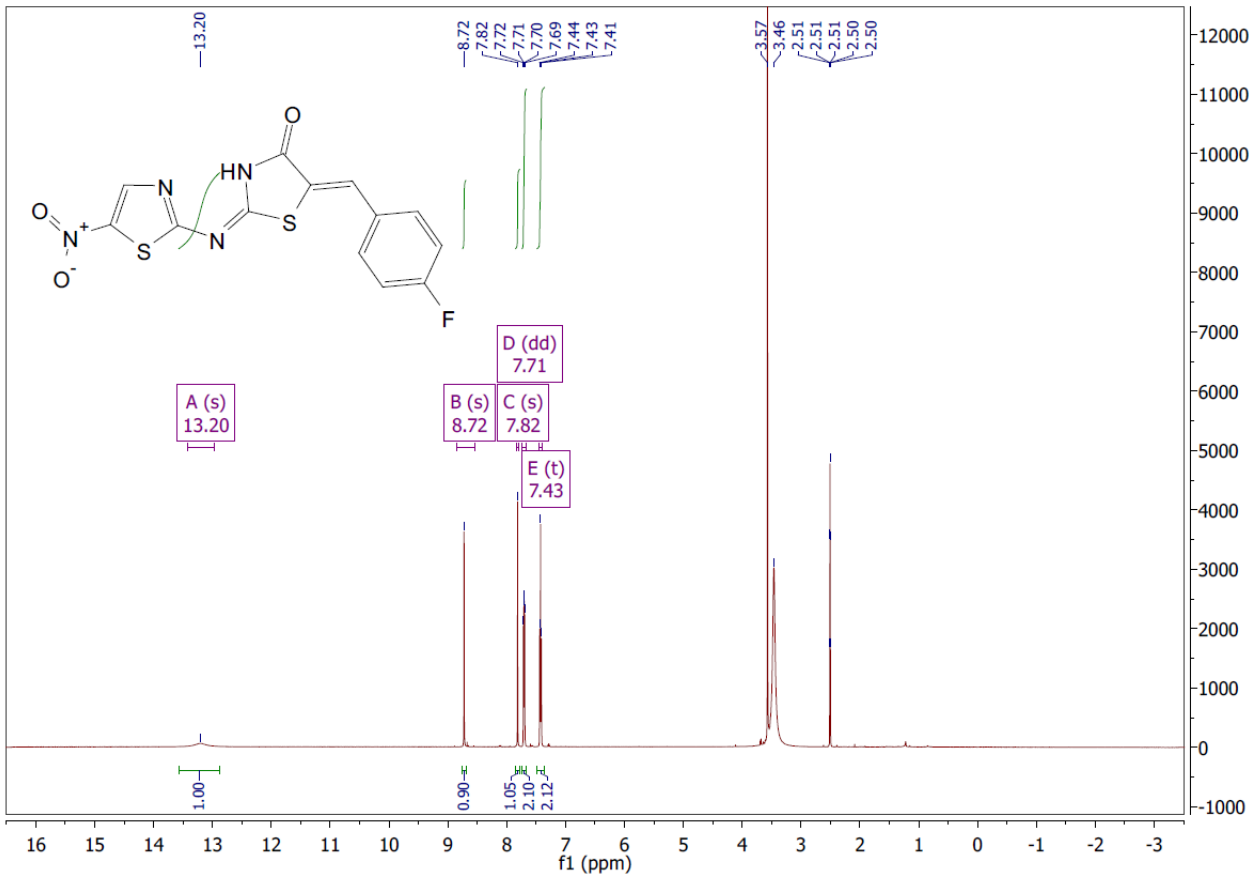
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
409.9169	1	C 13 H 7 Br N 4 O 3 S 2	100.00	409.9137	-3.1	-7.6	502.1	12.0	odd	ok
410.9241	1	C 13 H 8 Br N 4 O 3 S 2	100.00	410.9216	-2.6	-6.3	238.9	11.5	even	ok

HPLC

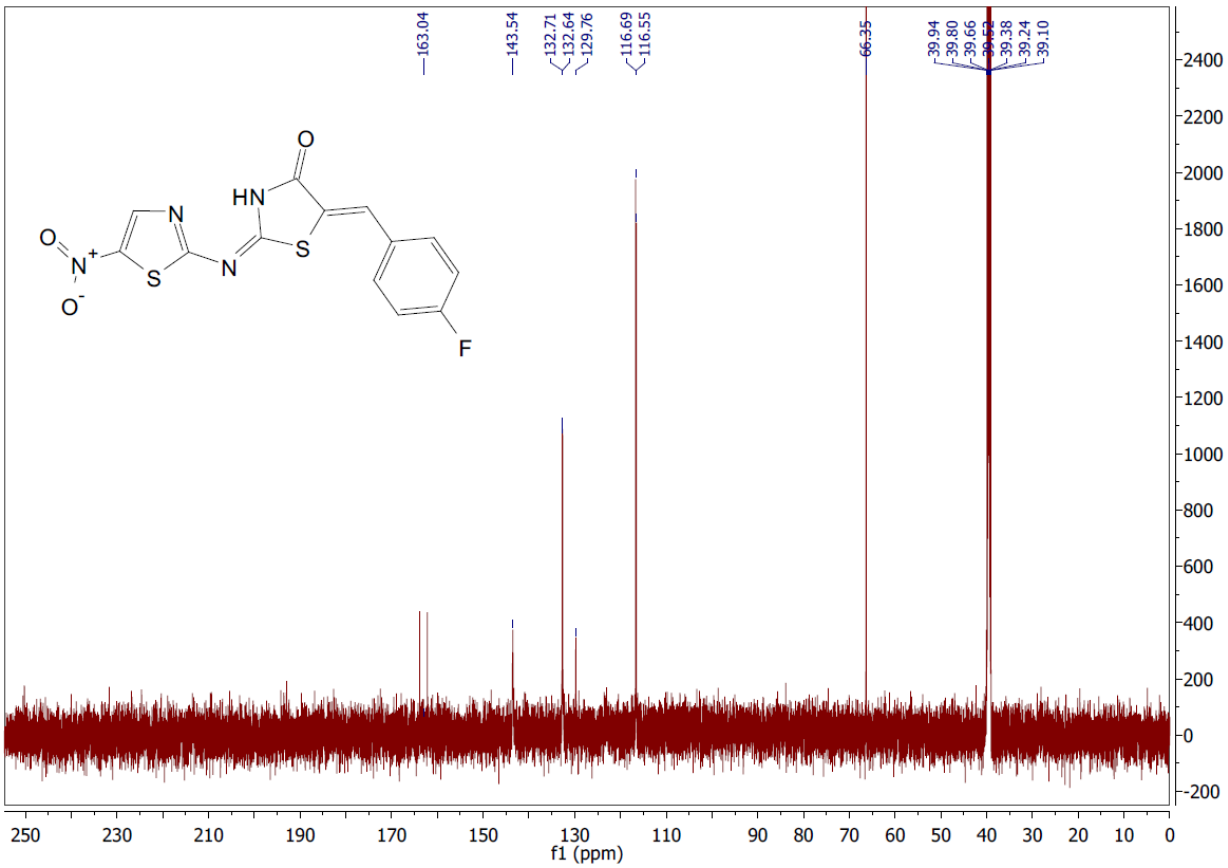

mAU
PDA Multi 1 280nm,4nm
250
200
150
100
50
0
7,107
7,261
8,950
9,558
9,977
10,807
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(4-fluorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3h**)

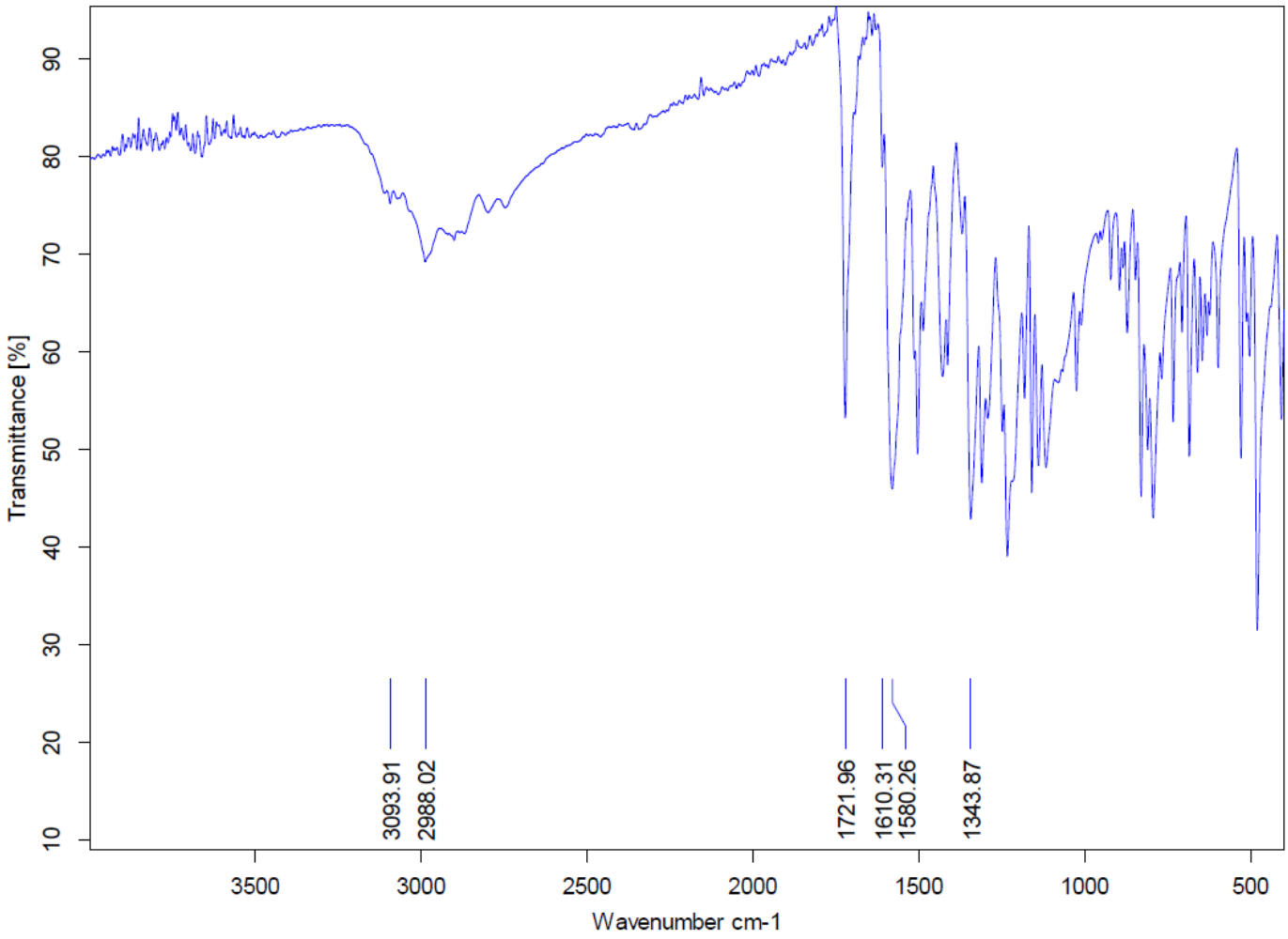
^{1}H NMR



^{13}C NMR



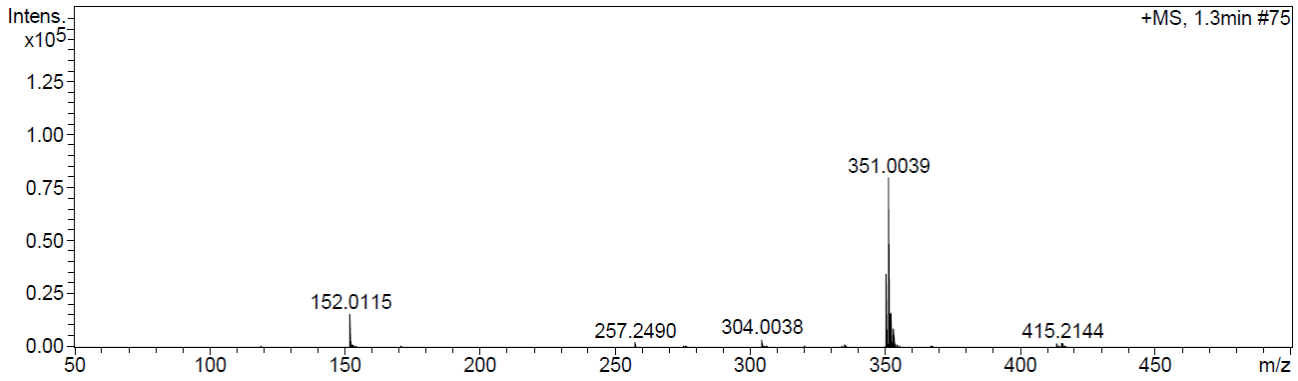
FTIR



HRMS

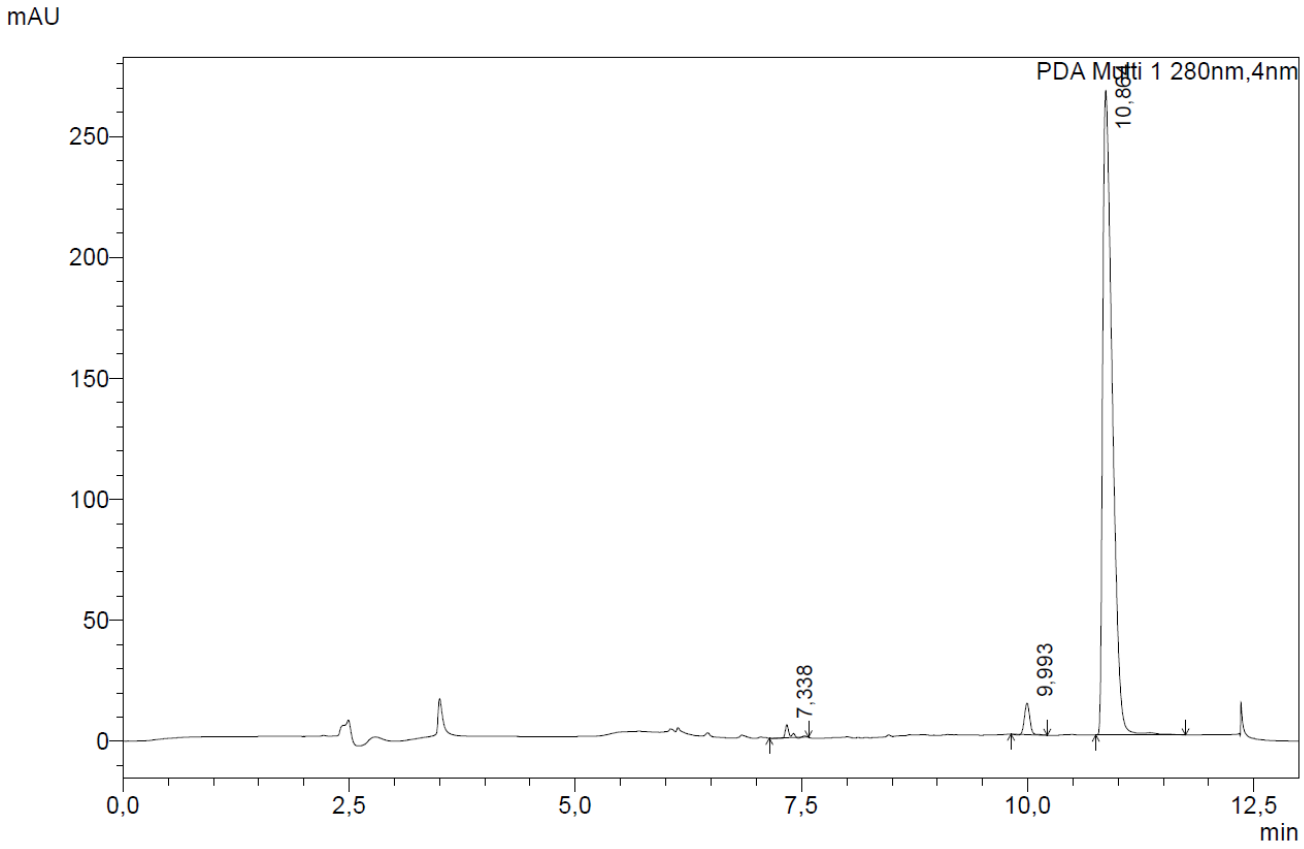
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



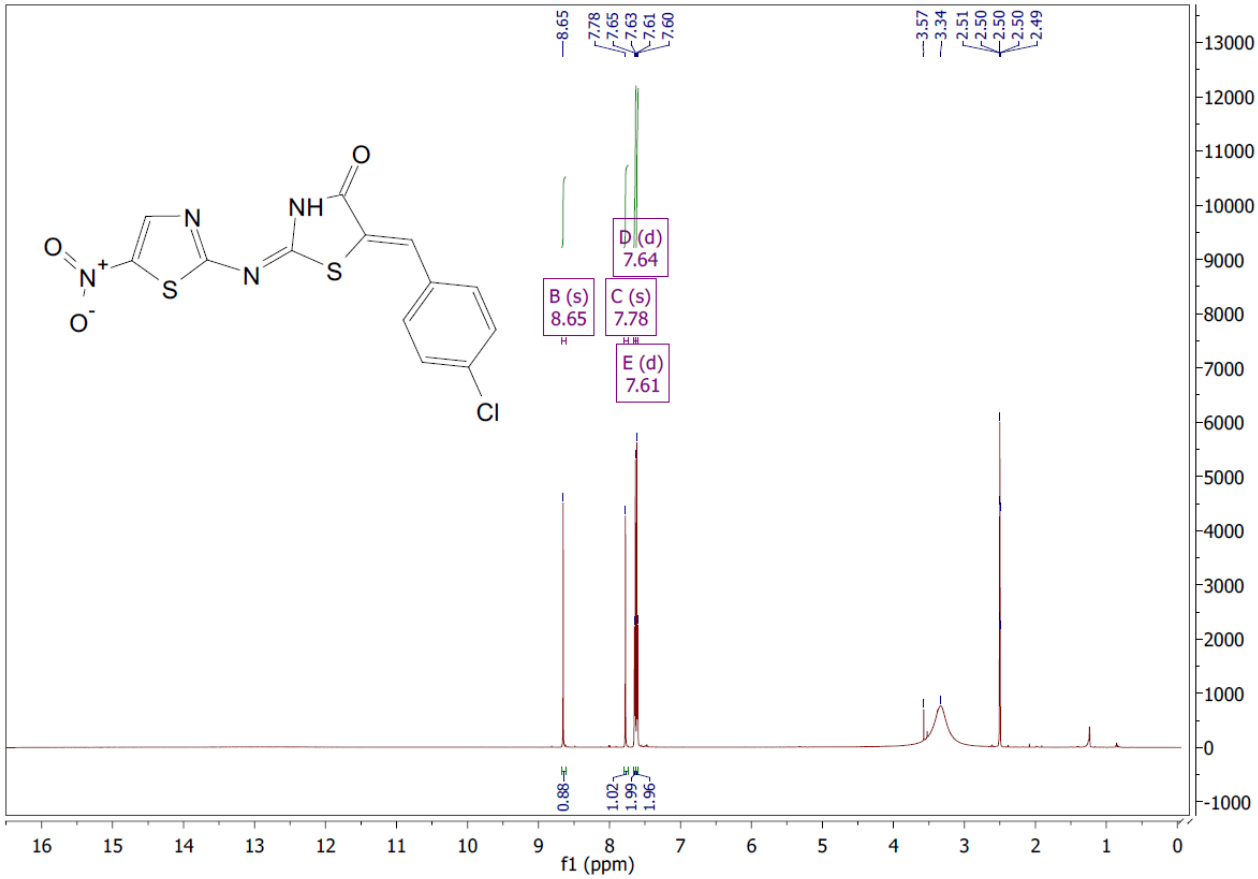
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
351.0039	1	C 13 H 8 F N 4 O 3 S 2	100.00	351.0016	-2.2	-6.4	16.5	11.5	even	ok

HPLC

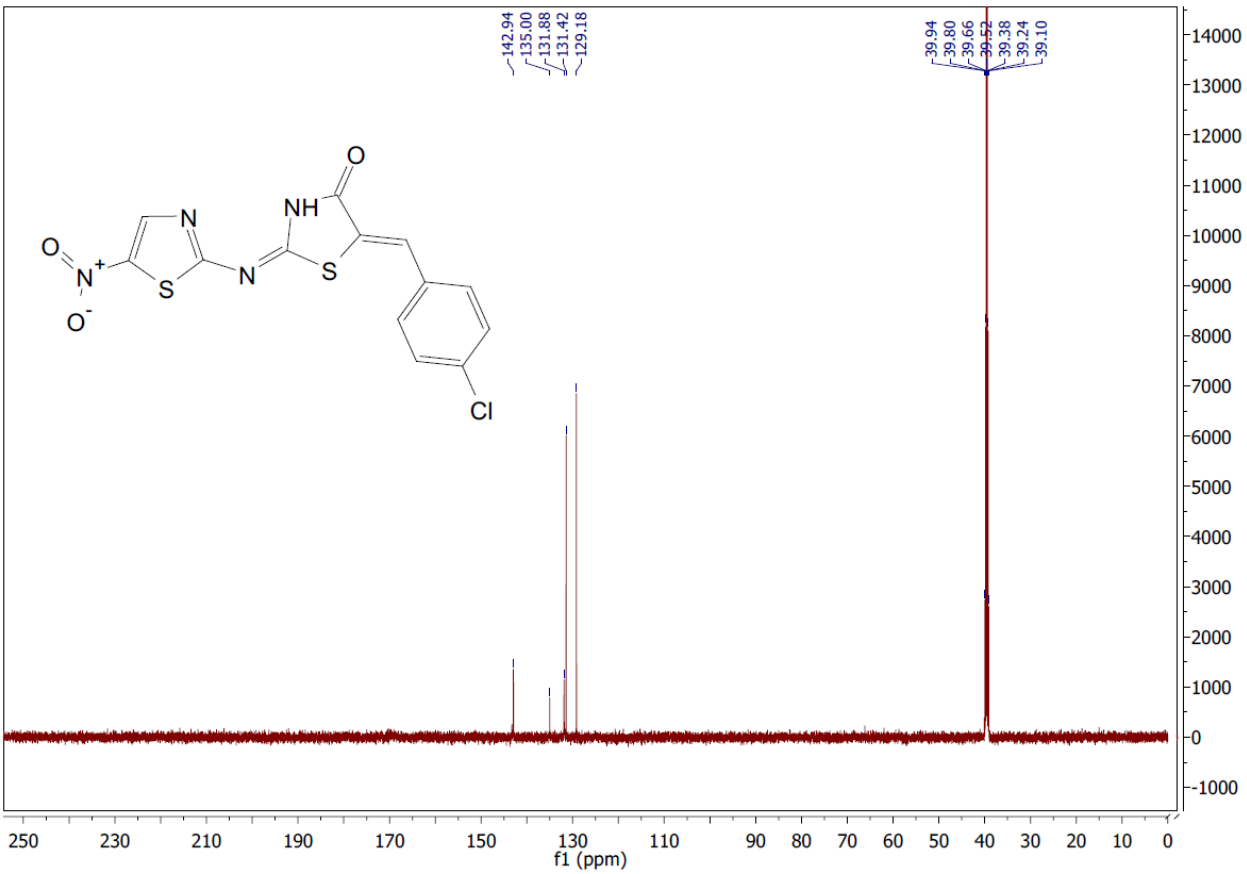

mAU
PDA Multi 1 280nm,4nm
10,804
7,338
9,993
250
200
150
100
50
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(4-chlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3i**)

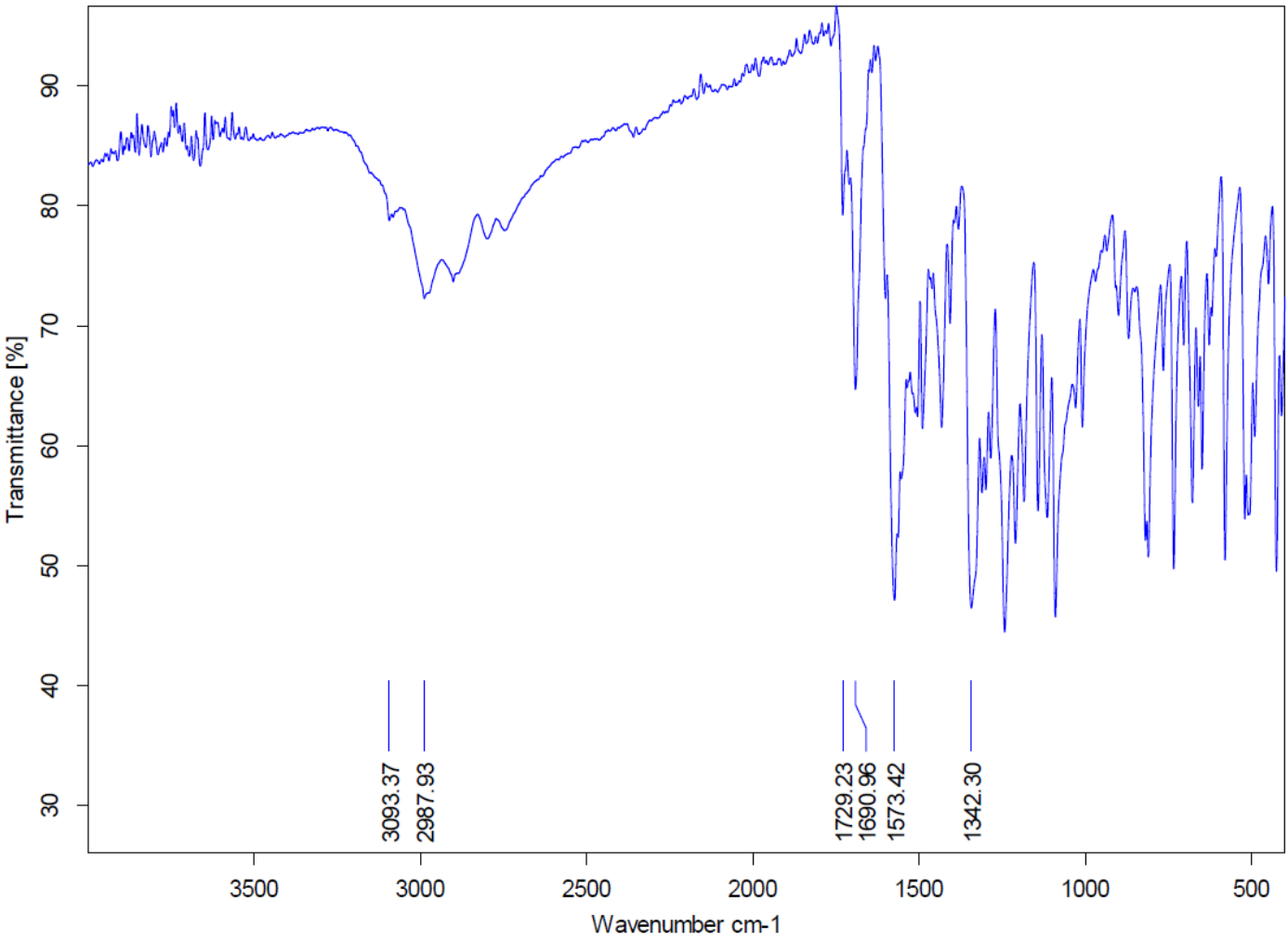
^{1}H NMR



^{13}C NMR



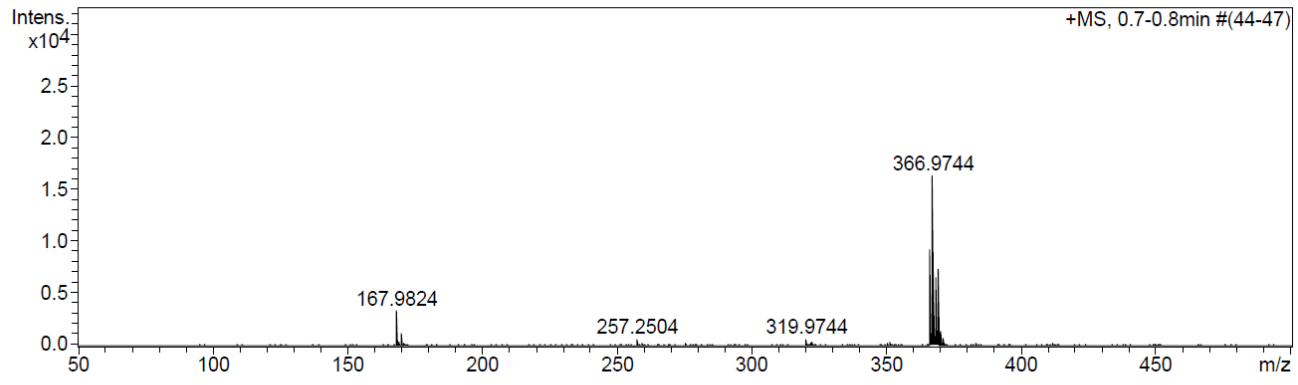
FTIR



HRMS

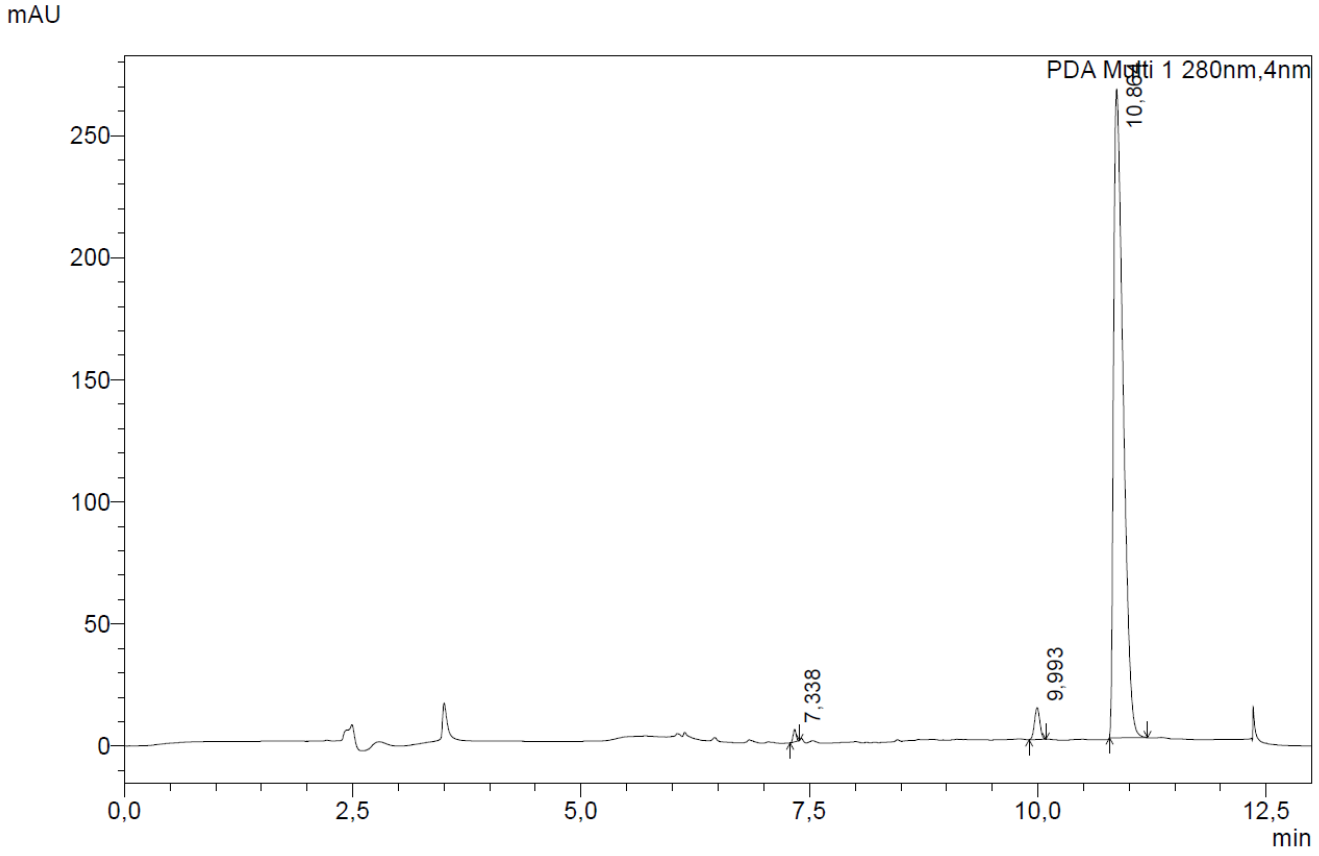
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



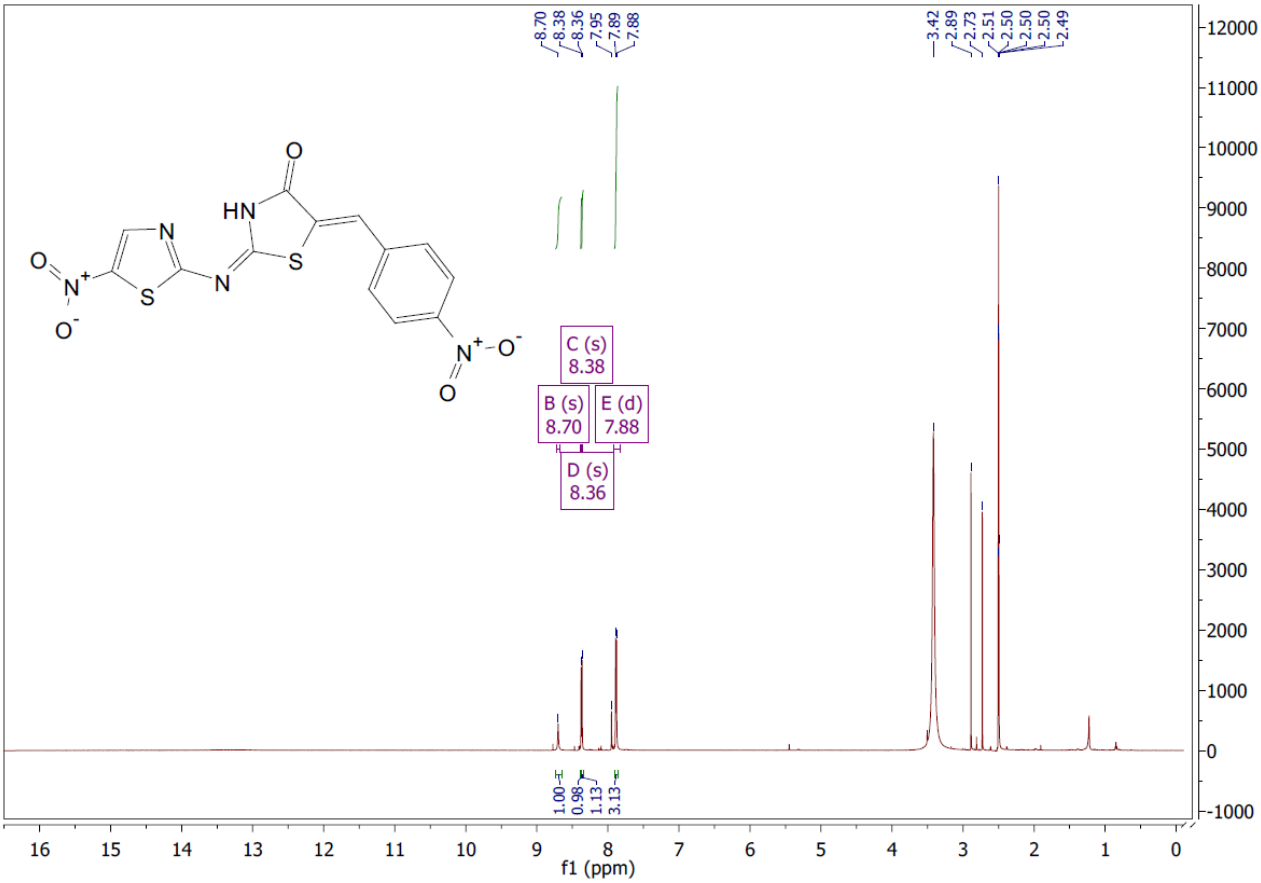
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
366.9744	1	C 13 H 8 Cl N 4 O 3 S 2	100.00	366.9721	-2.3	-6.2	93.4	11.5	even	ok

HPLC

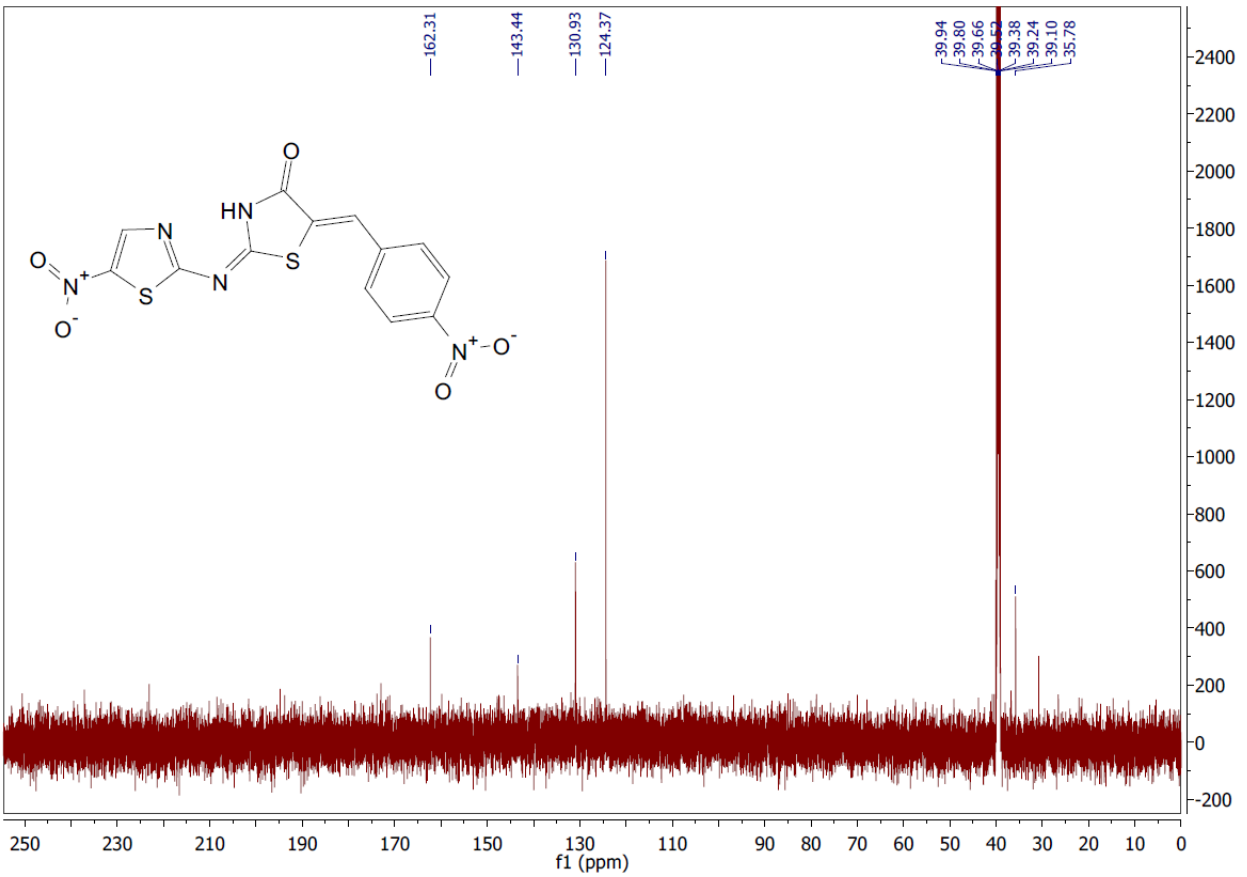

mAU
PDA Multi 1 280nm,4nm
10,864
7,338
9,993
250
200
150
100
50
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(4-nitrobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3j**)

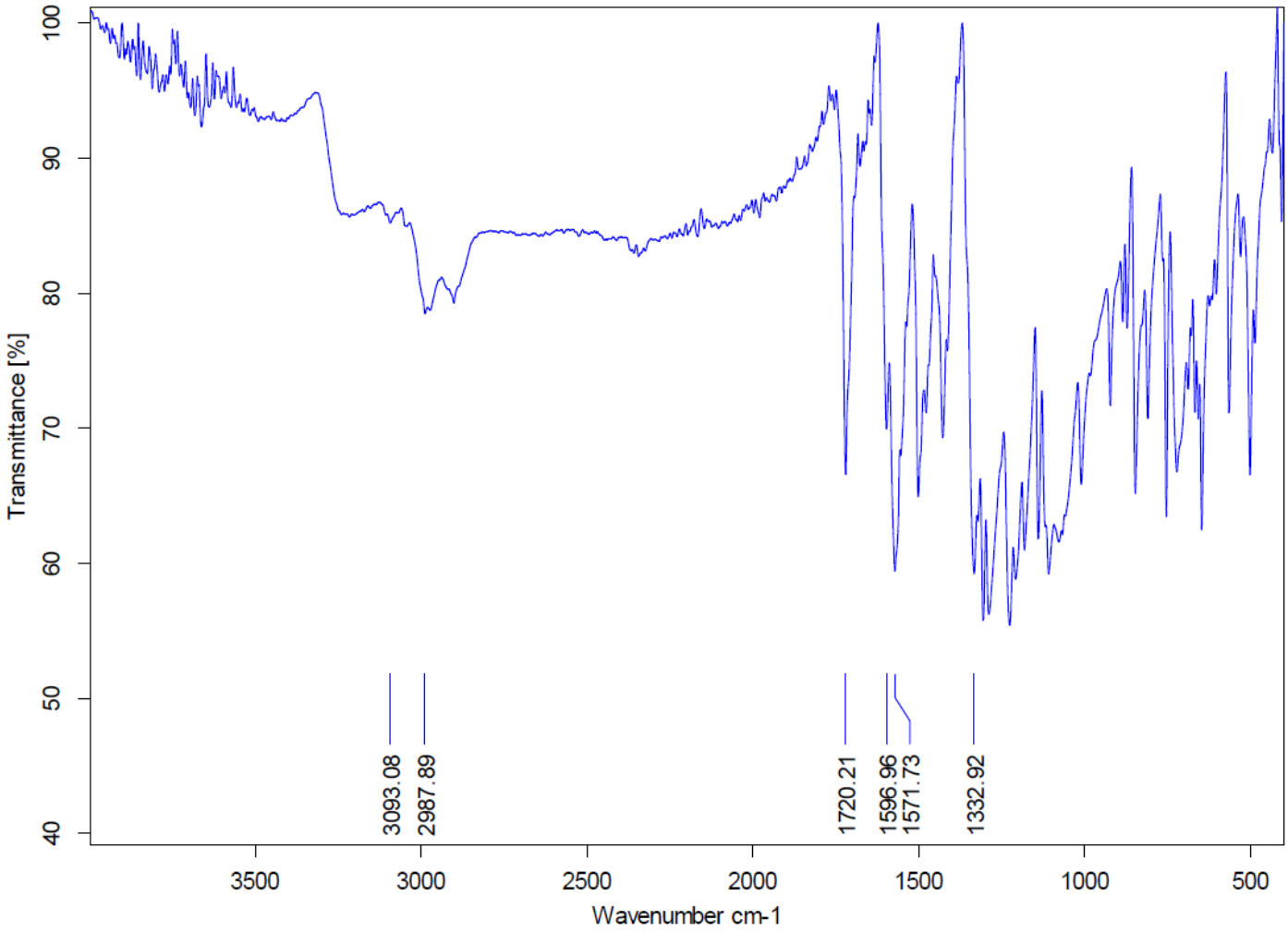
^{1}H NMR



^{13}C NMR



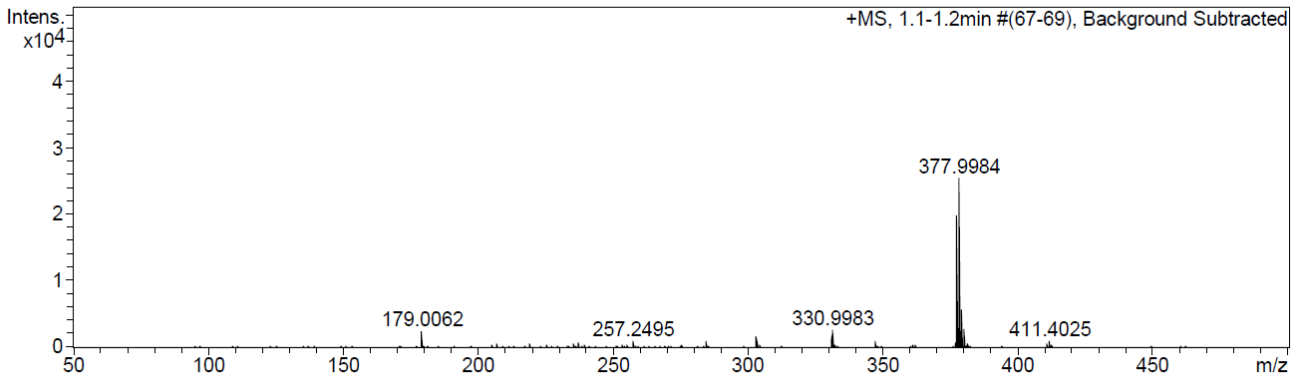
FTIR



HRMS

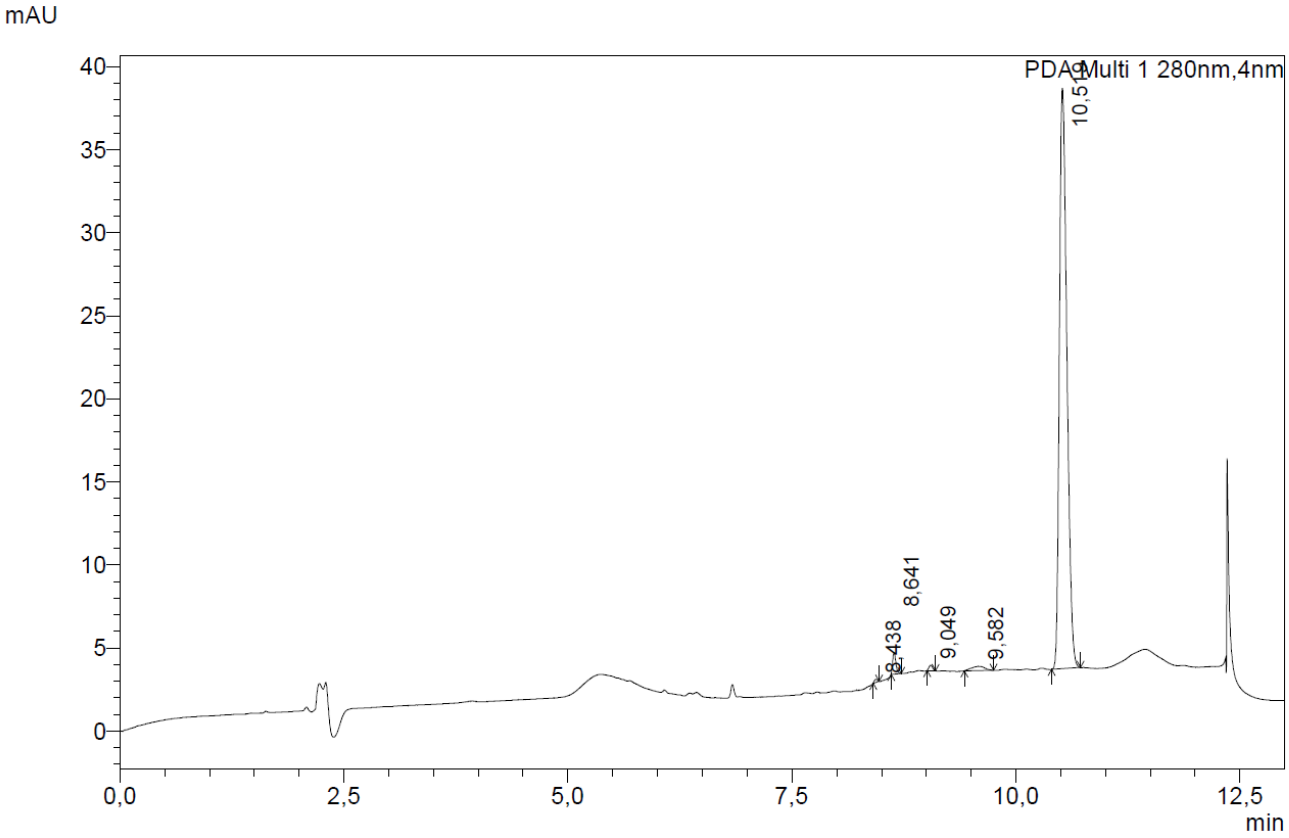
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
377.9984	1	C 13 H 8 N 5 O 5 S 2	100.00	377.9961	-2.2	-5.9	25.6	12.5	even	ok

HPLC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PDA Multi 1 280nm,4nm
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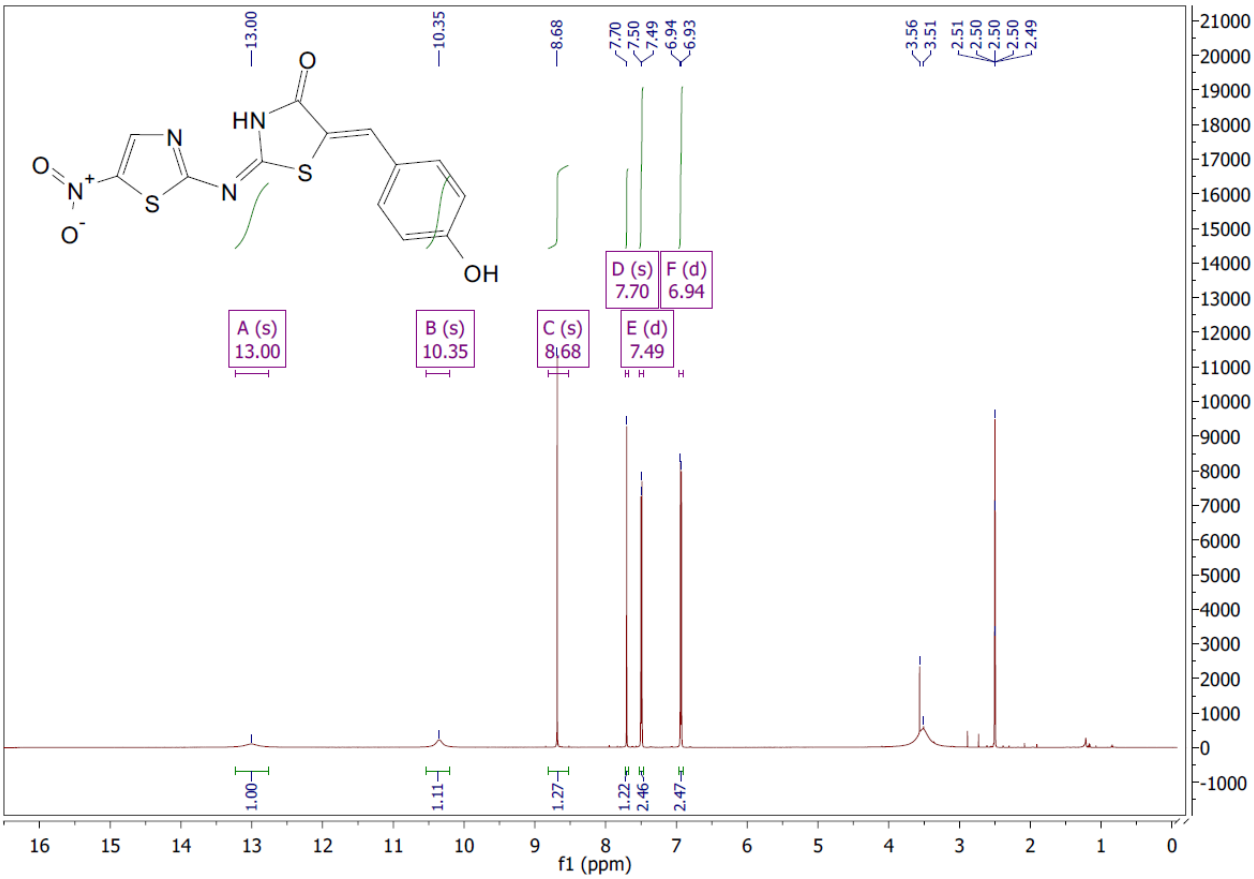
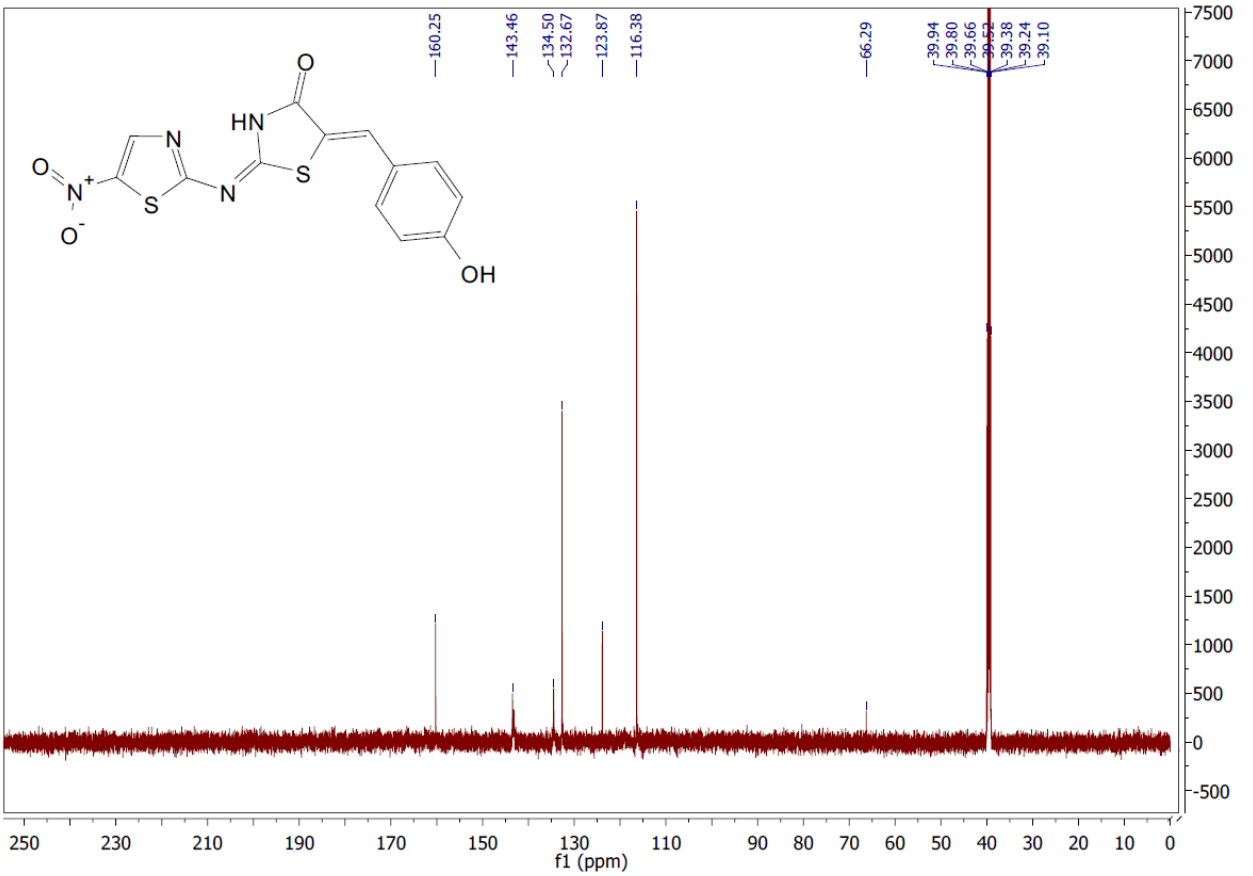
8,641
8,438
9,049
9,582
40
35
30
25
20
15
10
5
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(4-hydroxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3k**)

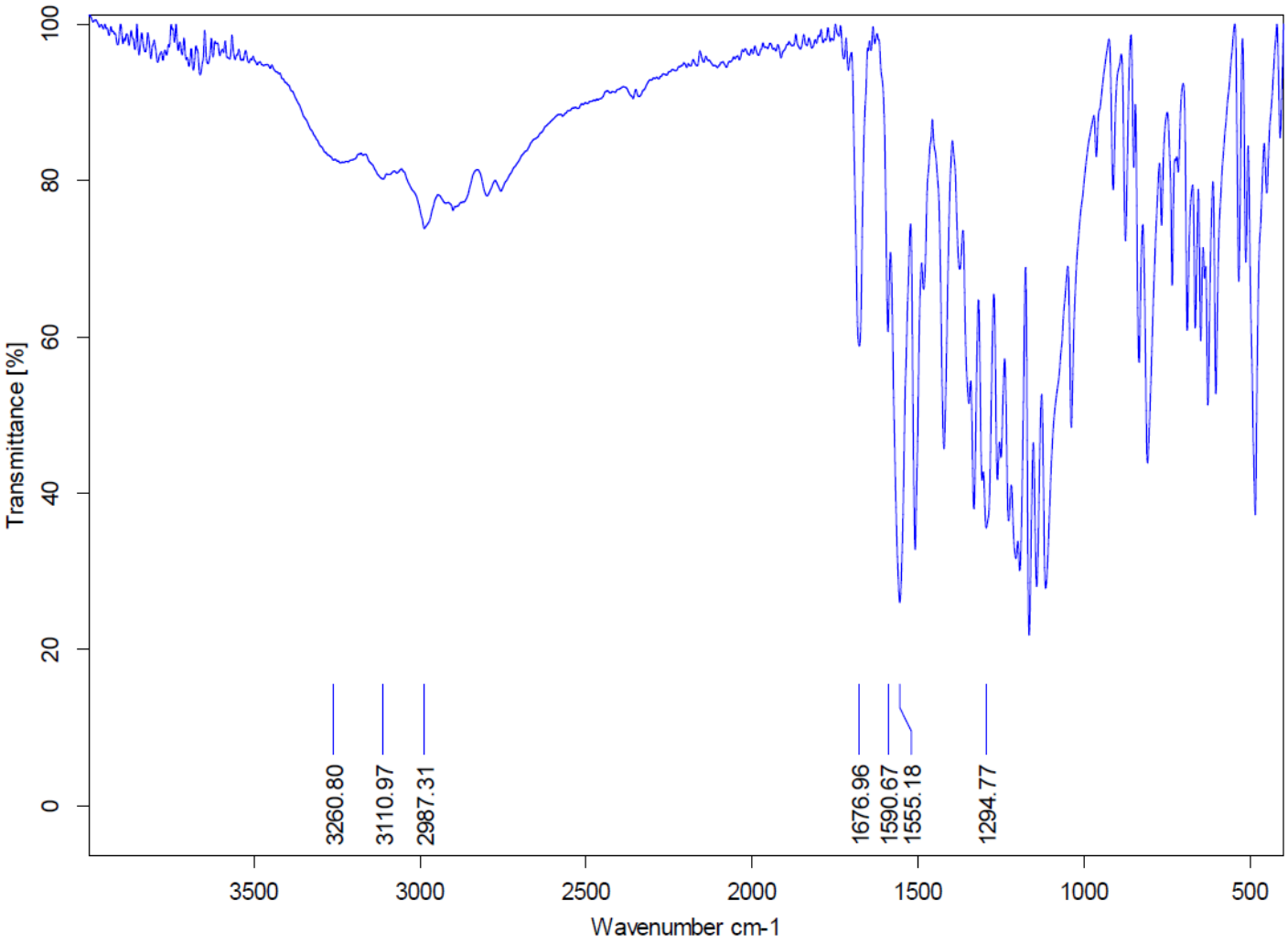
^{1}H NMR



^{13}C NMR



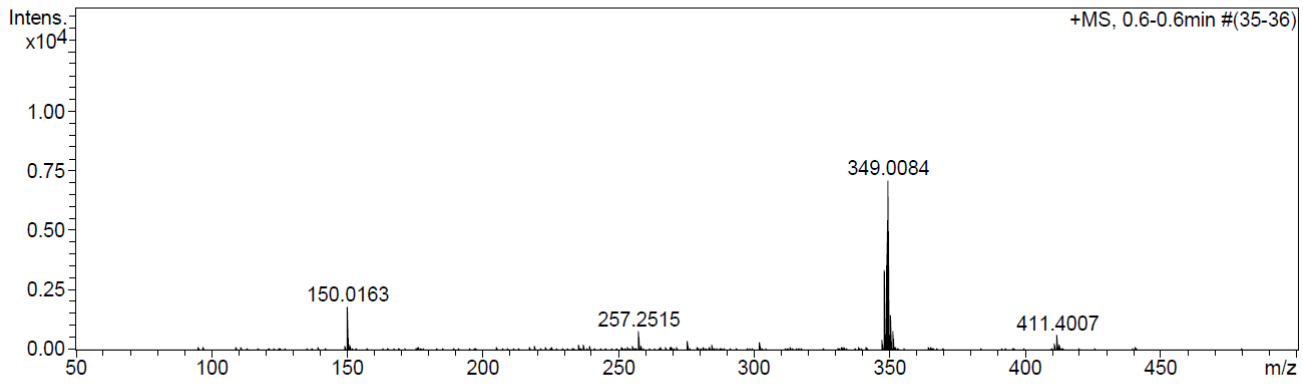
FTIR



HRMS

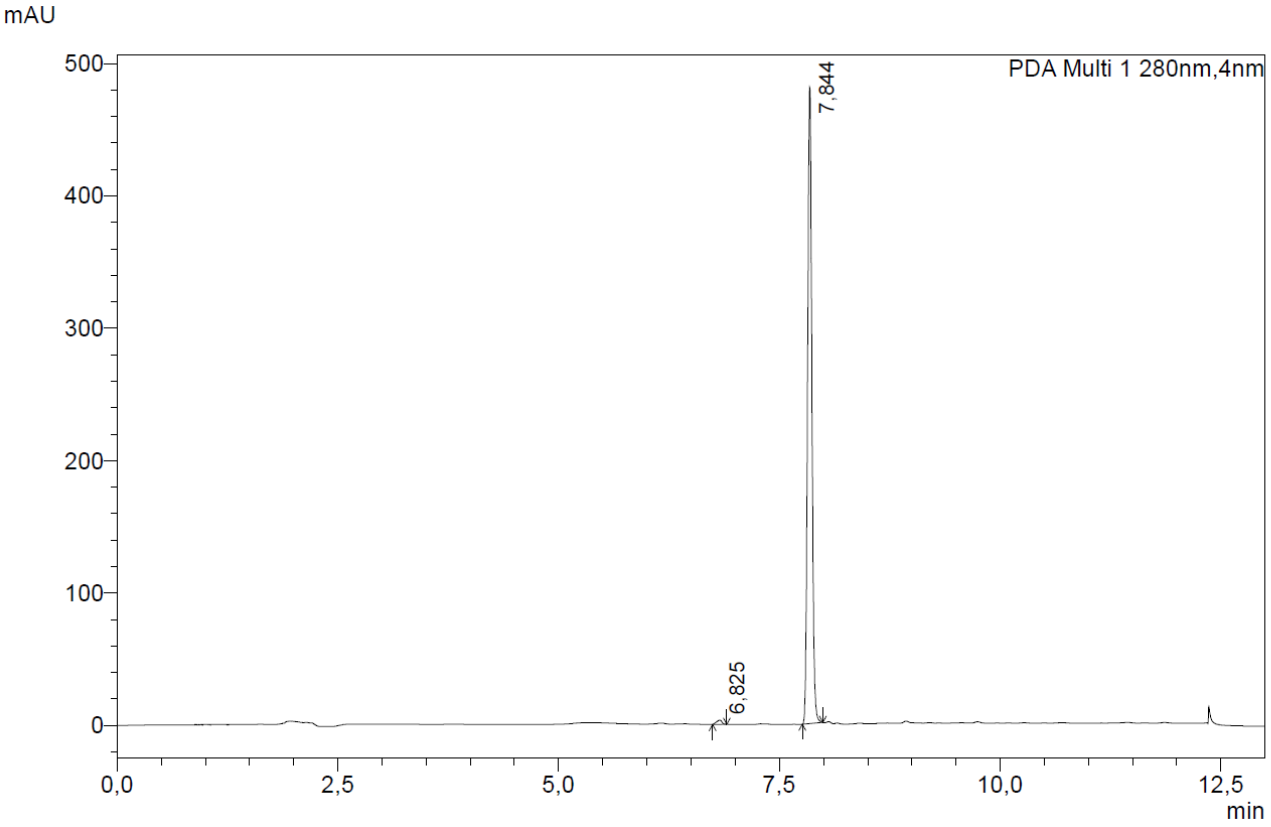
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



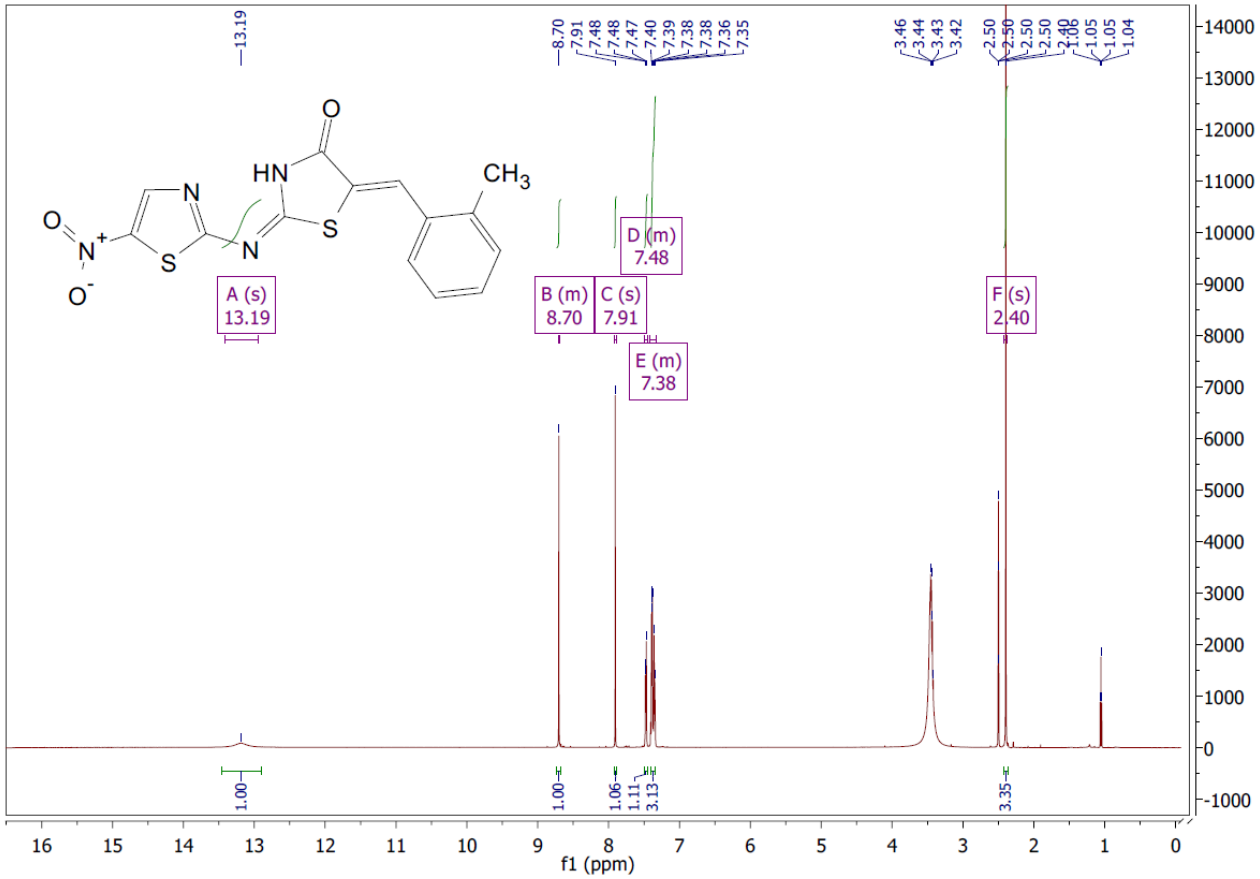
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
349.0084	1	C 13 H 9 N 4 O 4 S 2	100.00	349.0060	-2.4	-6.9	19.1	11.5	even	ok

HPLC

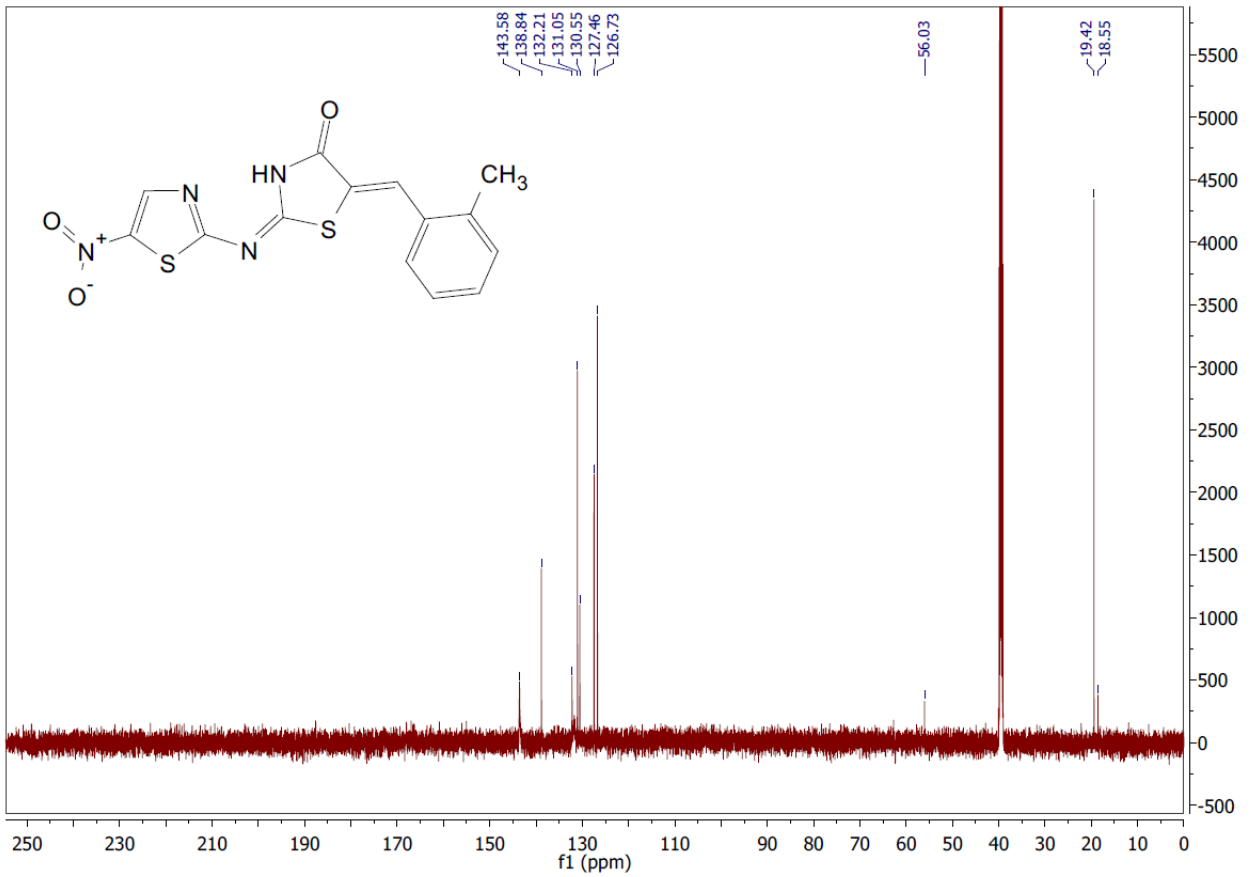

mAU
500
400
300
200
100
0
PDA Multi 1 280nm,4nm
7,844
6,825
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(2-methylbenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3l**)

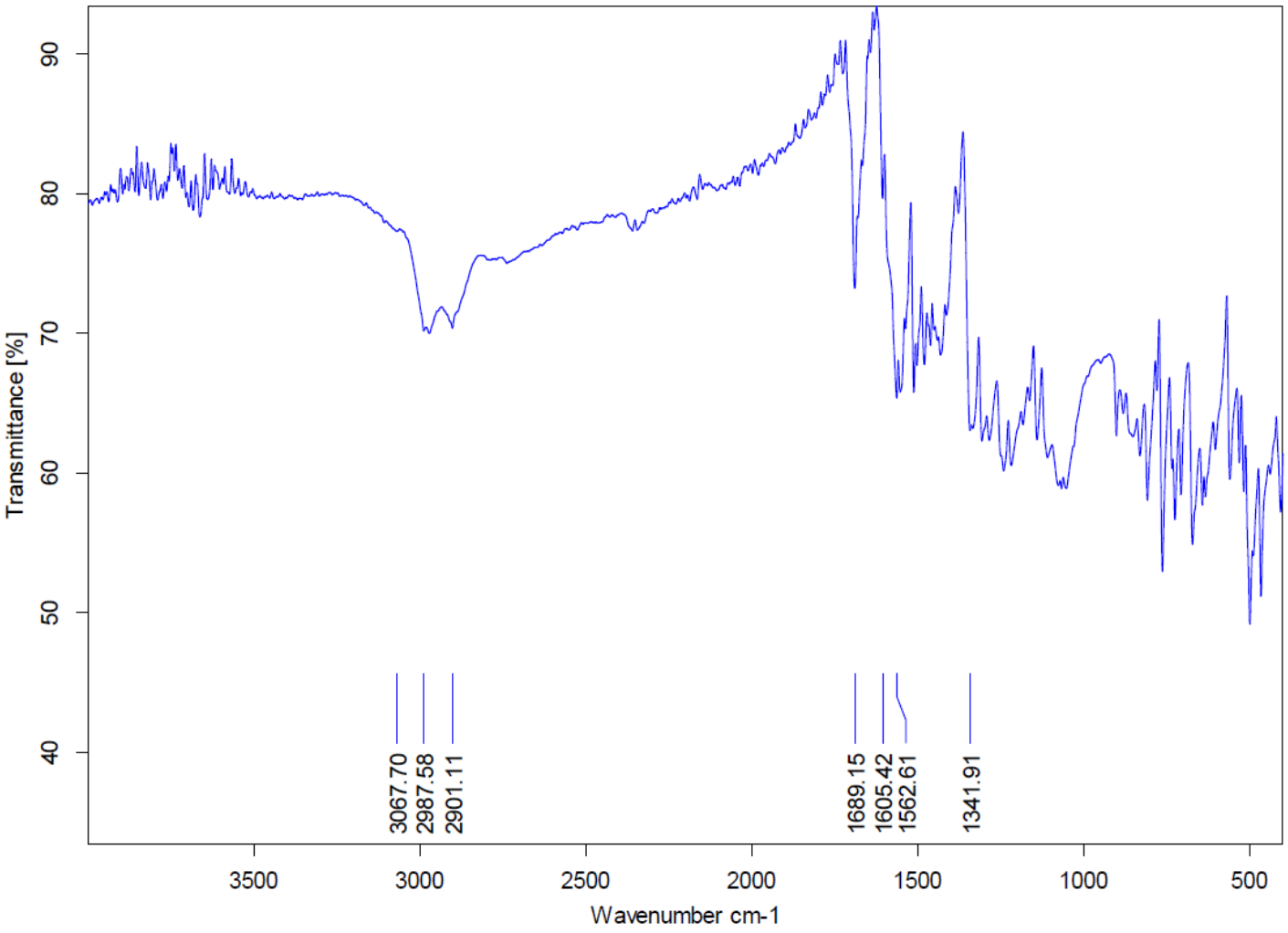
^{1}H NMR



^{13}C NMR



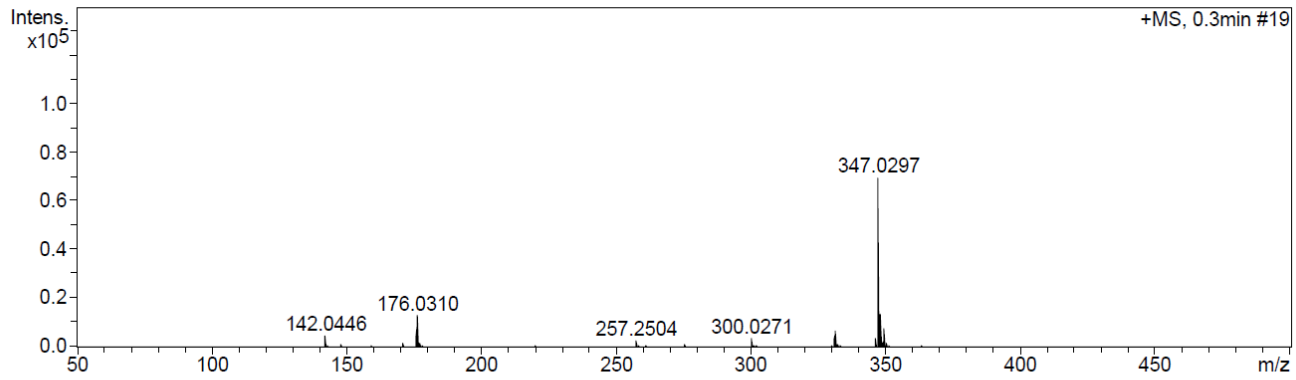
FTIR



HRMS

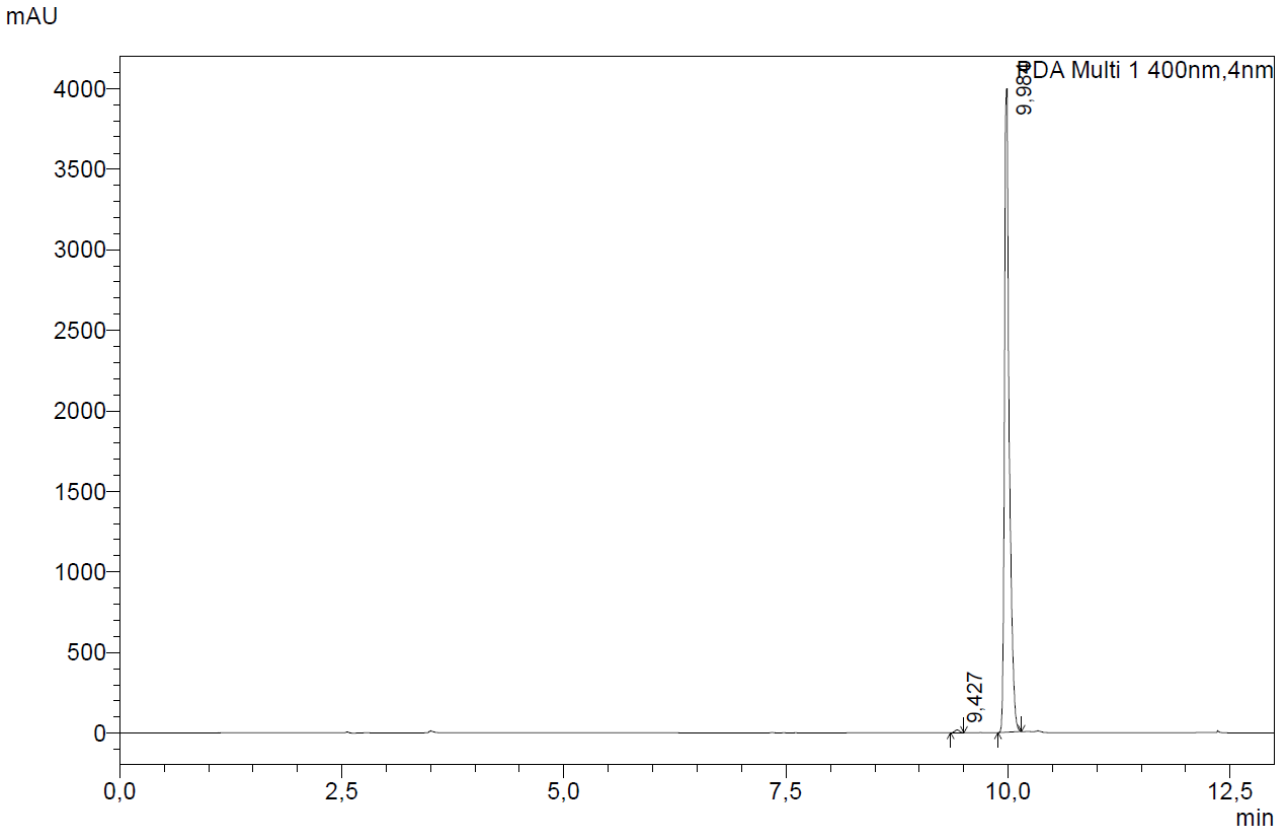
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



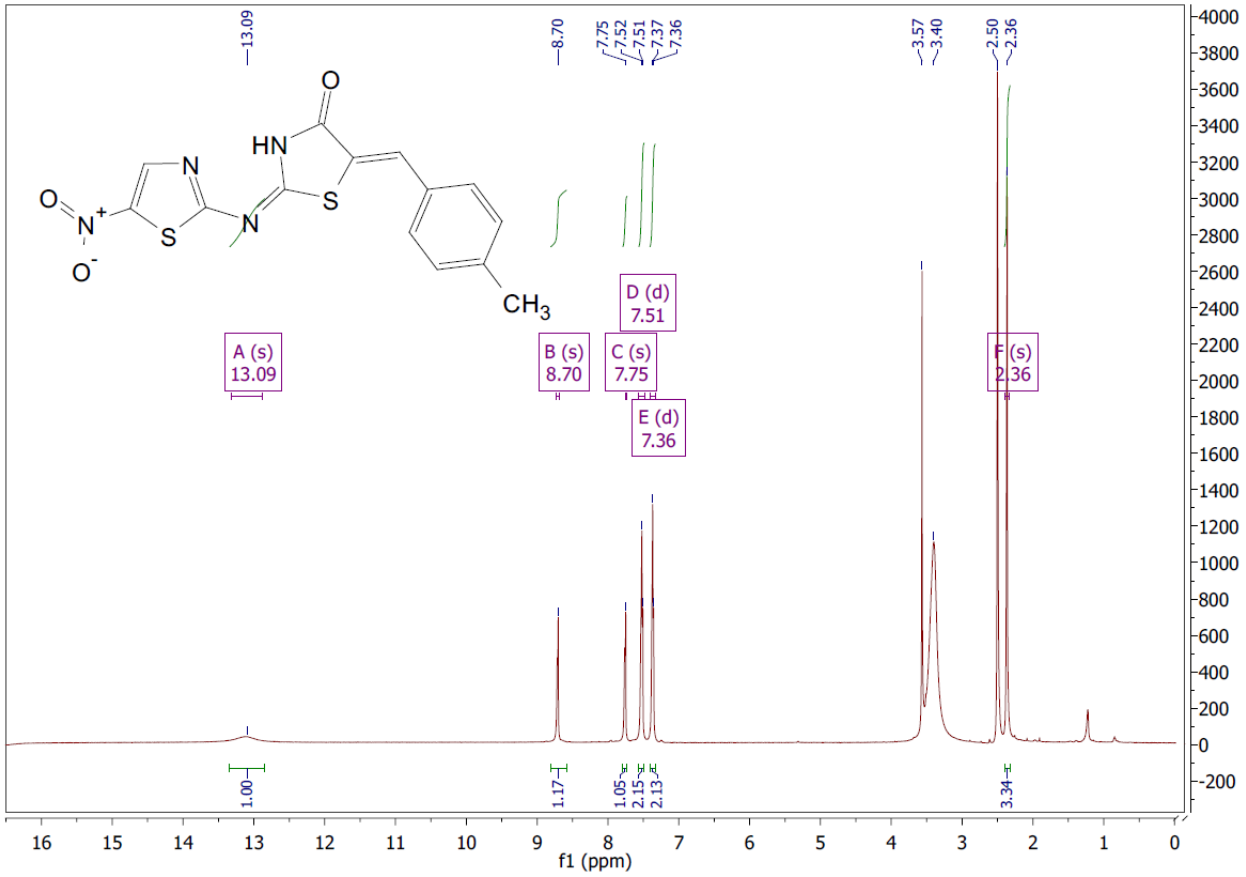
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
347.0297	1	C 14 H 11 N 4 O 3 S 2	100.00	347.0267	-3.0	-8.7	7.2	11.5	even	ok

HPLC

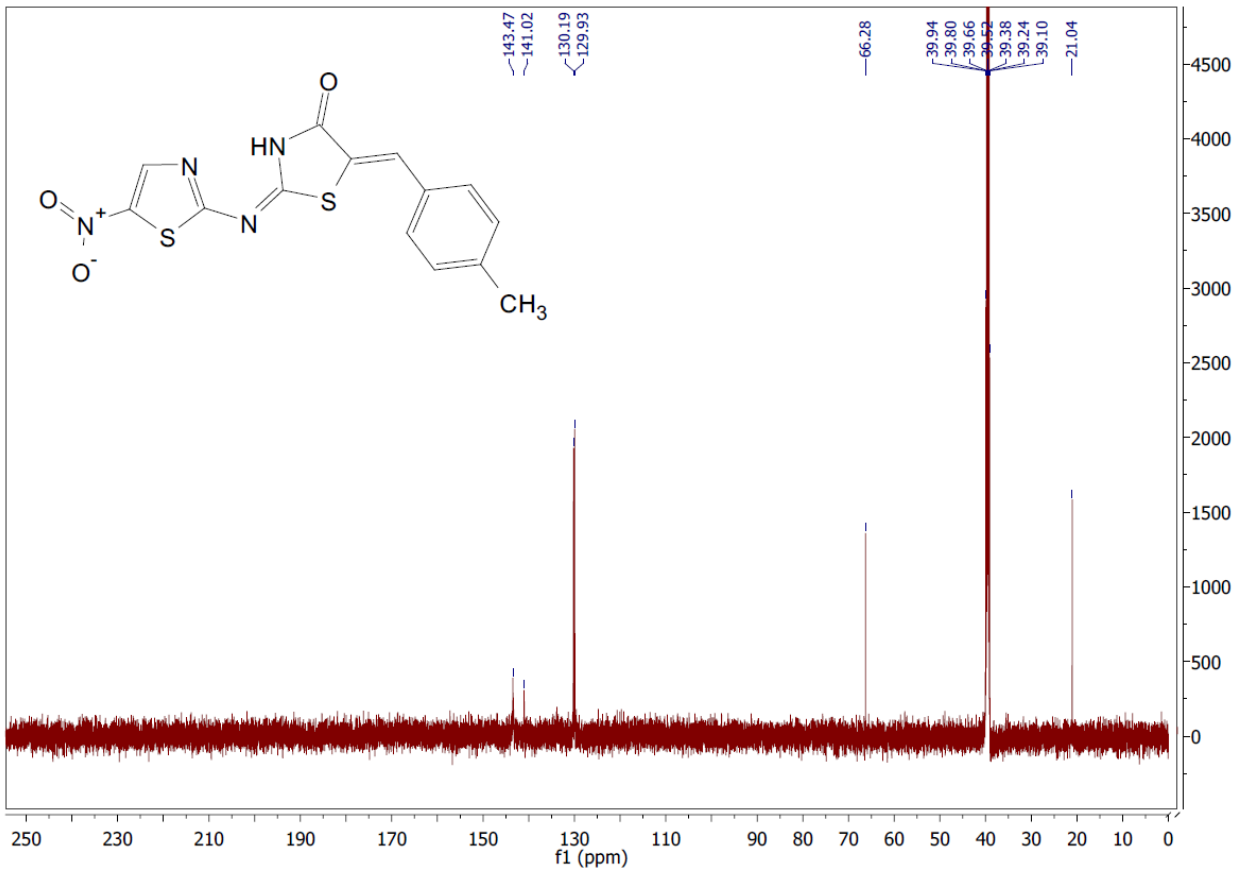

mAU
PDA Multi 1 400nm,4nm
9,984
9,427
4000
3500
3000
2500
2000
1500
1000
500
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(4-methylbenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3m**)

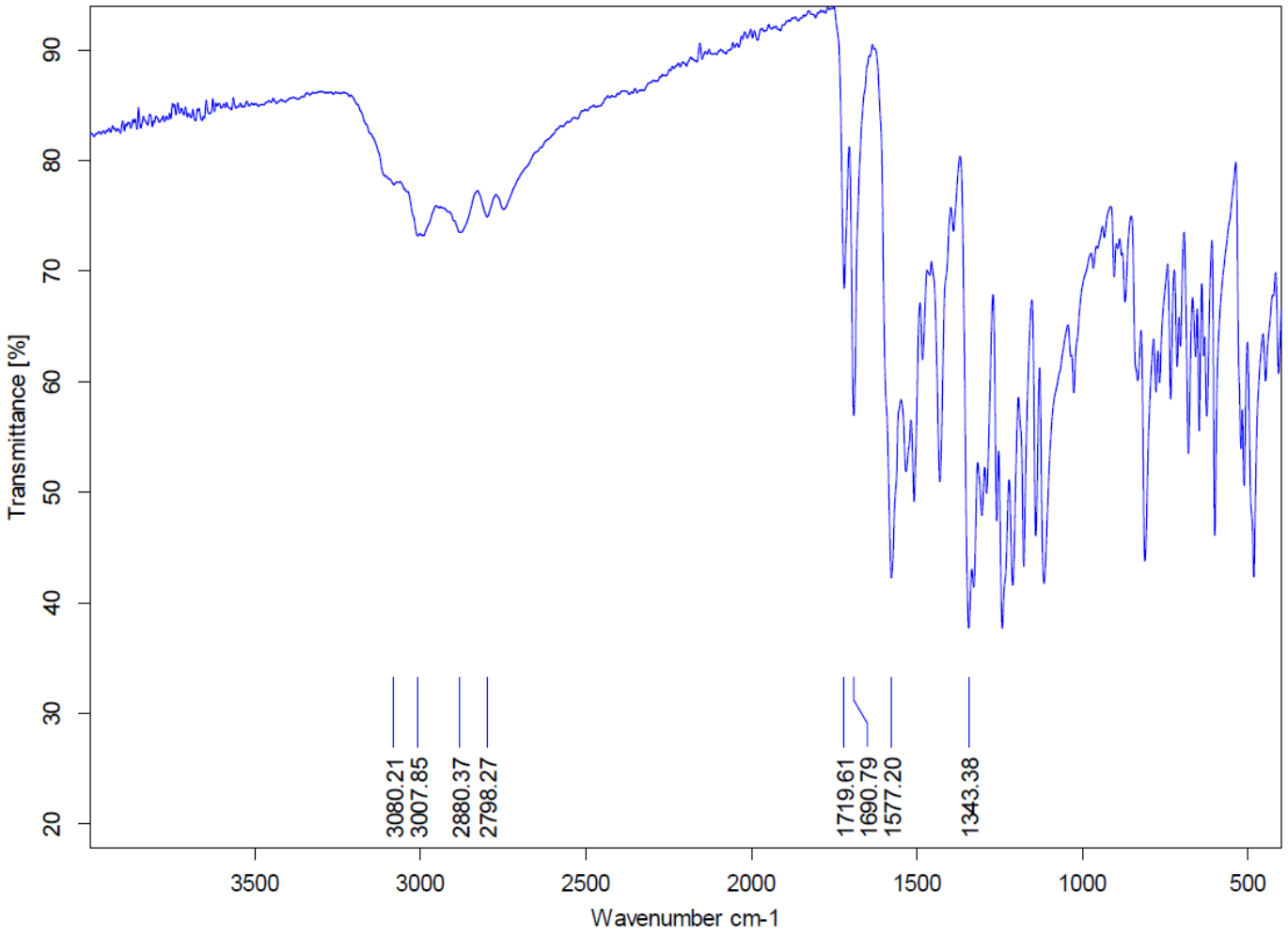
^{1}H NMR



^{13}C NMR



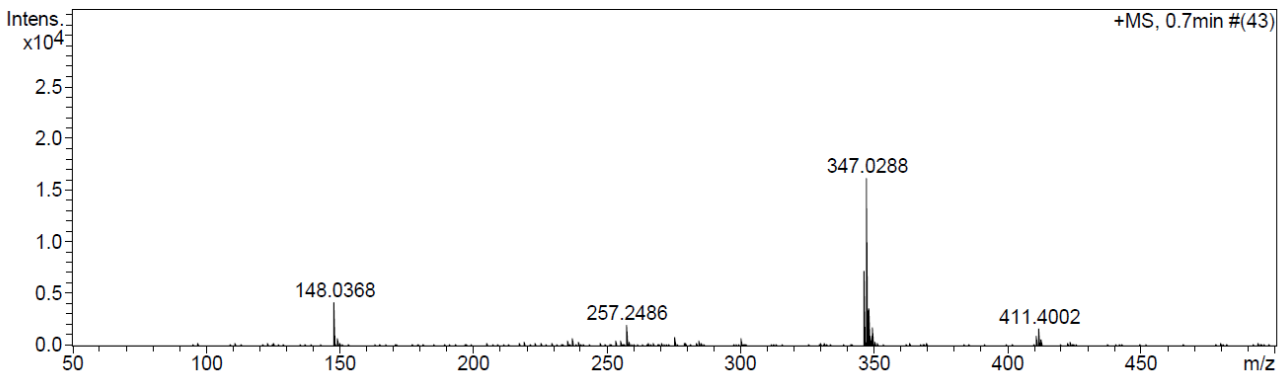
FTIR



HRMS

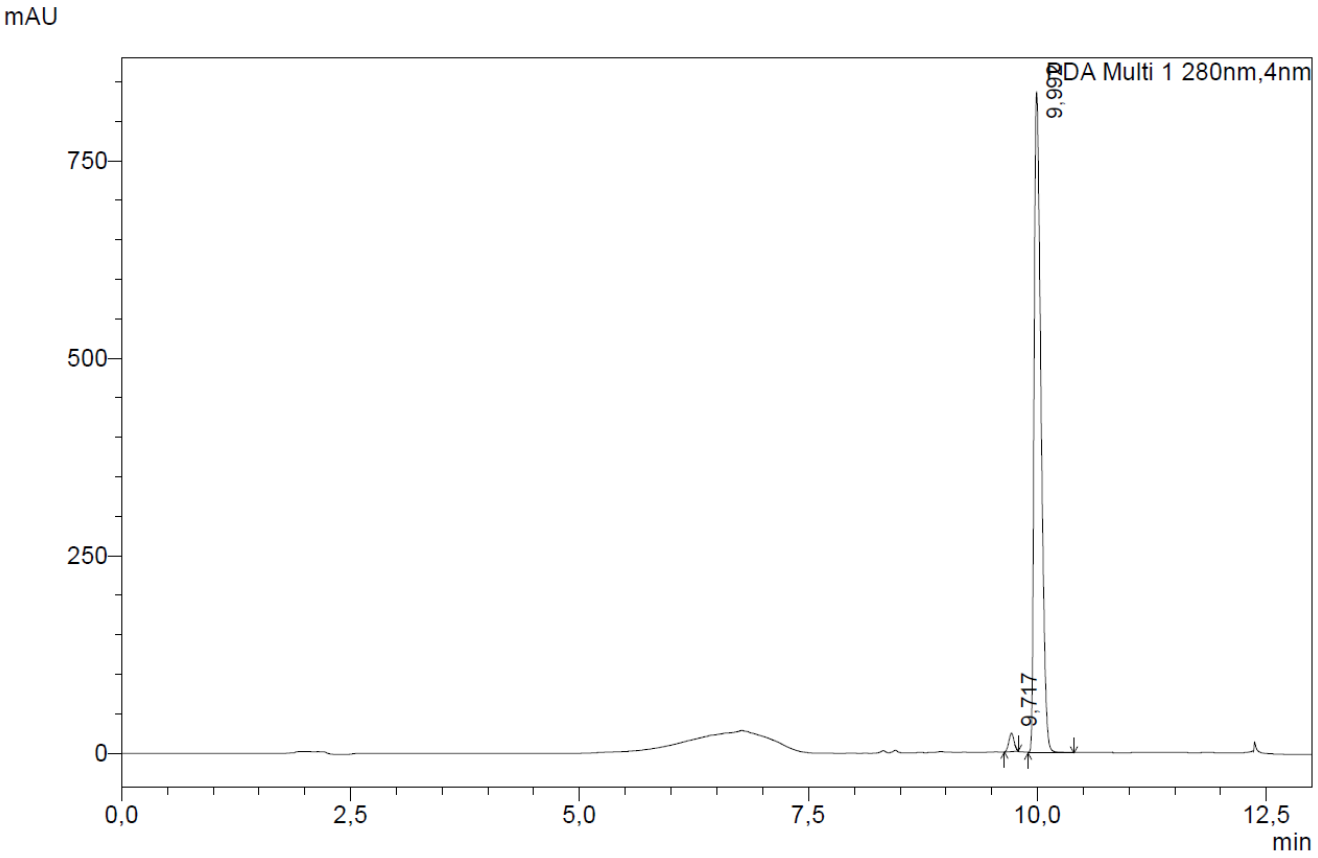
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



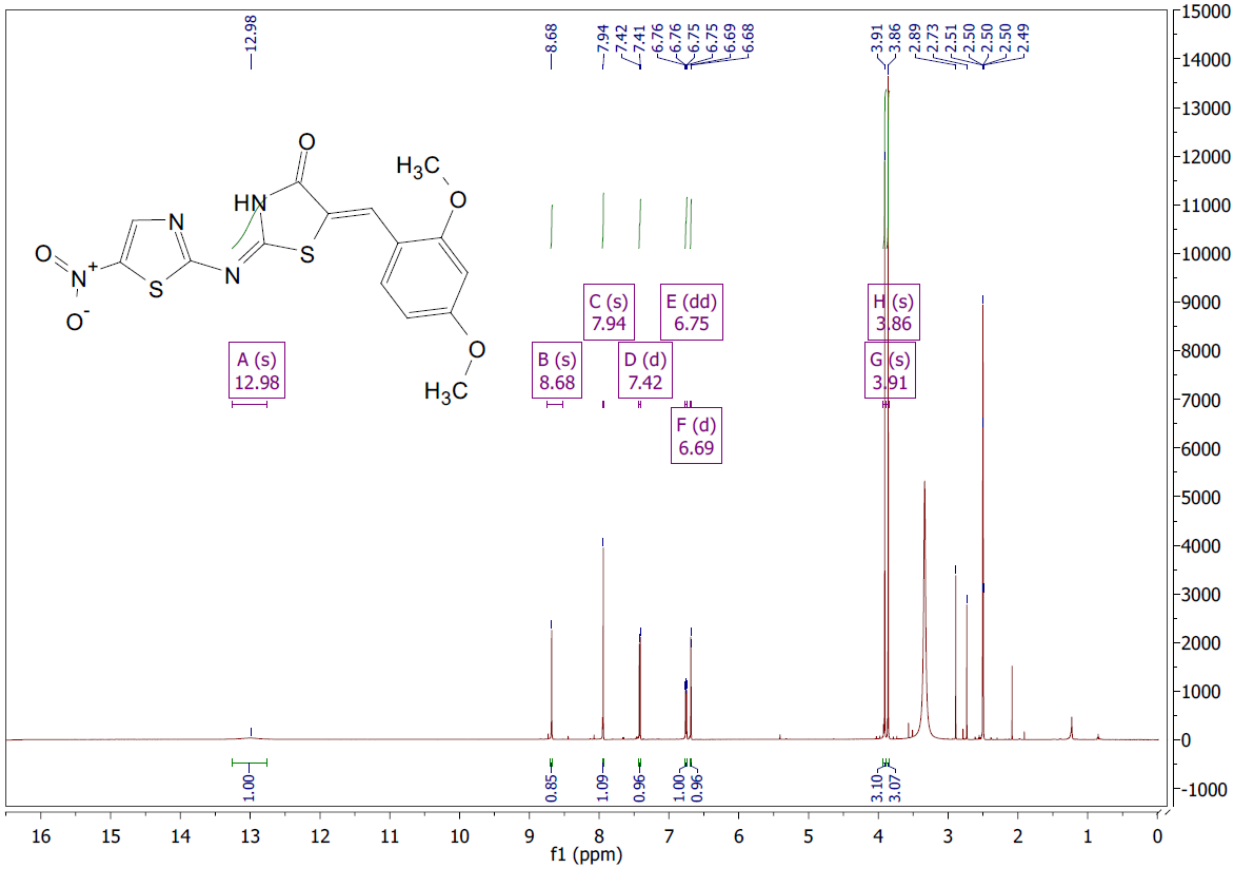
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
347.0288	1	C 14 H 11 N 4 O 3 S 2	100.00	347.0267	-2.1	-6.0	24.3	11.5	even	ok

HPLC

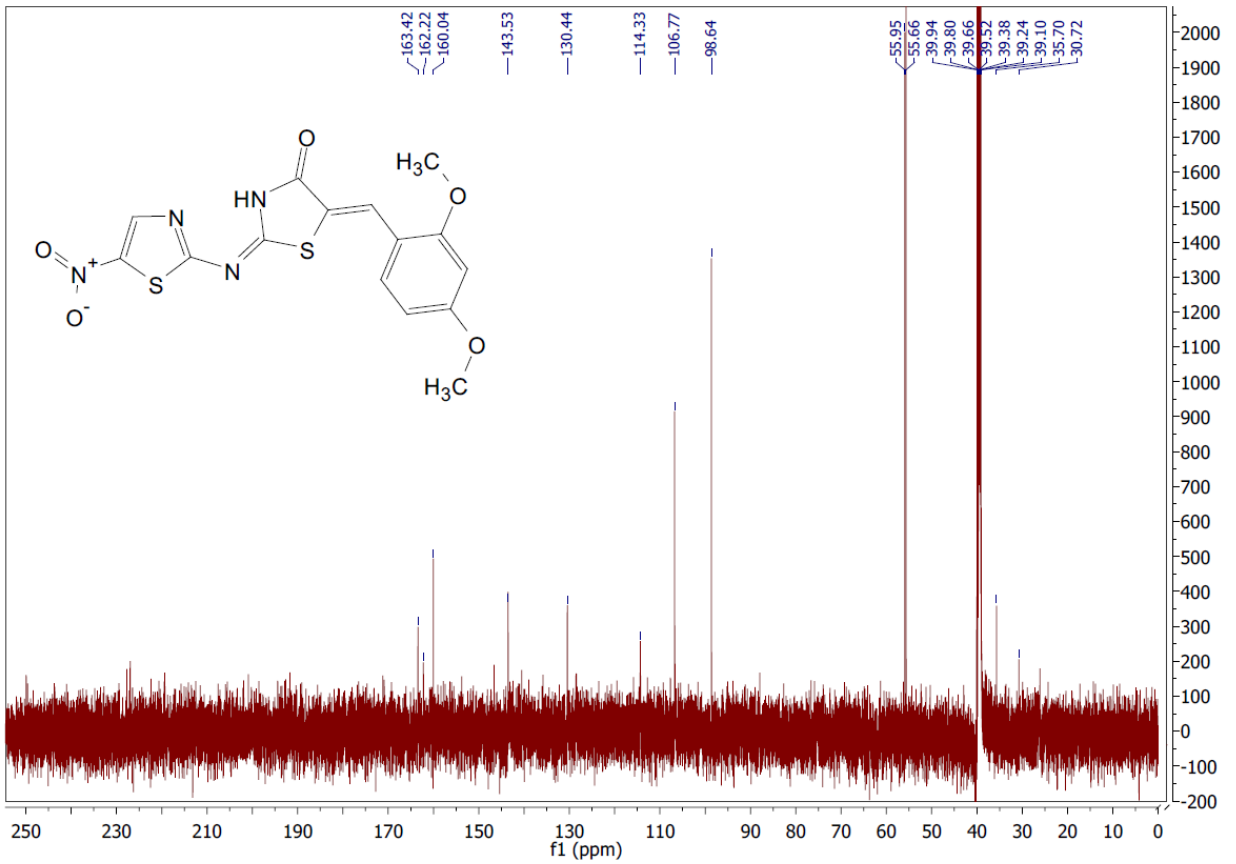

mAU
DA Multi 1 280nm,4nm
9,992
9,717
750
500
250
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(2,4-dimethoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3n**)

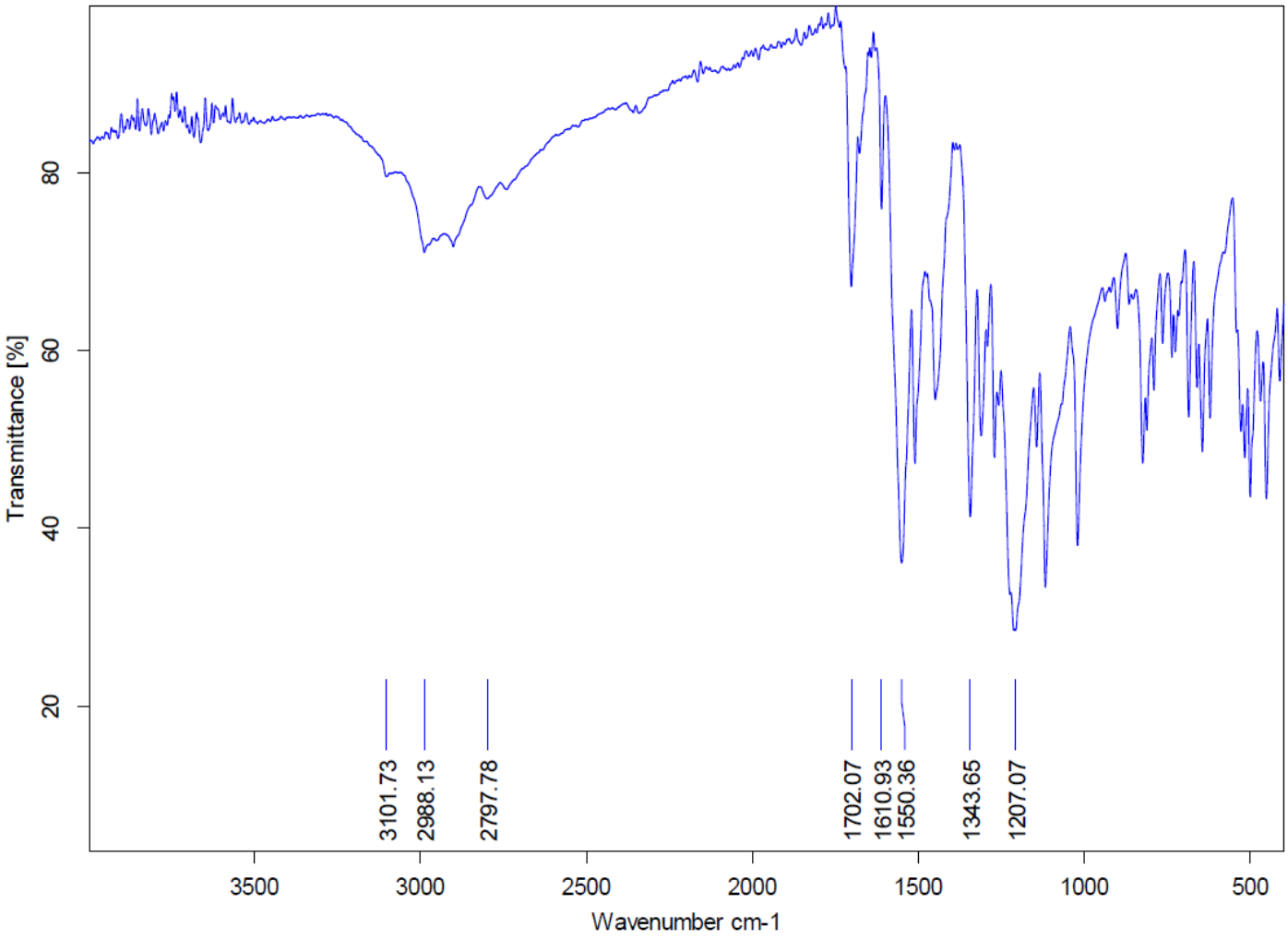
^{1}H NMR



^{13}C NMR



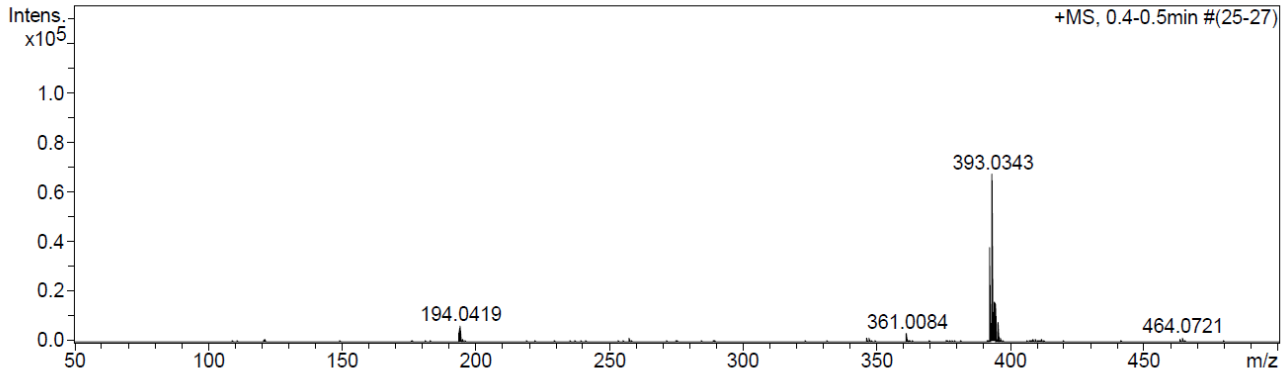
FTIR



HRMS

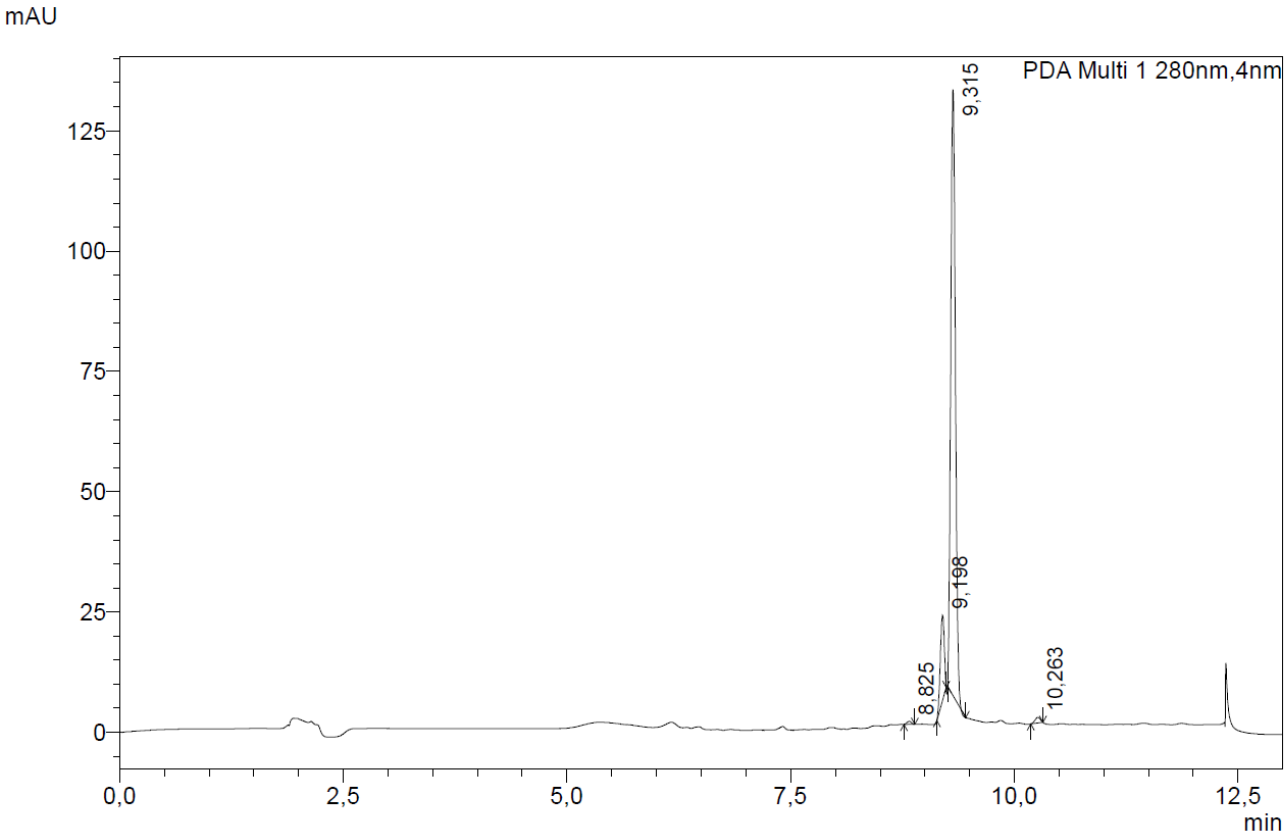
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



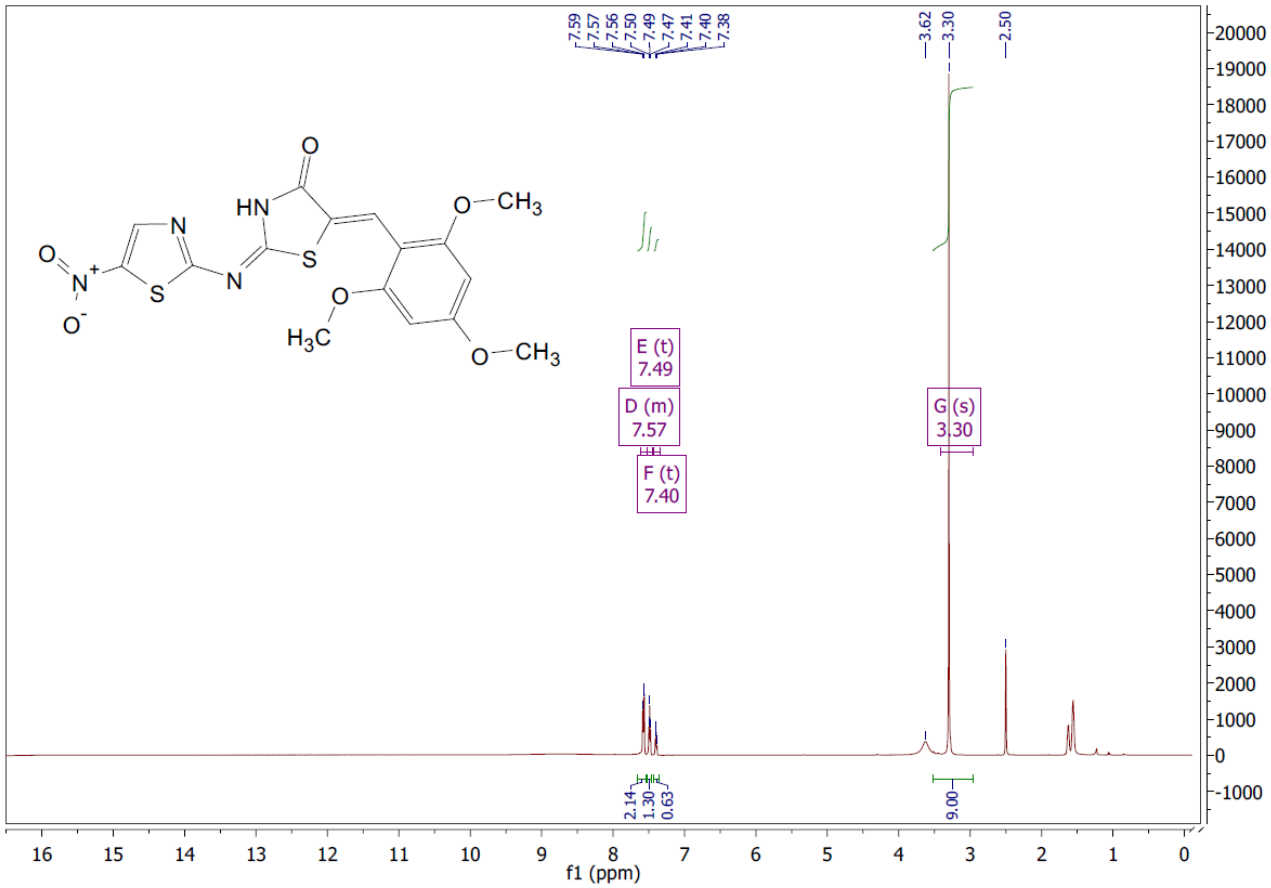
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
392.0274	1	C 12 H 16 N 4 O 5 S 3	100.00	392.0277	0.3	0.8	424.2	7.0	odd	ok
	2	C 15 H 12 N 4 O 5 S 2	0.01	392.0244	-3.1	-7.8	466.0	12.0	odd	ok
393.0343	1	C 15 H 13 N 4 O 5 S 2	100.00	393.0322	-2.1	-5.3	23.8	11.5	even	ok

HPLC

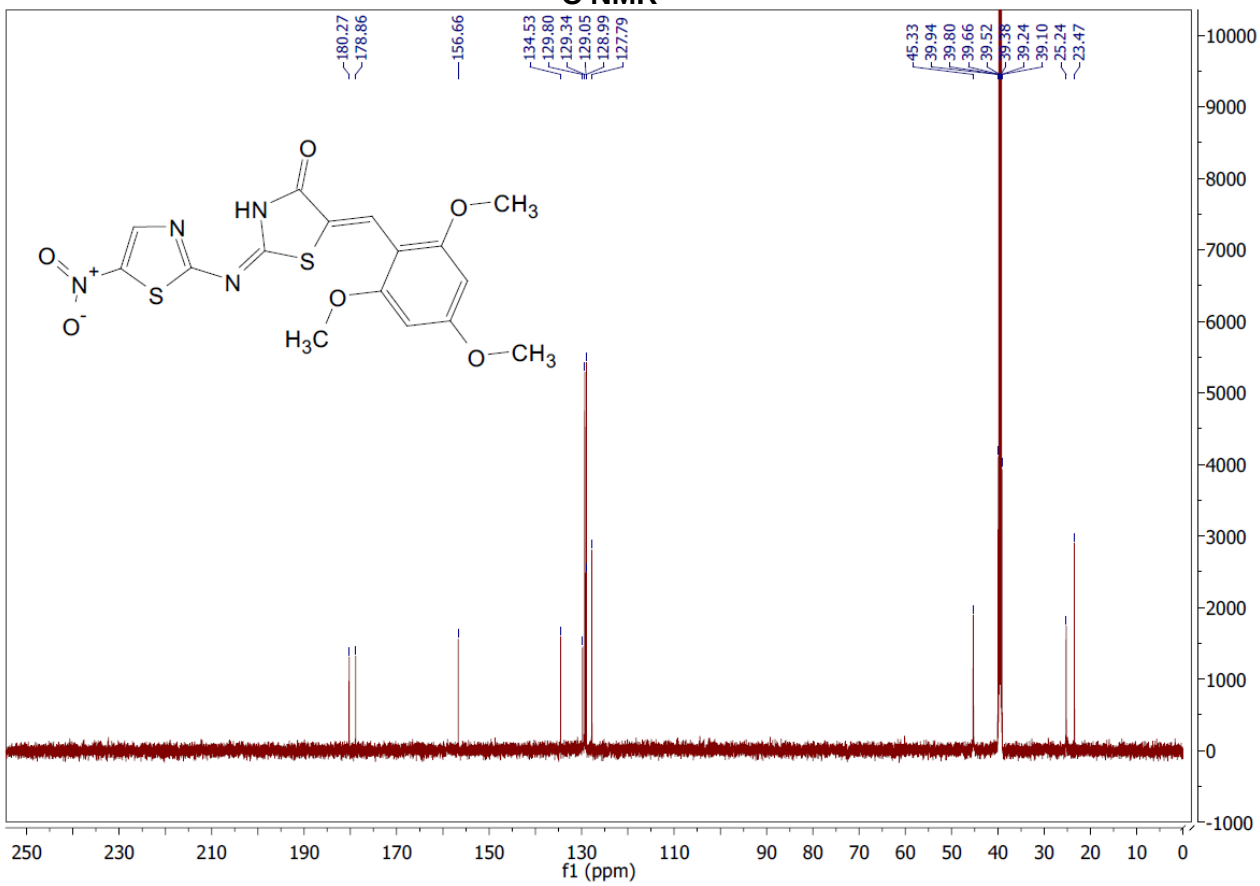

mAU
PDA Multi 1 280nm,4nm
9,315
9,198
8,825
10,263
125
100
75
50
25
0
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(2,4,6-trimethoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3o**)

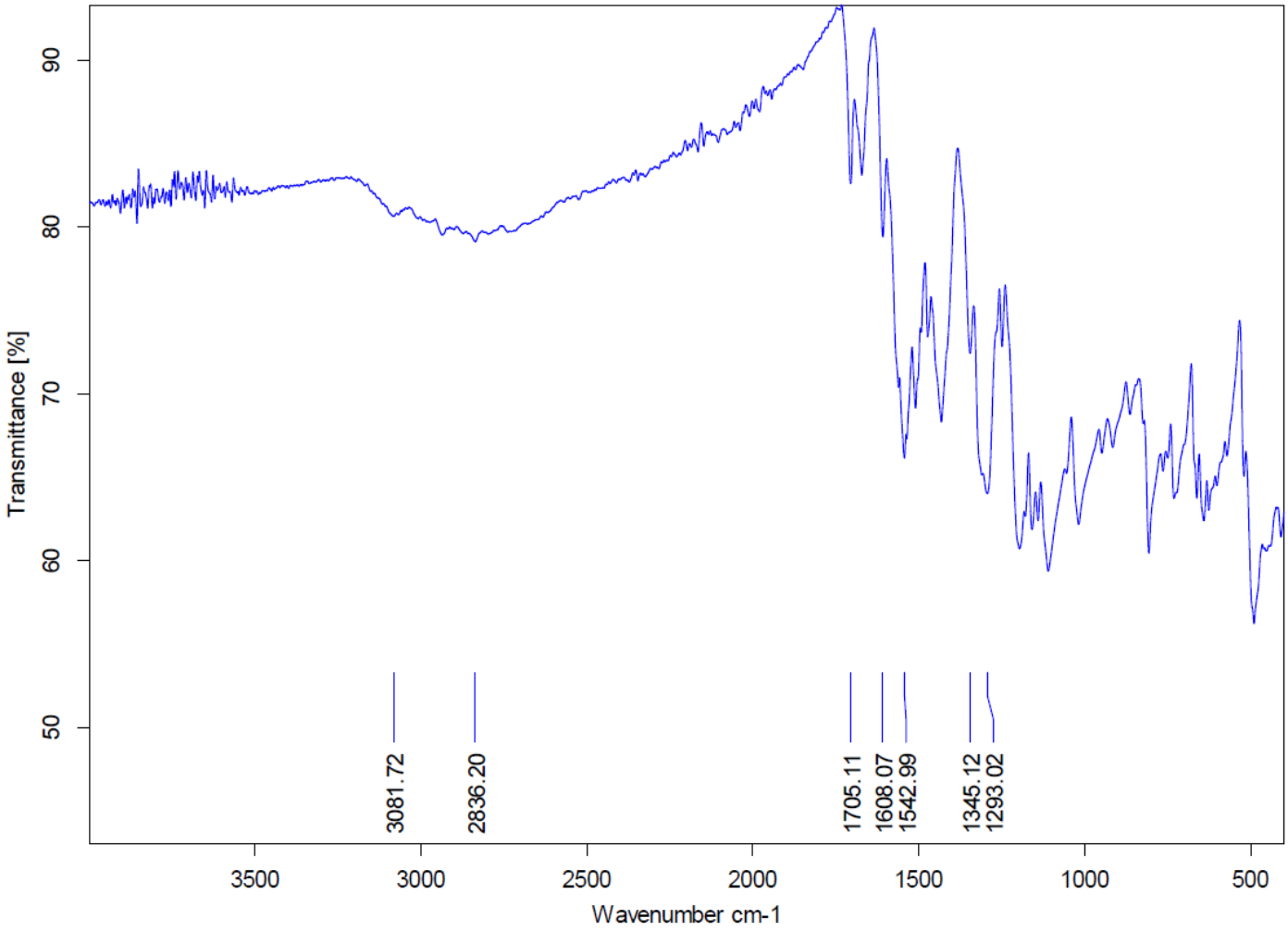
^{1}H NMR



^{13}C NMR



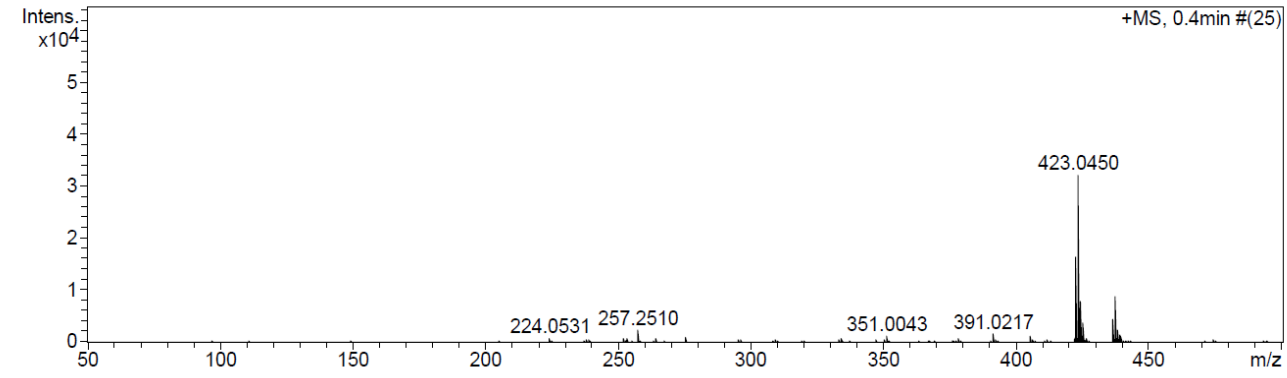
FTIR



HRMS

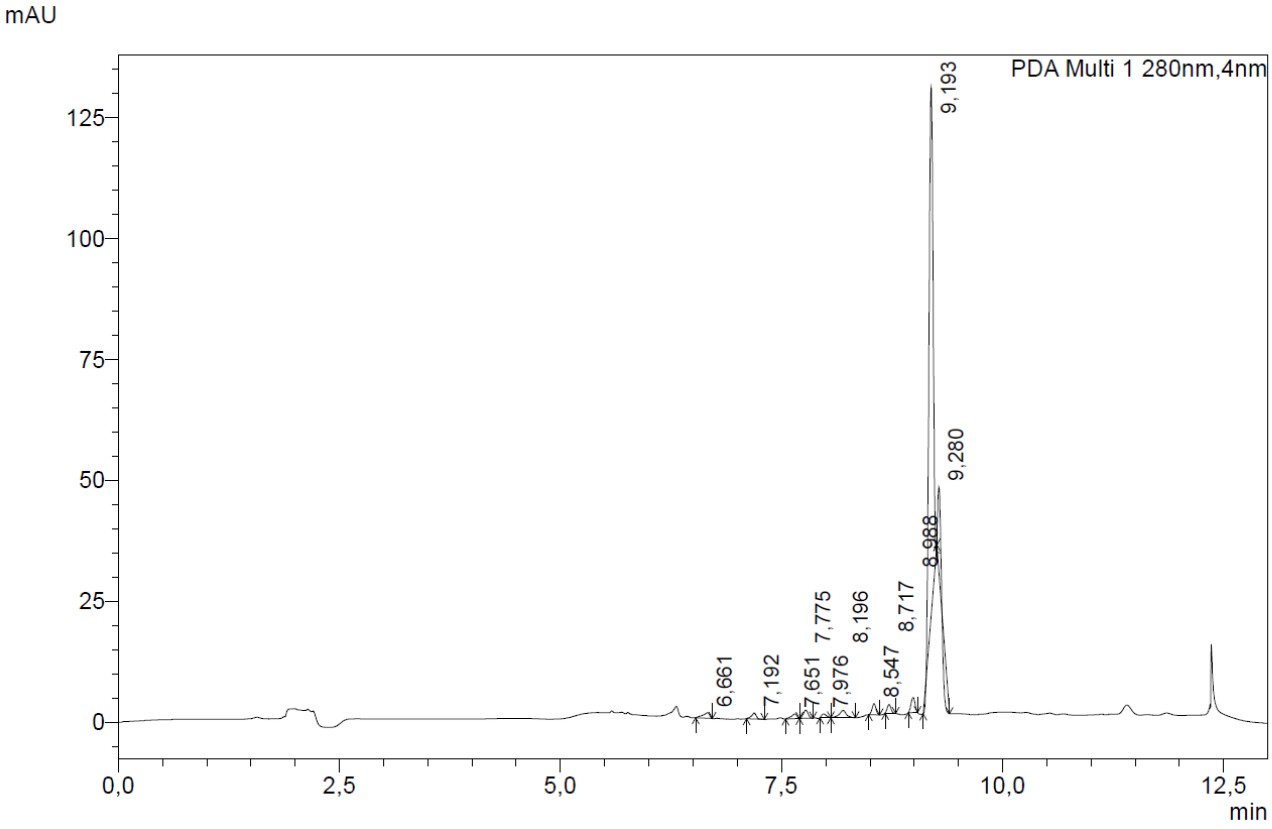
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



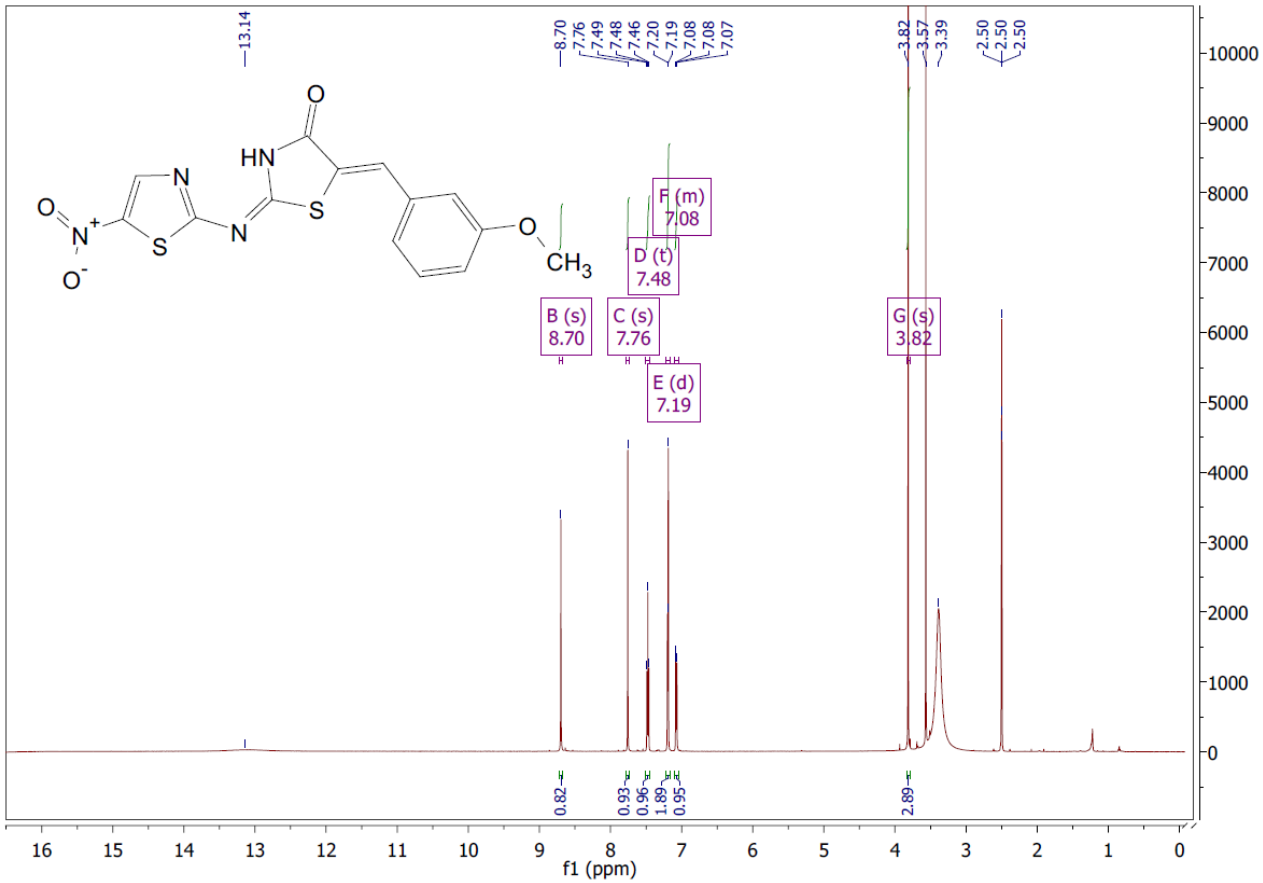
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
423.0450	1	C 16 H 15 N 4 O 6 S 2	100.00	423.0428	-2.2	-5.3	21.6	11.5	even	ok

HPLC

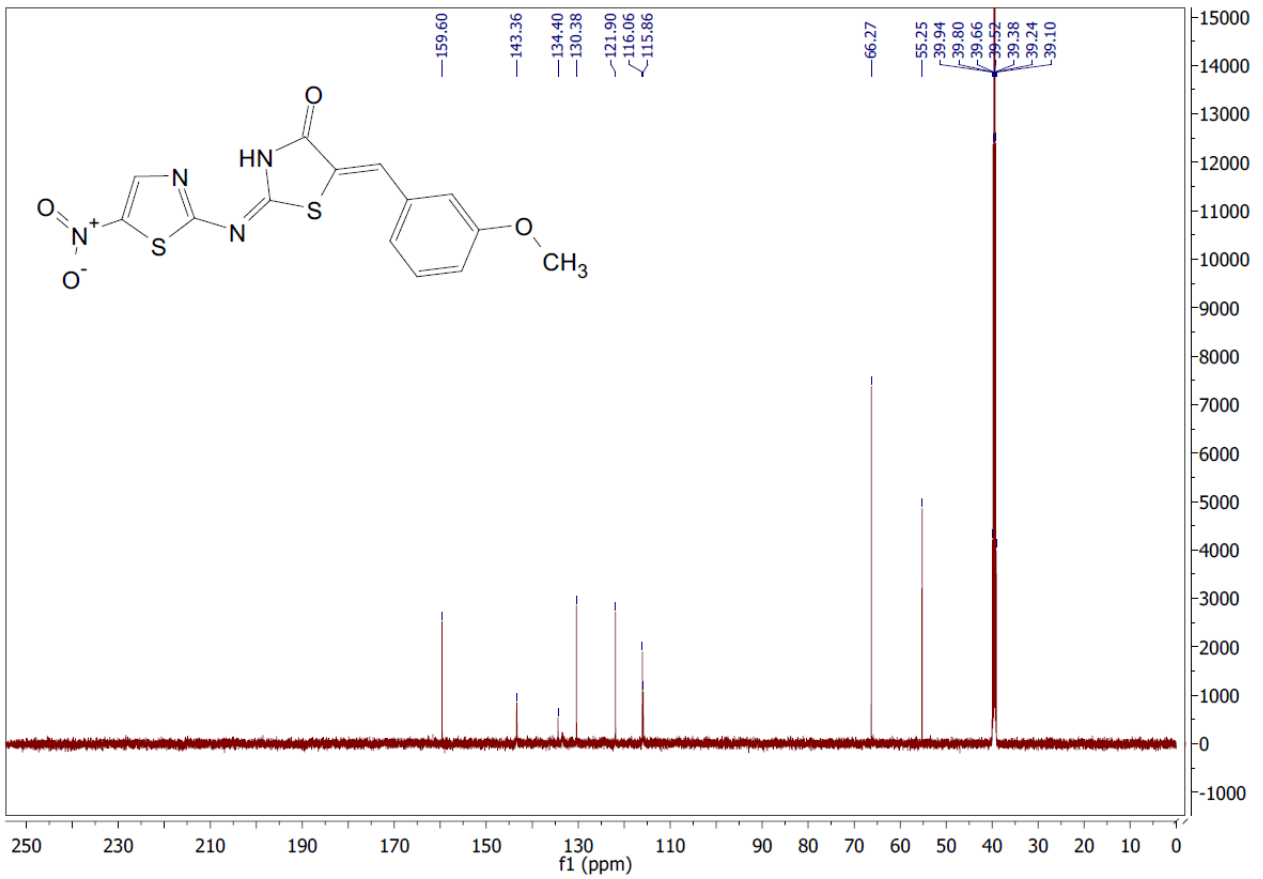

mAU
PDA Multi 1 280nm,4nm
125
100
75
50
25
0
0,0
2,5
5,0
7,5
10,0
12,5
min
6,661
7,192
7,651
7,775
7,976
8,196
8,547
8,717
8,988
9,193
9,280

(2E,5Z)-5-(3-methoxybenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3p**)

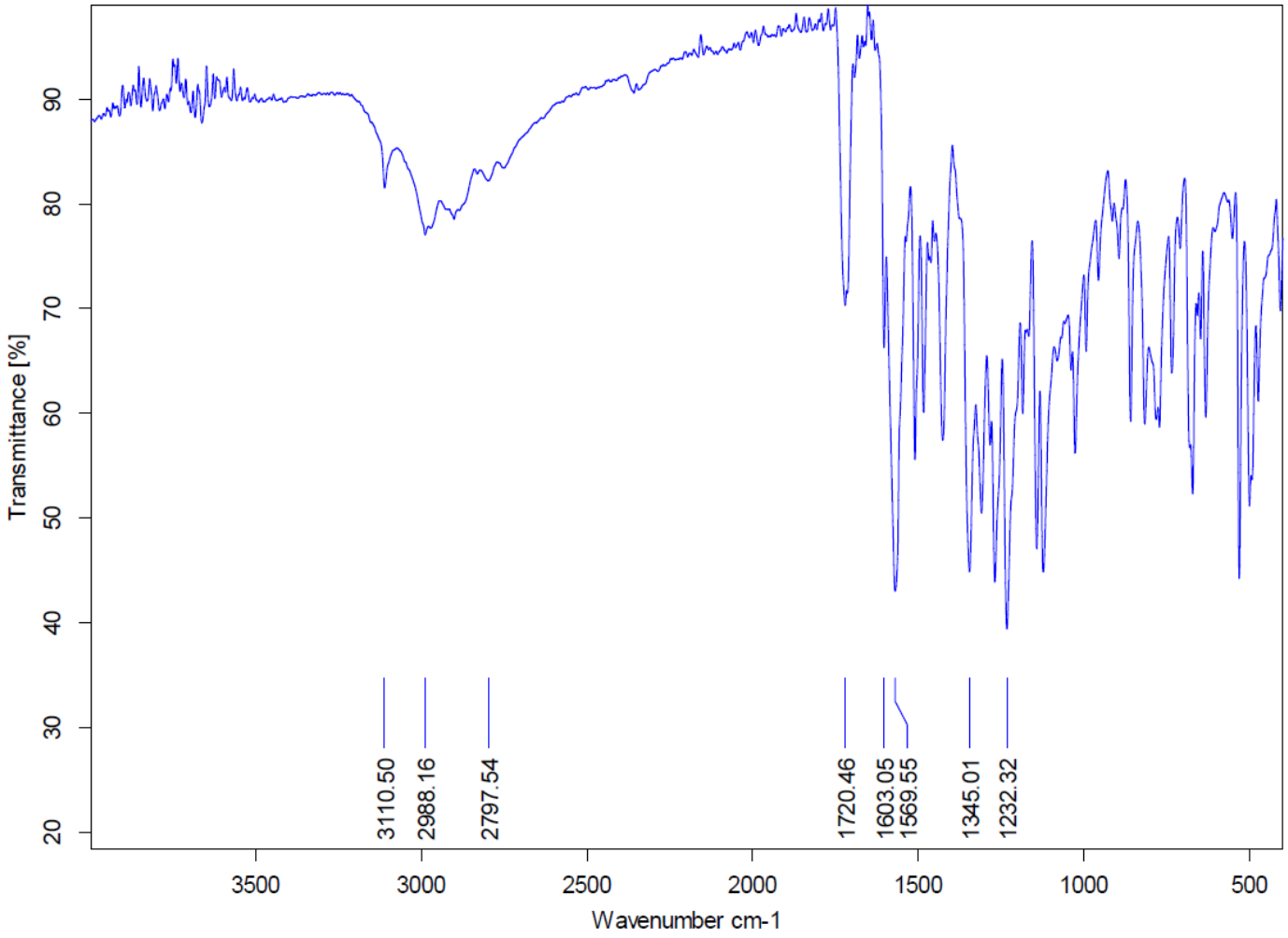
^{1}H NMR



^{13}C NMR



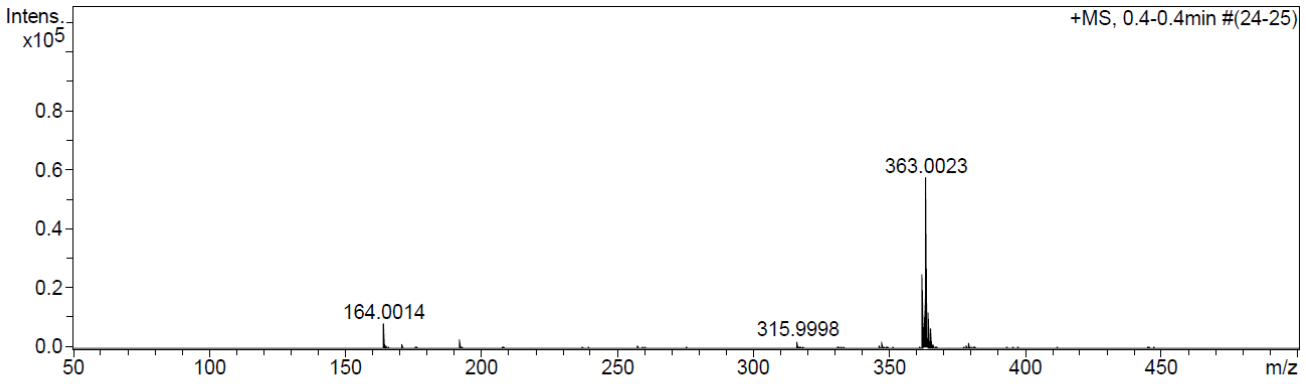
FTIR



HRMS

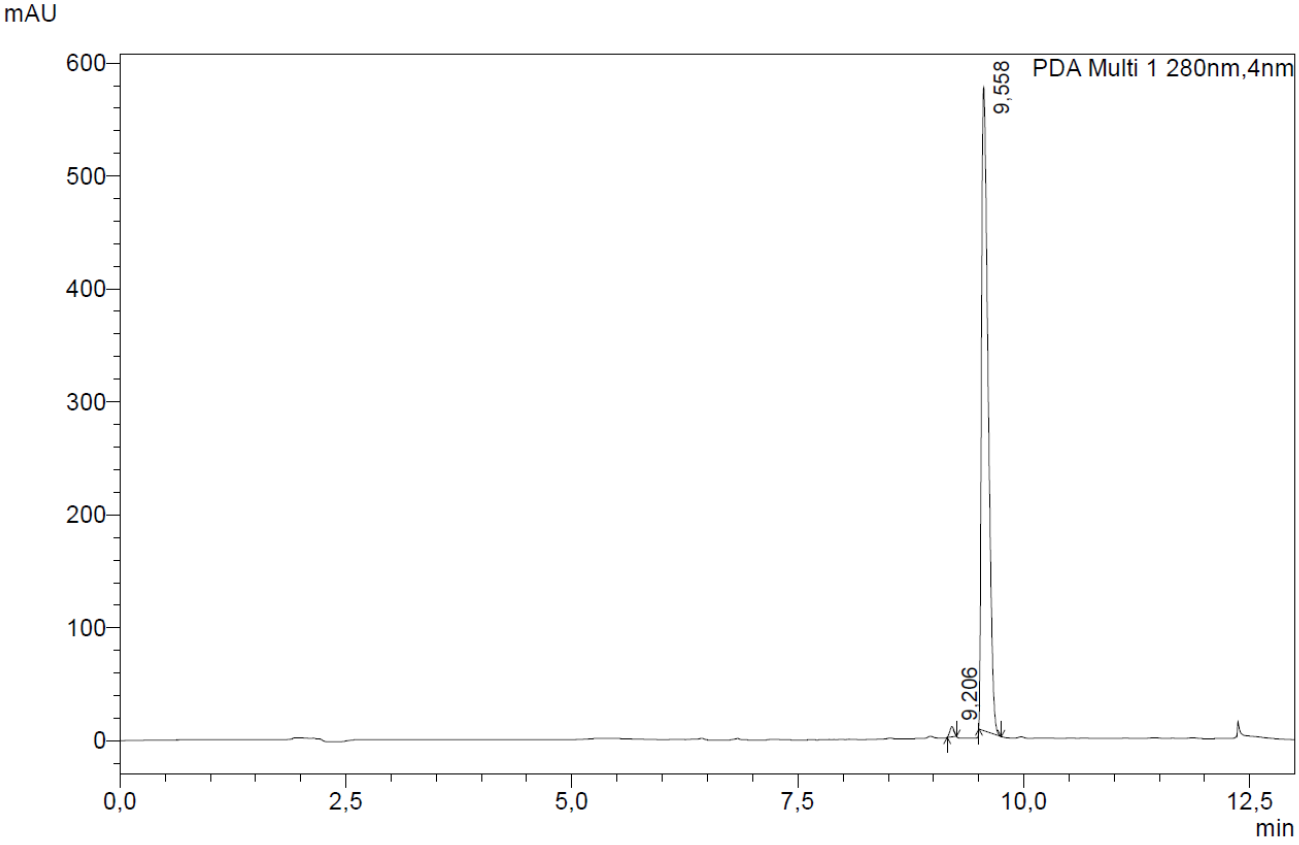
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



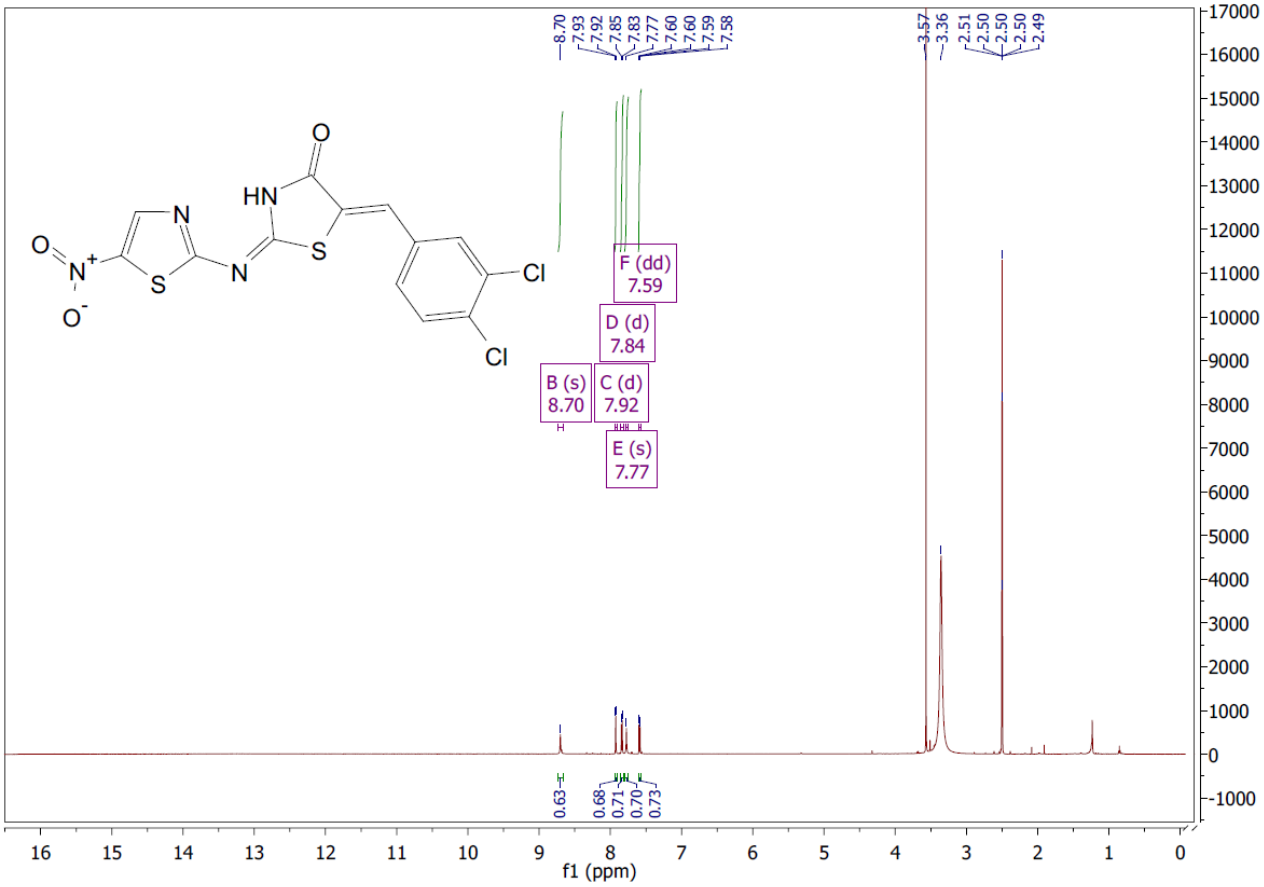
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
363.0023	1	C 14 H 11 N 4 O 2 S 3	100.00	363.0039	1.5	4.3	21.4	11.5	even	ok

HPLC

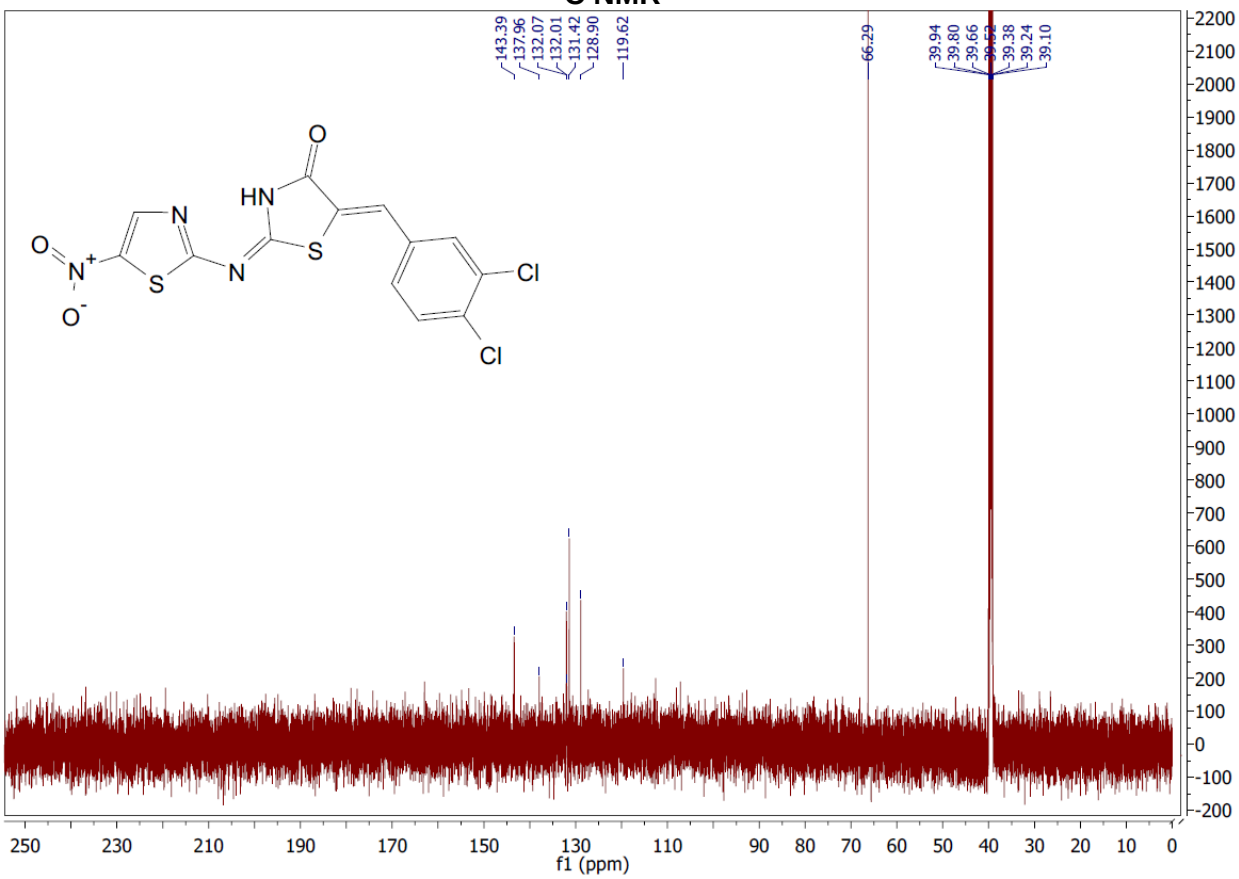

mAU
600
500
400
300
200
100
0
PDA Multi 1 280nm,4nm
9,558
9,206
0,0
2,5
5,0
7,5
10,0
12,5
min

(2E,5Z)-5-(3,4-dichlorobenzylidene)-2-((5-nitrothiazol-2-yl)imino)thiazolidin-4-one (**3q**)

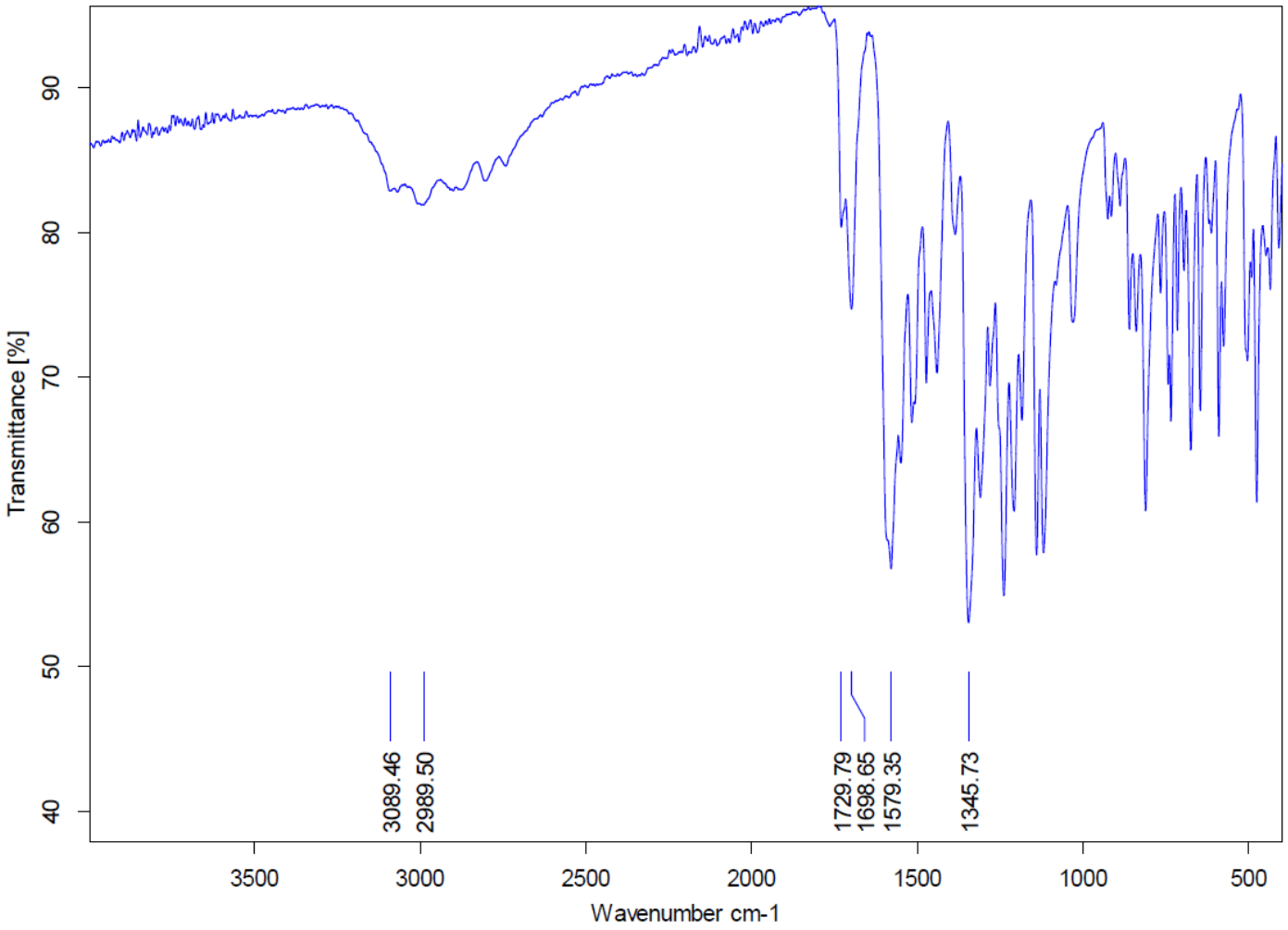
^{1}H NMR



^{13}C NMR



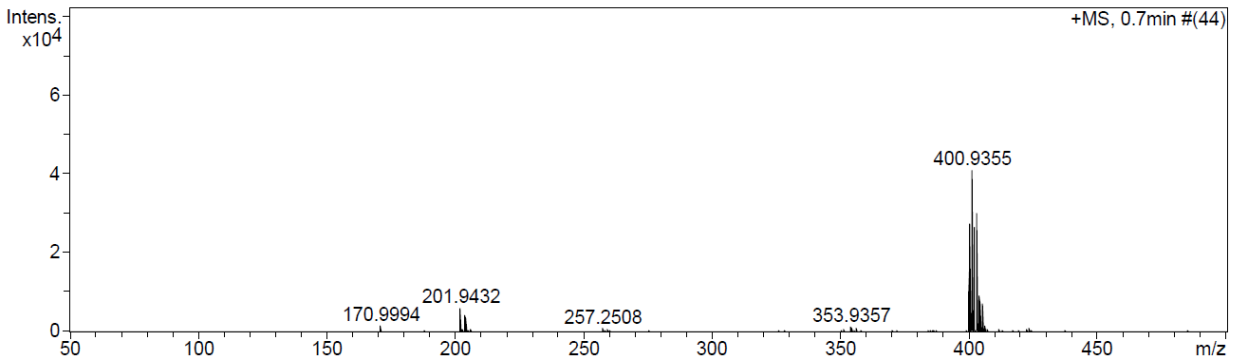
FTIR



HRMS

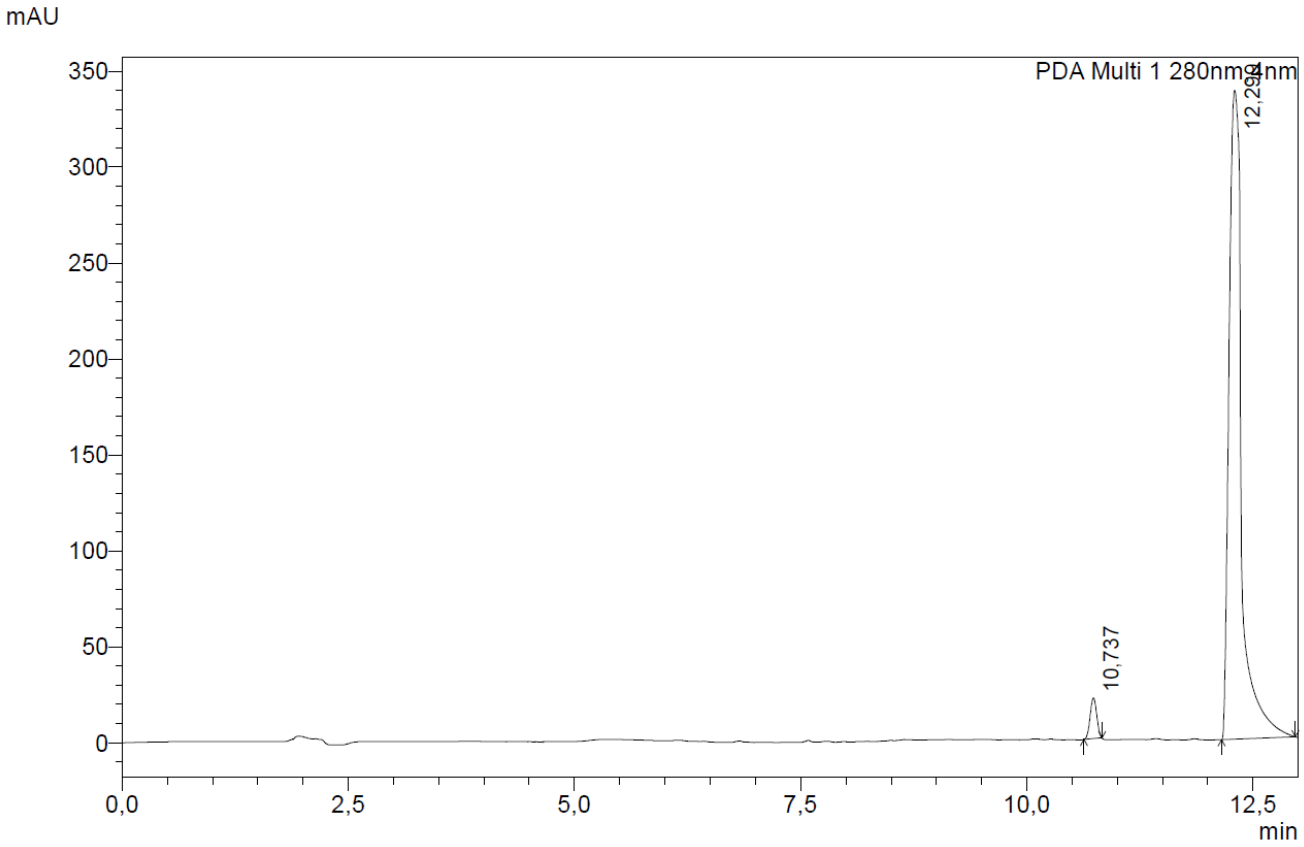
Acquisition Parameter

Source Type	APCI	Ion Polarity	Positive	Set Nebulizer	1.8 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	8.0 l/min
Scan End	1600 m/z	Set Collision Cell RF	150.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
399.9286	1	C 13 H 6 Cl 2 N 4 O 3 S 2	100.00	399.9253	-3.3	-8.3	414.1	12.0	odd	ok
400.9355	1	C 13 H 7 Cl 2 N 4 O 3 S 2	100.00	400.9331	-2.4	-5.9	182.9	11.5	even	ok

HPLC


mAU
PDA Multi 1 280nm,4nm
12,294
10,737
350
300
250
200
150
100
50
0
0,0
2,5
5,0
7,5
10,0
12,5
min

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Licensed Content Date	Apr 23, 2020
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Institution name	North-West University, Potchefstroom
Expected presentation date	Jan 2023
Order reference number	DHFIG2
Portions	Fig 2 - Keto/enol and amine/imine
Requestor Location	Pharmacen 6 Elephant Walk Richards Bay, Kwa-Zulu-Natal 3900 South Africa Attn: Dylan Hart
Total	0.00 USD
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