Synthesis and antitubercular activity of triazole-linked 1,4-benzoquinone derivatives

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PREFACE

This thesis is submitted in article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University.

Chapter 3: Article for submission

Synthesis and antitubercular activity of triazole-linked 1,4-benzoquinone derivatives

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ABSTRACT

Tuberculosis (TB) has scourged humankind for hundreds of years. Not only are millions of people infected and killed by this disease annually, but more than a quarter of the world's population is living with latent TB. *Mycobacterium tuberculosis* (*Mtb*), the causative pathogen of TB, is effortlessly spread when a person with the active diseases coughs, spits, sings or sneezes, propelling the pathogen into the air. TB not only affected ten million people in 2017, but proved fatal to 1.6 million infected that same year, of which 0.3 million were co-infected with human immunodeficiency virus (HIV) making TB the leading killer of HIV-positive people.

TB disease is in fact curable, but it is the rise of multi- and extensively- drug-resistant strains of *Mtb* that renders the control and effective treatment of the disease challenging. Currently only 55 % of multidrug-resistant TB patients are treated successfully and, to make matters worse, second-line chemotherapy options used to treat these cases are not only expensive and toxic, but also extensive. Extensive regimens create an opening for various other disadvantages, such as patient non-adherence and therefore treatment failure and relapse. This can in turn lead to the emergence of drug. There is, therefore, an urgent need for novel effective and affordable anti-mycobacterial agents with better safety profiles to curb TB more efficiently.

In search for such agents a series of eleven novel hybrids linking directly hydroquinone and triazole moieties were investigated. The series was synthesised in a two-step process, starting with nucleophilic substitution SN2 reaction of commercial benzoquinone with sodium azide in acidic medium to form an azido intermediate. This was followed by Huisgen's copper-catalysed alkyne-azide cycloaddition 'click' chemistry of the intermediate with various alkynes to afford targeted hybrids in low to good yields (23 – 70 %). Routine characterisation techniques such as infrared spectrometry, nuclear magnetic resonance, and high resolution mass spectrometry, were used to confirm the structures of the hybrids. The purities were determined by means of high performance liquid chromatography and were found to be in the 92 – 98 % range.

The anti-mycobacterial activity of the hybrids was assessed *in vitro* against the human virulent H37Rv strain of *Mtb*. Cytotoxicity of the synthesised hybrids were evaluated using human embryonal kidney cells (HEK-293).

In general, the hybrids were nontoxic to the mammalian cells, but were either inactive or possessed poor anti-mycobacterial activity. Hybrid **14**, featuring a thiobenzyl substituent on the triazole ring and with cLogP 3.03, was the most active of all. It possessed MIC_{90} 16 µM and showed no toxicity to kidney cells, but was poorly selective for mycobacteria with a selectivity index, SI = 6, which disqualifies it as a potential anti-mycobacterial hit.

A leading explanation to the overall insignificant anti-mycobacterial activities of these hybrids could be attributed to their structural rigidity conferred by the lack of linker between the quinol and triazole rings. It is this rigidity that obstructs the passage of the hybrids through the bacterium cell wall, thus preventing them from reaching the targeted site within *Mtb*. The impact of the linker on the biological activity may be elucidated through future investigation of flexible hybrids.

Keywords: Tuberculosis, *Mycobacterium tuberculosis*, hybridisation, hydroquinone, triazole, click-chemistry

ABBREVIATIONS

ahpC	Alkyl hydroperoxide reductase C
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
APCI	Atmospheric pressure chemical ionisation
BCG	Bacillus Calmette-Guérin
bp	Base pair
BQ	Benzoquinone
CAS	Casitone
CFP-10	Culture filtrate protein 10
CMI	Cell-mediated immunity
CNS	Central Nervous System
CT	Computer tomography
CuCAAC	Copper catalysed alkyne-azide cycloaddition
CXR	Chest x-rays
DCM	Dichloromethane
DHFR	Dihydrofolate reductase
DHFS	Dihydrofolate synthase
DHPS	Dihydropteroate synthse
DMSO	Dimethyl sulfoxide
DMSO-d_6	Dimethyl sulfoxide-d_6
DNA	Deoxyribonucleic acid
ECG	Electrocardiogram
EMA	European Medicines Agency
EMB	Ethambutol
EPTB	Extrapulmonary tuberculosis
ESTAT-6	Early secretory antigenic target-6
FDA	Food and Drug Administration

FQ	Fluoroquinolones
GFP	Green florescent protein
GLU	Glucose
H37Rv	Virulent culture strain of *Mycobacterium tuberculosis*
HEK-293	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
HQ	Hydroquinone
I-A09	Benzofuran salicylic acid
IC_{50}	50 % inhibitory concentration
IFN-y	Interferon-gamma
IGRA	Interferon-gamma release assay
INH	Isoniazid
inhA	Enoyl-acyl carrier protein reductase
IR	Infrared
kasA	3-oxoacyl-[acyl-carrier-protein] synthase 1
KatG	*Mycobacterium tuberculosis* catalase-peroxidase
LAM	Lipoarabinomannan
LM	Lipomannan
LPA	Line probe assay
LTBI	Latent tuberculosis infection
MA	Mycolic acid
mAGP	Mycolyl-arabinoglactan
MDR-TB	Multi-drug resistant tuberculosis
MHWI	Ministry of Health, Welfare and Labor
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA

Mta	*Mycobacterium avium*
Mts	*Mycobacterium smegmatis*
Mtb	*Mycobacterium tuberculosis*
NAA	Nucleic acid amplification
NaAsc	Sodium ascorbate
NADH	Nicotinamide adenine dinucleotide
NADH-2	Nicotinamide adenine dinucleotide dehydrogenase
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
PABA	*p*-aminobenzoic acid
PAS	*p*-aminosalicylic acid
PCR	Polymerase chain reaction
PIMs	Phosphatidylinositol mannosides
POA	Pyrazinoic acid
PPD	Purified protein derivative
ppm	Parts per million
PYZ	Pyrazinamide
QFT-GIT	QuantiFERON-TB Gold in-tube test
QT interval	Distance between the start of the Q wave and end of the T wave on the heart's electrical cycle
RF	Radio frequency
RibD	Riboflavin biosynthesis protein
RIF	Rifampicin
RNA	Ribonucleic acid
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
rpoB	RNA polymerase
rRNA	Ribose RNA
rt	Room temperature

SA	South Africa
SAR	Structure activity relationship
SEM	Standard error of the means
SI	Selectivity index
Sol	Solute
SSM	Sputum smear microscopy
TB	Tuberculosis
THF	Tetrahydrofuran
TLC	Thin layer chromatography
tRNA	Transfer RNA
T-spot	T-SPOT TB test
TST	Tuberculin skin test
Tx	Tyloxapol
WHO	World Health Organisation
XDR-TB	Extensively drug-resistant tuberculosis

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CHAPTER 1:

INTRODUCTION

1.1 Introduction and literature background

Tuberculosis (TB) is an infectious disease caused by the *Mycobacterium tuberculosis* (*Mtb*) bacterium, one of the world's most lethal infections to humans (Davies & Quah, 2017). It spreads from person to person when an individual with the active respiratory disease coughs or sneezes, expelling droplets containing the bacterium into the air (CDC, 2018*a*). The lungs (pulmonary TB) and other parts of the body (extrapulmonary TB) are typically affected by the bacterium (WHO, 2018*a*). However, only 5 – 15 % of the estimated two – three billion people infected essentially develop TB throughout their lifetime, because a healthy individual's immune system acts to separate or "wall off" (WHO, 2015) the bacteria (WHO, 2018*a*).

In 2017 alone, an estimated ten million people contracted TB globally, with 1.7 billion people (23 %) of the world's population infected with latent TB (WHO, 2018*a*). Eight countries accounted for two-thirds of the total number of new incidences, with India having the largest number of incidences and South Africa the eighth largest (WHO, 2018*b*). TB is the leading killer of human immunodeficiency virus (HIV)-positive people, with approximately 0.3 million people dying of HIV-associated TB in 2017. HIV-positive patients are 20 – 30 times more likely to develop active TB. Without proper treatment, nearly all HIV-positive people co-infected with TB pass away, compared to only 45 % of HIV-negative people succumbing to the disease without proper treatment. In 2017 there were approximately 0.9 million new cases of TB amongst HIV-positive people, of which seventy-two percent (72 %) were living in Africa (WHO, 2018*b*).

In order to end the global TB epidemic, the World Health Organisation (WHO) established an "End TB Strategy" that aims to reduce the absolute number of TB incidences and related deaths identified in 2015 by 90 % and 95 %, respectively, by the year 2035 (WHO, 2018*a*). Despite the steady annual decline in incidence, WHO still reported a global mortality of 1.6 million people in 2017 due to TB (WHO, 2018*b*). This puts TB, along with HIV-acquired immunodeficiency syndrome (AIDS), as the leading causes of death from a single infectious disease (Chetty *et al.*, 2017).

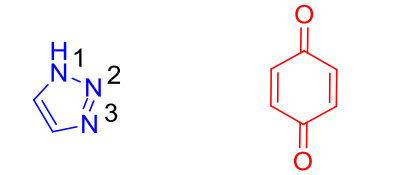
A growing problem in the treatment of TB is drug resistance, and it is threatening to return civilization to an era where the diagnosis of TB was a death sentence (Goldberg *et al.*, 2012). WHO estimates that there were 558 000 new cases of rifampicin resistance in 2017, of which 82 % were multidrug-resistant TB (MDR-TB) and about 8.5 % of MDR-TB cases were extensively drug resistant (WHO, 2018*b*).

The treatment of TB is divided into two phases, namely the intensive and the continuation phase. The intensive phase lasts two months, and consists of a four-drug regimen (Nahid & Hopewell, 2008; CDC, 2018*b*), namely isoniazid, rifampicin, pyrazinamide, and ethambutol, that rapidly kill the tubercle bacilli. The continuation phase of TB treatment follows the intensive, lasts four – seven months and consists of only two drugs, namely isoniazid and rifampicin. These drugs are bactericidal, eliminating the remaining bacilli and, in so doing, prevents relapse of the disease (DoHSA, 2014; CDC,2018*b*). TB remains a global emergency (Zumla *et al.*, 2013) and significant challenges exist with the current therapy. Treatment interruptions and/or changes to current regimens are often needed due to the development of drug intolerance, drug toxicities, and pharmacokinetic drug-drug interactions, especially in TB patients co-infected with HIV. The long-lasting six-month treatment period has a grave effect on patient adherence (Zumla *et al.*, 2013), and the emergence of drug-resistant TB strains further complicate therapy (Sandgren *et al.*, 2009).

Spontaneous and random mutations in the bacterial chromosome of *Mtb* lead to the emergence of MDR-TB and extensively drug-resistant TB (XDR-TB) (Nachega & Chaisson, 2003). MDR-TB refers to resistance to at least rifampicin and isoniazid, the two most effective anti-TB drugs (WHO, 2018*c*). XDR-TB refers to MDR-TB with further resistance to any of the fluoroquinolones and at least one of the three injectable second-line anti-TB drugs (WHO, 2018*d*). The rise of drug-resistant TB is undermining the control over the treatment of TB and it is, therefore, crucial to develop new drugs and treatment regimens (Bark *et al.*, 2011).

An established strategy in the discovery of new drugs is the molecular hybridisation of two or more pharmacologically active scaffolds. The hybridisation strategy entails the linking of two different pharmacophores, with different biological functions, that do not necessarily act on the same biological target. This results in a molecule with a dual mode of action that can kill multi-resistant strains (Meunier, 2007).

This study investigates whether the molecular hybridisation of 1,2,3-triazole and 1,4-benzoquinone pharmacophores (Figure 1.1) via copper-catalysed azide-alkyne cycloaddition (CuCAAC), will result in hybrid molecules that may be effective against *Mtb*.

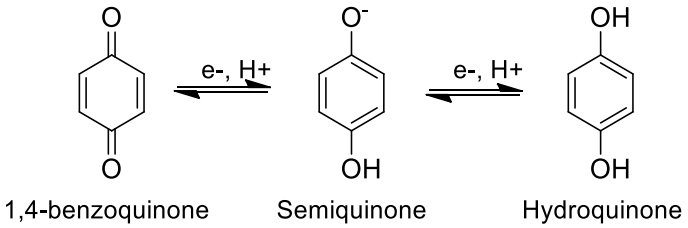


1,2,3-triazole 1,4-benzoquinone

Figure 1-1: Structures of 1,2,3-triazole and 1,4-benzoquinone scaffolds.

Significant development has been made in 1,2,3-triazole derivative research due to the wide range biological properties this moiety endows. These include anti-HIV (Gill *et al*., 2008), anti-mycobacterial (Gill *et* al., 2008; Boechat *et al.*, 2011; Dixit *et al.*, 2016), and anti-inflammatory (Costa *et al*., 2006) activity, as well as the inhibition of histidine biosynthesis (Gill *et al*., 2008). Examples of such triazole derivatives include; 2-(3-fluoro-phenyl)-1-[1-(substituted-phenyl)-1-H-[1,2,3]-triazol-4-yl-methyl)-1*H*-benzo[d] imidazole and N-substituted-phenyl-1,2,3-triazole-4-carbaldehydes derivatives. 1,2,3-Triazoles are also used in agrochemicals as fungicides and plant growth regulators as well as in dyes, corrosion inhibitors and photostabilisers in industrial applications (Gill *et al.*, 2008).

The partner pharmacophore to 1,2,3-triazole in this study is 1,4-benzoquinone (*p*-benzoquinone, BQ). Quinones, the class of compounds to which BQ belongs, also display broad pharmaceutical applications, i.e. antifungal (Tasdemir *et al.*, 2006), antimalarial (Tran *et* al., 2004; Tasdemir *et al.*, 2006), anticancer (Tasdemir *et al.*, 2006) and broad-spectrum anti-bacterial agents (Tran *et* al., 2004; Tasdemir *et al.*, 2006). Examples of such quinones include; plumbagin, juglone and primin. However, various published reports assert a constant interconversion between hydroquinone (HQ) and BQ (Scheme 1.1) in aqueous medium. The reaction is orientated more to the production of HQ in an acidic environment, and in the presence of a complete microsomal system (Souček *et al.*, 2000; McGregor, 2007; HCotN, 2012). By taking the effect that environmental conditions play on the generation of either HQ or BQ into account, investigation followed the synthesis of 1,4-benzoquinone/hydroquinone-1,2,3-triazole derivatives.



Scheme 1-1: Interconversion of *p*-benzoquinone and hydroquinone.

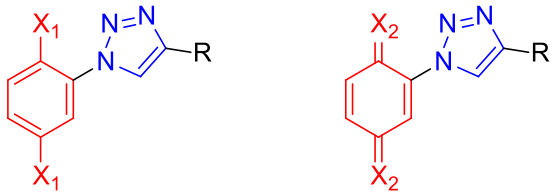
Limited research has been conducted on the anti-TB activity of BQ- and HQ-derived synthetic compounds, BQ/HQ-linked 1,2,3-triazole molecules in particular. However, the limited research reports that both BQ and 1,2,3-triazole containing compounds are biologically effective against *Mtb* (Tran *et al.*, 2004; Gill *et al.*, 2008; Boechat *et al.*, 2014). This allows one to hypothesise that chemically linking the two pharmacophores might result in the development of a new hybrid molecule that has improved efficacy against TB in comparison to current available anti-TB medicine.

1.2 Aim and objectives

The aim of this study is to develop novel molecular entities, via the molecular hybridisation of 1,4-benzoquinone and various substituted 1,2,3-triazole moieties, that may have enhanced effectiveness against *Mtb* and improved safety profiles in comparison to the current existing drugs used in the treatment of TB.

The objectives of this study are:

- To synthesise a series of novel benzo/hydroquinone-triazole hybrids with the general structure depicted in Figure 1.2.



where X_1 is = OH and X_2 = O,
and R is alkyl, aryl etc.

Figure 1-2: General structure of target hydroquinone- and benzoquinone-triazole hybrids.

- To characterise the synthesised compounds by means of routine techniques such as Nuclear magnetic resonance (NMR), Mass spectrometry (MS) and Infrared (IR) spectroscopy.

- To assess the *in vitro* cytotoxicity of the synthesised compounds using mammalian cell lines.

- To assess the *in vitro* anti-tubercular activity of the synthesised compounds against the MDR-TB strain, *Mtb* H37Rv strain.

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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Tuberculosis (TB) is a disease that has been around for hundreds of years, plaguing humankind through history and human prehistory. *Mtb* may have resulted in more deaths than any other pathogen (Daniel, 2006). In 1944 the search to find a cure for TB finally ended with the discovery of streptomycin. A few years later, TB became a treatable disease with the introduction of more effective drugs including isoniazid (INH) (1952) and pyrazinamide (PZA) (1952) (Zhang, 2005). However, despite over 60 years of anti-TB chemotherapy, millions of new cases of active TB are still registered each year, with nearly a quarter of the human population living with latent TB (Gomez & McKinney, 2004; WHO, 2018*a*).

The rise of drug-resistant strains of TB has made the treatment of TB virtually untreatable. The treatment of drug-susceptible TB is already a lengthy and complex process that is further complicated with the appearance of multidrug-resistant strains of *Mtb*. Inappropriate management of drug-resistant TB could have life-threatening results since many of the second-line drugs have toxic side effects. Drug-resistant TB should always be managed by direct observation or close consultation (CDC, 2018*a*) that may result in infection of healthier individuals in countries equipped with poor health care facilities. Statistics show that 82 % of the 558 000 new cases of TB reported in 2017, were multidrug-resistant and that only 55 % of these cases were successfully treated (WHO, 2018*a*). The need for new and effective anti-mycobacterial drugs has been one of the main driving forces pushing research to find novel strategies in drug development in the struggle against TB (Bark *et al.*, 2011).

In this chapter, current TB statistics, treatment control and drug resistance, as well as the challenges that drug-resistant TB bring to TB chemotherapy will be discussed. Chapter 2 will also discuss the strategy this study will embark on for the discovery of novel anti-TB drugs.

2.2 Epidemiology of tuberculosis

TB has been the leading cause of human deaths from a single infectious disease for the last five years, ranking above AIDS (WHO, 2018*b*). Not only is 23 % of the world's population infected with latent TB, but an estimated ten million new people fell ill with TB in 2017. In that year 1.6 million (including 0.3 million co-infected with HIV) succumbed to the disease (WHO, 2018*a*). Of the ten million new cases 5.8 million were men, 3.2 million were women, and one million were children (WHO, 2018*b*). The two-fold difference between males and females affected may

suggest that TB is primarily a disease of men and/or that the epidemiological differences between man and woman may play a role in both exposure to infection and in susceptibility to develop the active disease (Dye, 2006; WHO, 2018*b*). Eight countries accounted for two thirds of all the TB cases reported worldwide, with India leading the count, followed by China, Indonesia, Philippines, Pakistan, Nigeria, Bangladesh and South Africa (Figure 2.1) (WHO, 2018*a*).

However, when comparing statistics per 100 000 population Pakistan, South Africa and Mozambique have a much higher TB incidence rate (± 0.005 %) compared to India and China (0.002 and 0.0006 %, respectively.) (WHO, 2018*b*).

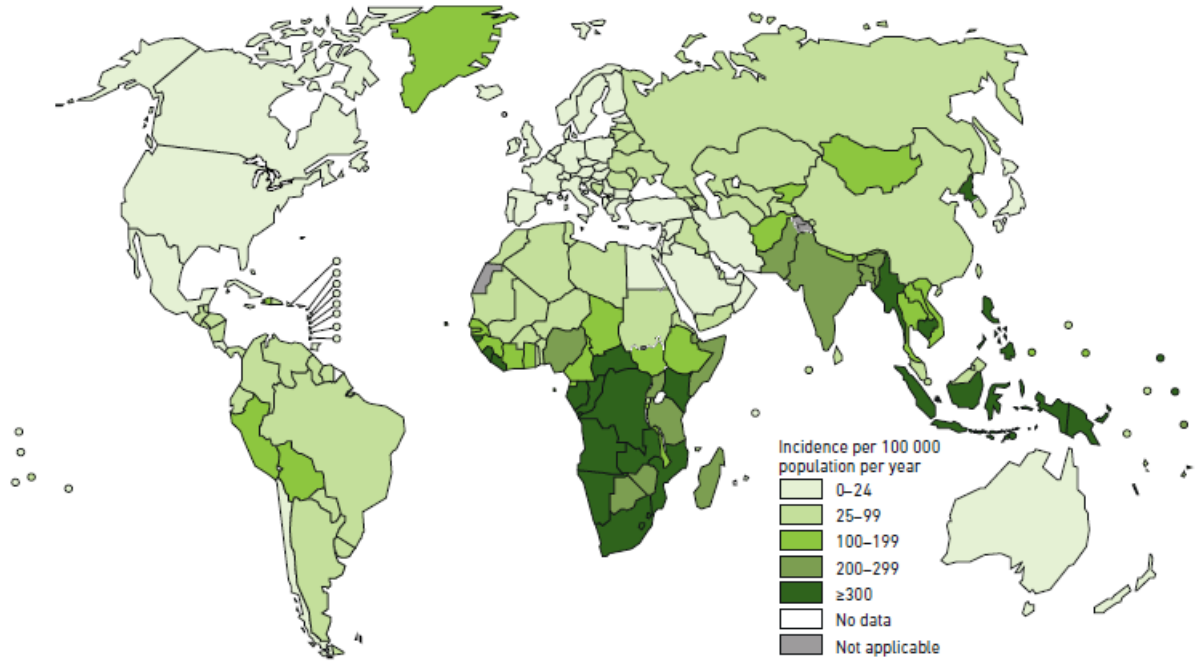

Figure 2-1: Global tuberculosis prevalence, 2017(WHO, 2018*b*).

Poverty stricken regions are the most affected by TB; with the most estimated cases occurring in the World Health Organisation (WHO) South-East Asia (44 %), African Region (25 %), and Western Pacific (18 %) regions in 2017. Smaller proportions of cases were registered in the WHO Eastern Mediterranean (7.7 %), Americas (2.8 %) and European region (2.7 %). The WHO African Region also had the highest number of TB cases co-infected with HIV, with parts of southern Africa exceeding 50 % (WHO, 2018*b*).

The “End TB Strategy” was established by WHO with the aim to end the global TB epidemic by 2035. The 2035 targets are a 95 % and 90% reduction in TB mortality and TB incidence rate, respectively. Interim milestones set for 2020 are a 35 % and 20 % reduction in TB mortality and TB incidence rate, respectively, compared with levels in 2015. From 2000 to 2017, there has been an estimated three percent annual decline in TB incidences. However, to reach the first (2020)

milestone of the “End TB Strategy” the rate of annual decline needs to be accelerated to four – five percent (WHO, 2018*a*; WHO, 2018*b*).

The WHO European and African Regions had the fastest decline in TB incidence with five and four percent per year, respectively from 2013 – 2017. The areas with the fastest decline in mortality rate were the WHO European and South-East Asia Regions with 11 % and 4.3 %, respectively, since 2013 and WHO African Region had the slowest rate of decline at 1.7 % per year (WHO, 2018*b*).

Drug resistance is undermining the control over TB. In 2017 there was an estimate of 558 000 new cases of rifampicin (RIF) resistance, of which 82 % had MDR-TB and 8.5 % of all MDR-TB cases had XDR-TB (WHO, 2018*a*). MDR-TB is defined as TB that is resistant to the two most effective first-line drugs, i.e. INH and RIF (WHO, 2018*c*). XDR-TB is defined as MDR-TB that is resistant to any fluoroquinolone, and at least one of the three injectable second-line drugs; amikacin, capreomycin, or kanamycin (WHO, 2018*e*). Drug-resistant TB is a growing problem that must be dealt with, or it will threaten to send civilization back into an era where the positive diagnosis of TB is a death sentence (Goldberg *et al.*, 2012). However, despite the fact that the 2018 WHO Global Tuberculosis Report revealed a high number of new TB incidences, the data also showed that the global mortality rate of TB had decreased overall by 42 % between the years 2000 and 2017 (WHO, 2018*b*).

2.3 Tuberculosis in South Africa

TB remains a major health problem in South Africa (SA). SA is one of the top 30 high TB burden countries, with an annual TB incidence of over 500 per 100 000 population (WHO, 2018*b*).TB is also the leading cause of death in the country (Kanabus, 2018*a*). In 2015 the Eastern Cape was recorded to have the highest incidence rate in the country with 692 per 100 000, followed by KwaZulu-Natal, and Western Cape with 685 and 681 per 100 000, respectively. The KwaZulu-Natal incidence rate has decreased over the last five years from 1 185 to 685 per 100 000. The average rate of TB/HIV co-infection in 2015 across SA was 56.7 %, with Gauteng having the highest number of co-infections at 68.4 % (Massyn *et al.*, 2016).

To control the rate of TB incidences in SA, four key aspects have been identified and need to be prioritised:(1) improve the cure rate of TB to ensure an interruption in the transmission of the disease; (2) improve the case detection rate of TB to ensure that fewer cases remain undiagnosed in the community to infect healthy individuals; (3) integrate TB and HIV services to ensure that 90 % of HIV-positive patients are screened for active TB and 90 % of TB patients are offered an HIV test (4) improve the identification and treatment of drug-resistant TB (Karim *et al.*, 2009).

2.4 Transmission and pathology of tuberculosis

2.4.1 Transmission

Mtb, the causative pathogen of TB, is transmitted inter-individually through the air by small droplet nuclei that can stay in the air for several hours (Russell *et al.*, 2010). These droplets are spread when a person with pulmonary of laryngeal TB coughs, sneezes, spits or sings, propelling the pathogen in to the air, causing people in the surrounding area to inhale the bacteria and become infected (CDC, 2018*b*; WHO, 2018*a*). Only 5 – 15 % of infected people develop the active disease, though patients with compromised immune systems are at a much higher risk of developing the active disease (WHO, 2018*a*).

2.4.2 *Mycobacterium tuberculosis* bacterium description and cell wall

Mtb is a rod-shaped, non-spore-forming, aerobic bacterium that is classified as a, acid-fast bacillus. It characteristically measures at 0.5 – 3 µm and has a unique, well developed and lipid rich cell wall structure that is fundamental to its survival (Glickman & Jacobs, 2001; Knechel, 2009). *Mtb* is visualised by acid-fast (Ziehl-Neelsen) staining due to the lipid rich cell wall which is capable of retaining carbol fuchsin dye, even in the presence of acidic alcohol (Glickman & Jacobs, 2001; Gengenbacher & Kaufmann, 2012).

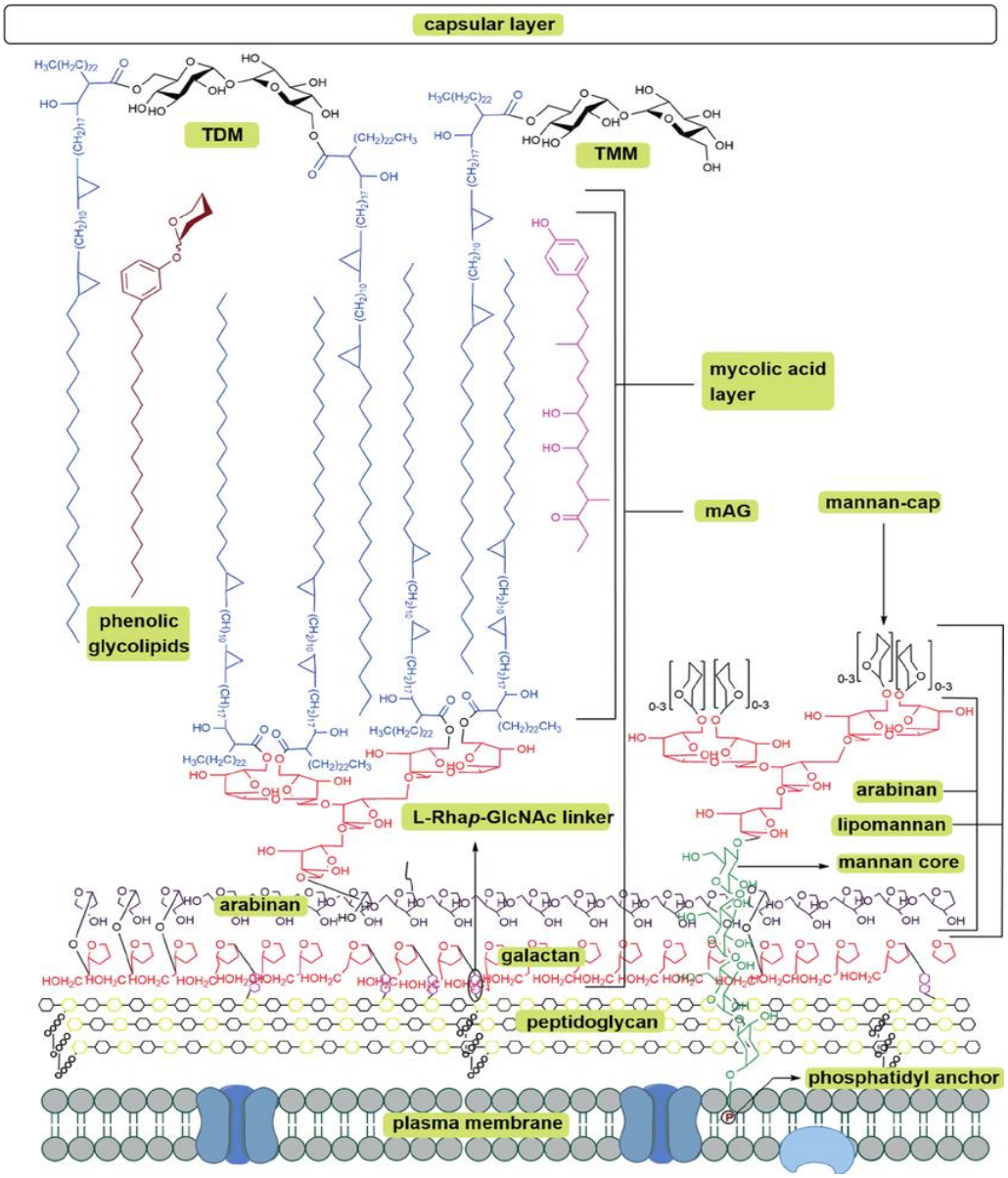

Figure 2-2: Schematic representation of *Mycobacterium tuberculosis* cell wall. mAG = mycolyl-arabinogalactan, AG = arabinogalactan, TMM = trehalose monomycolate, TDM = trehalose dimycolate (Thanna & Sucheck, 2016).

Mtb's cell wall is divided into upper and lower segments. The lower segment, termed "cell wall core", consists of a mycolyl-arabinogalactan-peptidoglycan (mAGP) complex. Here mycolic acid (MA), a long chain fatty acid, is covalently attached to the underlying peptidoglycan-bound polysaccharide arabinogalactan, generating an effective lipid barrier (Figure 2.2) (Knechel, 2009). The upper segment is composed of free lipids and scattered cell wall components including phosphatidylinositol mannosides (PIMs), phthiocerol containing lipid, lipomannan (LM), and lipoarabinomannan (LAM) (Brennan, 2003). LAM is immunogenic and facilitates the survival of the bacterium within the macrophage. The architectural arrangement of the upper segment increases the bacilli's resistance to degradation by host enzymes, its impermeability to toxic macromolecules, and the inactivation of reactive oxygen and nitrogen derivatives. (Korf *et al.*,

2005). Disruption of the cell wall leads to the solubilisation of the upper segment (the free lipids, proteins, LAM, and PIMs). The lower segment, the mAGP complex, remains as an insoluble residue that is essential in the viability of the cell (Brennan, 2003).

2.4.3 The life cycle of tuberculosis

The expectorated pathogen-containing droplets are inhaled and carried to the lungs. Due to the small size of the droplet nuclei (1 – 5 µm) the tubercle bacilli are able to reach the alveolar spaces where it replicates (Figure 2.3) (Ahmad, 2010; Wani, 2013). Here the bacteria is ingested by alveolar macrophages and ultimately invade the subtending epithelial layer (Bermudez & Goodman, 1996; Gengenbacher & Kaufmann, 2012). *Mtb* has the ability to persist, survive and replicate in this extreme microbicidal environment of macrophages. The pathogen is able to elude most macrophage effector functions, such as inhibiting phagosome-lysosome fusion by inhibiting the acidification of phagosomes (Hingley-Wilson *et al.*, 2003; Korf *et al.*, 2005). The pathogen retards phagosome maturation (Russell, 1995) and shields itself from toxic oxidative burst caused by reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the macrophages as part of their antimicrobial response (Piddington *et al.*, 2001).

Intracellular replication of the bacteria at the initial pulmonary site of infection, the spread to lymph nodes in the lungs, and the simultaneous dissemination of the infection to extrapulmonary sites in the body occur before the development of the adaptive immune response (Glickman & Jacobs, 2001; Ahmad, 2010). The infected host cells induce a localised pro-inflammatory response that attracts T lymphocytes and mononuclear cells to build up a granuloma, a defining tissue reaction of TB (Gengenbacher & Kaufmann, 2012). At the beginning, the granuloma is an amorphous mass of macrophages, neutrophils and monocytes. The macrophages later differentiate into several specialised cells, namely; foamy- and epithelioid macrophages and multi-nucleated giant cells. The granuloma becomes more organised and stratified after the initiation of an acquired immune response and the arrival of lymphocytes. A mantle of lymphocytes surrounds the macrophage-rich centre that may then later be enclosed in a fibrous cuff that marks the periphery of the structure (Russell *et al.*, 2010).

The granuloma acts to wall off the growing necrotic tissue caused by the pathogen, and in so doing limit the spread and replication of the pathogen (Ahmad, 2010). The immune response can normally eradicate virtually all of the *Mtb* in the caseating granulomas, halting the progression of the disease. However, the pathogen is very rarely completely eradicated as it has evolved to evade the immune response, survive and persist in the host in a non-replicating state (latent TB) (Glickman & Jacobs, 2001; Frieden *et al.*, 2003; Ahmad, 2010).

The host's immune system is, therefore, either able to take successful control over the infection, leading to a latent infection if not all bacteria are eradicated, or is not able to take effective control and the infection progresses to the active disease (primary progressive TB) (Frieden *et al.*, 2003). An effective cell-mediated immunity (CMI), in infected people, usually develops two – eight weeks after infection (Frieden *et al.*, 2003). The *Mtb* bacilli will continue to replicate in the host's system until an effective CMI has been developed. If the host fails to mount an effective CMI and damaged tissue is not repaired, progressive destruction of the lungs will take place (Wani, 2013).

Upon failure of eliminating the infection *Mtb* bacilli proliferate inside the alveolar macrophages, killing the cells. Cytokines and chemokines are produced by the infected macrophages, attracting other phagocytic cells, such as other alveolar macrophages, monocytes and neutrophils. A nodular granulomatous structure, called a tubercle, is eventually formed. If the replication of the pathogen is not controlled the tubercle enlarges and the bacilli enter local draining lymph nodes, causing lymphadenopathy (a prominent characteristic of active TB), and the active disease occurs (Ahmad, 2010; Wani, 2013).

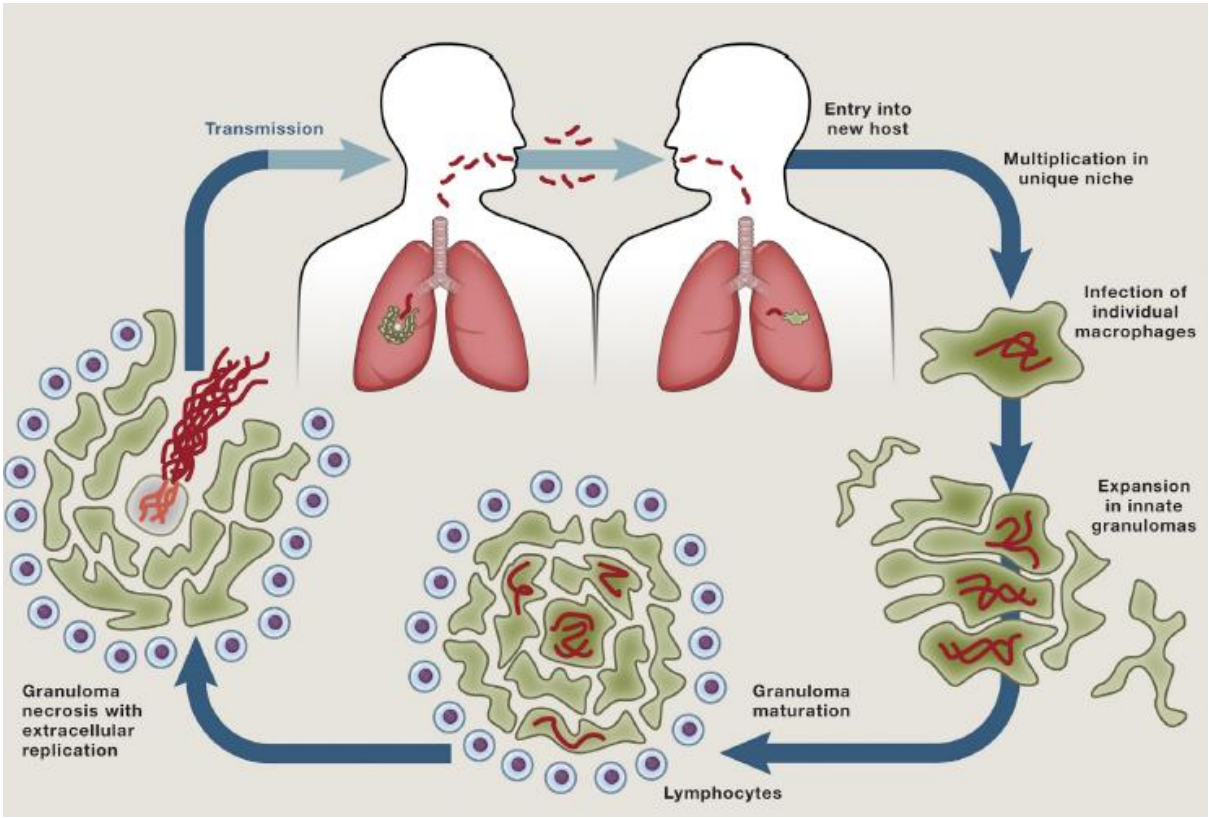

Figure 2-3: Life cycle of *Mycobacterium tuberculosis* (Cambier *et al*., 2014).

2.5 Clinical manifestation of tuberculosis

The development of TB disease depends on the immune system of every patient and the disease may, therefore, present differently in each patient. Each stage of TB also has its own clinical

manifestation (Knechel, 2009), with the most common symptoms being coughing, chest pains, weight loss, fever, weakness, fatigue, malaise and night sweats (ATS, 1999; WHO, 2018*b*). These symptoms are non-specific, which sometimes results in misdiagnosis and/or delayed diagnosis (Babajide & Mukadi Ya, 2006). TB disease associated with the lungs of a patient is referred to as pulmonary TB, while TB disease in any part of the body (*e.g.* the spine, kidneys, or lymph nodes) is classified as extrapulmonary TB (CDC, 2018*c*). The risk of developing extrapulmonary TB increases with immunosuppression (CDC, 2018*d*).

2.5.1 Primary pulmonary tuberculosis

The primary disease essentially exists sub-clinically and is often asymptomatic, with the only evidence of disease being the diagnostic result, and some self-limiting findings such as paratracheal lymphadenopathy and pleural effusion being noticed during an assessment. (Knechel, 2009). As mentioned in paragraph 2.4.1, only 5 – 15 % of infected people develop the active disease, though patients with compromised immune systems are at a much higher risk of developing the active disease (WHO, 2018*a*).

The *Mtb* bacilli spread through the lymphatic system from the lungs, causing paratracheal lymphadenopathy. Pleural effusion may develop due to the bacilli infiltrating the pleural space. When the effusion becomes large enough, it induces symptoms such as pleuritic chest pain, fever, and dyspnea. Affected lung tissue with poor gas exchange causes dyspnea (Knechel, 2009).

As mentioned in paragraph 2.4.3, if the host's immune system is unable to take successful control over the infection the mycobacteria multiply and grow in the host, leading to primary progressive (active) TB. Early stages of the disease are non-specific, with the most common symptoms being; malaise, progressive fatigue, low-grade fever, weight-loss, chills and night sweats (ATS, 1999; Knechel, 2009).

Inflammatory and immune responses caused by pulmonary TB create a lack of appetite and an altered metabolism that results in wasting. Wasting is the loss of both lean and fat tissue, and it is a classic feature of TB (MacAllan *et al.*, 1998; Paton *et al.*, 2004). Another definitive feature of pulmonary TB is a bad cough that lasts for three weeks or more (CDC, 2018*e*). The coughing may at first be non-productive but can develop to a productive cough with purulent sputum that may be streaked with blood (haemoptysis). There are numerous reasons for haemoptysis, including the destruction of a patent vessel positioned in the wall of the cavity, the formation of an aspergilloma in an old cavity, and/or the rupture of a dilated vessel in the cavity. Pleuritic chest pain is attributed to inflamed parenchyma (Knechel, 2009).

2.5.2 Extrapulmonary tuberculosis

Pulmonary TB is the most common form of TB, but when the infection disseminates to other body parts the disease is classified as extrapulmonary TB (EPTB) (Peirse & Houston, 2017). EPTB is, therefore, the infection of any organ by *Mtb*, excluding the lungs. EPTB is caused by the reactivation of a latent infection and the dissemination of the bacteria through the body to various organs. Immunodeficiency (especially in HIV-positive individuals) increases the risk of developing EPTB. Other important risk factors of EPTB include corticosteroids, malignancy, tumour necrosis factor-α antagonists (infliximab), not smoking and female gender (Peirse & Houston, 2017) as opposed to the primary disease being more prominent with the male gender (see paragraph 2.2).

Patients with EPTB have non-specific symptoms, such as anorexia, fever, weight-loss, fatigue, and malaise, and symptoms can differ vastly depending on the affected organ(s) (Sharma & Mohan, 2004). The most severe signs and symptoms of EPTB are those implicating the involvement of the central nervous system (CNS) (Knechel, 2009). Infection of the CNS can result in meningitis (infection of the meninges) or space-occupying lesions (tuberculomas) of the brain (Knechel, 2009). Infection of the blood stream by *Mtb* (disseminated or miliary TB) is another fatal form of EPTB (Knechel, 2009).

Other possible locations for infection EPTB include the bones or joints (the spine being the most common structure affected) (Frieden *et al.*, 2003), genitourinary system (although uncommon, and difficult to distinguish from other genitourinary tract infections) (Frieden *et al.*, 2003), and the lymphatic system (the most common form of EPTB) (Knechel, 2009).

2.5.3 Miliary tuberculosis

Miliary TB develops when the host's immune system becomes suppressed, resulting in the proliferation and dissemination of the organism throughout the body (Sharma *et al.*, 2005). Two methods by which miliary TB can occur include (1) lympho-haematogenous dissemination of the bacteria through the body from an extrapulmonary focus and embolisation to the vascular beds of several organs, and (2) less commonly, the reactivation of several foci in various organs.

Miliary TB accounts for three percent of EPTB and can affect any organ in the body (Babajide & Mukadi Ya, 2006; Peirse & Houston, 2017). The diagnosis of miliary TB is made when diffuse miliary infiltrate is present on high-resolution computer tomography (CT) scans or chest radiographs, or when miliary tubercles are observed in several organs during laparoscopy, autopsy, or open surgery (Sharma *et al.*, 2005). Previously, miliary TB was often only diagnosed during autopsies and it has been revealed that the disease most frequently affects organs with a high blood flow (*e.g.* spleen, bone marrow, lungs, liver, adrenals, and kidneys). The availability of high-resolution CT scans has made it possible to diagnose that condition in living patients. Miliary

TB, affecting almost all organs, is most often asymptomatic or accompanied by protean and non-specific symptoms such as fever, weight-loss, anorexia, coughing, and lethargy. Mental status changes and headaches are more severe forms of symptoms and could suggest meningeal involvement (Sharma *et al.*, 2005).

2.5.4 Latent tuberculosis infection

Latent TB infection (LTBI) occurs when an individual infected with *Mtb* has an immune response controlling the pathogen, forcing it into a dormant state (Parrish *et al.*, 1998). Latent TB individuals have no symptoms and cannot spread the pathogen to others, yet generally have a positive TB blood or skin test reaction (refer to paragraph 2.6). LTBI does not always develop into the active disease as it can remain inside the host, inactive for a lifetime never causing disease (CDC, 2018*d*). A person with latent TB has a 5 –15 % lifetime risk of TB reactivation and the risk increases considerably in the presence of predisposing factors, such as weakened immune system (WHO, 2018*a*), critical illness (Knechel, 2009), HIV co-infection (the greatest risk factor for reactivation) (Frieden *et al.*, 2003), malnutrition, cancer, drug abuse, diabetes, immunosuppressive drug therapy and chronic renal infection (Parrish *et al.*, 1998).

2.6 Diagnosis of tuberculosis

There are several tests that can be used to diagnose TB. However, diagnosing a patient with TB is often difficult, with most tests being inaccurate and/or time consuming. Diagnostic tests for TB vary in specificity (the ability to correctly detect people with TB – leading to false positives), sensitivity (the ability to correctly detect people who do not have TB – leading to false negatives), cost, and speed (Frieden *et al.*, 2003; Kanabus, 2018*b*). The most commonly used tests are discussed in paragraph 2.6.

2.6.1 Tuberculosis sputum smear microscopy

The sputum smear microscopy (SSM) method is still used in many countries as the cornerstone method of TB diagnosis, especially in low to middle-income countries (Dorman, 2010) or in countries where there is a high TB morbidity rate (WHO, 2018*b*). Ziehl-Neelsen, Fluorochrome, and Kinyoun staining methods can all be used in the sputum smear test and these methods are regarded as relatively inexpensive rapid tests (Frieden *et al.*, 2003). The SSM method includes a few limitations, such as: (1) only half of the number of TB cases are accurately detected; (2) all mycobacteria are acid-fast and morphologically similar, making it difficult for technicians to distinguish between non-pathogenic and pathogenic mycobacteria; (3) SSM cannot detect drug-resistant mycobacteria. This method requires trained laboratory technicians to examine sputum samples under a microscope and determine whether the *Mtb* bacteria are present in the samples (Van Deun, 2004; WHO, 2018*b*). A collected sputum smear sample is placed on a microscope

slide and is stained with the primary stain dye, Carbol fuchsin, to detect acid-fast bacteria, which stains red. The sputum smear is then decolourised, using three percent acid-alcohol or 25 % sulphuric acid solutions, and is then treated with a secondary stain, methylene blue that stains non-acid fast bacteria blue. *Mycobacteria* is acid-fast and, therefore, retains the red colour despite the decolouration steps, leading to its identification (Rieder *et al.*, 2007; Dezemon *et al.*, 2014).

2.6.2 Tuberculosis skin test

The Mantoux tuberculin skin test (TST) requires two visits to a health care provider. Upon the first visit the patient receives an intradermal injection, containing a tuberculin-purified protein derivative (PPD), into the lower part of their arm. The patient must return within 48 – 72 hours to examine the reaction on the arm and determine the results of the test (CDC, 2018*f*). The test results depend on the size of the raised, hard area or swelling (erythema is not included when size is measured). If the TST tests positive, it indicates that the patient is infected with *Mtb*, but does not show whether the patient has latent TB or the active disease. False-positives are also high for patients infected with non-TB mycobacteria and people who have had the bacilli Calmette-Guérin (BCG) vaccine. A negative test result suggests that latent TB or active TB disease is highly unlikely. The TST is the preferred TB test for children younger than five years (CDC, 2018*f*).

2.6.3 Tuberculosis Interferon-gamma release assays

There are two TB blood tests available and both are interferon-gamma release assays (IGRAs): the QuantiFERON-TB Gold in-tube test (QFT-GIT) and the T-SPOT TB test (T-spot). IGRAs assess a patient's cell-mediated immune reactivity to *Mtb* and requires the health care provider to take a single draw of blood from the patient. The lymphocytes of most patients infected with *Mtb* release interferon-gamma (IFN-*y*) when the blood is mixed with certain antigens *viz.* culture filtrate protein 10 (CFP-10) and early secretory antigenic target-6 (ESTAT-6) derived from *Mtb* (Mazurek *et al.*, 2010; Belknap & Daley, 2014; CDC, 2018*g*). IGRAs were developed to replace TST, as the antigens used are specific to *Mtb* and are not present in most non-TB mycobacteria or BCG strains (Belknap & Daley, 2014), making this method of diagnosis more specific. Even though these tests were developed as replacement tests, they are not useful when used alone in the diagnosis of active TB in both HIV-negative and positive patients (Ampath, 2012). IGRAs are the preferred TB test for people who have received the TB vaccine (BCG) and people who are not able to return for a second appointment, as is required for the TST (CDC, 2018*g*).

2.6.4 Chest X-rays

Chest abnormalities are identified by using a posterior-anterior chest radiograph. Lesions in the lungs differ in shape, size, cavitation and density and can appear anywhere in the lungs. This

method of diagnosis is very low in specificity, as the appearance of the chest x-rays (CXR) are never typical of TB. They either present as classical (mildly immunocompromised patients) or atypical patterns, especially in the case of severely immunocompromised patients, creating a window for misdiagnosis as many other lung diseases present CXR patterns similar to TB. CXR can also result in an over diagnosis of pulmonary TB because of lung fibrosis/destruction caused by old TB. The sensitivity of CXR is also low for HIV-positive patients, due to the lung cavities being less pronounced (Harries *et al.*, 2005; Van Cleeff *et al.*, 2005; DoHSA, 2014). Regardless of the low specificity and sensitivity of CXR, it is still widely used as a method of diagnosis of pulmonary TB. However, due to the limitations of this method, CXR cannot be used as a definitive diagnosis for TB. It is recommended that further tests be done to increase sensitivity and specificity, such as TST and IGRAs, to ensure a correct diagnosis (du Preez & Loots, 2014; CDC, 2018*d*).

2.6.5 Tuberculosis culture test

A culture test is used to determine whether specific bacteria is present in a patient. The bacteria is grown on different media, either on solid culture plates or in liquid culture broths. TB culture tests are used to determine drug resistance and can also identify *Mycobacterium* complex species other than TB. TB drug resistance is tested by growing the *Mtb* bacteria in the culture medium in the presence of anti-TB drugs (LL, 2016; Kanabus, 2018*b*). If bacterial growth continues, it means that the bacteria is resistant to the drug present in the growing medium. If there is no bacterial growth, there is no drug resistance and the drugs are effective against the bacteria. A large advantage that this diagnosis method has over the other methods is that cultures provide a very definitive and accurate diagnosis of TB, with high sensitivity (80 %) and specificity (98 %) values. Significant disadvantages of this method are that the final results are only obtained after two – six weeks and that it is an expensive procedure, as more sophisticated equipment and laboratory facilities are required (LL, 2016; Kanabus, 2018*b*).

2.6.6 Tuberculosis molecular tests

Molecular TB tests have developed drastically over the last two decades in an effort to improve the early detection of TB and MDR-TB. Two molecular test methods that are currently recognised by the WHO are the Xpert MTB/RIF assay and line probe assays (Noor *et al.*, 2015).

2.6.6.1 Xpert MTB/RIF assay

The Xpert MTB/RIF assay is a fully automated real-time cartridge-based polymerase chain reaction (PCR) test that is able to detect both TB and RIF resistance within 2 hours (Weyer *et al.*, 2013; WHO, 2013). The specificity and sensitivity of Xpert MTB/RIF assays are greater than that of TB culture tests, with a pooled sensitivity of 88 %, and specificity of 99 % (WHO, 2013). Xpert

MTB/RIF is also able to detect RIF resistance with a sensitivity of 95 % and a specificity of 98 % (WHO, 2013). It requires only minimally trained staff and, because the assay is enclosed in a self-enclosed unit, contamination is minimal. However, the equipment used is sensitive and requires protection, and the cost of the assay is also relatively expensive (Boyle & Pai, 2014).

2.6.6.2 Line probe assay

Line probe assays (LPA) were the first TB molecular tests endorsed by the WHO (Noor *et al.*, 2015). Both the LPA and Xpert MTB/RIF assays target the ribonucleic acid (RNA) polymerase (*rpoB)* gene (Rufai *et al.*, 2014). LPA are centred on reverse hybridisation and involves the extraction of deoxyribonucleic acid (DNA), followed by PCR amplification of the *rpoB* gene (WHO, 2008). The PCR products are then hybridised by specific oligonucleotide probes (Rufai *et al.*, 2014; Noor *et al.*, 2015). The sensitivity and specificity of LPA in the detection of RIF resistance are high, with 97 % sensitivity and 99 % specificity, and test results are available rapidly (within 48 hours) (Noor *et al.*, 2015). LPA are able to detect both RIF and INH resistance (DoHSA, 2014). Disadvantages of this method include a higher risk of cross-contamination (open system PCR), the requirement of highly trained and skilled personnel, and the fact that the tests have to be performed in a laboratory with prerequisite biosafety level precautions (WHO, 2008; Noor *et al.*, 2015).

2.6.7 Diagnostic test for drug-resistant tuberculosis

Drug resistance develops spontaneously and at random. It is a growing problem in the treatment of TB disease, rendering strategy for the control of TB difficult. The faster and more accurately drug resistance is identified, the better and more effective treatment a patient can receive (LoBue *et al.*, 2009; Sandgren *et al.*, 2009).

The use of the Xpert MTB/RIF assay (paragraph 2.6.6.1) has expanded considerably in the last seven years. The test can simultaneously detect *Mtb* and RIF resistance within two hours, much faster than the standard two – six weeks taken by conventional diagnostic tests (CDC, 2018*i*; WHO, 2018*a*; WHO, 2018*b*). The Xpert MTB/RIF assay is a nucleic acid amplification (NAA) test, the test is carried out by collecting sputum from a suspected TB patient. The sputum is mixed with the reagent provided with the assay, and this mixture is placed in the GeneXpert machine (CDC, 2018*i*).

The diagnostic test, named MTBDRs1, is the most reliable way to rule out second-line drug resistance. It is a DNA-based test that is able to identify the genetic mutations that made the MDR-TB bacteria resistant to fluoroquinolones and injectable second-line TB drugs. The MTBDRs1 test yields results within 24 – 48 hours, much faster than the current period of 3 months or longer. Therefore, patients are diagnosed quicker and can receive the correct second-line

regimes from the start. Faster and more accurate diagnosis is a high priority, as the WHO reports that less than 20 % of the 480 000 estimated MDR-TB patients are being treated properly (WHO, 2018*f*).

2.7 Tuberculosis vaccine

Currently there is only one vaccine for TB, namely the BCG vaccine. The BCG vaccine is made up of a live-attenuated strain of *Mycobacterium bovis* (Mahairas *et al.*, 1996). It triggers an immune response to ensure that the patients who receive the vaccine have a good immunity towards TB, but do not actually develop the disease as the live strain is too weak (Iqbal & Hussain, 2014). The vaccine is often given to infants and small children to prevent childhood TB meningitis and miliary disease, but only in countries where the prevalence of TB is high. Due to the potential interference of the vaccine with TST reactivity and the poor effectiveness of the vaccine against adult pulmonary TB, countries such as the United States, where the prevalence of TB is low, do not widely administer the vaccine (CDC, 2018*h*).

There were, originally, concerns about the efficiency of the BCG vaccine with various clinical trials showing BCG effectiveness ranging between 0 – 80 % (Tuberculosis Prevention Trial, 1980). However, a meta-analysis of published literature done by Colditz *et al*. (1994) and Brewer (2000), confirmed that a BCG vaccination does in fact significantly reduce the risk of TB infection (by an average of 50 %), pulmonary TB, and extrapulmonary disease (Colditz *et al.*, 1994; Brewer, 2000). A limitation of the vaccine is that it does not prevent TB infection or the reactivation of LTBI. Due to the inconsistent effectiveness of the BCG vaccine, there has been rapid advancements in new experimental vaccines for TB, especially in areas such as mycobacterial genomics and immunology (WHO, 2018*d*), DNA and recombinant vaccines (Orme *et al.*, 2001)

2.8 Treatment of tuberculosis

Effective treatment of TB has been available for over 60 years, but a prolonged treatment regimen (six – nine months), poor patient compliance, and the increasing rate of drug resistance threaten the successful treatment of TB (Maher *et al.*, 2003; Horsburgh *et al.*, 2015). Medications used in the treatment of TB are divided into two sections, namely first-line and second-line treatment antimicrobial drugs. Both active- and latent-TB diseases can be cured by strictly following a standard regimen of a combination of first-line drugs for six – nine months (CDC, 2018*a*). INH and RIF are the two most powerful first-line antimicrobials and form the core of standard TB regimens. Other first-line agents include ethambutol (EMB) and PZA (WHO, 2018*a*).

However, the emergence of TB strains resistant to one or more of the first-line anti-TB drugs weakens the probability of successful treatment, with only 55 % of MDR-TB patients receiving

successful treatment in 2017(WHO, 2018*a*).This necessitates a regimen of at least five effective second-line agents. Second-line agents are divided into groups A – D, where: Group A includes all the fluoroquinolones; Group B is second-line injectable agents (aminoglycosides); Group C is other core second-line agents (Ethionamide /Prothionamide, Cycloserine/Terizidone, Linezolid, and Clofazimine); and Group D includes all the add-on agents that are not part of the core drug-resistant treatment regimen (WHO, 2010; WHO, 2016)

2.8.1 Treatment of drug susceptible tuberculosis

TB is treatable and curable with 54 million lives saved between 2000 and 2017 through its diagnosis and treatment. Active, drug-susceptible TB is treated by a four-drug (INH, RIF, EMB, and PZA) regimen for six – nine months. Patient adherence is often difficult so support is frequently provided to the patient (health worker or trained volunteer) to ensure proper medicine consumption. When anti-mycobacterial drugs are taken incorrectly the bacteria that survive the treatment develop resistance to those drugs, which then further complicate therapy (CDC, 2018*a*; WHO, 2018*a*).

2.8.1.1 Isoniazid

INH (isonicotinic acid hydrazide, **1**), along with RIF (discussed in paragraph 2.8.1.2), is one of the most active drugs against drug susceptible TB (Ahmand & Mokaddas, 2009) and has been used against TB since 1952 (Zhang, 2005). INH comprises of a hydrazine bond and carbonyl group attached to a pyridine ring (Arbex *et al.*, 2010) as seen in Figure 2.4. It is also bactericidal against actively growing tubercle bacilli. INH has a minimum inhibitory concentration (MIC) of 0.2 µg/mL or less against most tubercle bacilli (Deck & Winston, 2012*a*), with a MIC against susceptible strains of less than 0.02 – 0.05 µg/mL (Musser, 1995; Ramaswamy & Musser, 1998). INH is freely water soluble, and is readily absorbed from the gastrointestinal tract, with peak plasma concentrations being achieved within one – two hours (half-life of 1 hour) post-administration (Deck & Winston, 2012*a*). The half-life of a drug is the period of time required for the concentration of drug in the body to be reduced by one-half, also known as the duration of action (Wharrad, 2015). INH is metabolised in the liver and is, therefore, potentially hepatotoxic (Tostmann *et al.*, 2008).

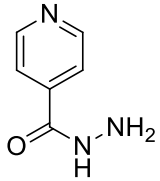

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Figure 2-4: Structure of isoniazid (1).

INH is a prodrug that is activated *in vivo* by the *Mtb* catalase-peroxidase (KatG) enzyme. After activation, INH mainly targets NADH-specific mycobacterial proteins (such as InhA), that are essential for the synthesis of MA (discussed in paragraph 2.4.2). Depletion of MA causes DNA damage and, subsequently, bacilli cell death (Arbex *et al.*, 2010; Gumbo, 2011; Ahmand & Mokaddas, 2009).

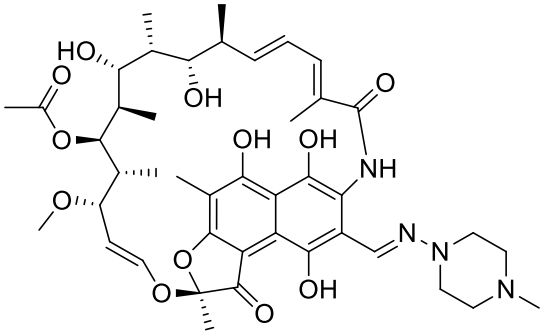
The prevalence of INH drug-resistant isolates are 1 in 10^6 bacilli (Gumbo, 2011). Resistance against INH is most often the result of a mutation and/or depletion of the catalase-peroxidase (*katG*) gene, or mutations of the *inhA* operon causing an increase in drug target. The KatG enzyme is the only *Mtb* enzyme that is able to activate the INH prodrug (Sherman *et al.*, 1996) and KatG mutants, therefore, have a high level of INH resistance (Deck & Winston, 2012*a*). Alterations to the *katG* gene is most often attributed to mutations coding for Ser315Thr shift (Marttila *et al.*, 1998) that alters the structure of the catalase-peroxidase enzymes (Ramaswamy & Musser, 1998; Somoskovi *et al.*, 2001). The altered enzyme no longer effectively metabolizes INH to its biologically active form (Ramaswamy & Musser, 1998). Consequently, MA synthesis is no longer inhibited and the bacteria survives the treatment with INH.

INH resistance can also occur through: (1) promoter mutations that result in the overexpression of alkyl hydroperoxide reductase C (*ahpC),* a gene that protects the tubercle cell against oxidative stress, and (2) mutations in 3–oxoacyl-[acyl-carrier-protein] synthase 1 (*kasA*) (Deck & Winston, 2012*a*), an enzyme that is also involved in MA synthesis (Somoskovi *et al.*, 2001). Administration of INH in isolation rarely causes adverse effects. However, INH hepatotoxicity is potentiated when used in combination with RIF. RIF is a potent CYP2E1 inducer, an enzyme that converts INH to hepatotoxic metabolites (Arbex *et al.*, 2010; Gumbo, 2011). Neurological toxicities can include convulsions, especially in patients suffering from seizure disorders. Patients may also develop haematological reactions and hypersensitivity to isoniazid. Arthritic symptoms such as arthralgia of the knees, wrists, and elbows, back pain, and the “shoulder-hand” syndrome are also attributed to INH therapy (Gumbo, 2011). Other minor effects include fever, nausea, vomiting, headache, and acne (Arbex *et al.*, 2010). The administration of INH to persons predisposed to pyridoxine-deficiency anaemia may result in dramatic anaemia, which can be countered by treatments of large doses of vitamin B_6 (Gumbo, 2011).

2.8.1.2 Rifampicin

RIF (rifampin, **2**) was first developed in 1966 (Zhang, 2005), and was only used as anti-TB therapy in the 1970s (Musser, 1995; Niemi *et al.*, 2003). RIF is used in combination with INH as first-line treatment against TB, as it is bactericidal to mycobacteria. RIF is well absorbed after oral administration, with peak plasma concentration reached after two – four hours (half-life of 3.5 hours). The CYP450 liver system metabolises approximately 85 % of RIF (making it a potential

hepatotoxic drug) (Tostmann *et al.*, 2008) and it is excreted mainly via the biliary duct (Arbex *et al.*, 2010; Deck & Winston, 2012*a*). Food intake reduces the absorption of RIF by as much as 26 % and it is, therefore, advised that RIF should be taken on an empty stomach (Arbex *et al.*, 2010; Gumbo, 2011). RIF has a MIC ranging between 0.05 – 0.5 µg/mL against *Mtb* (Zhang, 2005), is poorly water soluble and is characterised by a long aliphatic bridge spanning a chromophoric naphthohydroquinone group, and an acetyl group at C25 (Gumbo, 2011), as seen in Figure 2.5.



2

Figure 2-5: Structure of rifampicin (2).

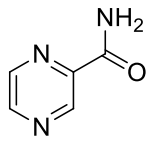
RIF brings about its effect by binding to the β subunit of bacterial DNA-dependant *rpoB*, forming a drug-enzyme complex that suppresses chain formation in RNA synthesis, thus inhibiting mycobacterial transcription and causing cell death (Somoskovi *et al.*, 2001; Gumbo, 2011; Deck & Winston, 2012*a*). Resistance to RIF occurs as a result of mutations on any of the possible points in the *rpoB* gene. Such mutations lead to a reduced binding ability of RIF to RNA polymerase, thereby deterring the effect of RIF on tubercle bacilli (Deck & Winston, 2012*a*). The prevalence of RIF drug-resistant isolates is one in every 10^7 to 10^8 bacilli (Gumbo, 2011). Almost all (more than 98 %) RIF-resistant strains have a mutation in the 81 base pair (bp) region of *rpoB*, with resistance due to mutations at codon 526 and 531 in the 81 bp area accounting for 86 % of cases (Somoskovi *et al.*, 2001).

RIF is generally well tolerated with more than 4 % of patients developing adverse effects commonly including fever, rash, nausea and vomiting (Gumbo, 2011), and a harmless orange colour change of urine, sweat and tears (Deck & Winston, 2012*a*).

2.8.1.3 Pyrazinamide

PZA (**3**) not only bares many similarities to INH, it was also discovered the same year (1952) (Zhang, 2005). PZA is also related to nicotinamide and requires activation to a bioactive form to

produce pharmacological effects. No cross-resistance of *Mtb* exists between INH and PZA (Arbex *et al.*, 2010; Deck & Winston, 2012*a*). The structure of PZA is seen in Figure 2.6.



3

Figure 2-6: Structure of pyrazinamide (3).

PZA is only slightly water soluble, but well absorbed after oral administration, with peak plasma concentrations reached after two hours (Arbex *et al.*, 2010). PZA is metabolised by liver enzymes and has a very long half-life of 8 – 11 hours. It is therefore recommended that PZA be administered three times a week, and not daily, in patients on haemodialysis and patients with a creatinine clearance less than 30 mL/min (Deck & Winston, 2012*a*). PZA is bactericidal to *Mtb* and has a strong sterilising effect on semi-dormant bacilli. PZA has a MIC of 6.25 – 50.0 µg/mL at a pH of 5.5 (Arbex *et al.*, 2010).

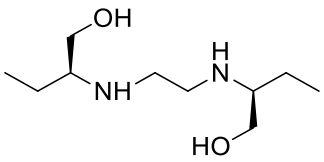
PZA is converted to its active form, pyrazinoic acid (POA), by a mycobacterial pyrazinamidase encoded by *pncA*. It is inactive at neutral pH and is activated in an acidic environment. PZA passively enters the *Mtb* bacillus where it is converted to POA. Due to an ineffective efflux system, POA concentrations in the bacterial cytoplasm increases with rising concentrations, lowering the intracellular pH to suboptimal levels that result in the inactivation of vital enzyme, fatty acid synthase 1. This enzyme plays a vital role in the synthesis of fatty acids and its inactivation consequently impairs MA synthesis (Somoskovi *et al.*, 2001; Arbex *et al.*, 2010; Deck & Winston, 2012*a*).

Resistance to PZA arises as a result of mutations on the *pncA* gene that encodes the mycobacterial pyrazinamidase enzyme needed in the activation of PZA. Pyrazinamidase then has a reduced affinity for PZA, leading to a decrease in the conversion to POA. Single point mutations in the *pncA* gene constitute 70 % of resistant isolates (Arbex *et al.*, 2010; Gumbo, 2011).

Hepatotoxicity is the most serious side effect of PZA and PZA should, therefore, only be given to patients with hepatic dysfunction if it is unavoidable. Other adverse effects of PZA include drug fever (Gumbo, 2011; Deck & Winston, 2012*a*), nausea and vomiting (Gumbo, 2011; Deck & Winston, 2012*a*), anorexia (Gumbo, 2011), malaise (Gumbo, 2011) and hyperuricemia (Deck & Winston, 2012*a*).

2.8.1.4 Ethambutol

EMB (**4**) as seen in Figure 2.7, was discovered in1961 (Zhang, 2005). EMB is a dihydrochloride salt, that is synthetic, heat-stable, and water-soluble (Arbex *et al.*, 2010; Deck & Winston, 2012*a*). EMB is bacteriostatic (stops the growth) against *Mtb*, especially rapidly growing bacilli. EMB is well absorbed after administration, with peak plasma concentrations reached after two – four hours. It has a half-life of 4 hours and 50 % of the drug is excreted unchanged in the urine (Deck & Winston, 2012*a*). In patients with severe kidney failure the half-life of the drug can be as long as ten hours (Arbex *et al.*, 2010). The dosage is, therefore, reduced by half for patients with a creatinine clearance of less than 10 mL/min (Deck & Winston, 2012*a*). The MIC of EMB against susceptible *Mtb* strains is 1 – 5 µg/mL (Deck & Winston, 2012*a*).



4

Figure 2-7: Structure of ethambutol (4).

EMB disrupts the biosynthesis of the principal polysaccharide on the mycobacterial cell wall, arabinogalactan. EMB inhibits the arabinosyl transferase enzyme that mediates the polymerisation of arabinose into arabinogalactan. Arabinosyl transferase is encoded by the *embCAB* operon and resistance to EMB is, therefore, most likely due to mutations within the *embB* gene or mutations that cause an overexpression of *emb* gene products (Arbex *et al.*, 2010; Gumbo, 2011; Deck & Winston, 2012*a*). EMB resistance develops rapidly if the drug is used as monotherapy. Mutations that occur at codon 306 of the *embB* gene accounts for 30 – 70 % of clinical isolates that are resistant to EMB. Another cause of resistance to both EMB and INH could be attributed to an enhanced efflux pump activity (Gumbo, 2011).

EMB is generally well tolerated with very few serious adverse effects, the most important being retrobulbar optic neuritis. Retrobulbar optic neuritis results in the diminution of visual acuity and red-green colour blindness. Its severity is dependent on the dose and duration of administration and is reversible (Arbex *et al.*, 2010; Gumbo, 2011; Deck & Winston, 2012*a*). Other negative effects of EMB include gastrointestinal effects (nausea, vomiting, hepatotoxicity and abdominal pain), skin rash and drug fever (Arbex *et al.*, 2010; Gumbo, 2011).

2.8.2 Treatment of drug-resistant tuberculosis

Only 55 % of current MDR-TB cases worldwide are treated successfully. In 2016 the WHO approved the use of a short, 9 – 12 month, standardised regimen for patients with MDR-TB who

are not additionally resistant to second-line anti-mycobacterial drugs. The conventional treatment regimen for MDR-TB can take up to 2 years, making the standardised regimen less expensive as well. Patients that are resistant to second-line agents or have XDR-TB cannot use this regimen and have to follow a longer MDR-TB regimen to which either delamanid and bedaquiline may be added (WHO, 2018*a*).

According to the new WHO treatment guidelines for drug-resistant TB, patients with RIF resistance or MDR-TB should follow a regimen that is comprised of at least five effective TB medicines during the initiation phase. This should include PZA and four core second-line antimicrobials – one from both group A and B, and at least two from group C. In the scenario of impossibility to compose a regimen as stated above, an agent from group D2 and other agents from group D3 may be added to bring the total to five effective medicines (WHO, 2016; Tiberi *et al.*, 2017). In the case where PZA is compromised, the regimen can be reinforced with a drug from either group C or group D (preferably D2, if that is not possible, from D3). Group D1 agents are only added if they are believed to add benefit (Tiberi *et al.*, 2017). The core second-line drugs, in their respective groups, are summarised in Table 2.1.

Furthermore, the WHO recommends that the standard treatment regimen for patients with RIF resistance, MDR-TB or patients without a high-level INH resistance be further strengthened with a high-dose of EMB and/or INH (WHO, 2016; Tiberi *et al.*, 2017).

Table 2-1: Second-line anti-tuberculosis agents shown in descending order of preference for use (WHO, 2016).

<table>
<tr><th colspan="4">Core second-line agents</th></tr>
<tr><th>Group A:
Fluoroquinolones</th><th>Group B:
Aminoglycosides</th><th>Group C:
Other core second-line agents</th><th>Group D:
Add-on agents</th></tr>
<tr><td rowspan="4">Levofloxacin
Moxifloxacin
Gatifloxacin
Ciprofloxacin</td><td rowspan="4">Amikacin
Capreomycin
Kanamycin
Streptomycin</td><td>Ethionamide/Prothionamide</td><td>D1:
Pyrazinamide
Ethambutol
High-dose isoniazid</td></tr>
<tr><td>Cycloserine/Terizidone</td><td>D2:
Bedaquiline
Delamanid</td></tr>
<tr><td>Clofazimine</td><td rowspan="2">D3:
p-aminosalicylic acid
Imipenem-cilastatin
Meropenem
Amoxicillin-clavulanate
Thioacetazone</td></tr>
<tr><td>Linezolid</td></tr>
</table>

2.8.2.1 Group A – Fluoroquinolones

Group A core second-line anti-TB agents are the fluoroquinolones (FQs). This group includes ciprofloxacin (**5**), gatifloxacin (**6**), moxifloxacin (**7**), and levofloxacin (**8**), seen in Figure 2.8. The favourable pharmacokinetic, microbiological, drug interaction, and toxicity profile of FQs have made this class of medication promising agents against TB (Berning, 2001). FQs are synthetic fluorinated analogues of nalidixic acid that act by inhibiting DNA gyrase (Gumbo, 2011; Deck & Winston, 2012*b*). FQ are generally well absorbed after oral administration, with peak plasma concentrations reached after one – two hours. Oral absorption of FQ is reduced by concomitant uptake of food and di- and trivalent cations. FQs exhibit a concentration-dependant effect, have a MIC of less than 2 µg/mL, and a half-life of three – ten hours, which permits once-daily dosing (Berning, 2001; Deck & Winston, 2012*b*).

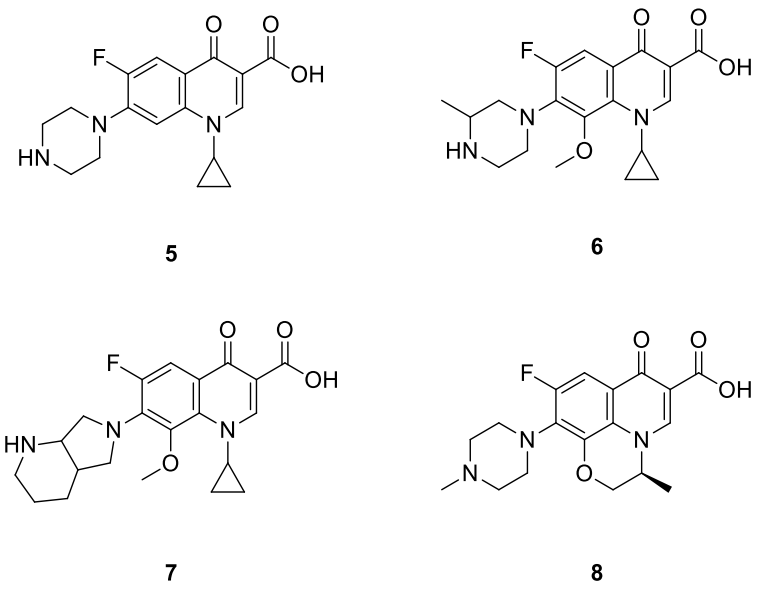


Figure 2-8: Structure of anti-tuberculosis clinical fluoroquinolones; ciprofloxacin (**5**), gatiflaxacin (**6**), moxiflaxacin (**7**), and levofloxacin (**8**).

FQs act by obstructing bacterial DNA synthesis through the inhibition of DNA gyrase (topoisomerase II) and topoisomerase IV enzymes. DNA gyrase is composed of two A and two B subunits, encoded by the genes *gyrA* and *gyrB,* respectively (Musser, 1995; Petri, 2011). DNA gyrase inhibition disrupts normal transcription and replication by preventing the relaxation of supercoiled DNA. Topoisomerase IV inhibition hinders cell division by interfering with the separation of the replicated chromosomal DNA into respective daughter strands (Deck & Winston, 2012*b*). Resistance to FQs arise due to a decrease in the permeability of the mycobacterium cell wall or one or more point mutations in the gyrase A subunit. Resistance to one FQ generally confers cross-resistance to all other FQs, especially if the level of resistance is high (Deck & Winston, 2012*b*).

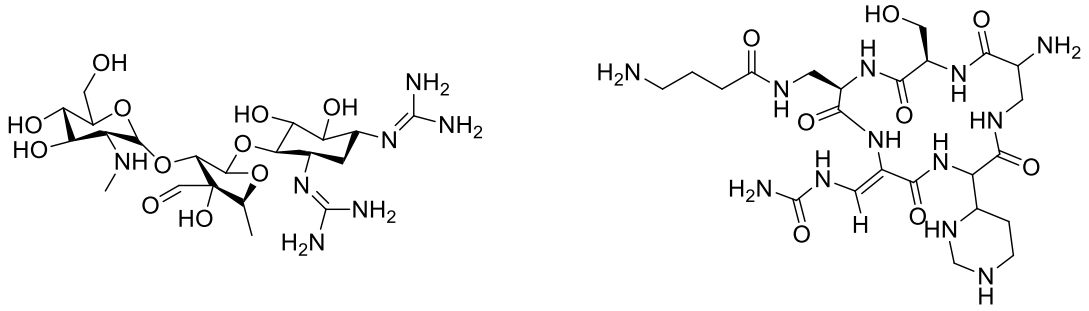
FQs are generally well tolerated with the most common adverse effects being gastrointestinal (diarrhoea, nausea and vomiting) and the occasional headache, skin rash, dizziness, and insomnia (Deck & Winston, 2012*b*). Other, more severe adverse effects include tendonitis and tendon rupture (Ginsburg *et al.*, 2003), seizures (Ginsburg *et al.*, 2003) and prolongation of the QT interval (distance between the start of the Q wave and end of the T wave on the electrocardiogram (ECG)), triggering life threatening ventricular arrhythmias known as Torsades de Pointes (Isbister, 2015) (especially with moxifloxacin, levofloxacin and gatifloxacin (Deck & Winston, 2012*b*)). FQs are not routinely recommended to patients under the age of 18 years. This restriction is due to the possibility of cartilage damage, as FQ may damage growing cartilage and cause arthropathy (Ginsburg *et al.*, 2003; Deck & Winston, 2012*b*)

2.8.2.2 Group B – Second-line injectable agents: Aminoglycosides

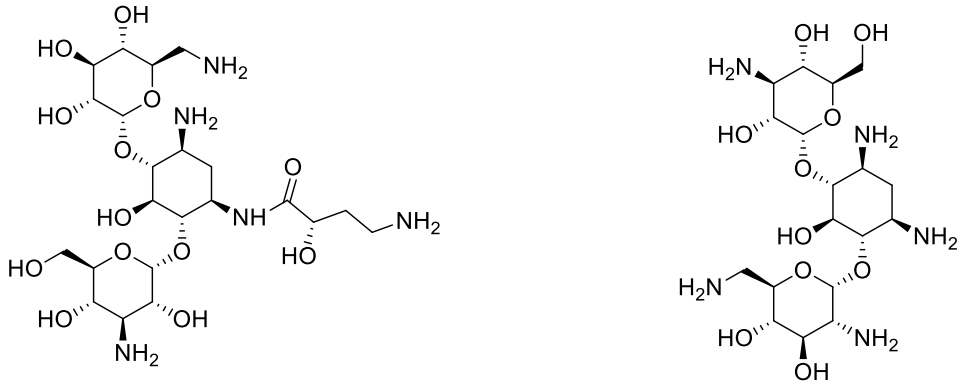
Group B core agents are aminoglycosides and include second-line anti-TB injectable agents, streptomycin (**9**), capreomycin (**10**), amikacin (**11**), and kanamycin (**12**), seen in Figure 2.9. They are important in the treatment of TB, especially MDR-TB (Deck & Winston, 2012*a*). The intramuscular route is the adopted route of administration, due to very poor absorption from the gastrointestinal tract and an almost complete excretion in the faeces after oral administration. Peak plasma concentrations are reached within 30 – 90 minutes after intramuscular injection. Aminoglycosides inhibit protein synthesis by binding to the 30S ribosomal subunit, resulting in the misinterpretation of the genetic code during translation and, ultimately, to irreversible protein synthesis inhibition (Deck & Winston, 2012*b*). There are three mechanisms of action by which aminoglycosides inhibit protein synthesis: (1) interference with the initiation complex of peptide formation; (2) aminoglycosides cause the break-up of polysomes into non-functional monosomes; and (3) misinterpretation during translation (therefore mRNA) that results in the incorporation of incorrect amino acids in the peptide and the formation of toxic or non-functional proteins. Monotherapy of aminoglycosides is not recommended but always in combination therapy with at least one, and preferably two or three other *Mtb* is susceptible drugs (Deck & Winston, 2012*b*).

As with the mechanism of action, there are also three principal mechanisms of resistance to aminoglycosides (Deck & Winston, 2012*a*; Deck & Winston, 2012*b*): (1) diminished entry of aminoglycoside into the cell; (2) alteration or deletion of the receptor protein on the 30S ribosomal subunit due to mutation. Mutations that take place in either the *rrs* gene that encode 16S ribosomal rRNA, or in the *rpsL* gene that encode S12 ribosomal protein cause such receptor protein alterations. (3) Production of transferase enzymes or enzymes which cause the inactivation of aminoglycoside by acetylation, adenylylation, or phosphorylation. Amikacin is usually indicated in streptomycin-resistant TB, as there is no cross-resistance between amikacin and streptomycin. However, kanamycin resistance is often indicative of amikacin resistance (Deck & Winston, 2012*a*; Deck & Winston, 2012*b*).

Aminoglycosides are ototoxic and nephrotoxic, especially if the therapy is continued for longer than five days. Other adverse effects of aminoglycoside include deafness, tinnitus and vestibular disturbances (Deck & Winston, 2012*a*).



9 **10**



11 **12**

Figure 2-9: Structure of clinical anti-TB aminoglycosides; streptomycin (**9**), capreomycin (**10**), amikacin (**11**), and kanamycin (**12**).

2.8.2.3 Group C – Other core second-line drugs

Group C agents include all other core second-line agents, i.e. ethionamide/prothionamide, cycloserine/terizidone, linezolid and clofazimine. Figure 2.10 include the structures of Group C agents.

Linezolid (**13**) , an oxazolidinone antibiotic (Sotgiu *et al.*, 2012), is a core anti-TB drug with both bactericidal and bacteriostatic effects on the bacterium (Caminero & Scardigli, 2015). Linezolid brings about its effect by binding onto 23S rRNA (Dooley *et al.*, 2013) on the 50S ribosomal subunit, preventing the bacteria from assembling ribosomes from their dissociated subunits and ultimately inhibiting ribosomal protein synthesis (Lin *et al.*, 1997; Moellering, 2003). Consequently, mutations affecting 23S rRNA gene confer a high-level of linezolid resistance (Dooley *et al.*, 2013).

The limited information available on Linezolid suggests that it is very active against *Mtb*, but unfortunately its safety and tolerability profile is not as favourable, with many patients experiencing major adverse effects (Sotgiu *et al.*, 2012; Sotgiu *et al.*, 2015). The main adverse

effects include anaemia, gastrointestinal disorders, peripheral neuropathy, optic neuritis and thrombocytopenia (Sotgiu *et al.*, 2012). In an effort to reduce or prevent the adverse effects experienced, the standard 600 mg taken twice daily for gram-positive infections has been adjusted to 300 – 600 mg once daily for drug-resistant TB (Schecter *et al.*, 2010; Dooley *et al.*, 2013; Sotgiu *et al.*, 2015).

Ethionamide (**14**)/*Prothionamide* (**15**) are classified as thionamide drugs that are used interchangeably in the treatment of multibacillary leprosy (WHO, 2018*g*) and MDR-TB (WHO, 2016). The biological and structural properties and therapeutic potencies of ethionamide and prothionamide are very similar (WHO, 2018*g*). Prothionamide has a propyl group in the 2nd position of the pyridine ring, as seen in Figure 2.10.

Not only is ethionamide and prothionamide structurally similar to INH, but the mechanism of action is also similar to that of INH. Ethionamide brings about its effect by inhibiting InhA (Wang *et al.*, 2007), and thereby inhibiting mycolic acid synthesis (Caminero *et al.*, 2010). The similar mechanism of action could possibly confer resistance to both INH and ethionamide (Wang *et al.*, 2007; Caminero *et al.*, 2010). Ethionamide is a prodrug that must be activated first to bring about its effect. However, unlike INH that is activated by KatG, ethionamide is activated by FAD-containing monooxygenase (EthA) enzyme, encoded by the *ethA* gene. Therefore, KatG mutant strains that are resistant to INH retain their sensitivity to ethionamide (DeBarber *et al.*, 2000; Wang *et al.*, 2007). Resistance to ethionamide occurs when there is a mutation of *inhA* or *ethA* (Wang *et al.*, 2007).

Adverse effects of ethionamide and prothionamide are rarely reported, but 8.2 % of patients experience serious effects, including gastro-intestinal disturbances and hypothyroidism, especially when used in combination with *p*-aminosalicylic acid (WHO, 2016).

Clofazimine (**16**) is a riminophenazine that was specifically developed to treat TB, but inconsistent animal models delayed its development into anti-TB drug. However, extensive research revealed its usefulness against other mycobacterial infections, such as *Mycobacterium leprae* (Reddy *et al.*, 1999; Dooley *et al.*, 2013). The mechanism of action of clofazimine remains unclear, but existing evidence suggests a redox cycling pathway where clofazimine is enzymatically reduced by nicotinamide adenine dinucleotide (NADH) dehydrogenase (NDH-2), and then spontaneously re-oxidised by oxygen to release ROS (Yano *et al.*, 2011; Hartkoorn *et al.*, 2014). Very little is known about the mechanism of resistance to clofazimine, but mutations in the *rv0678* gene are suggested to be a confounding factor (Hartkoorn *et al.*, 2014).

Clofazimine has a sterilising activity (Tiberi *et al.*, 2017) and is generally well tolerated, with the main adverse effect being reversible red-black skin discolouration occurring in virtually all patients (Dooley *et al.*, 2013; WHO, 2016).

Cycloserine (**17**)/*Terizidone* (**18**) are used interchangeably in the treatment of drug-resistant TB (WHO, 2016). Cycloserine (D-cycloserine) is a structural analogue of D-alanine and is bacteriostatic against mycobacteria (Chopra & Brennan, 1998). It acts by competitively blocking peptidoglycan biosynthesis, a building block in the cell wall of *Mtb*. D-cycloserine competitively inhibits both D-alanine racemase (which racemizes L-alanine to D-alanine), and D-alanyl-D-alanine synthase (which catalyses the formation of dipeptide D-alanine-D-alanine) (David *et al.*, 1970; Chopra & Brennan, 1998). The mechanism of resistance to cycloserine is still unclear, but genetic analysis has clearly demonstrated that the overexpression of *alrA* confers it (Chopra & Brennan, 1998). Terizidone results from the chemical combination of two cycloserine molecules. It was developed to improve the toxicity profile of this group of drugs. Psychiatric adverse events (i.e. psychotic reactions with suicidal tendencies) are some of the major drawbacks of these medicines, especially cycloserine (Caminero *et al.*, 2010).

Other symptoms include anxiety, dizziness and slurred speech, and treatment should be halted immediately if a patient is suicidal or psychotic (Tomlinson, 2011).

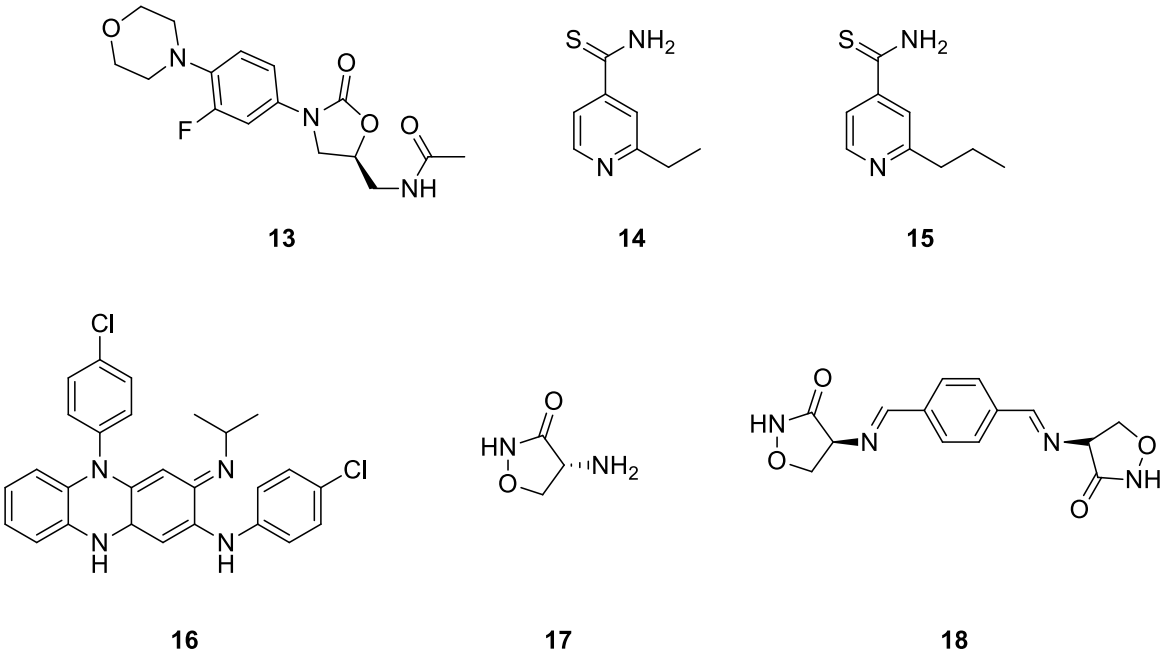


Figure 2-10: Structure of all Group C clinical anti-TB agents; linezolid (**13**), ethionamide (**14**), prothionamide (**15**), clofazimine (**16**), cycloserine (**17**), and terizidone (**18**).

2.8.2.4 Group D – Other add-on agents

Group D agents are add-on agents and not core MDR-TB regimen components. This group includes; bedaquiline, delamanid, *p*-aminosalicylic acid, thioacetazone and carbapenems (e.g. imipenem and meropenem).

2.8.2.4.1 Group D2

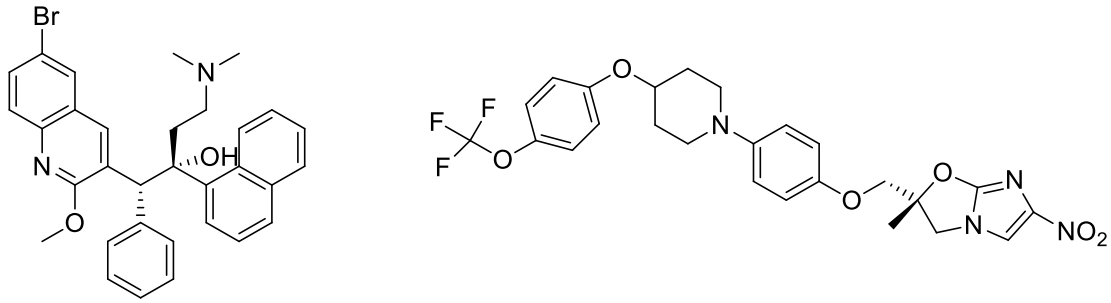
Bedaquiline (**19**) and delamanid (see Figure 2.11) are two of the first drugs approved for the treatment of drug-resistant TB in over 40 years (Mahajan, 2013). Bedaquiline is a diarylquinoline (Diacon *et al.*, 2014) that targets both dormant and actively replicating TB bacilli (Caminero & Scardigli, 2015; Tiberi *et al.*, 2017). Bedaquiline brings about its effect by inhibiting the proton pump of adenosine triphosphate (ATP) synthase. Bedaquiline binds to the oligomeric and proteolipid subunit-c of mycobacterial ATP synthase, thus causing the inhibition of ATP synthesis, which subsequently leads to bacterial cell death (Mahajan, 2013; Andries *et al.*, 2014). One method by which bedaquiline resistance develop is as a result of mutations in the *atpE* gene (the gene that encodes the subunit-c of ATP synthase) (Mahajan, 2013). Another mechanism of resistance, and a reason for the cross-resistance between bedaquiline and clofazimine, are mutations in the *Rv0678* gene, involved in the encoding of the MmpS5-MmpL5 efflux pump (Andries *et al.*, 2014).

The Food and Drug Administration (FDA) has voiced its concern over the use of bedaquiline and a concomitant increase in number of deaths. Only 2.5 % of patients taking placebos during the clinical trials died, compared to 11.4 % of patients, who used bedaquiline.(Mahajan, 2013). Other adverse effects caused by bedaquiline include, nausea, vomiting, and arthralgia (Diacon *et al.*, 2014) and with the most common effect being prolongation of the QT interval (Mahajan, 2013; Caminero & Scardigli, 2015). Bedaquiline and delamanid both have the characteristics of core drugs, but with limited data available on the effectiveness of the drugs, it is only used in the first six months of treatment (Caminero & Scardigli, 2015).

Delamanid (**20**) is a dihydro-imidazooxazole that has already been approved by the Japanese Ministry of Health, Welfare and Labor (MHWL) and the European Medicines Agency (EMA) for the treatment of MDR-TB (Lewis & Sloan, 2015). Delamanid has both bactericidal and sterilising activity, and its absorption is increased two-fold when co-administered with food (Caminero & Scardigli, 2015; Szumowski & Lynch, 2015). The mechanism of action of delamanid is not completely understood, but it is thought to inhibit the synthesis of mycolic acid synthesis, primarily keto-mycolic and methoxy-mycolic acid (components in the mycobacterial cell wall). Delamanid is a prodrug that is activated by the mycobacterial F420 co-enzyme system. Therefore, resistance to delamanid is thought to arise when there are mutations to the mycobacterial F420 genes, *fgd,*

Rv3547, fbiA, fbiB, and *fbiC*, involved in the activation of the drug (Lewis & Sloan, 2015; Szumowski & Lynch, 2015). However, no cross-resistance exists between delamanid and other anti-TB drugs as of yet (Tiberi *et al*., 2017).

Delamanid is generally well tolerated, with the most frequent adverse effects being nausea, vomiting, dizziness, paraesthesia, tremor, anxiety, and the most severe being QT interval prolongation. As with bedaquiline, delamanid requires significant efficacy and safety data before it can be used during the entire length of treatment against TB, and not just six months (Caminero & Scardigli, 2015).



19 **20**

Figure 2-11: Structures of bedaquiline (19) and delamanid (20).

2.8.2.4.2 Group D3

The anti-TB Group D3 drugs include thioacetazone (**21**), carbapenems (e.g. imipenem (**22**) and meropenem (**23**)) and *p*-aminosalicylic acid (**24**), see Figure 2.12. Group D3 agents are only used in MDR- and XDR-TB treatment as a last resort. Therefore, if the minimum effective TB treatment regimen cannot be composed of one drug from group A, one from group B, and at least two drugs from group C, only then should one drug from group D2 and other drugs from group D3 be included to make up the five drug regimen recommended by WHO (WHO, 2016).

The use of thioacetazone as part of the first-line combination treatment of TB was restricted in the 1990s due to severe life threatening skin reactions (such as Stevens-Johnson syndrome and toxic epidermal necrolysis) and the widespread availability of safer, more affordable alternative medicines (WHO, 2016). Thioacetazone is a bacteriostatic prodrug that is activated by the same mycobacterial monooxygenase that activates ethionamide, EthA, leading to the inhibition of mycolic acid synthesis (Alahari *et al.*, 2007).

Although carbapenems are currently used in the treatment of MDR- and XDR-TB, evidence on their safety, efficacy, and tolerability are still anecdotal (Sotgiu *et al.*, 2016). However, reports have shown that carbapenems, a subclass of β-lactam antibiotics, also exhibit high antimicrobial

potency against *Mtb* and should, therefore, be effective in the treatment of TB (Kaushik *et al.*, 2015). WHO recommends that carbapenems always be administered in combination with amoxicillin-clavulanate (WHO, 2016).

p-aminosalicylic acid

The anti-TB activity of *para*-aminosalicylic acid (PAS) was discovered in 1943 by Swedish chemist Jörgen Lehmann (O'Connor, 1948). However, after the introduction of better, more effective anti-TB drugs (RIF and PZA), PAS therapy was mainly discontinued (Mathys *et al.*, 2009) and is currently used only for drug-resistant TB. Even though the drug has been used clinically for over 60 years, its exact mechanism of action is elusive. Due to the structural similarities of PAS to *para*-aminobenzoic acid (PABA), it is speculated that PAS is also involved in the folate biosynthetic pathway (Zheng *et al.*, 2013). The folate metabolic pathway in prokaryotes and eukaryotes generates tetrahydrofolate that is needed for the synthesis of formylmethionyl $tRNA^{fMet}$, an essential component in the initiation of protein synthesis. Upon incorporation into the folate pathway, the prodrug PAS interacts with dihydrofolate synthase (DHFS) and dihydropteroate synthase (DHPS) to form a hydroxyl dihydrofolate antimetabolite that inhibits the enzymatic activity of dihydrofolate reductase (DHFR) (Zheng *et al.*, 2013).

Mycobacterial resistance to PAS has been associated with mutations to the *thyA* gene that encodes thymidylate synthase in the folate pathway (Mathys *et al.*, 2009; Zhao *et al.*, 2014). As PAS is a prodrug that targets the activity of DHFR, mutations to or overexpression of genes involved in the conversion of PAS to its active form in the folate pathway also result in resistance to PAS. Therefore, the overexpression of *dfrA* (the gene that encodes DHFR in *Mtb* (Zheng *et al.*, 2013)) and mutations to *folC* (the gene that encodes DHFS in *Mtb* (Zhao *et al.*, 2014)) are responsible for PAS resistance. In addition to the above mentioned mechanisms of resistance, it has been discovered that the overexpression of the enzyme riboflavin biosynthesis protein (RibD), a functional analogue of DHFR, conferred PAS resistance as well (Zheng *et al.*, 2013).

The effectiveness of PAS against TB is very poor. It is expensive and poorly tolerated with a high frequency of adverse effects (gastro-intestinal disturbances, and hypothyroidism, especially when used in combination with ethionamide/prothionamide) (WHO, 2016). Regardless of the drawbacks, PAS is an important drug in the treatment of MDR and XDR TB, but should still be reserved for cases where there are no other options of drugs to use (Caminero *et al.*, 2010; WHO, 2016).

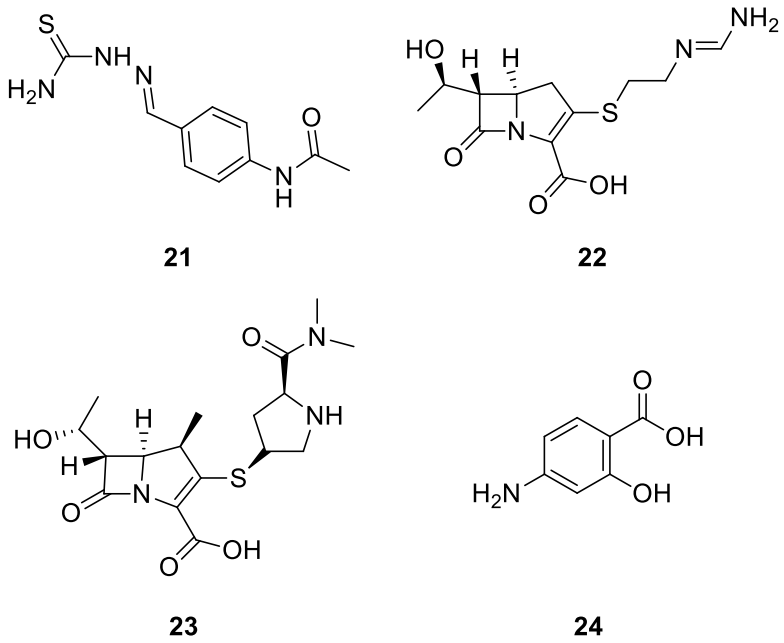


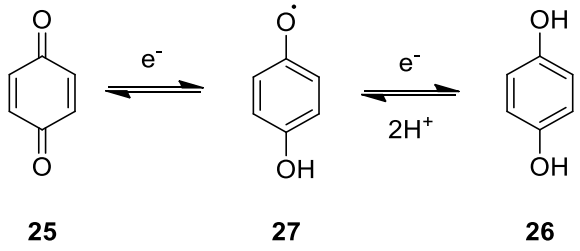
Figure 2-12: Structures of all Group D3 clinical anti-TB agents; thioacetazone (**21**), imipenem (**22**), meropenem (**23**), and *p*-aminosalicylic acid (**24**).

2.9 Drug rationale

In this project 1,4-benzoquinone and the pharmacophore 1,2,3-triazole are hybridised to form various 1,2,3-triazole linked quinone derivatives to be utilised in drug development against TB. Discussed further is the justification for the use of these compounds.

2.9.1 Benzoquinone-hydroquinone

Benzoquinone (BQ, **25**) and hydroquinone (HQ, **26**) are both members of naturally occurring quinones (Kim *et al.*, 2010). When present in an aqueous solution BQ and HQ are both susceptible to spontaneous and enzymatically mediated redox cycling and acid-base transformations. This leads to the reversible interconversion of HQ and BQ, respectively, as seen in Scheme 2.1.



Scheme 2.1: The interconversion of 1,4-benzoquinone (**25**) to hydroquinone (**26**), via semiquinone (**27**).

In an aqueous solution, HQ undergoes autoxidation to form BQ, via a semiquinone radical (Souček *et al.*, 2000). The reaction is exerted by cytochrome P450 and various peroxidases (e.g. myeloperoxidase, prostaglandin H synthase and horseradish peroxidase) (McGregor, 2007; HCotN, 2012). BQ, however, can either be reduced via a one-electron transfer process (by enzymes such as cytochrome P450 reductase and ubiquinone oxidoreductase), or a two-electron process (catalysed by flavoproteins NAD(P)H-quinone oxidoreductases, NQO1 and NQO2) to produce hydroquinone. Since BQ and HQ are interconverted to each other in an aqueous medium, observations made for one compound might also be relevant to the other compound. The biotransformation of the two compounds are, therefore, combined (HCotN, 2012).

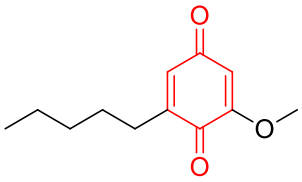
The rate of oxidation and reduction of HQ to BQ and BQ to HQ, respectively, is influenced by the presence of a microsomal system (microsomes plus NADPH) (Souček *et al.*, 2000). The rate of HQ oxidation can be significantly slowed down by the presence of NADPH and completely stopped in the presence of a complete microsomal system. For BQ, the rate of reduction of BQ to HQ is significantly faster when stimulated by a complete microsomal system (less than five minutes). The microsomal environment exerts a reducing action and strongly influences the redox cycling of quinones. The autoxidation of HQ is also influenced by the pH of the medium. HQ rapidly autoxidises to BQ under alkaline conditions, and slowly under acidic conditions, with the opposite being true for the autoreduction of BQ to HQ (HCotN, 2012).

The naturally occurring BQ is found primarily in higher plants, bacteria, fungi and small parts of the animal kingdom. BQ is a poorly water soluble substance that possesses several pharmacological properties, such an anti-inflammatory (Sagnou *et al.*, 2009), anticancer (Lindsey *et al.*, 2004), antiviral (Bogdanova *et al.*, 1970) and antimicrobial (Yezerski *et al.*, 2007) activities. An additional major use of BQ is its transformation into HQ (HCotN, 2012). Other effects of BQ include gene mutation (Ludewig *et al.*, 1989) and the inhibition of amyloid fibril formation of lysosomes (Wang *et al.*, 2006). BQ has shown dose dependant inhibitory effects against fibrillogenesis and is believed to help in the understanding and future prevention of amyloidogenic disease (Wang *et al.*, 2006). Nevertheless, BQ was never tested against TB in these studies. BQ is readily absorbed from the gastro-intestinal tract and via subcutaneous tissue (HCotN, 2012).

HQ is a crystalline structure that is water soluble and is almost exclusively used as an industrial chemical. It is also used as a photographic chemical, polymerisation inhibitor, tanning agent (HCotN, 2012), antioxidant in the rubber industry, and as a stabiliser in paints, motor fuels, oils and varnishes (McGregor, 2007). HQ is rapidly and extensively absorbed after oral administration, but absorption through the skin is much slower (HCotN, 2012).

In a study done by Kim *et al*. (2010), BQ has shown significant antibacterial activity (Kim *et al.*, 2010), especially against pathogens that cause food poisoning, as well as antioxidant and cytotoxic activities (Lana *et al.*, 2006).

In another study conducted by Tasdemir *et al*. (2006), primin, **28**, (a natural BQ) was synthesised and its anti-mycobacterial activity, as well as activity against other pathogens, was investigated. The study revealed that primin has only moderate inhibitory activity against *Mtb* (MIC 60.3 µM or 12.55 µg/mL) (see Figure 2.13), but significant *in vitro* anti-leishmanicidal potential half-maximum inhibitory concentration (IC_{50} 0.711 µM) compared to reference drug miltefosine (IC_{50} 0.373 µM) (Tasdemir *et al.*, 2006). The IC_{50} value indicates the concentration of a particular drug (inhibitor) that is needed to inhibit a given biological process by half (Aykul & Martinez-Hackert, 2016).



28
Mtb MIC = 12.55 µg/mL

Figure 2-13: Structure of primin (**28**), a natural benzoquinone (Tasdemir *et al.*, 2006).

Tran and co-workers conducted a study to determine the possibility of quinones as anti-mycobacterial agents. With MIC values of 50, 100 and 25 µg/mL against *M. smegmatis* (*Mts*), *M. avium* (*Mta*) and *Mtb*, respectively, 1,4-Benzoquinone has been found to possess anti-mycobacterial activity (Tran *et al.*, 2004). Nonetheless, the extent of research done on BQ and, more so, on BQ-linked 1,2,3-triazole compounds and their activity against *Mtb* remain limited.

In a study done by Jyoti *et al*. (2016), ursolic acid and HQ were extracted from the plant *Artemisia capillaris*, and tested against various strains of *Mtb* to determine their inhibitory effects. It has been determined that HQ did in fact inhibit several strains (resistant and susceptible) of TB with a MIC ranging between 12.5 to 25 µg/mL. The mode of action of HQ is still unclear, it has been suggested that HQ disrupts intracellular components, such as RNA, DNA, internal proteins or other organelles, affecting *Mtb* cells (Jyoti *et al.*, 2016).

2.9.2 1,2,3-Triazole

Triazoles are five membered heterocyclic compounds, containing three nitrogen atoms and two carbon atoms (Dheer *et al.*, 2017), that have gained significant interest over the last few years as part of compounds with extensive biological activities (Emmadi *et al.*, 2015; Dheer *et al.*, 2017). Triazoles are capable of hydrogen bonding and the binding of biomolecular targets that improve

the solubility (Ali *et al.*, 2017). There are two isomeric triazoles, namely 1,2,3-triazole and 1,2,4-triazole, but for the purpose of this study we focused on the 1,2,3-triazole moiety. The broad spectrum of pharmaceutical and therapeutic applications of the 1,2,3-triazole moiety include anticancer (carboxyamidotriazole, **29**), anti-HIV (TSAO, **30**), anti-bacterial activities (tazobactum, **31**, and cefatrizine, **32**) and benzofuran salicylic acid derivative (I-A09, **33**), a leading anti-tubercular agent currently on the market (Ali *et al.*, 2017; Zhang *et al.*, 2017). Structures of these compounds are seen in Figure 2.14. Additionally, 1,2,3-triazole derivatives have been used as enzyme inhibitors, such as histone deacetylase and alkaline phosphate. It also serves as a key synthetic intermediate in many industrial applications such as corrosion inhibitors, additives, agrochemicals, photostabilisers, liquid crystals, and metal chelators (Dheer *et al.*, 2017).

The moiety's enhanced biological activities are due to the favourable properties of the triazole ring (moderate dipole character, rigidity, hydrogen bonding capability, and stability under *in vivo* conditions) (Zhang *et al.*, 2017). The mechanism of action of triazoles is similar to that of INH, i.e. the inhibition of cell wall synthesis by blocking lipid biosynthesis (Kumar *et al.*, 2014; Zhang *et al.*, 2017).

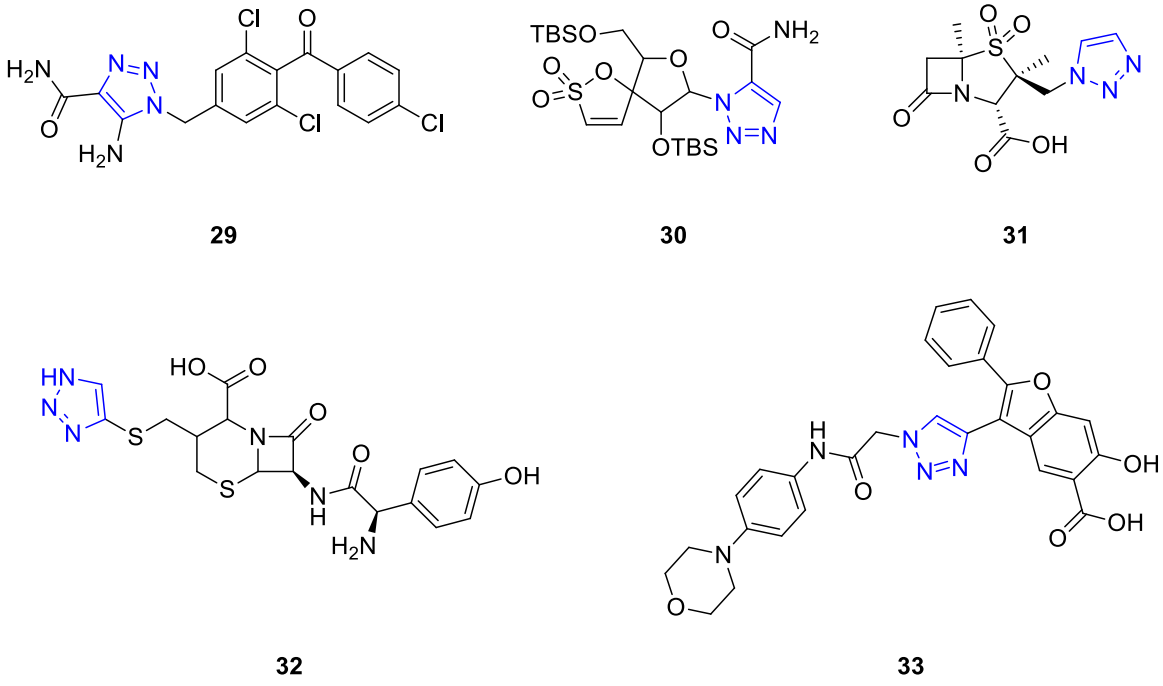


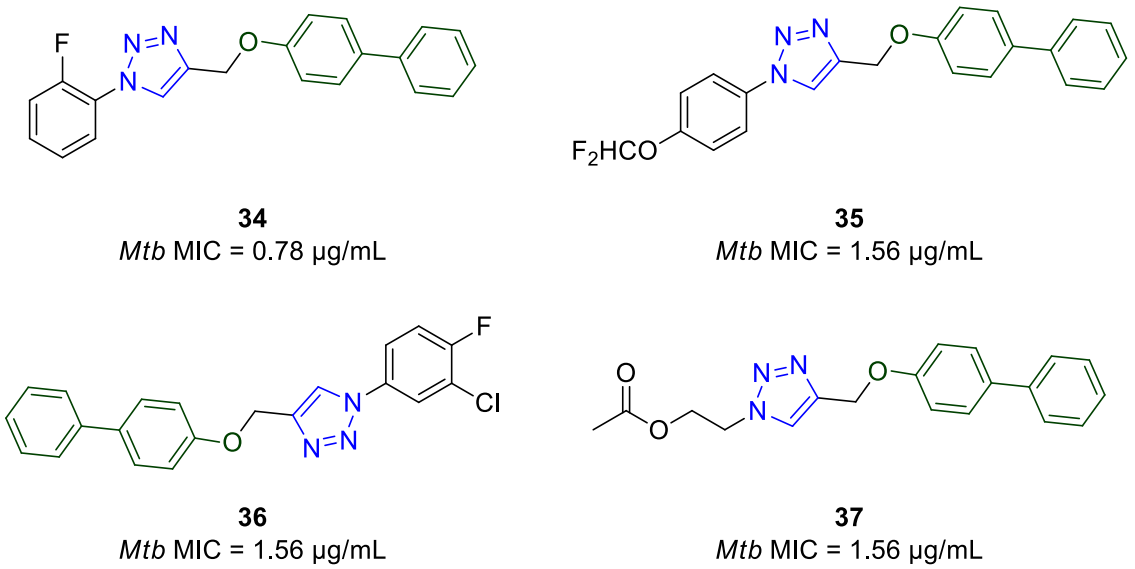
Figure 2-14: Structures of triazole derivatives currently on market; carboxyamidotriazole (**29**), TSAO (**30**), tazobactum (**31**), cefatrizine (**32**), and I-A09 (**33**).

2.9.3 Molecular hybridisation

Drug-resistant pathogens, in this study's case MDR- and XDR-TB, has created a continuous demand for the development of more selective, efficient, and economically accessible pharmaceutical agents (Viegas-Junior *et al.*, 2007; Ali *et al.*, 2017). This demand is pushing

researchers into the direction of molecular hybridisation, an emerging strategy in drug discovery and medicinal chemistry. The molecular hybridisation strategy is essentially based on the chemical combination of two or more pharmacophore moieties of different bioactivities. This results in the creation of a single hybrid molecule that possesses pre-selected characteristics of the initial pharmacophores, improving the efficacy and affinity compared to the parent drugs (Viegas-Junior *et al.*, 2007; Xu *et al.*, 2017). Molecular hybridisation also includes the modulation of undesirable effects into a hybrid, or the incorporation of two agents, with two different therapeutic profiles, into one potentially new dual-acting therapeutic agent. This strategy allows for the activation of different targets by a single molecule, thereby improving the bioavailability and the therapeutic efficacy profile. Molecular hybridisation is an important tool in the innovation of new drugs and the development of new hybrid molecules with novel mechanisms of action (Viegas-Junior *et al.*, 2007; Ali *et al.*, 2017).

In a study conducted by Ali *et al*. (2017), seventeen synthesised 1,2,3-triazole derivatives were screened for *in vitro* anti-TB activity against *Mtb* H37Ra. Six derivatives have shown good activity, with MIC ranging between 3.12 – 0.78 µg/mL and the remaining 11 compounds having MIC less than 12.5 µg/mL. Figure 2.15 indicates the structures of the four compounds (**34** – **37**) with the strongest MIC. The MIC of INH and EMB in the study were 0.025 and 2.00 µg/mL, respectively (Ali *et al.*, 2017).



34
Mtb MIC = 0.78 µg/mL

35
Mtb MIC = 1.56 µg/mL

36
Mtb MIC = 1.56 µg/mL

37
Mtb MIC = 1.56 µg/mL

Figure 2-15: Structures of synthesised 1,2,3-triazole derivatives with strong anti-TB activity (Ali *et al.*, 2017).

Emmadi *et al*. (2015) screened pyrazolo-1,2,3-triazole hybrids for anti-mycobacterial activity against *Mts*. The screening has revealed that some of the synthesised compounds showed promising anti-mycobacterial activity with the MIC values of the compounds ranging from 15 – 95 µg/mL. Compound **38**, **39**, and **40** showed the most promising anti-mycobacterial activity, MIC

15.34, 16.18 and 16.60 µg/mL, respectively, with compound **38** emerging as a potential anti-TB agent with low cytotoxicity (Emmadi *et al.*, 2015), see Figure 2.16.

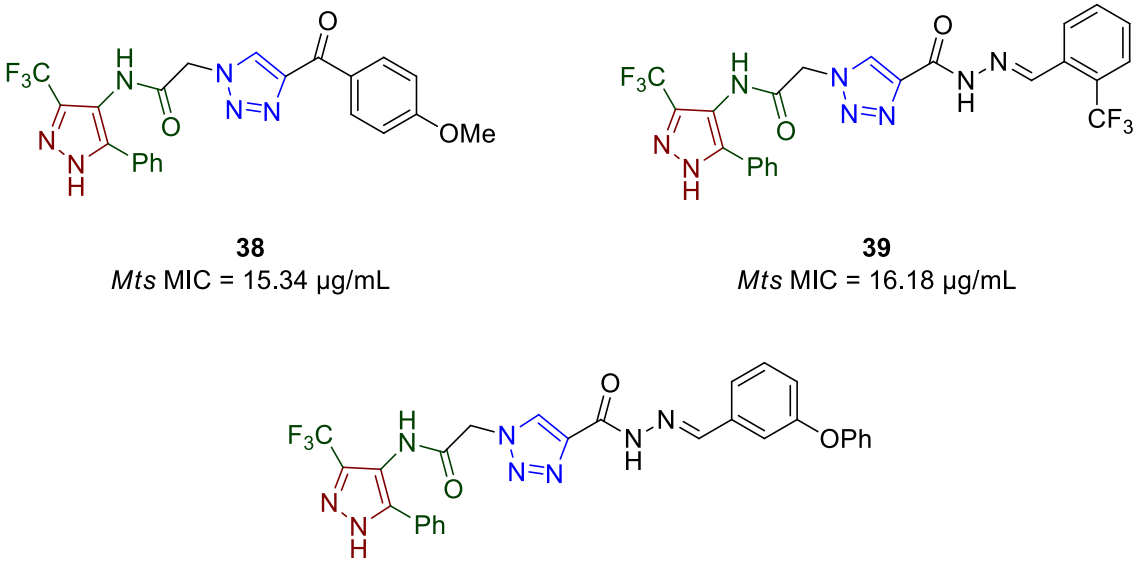


Figure 2-16: Structures of different pyrazolo-1,2,3-triazole hybrids showing promising anti-mycobacterial activity (Emmadi *et al.*, 2015).

Dheer *et al*. (2017), screened 1,2,3-triazole conjugates of 2-mercaptobenzothiazoles for activity against *Mtb* H37Rv. A few of the synthesised compounds (**41** – **43**), structures seen in Figure 2.17, has shown to be potent analogues against the *Mtb* H37Rv strain, with MIC of 8 µg/mL (Dheer *et al.*, 2017).

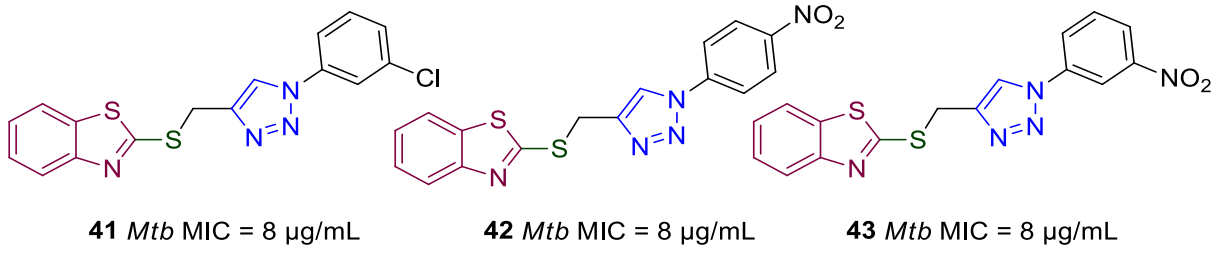
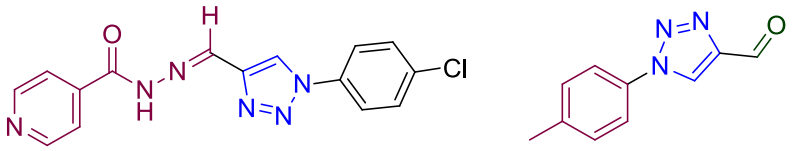


Figure 2-17: Structure of different 1,2,3-triazole conjugates of 2-mercaptobenzothiazoles (Dheer et al., 2017).

The synthesis of 1*H*-1,2.3-triazoles derived from the antifungal agent, econazole, led to the discovery of a different method for optimising *Mtb* specific agents (Dheer *et al.*, 2017). The hydroxyl-triazole compound no longer had antifungal effects, but rather anti-TB activity that is two times more potent than econazole, suggesting that this 1*H*-1,2,3-triazole scaffold could be further utilised to optimise anti-TB agents (Dheer *et al.*, 2017).

Boetchat *et* al. (2011), has shown that various phenyl-1,2,3-triazole derivatives, especially those with isonicotinoyl hydrazide functional groups (**44**), had tuberculostatic activity, with MIC values in the 2.5 – 0.62 µg/mL range (Boechat *et al.*, 2011). Costa *et al*. (2006), also published results on the tuberculostatic activity of a series of 1,2,3-triazoles, of which 1-(methylphenyl)-1,2,3-triazole-4-carbaldehyde (**45**) had the lowest MIC value (2.5 µg/mL) (Costa *et al.*, 2006), see Figure 2.18.

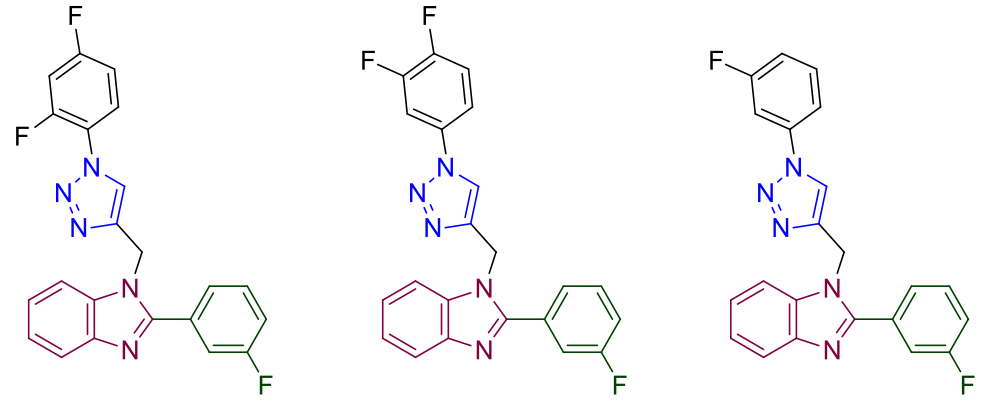


44
Mtb MIC = 0.62 µg/mL

45
Mtb MIC = 2.5 µg/mL

Figure 2-18: Structure of a substituted *N*-phenyl-1,2,3-triazole hybrid containing an isonicotinoyl hydrazide unit (**44**) (Boechat *et al.*, 2011) and 1-(methylphenyl)-1,2,3-triazole-4-carbaldehyde (**45**) (Costa *et al.*, 2006).

Gill *et* al. (2008), revealed the emergence of potent anti-mycobacterial derivatives, owing to the attachment of a 1,2,3-triazole ring to fluorine benzimidazole. The MIC of the most active compounds (**46** – **48**) has been found to be between 0.32 to 0.58 µM of which **46**, at 0.32 µM (0.129 µg/mL), was the most active compound (Gill *et al.*, 2008), see Figure 2.19.



46
Mtb MIC = 0.129 µg/mL

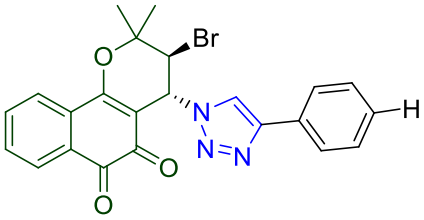
47
Mtb MIC = 0.137 µg/mL

48
Mtb MIC = 0.224 µg/mL

Figure 2-19: Structures of hybrids (**46**, **47** and **48**) from 1,2,3-triazole and benzimidazole pharmacophores (Gill et al., 2008).

Limited research has been done on benzoquinone-triazole linked hybrids and their anti-mycobacterial activity. However, reports on quinone-triazole linked hybrids are not as rare. Jardim *et* al. (2015) conducted a study where lapachone-based 1,2,3-triazoles compounds were

synthesised (**49**). Both the lapachol and β-lapachone were classified as quinones. The afforded compounds and their derivatives were evaluated for their effectiveness against *Mtb*. The β-lapachone-based 1,2,3-triazoles have demonstrated potent anti-mycobacterial activity, with MIC values less than 6.25 µg/mL (Jardim *et al.*, 2015), see Figure 2.20.



49
Mtb MIC < 0.62 µg/mL

Figure 2-20: Structure of β-lapachone-based 1,2,3-triazole hybrid (**49**) (Jardim *et al.*, 2015).

Due to these results, it is hypothesised that the hybridisation of 1,2,3-triazole and BQ scaffolds via copper catalysed azide-alkyne cycloaddition may result in a novel compound with a dual mode of action and enhanced pharmacological features. These features may possess the much-needed improved activity against *Mtb*.

Chapter 3 comprises of a manuscript for publication and reports the details of the synthetic, biological work, as well as the results, analysis, and conclusions of the synthesised anti-mycobacterial hybrids of this project.

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CHAPTER 3

ARTICLE FOR SUBMISSION

Chapter 3 comprises the manuscript of an article to be submitted to the European Journal of Pharmaceutical Sciences. Contained within the article is the Introduction, Materials and methods, Results, Discussion and Conclusion. The article is prepared according to the author's guidelines, accessible in Annexure B and available on the Journal's homepage:

https://www.elsevier.com/journals/european-journal-of-pharmaceutical-sciences/0928-0987/guide-for-authors

SYNTHESIS AND ANTI-TUBERCULAR ACTIVITY OF TRIAZOLE-LINKED 1,4-BENZOQUINONE DERIVATIVES

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The synthesis part of this project was conducted by C-M Horn under the guidance of Dr FJ Smit and Prof DD N'Da. The cytotoxicity was assessed by Dr J Aucamp while the antimycobacterial activity determination was conducted by Ms A Jordaan, Dr R Seldon and Prof Digby Warner.

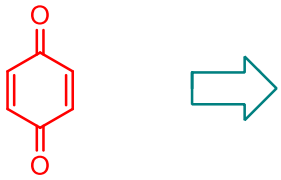
ABSTRACT

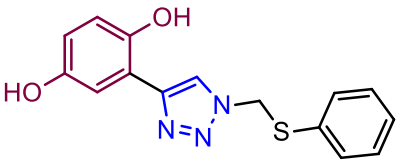
Today tuberculosis continues to reign as one of the world's most lethal infectious diseases, killing approximately 1.6 million people in 2017 and infecting a quarter of the world's population with its latent form. A major factor contributing to the poor control of tuberculosis is the increasing rise of multidrug-resistant strains of the pathogen *Mycobacterium tuberculosis* that has led to the pressing need to develop new and effective anti-tubercular drugs. This need for such agents has led to the investigation of a series of hydroquinone-triazole hybrids of which the design, synthesis and biological activity against the human virulent H37Rv strain of *Mtb* are herein reported. The synthesis of the hybrid molecules followed a two-step process, starting with the formation of an azido intermediate via aromatic nucleophilic substitution and followed by the reaction of the intermediate with various alkynes through 'click' chemistry to form the targeted hybrid molecules.

The hydroquinone-triazole hybrids were nontoxic towards human kidney embryonic (HEK-293) cells, but expressed poor cellular anti-mycobacterial activity. Hybrid **14**, with a strong electron withdrawing thiobenzyl R group and cLogP 3.03, expressed the best activity (MIC_{90} 16.4 µM) with a poor safety profile, being as toxic to mammalian cells as to mycobacteria.

Keywords: *Mycobacterium tuberculosis*, hydroquinone, 1,4-benzoquinone, 1,2,3-triazole, molecular hybridisation, click-chemistry

GRAPHICAL ABSTRACT





p-Benzoquinone

Mtb H37Rv MIC_{90} >125 μM
cLogP 0.20, HEK-293 IC_{50} >100 μM

Hybrid **14**

Mtb H37Rv MIC_{90} 16.38 μM
cLogP 3.03, HEK-293 IC_{50} >100 μM

3.1 Introduction

After decades of effective chemotherapeutic treatment, tuberculosis (TB) still affects and kills millions of people each year. TB is one of the top ten causes of mortality worldwide (WHO, 2018a) and is the leading cause of death from a single infectious disease, surpassing human immunodeficiency virus (HIV) (WHO, 2018b). In 2017 alone, ten million people contracted TB and 1.6 million fatalities were registered, of which more than 95 % occurred in low- and middle-income countries (CDC, 2018a; WHO, 2018a). The majority of the estimated incidence cases occurred in South-East Asia (44 %), and Africa (25 %) (WHO, 2018c), making TB a disease of the developing world.

The use of anti-tubercular drugs is currently the only viable option available for the control of TB, as other control measures (such as the use of Bacillus Calmette-Guérin (BCG) vaccine and TB chemoprophylaxis) appear to be unsatisfactory (du Toit et al., 2006). The existing anti-tubercular drugs, although of immense value in controlling the disease, have several limitations with the most important shortcoming being the emergence of drug-resistant TB strains that renders the frontline drugs inactive (Amir et al., 2014). Drug-resistant TB occurs when *Mycobacterium tuberculosis* (*Mtb*), the causative agent of TB, becomes resistant to at least one of the first-line anti-TB drugs. Multidrug-resistant TB (MDR-TB) occurs when *Mtb* becomes resistant to the two most effective anti-TB drugs, isoniazid and rifampicin (CDC, 2018b). Additionally, MDR-TB patients with further resistance to any fluoroquinolone and at least one of the three injectable second-line drugs (capreomycin, kanamycin, or amikacin) is classified as extensively drug-resistant TB (XDR-TB) (CDC, 2018b). In 2017 alone the WHO revealed an estimate of 558 000 new cases of rifampicin resistance with 82 % suffering from MDR-TB, of which 8.5 % were reported as XDR-TB cases. To make matters worse, only 55 % of MDR-TB cases were treated successfully (WHO, 2018a) during that year. The advent of drug-resistant TB has made the treatment and cure of TB even more complicated.

Drug susceptible TB chemotherapy is a lengthy regimen, spanning over a six – nine month period and consisting of a multidrug therapy that combines four anti-TB drugs, namely rifampicin (RIF), isoniazid (INH), pyrazinamide (PYZ) and ethambutol (EMB) (CDC, 2018c). In contrast, the treatment of drug-resistant TB requires a regimen that consists of at least five effective TB agents during the intensive phase. The combination should comprise PYZ and four core second-line TB agents, namely one fluoroquinolone, one aminoglycoside, one thionamide (ethionamide or prothionamide), and either cycloserine or terizidone (WHO, 2016).

However, apart from drug resistance the current anti-TB drugs possess several limitations, including a high prevalence of adverse side-effects and the inability to act upon latent forms of the *Mtb* bacillus (Amir et al., 2014), leading to patient non-compliance and ultimately to a

discontinuation of therapy with further implications. Adverse side-effects caused by drug therapy may incur substantial additional costs due to outpatient visits, additional tests and, in more serious cases, hospitalisation. Second-line agents often have more severe effects, are less effective, have worse toxicity profiles and are more expensive (Kumar et al., 2014a*;* Yee et al*.*, 2003). The treatment duration using second-line agents is often prolonged by two years (Kumar et al*.*, 2014a), adding additional challenges to patient compliance and resulting in a high risk of treatment failure and relapse (Yee et al., 2003).

These limitations and challenges necessitate an urgent need for new anti-TB drugs with novel mechanisms of action to achieve effective control over the disease (Sajja et al., 2017). A promising strategy for the discovery of such drugs is molecular hybridisation. It is defined as the chemical (covalent) linking of two or more pharmacophores to create a single new chemical entity, with two structural domains and biological functions that may act on different targets (dual drug action) or wherein one part may equipoise the side effects caused by another part (Kumar et al., 2014a*;* Smit et al., 2015). Hybrid molecules have improved efficacy and affinity (Viegas-Junior et al*.*, 2007) and are therapeutically and medicinally more effective than individual components (Smit et al*.*, 2015).

A well-known privileged nucleus that has drawn much attention in drug discovery is the triazole core (Dheer et al., 2017). 1,2,3-Triazole is a five-member *N*-heterocyclic compound (Ali et al*.*, 2017) which has attracted significant attention due to the wide range of biological properties of compounds containing this moiety, including anti-tubercular (Boechat et al*.*, 2011), anti-fungal (Dai et al., 2015), anti-HIV (Mohammed et al*.*, 2016), anti-malarial (Kumar et al., 2014b*;* Singh et al., 2017), and anti-inflammatory (Shafi et al*.*, 2012) activity. The moiety possesses hydrogen bonding capability, moderate dipole character, rigidity and stability under *in vivo* conditions, which all together are responsible for its enhanced biological properties (Zhang et al*.*, 2017).

Interestingly, some triazole containing compounds and isoniazid share a similar mechanism of action as they inhibit microbial cell wall synthesis by blocking lipid biosynthesis (Kumar et al*.*, 2014a*;* Zhang et al*.*, 2017). Drugs currently on the market that possess the 1,2,3-triazole moiety include cefatrizine and tazobactam (antibiotics) (Ali et al*.*, 2017; Zhang et al*.*, 2017), TSAO (anti-HIV) (Zhang et al*.*, 2017), and carboxyamidotriazole (anti-cancer) (Figure 3.1) (Ali et al., 2017; Zhang et al., 2017). Benzofuran salicylic acid derivative (I-A09), a synthesised 1,2,3-triazole derivative, is currently the lead anti-tubercular agent in clinical evaluations and may be used to treat TB in the near future (Ali et al*.*, 2017; Zhang et al., 2017).

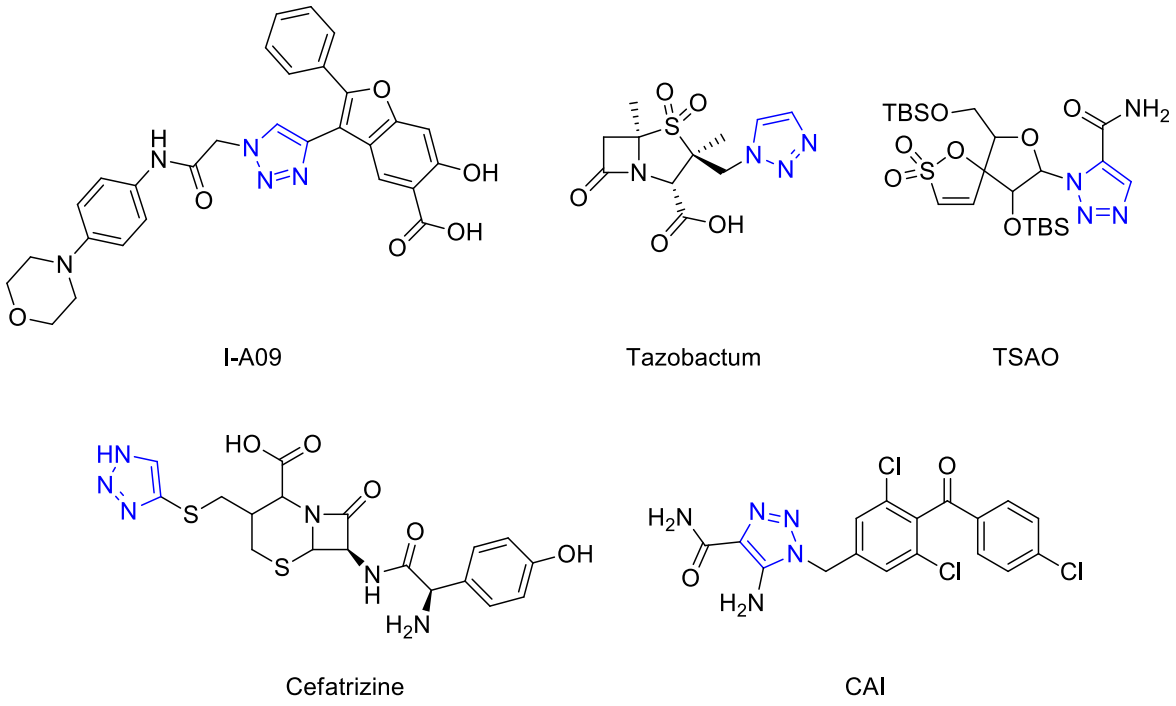
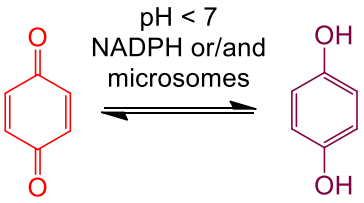


Figure 3-1: Structures of 1,2,3-triazoles currently on the market.

1,4-benzoquinone is the envisaged partner pharmacophore to 1,2,3-triazole investigated in this study. The reduction of benzoquinone to hydroquinone, via semiquinone, occurs spontaneously and by the action of various enzymes in a biological system (Scheme 3.1), the same also being valid for the reverse reaction (hydroquinone ↔ benzoquinone) (McGregor, 2007; Netherlands, 2012). In an aqueous solution 1,4-benzoquinone is reduced to hydroquinone at a significant rate, with the rate being further stimulated by the presence of nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and more so in the presence of NADPH and microsomes (a complete microsomal system). In contrast the autoxidation of hydroquinone is slow, the rate of oxidation is slowed down even further in the presence of NADPH, and stopped entirely by a complete microsomal system. Therefore, in an aqueous medium the main direction of conversion between benzoquinone and hydroquinone is the reduction of benzoquinone, with the complete reduction of benzoquinone to hydroquinone taking place within five minutes in a complete system (NADPH and microsomes) (Souček et al., 2000). Hydroquinone autoxidation in an aqueous medium is pH-dependent, occurring rapidly under alkaline conditions and slowly under acidic conditions (McGregor, 2007; Netherlands, 2012).



1 **2**

Scheme 3-1: The interconversion of benzoquinone (**1**) to hydroquinone (**2**) with conditions favouring the reduction of **1**.

Benzoquinone and hydroquinone are metabolites of each other, with the interconversion rate dependent in prevailing local conditions. Due to this constant interconversion between *p*-benzoquinone and hydroquinone in an aqueous medium (Netherlands, 2012), we justified synthesising hydroquinone-linked 1,2,3-triazoles instead of 1,4-benzoquinone-linked 1,2,3-triazoles.

Benzoquinone is mainly used in the production of hydroquinone, a compound used almost exclusively as an industrial chemical (Netherlands, 2012). Pharmacological properties of benzoquinone compounds include anti-inflammatory (Sagnou et al., 2009), anti-cancer (Lindsey et al., 2004; Tasdemir et al., 2006), anti-viral (Tasdemir et al., 2006), anti-mycobacterial (Jyoti et al., 2016; Tasdemir et al., 2006) and anti-malarial (Tasdemir et al., 2006) activities. Even though limited research has been done on *p*-benzoquinone and hydroquinone, the available literature does suggest that benzoquinone and hydroquinone both possess anti-mycobacterial activity (Jyoti et al., 2016; Tasdemir et al., 2006).

Quinones have a number of biological activities and, although the precise mechanism of action is not fully understood, most of their effects are attributed to redox cycling and the generation of reactive oxygen species (ROS), such as superoxide and hydrogen peroxide, that damage the cell (Tran et al., 2004). ROS directly combat infection by signalling cascades to prompt other protective cellular measures (such as apoptosis) or by causing sever oxidative stress within cells (Tasdemir et al., 2006). Likewise, hydroquinone might affect *Mtb* by disrupting intracellular components such as RNA, DNA, internal proteins, and other organelles (Jyoti et al., 2016).

In this article, we investigated whether the hybridisation of hydroquinone and 1,2,3-triazoles, both pharmacophores with reported anti-TB activity (Ali et al., 2017; Dheer et al., 2017; Emmadi et al., 2015; Jardim et al., 2015; Jyoti et al., 2016; Tasdemir et al., 2006; Tran et al., 2004), will result in derivatives with enhanced anti-mycobacterial potencies comparison to the parent pharmacophores.

3.2 Materials and methods

3.2.1 Materials

p-Benzoquinone, sodium azide (NaN_3), β-cyclodextrin, sodium ascorbate (NaAsc), copper ascorbate (CuAsc), copper sulfate ($CuSO_4$), cyclohexanol, 1-hexyne, 1-heptyne, 1-octyne, 1-decyne, phenylacetylene, tetrahydro-2-(2-propynyloxy)-2H-pyran, methyl propiolate, propargyl alcohol, 4-ethynyltoluene, phenyl propargyl sulphide, sodium nitrite ($NaNO_2$) magnesium sulphate ($MgSO_4$), sodium bicarbonate ($NaHCO_3$) were purchased from Sigma-Aldrich (South Africa). All solvents used – methanol (MeOH), acetone, tetrahydrofuran (THF), ethyl acetate (EtOAc), dichloromethane (DCM), hexane, dimethyl sulfoxide (DMSO) – were purchased from Associated Chemical Enterprises (ACE, South Africa) or from Sigma-Aldrich (South Africa). All chemicals and reagents were of analytical grade and were used without further purification. For inert reactions, DCM was distilled over calcium hydride and stored over 3 Å molecular sieves.

3.2.2 General procedures

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Burker Avance^{TM} III 600 spectrometer at a frequency of 150 MHz, respectively, in deuterated dimethyl sulfoxide (DMSO- d6). Chemical shifts are reported in parts per million (ppm) with the residual protons of the solvent as reference (^{1}H NMR (600 MHz, DMSO) δ 2.5 and ^{13}C NMR (151 MHz, DMSO) δ ^{13}C 40). The splitting pattern abbreviations are as follows: s (singlet), d (doublet), dd (doublet of doublet), t (triplet), q (quartet), p (pentet), and m (multiplet).

High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer that had an atmospheric pressure chemical ionisation (APCI) source set at 200 °C using Bruker Compass DataAnalysis 4.0 software. A full scan, ranging between 50 – 1500 m/z, was generated at a capillary voltage of 4500 V, an end plate offset voltage of 500 V and a collision cell radio frequency (RF) voltage of 100 Vpp.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Melting points (mp) were determined with a BÜCHI melting point B-545 instrument and were uncorrected. Thin layer chromatography (TLC) was performed using silica gel plates ($60F_{254}$), acquired from Merck (South Africa).

High performance liquid chromatography (HPLC) analysis of the final compounds were performed to determine purity. An Agilent 1100 HPLC system equipped with a quaternary pump and an Agilent 1100 series diode array detector were utilized. HPLC-grade acetonitrile (Merck) and Milli-Q water (Millipore) were used for chromatography. A Venusil XBP C18 column (4.60 x 150 mm, 5 µm), with an initial mobile phase (70 % MilliQ water: 30 % acetonitrile), was employed at a flow

rate of 1 ml/min. The concentration of acetonitrile in the mobile phase was linearly increased over a period of five minutes to a final concentration of 85 %. The time allowed for equilibration between runs was five minutes and the duration of each HPLC run was 15 minutes. The concentration of the test compounds injected varied (20 µl of 1 mM to 20 µl of 0.25 mM). The eluent was monitored at wavelengths of 210, 254, and 300 nm.

3.2.3 Synthesis

3.2.3.1 Synthesis of azido intermediate (**3**)

In a single neck round bottom flask, *p*-benzoquinone **1** (18.6 mmol, 2.0 g, 1.00 eq) was dissolved in MeOH (80 mL) upon stirring at -78 ^{o}C (on dry ice), and flushed with argon. Sodium azide, (74.0 mmol, 4.8 g, 4.00 eq) was dissolved in MeOH (30 mL) and pH of the solution was adjusted to 4 using 1M HCl then added portionwise to benzoquinone solution at regular intervals (5 x 10 min) (Scheme 3.2, step i). The resulting mixture was then stirred at -78 ^{o}C under argon for 1.5 h to produce the azide intermediate. The progress of the reaction was monitored using TLC. Upon completion, the solvent was evaporated and $NaHCO_3$ (50 mL) was added to the residue. The crude organic layer was successively extracted with EtOAc (150 mL) and DCM (150 mL). The resulting organic layers were dried over anhydrous magnesium sulphate ($MgSO_4$), filtered, evaporated to dryness. The resulting residue was purified by column chromatography on silica gel eluting DCM/MeOH (9:1, v/v) to yield the isolated azide intermediate.

Dark brown powder; yield: 89 %; ^{1}H NMR (600 MHz, DMSO) δ 9.21 (s, 1H, H-1a), 8.96 (s, 1H, H-4a), 6.67 (d, *J* = 8.7 Hz, 1H, H-3), 6.41 (dd, *J* = 8.7, 2.8 Hz, 1H, H-5), 6.33 (d, *J* = 2.8 Hz, 1H, H-6); ^{13}C NMR (151 MHz, DMSO) δ 150.77 (C-4), 143.08 (C-1), 125.99 (C-2), 117.43 (C-5), 112.80 (C-6), 107.54 (C-3).

3.2.3.2 Synthesis of compounds **4** – **14**

Compounds **4** – **14** were prepared in accordance with the general procedure depicted in Scheme 3.2, step ii and is described as follows:

In a single neck flat bottom flask, alkyne (1.20 eq) was mixed in THF (6 mL), MeOH (6 mL) and distilled H_2O (6 mL). β-Cyclodextrin (0.02 eq), sodium ascorbate (0.20 eq), intermediate **3** (1.00 eq), and copper sulphate (0.10 eq) was consecutively added to the flask. The reaction was left to stir at room temperature for 28 – 29 h (Scheme 1, step ii). The progress of the reaction was monitored using TLC. Afterwards, the solvent was evaporated, the product was extracted with hot EtOAc, washed with $NaHCO_3$, dried over anhydrous $MgSO_4$, and recrystallized using hot EtOAc and hexane, to produce the desired pure compound.

During the optimisation of step (ii) various attempts were made to determine the correct order of addition of the reagents. The reaction temperature and the length of (step (ii)) reaction had to be adjusted to improve the yield. To optimise the yield after purification, purification by column chromatography and recrystallization was attempted, with purification by recrystallization resulting in a greater yield of product.

3.2.3.2.1 2-(4-butyl-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **4**

The reaction with 1-Hexyne afforded hybrid **4** as brown crystals; yield: 42 %; m.p. 159.4 – 162.4 ^{o}C IR ν_{max}: 3185, 3079, 2955, 2579, 1614, 1533, 1467, 1432, 1407 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.71 (s, 1H, H-4a), 9.24 (s, 1H, H-1a), 8.21 (s, 1H, H-11), 7.04 (d, *J* = 2.9 Hz, 1H, H-3), 6.92 (d, *J* = 8.8 Hz, 1H, H-6), 6.74 (dd, *J* = 2.9 Hz, 1H, H-5), 2.69 (t, *J* = 7.6 Hz, 2H, H-12), 1.63 (m, 2H, H-13), 1.36 (dd, *J* = 14.9, 7.4 Hz, 2H, H-14), 0.92 (t, *J* = 7.4 Hz, 3H, H-15); ^{13}C NMR (151 MHz, DMSO) δ 150.55 (C-10), 147.10 (C-4), 141.88 (C-1), 125.09 (C-2), 123.59 (C-11), 118.33 (C-5), 116.88 (C-6), 111.15 (C-3), 31.59 (C-13), 25.09 (C-12), 22.22 (C-14), 14.19 (C-15); HRMS (APCI) *m/z*: $[M+H]^+$ 234.1233 (calcd for $C_{12}H_{16}N_3O_2$: 234.1243); Purity (HPLC): 96 %.

3.2.3.2.2 2-(4-pentyl-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **5**

The reaction with 1-Heptyne gave hybrid **5** as off white crystals; yield: 47 %; m.p. 156.3 – 158.3 ^{o}C; IR ν_{max}: 3180, 3080, 3024, 2588, 1614, 1529, 1473, 1439, 1380 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.70 (s, 1H, H-4a), 9.23 (m, 1H, H-1a), 8.21 (m, 1H, H-11), 7.05 (d, *J* = 2.9 Hz, 1H, H-3), 6.92 (d, *J* = 8.8 Hz, 1H, H-6), 6.73 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 2.69 (t, *J* = 7.7 Hz, 2H, H-12), 1.64 (m, 2H, H-15), 1.33 (q, *J* = 7.1, 3.3 Hz, 4H, H-13, H-14), 0.88 (t, *J* = 7.0 Hz, 3H, H-16); ^{13}C NMR (151 MHz, DMSO) δ 150.56 (C-10), 147.16 (C-4), 141.88 (C-1), 125.10 (C-2), 123.57 (C-11), 118.35 (C-5), 116.89 (C-6), 111.15 (C-3), 31.34 (C-14), 29.12 (C-12), 25.38 (C-13), 22.34 (C-15), 14.38 (C-16); HRMS (APCI) *m/z*: $[M+H]^+$ 248.1411 (calcd for $C_{13}H_{18}N_3O_2$: 248.1399); Purity (HPLC): 97 %.

3.2.3.2.3 2-(4-hexyl-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **6**

The reaction with 1-Octyne produced hybrid **6** as brown crystals; yield: 46 %; m.p. 157.4 – 159.5 ^{o}C; IR ν_{max}: 3180, 3081, 3020, 2583, 1614, 1472, 1388 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.72 (s, 1H, H-4a), 9.24 (s, 1H, H-1a), 8.21 (s, 1H, H-11), 7.06 (d, *J* = 17.9, 2.9 Hz, 1H, H-3), 6.93 (d, *J* = 13.1 Hz, 1H, H-6), 6.74 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 2.68 (t, *J* = 7.6 Hz, 2H, H-12), 1.64 (m, 2H, H-13), 1.29 (m, 6H, H-14, H-15, H-16), 0.87 (t, *J* = 6.9 Hz, 3H, H-17); ^{13}C NMR (151 MHz, DMSO) δ 150.55 (C-10), 147.14 (C-4), 141.87 (C-1), 125.08 (C-2), 123.59 (C-11), 118.33 (C-5), 116.88 (C-6), 111.13 (C-3), 31.50 (C-15), 29.42 (C-14), 28.79 (C-12), 25.42 (C-13), 22.53 (C-16), 14.43 (C-17); HRMS (APCI) *m/z*: $[M+H]^+$ 262.1561 (calcd for $C_{14}H_{20}N_3O_2$: 262.1556); Purity (HPLC): 96 %.

3.2.3.2.4 2-(4-octyl-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **7**

The reaction with 1-Decyne gave hybrid **7** as off white crystals; yield: 52 %; m.p. 146.3 – 150.3 ^{o}C; IR ν_{max}: 3173, 3042, 2958, 2592, 1614, 1477, 1440, 1219 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.71 (s, 1H, H-4a), 9.24 (s, 1H, H-1a), 8.21 (s, 1H, H-11), 7.04 (d, *J* = 2.9 Hz, 1H, H-3), 6.92 (d, *J* = 8.8 Hz, 1H, H-6), 6.73 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 2.68 (t, *J* = 7.6 Hz, 2H, H-12), 1.64 (m, 2H, H-18), 1.29 (m, 10H, H-13, H-14, H-15, H-16, H-17), 0.86 (t, *J* = 7.0 Hz, 3H, H-19); ^{13}C NMR (151 MHz, DMSO) δ 150.55 (C-10), 147.19 (C-4), 141.86 (C-1), 125.08 (C-2), 123.60 (C-11), 118.32 (C-5), 116.87 C-6), 111.12 (C-3), 31.76 (C-17), 29.46 (C-16), 29.26 – 29.23 (C-15), 29.20 (C-14), 29.14 (C-12), 25.52 – 25.33 (C-13), 22.58 (C-18), 14.44 (C-19); HRMS (APCI) *m/z*: $[M+H]^+$ 290.1862 (calcd for $C_{16}H_{24}N_3O_2$: 290.1869); Purity (HPLC): 97 %.

3.2.3.2.5 2-(4-phenyl-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **8**

The reaction with phenylacetylene afforded hybrid **8** as brown-yellow crystals; yield: 70 %; m.p.257.0 – 261.7 ^{o}C; IR ν_{max}: 3194, 3080, 3028, 2584, 1610 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.81 (s, 1H, H-4a), 9.30 (s, 1H, H-1a), 8.91 (s, 1H, H-11), 7.95 (m, 2H, H-13, H-17), 7.48 (t, *J* = 7.7 Hz, 2H, H-14, H-16), 7.37 (t, *J* = 7.4 Hz,1H, H-15), 7.08 (d, *J* = 2.9 Hz, 1H, H-3), 6.98 (d, *J* = 8.8 Hz, 1H, H-6), 6.81 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5); ^{13}C NMR (151 MHz, DMSO) δ 150.58 (C-10), 146.53 (C-4), 142.42 (C-1), 131.05 (C-12), 129.41 (C-2), 128.44 (C-14, C-16), 125.80 (C-15), 124.90 (C-13, C-17), 123.27 (C-11), 118.36 (C-5), 117.51 (C-6), 111.66 (C-3); HRMS (APCI) *m/z*: $[M+H]^+$ 254.0917 (calcd for $C_{14}H_{12}N_3O_2$: 254.0930); Purity (HPLC): 98 %.

3.2.3.2.6 2-(4-(*p*-tolyl)-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **9**

The reaction with 4-ethynyltoluene gave hybrid **9** as brown crystals; yield: 58 %; m.p. 275.7 – 276.7 ^{o}C; IR ν_{max}: 3188, 3074, 3025, 2581, 1615, 1474 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.81 (s, 1H, H-4a), 9.31 (s, 1H, H-1a), 8.85 (s, 1H, H-11), 7.84 (d, *J* = 8.1 Hz, 2H, H-13, H-17), 7.29 (d, *J* = 7.9 Hz, 2H, H-14, H-16), 7.07 (d, *J* = 2.9 Hz, 1H, H-3), 6.97 (d, *J* = 8.8 Hz, 1H, H-6), 6.80 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 2.35 (s, 3H, H-18); ^{13}C NMR (151 MHz, DMSO) δ 150.55 (C-10), 146.58 (C-4), 142.39 (C-1), 137.75 (C-15), 129.96 (C-12), 128.26 (C-2), 125.72 (C-14, C-16), 124.91 (C-13, C-17), 122.84 (C-11), 118.32 (C-5), 117.43 (C-6), 111.63 (C-3), 21.35 (C-18); HRMS (APCI) *m/z*: $[M+H]^+$ 268.1096 (calcd for $C_{15}H_{14}N_3O_2$: 268.1086); Purity (HPLC): 98 %.

3.2.3.2.7 Methyl 1-(2,5-dihydroxyphenyl)-1*H*-1,2,3-triazole-4-carboxylate; **10**

The reaction with methyl propiolate produced hybrid **10** as fluffy off white crystals; yield: 69 %; m.p. 238.0 – 238.4 ^{o}C; IR ν_{max}: 3177, 2953, 1693, 1556, 1475, 1212 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.97 (s, 1H, H-4a), 9.36 (s, 1H, H-1a), 9.02 (s, 1H, H-11), 7.07 (d, *J* = 2.9 Hz, 1H, H-3), 6.96 (d, *J* = 8.8 Hz, 1H, H-6), 6.82 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 3.88 (s, 3H, H-14); ^{13}C NMR

(151 MHz, DMSO) δ 161.17 (C-12), 150.57 (C-4), 142.40 (C-1), 138.87 (C-10), 130.63 (C-2), 124.09 (C-11), 118.39 (C-5), 118.07 (C-6), 111.48 (C-3), 52.39 (C-14); HRMS (APCI) *m/z*: $[M+H]^+$ 236.0668 (calcd for $C_{10}H_{10}N_3O_4$: 236.0671); Purity (HPLC): 96 %.

3.2.3.2.8 2-(4-(hydroxymethyl)-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **11**

The reaction with propargyl alcohol gave hybrid 11 as dark brown crystals; yield: 29 %; m.p. 228.0 – 233.6 ^oC; IR ν_{max}: 3357, 3183, 3085, 1615, 1472 cm^{-1}; ^1H NMR (600 MHz, DMSO) δ 9.77 (s, 1H, H-4a), 9.25 (s, 1H, H-1a), 8.33 (s, 1H, H-11), 7.06 (d, *J* = 2.9 Hz, 1H, H-3), 6.93 (d, *J* = 8.8 Hz, 1H, H-6), 6.75 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 5.26 (t, *J* = 5.3 Hz, 1H, H-13), 4.60 (d, *J* = 4.8 Hz, 2H, H-12); ^{13}C NMR (151 MHz, DMSO) δ 150.58 (C-10), 148.16 (C-4), 141.92 (C-1), 124.98 (C-2), 124.49 (C-5), 118.37 (C-11), 117.04 (C-2), 111.17 (C-3), 55.43 (C-12); HRMS (APCI) *m/z*: $[M+H]^+$ 208.0727 (calcd for $C_9H10N_3O_3$: 208.0722); Purity (HPLC): 98 %.

3.2.3.2.9 2-(4-(1-hydroxycyclohexyl)-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **12**

The reaction with cyclohexanol produced hybrid **12** as fluffy off white crystals; yield:43 %; m.p. 46.2 – 46.3 ^oC; IR ν_{max}: 3263, 3171, 3074, 3025, 2857, 1614, 1494, 1473 cm^{-1}; ^1H NMR (600 MHz, DMSO) δ 9.76 (s, 1H, H-4a), 9.25 (s, 1H, H-1a), 8.25 (s, 1H, H-11), 7.08 (d, *J* = 2.9 Hz, 1H, H-3), 6.93 (d, *J* = 8.8 Hz, 1H, H-6), 6.74 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 4.98 (s, 1H, H-12a), 1.92 (m, 2H, H-15), 1.72 (m, 4H, H13, H-17), 1.49 (m, 4H, H-14, H-16); ^{13}C NMR (151 MHz, DMSO) δ 156.15 (C-4), 150.59 (C-10), 141.68 (C-1), 125.03 (C-2), 122.56 (C-11), 118.42 (C-5), 116.86 (C-6), 110.93 (C-3), 68.51 (C-12), 38.26 (C-13, C-17), 25.72 (C-15), 22.10 (C-14, C-16); HRMS (APCI) *m/z*: $[M+H]^+$ 276.1334 (calcd for $C_{14}H_{18}N_3O_3$: 276.1348); Purity (HPLC): 97 %.

3.2.3.2.10 2-(4-(((tertahydro-2*H*-pyran-2-yl)oxy)methyl)-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **13**

The reaction with tetrahydro-2-(2-propynyloxy)-2*H*-pyran gave hybrid **13** as brown-red crystals; yield: 45 %; m.p. 140.1 – 141.3 ^oC; IR ν_{max}: 3179, 3084, 2584, 1720, 1614, 1473, 1019 cm^{-1}; ^1H NMR (600 MHz, DMSO) δ 9.81 (s, 1H, H-4a), 9.29 (s, 1H, H-1a), 8.45 (s, 1H, H-11), 7.05 (d, *J* = 2.9 Hz, 1H, H-3), 6.93 (d, *J* = 8.8 Hz, 1H, H-6), 6.76 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 4.77 (d, *J* = 12.2 Hz, 2H, H-12), 4.58 (d, *J* = 12.1 Hz, 1H, H-14), 3.83 (t, 2H, H-16), 3.50 (t, 2H, H-16), 1.67 (m, 2H, H-19), 1.50 (m, 4H, H-17, H-18); ^{13}C NMR (151 MHz, DMSO) δ 150.56 (C-4), 144.07 (C-10), 142.04 (C-1), 125.85 (C-2), 124.84 (C-11), 118.33 (C-5), 117.22 (C-6), 111.32 (C-3), 97.69 (C-14), 61.78 (C-16), 59.89 (C-12), 30.55 (C-19), 25.47 (C-17), 19.47 (C-18); HRMS (APCI) *m/z*: $[M+H]^+$ 292.1314 (calcd for $C_{14}H_{18}N_3O_4$: 292.1297); Purity (HPLC): 97 %.

3.2.3.2.11 2-(4-((phenylthio)methyl)-1*H*-1,2,3-triazol-1-yl)benzene-1,4-diol; **14**

The reaction with phenyl propargyl sulphide afforded hybrid **14** as brown-black crystals; yield: 23 %; m.p. 151.8 – 153.0 ^{o}C; IR v_{max}: 3177, 3074, 2595, 1719, 1614 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ 9.79 (s, 1H, H-4a), 9.26 (s, 1H, H-1a), 8.33 (s, 1H, H-11), 7.42 (d, *J* = 7.7 Hz, 2H, H-14, H-18), 7.33 (t, *J* = 7.7 Hz, 2H, H-15, H-17), 7.20 (t, *J* = 7.4 Hz, 1H, H-16), 7.05 (d, *J* = 2.9 Hz, 1H, H-3), 6.92 (d, *J* = 8.8 Hz, 1H, H-6), 6.75 (dd, *J* = 8.8, 2.9 Hz, 1H, H-5), 4.37 (s, 2H, H-12); ^{13}C NMR (151 MHz, DMSO) δ 150.59 (C-4), 143.77 (C-1), 141.78 (C-10), 136.30 (C-13), 129.49 (C-14, C-18), 128.75 (C-15, C-17), 126.42 (C-2), 124.94 (C-16), 124.74 (C-11), 118.40 (C-5), 117.17 (C-6), 110.95 (C-3), 27.58 (C-12); HRMS (APCI) *m/z*: $[M+H]^{+}$ 300.0808 (calcd for $C_{15}H_{14}N_3O_2S$: 300.0807); Purity (HPLC): 92 %.

3.3 Biological evaluation

3.3.1 *In vitro* anti-mycobacterial assay

The *in vitro* anti-mycobacterial activity assay of the synthesised compounds was carried out in one culture medium, 7H9 CAS Tx, using reported literature (Stringer et al., 2017). The 7H9 GLU CAS Tx medium is comprised of a standard 7H9 base, supplemented with casitone (CAS) and tyloxapol (Tx) as surfactant. CAS does not contain albumin, therefore there is no proxy for protein binding.

3.3.2 *In vitro* cytotoxicity assay

Human embryonal kidney (HEK-293) cells were cultured in Hyclone Dulbecco's modified Eagle's medium with high glucose supplemented with 10 % fetal bovine serum (Thermofishe Scientific) and 1 % L-glutamine (Lonza), penicillin-streptomycin (Lonza), amphotericin B (Lonza) and non-essential amino acids (Lonza). The cells are maintained in a humidified atmosphere at 37 °C and 5 % CO_2. For the MTT assay, 96 well plates were prepared with 200 µL of cell suspension (25 000 cells/mL) and incubated for 24 hours. The growth medium was then removed and the cells treated with: (1) 100 µL of emetine dihydrochloride solution diluted with growth medium to the necessary concentrations (positive control); (2) 80 µL of growth medium and 20 µL of solvent (negative control to compensate for possible solvent effects); (3) 80 µL of growth medium and 20 µL of experimental compound solutions. Blanks contained growth medium without cells. The treated plates were incubated for 48 hours.

Due to light sensitivity of MTT reagent, the assay was performed in the dark. Sterile-filtered MTT solution (20 µL of 1 mg/mL solution in PBS) was added to initiate the MTT assay, the plates covered with aluminium foil and incubated for four hours. The growth medium-MTT mixture was then aspirated and 100 µL of 2-propanol added to dissolve purple formazan crystals. The contents

were gently mixed for 5 minutes at room temperature. Absorbance was measured at 560 and 650 nm using the Thermofisher Scientific GO Multiscan plate reader. Data analysis was performed for each biological replicate using SkanIt 4.0 Research Edition software. Background absorbance (650 nm) was subtracted from absorbance values (560 nm), the mean absorbance calculated and the percentage cell viability was determined by the following equation:

Cell viability % = (Δ Abs sample – Δ Abs blank) / (Δ Abs neg control – Δ Abs blank) X 100.

For the final IC_{50} of each compound, the mean IC_{50} of three biological replicates were calculated in GraphPad Prism 5.

3.4 Results and discussion

3.4.1 Chemistry

We envisaged to investigate a series of benzoquinone-1,2,3-triazole hybrids by primarily focusing on compounds resulting from the direct binding of both scaffolds. Three possible synthetic routes to bring about these hybrids were considered. First, an 2-azidobenzophenone intermediate is formed from commercially available benzoquinone, then the intermediate is subjected to click chemistry with various alkynes to afford the target hybrids in an overall two-step process. Second, 2-azido hydroquinone intermediate is formed from commercial hydroquinone. It then undergoes click chemistry with various alkynes to give hydroquinone-triazole hybrids, which are further oxidised into the benzoquinone-triazole compounds – an overall three-step process (Guimarães et al., 2013; Dixit et al., 2012). However, considering the interconversion between benzoquinone and hydroquinone (Netherlands, 2012) and the non-availability of commercial hydroquinone, a third synthetic route combining the first two routes was adopted. It is depicted in Scheme 3.2 and is described as follows: a series of hydroquinone-1,2,3-triazole derivatives were synthesised using a two-step process. In the first step 2-azidohydroquinone, also referred to as azidoquinol intermediate, was synthesised via acid-catalyzed aromatic nucleophilic substitution and reduction using a modified method (Guimarães et al., 2013). The reaction was performed in methanolic acid medium (pH 4) that favoured the reduction of the benzoquinone moiety (Netherlands, 2012).

Before a final and successful method for step (i) was adopted, various modifications were introduced to the published method; room temperature revealed to be too exothermic thus the reaction was run at -78 ^{o}C.

Likewise, the volume of the solvent (MeOH) and the reaction time of step (i) were adjusted and optimal yield of the azido-intermediate was obtained with the adopted solvent volume and time figures.

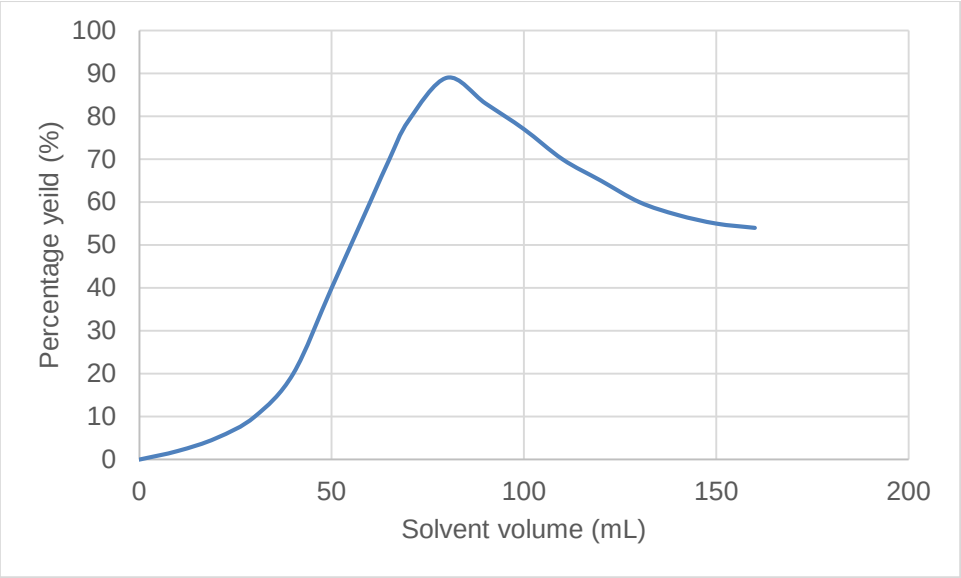


Figure 3-2: The effect solvent volume on the optimisation of step (i).

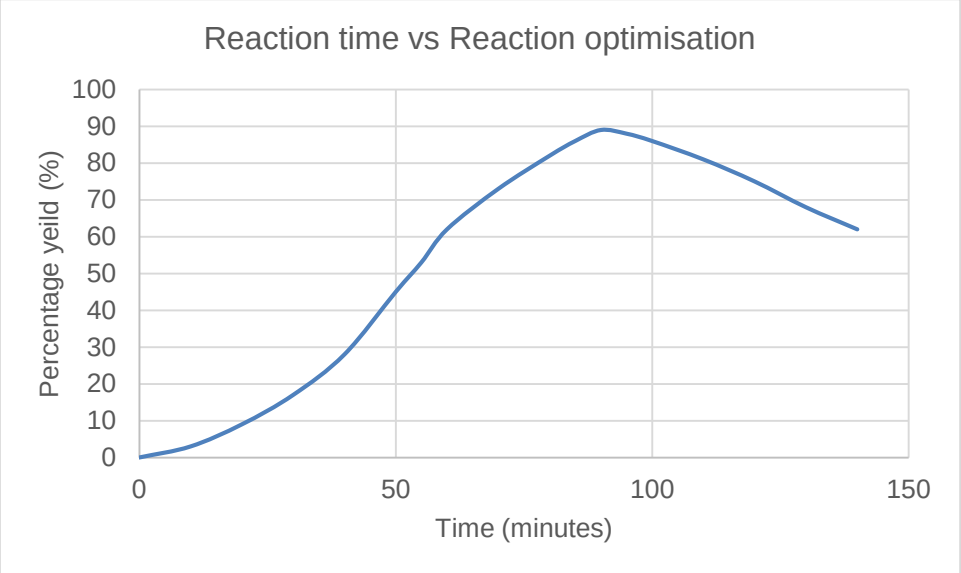
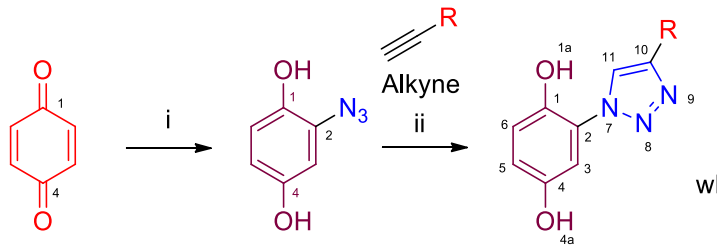


Figure 3-3: The effect of reaction time on the optimisation of step (i).

In the second step the azido intermediate was reacted with various individual alkynes through Huisgen's copper-catalysed alkyne-azide cycloaddition (CuCAAC) reaction, or otherwise referred to as click-chemistry, to produce different hydroquinone-1,2,3-triazole hybrids. The CuCAAC reaction employed is adapted from a procedure previously reported (Dixit et al., 2012). All synthesised hybrids (Table 3.1) were produced in poor to good yields (23 – 70 %) after purification by recrystallization with hexane. Structurally, all hybrids differ by the substituent at C-10 of the triazole ring.

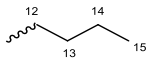
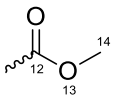
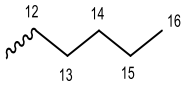
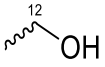
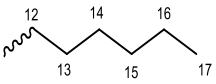
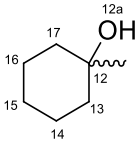
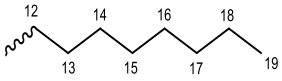
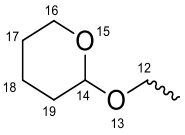
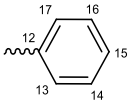
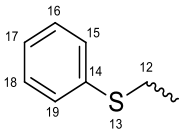
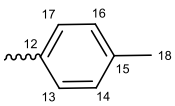


1 **3** **4 - 14**

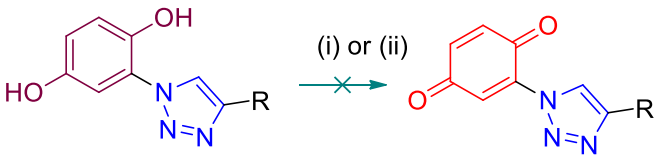
1,4-*p*-Benzoquinone 2-Azido hydroquinone Hybrids

Scheme 3-2: General reaction procedure of hydroquinone-1,2,3-triazole hybrids.

Reagents and conditions: (i) **1**: *p*-benzoquinone (1.00 eq), MeOH, -78 °C, NaN_3 (4.00 eq)/MeOH sol. (pH~4), 1.5 h. (ii) **4** – **14**: alkyne (1.20 eq), THF, MeOH, H_2O, β-cyclodextrin (0.02 eq), sodium ascorbate (0.20 eq), **3** (1 eq) and $CuSO_4$ (0.10 eq), rt, 28 h.

Compd	R	Compd	R
4		**10**	
5		**11**	
6		**12**	
7		**13**	
8		**14**	
9			

Two oxidative routes were attempted to convert the hydroquinone-triazole hybrids **4** – **14** to benzoquinone hybrids (Scheme 3.3). These include the copper-catalysed hydroquinone oxidation (Li & Trush, 1993) and oxidation by nitrogen oxide (Bosch et al., 1994), as illustrated below.



Scheme 3-3: Attempted routes of oxidative conversion of hydroquinone to benzoquinone hybrids.

Reagents and conditions: (i) MeOH, CuAsc (1.00 eq), O_2, reflux, 1 h, 60 ^oC then 4 h, rt; (ii) THF, $NaNO_2$, O_2, HCl 32 % (two drops), 46 h, rt.

p-Hydroquinone is electron rich and serves as a potential electron donor (Burner et al., 2000). The 1,2,3-Triazole ring, on the other hand, is a π-conjugated electron-deficient system; therefore, an electron withdrawing group by resonance. Thus, the direct conjugation of triazole to the quinol ring should logically favour oxidation of the latter, especially where R is also electron-withdrawing by induction. However, following the failure of these reactions, the investigation was pursued with the hydroquinone-triazole hybrids.

The formation of the target compounds was confirmed by routine chemical structure elucidation techniques, NMR, HRMS and IR.

The ^1H NMR spectra of all title compounds were thoroughly examined for characteristic signals, evidence of the hydroquinone and triazole scaffolds. The hydroquinone moiety was clearly identified in the ^1H spectra of all hybrids by the presence of two singlets at 9.7 and 9.2 ppm, attributed to quinolic protons H-4a and H-1a, respectively. Additionally, two doublets at 7.0 and 6.9 ppm attributed to H-3 and H-6, in *m*- and *o*-position, respectively of the benzene ring, and a doublet of doublets *ca* 6.7 ppm, attributed to the resonance of H-5, caused by coupling with proton H-3 and H-6, were also present in the spectra. The triazole scaffold is clearly identified by the singlet peak at 8.2 ppm, attributed to the resonance of the triazolyl proton H-11.

The ^{13}C NMR spectra further confirmed the quinol moiety in the structures of the hybrids as evidenced by the presence of six pronounced peaks at 147.1, 141.6, 125.0, 118.3, 116.8, and 111.1 ppm attributed to the resonance of aryl carbons, C-4, C-1, C-2, C-5, C-6, and C-3 of hydroquinone moiety. Further evidence of the total conversion of benzoquinone to hydroquinone is the absence of carbonyl carbons peaks in the spectra. The ^{13}C NMR spectra, likewise, further support the presence of the triazole with two singlet peaks at 150.5 and 122.5 ppm pertaining to the resonance of triazolyl carbons, C-10 and C-11.

The IR-spectra of all compounds were also inspected for the presence of characteristic absorptions, allowing for the identification of functional groups. The IR spectra clearly illustrate the vibration of the alcohol OH functional groups of quinol indicated as variable broad and sharp peaks at 3000 – 3200 cm^{-1} and a strong stretch, corresponding with the C=C bond of the triazole moiety, in 1550 – 1650 cm^{-1}.

HRMS using APCI ion source show the presence of $[M+H]^+$ in the spectra that confirmed the presence of M^+ molecular ion of each hybrid. The experimental *m/z* values of compound corresponded to the exact mass calculated via ChemBioDraw.

3.4.2 Physiochemical properties

Transmembrane transport gives a good indication of the biological properties, such as oral bioavailability, cellular uptake, receptor affinity, protein binding, toxicity, and pharmacological activity of a compound (Gombar & Enslein, 1996). An ideal drug must possess well-equipoised hydrophilic/lipophilic properties, so as to efficiently permeate biological membranes and be absorbed into the systemic circulation (Lipinski et al., 1997) . The *n*-octanol/water partition coefficient (logP) is a key parameter used in the measurement of the hydrophilicity and lipophilicity of a chemical, allowing one to predict the transport characteristics of a substance across biological membranes through passive diffusion (Gombar & Enslein, 1996). LogP values between one and five are often targeted, with values between one and three being ideal (Lipinski et al., 1997).

All compounds, **4 – 15**, showed good drug-like properties with cLogP values within the targeted range. However, these cLogP values were merely an estimate of the lipophilic/hydrophilic characteristics of the synthesised hybrids. This implies that with the cLogP values in the targeted range, these hybrids may still be inactive as biological activity is dependent of many parameters aside from physicochemical properties (Pop et al., 2004)

3.4.3 Biological activities

Before a novel drug can advance to the use in humans, a series of preclinical studies (*viz*. a sequence of *in vitro* assays, followed by a series of *in vivo* assays) must be completed (Franzblau et al., 2012). The mycobacterium growth inhibitory potential of the hybrids was assessed using a Green Fluorescent Protein-expression (GFP) assay. The GFP assay used a glucose-based Middlebrook 7H9-CAS broth base as growth medium for tubercle bacilli.

The minimum inhibitory concentration of each hybrid that is required to inhibit the growth of 90 % of mycobacteria of H37Rv, expressed as MIC_{90}, is shown in Table 3.2, alongside rifampicin (RIF) as anti-tubercular standard. In addition, HEK-293 cells were used to determine the cytotoxicity of the compounds, alongside cytotoxic drug emetine as reference (Table 3.1).

Table 3-1: ***In vitro*** **anti-mycobacterial activities as well as cytotoxicity of hybrids 4 – 14, benzoquinone (1) and hydroquinone (2) against H37Rv strain using GFP assay in 7H9 GLU CAS medium.**

Compd	cLogP[a]	Anti-mycobacterial activity, MIC_{90} (μM)[b]	Cytotoxicity IC_{50} (μM)[c] HEK-293	Selectivity index, SI[d]
RIF	3.71	0.075		
1	0.20	>125	>100	
2	0.80	>125	>100	
4	2.76	>125	>100	
5	3.29	>125	>100	
6	3.82	>125	17.5 ± 2.1	
7	4.88	>125	71.0± 7.7	
8	3.00	>125	77.6 ± 7.2	
9	3.50	33.84	41.9 ± 7.9	1
10	1.21	>125	>100	
11	-0.12	>125	>100	
12	1.89	>125	>100	
13	1.20	>125	>100	
14	3.03	16.38	>100	6
EM			0.01± 0.001	

[a]cLogP values calculated using ChemBioDraw Ultra Version 12.0; [b]compounds screened in media: 7H9 GLU CAS; [c]HEK-293 cell line; [d]Selectivity Index (SI) = HEK293/H37Rv; EM: emetine.

None of the hybrids in the series possessed notable anti-mycobacterial activity. All *n*-alkyl chain hybrids were inactive and so were the precursor scaffolds, *p*-benzoquinone and hydroquinone with MIC_{90} values greater than 125 μM. Only compounds **9** and **14** had MIC_{90} values below 125 μM and thus were considered active. Hybrids **14** and **9** with MIC_{90} 16.38 and 33.84 μM, respectively, were the first and second most active hybrids in the series, respectively. Anti-mycobacterial active hybrid **9** had a *para*-benzene substituted electron donating methyl group. However, removal of this methyl group resulted in hybrid **8** that was found to be completely inactive, having MIC_{90} greater than 125 μM. Hybrid **14**, on the other hand, had a thiobenzyl

substituent that is the strongest electron withdrawing group. Hybrids **10** and **13** possessed strong electron withdrawing and donating groups, respectively, and yet were inactive. This leads to the inference that the biological activity of these compounds might not have been controlled by electrochemical properties. However, due to the limited number of active compounds relative to inactive compounds it is difficult to make any definitive conclusions.

All synthesised hybrids showed favourable drug-like properties, with all hybrids except **11** having cLogP values in the targeted area (one – five) and thus were expected to be efficiently transported into the mycobacterium through passive diffusion. However, none of the hybrids showed any activity against *Mtb*. The unique, lipid rich (Glickman & Jacobs, 2001; Knechel, 2009) cell wall structure of mycobacteria is crucial to the pathogen's survival (Knechel, 2009). Therefore, lipophilicity of a drug is an imperative consideration in the design and activity of novel anti-mycobacterial drugs (Suresh et al., 2014). The anti-mycobacterial activity could, therefore, be enhanced by improving the lipophilic property of a drug *viz.* the attachment of a bulky lipophilic moiety facilitating diffusion through bio-membranes. However, similarly to the current study, hybrids that possessed a good lipophilic character, such as bis-1,2,3-triazole, exhibited poor anti-TB activities, suggesting that lipophilicity might not be the sole parameter modulating anti-TB activity (Zhang et al., 2017).

A structure activity relationship (SAR) study done by Nagesh et al., (2013) on various 6-(4-((substituted-1*H* -1,2,3- triazol-4-yl)methyl)piperazin-1-yl)phenanthridine derivatives revealed that the addition of a functional group that is capable of acting as a hydrogen bond acceptor, preferably through its lone pair, might enhance binding interactions and, therefore, improve anti-mycobacterial activity (Gill et al., 2008; Nagesh et al., 2013), presuming it is inhibiting the same target. Hybrid **14**, through its thiobenzyl group of which the S atom acts as H-acceptor, corroborates this early finding.

However, it is hypothesised that the inactivity of the compounds could most likely be attributed to their structural rigidity that obstructs the hybrid at one of two places, namely (a) the binding site of the compound or, more plausibly, (b) its permeation through the bacterium cell wall. Indeed, with regards to (a), as previously stated, triazoles and isoniazid share a similar mechanism of action that inhibits the growth of bacteria by blocking lipid biosynthesis (Zhang et al., 2017) through inhibition of enoyl-acyl carrier protein reductase (inhA) protein (Dheer et al., 2017). Site II, a key region in *InhA* binding site has a flexible hydrophobic pocket that accommodates long alkyl chains. This flexible pocket allows for significant movement of alkyl side chain (Campaniço et al., 2018). The extension of InhA substrate-competitive inhibitors into the hydrophobic pocket increases the potency and significantly enhances lipophilicity of synthesised inhibitors (Shirude et al., 2013). The triazole moiety, however, is rigid (Zhang et al., 2017), so is the quinol, and ultimately the synthesised hybrids. The poor anti-mycobacterial activity of the synthesised hybrids

may thus be ascribed to their commonly shared rigidity. No significant movement is able to take place within the compound and, therefore, interaction with the binding site is impeded. The addition of a linker between the two pharmacophores could improve the flexibility and, therefore, the interaction of the hybrids with the binding site, which may result in an enhanced, more potent anti-mycobacterial effect.

Furthermore, with regards to (b), before anti-mycobacterial drugs can reach their respective target sites within *Mtb* they must first penetrate the *Mtb* cell wall. The architecture of the *Mtb* cell wall is dominated by a variety of lipids and carbohydrates that form an impermeable barrier, especially to hydrophilic anti-microbials (Bhat et al., 2017). A method by which solutes penetrate the cell wall is through water-filled porin channels. Nevertheless, the influx of solutes through porins pose several restrictions. Only solutes that are long and flexible with high molecular weights and small cross-sections are able to slowly pass through porin channels, twisting their way through the narrow channel (Nikaido, 2001). The rigidity of the synthesised hybrids may, therefore, impede their transportation across the porin channel, which may also explain the observed activity.

Benzoquinone (**1**), hydroquinone (**2**) and the hybrids (exception of **6** – **9**) in general showed no toxicity to the HEK-293 cells, whereas hybrids **6** – **9** displayed mild to moderate toxicity. This may be ascribed to their relatively higher lipophilicity with cLogP values, varying in the three – five range. In the alkyl chain-substituted sub-series**,** the short chain containing hybrids **4** (n = 4) and **5** (n = 5) were found to be non-cytotoxic. However, a further increase in chain length resulted in a cytotoxicity decrease, with **7** (n = 8; IC_{50} 71.0 µM) being less toxic than **6** (n = 6; IC_{50} 17.5 µM). Hybrids **6** and **7** possessed mild and moderate toxicities, respectively.

The most anti-mycobacterial active hybrid **14** (MIC_{90} 16.38 µM) showed no toxicity to HEK-293 cells, but had a poor selectivity towards the bacteria (SI = 6). All together, these biological features disqualify this hybrid as a potential anti-mycobacterial hit (Katsuno et al., 2015).

3.5 Conclusion

A series of benzoquinone-triazole hybrids linking directly *p*-1,4-benzoquinone and 1,2,3-triazole scaffolds were initially targeted. However, the adoption of acidic experimental medium coupled with unsuccessful oxidation of hydroquinone to benzoquinone ultimately resulted in the investigation of hydroquinone-1,2,3-triazole hybrids. A series of novel quinone-1,2,3-triazole derivatives were synthesised in poor to moderate yields following a two-step process that included an aromatic nucleophilic substitution in methanolic acid medium from commercial benzoquinone, followed by Huisgen's copper-catalysed azide-alkyne cycloaddition.

Routine characterisation techniques (NMR, IR, HRMS) served to confirm the structures. The anti-mycobacterial activity of the hybrids was evaluated *in vitro* against human virulent *Mtb* H37Rv

strain using a GFP assay in Middlebrook 7H9 broth media. The cytotoxicity of the compounds was also assessed using HEK-293 cells. Even though all hybrids showed good drug-like properties, they were found to be mostly inactive. The most active hybrid, **14** featuring *para*-methylbenzyl substituent, although non-toxic to mammalian cells, possessed moderate anti-mycobacterial activity (MIC_{90} 16.38 µM) and poor bacterial selective action. Thus, no anti-tubercular hit was discovered during this study.

Upon analysis of the biological data, it could be deduced that *Mtb* activity was structure-specific. The hybrids were all rigid in structure; therefore, the inactivity could be linked this rigidity as a consequence of their inability to permeate through the bacterium cell wall. It could be speculated that the addition of a linker between the two pharmacophores to improve the flexibility might endow the resulting hybrids with anti-mycobacterial activity, effective cell wall permeation and better interaction with the binding site.

Disclaimer

Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and therefore the NRF does not accept any liability in regard thereto.

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CHAPTER 4

SUMMARY AND CONCLUSION

Tuberculosis (TB) is a global problem, instilling an enormous burden on the general health of the public, affecting ten million and killing 1.6 million people in 2017 alone. Furthermore, approximately a quarter of the world's population is living with latent TB, with a 5 – 15 % lifetime risk of developing the active disease (WHO, 2018*a*). TB is predominantly found in poverty stricken areas with 44 % of estimated cases in 2017 occurring in WHO South-East Asia region and 25 % in WHO African region (WHO, 2018*b*).

Mycobacterium tuberculosis (*Mtb*) is spread when a person with the active disease sneezes, coughs or spits, propelling the pathogen into the air for it to be inhaled by someone else and become infected. TB, in general, affects the lungs (pulmonary TB) and is communicable in this form (WHO, 2018*a*). When other organ systems of the body are affected, the disease is referred to as extrapulmonary TB (CDC, 2018*a*).

Even with the abysmal statistics, TB remains a treatable and preventable disease. Active, drug-susceptible TB is treated with four antimicrobial drugs (*viz*. isoniazid, rifampicin, ethambutol and pyrazinamide) for six – nine months. A combination of the four drugs are given during the first two months, followed by isoniazid and rifampicin for four – seven months. However, infected individuals that do not comply to the treatments result in the rise of *Mtb* strains resistant to available anti-mycobacterial drugs, a growing problem in the management of the disease (Sandgren *et al.*, 2009). The treatment and cure of drug-resistant TB is complex, with inappropriate treatment having life-threatening results (CDC, 2018*b*). Presently only 55 % of all multidrug-resistant cases are being treated successfully (WHO, 2018*a*), creating an urgent need for the development of effective novel anti-mycobacterial drugs.

An innovative strategy for the discovery of more selective and efficient therapeutic agents is molecular hybridisation. Molecular hybridisation is the formation of a single molecule through the adequate fusion of two or more bioactive pharmacophores. The synthesised hybrid maintains the carefully chosen characteristics, such as biological function and structural domains of the parent molecules, thereby improving its affinity and efficacy compared to the parent drugs (Viegas-Junior *et al.*, 2007; Xu *et al.*, 2017). The molecular hybridisation strategy was adopted in the search for new anti-tubercular drugs in the framework of this study and involved 1,4-benzoquinone and 1,2,3-triazole scaffolds.

The 1,2,3-triazole scaffold possesses a wide range of biological activities (*viz*. anti-malaria (Kumar *et al.*, 2014; Singh *et al.*, 2017), anti-TB (Boechat *et al.*, 2011), anti-HIV (Mohammed *et al.*, 2016), anti-fungal (Dai *et al.*, 2015), and anti-inflammatory (Shafi *et al.*, 2012) properties), that have attracted a considerable amount of attention towards this pharmacophore (Ali *et al.*, 2017).

The partner pharmacophore to triazole is 1,4-bezoquinone. Benzoquinone and hydroquinone are metabolites of one another. There is a constant interconversion of each metabolite into the other, with the rate of interconversion dependant on a number of factors, including medium, pH of medium, and the presence of substances such nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) and/or microsomes (HCotN, 2012).

Both benzoquinone and hydroquinone possess a wide range of biological activities such as anti-cancer (Lindsey *et al.*, 2004; Tasdemir *et al.*, 2006), anti-viral (Tasdemir *et al.*, 2006), anti-malarial (Tasdemir *et al.*, 2006), anti-inflammatory (Sagnou *et al.*, 2009), and anti-mycobacterial (Tasdemir *et al.*, 2006; Jyoti *et al.*, 2016) activity. However, benzoquinone is largely used as an industrial chemical in the production of hydroquinone and hydroquinone(HCotN, 2012).

The aim of this study was to synthesise novel 1,4-benzoquinone/hydroquinone-linked 1,2,3-triazoles, via molecular hybridisation, with the ultimate goal that the afforded compounds have enhanced effectiveness against *Mtb* and an improved safety profile compared to current anti-mycobacterial drugs.

The following objectives were set to help achieve the aim of the study:

- To synthesise a series of novel benzo/hydroquinone-triazole hybrids
- To characterise the synthesised compounds by means of routine techniques such as nuclear magnetic resonance (NMR), mass spectrometry (MS) and infrared spectroscopy (IR).
- To assess the *in vitro* cytotoxicity of synthesised compounds using mammalian cell lines.
- To assess the *in vitro* anti-tubercular activity against the MDR-TB strain, *Mtb* H37Rv strain.

The hybrids were synthesised following a two-step process; starting with an aromatic nucleophilic substitution reaction using a modified method (Guimarães *et al.*, 2013) of sodium azide and commercially available benzoquinone to form the 2-azidohydroquinone intermediate. Secondly, various individual alkynes were reacted with the azido intermediate through click-chemistry to

produce different hydroquinone-linked 1,2,3-triazole hybrids. The click-chemistry reaction method used was adapted and modified from a literature reported method (Dixit *et al.*, 2012). All hybrids were subjected to purification by recrystallization from hexane and were isolated in poor to good yields (23 – 70 %). The structures of all the synthesised hybrids were confirmed by IR, HRSM, NMR techniques and the purity was assessed by HPLC and was found to be in the 92 – 98 % range.

The *in vitro* anti-mycobacterial activity of the synthesised hybrids was assessed against *Mtb* H37Rv using a Green Fluorescent Protein (GFP) assay in Middlebrook 7H9-CAS broth media. Dejectedly, none of the synthesised hybrids possessed notable anti-mycobacterial activity, with MIC_{90} values all significantly higher than 10 µM. Compounds **9** and **14** had the lowest MIC_{90} values 34 and 16 µM, respectively.

Overall the synthesised hybrids expressed poor toxicity against human embryonic kidney cells, with only hybrids **6** – **9** displaying mild to moderate toxicity. The most active compound, **14** (MIC_{90} 16 µM), expressed no toxicity against the mammalian cells, but was poorly selective towards *Mtb* (SI = 6), which ruled it out as a potential anti-mycobacterial hit.

The hybrids generally possessed favourable drug-like properties, with cLog*P* values ranging from one to five. It was thus anticipated that they would be efficiently transported across the bio-membranes of *Mtb* through passive diffusion and be anti-mycobacterial active. However, none of the hybrids showed noteworthy activity. The calculated cLog*P* values are merely an estimate of drug-likeness, other parameters exist that influence the uptake of a drug and, therefore, its overall activity (Pop *et al.*, 2004; Chereson, 2009). With the cell wall of *Mtb* being lipid rich in nature, (Glickman & Jacobs, 2001; Knechel, 2009) lipophilicity should be imperative in the design and activity of anti-mycobacterial drugs (Suresh *et al.*, 2014).

The overall poor activity of the hybrids may be attributed to their structural rigidity. Flexibility of a compound is vital in order to permeate the bacterium cell wall (Nikaido, 2001) and to interact with the binding site (Shirude *et al.*, 2013). Therefore, the addition of a linker between the triazole and hydroquinone moieties, creating a more flexible hybrid that can efficiently permeate the *Mtb* cell wall and interact with the binding site, might enhance the anti-mycobacterial activity.

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ANNEXURE A: ANALYTICAL DATA FOR CHAPTER 3

Azido Intermediate; **3**

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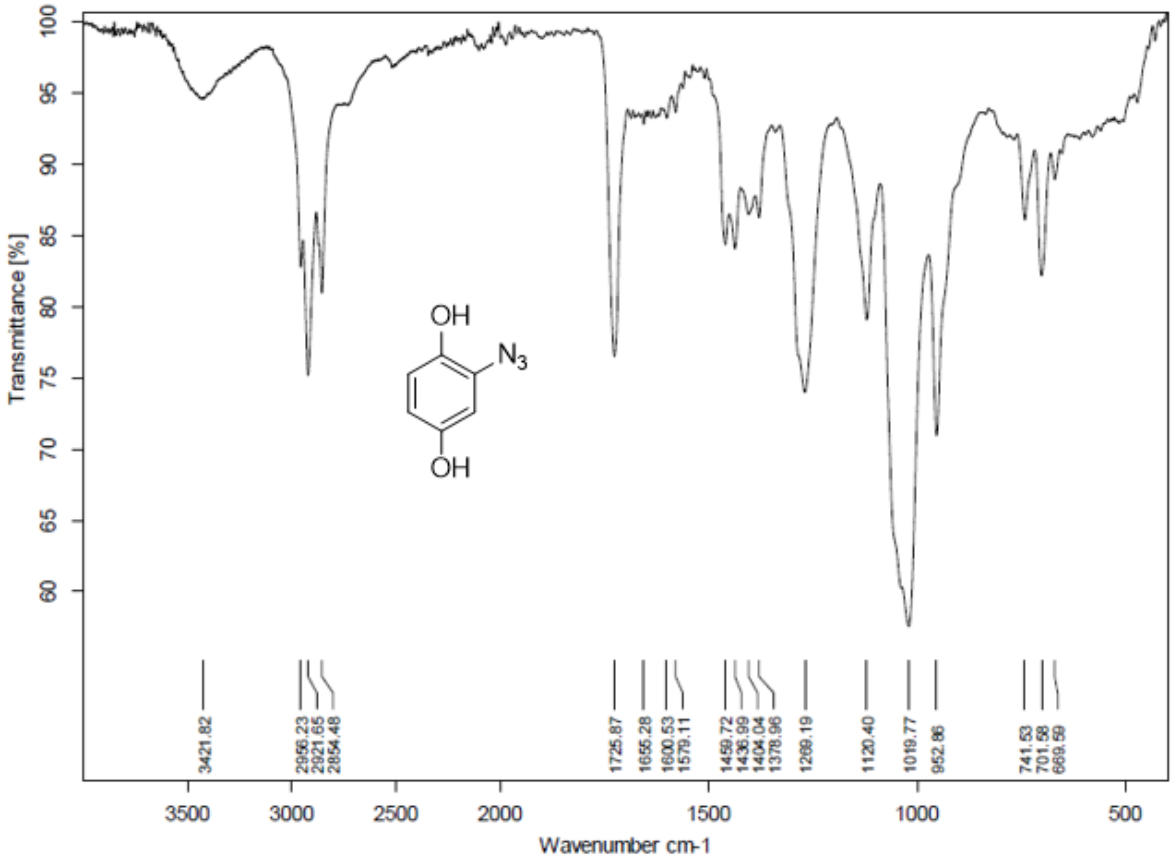

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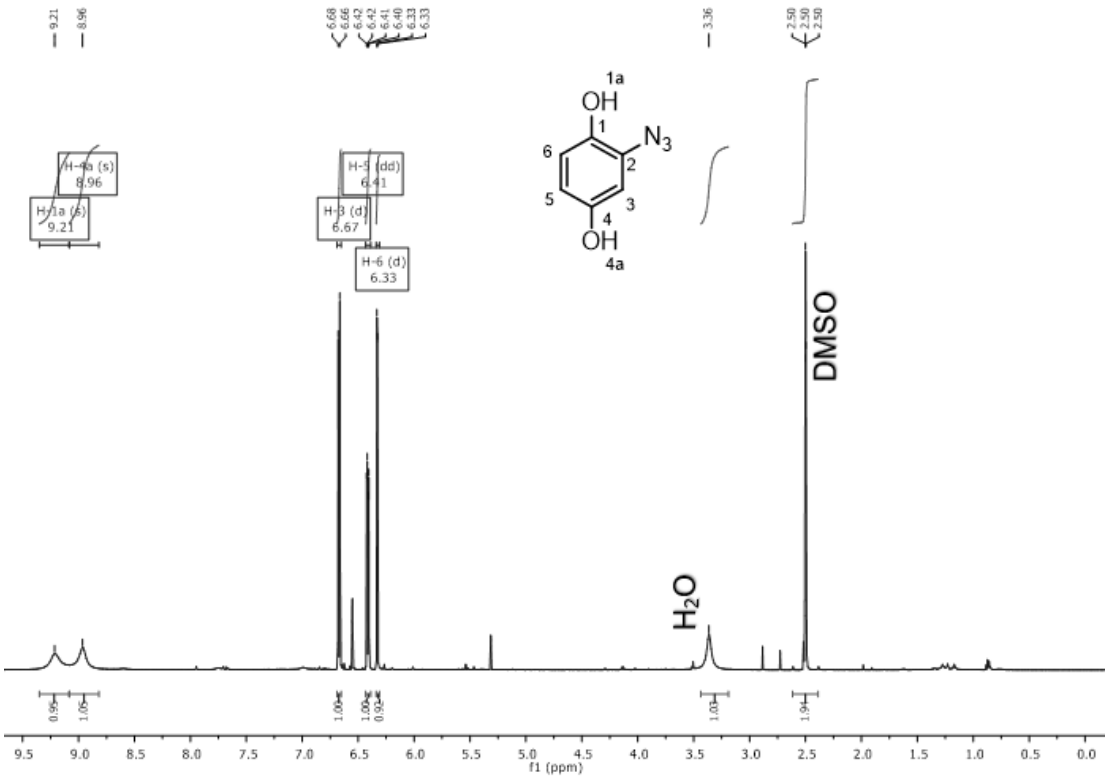

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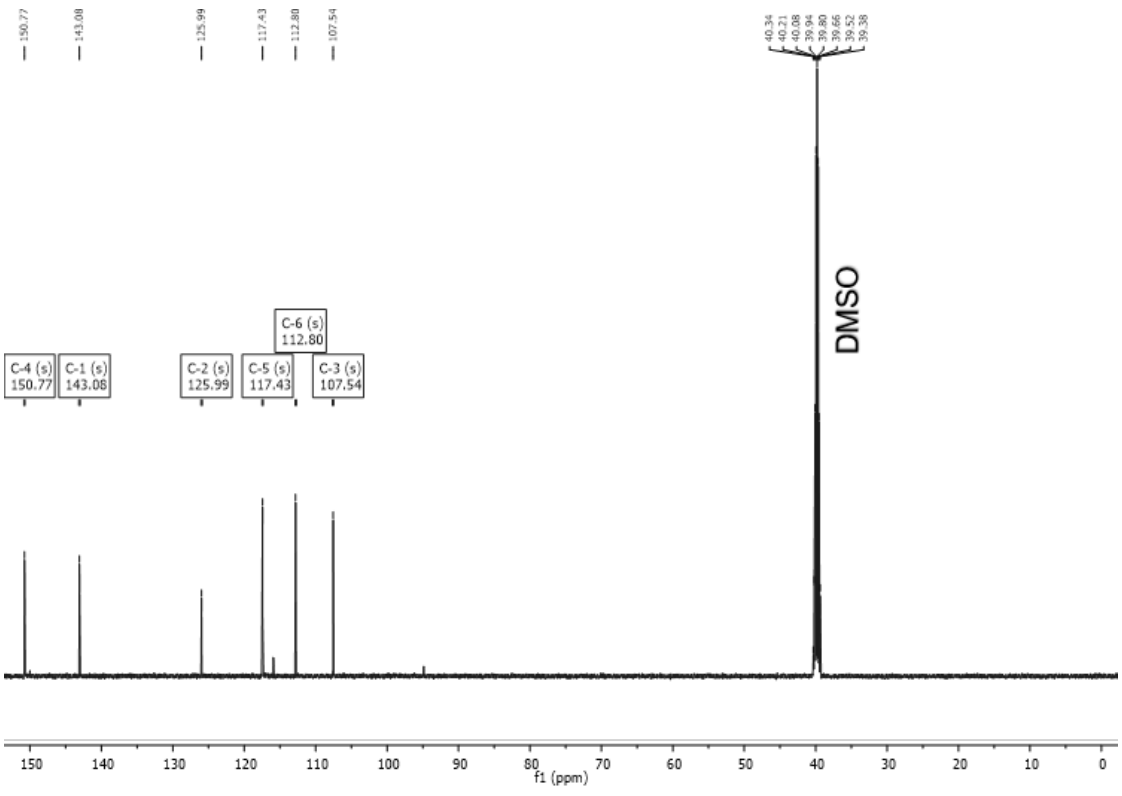

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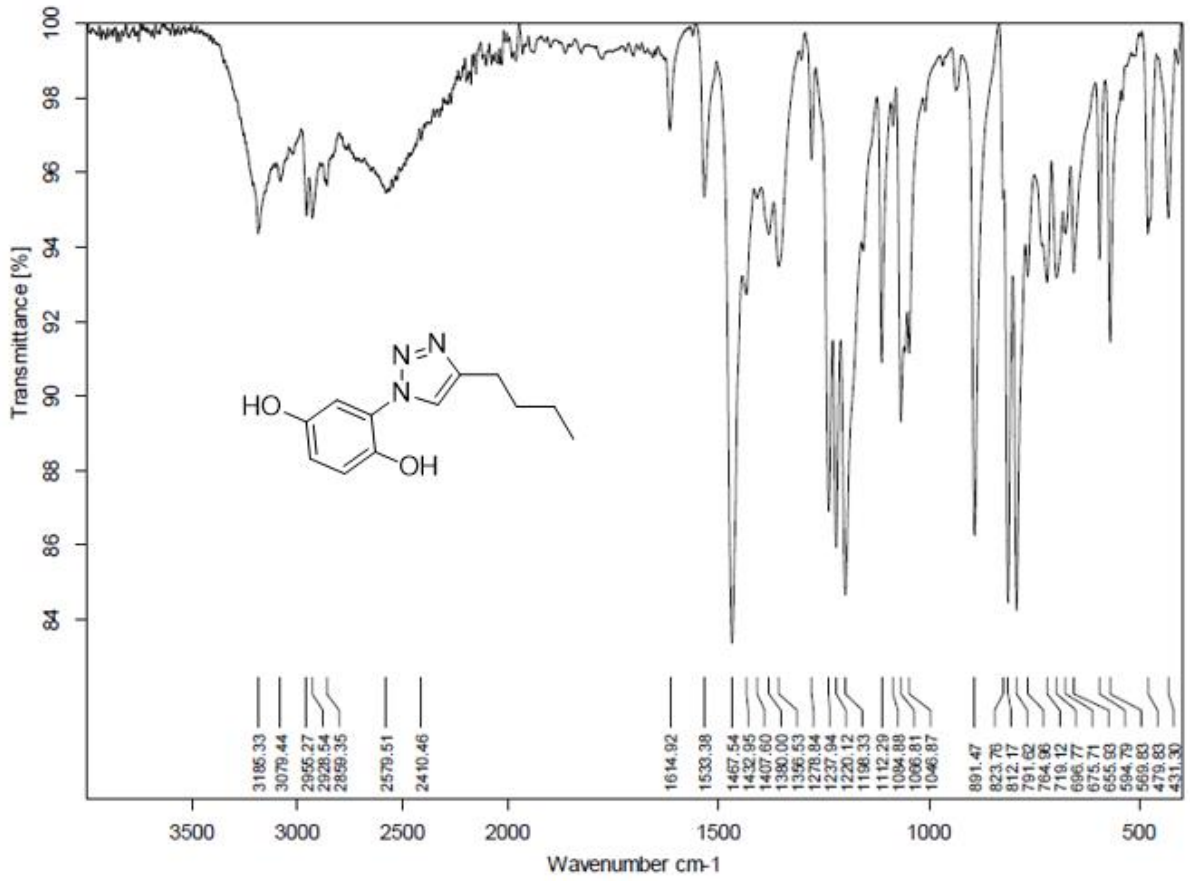

HRMS

Acquisition Parameter					
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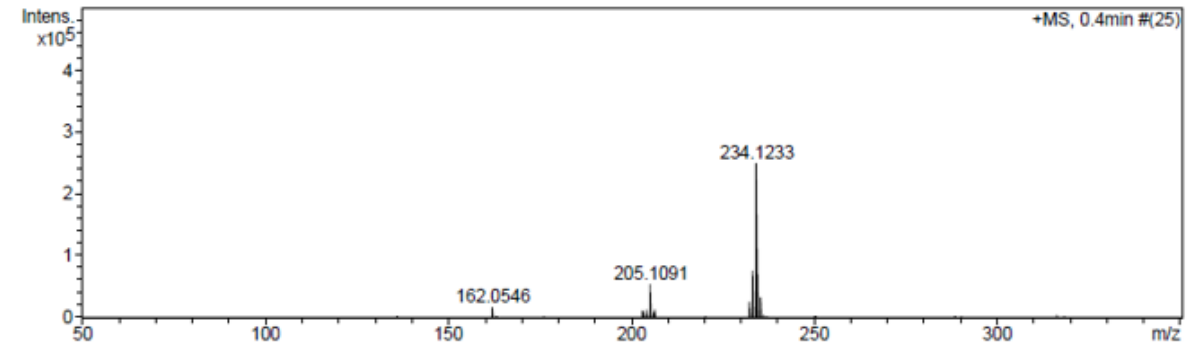
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



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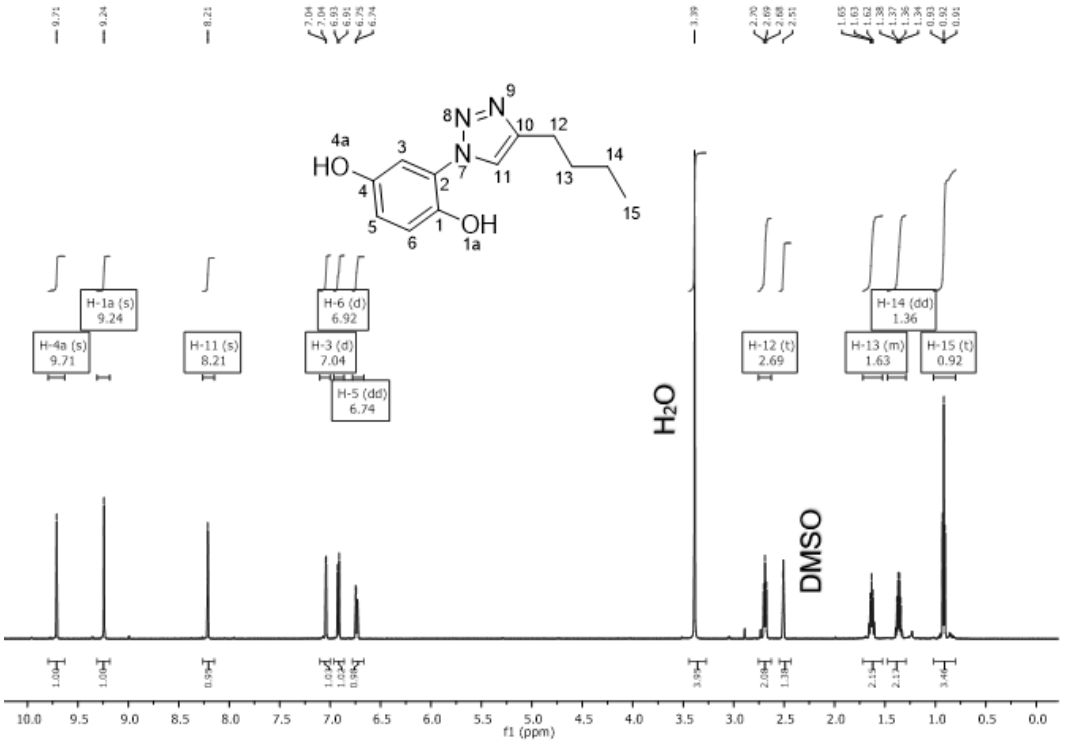

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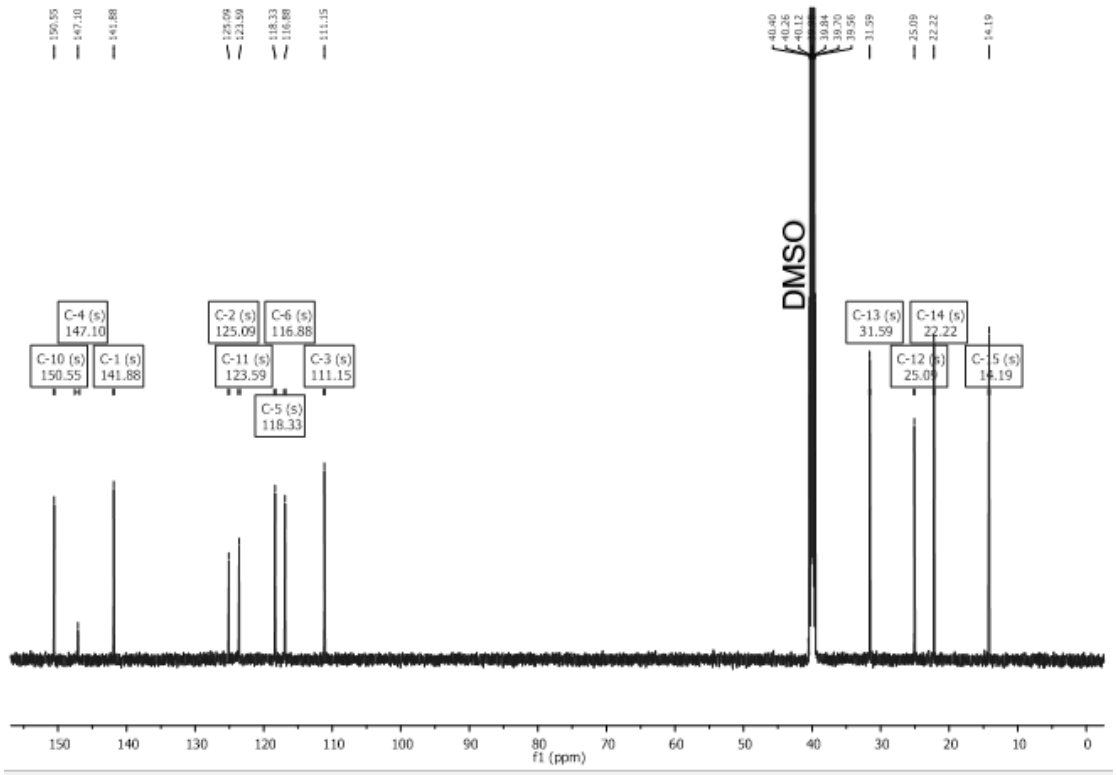

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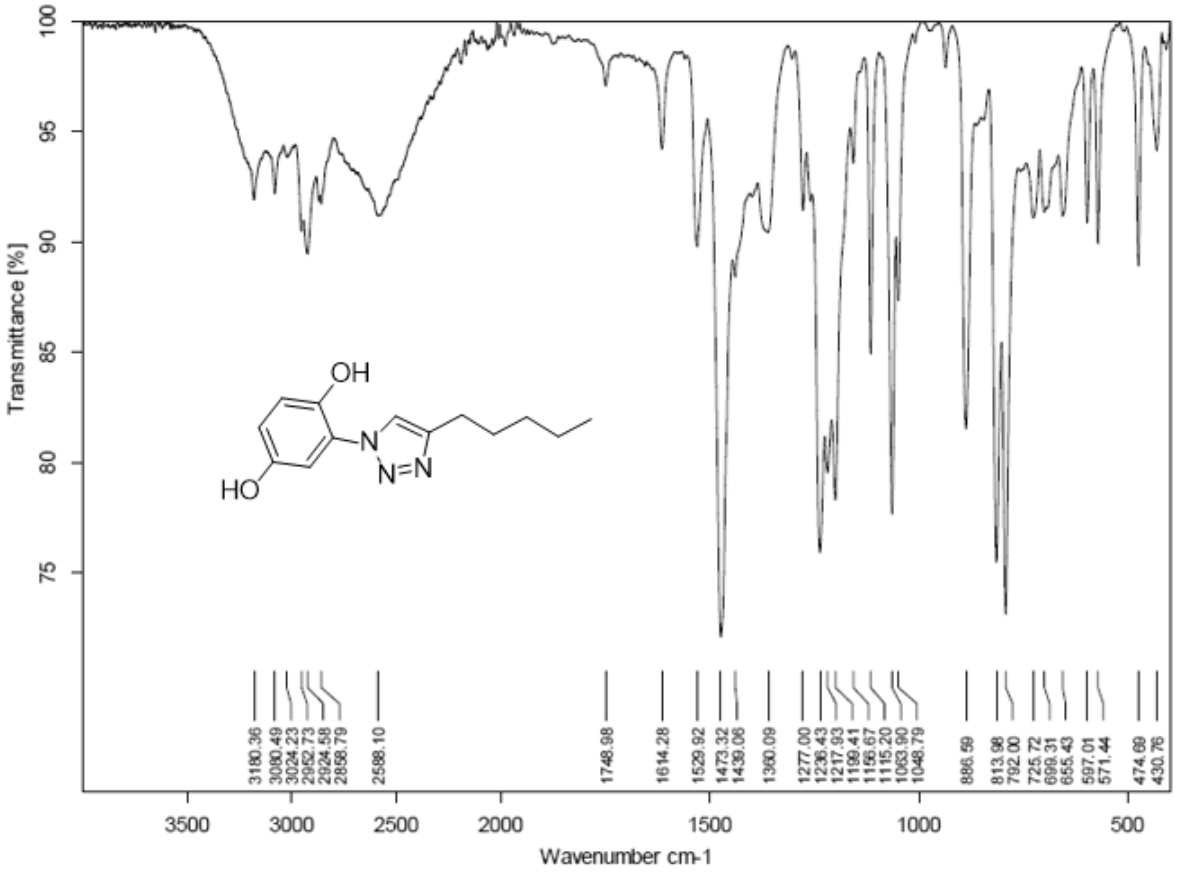

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Acquisition Parameter					
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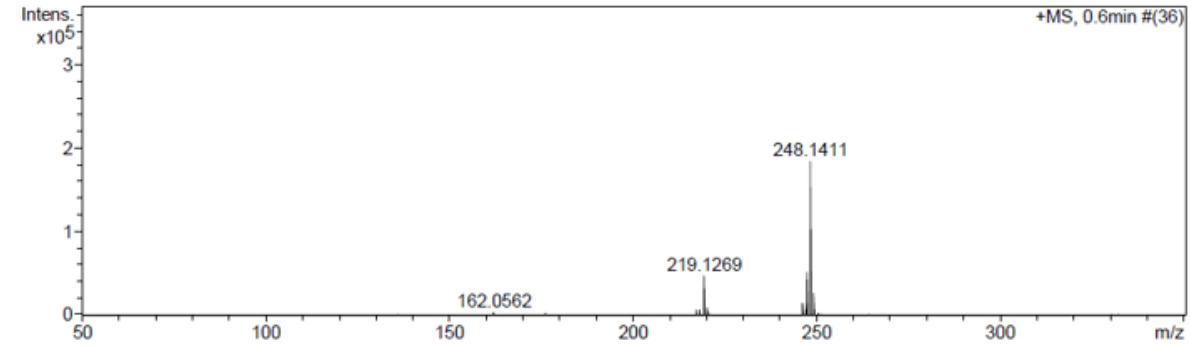

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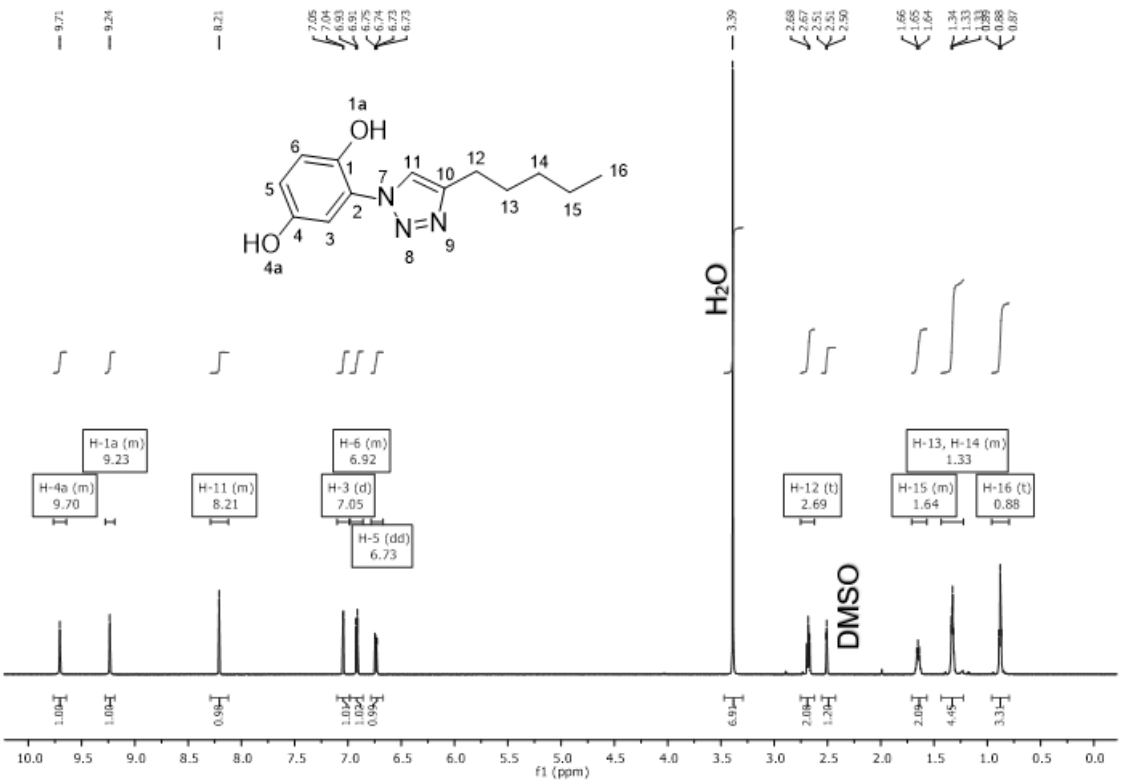

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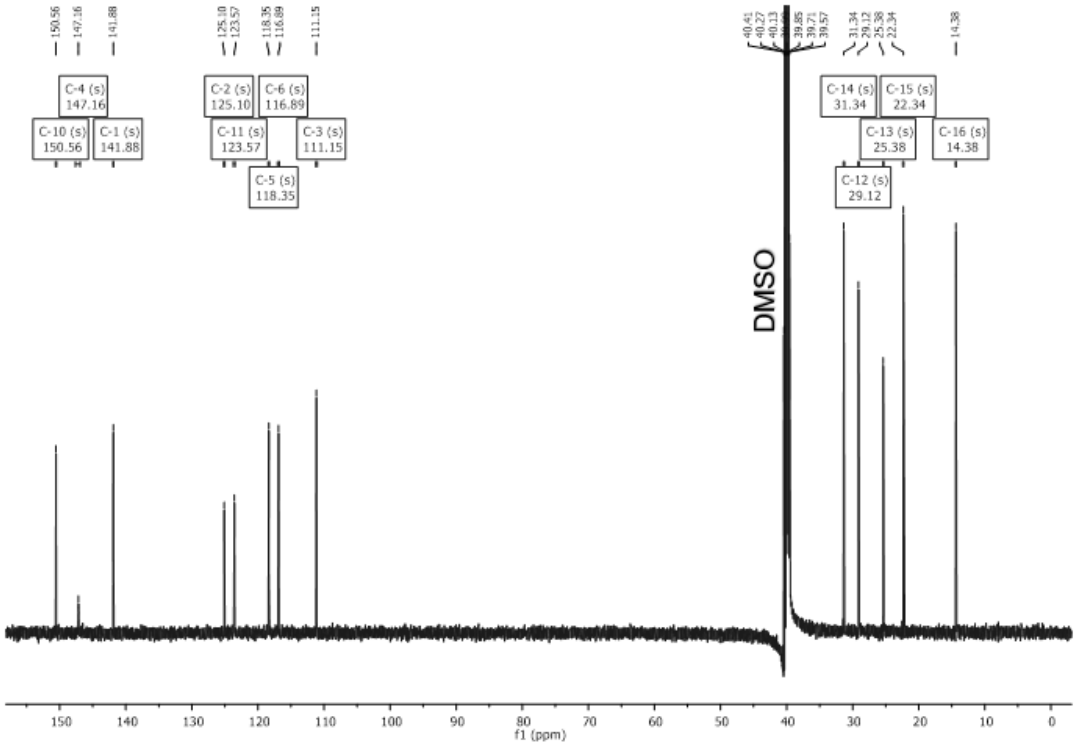

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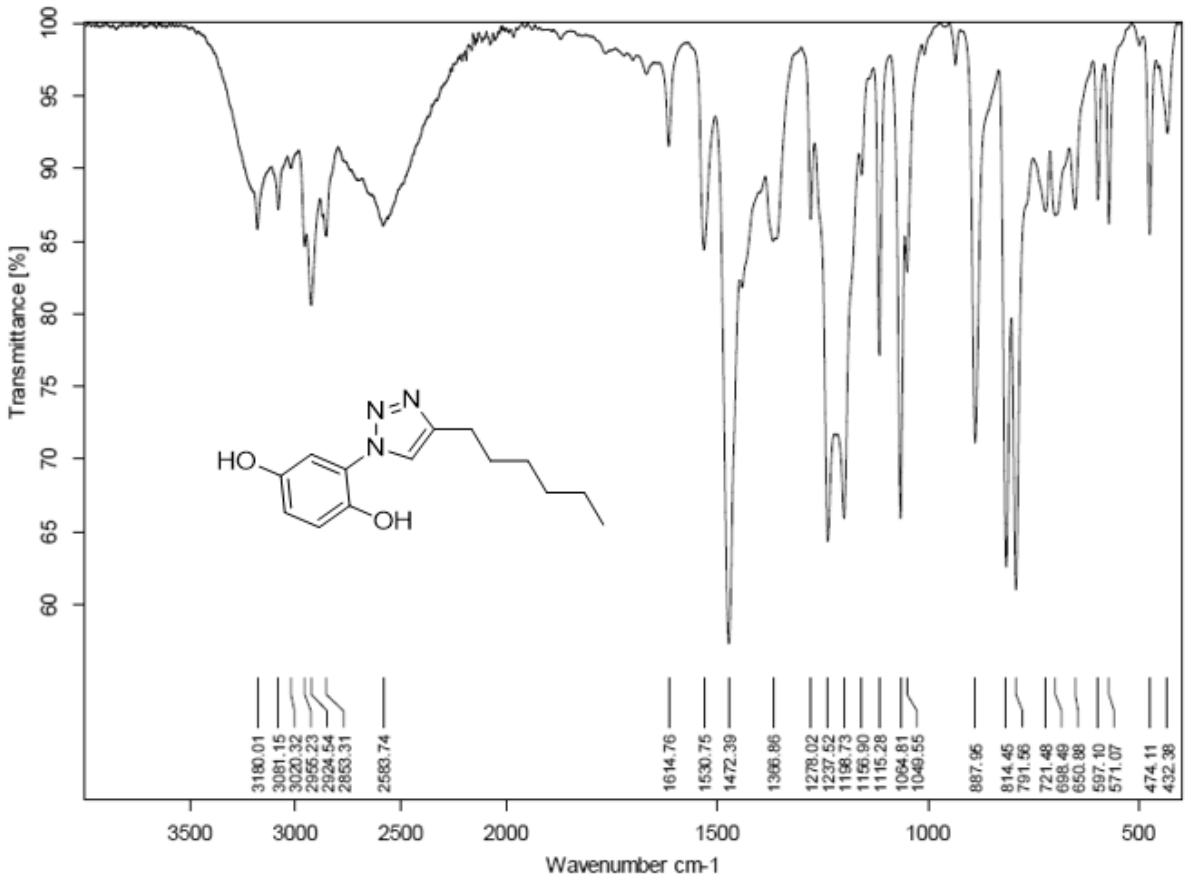

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Acquisition Parameter

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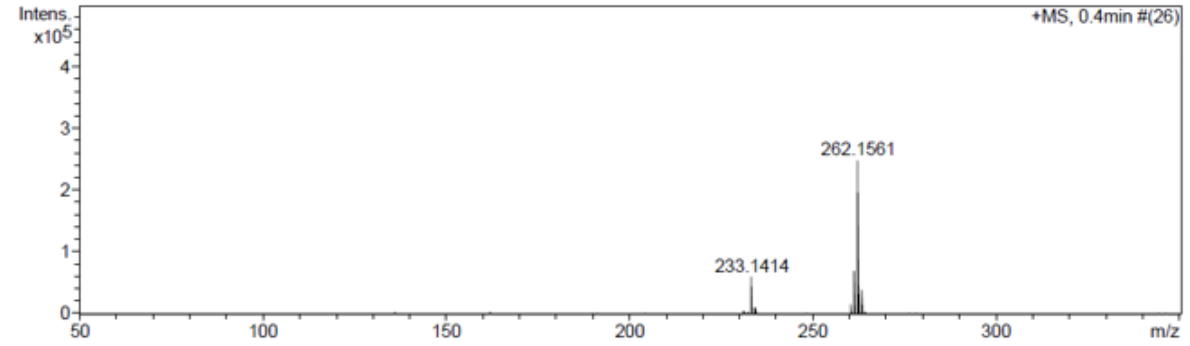

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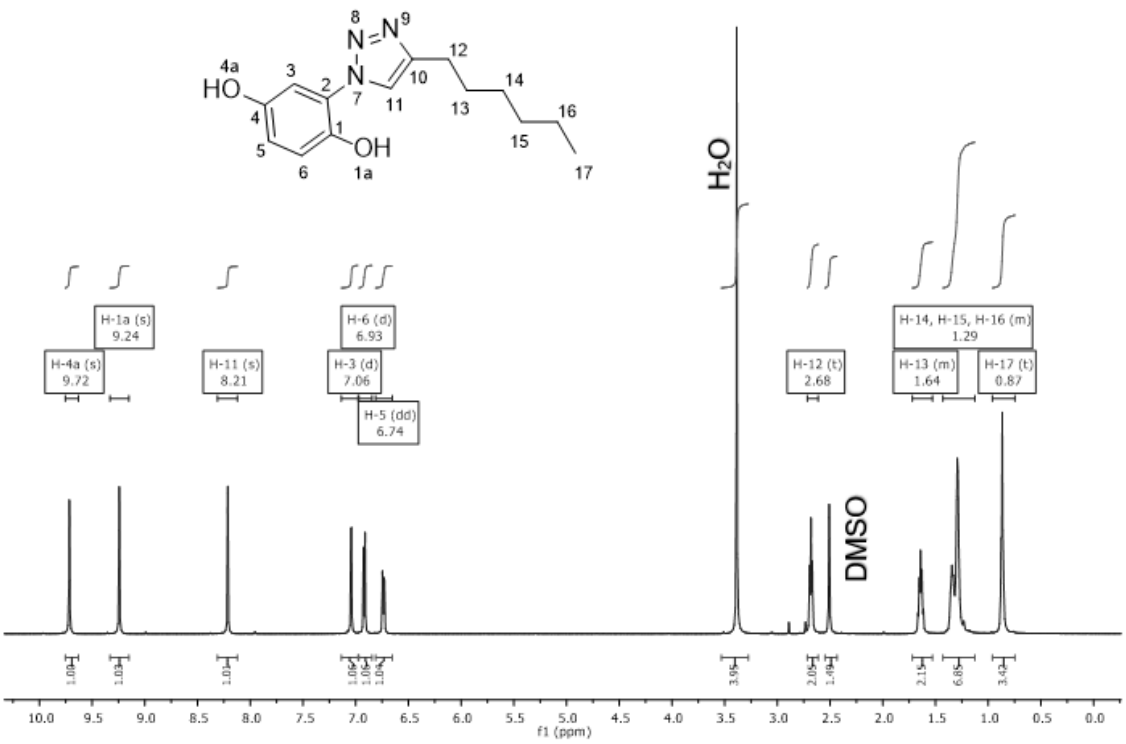

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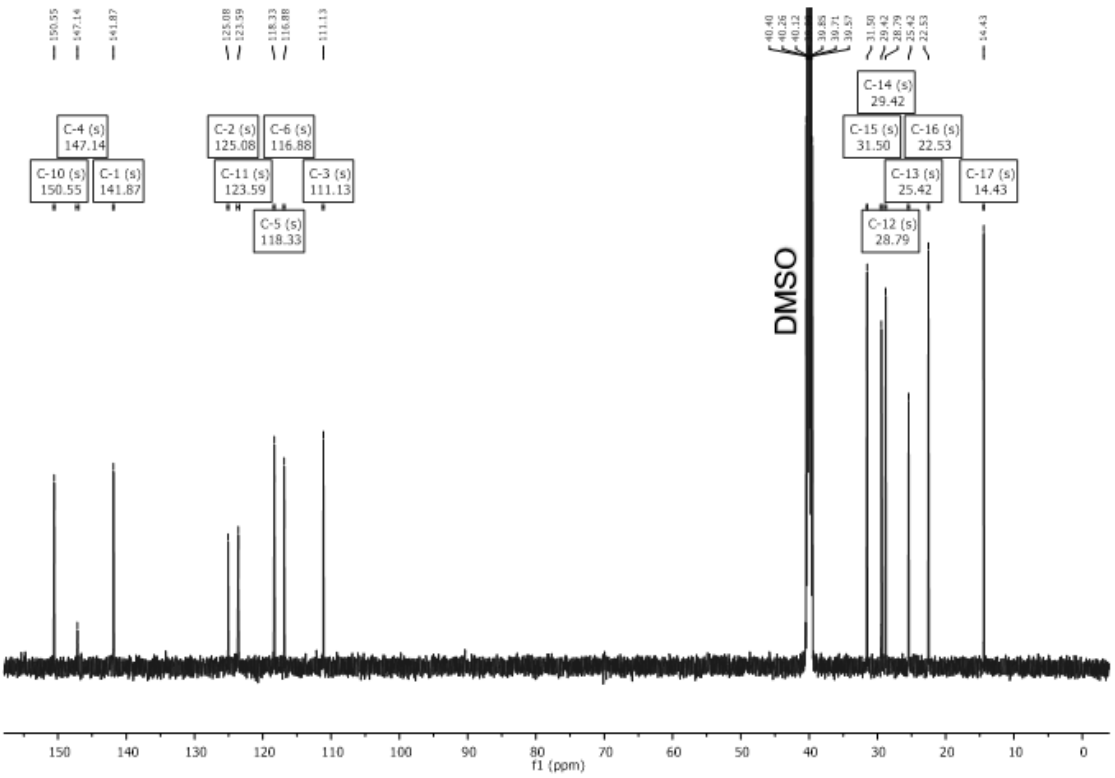

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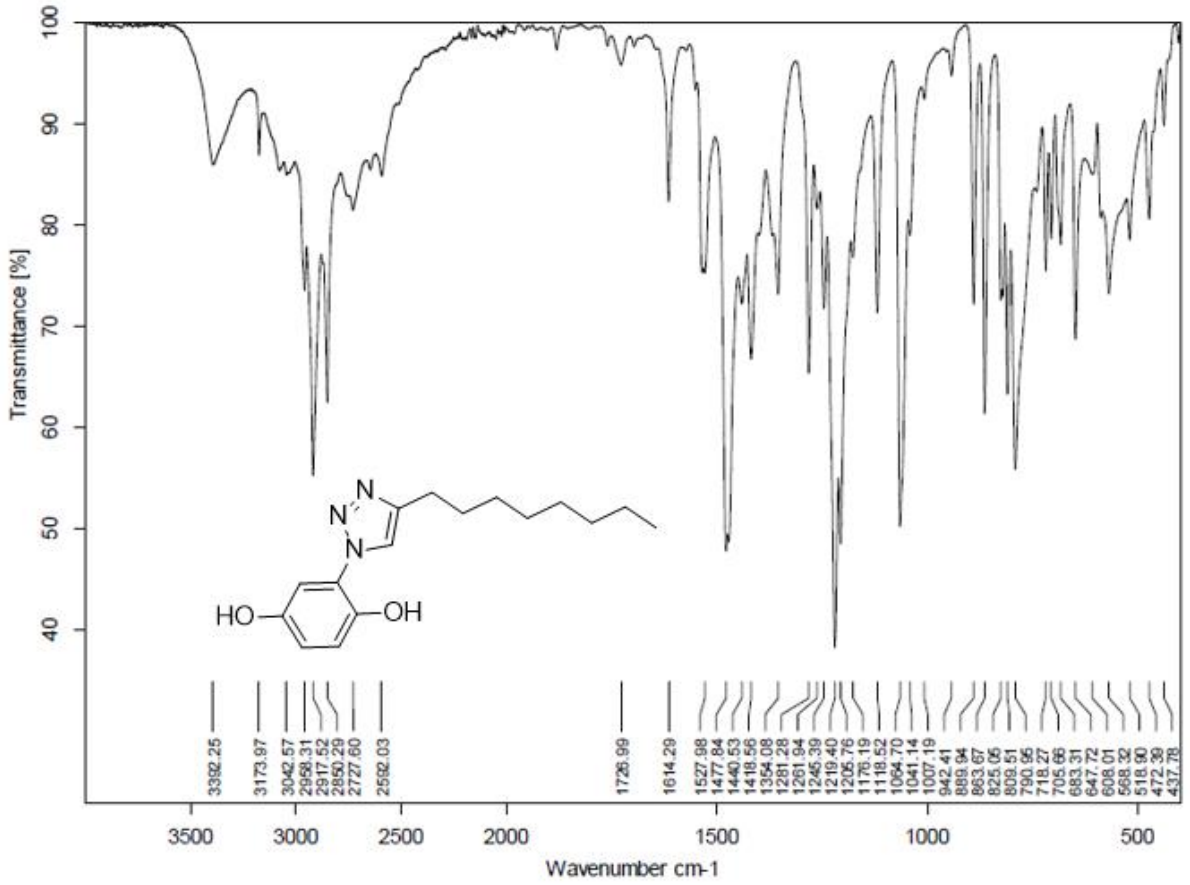

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Acquisition Parameter					
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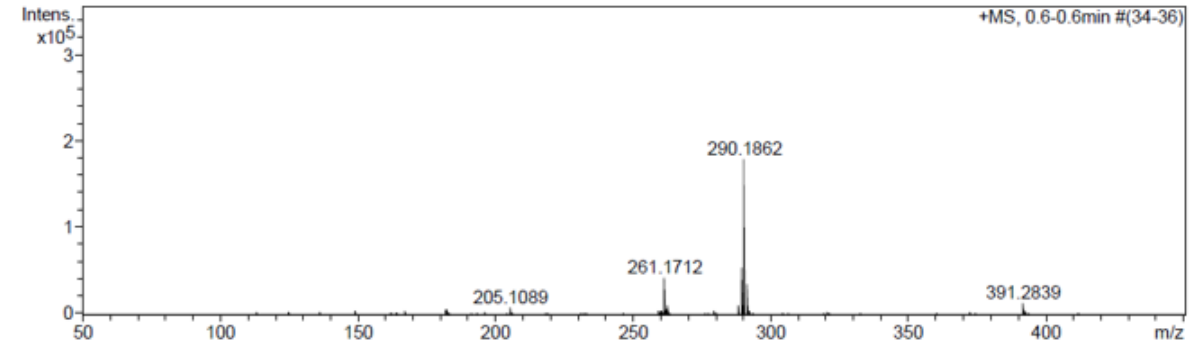

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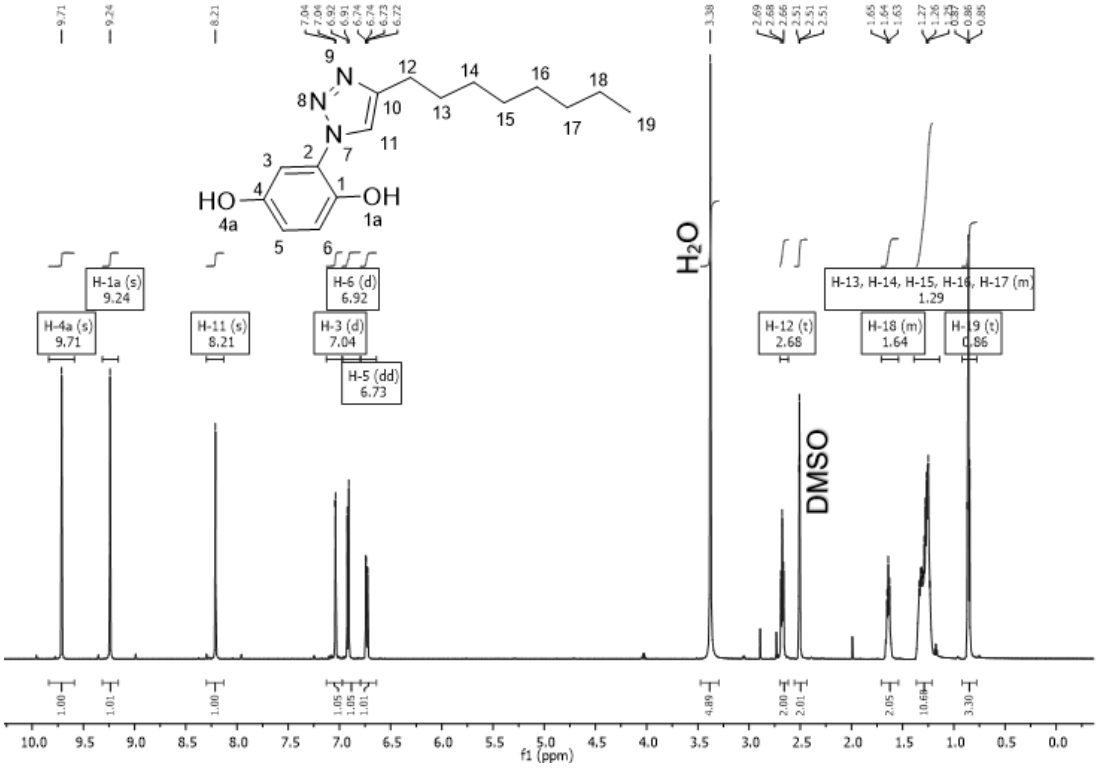

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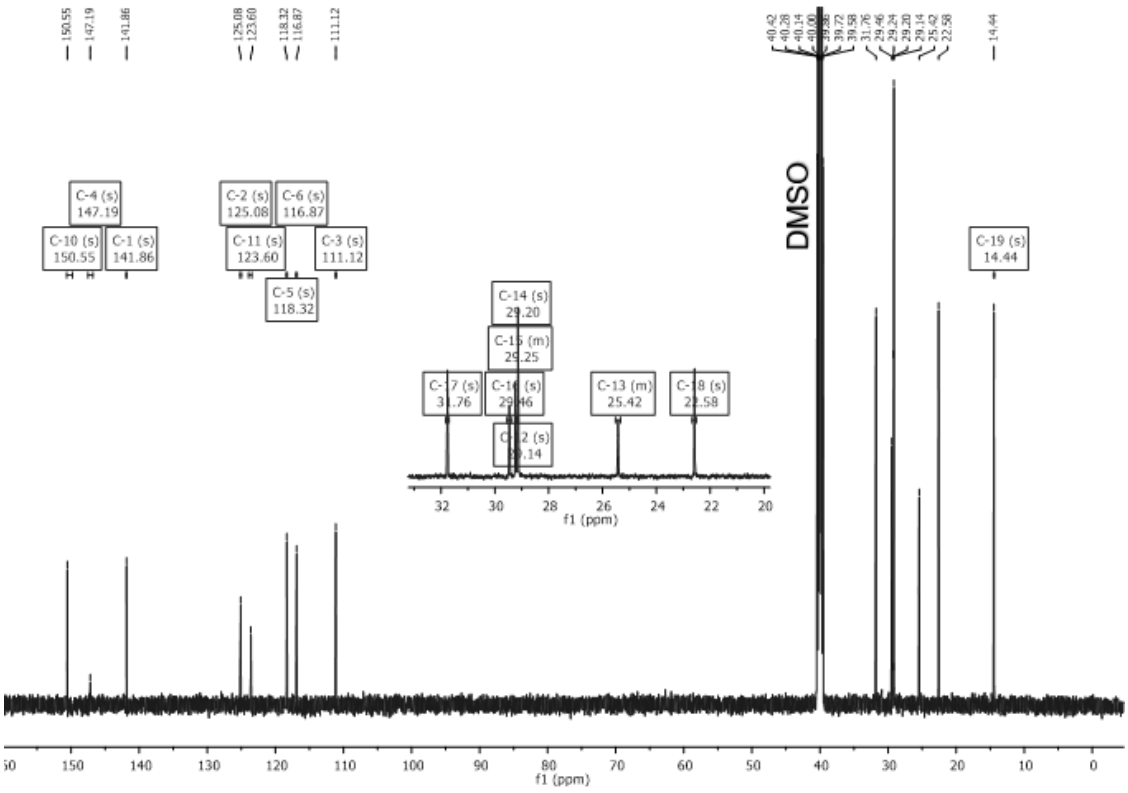

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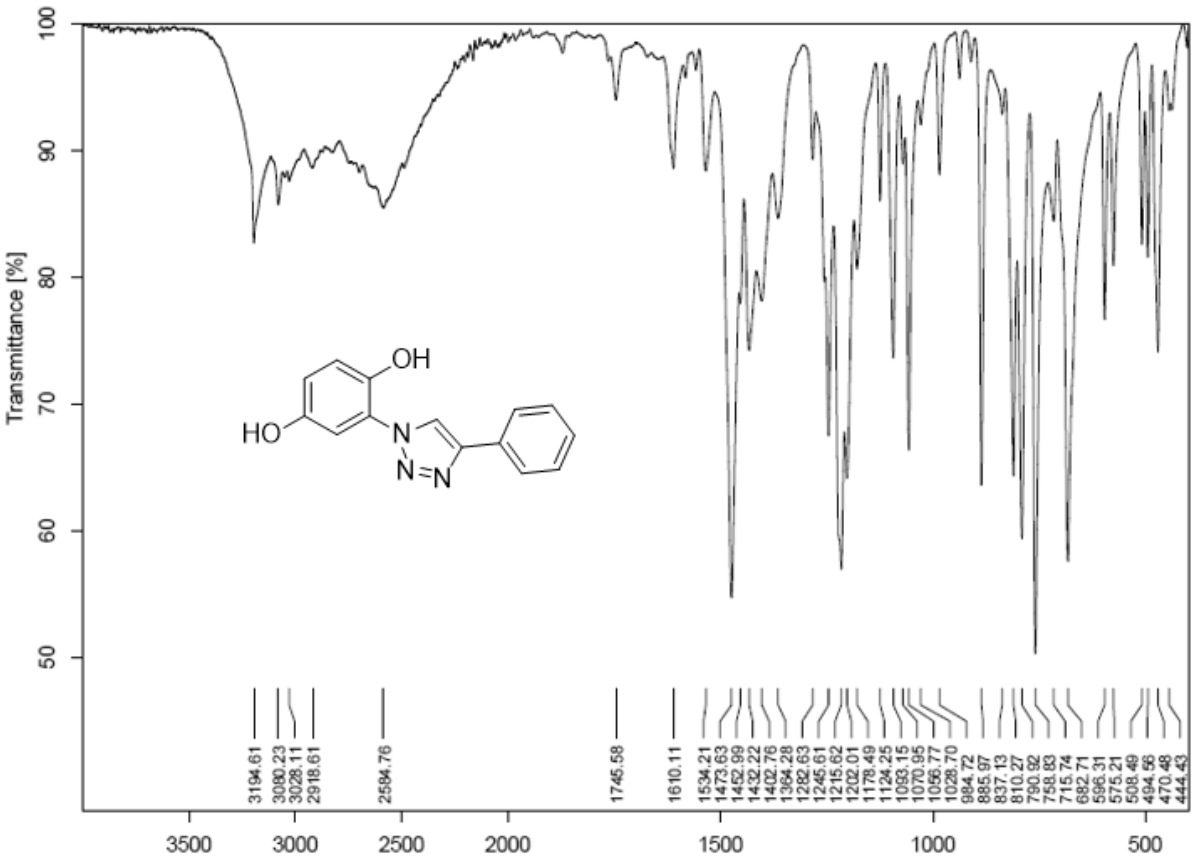

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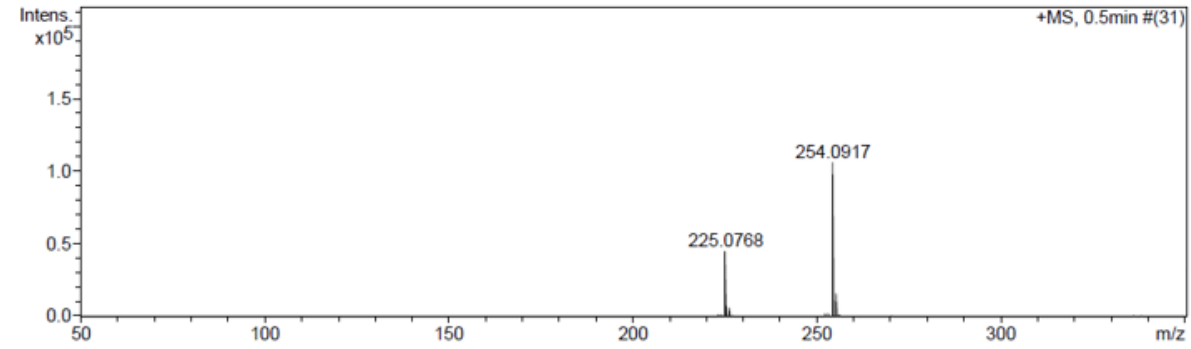

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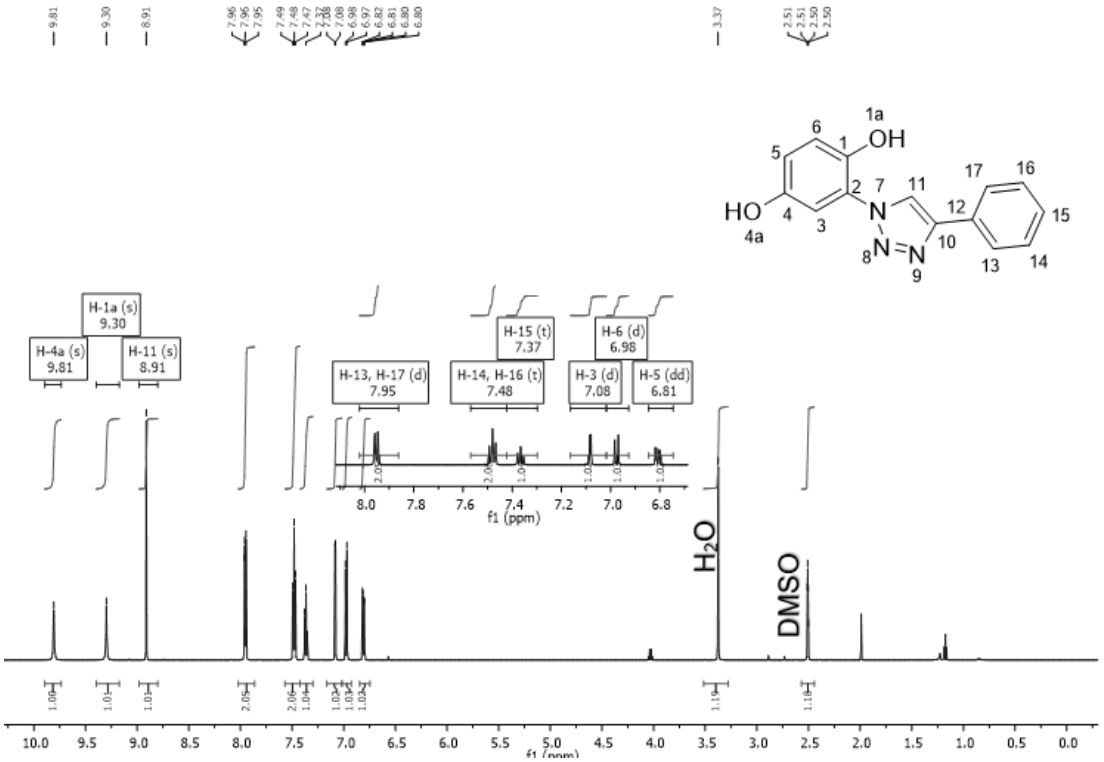

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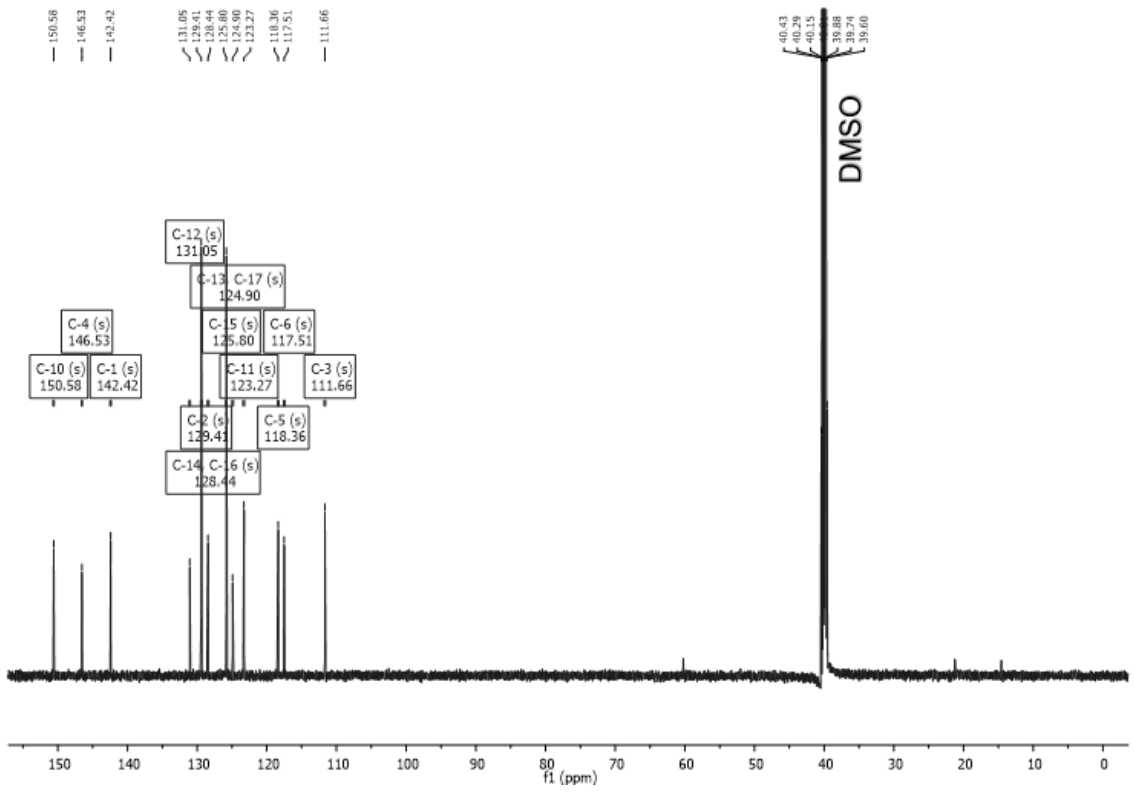

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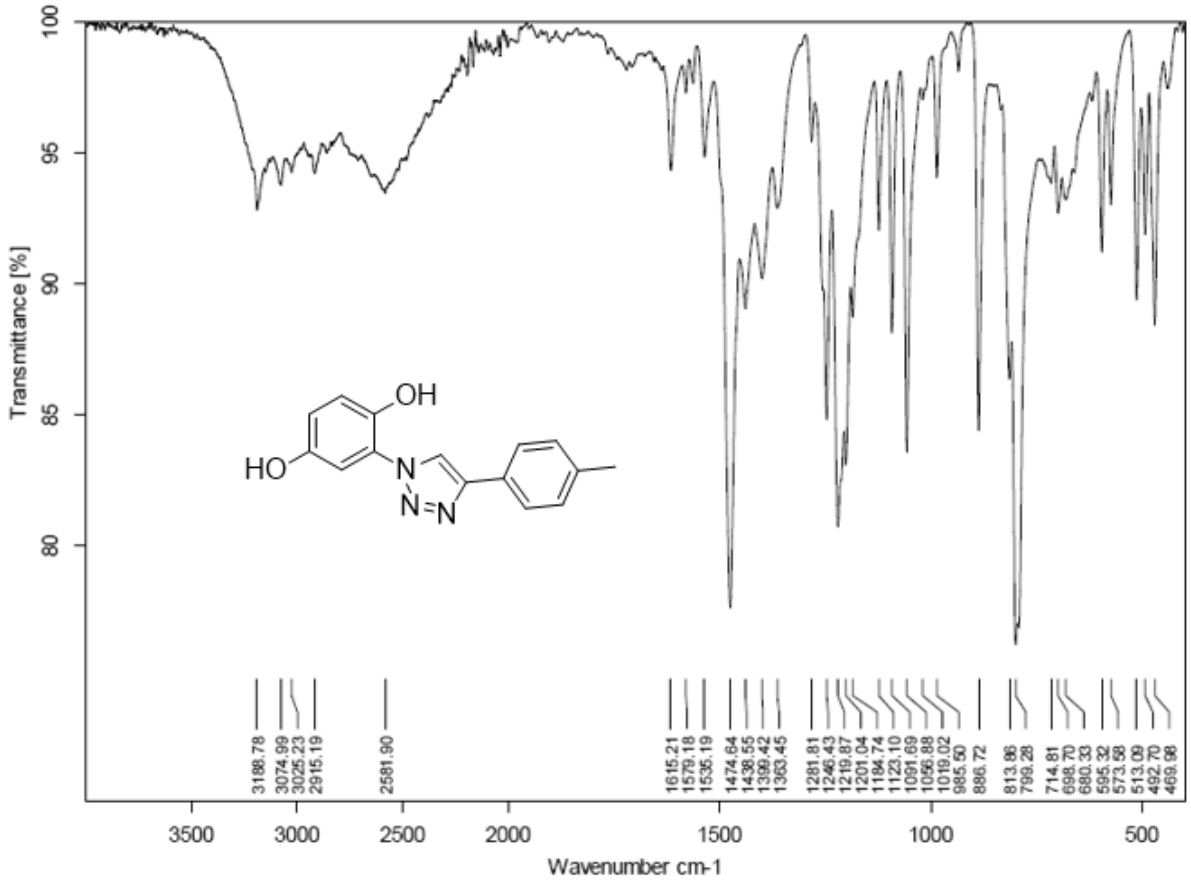

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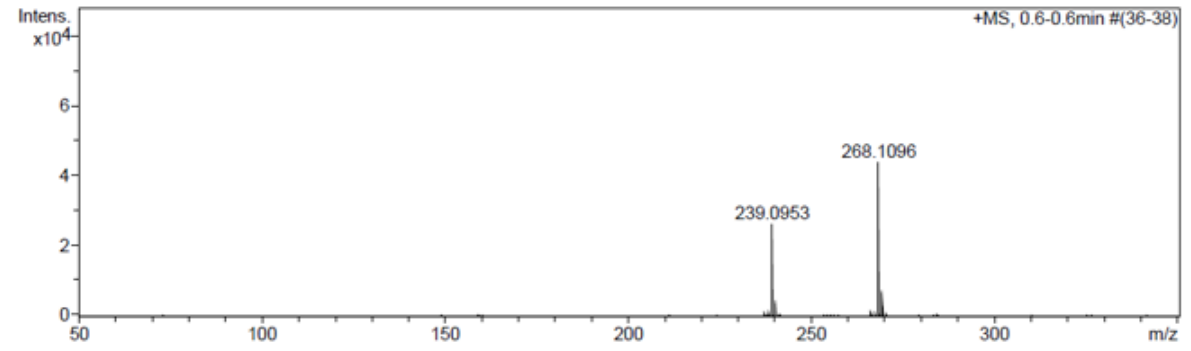

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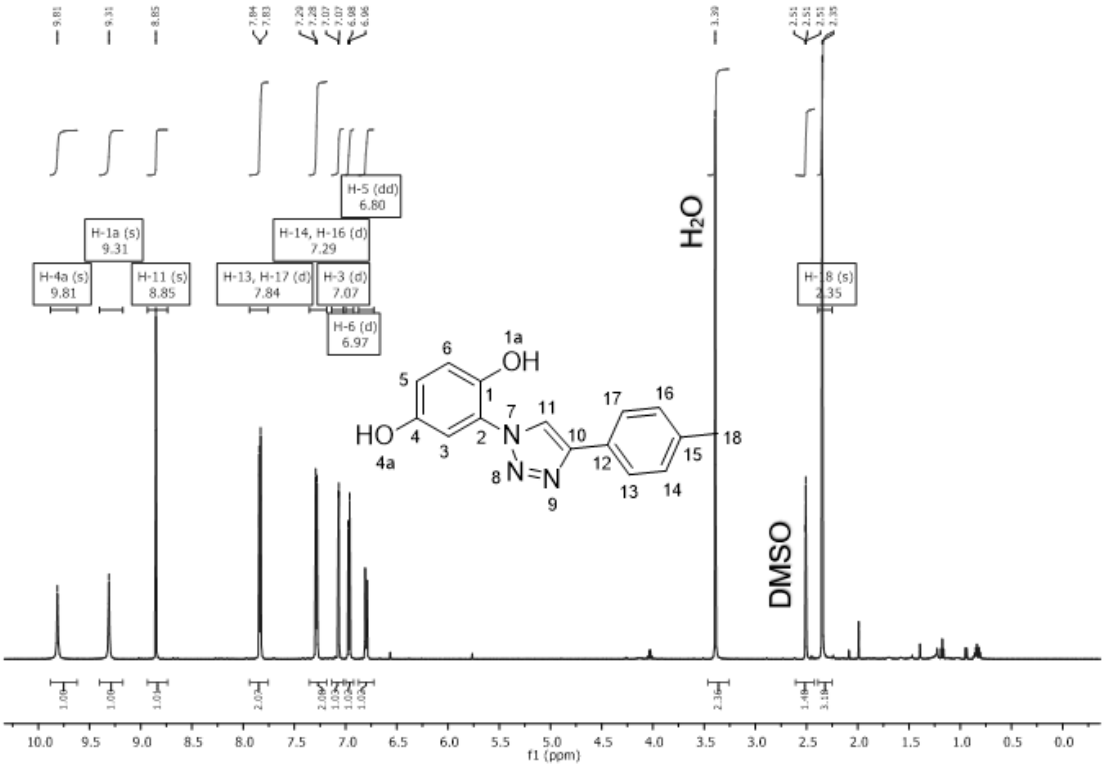

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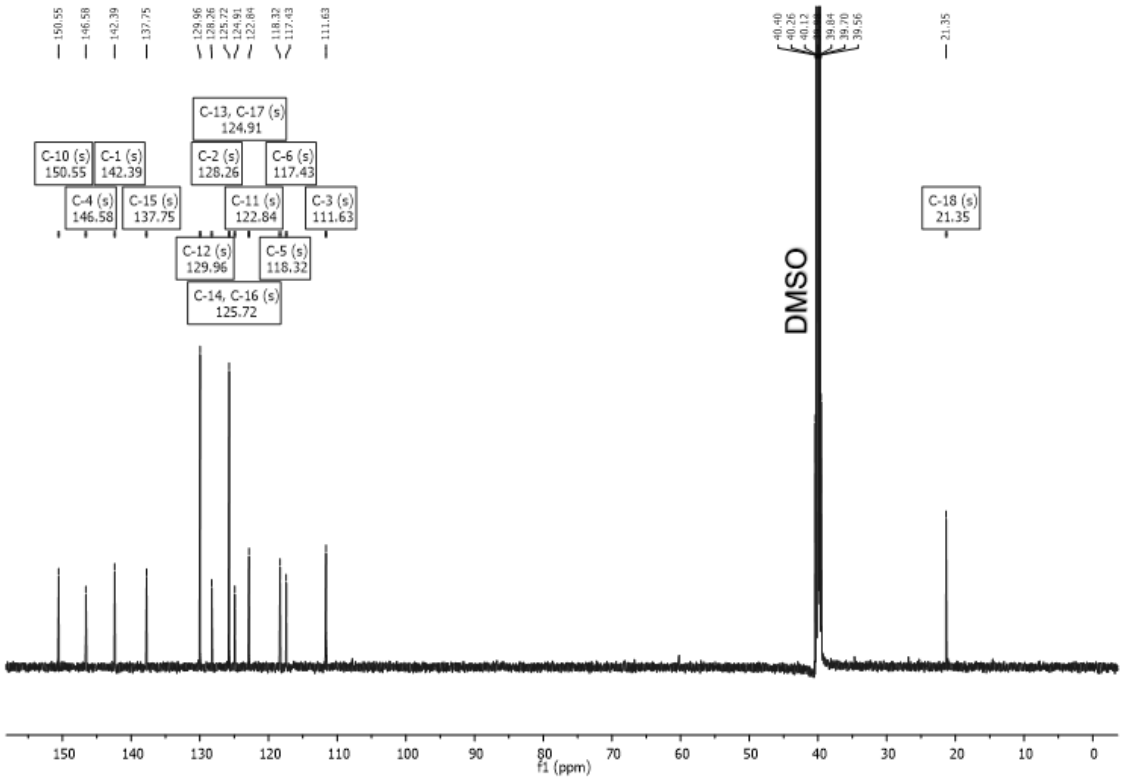

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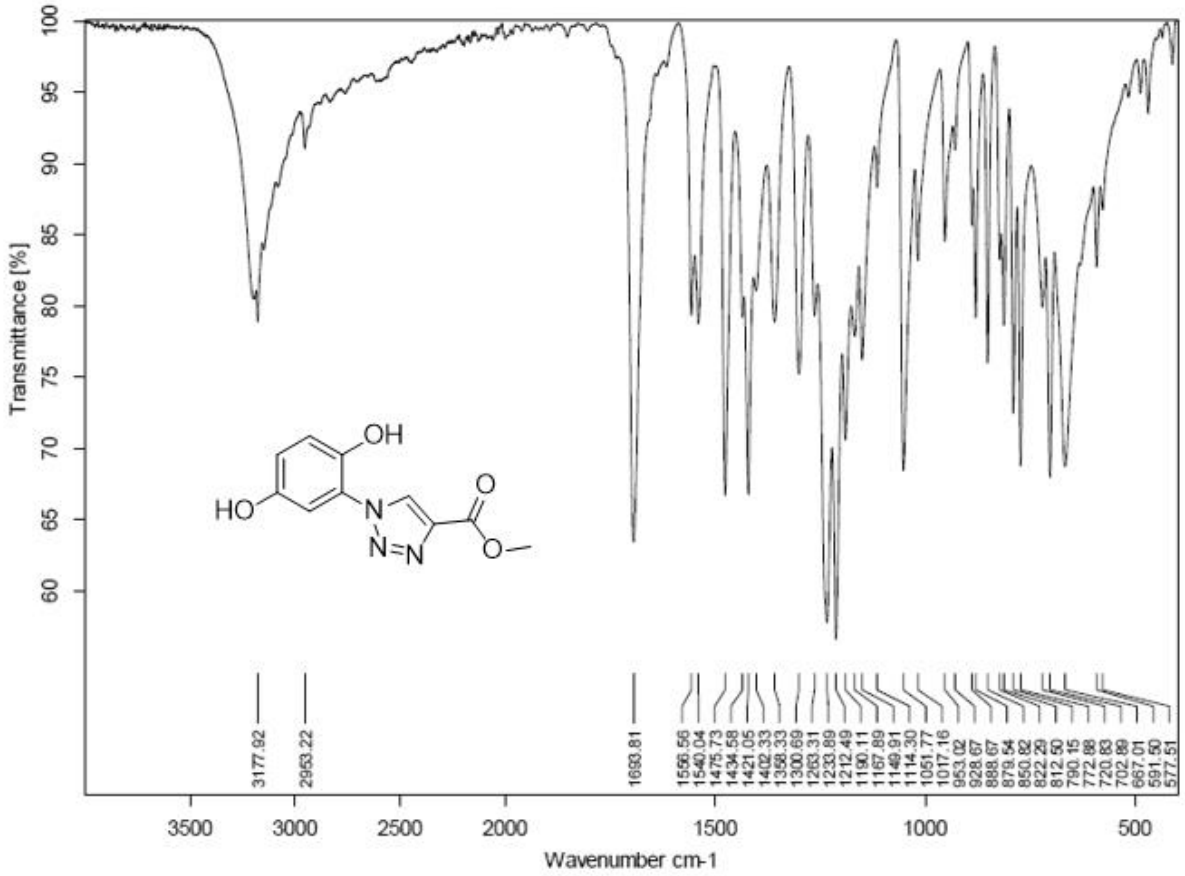

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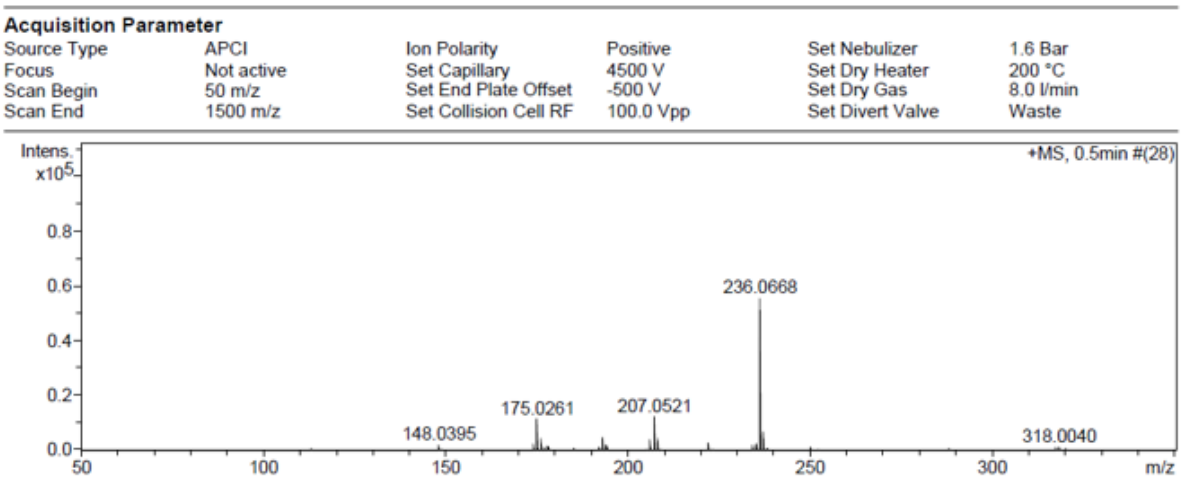

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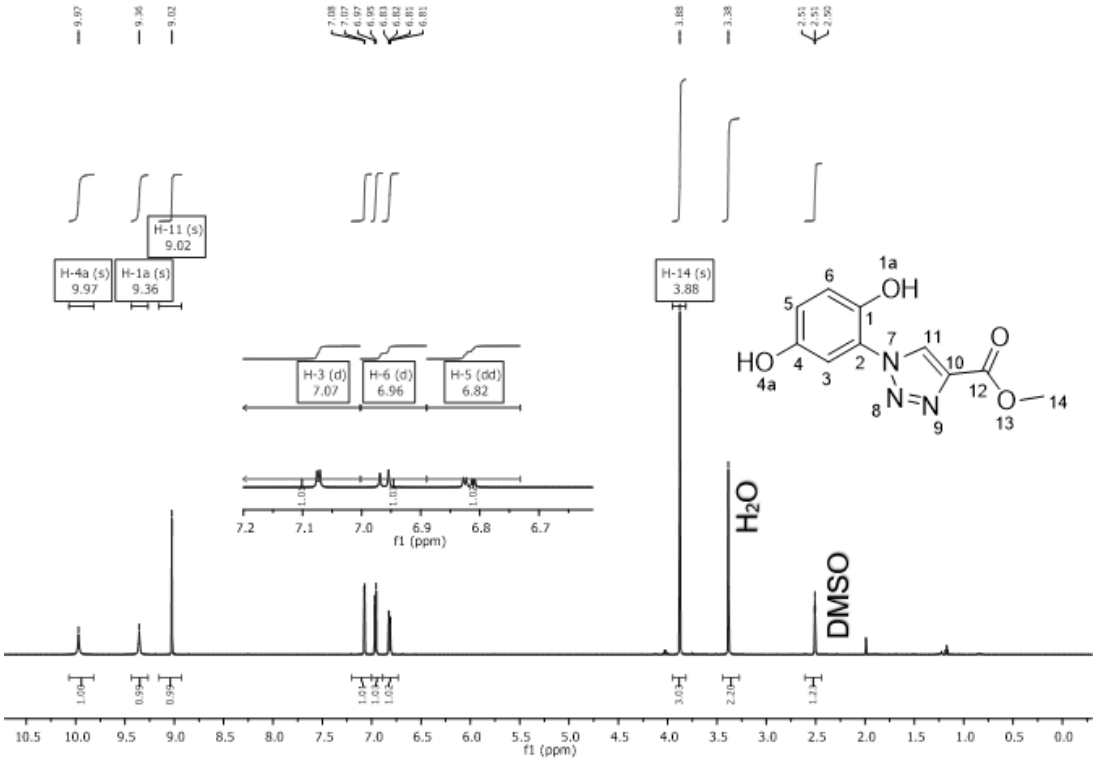

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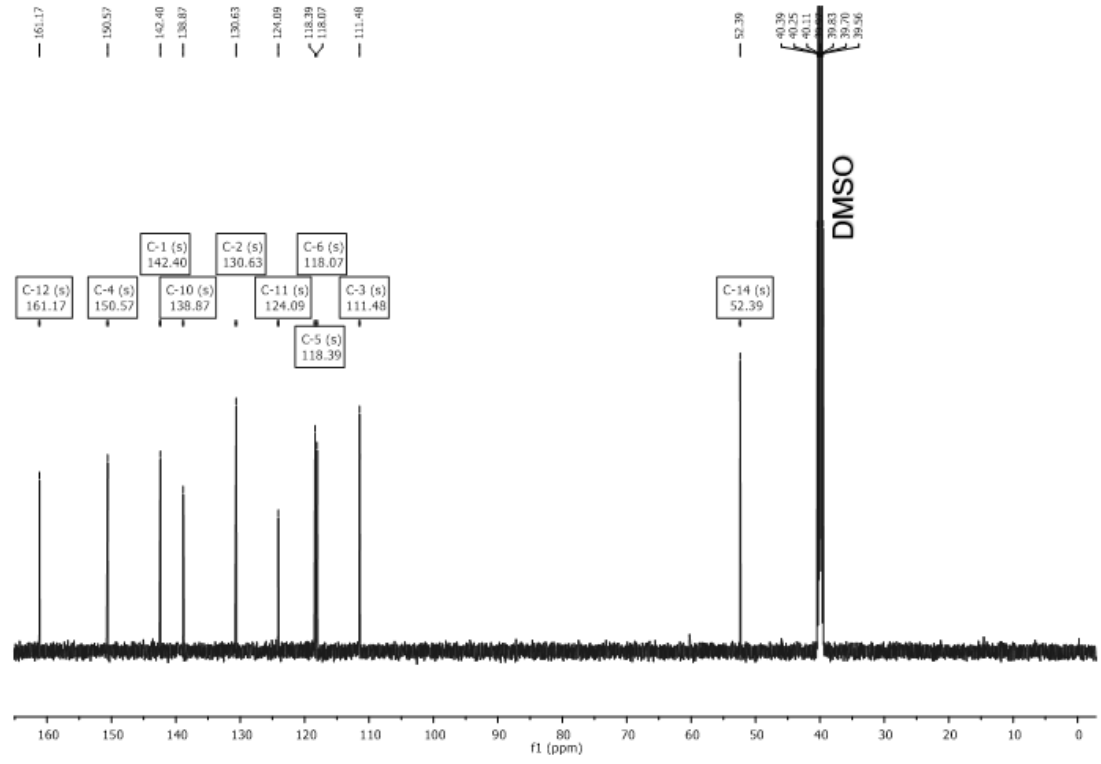

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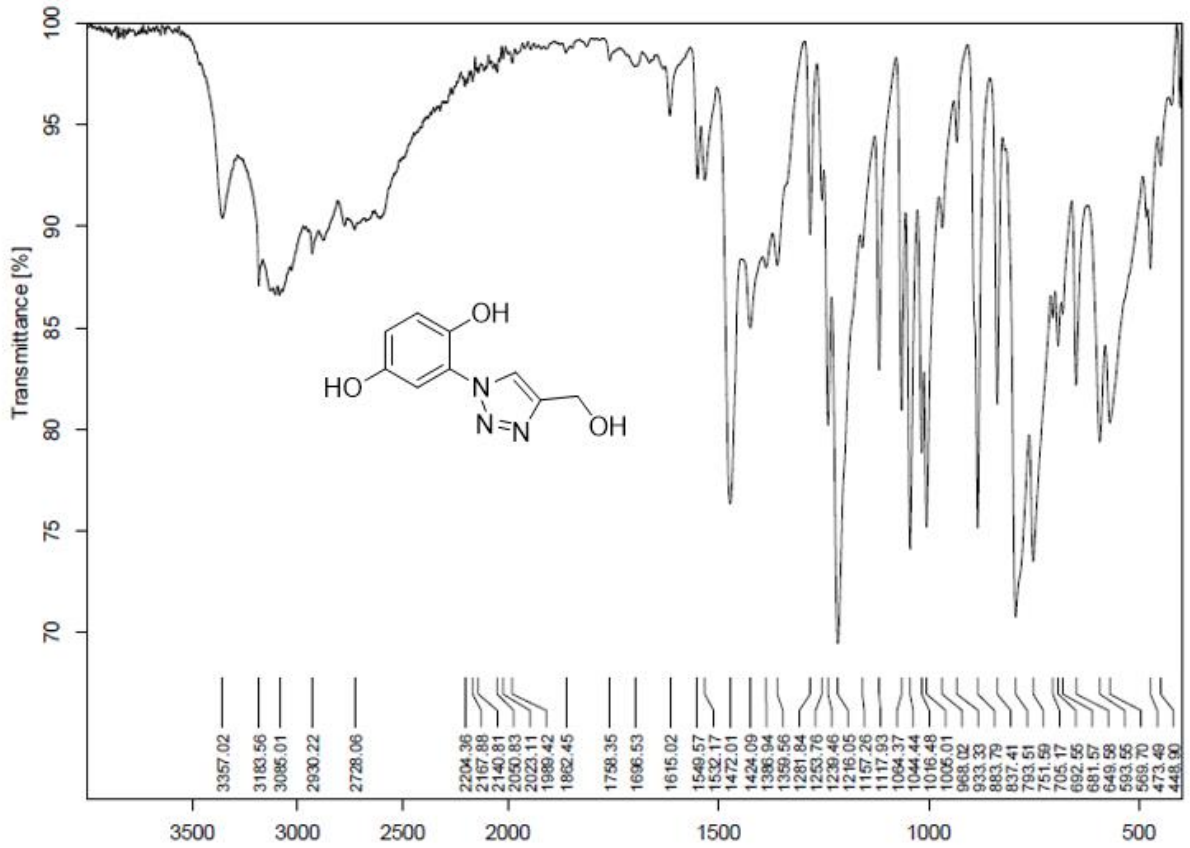

HRMS

Acquisition Parameter

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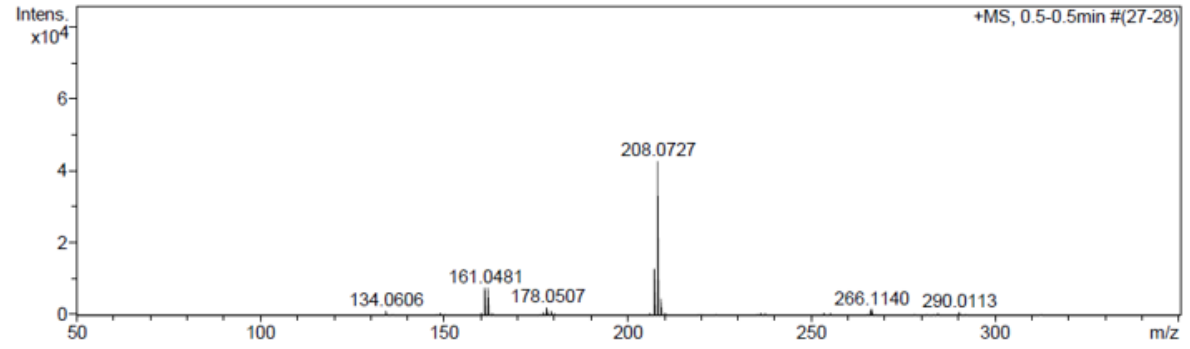

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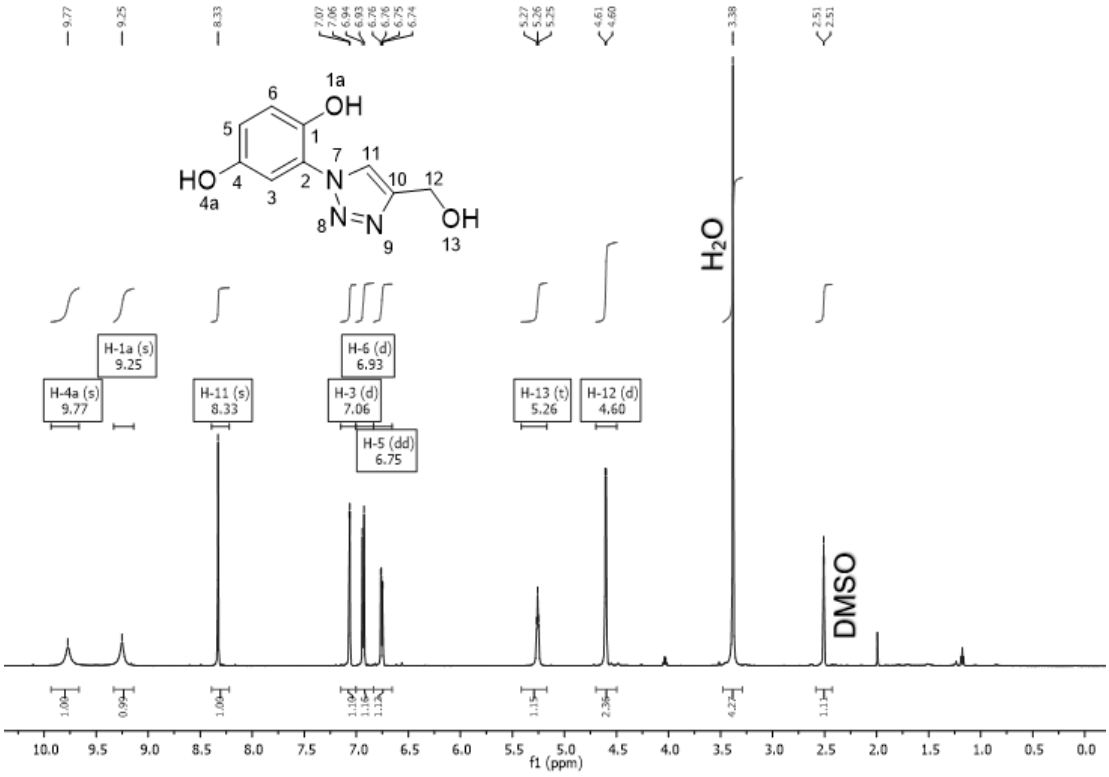

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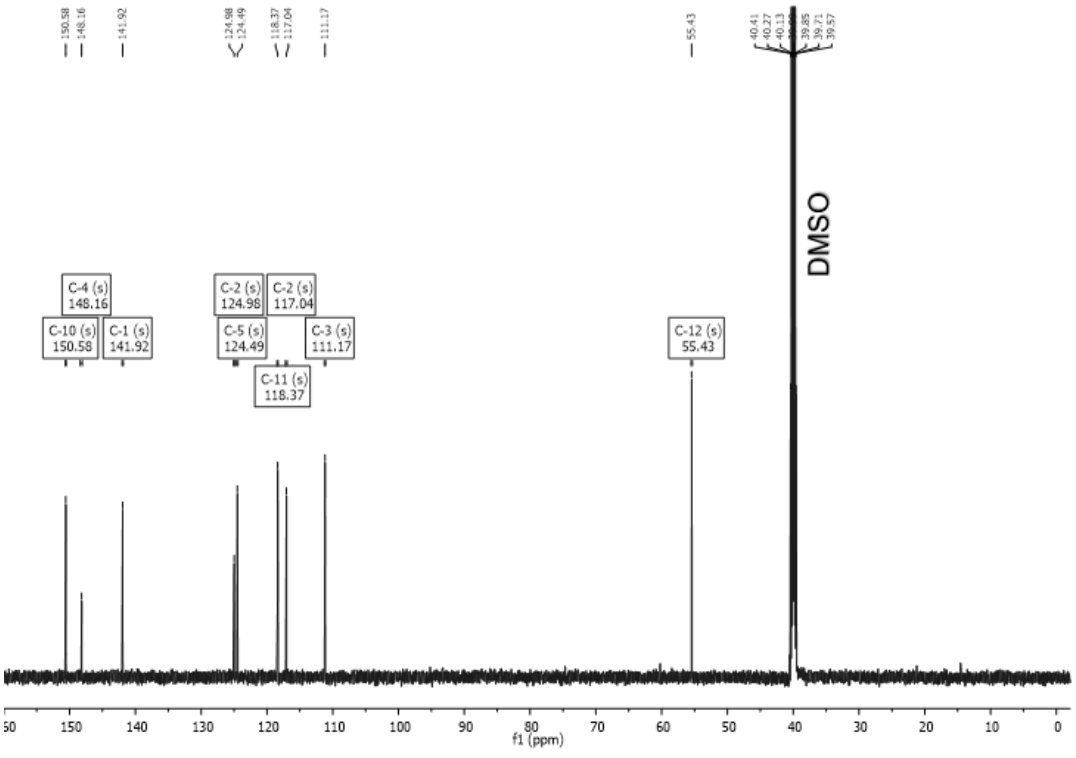

Compound **12**

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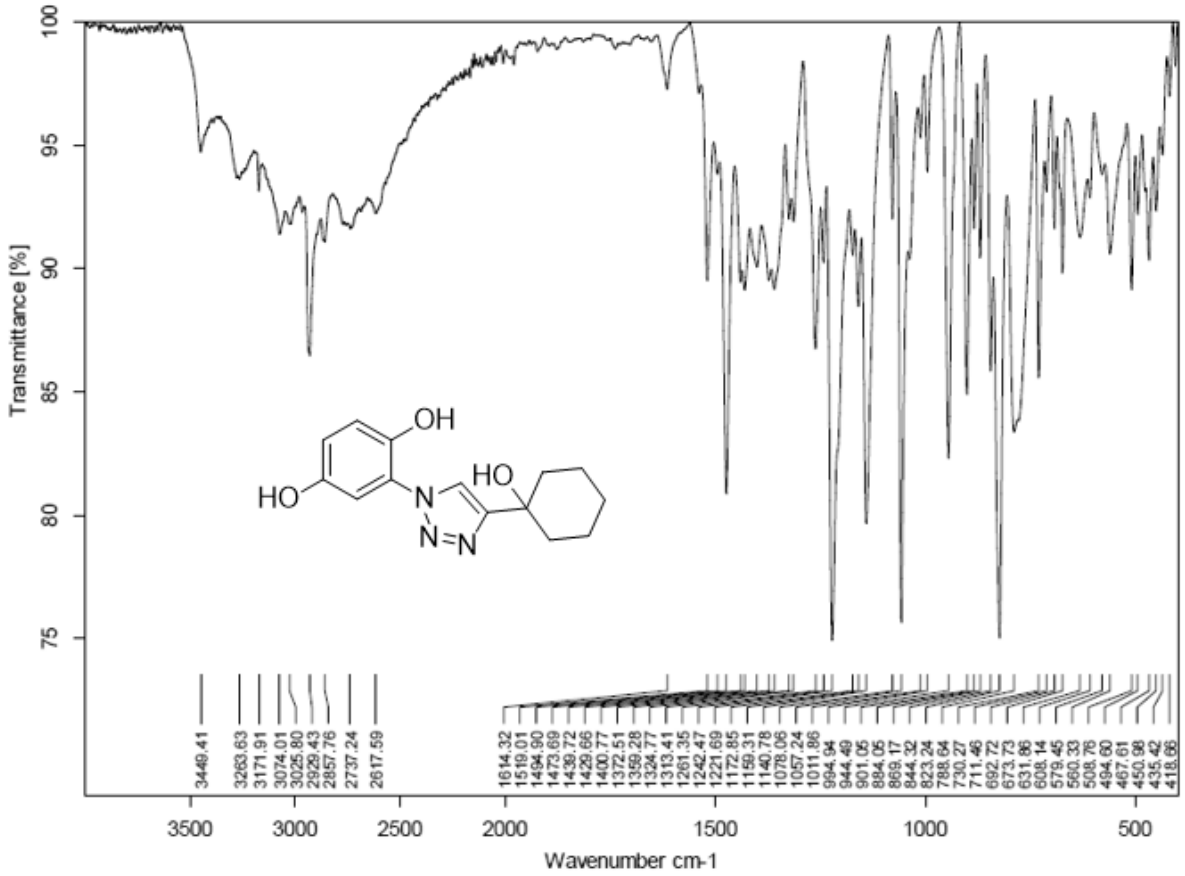

HRMS

Acquisition Parameter

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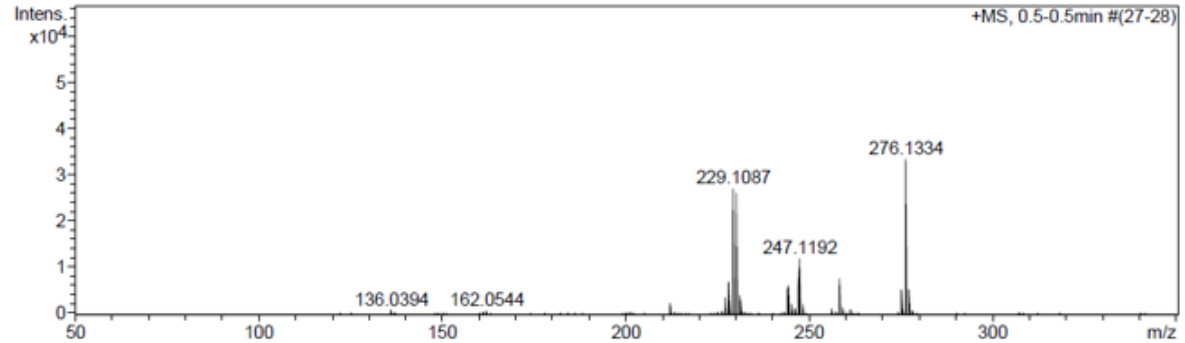

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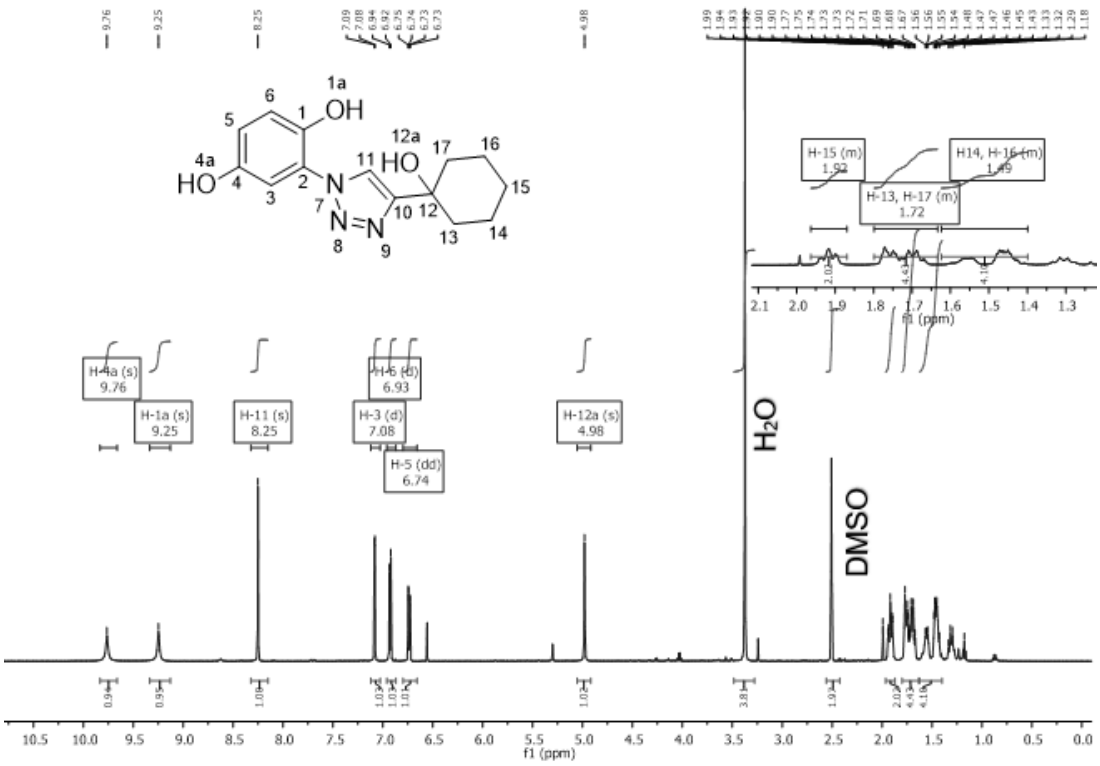
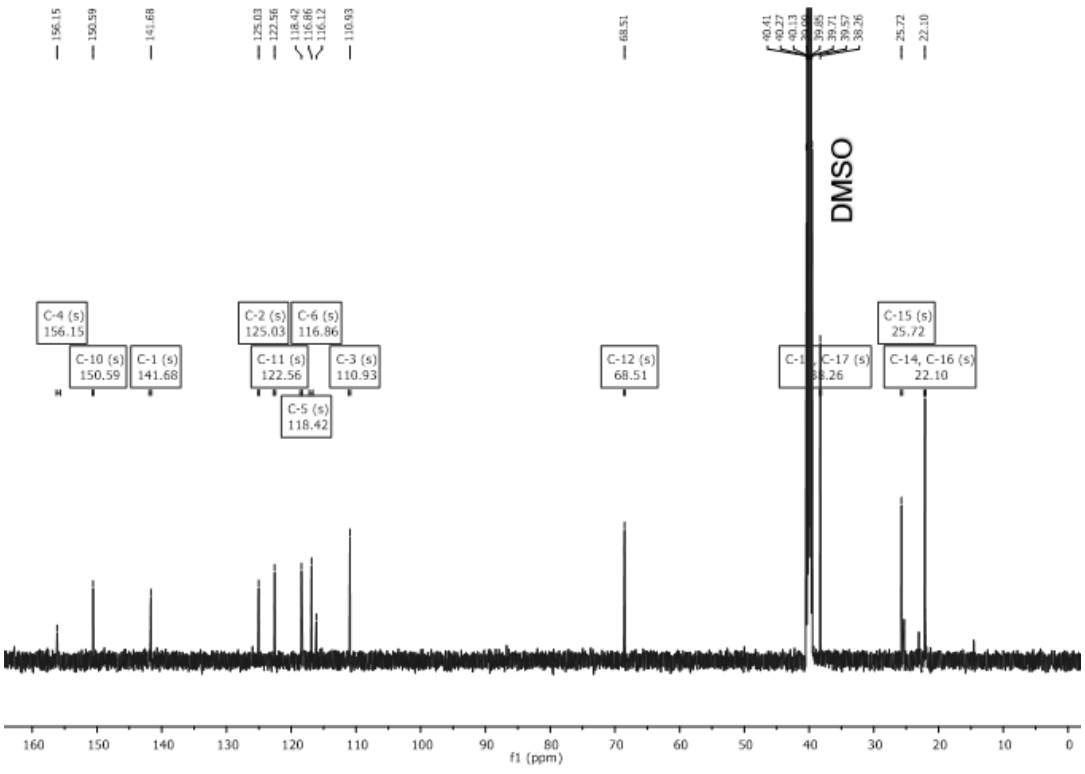
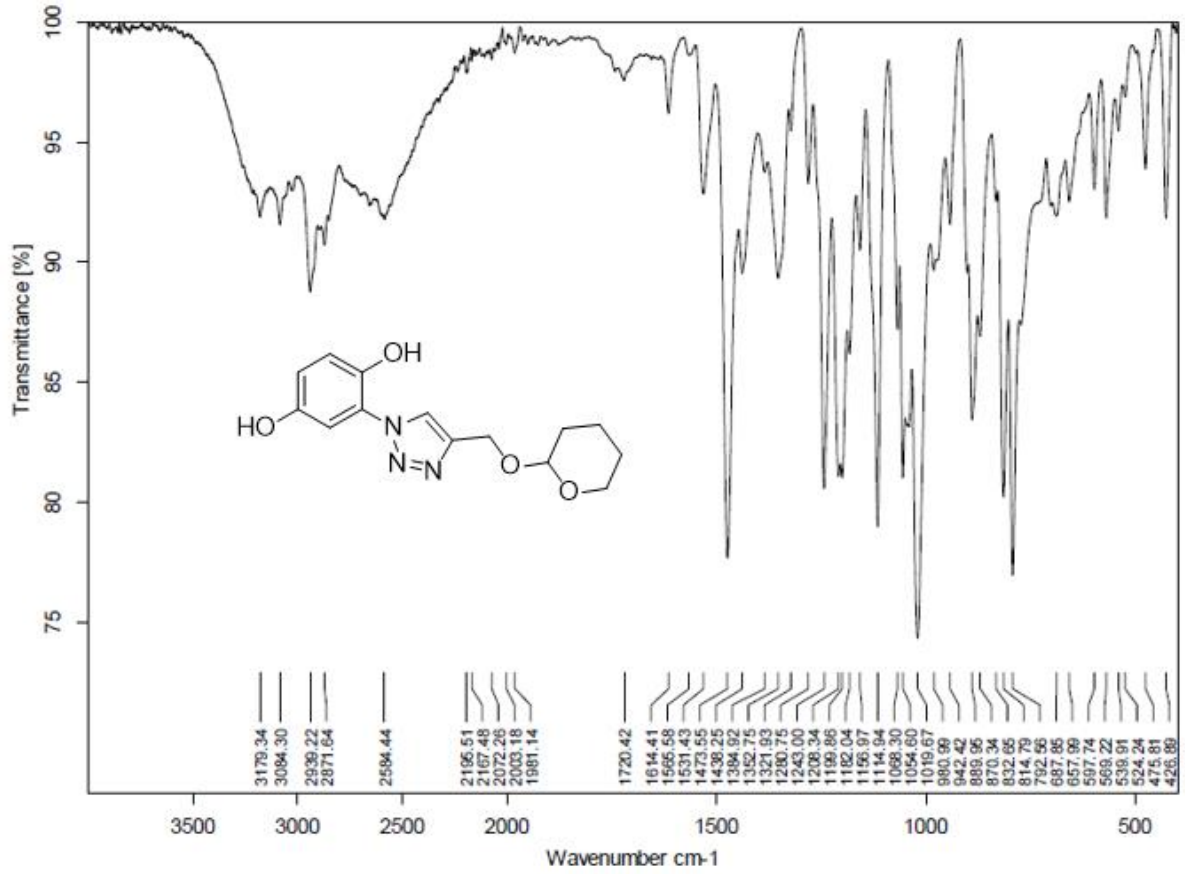


^{13}C in DMSO

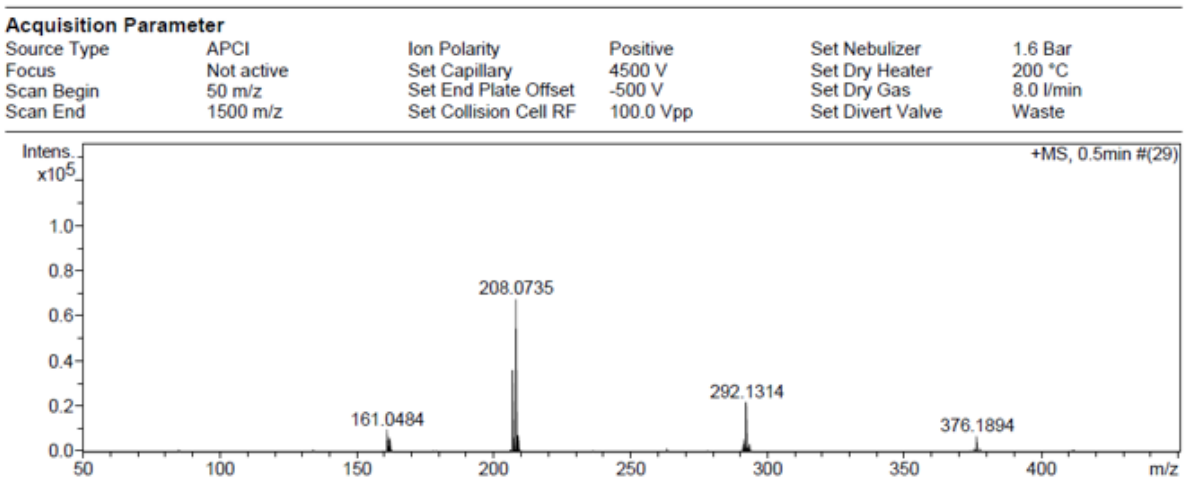


Compound **13**

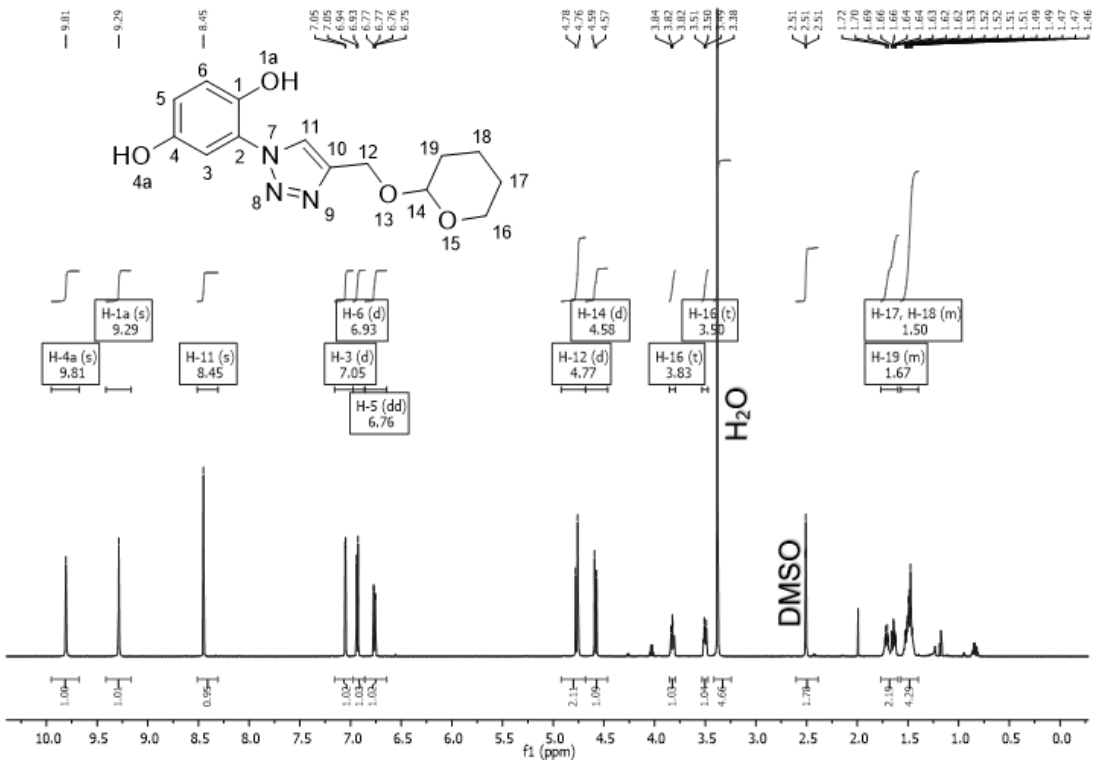
IR



HRMS



^{1}H in DMSO



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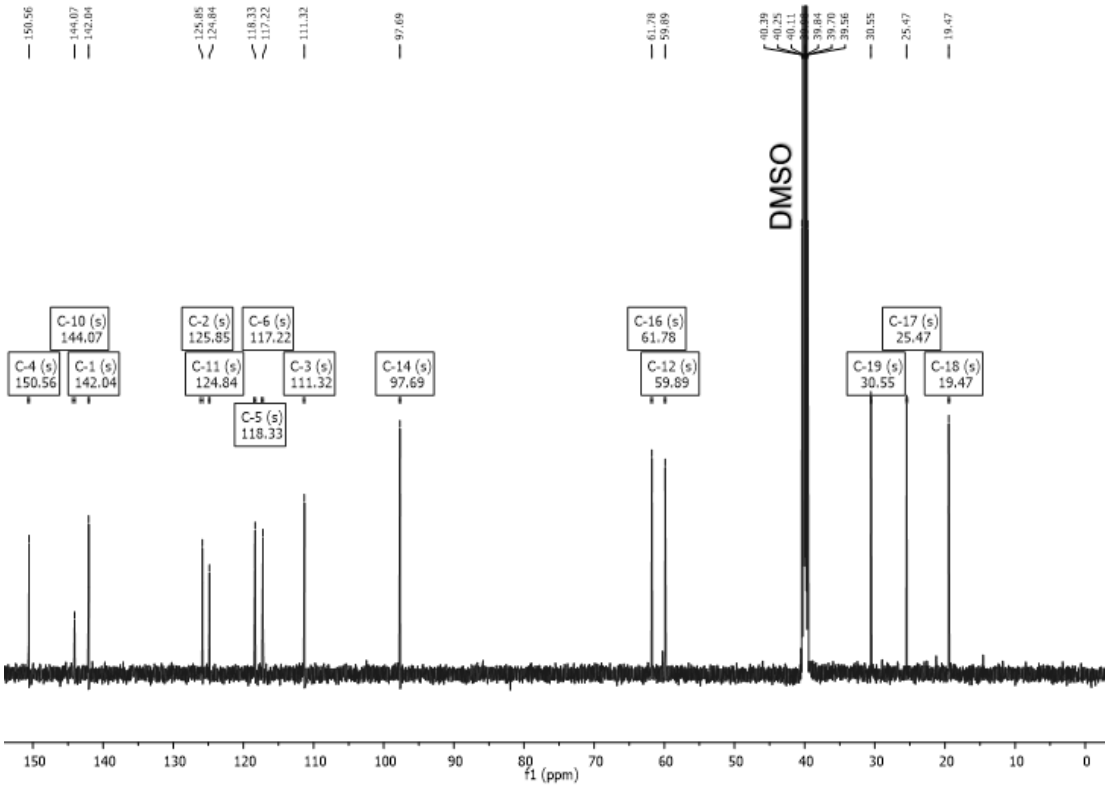
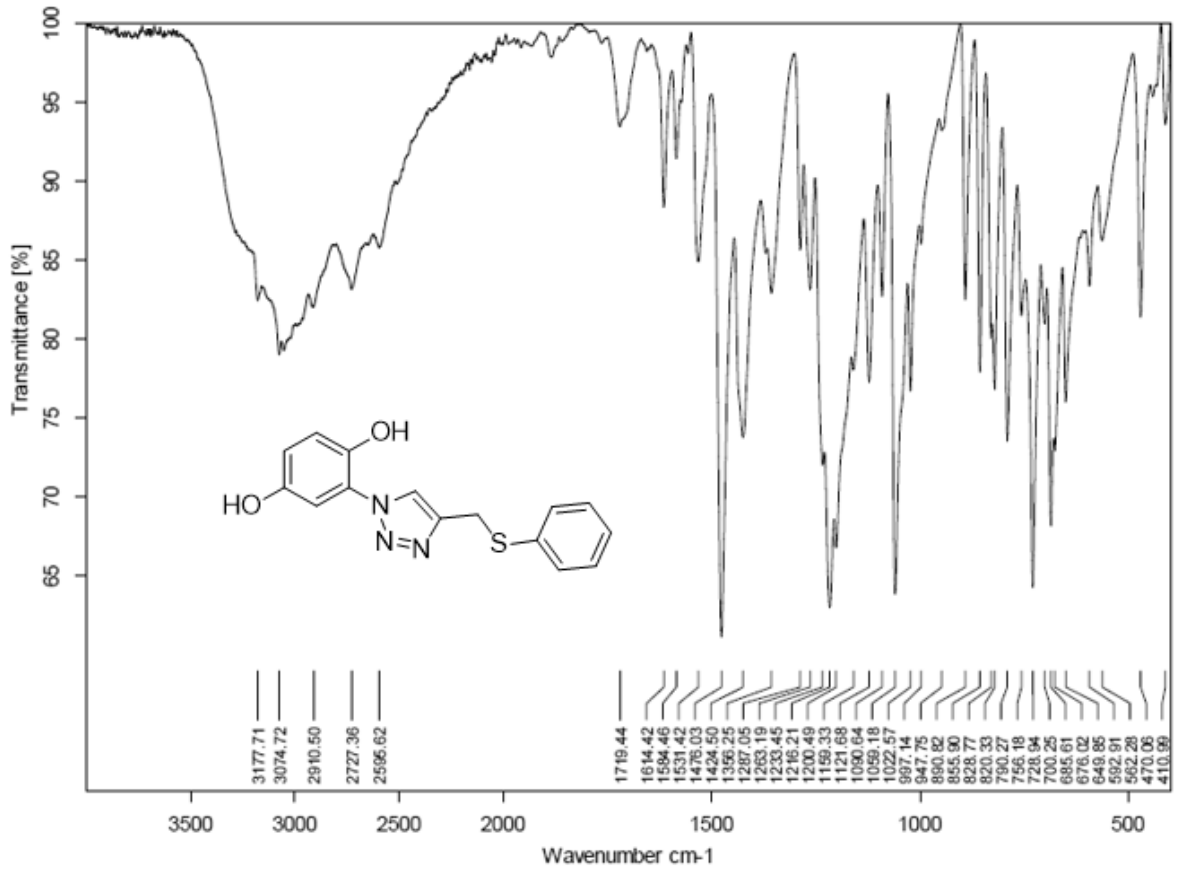
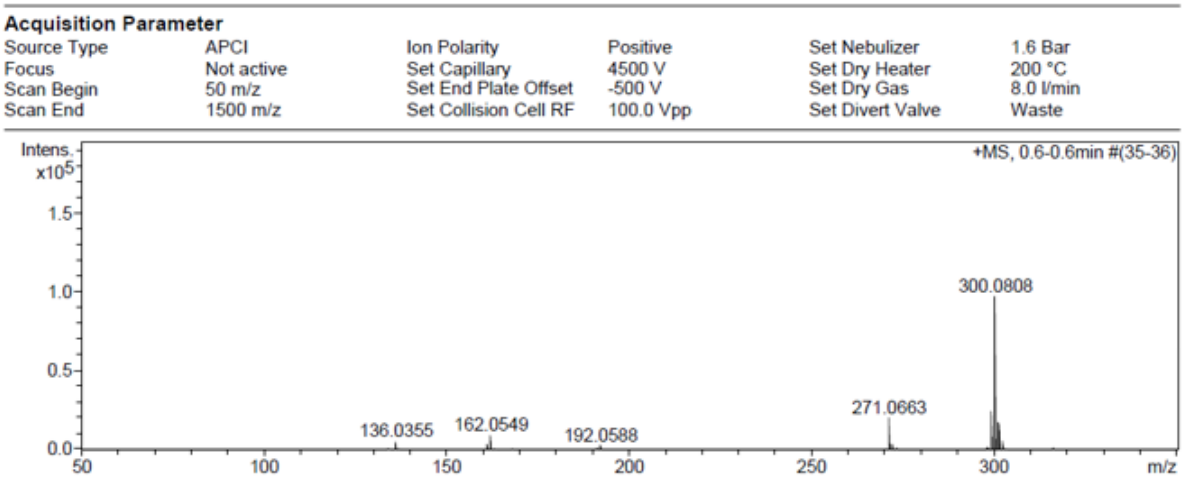


Compound **14**

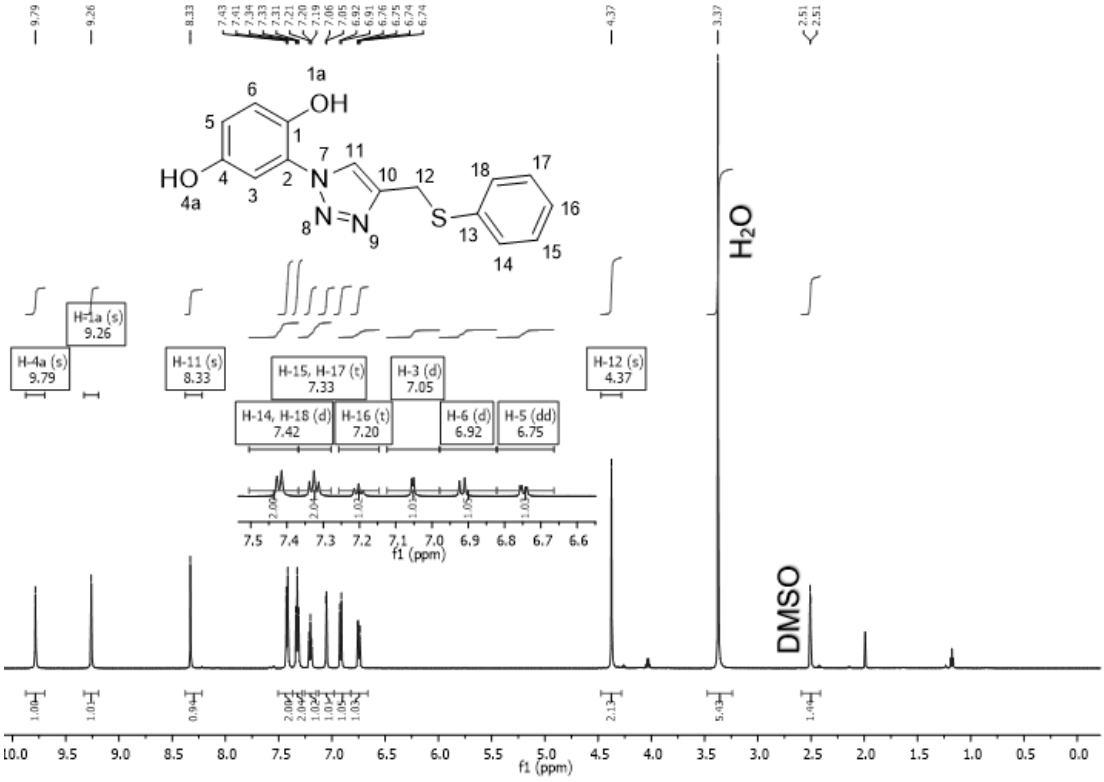
IR



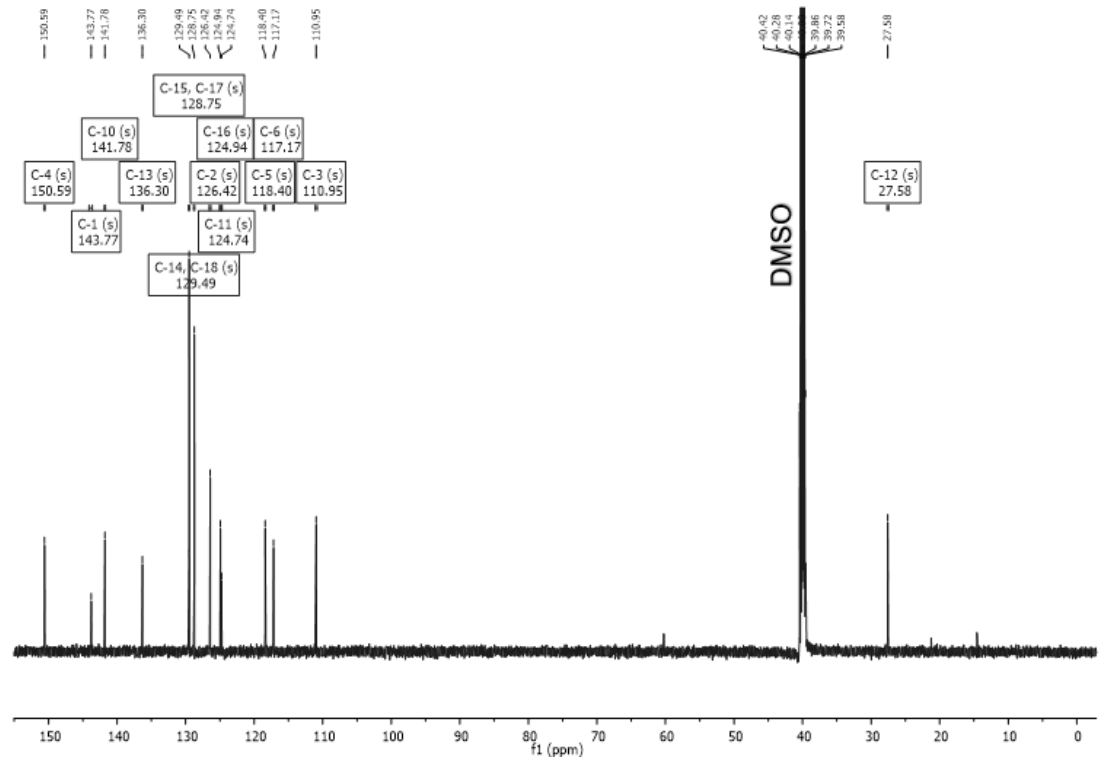
HRMS



^{1}H in DMSO



^{13}C in DMSO



ANNEXURE B: GUIDE FOR AUTHORS

EUROPEAN JOURNAL OF PHARMACEUTICAL SCIENCES

Official Journal of the European Federation for Pharmaceutical Sciences (EUFEPS)

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

ISSN: 0928-0987

DESCRIPTION

The journal publishes research articles, review articles and scientific commentaries on all aspects of the pharmaceutical sciences with emphasis on conceptual novelty and scientific quality. The Editors welcome articles in this multidisciplinary field, with a focus on topics relevant for drug discovery and development.

More specifically, the Journal publishes reports on medicinal chemistry, pharmacology, drug absorption and metabolism, pharmacokinetics and pharmacodynamics, pharmaceutical and biomedical analysis, drug delivery (including gene delivery), drug targeting, pharmaceutical technology, pharmaceutical biotechnology and clinical drug evaluation. The journal will typically not give priority to manuscripts focusing primarily on organic synthesis, natural products, adaptation of analytical approaches, or discussions pertaining to drug policy making.

Scientific commentaries and review articles are generally by invitation only or by consent of the Editors. Proceedings of scientific meetings may be published as special issues or supplements to the Journal.

Manuscripts submitted to the Journal are only accepted on the understanding that (a) they are subject to editorial review (generally by two independent reviewers); (b) they have not been, and will not be, published in whole or in part in any other journal; (c) the recommendations of the most recent version of the Declaration of Helsinki, for humans, and the European Community guidelines as accepted principles for the use of experimental animals have been adhered to.

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GUIDE FOR AUTHORS

INTRODUCTION

Manuscripts submitted to the journal are accepted on the understanding that: (1) they are subject to editorial review, (2) they have not been and will not be published in whole or in part in any other journal and (3) the recommendations of the most recent version of the Declaration of Helsinki, for humans, and the European Community guidelines as accepted principles for the use of experimental animals, have been adhered to. *The European Journal of Pharmaceutical Sciences* will, therefore, only consider manuscripts that describe experiments which have been carried out under approval of an institutional or local ethics committee.

Types of Paper

Research articles

The *European Journal of Pharmaceutical Sciences* publishes research articles in the multidisciplinary field of pharmaceutical sciences, with a focus on topics relevant for drug discovery and development.

More specifically, the Journal publishes reports on medicinal chemistry, pharmacology, drug absorption and metabolism, pharmacokinetics and pharmacodynamics, pharmaceutical and biomedical analysis, drug delivery (including gene delivery), drug targeting, pharmaceutical technology, pharmaceutical biotechnology and clinical drug evaluation.

The journal will typically not give priority to manuscripts focusing primarily on organic synthesis, natural products, adaptation of analytical approaches, or discussions pertaining to drug policy making.

Important other criteria for manuscript acceptance are conceptual novelty, scientific rigorousness of the experiments, relevance for a broad readership beyond the specific topic of the manuscript, and adherence to high ethics standards of experimentation. Research articles should comply with the format requirements set forth in the section "Article Structure below".

Review articles

The manuscript of a review article should be arranged as described for research articles but according to the following sections: title page, abstract and keywords (indexing terms, normally 3-6 items), Introduction, Specific sections determined by the author, Conclusions, Acknowledgements, References, Figure legends and Figures, Tables. Sections ranging from the Introduction to the Conclusions should be numbered. Subdivisions within a section should also be numbered within that section: 2.1., 2.2., 2.3. etc. All pages should be numbered consecutively, the title page being p.1.

Commentaries and Mini-reviews

One page suggestions for comprehensive reviews, commentaries or mini-reviews should be sent to the Editor-in-Chief at ejps@sdu.dk for consideration. Please see detailed information on commentaries and mini-reviews below.

Commentaries (Guidance)

The definition of a Commentary for EJPS is three-fold. Firstly, it can be an argued piece of provocative scientific writing purporting to take a balanced position on a controversial pharmaceutical science topic. A second option is for the author to approach the topic from a particular viewpoint on one side of an argument. A third option is to provide a topical update on a hot topic in Pharmaceutical Sciences and this can be more informative than controversial.

Commentaries will be commissioned by the editors in advance or invited from non-commissioned authors if they wish to initially submit a one page summary of the intended Commentary to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The journal is looking for a stimulating and provoking essays, with referenced material, but without an extensive reference list. Commentaries can contain one summary figure and/or table and should have no more than 30 references to preferably recent peer-reviewed material. The word count should be approximately 2,000 words maximum.
The commentary should have a short abstract summary of 150 to 200 words and 4-5 key words should be included, The text should be broken down into 4-5 numbered sections beginning with an Introduction and ending with a Conclusions section. A model of the structures is to be found in Eur. J. Pharm. Sci. 19, 1-11 by R.D. Combes.

Mini-review (Guidance)

Mini-reviews are thought provoking reviews of contemporary pharmaceutical research. Themes are as described in the Scope of the Journal section.

Mini-reviews will usually be commissioned by the editors in advance, but contributions are invited from non-commissioned authors if they wish to initially submit a one page summary of the intended review to the editors in advance. All manuscripts will be assessed by 2-3 independent referees.

The structure of the mini-review is as follows: a title page followed by a 200-300 word abstract with 4-5 key words. The text is then divided into numbered sections finishing with a Summary section. References should be kept to a maximum of 60 and should be mostly to recent peer-reviewed material. There is a combined maximum of 5 figures / tables. Authors are encouraged to submit their original unpublished work as part of the review if appropriate. The total length of the review should be a maximum of 4,000 words.

Submission checklist

You can use this list to carry out a final check of your submission before you send it to the journal for review. Please check the relevant section in this Guide for Authors for more details.

Ensure that the following items are present:

One author has been designated as the corresponding author with contact details:
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Supplemental files (where applicable)

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BEFORE YOU BEGIN

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Author contributions

For transparency, we encourage authors to submit an author statement file outlining their individual contributions to the paper using the relevant CRediT roles: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Software; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing. Authorship statements should be formatted with the names of authors first and CRediT role(s) following. More details and an example

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Introduction

State the objectives of the work and provide an adequate background, avoiding a detailed literature survey or a summary of the results.

Material and methods
Provide sufficient details to allow the work to be reproduced by an independent researcher. Methods that are already published should be summarized, and indicated by a reference. If quoting directly from a previously published method, use quotation marks and also cite the source. Any modifications to existing methods should also be described.

Results
Results should be clear and concise.

Text, tables and figures must show minimal overlap, and must be internally consistent.

Discussion
This should explore the significance of the results of the work, not repeat them. A combined Results and Discussion section is often appropriate. Avoid extensive citations and discussion of published literature.

Conclusions
The main conclusions of the study may be presented in a short Conclusions section, which may stand alone or form a subsection of a Discussion or Results and Discussion section.

Appendices
If there is more than one appendix, they should be identified as A, B, etc. Formulae and equations in appendices should be given separate numbering: Eq. (A.1), Eq. (A.2), etc.; in a subsequent appendix, Eq. (B.1) and so on. Similarly for tables and figures: Table A.1; Fig. A.1, etc.

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A concise and factual abstract is required. The abstract should state briefly the purpose of the research, the principal results and major conclusions. An abstract is often presented separately from the article, so it must be able to stand alone. For this reason, References should be avoided, but if essential, then cite the author(s) and year(s). Also, non-standard or uncommon abbreviations should be avoided, but if essential they must be defined at their first mention in the abstract itself.

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Abbreviations

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Abbreviations of units of measurements and other terms are as follows:

Units of mass

1 kilogramkg gramg milligrammg microgramμg nanogramng mole (gram-molecule)mol millimolemmol micromoleμmol nanomolenmol picomolepmol femtomolefmol equivalenteq

Units of time

1 hourh minutemin seconds millisecondms microsecondμs

Units of volume

1 litrel millilitreml microlitreμl

Units of length

1 metrem centimetrecm millimetremm micrometreμm nanometrenm

Units of concentration

1 molar (mol/l)M millimolarmM micromolarμM nanomolarnM picomolarpM

Units of heat, energy, electricity

1 jouleJ degree Celsius (centigrade)°C coulombC ampereA voltV ohmΩ siemensS

Units of radiation

1 curieCi counts per minutecpm disintegrations per minutedpm becquerelBq

Miscellaneous

1 gravity*g* dissociation constantK_d median dosesLD_{50}, ED_{50} probability*P* routes of drug administrationi.v., i.p., s.c., i.m. square centimetrecm^2 standard deviationS.D. standard error of the meanS.E.M. Svedberg unit of sedimentation coefficientS Hill coefficientn_H

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