Synthesis and antimycobacterial activity of ciprofloxacin-triazole hybrids

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PREFACE

This thesis is submitted in an article format in accordance with the General Academic Rules (A.13.7.3) of the North-West University. An article in the form of a manuscript is included in this dissertation:

Chapter 3: Article for submission

Design, synthesis and *in vitro* antimycobacterial activity of novel ciprofloxacin derivatives

This article will be submitted to the European Journal of Medicinal Chemistry and was prepared according to the author's guidelines, available in the author information pack on the Journal's homepage:

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ABSTRACT

Tuberculosis (TB) is a public health crisis – infecting a quarter of the world's population, also, inflicting 10.4 million morbidities and 1.7 mortalities in 2016. TB is effortlessly acquired through the inhalation of droplets containing the bacilli *Mycobacterium tuberculosis* (*Mtb*) from an infected individual. The occurrence of multi-, extensively-, and totally drug-resistant strains of *Mtb*, co-infection with human immunodeficiency virus (HIV), and the complex, prolonged and substantial treatment regimen with its associated side-effects result in poor patient compliance and creates an irrefutable demand for the development of novel antimycobacterial agents to address these issues.

Within this body of work, the skeleton of a second-line antimycobacterial agent, i.e. ciprofloxacin (CPX), was utilised to devise a new chemical entity with potent antimycobacterial activity. However, poor solubility and the rise of resistance against the fluoroquinolones class of drugs was recognised as a major drawback associated with CPX that requires improvement. Two series of CPX derivatives were synthesised, with each series a different strategy in mind for targeting *Mtb*. In Series 1 (analogues), CPX was derivatised in position N-15 and/or further modifications were made by replacing the hydroxyl-group in position C-11 of the carboxylic acid, with either an ester or an amide. In Series 2 (hybrids), hybrid drugs – consisting of CPX and 1,2,3-traizole (chosen for its reported antimycobacterial activity) – were prepared when utilising Huisgen's copper alkyne-azide cycloaddition ''click'' reaction. In both series, cholesterol was employed as targeting moiety since it gets taken up by *Mtb* who uses it as energy (carbon) source after metabolism. Anchoring CPX to cholesterol will result in the transport of the active drug into the bacterium through the *mce4*-transporter. Characterisation of all synthesised compounds was achieved by means of nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared spectrometry (IR), and the purity of compounds determined with the help of high performance liquid chromatography (HPLC).

Both series of compounds were assessed *in vitro* for their antimycobacterial activity against the human virulent H37Rv strain of *Mtb* in the commonly employed Middlebrook 7H9 Broth base media, using either a GFP or MABA reported assay. Overall, analogues with the carboxylic acid retained in position C-3 were mostly found to be active (MIC_{90} < 10 µM), the amides inactive (MIC_{90} >125 µM) and the esters had mixed activities (MIC_{90} 1 – 125 µM). The analogues containing the highly lipophilic cholesteryl moiety were mostly inactive. The activity of these fluoroquinolones was revealed in fact to be structure specific. This was equally seen with the hybrids. Additionally, the structure-activity relationship (SAR) revealed the least electron donating or withdrawing the substituent on the phenyl ring the more anti-*Mtb* active the hybrid.

Cytotoxicitities of synthesised compounds were assessed using normal human fetal lung fibroblast (WI38), human embryonic kidney cells (HEK-293), and Chinese hamster ovary (CHO) mammalian cell lines. The active analogues **9**, **10**, **11** and **13**, and the hybrids **26**, **28**, **29**, **30** and **32** were nontoxic towards these cell lines, with selectivity indices (SI) greater than 10 – showing high specificity towards *Mtb*. On account of the efficacy and safety criteria, analogues **10** and **11** and hybrid **28** with MIC_{90} below 10 µM, and SI-values greater 27 were the most promising hit candidates lending themselves for further development.

Keywords: *Mycobacterium tuberculosis*, ciprofloxacin, 1,2,3-triazole, click-chemistry, hybrids

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ABBREVIATIONS

µM	Micromolar
abs	Absolute
ACN	Acetonitrile
ADC	Albumin-dextrose-catalase
AIDS	Acquired immunodeficiency syndrome
APCI	Atmospheric pressure chemical ionisation
BCG	Bacille Calmette-Gièrin
CDC	Centers for Disease Control
$CDCl_3$	Chloroform-*d*
CHO	Chinese hamster ovary cell line
Cmax	Maximum serum concentration
CNS	Central nervous system
CPX	Ciprofloxacin
CYP450	Cytochrome P450
DCM	Dichloromethane
DE	Diethyl ether
DHFR	Dihydrofolate reductase
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DMSO-d_6	Dimethyl sulfoxide-d_6

DOT	Directly observed therapy
DST	Drug susceptibility testing
EA	Ethyl acetate
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EDG	Electron donating group
EGT	Ergothioneine
EM	Emetine
EPTB	Extra-pulmonary tuberculosis
ESI	Electrospray ionisation
ETB	Ethambutol
EtOH	Ethanol
EWG	Electron withdrawing group
FAD	Flavin adenine dinucleotide
GFP	Green fluorescent protein
GLU	Glucose
H37Rv	Virulent culture strain of *Mycobacterium tuberculosis*
HBTU	*N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate,
HEK-293	Human embryonic kidney cells
HIV	Human immunodeficiency virus
HIV/TB	Human immunodeficiency virus and tuberculosis
HPLC	High-performance liquid chromatography
Hz	Hertz

IC_{50}	50% Inhibitory concentration
INH	Isoniazid
IR	Infrared
LTBI	Latent tuberculosis infection
m.p.	Melting point
MABA	Microplate Alamar Blue Assay
MDR-TB	Multi-drug resistant tuberculosis
MeOH	Methanol
$MgSO_4$	Magnesium sulphate
MIC	Minimum inhibitory concentration
mPz	methylpiperazine
mRNA	messenger ribonucleic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass spectrometry
MSH	Mycothiol
MSSA	Methicillin-sensitive *Staphylococcus aureus*
Mtb	*Mycobacterium tuberculosis*
NaAsc	Sodium ascorbate
$NaHCO_3$	Sodium bicarbonate
NaN_3	Sodium azide
NH_4C	Ammonium chloride
NMR	Nuclear Magnetic Resonance
NRF	South African National Research Foundation

PABA	*para*-Aminobenzoic acid
PAS	*para*-Aminosalicylic acid
POA	Pyrazinoic acid
PZA	Pyrazinamide
QT-interval	Start of the Q-wave and the end of the T-wave in the hearts electrical cycle
RIF	Rifampicin
RNA	Ribonucleic acid
ROS	Reactive species of oxygen
rRNA	Ribosomal ribonucleic acid
RR-TB	Rifampicin-resistant tuberculosis
SA	South Africa
SAE	Serious adverse events
SAR	Structure-activity relationship
SI	Selectivity index
SJS	Stevens–Johnson syndrome
TB	Tuberculosis
TDR-TB	Totally drug-resistant tuberculosis
TEA	Triethylamine
THF	Tetrahydrofuran
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor
TST	Tuberculin skin test

TW	Tween 80
VRE	Vancomycin-resistant enterococci
WHO	World Health Organization
WI-38	Normal human fetal lung fibroblast cell line
XDR-TB	Extensively drug-resistant tuberculosis
XXDR-TB	Extremely drug-resistant tuberculosis

CHAPTER 1:
INTRODUCTION

1.1 General background and problem statement

Tuberculosis (TB), malaria and human immunodeficiency virus (HIV)-1 infections are the most lethal infectious diseases worldwide (Goldberg *et al.*, 2012). TB is caused by a bacterium, the bacillus *Mycobacterium tuberculosis* (*Mtb*), and is spread through the air when an infected individual with pulmonary TB expels the bacteria *via* cough or sneeze (WHO, 2018c). According to the World Health Organization (WHO), one-quarter of the world's population is living with latent TB (WHO, 2018c) and an additional 10.4 million new TB cases were reported in 2016 (WHO, 2017b). What's more, TB accounted for 1.7 million deaths in 2016 (WHO, 2017b); in addition, 0.4- and 1.0 million people succumbed to malaria and HIV, respectively (WHO, 2017c; WHO, 2017e). This places TB as the leading infectious killer worldwide (WHO, 2017b).

However, TB is a preventable, treatable, and curable disease (WHO, 2018c); provided that a standard six-month course with first-line drugs be strictly followed. This regimen consists of a two-month course, known as the intensive phase, with isoniazid, rifampicin, pyrazinamide, and ethambutol and a continuation phase of four months with isoniazid and rifampicin (National Department of Health, 2014). The intensive phase is intended for the rapid killing of the tubercle bacilli, whilst the continuation phase eliminates any reaming bacilli to prevent relapse (Rossiter *et al.*, 2012). Unfavourable interactions between different TB drugs (Goldberg *et al.*, 2012) and a lengthy treatment regimen may lead to side effects, poor patient compliance, and eventually the emergence of drug-resistant TB (Krishna *et al.*, 2014). TB was reported to be the leading cause of death due to drug-resistance (WHO, 2017b). Furthermore, treatment success gets considerably smaller from drug susceptible TB to drug-resistant TB (between 30 to 54%) (WHO, 2018c).

The first form of drug-resistance due to failure of the two most potent anti-TB agents, i.e. rifampicin and/or isoniazid, is known as multi-drug resistant TB (MDR-TB) (WHO, 2018d) which resulted in 490 000 new cases of MDR-TB and accounted for 240 000 deaths in 2016 (WHO, 2017d). Treatment options of resistant TB with second-line anti-TB agents are substantially longer (up to two years), limited availability, expensive, and the incidence of serious adverse effects are increased which makes it all the harder to treat (WHO, 2018d). Aforementioned factors may all lead to incomplete adherence to treatment (WHO, 2018d) and consequently to the development of more severe forms of resistant TB. For instance, the fluoroquinolone class of drugs including norfloxacin, ciprofloxacin, levofloxacin, gemifloxacin, and moxifloxacin are the second-line drugs

for TB treatment known to elicit serious adverse events during extended use (Olcay *et al.*, 2011; Stahlmann & Lode, 2013; Verma *et al.*, 2009). Indeed, the Food and Drug Administration (FDA) issued a *Boxed Warning* due to tendon rupture, irreversible peripheral neuropathy, and central nervous system effects associated with the use of these drugs (FDA, 2017).

Extensively drug-resistant TB (XDR-TB) is defined as MDR-TB with added resistance to a fluoroquinolone and an injectable agent (e.g. amikacin, capreomycin, kanamycin, or streptomycin) (Pooran *et al.*, 2013). There were 8 000 reported cases of XDR-TB in 2016 (WHO, 2017d). In addition, *Mtb* that does not respond to any of the first – and second-line anti-TB agents are known as totally/extremely drug-resistant TB (TDR/XXDR-TB) (WHO, 2018b). The first reported cases of TDR-TB were in Iran and another four in India, back in 2009 (WHO, 2018a). Controversy regarding the term TDR-TB exist, as clarification is needed. The WHO currently do not accept this term due to challenges linked to drug susceptibility testing and limitations on the interpretation of results, as well as new drugs (i.e. bedaquiline, and delamanid) that was introduced in 2016 (WHO, 2018a). These new drugs effectiveness has not yet been established against TDR-TB, which nullifies the term TDR-TB (WHO, 2018a).

Developing countries carries almost exclusively (>95%) the burden of TB cases and deaths, even though TB can occur in any part of the world (WHO, 2018c). In 2016, Asia and Africa accounted collectively for 87% of all newly reported TB cases, where countries like India, Indonesia, China, Philippines, Pakistan, Nigeria and South Africa stood out as the seven countries bearing the brunt (WHO, 2018c). And once again South Africa accounted for the majority (41%) of HIV-positives who started TB treatment (WHO, 2017b). Co-infection with HIV causes a further concern to TB control (Xia *et al.*, 2017) and lead to 0.4 million deaths due to HIV-associated TB (WHO, 2018c). TB was also the leading cause of death in people living with HIV (WHO, 2017b).

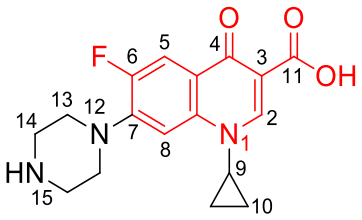
Overall, whilst TB, malaria, and HIV are caused by vastly different pathogens, these diseases share a common problem; the emergence of drug resistant strains of the causative organisms, which further complicates the eradication/control of these diseases. It is therefore crucial to develop new agents or combinations to treat both drug-sensitive and drug-resistant organisms (Goldberg *et al.*, 2012).

1.2 Rationale

The development of clinically useful drugs is complex, time consuming, risky (Forum on Neuroscience and Nervous System Disorders *et al.*, 2014), and expensive (Projan, 2003). Exploring the structure-activity relationship (SAR) of lead compounds forms the cornerstone of drug discovery (Guha, 2013), compared to the cumbersome discovery and establishment of

innovative lead compounds. Encouraged by the structure-activity relationship, the fluoroquinolones are a promising class of drugs that will be exploited, through the investigation of novel ciprofloxacin-derivatives, as they are all currently used to treat MDR-TB (WHO, 2016). The fluoroquinolones' relative simple pharmacophore (Figure 1-1) makes it convenient for an array of structural amendments (Sharma *et al.*, 2009). Investigation of the SAR of the fluoroquinolones indicated that modifications made in position C-7 greatly control their spectrum, potency, and pharmacokinetics (Andersson & MacGowan, 2003; Rajulu *et al.*, 2013). Also, replacement or deprotonation of the carboxylic acid, in position C-3, with either an isothiazole group or certain esters affords derivatives with increased antibacterial activity to the parent drugs, e.g. ciprofloxacin (Sharma *et al.*, 2009).

Ciprofloxacin (CPX) can be recognised as the paragon of the fluoroquinolone class of agents, as most newer drugs (e.g. moxifloxacin and gatifloxacin) in this class, are built on its scaffold and also due to it being commonly prescribed (Asif, 2014; Sharma *et al.*, 2009). In fact, the WHO considers this antibiotic as an essential drug (Breda *et al.*, 2009). CPX contains the quinolone pharmacophore and a 6-fluoro moiety; which awards it its broad spectrum of activity (Andersson & MacGowan, 2003). Additionally, the cyclopropyl and piperazine rings in positions N-1 and C-7 (Figure 1-1), further potentiates its activities (Sharma *et al.*, 2009). However, CPX is also known for its lowest in-class solubility (Firestone *et al.*, 1998) as it is zwitterionic – leading to a restriction in bioavailability (Surov *et al.*, 2015).



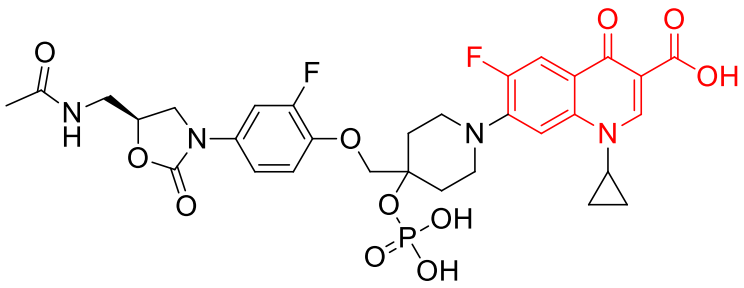
Ciprofloxacin (CPX)

Figure 1-1: Structure of ciprofloxacin (in red is the fluoroquinolone pharmacophore).

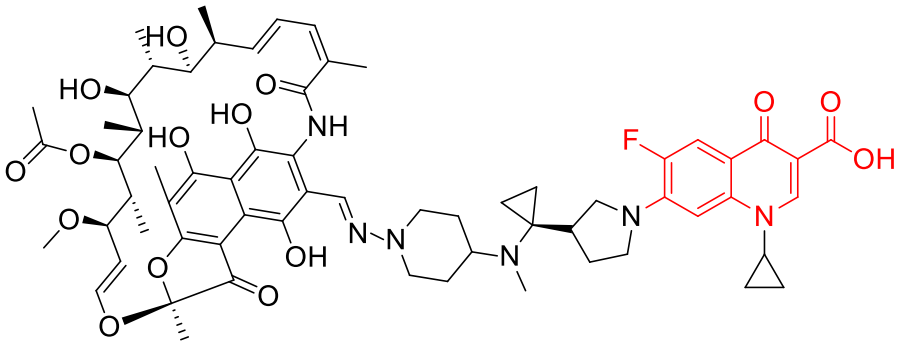
Thus, by building on the nucleus of CPX and with all the SAR considered, other substituents will be explored in this study in an effort to enhance this agent's antimycobacterial activity whilst reducing associated side-effects and warrant sufficient bioavailability.

Another common and well-established strategy in the search for new drugs is molecular hybridisation. A hybrid molecule is a single entity obtained by covalently linking two distinct chemical pharmacophores with multiple effects. These molecules are introduced in anticipation

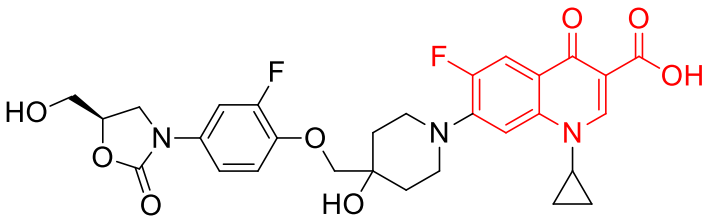
that they may overcome drug resistance by working in synergy (Meunier, 2007). New agents with improved activity have successfully been synthesised based on this concept, and some such as MCB-3837 (oxazolidinone-fluoroquinolone hybrid), TNP-2092 (rifamycin-fluoroquinolone hybrid) have already entered the clinical trials and are both in Phase-I. Cadazolid, a hybrid of oxazolidinone and fluoroquinolone pharmacophores, has entered Phase-III clinical development against various WHO priority pathogens such as *Mtb* and *Clostridium difficile* (Bellot *et al.*, 2010; Dixit *et al.*, 2016; Manohar *et al.*, 2010; Manohar *et al.*, 2011; WHO, 2017a). Figure 1-2 illustrates each hybrid's chemical architecture, with the fluoroquinolone pharmacophore (outlined in red) part of their design.



MCB-3837



TNP-2092



Cadazolid

Figure 1-2: Structures of hybrid molecules containing the fluoroquinolone core that are currently in clinical trials.

Based on the above-mentioned strategy/technique, and in the midst of addressing the drug-resistance issue, the commonly used anti-TB agent (i.e. ciprofloxacin) and a proven TB active scaffold (i.e. 1,2,3-triazole) will be covalently linked in an attempt to generate hybrid molecules with increased activity, in comparison to the individual active component, and reduced side-effects associated with fluoroquinolones, which will in-turn have an impact on resistance.

Triazoles are heterocyclic compounds with three nitrogen atoms as part of their aromatic five-membered ring. They exist as a pair of isomers namely 1,2,3- (Figure 1-3) and 1,2,4-triazoles, however notice has been taken of the 1,2,3-isomer due to its broad biological activities

(antimicrobial, anti-HIV, anticancer, anti-inflammatory and antitubercular) (Emmadi *et al.*, 2015). Moreover, synthetic molecules containing the 1,2,3-triazole ring have shown among others antitubercular activities – I-A09 and radezolid (Figure 1-3) are both examples of anti-TB agents containing the 1,2,3-triazole moiety and are both being assessed for activity against drug-resistant TB (Bhukya & Tangutur, 2017; Xia *et al.*, 2017).

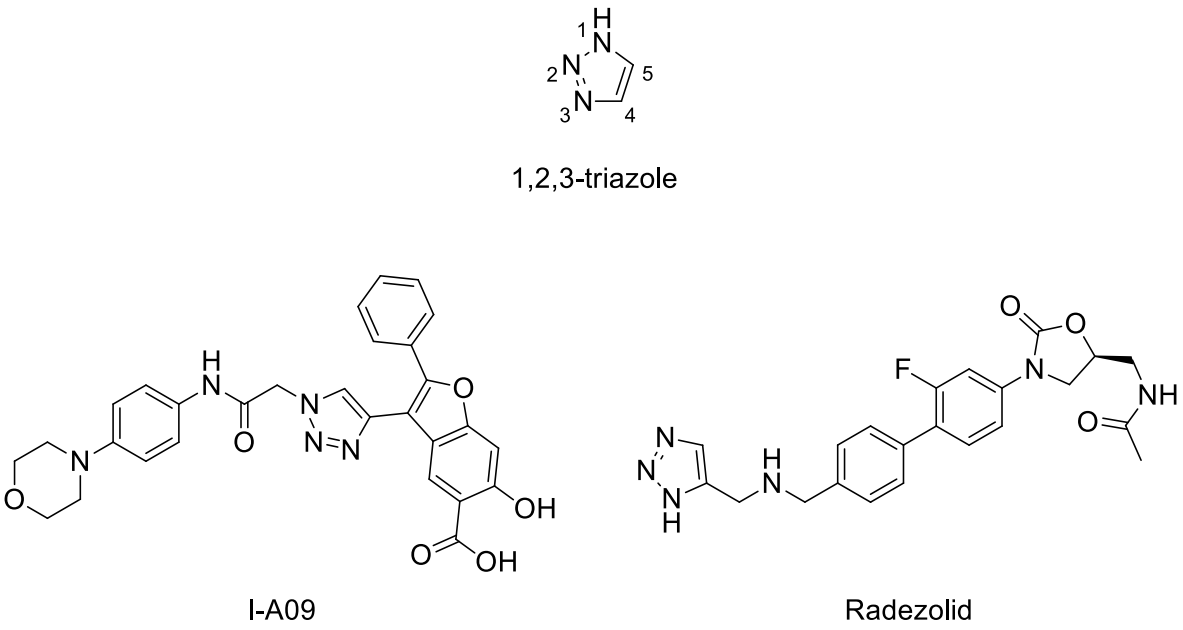


Figure 1-3: Structures of the 1,2,3-triazole moiety, also the I-A09 derivative and drug, radezolid, containing the 1,2,3-triazole ring.

Furthermore, recent studies have implicated cholesterol as a major carbon source and a driving factor for virulence of TB as reported by Brzostek *et al.* (2007). The pathogenic effect of cholesterol in *Mtb* virulence has been demonstrated in mice infected with *Mtb* strains lacking the *Mtb* cholesterol transporter, *mce4*. These mice survived the infection, suggesting an attenuated virulence of *Mtb*. The lack of this transporter limits bacterial growth when cholesterol is used as a limited carbon source (Pandey & Sassetti, 2008). Therefore, incorporating cholesterol into drug molecules may thus improve transport of the latter into the bacterium. This will allow penetration through the highly lipophilic cell wall of the *Mtb* and assist in transporting hydrophilic molecules into the *Mtb*. The oxidative and nutrient deprived environments of the macrophage and the granuloma can be exploited as potential routes for new anti-TB drugs. Thus, in the framework of this CPX-project, analogues and hybrids may contain a cholesterol moiety as carrier to facilitate their transport across the lipophilic bacteria wall.

The latest global TB statistics galvanised this project into delving for new drugs that are more potent than existing drugs, together with a safe pharmacokinetic profile. The excellent bactericidal

effect of fluoroquinolones and the fact that this class of drugs are recommended by WHO as second-line anti-TB agents cemented our choice of fluoroquinolone, the scaffold of CPX in the design of novel anti-TB agents. Increasing the lipophilic character of CPX may theoretically improve its activity towards *Mtb*, as infiltration of fluoroquinolones into bacterial cells are greatly controlled by their lipophilicity (Xia *et al.*, 2017). For the design of novel drugs, emphasis will be placed on the SAR of fluoroquinolones, and on the integration of another pharmacophore (1,2,3-triazole) and/or a dietary molecule (cholesterol) to CPX; in an attempt to address the issues such as poor solubility, toxicity, and ultimately resistance that are associated with fluoroquinolone therapy.

1.3 Hypothesis

SAR studies of the fluoroquinolone molecule have determined that substituent changes made in positions N-15 and C-11 greatly controls antibacterial spectrum, potency, and pharmacokinetic profile (Andersson & MacGowan, 2003; Asif, 2014; Chu & Fernandes, 1989; Tillotson, 1996). Also, five or six membered heterocyclic rings containing nitrogen boosts activity when substituted in position C-7 of fluoroquinolones (Tillotson, 1996). Thus, based on the fact that fluoroquinolones are already in use against drug resistant strains of *Mtb* (WHO, 2016), it can be hypothesised that CPX's scaffold will be a promising lead for substituent changes made in positions N-15 and C-11, to yield fluoroquinolone-analogues that have heightened activity towards *Mtb* and reveal the structure-activity relationship that control *Mtb* activity due to improved solubility.

Hybridisation of biologically active pharmacophores is a powerful tool to produce a single multi-functional agent that may lead to a powerful therapeutic due to the synergism that these molecules produce (Bérubé, 2016). Therefore, based on the fact that the second-line anti-TB agent CPX and 1,2,3-triazole moiety are proven active against *Mtb*; it can further be postulated that the hybridisation of these scaffolds will result in a single chemical entity that possesses enhanced antimycobacterial activity due to synergism.

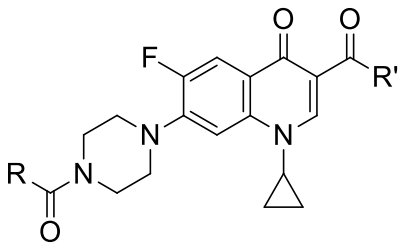
Cholesterol forms an integral part of *Mtb* pathogenicity/virulence, since *Mtb* utilises cholesterol as a carbon source through the mce4-transporter (Brzostek *et al.*, 2007). Thus, cholesterol will be joined to CPX and the hybrid drug. The hypothesis is that *Mtb* will utilise the dietary molecule (i.e cholesterol) and so mistakenly facilitate the transport of the CPX and/or triazole component into the bacterium with the intention of each pharmacophore to initiate its working mechanism, and ultimately lead to *Mtb* death.

1.4 Aims and objectives of this project

The aim of this project is to investigate novel ciprofloxacin derivatives through the design and synthesis of analogues and hybrid molecules, also their *in vitro* biological evaluation against the human virulent strain H37Rv of *Mycobacterium tuberculosis*; with the ultimate goal to develop potent, effective, safer, and affordable new antitubercular agents as potential replacements for the current drugs in clinical use.

Set of objectives for this study to achieve these aims are:

- Synthesis and characterisation of novel analogues of ciprofloxacin; with the general structure depicted in Figure 1-4. Investigation of this series is intended to get insight into the impact of improved aqueous solubility on the antitubercular activity. Furthermore, the SAR of this series will shed light on the best C-11 functional group resulting in increased activity.

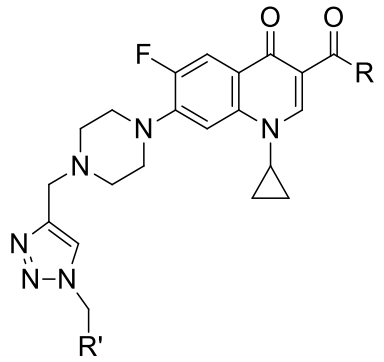


Where R is n-alkyl, cycloamine, heterocyclic, or cholesteryl

and R' is a hydoxyl (OH), etoxy (O),

or methylpiperazine moiety (N N).

Figure 1-4: General structure of ciprofloxacin analogues.



Where R is hydoxyl (OH), etoxy (O),

or methylpiperazine (N N),

and R' is aryl, and cholesteryl.

Figure 1-5: General structure of ciprofloxacin hybrids.

- Characterisation of the synthesised compounds will be done by means of nuclear magnetic resonance (NMR), mass spectrometry (MS), infrared spectrometry (IR), and determining the melting points of each compound. The purity of compounds will also be determined by means of high performance liquid chromatography (HPLC).

- Assessment of the *in vitro*, antitubercular activity of the synthesised compounds, against the laboratory virulent Mtb H37Rv strain in Middlebrook 7H9 Broth Base media, using green-fluorescent protein (GFP) assay or microplate alamar blue assay (MABA).

- Evaluation of the cytotoxicity profiles of the active compounds using mammalian cell lines, such as human fetal lung fibroblast - (WI38), human embryonic kidney cells (HEK-293), and Chinese hamster ovary cell lines (CHO).

BIBLIOGRAPHY

Andersson, M.I. & MacGowan, A.P. 2003. Development of the quinolones. *Journal of antimicrobial chemotherapy*, 51(1):1-11.

Asif, M. 2014. A mini review on the study of new broad-spectrum antimicrobial fluoroquinolone JNJ-Q2. *Journal of chemistry & applications*, 1(1):5-10.

Bellot, F., Coslédan, F., Vendier, L., Brocard, J., Meunier, B. & Robert, A. 2010. Trioxaferroquines as new hybrid antimalarial drugs. *Journal of medicinal chemistry*, 53(10):4103-4109.

Bérubé, G. 2016. An overview of molecular hybrids in drug discovery. *Expert opinion on drug discovery*, 11(3):281-305.

Bhukya, B. & Tangutur, A.D. 2017. Microbial biotechnology: technological challenges and developmental trends. Waretown, NJ: Apple Academic Press.

Breda, S.A., Jimenez-Kairuz, A.F., Manzo, R.H. & Olivera, M.E. 2009. Solubility behavior and biopharmaceutical classification of novel high-solubility ciprofloxacin and norfloxacin pharmaceutical derivatives. *International journal of pharmaceutics*, 371(1):106-113.

Brzostek, A., Dziadek, B., Rumijowska-Galewicz, A., Pawelczyk, J. & Dziadek, J. 2007. Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*. *Federation of european microbiological societies: microbiology letters*, 275(1):106-112.

Chu, D. & Fernandes, P.B. 1989. Structure-activity relationships of the fluoroquinolones. *Antimicrobial agents and chemotherapy*, 33(2):131-135.

Dixit, P.P., Dixit, P.P. & Thore, S.N. 2016. Hybrid triazoles: design and synthesis as potential dual inhibitor of growth and efflux inhibition in tuberculosis. *European journal of medicinal chemistry*, 107:38-47.

Emmadi, N.R., Bingi, C., Kotapalli, S.S., Ummanni, R., Nanubolu, J.B. & Atmakur, K. 2015. Synthesis and evaluation of novel fluorinated pyrazolo-1, 2, 3-triazole hybrids as antimycobacterial agents. *Bioorganic & medicinal chemistry letters*, 25(15):2918-2922.

FDA. 2017. FDA drug safety communication: FDA updates warnings for oral and injectable fluoroquinolone antibiotics due to disabling side effects. https://www.fda.gov/Drugs/DrugSafety/ucm511530.htm Date of access: 16 Jan. 2018.

Firestone, B.A., Dickason, M.A. & Tran, T. 1998. Solubility characteristics of three fluoroquinolone ophthalmic solutions in an in vitro tear model. *International journal of pharmaceutics*, 164(1):119-128.

Forum on Neuroscience and Nervous System Disorders, Board on Health Sciences Policy & Institute of Medicine. 2014. Drug development challenges. *(In* Altevogt, B.M., Davis, M., Pankevich, D.E. & Norris, S.M.P., *eds*. Improving and accelerating therapeutic development for nervous system disorders: workshop summary. Washington, DC: National academies press. p. 9).

Goldberg, D.E., Siliciano, R.F. & Jacobs, W.R. 2012. Outwitting evolution: fighting drug-resistant TB, malaria, and HIV. *Cell*, 148(6):1271-1283.

Guha, R. 2013. On Exploring Structure Activity Relationships. *Methods molecular biolology*, 993:81-94.

Krishna, K.M., Inturi, B., Pujar, G.V., Purohit, M.N. & Vijaykumar, G. 2014. Design, synthesis and 3D-QSAR studies of new diphenylamine containing 1, 2, 4-triazoles as potential antitubercular agents. *European journal of medicinal chemistry*, 84:516-529.

Manohar, S., Khan, S.I. & Rawat, D.S. 2010. Synthesis, antimalarial activity and cytotoxicity of 4-aminoquinoline–triazine conjugates. *Bioorganic and medicinal chemistry letters*, 20(1):322-325.

Manohar, S., Khan, S.I. & Rawat, D.S. 2011. Synthesis of 4-aminoquinoline-1, 2, 3-triazole and 4-aminoquinoline-1, 2, 3-triazole-1, 3, 5-triazine hybrids as potential antimalarial agents. *Chemical biology and drug design*, 78(1):124-136.

Meunier, B. 2007. Hybrid molecules with a dual mode of action: dream or reality? *Accounts of chemical research*, 41(1):69-77.

National Department of Health, S.A. 2014. National Tuberculosis Management Guidelines. https://www.health-e.org.za/2014/06/10/guidelines-national-tuberculosis-management-guidelines/ Date of access: 1 Nov. 2017.

Olcay, E., Beytemur, O., Kaleagasioglu, F., Gulmez, T., Mutlu, Z. & Olgac, V. 2011. Oral toxicity of pefloxacin, norfloxacin, ofloxacin and ciprofloxacin: comparison of biomechanical and histopathological effects on Achilles tendon in rats. *The Journal of toxicological sciences*, 36(3):339-345.

Pandey, A.K. & Sassetti, C.M. 2008. Mycobacterial persistence requires the utilization of host cholesterol. *Proceedings of the national academy of sciences*, 105(11):4376-4380.

Pooran, A., Pieterson, E., Davids, M., Theron, G. & Dheda, K. 2013. What is the cost of diagnosis and management of drug resistant tuberculosis in South Africa? *Public library of sciences*, 8(1):e54587.

Projan, S.J. 2003. Why is big Pharma getting out of antibacterial drug discovery? *Current opinion in microbiology*, 6(5):427-430.

Rajulu, G.G., Naik, H.S.B., Viswanathan, A., Agarwal, D.S., Sambasivam, G. & Koppolu, K.P. 2013. Design and synthesis of new N-substituted amino methyl-[1, 2, 3] triazolyl moieties of fluoroquinolones as antibacterial agents. *Medicinal chemistry research*, 22(8):3843-3856.

Rossiter, D., University of Cape, T. & Division of Clinical, P. 2012. South African medicines formulary. Rondebosch, South Africa: Health and Medical Publishing Group.

Sharma, P.C., Jain, A. & Jain, S. 2009. Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. *Acta poloniae pharmaceutica*, 66(6):587-604.

Stahlmann, R. & Lode, H.M. 2013. Risks associated with the therapeutic use of fluoroquinolones. *Expert opinion on drug safety*, 12(4):497-505.

Surov, A.O., Manin, A.N., Voronin, A.P., Drozd, K.V., Simagina, A.A., Churakov, A.V. & Perlovich, G.L. 2015. Pharmaceutical salts of ciprofloxacin with dicarboxylic acids. *European journal of pharmaceutical sciences*, 77:112-121.

Tillotson, G. 1996. Quinolones: structure-activity relationships and future predictions. *Journal of medical microbiology*, 44(5):320-324.

Verma, R., Dhamija, R., Batts, D.H., Ross, S.C. & Loehrke, M.E. 2009. Moxifloxacin induced fatal hepatotoxicity in a 72-year-old man: a case report. *Cases journal*, 2(1):8063.

WHO. 2016. WHO treatment guidelines for drug-resistant tuberculosis. http://apps.who.int/iris/handle/10665/250125/ Date of access: 15 Sept. 2017.

WHO. 2017a. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. http://webcache.googleusercontent.com/search?q=cache:f28U3WiFYVwJ:www.who.int/medicines/news/2017/IAU_AntibacterialAgentsClinicalDevelopment_webfinal_2017_09_19.pdf+&cd=5&hl=en&ct=clnk&gl=za&client=firefox-b Date of access: 30 Jan. 2018.

WHO. 2017b. Global tuberculosis report 2017. http://www.who.int/tb/publications/global_report/en/ Date of access: 18 Nov. 2017.

WHO. 2017c. Media centre: HIV/AIDS fact sheet. http://www.who.int/mediacentre/factsheets/fs360/en/ Date of access: 3 Feb. 2018.

WHO. 2017d. Multidrug-resistant tuberculosis (MDR-TB), 2017 update www.who.int/entity/tb/challenges/mdr/MDR-RR_TB_factsheet_2017.pdf?ua=1 Date of access: 15 Jan. 2018.

WHO. 2017e. World malaria report 2017. http://www.who.int/malaria/publications/world_malaria_report/en/ Date of access: 3 Feb. 2018.

WHO. 2018a. Drug-resistant TB: Totally drug-resistant TB FAQ. http://www.who.int/tb/areas-of-work/drug-resistant-tb/totally-drug-resistant-tb-faq/en/ Date of access: 16 Jan. 2018.

WHO. 2018b. Drug-resistant TB: XDR-TB frequently asked questions. www.who.int/tb/areas-of-work/drug-resistant-tb/xdr-tb-faq/en/ Date of access: 16 Jan. 2018.

WHO. 2018c. Media center: tuberculosis key facts. http://www.who.int/mediacentre/factsheets/fs104/en/ Date of access: 25 Jan. 2018.

WHO. 2018d. What is multidrug-resistant tuberculosis (MDR-TB) and how do we control it? http://www.who.int/features/qa/79/en/ Date of access: 15 Jan. 2018.

Xia, X., Zhang, Q., Zhao, L. & Hu, Y. 2017. Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: Design, synthesis and *in vitro* anti-mycobacterial evaluation. *European journal of medicinal chemistry*, 138:66-71.

CHAPTER 2:
TUBERCULOSIS

2.1 Introduction

Tuberculosis (TB) persist, to this day, as the scourge of humankind (Goldberg *et al.*, 2012). To date, the absolute scale/breath of morbidity and mortality this disease inflicted on humanity are like no other disease in history (Sharma & Mohan, 2013). The ability of *Mycobacterium tuberculosis* to conceal itself and adapt to environmental changes (such as nutrient deprivation, hypoxia, various exogenous stress conditions, and the intraphagosomal environment) are commonly ascribed to the success of this pathogen (Cook *et al.*, 2009).

Most people are infected with TB without them knowing it, especially in countries where TB is highly prevalent (like India and South Africa) (Kanabus, 2017a). This is due to TB's communicability being highly contagious and airborne (WHO, 2017a). TB is also opportunistic in nature, arising in individuals when their immune system is compromised (WHO, 2018b). Co-infection with other infectious diseases are widespread and can increase the risk of the other, viz. human immunodeficiency virus (HIV) infection-TB (Gray & Cohn, 2013), and TB-malaria (Li & Zhou, 2013). The emergence of the HIV/acquired immunodeficiency syndrome (AIDS) pandemic in particular are linked to an alarming rise of TB (Sharma & Mohan, 2013).

Fortunately, most people can be treated and cured from TB with a combination of various antitubercular drugs (Kanabus, 2017d). However, most of these antitubercular drugs were developed years ago (Kanabus, 2017b) and pathogen resistance to almost all available drugs has been established and recognised. Treatment of drug-resistant TB is complex, prolonged, and the chemotherapeutic options are limited. Drug-resistance drastically worsens the TB-mortality rate further. Thus, a lengthy treatment regimen (in both drug-susceptible and –resistant TB), a handful of drugs required daily for treatment and resistance create an overwhelming demand for novel drugs with a new mechanism of action to target TB (Wright & Sutherland, 2007). The main goal thus is to shorten treatment with a single effective drug that is potent.

In this chapter (Chapter 2), a review of the current TB statistics and a layout of TB control measures, together with, the challenges faced with TB-chemotherapy are elaborated on. This chapter will also discuss the strategies this project will embark on for novel TB drug discovery.

2.2 History of tuberculosis

It may be said that the history of TB is interwoven with the history of man; since skeletal remains – thousands of years old – contain the causative agent of TB, namely *Mycobacterium tuberculosis* (*M. tuberculosis*) (Daniel, 2006; Delogu *et al.*, 2013; Jordao & Vieira, 2011).

The age-old infectious disease tuberculosis (in short TB, meaning *tubercle bacillus*) was accurately described by John Bunyan in "The Life and Death of Mr.Badman as "the captain of all these men of death". During the course of history, TB was also known as the great white plague" (seeing as its sufferers were anaemic) as well as *phtisis*, the Greek word for consumption (pertaining to weight loss of its sufferers) (Daniel, 2006; Jordao & Vieira, 2011). Interestingly, this infectious disease was romanticised by early society; artists, composers, novelists, and poets associated TB with artistic qualities – given that the signs and symptoms (fever, chills, night sweats and so forth) of the disease supposedly facilitated a clear view of life (Daniel, 2006).

The discovery of the *tubercle bacillus* responsible for TB was made by Hermann Heinrich Robert Koch on 24 March 1882. Koch presented his findings, *"Die Aetiologie der Tuberculose"*, to the Berlin Physiological Society (Daniel, 2006) and, as a result, 24 March was proclaimed World TB Day since 1982 to raise awareness of this disease (WHO, 2017b). The Bacille Calmette-Guérin (BCG) vaccine against TB was developed over 13 years (from 1908 to 1921) (Hansen-Flaschen, 2018) by Albert Calmette and Camille Guérin at the Pasteur Institute of Lille, France. In 1921 the BCG vaccine was first administered to a new-born baby of a mother dying of TB and in the care of a TB infected grandmother – the baby lived and did not contract TB (Daniel, 2006). Almost three decades later, in 1944, effective and efficient TB treatment came to be, on account of the bactericidal antibiotic agent, namely streptomycin by Albert Schatz, Elizabeth Bugie, and Selman Waksman (Daniel, 2006; Jordao & Vieira, 2011). Isoniazid, the first oral mycobactericidal drug, and rifamycin followed in 1952 and 1957, respectively (Daniel, 2006).

2.3 Epidemiology

TB was the deadliest infectious disease in 2016 and ranked in ninth place as leading cause of death globally. According to the World Health Organisation (WHO), roughly 2 billion people are infected with TB, of which 10.4 million people developed active TB (incident cases), and 1.7 million people succumb to this devastating disease in 2016 (WHO, 2017a).

Adults in their most productive years were primarily affected by TB (90% incident cases) in 2016, of which 6.2 million cases prevailed in men and 3.2 million in women. However, TB can affect any age-group; one million children (<15 years of age) became ill and 250 000 died due to TB. The immune-compromised is at greater risk of developing active TB, which is evident in statistics. For

instance, malnourishment accounted for 1.9 million, diabetes for 0.8 million, smoking 0.8 million, and HIV infection 1 million of reported TB cases in 2016 alone (WHO, 2017a).

TB is a common found illness in those living with HIV and a major cause of death (0.4 million in 2016) among people with HIV. Eighty seven percent of the dual epidemic was concentrated in Sub-Saharan Africa (WHO, 2017a).

TB has a worldwide occurrence. However, all figures (64% of new cases and >95% of deaths) are mostly representative of developing countries. The seven countries (Figure 2-1) that carried the brunt of TB were India, Indonesia, China, Philippines, Pakistan, Nigeria, and South Africa (WHO, 2017a).

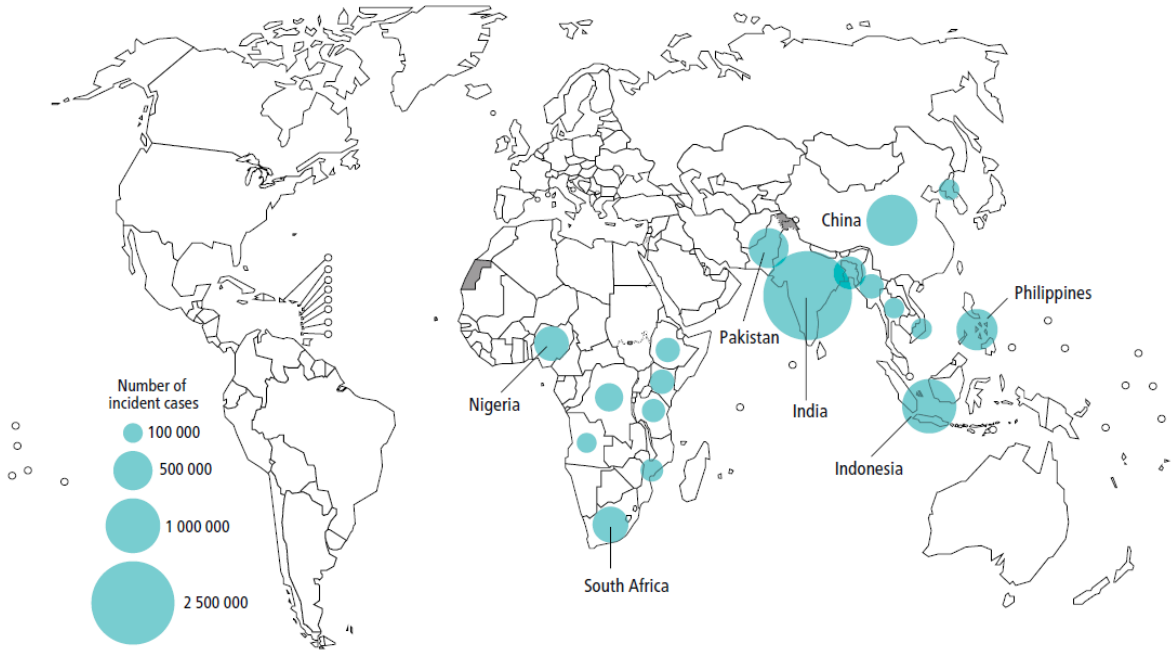


Figure 2-1: Graphical representation of TB incidence cases in high burden countries in 2016 (WHO, 2017a).

2.3.1 Tuberculosis in South Africa

Africa showed an increase in TB incidences, as a result of the HIV-TB syndemic (Frieden *et al.*, 2003). South Africa is one of the principal TB burdened countries where there is a considerably large overlap in TB, drug-resistant TB, and TB-HIV co-infections (WHO, 2017a). South Africa accounted for 4% of the global total of active TB cases in 2016 (WHO, 2017a). More specifically, the Eastern Cape, KwaZulu-Natal, and the Western Cape were the provinces with the highest incidence rates (Kanabus, 2017c). Mozambique and Lesotho are also some of the neighbouring countries heavily affected by TB. TB remains the primary cause of death in South Africa

(Kanabus, 2017c). The WHO reported that 25 000 people of the 54 million population of South Africa, have died of TB in 2015 (Kanabus, 2017c).

2.4 Aetiology

TB is caused by strains of five species of the *Mycobacterium tuberculosis* complex, including *Mycobacterium tuberculosis*, *M. africanum*, *M. bovis*, *M. canetti*, and *M. microti* as well as two sub-species, namely *M. caprae* and *M. pinnipedii* (Delogu, 2013; Jordae, 2011). The said mycobacteria are 99.9% similar in terms of nucleotides and practically identical pertaining to 16S rRNA sequences, yet, these mycobacteria differ with respect to host tropisms, phenotypes, and pathogenicity (Jordao & Vieira, 2011).

M. tuberculosis is the most common causative pathogen of TB in humans. However. *M. africanum* is endemic in West Africa, and responsible for 50% of TB cases in that region (de Jong *et al.*, 2010; Jordao & Vieira, 2011). *M. bovis* is the causative agent of bovine TB and the source of only 5 – 10% of TB in humans, thanks to pasteurization of milk (Jordao & Vieira, 2011).

M. tuberculosis is a slow growing mycobacterium since under optimal conditions, it only divides every 12 – 24 h (relative to other bacteria which divide in less than an hour). Additionally, the cell wall structure of *M. tuberculosis* is unique and forms a strong impermeable barrier to toxic agents and drugs – making TB infection quite virulent. Mycobacteria cell wall structures consist of an outer membrane, which contains glycolipids and waxy components as well as an inner membrane, with an asymmetric lipid bilayer made up of long fatty acids. The outer and inner membranes form a periplasmic space; containing peptidoglycan covalently bound to arabinogalactan and lipoarabinomannan, which, in turn, are bound to mycolic acids (Delogu *et al.*, 2013). Another contributing factor that makes pathogenic bacteria virulent is protein secretion systems and the mycobacteria, specifically *M. tuberculosis* possesses five type 7 protein secretion systems (Delogu *et al.*, 2013; Jordao & Vieira, 2011).

Certain risk factors, such as age, malnutrition, co-morbidity with other diseases (such as silicosis, diabetes mellitus, alcoholism, and HIV-infection), cigarette smoking, and overcrowding can all increase the risk of developing active TB (WHO, 2018c). Most risk factors are due to a weakened immune system. The use of certain medications, e.g. corticosteroids, can also compromise the immune system and lead the emergence of TB (CDC, 2016c).

2.5 Transmission and life cycle of tuberculosis

This communicable disease gets transmitted from people with respiratory/laryngeal TB to a susceptible person through careless coughing, talking, or sneezing of droplets, containing the

bacillus *M. tuberculosis*. Droplets are small, usually 1 – 5 microns in diameter, and remains viable in the environment for several hours (Frieden *et al.*, 2003; Saeed & Hasan, 2015).

With the infected droplet lodged in the alveoli of the distal airway(s), the immune system responds with phagotism by alveolar macrophages, in an attempt to contain the infection. This subsequently leads to granuloma formation (tubercle), the hallmark of TB. For further containment, mononuclear cells get recruited from neighbouring blood vessels and the granuloma forms as more cells accumulate. Infected macrophages, also, differentiate into multinucleated giant cells, epithelioid cells, and lipid-rich, giant foamy cells as the granuloma develops. A calcified Ghon focus (visible on chest X-ray) forms, in the site of the initial infection. If containment was successful, active TB may never develop.

Cell-mediated immunity develops after 2 – 8 weeks of infection, through infected macrophages releasing cytokines (interleukin 12 and 18) and activating T-lymphocytes, to release interferon *y,* which in turn stimulates phagocytosis, as well as, the release of tumour necrosis factor (TNF-α) by macrophages. TNF signalling is needed for chemotaxis (i.e. the recruitment and retention of cells).

In 10% of patients (e.g. HIV co-infected, alcoholics), there is a perturbation of the immune system, and active TB develops due to containment failure. TB reactivation transpires as caseation occurs, necrosis of the granuloma and cavitation, discharging thousands of the infectious bacilli into the airways. Cavitation of the airways initiates the development of a productive cough, promoting aerosolisation of the infectious bacilli (Figure 2-2) (Elkington, 2013; Frieden *et al.*, 2003; Russell, 2001; Russell *et al.*, 2009; Russell *et al.*, 2010).

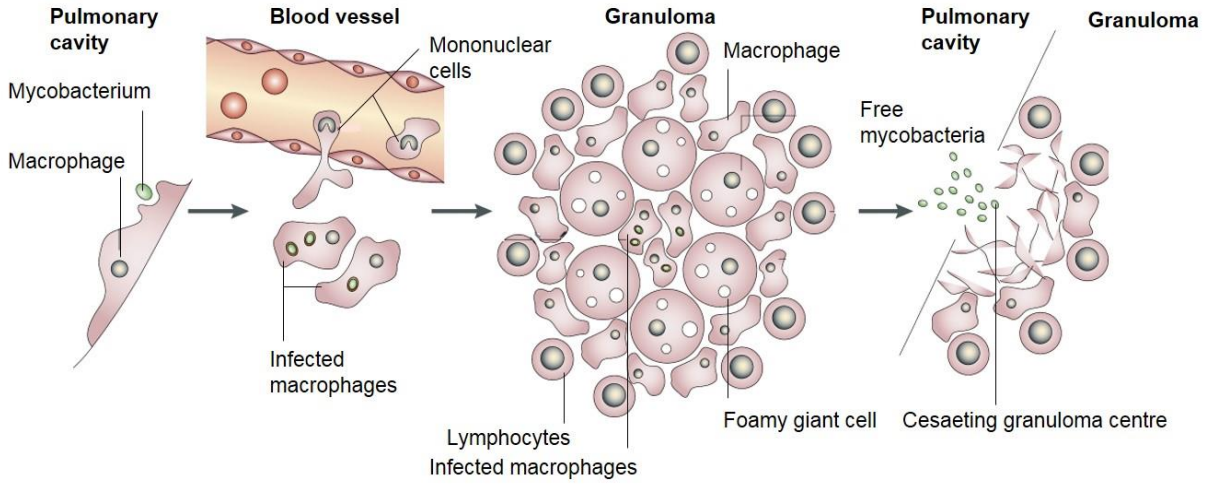


Figure 2-2: Pathogenesis of TB. Figure adapted from Russell (2001), with permission from Nature Publishing Group.

2.6 Clinical features

TB may be pulmonary (present inside the lungs) and/or extra-pulmonary (present outside the lungs, such as in the pleura, central nervous system, genitourinary system, lymphatic system, and the bones and joints); although the lungs are generally the organs infected by *M. tuberculosis*. Various organ systems may be susceptible, and pulmonary and extra-pulmonary TB may well co-exist. General signs and symptoms of this infectious disease include: fever (> two weeks), chills, drenching night sweats, unexplained weight loss (> 1.5 kg in one month), loss of appetite, and fatigue as well as nail clubbing (National Department of Health, 2014; Saeed & Hasan, 2015; Zumla *et al.*, 2013).

2.6.1 Pulmonary TB

Symptomatic pulmonary TB involves, in addition to the general signs and symptoms, persistent chest pain and persistent coughing (> 2 weeks), with sputum and, occasionally, pulmonary TB patients experience haemoptysis (National Department of Health, 2014; Saeed & Hasan, 2015; Zumla *et al.*, 2013). Moreover, scarring of the lungs occurs with chronic TB and the upper lobes of the lungs are more frequently affected than the lower lobes (Saeed & Hasan, 2015) due to it being a more favourable environment for bacterial growth (National Department of Health, 2014).

2.6.2 Extra-pulmonary TB (EPTB)

Extra-pulmonary TB (EPTB) may infect any organ system in the human body and exhibits diverse and erratic clinical features (Zumla *et al.*, 2013). 10 to 25% of patients with tuberculosis may develop EPTB (Müller, 2016). The two most common and fatal EPTB types are disseminated/miliary tuberculosis and TB meningitis (National Department of Health, 2014). Both, miliary tuberculosis and TB meningitis, are acute, developing soon after the first exposure to the tubercle bacilli (primary infection) and are commonly associated with children (National Department of Health, 2014). EPTB usually presents with similar symptoms as those of pulmonary TB. Additionally, specific symptoms develop, depending on the infected organ (Haley, 2013). For example, patients with TB meningitis commonly present with headache, malaise, confusion, reduced consciousness, and with neck stiffness (National Department of Health, 2014). Miliary TB usually presents as a result of an eroding tubercular lesion into the bloodstream, spreading bacilli to adjacent organs (e.g. bone marrow) (National Department of Health, 2014). Symptoms may include anaemia or pancytopenia (Tierney, 2014).

2.7 Diagnosis

Latent TB is a condition (WHO, 2018a) where individuals are infected with *M. tuberculosis* but not diseased (thus asymptomatic and not contagious) (CDC, 2014). Moreover, *M. tuberculosis* is in a dormant state. 5 to 10% of latent TB cases can progress to active TB (CDC, 2014). Active TB marks the start of characteristic symptoms associated with TB due to *M. tuberculosis* that prevailed over the host immune system and replicates (CDC, 2014). Patients suffering from active TB are contagious and symptoms, such as coughing causes TB to spread to other individuals (CDC, 2014).

2.7.1 Diagnosing latent tuberculosis

Screening for latent *M. tuberculosis* infection (LTBI) is indicated for those at high risk of contracting TB. These risk factors are: foreigners from areas associated with endemic TB, patients with HIV, or diabetes mellitus, patients receiving immunosuppressive therapy, and/or people in contact with TB patients. Latent TB may be diagnosed with either a tuberculin skin test or an interferon-gamma release assay (Delogu *et al.*, 2013; Saeed & Hasan, 2015; Zumla *et al.*, 2013). However, previously immunised patients may give a false positive with the tuberculin skin test, while a false negative is associated with active tuberculosis, Hodgin's lymphoma, malnutrition, and sarcoidosis (Zumla *et al.*, 2013). An interferon-gamma release assay is done on a blood sample after a positive skin test, as it is unaffected by immunisation or most environmental mycobacteria, leading to fewer false positives. Regrettably, it is affected by *M. szulgai*, *M. marinum* and, *M. kansasii.* Interferon-gamma release assays are more sensitive when used in conjunction with the tuberculin skin test, and less sensitive than the tuberculin skin test when used alone (Saeed & Hasan, 2015; Zumla *et al.*, 2013).

2.7.2 Diagnosing active- and extra-pulmonary tuberculosis

Diagnosis of active tuberculosis based only on signs and symptoms of the infectious disease is difficult, especially in those who are immunosuppressed (Zumla *et al.*, 2013). TB needs to be a differential diagnosis in patients with signs and symptoms of lung disease, lasting longer than two weeks (Zumla *et al.*, 2013). Radiography may be part of the primary evaluation for active tuberculosis (Saeed & Hasan, 2015).

Sputum microscopy and culture in liquid medium with subsequent drug-susceptibility testing (DST) are standard diagnostic methods for active tuberculosis (Saeed & Hasan, 2015; Zumla *et al.*, 2013). Additionally, nucleic acid amplification tests, imaging, and histopathological examination of biopsy samples supplement the said evaluations. A novel molecular diagnostic test, namely Xpert MTB/RIF, detects *M. tuberculosis* complex within two hours and is more

sensitive than smear microscopy. The tuberculin skin test and interferon-gamma release assays is not relevant to the diagnosis of active tuberculosis (Zumla *et al.*, 2013).

Diagnosis of EPTB is complicated as this infection mimics other diseases and due to affected sites/organs being inaccessible for biopsy, and only few bacilli being present (paucibacillary), which may reduce the sensitivity of diagnostic tests (Haley, 2013; Lee, 2015). However, tissue biopsy remains the mainstay of diagnosing EPTB, although it is intrusive and occasionally inaccessible (Lee, 2015). The examination of bodily fluids, such as pleural, peritoneal, and pericardial fluids can serve as diagnostic clues for diagnosing EPTB (Lee, 2015). Diagnosis of meningeal TB depends on the examination of cerebrospinal fluid (CSF) through a lumbar puncture, while miliary TB diagnosis relies on chest X-rays, scrutinising for a distinctive pattern of small nodules that are diffuse and evenly distributed and resemble those of millet seeds (National Department of Health, 2014).

2.8 Prevention

2.8.1 Vaccination

The Bacilli Calmette-Guérin (BCG) vaccine is the only licenced anti-TB vaccine that is available today (Davenne & McShane, 2016; University of Oxford, 2018). It contains a live, attenuated strain of *M. bovis* (Davenne & McShane, 2016) that encourages immunity against *M. tuberculosis*.

The BCG vaccine is administered to newborn babies in endemic TB areas and is believed to be partially effective (50%) in the prevention of severe forms of TB (Zumla *et al.*, 2013), especially meningeal- and military TB (Rossiter *et al.*, 2012). The BCG vaccine is contra-indicated in HIV-infected newborn babies (Zumla *et al.*, 2013) due to disseminated BCG disease (BCGosis) – a rare but serious complication, prompting the use of first-line antitubercular agents (Shahmohammadi *et al.*, 2014).

Vaccination of adults/adolescents is not recommended as it have variable effectiveness/protection against adult pulmonary TB and may lead to a false positive with tuberculin skin test (TST) (CDC, 2016a; WHO, 2018e). Furthermore, BCG vaccination is contraindicated for the immunosuppressed (e.g. HIV-infected or organ transplant patient), during pregnancy (CDC, 2016a) and individuals previously infected with TB (University of Oxford, 2018). In the latter, immunity already built-up against TB and exposure to the vaccine might cause adverse effects (University of Oxford, 2018).

2.9 Treatment of tuberculosis

Antimicrobials form the foundation for TB treatment (Mayo Clinic, 2018); which is aimed at curing diseased patients to abate morbidity and mortality, prevent development of drug-resistance, and to limit the spread of *M. tuberculosis* (CDC, 2016b). The current armamentarium of available chemotherapy for TB can be divided into first-line- and second-line drugs. Active- and latent TB can be cured if a standard regimen of several months with first-line antimicrobial agents (WHO, 2018b) are strictly followed. Isoniazid and rifampicin form the core for standard treatment regimens (CDC, 2011) as they are powerful and highly effective (Frieden *et al.*, 2003). Other first-line agents include ethambutol and pyrazinamide.

However, the rise of resistance against an individual or multiple first-line drugs necessitates the use of a combination of at least five effective second-line agents (Kanabus, 2017e). Second-line agents are further divided into four groups (Group A to D). These second-line agents include fluoroquinolones (Group A), aminoglycosides (Group B); as well as ethionamide, cycloserine, linezolid, clofazimine (Group C), and other add agents such as pyrazinamide, *p*-aminosalicylic acid, and two newly added drugs, namely bedaquiline and delamanid (Group D).

A new unconventional strategy for treatment of drug-resistant TB is the partial resection of the affected lung (WHO, 2016b). Evident in this strategy of treatment is that the arsenal of anti-TB drugs is too old (40 years to be exact) (Mahajan, 2013) and the last resort is surgery due to drug-resistant TB that is unaffected by chemotherapy (WHO, 2016b). Though, from May 2016 the WHO changed their recommendations on the use of second-line anti-TB agents and introduced two new agents (e.g. bedaquiline and delamanid), with the intention of overcoming drug-resistance.

Listed below is a review of the current chemotherapy for TB; discussed in relation to the different conditions of TB, namely latent-, active drug-susceptible-, and drug-resistant TB.

2.9.1 Latent tuberculosis

It is estimated that a quarter of the global population is carrying a *M. tuberculosis* infection, not showing any signs or symptoms of the disease, and thus is unable to transmit the disease (WHO, 2018b) as result of the hosts immune system successfully supressing the bacilli from proliferating and thus unable to cause morbidity. Individuals with latent TB infection (LTBI) are at high risk (5 – 15% (WHO, 2018b)) of developing active tuberculosis, especially when they are immunocompromised, and should therefore be swiftly identified/diagnosed to commence the recommended regime of 300 mg isoniazid (White *et al.*, 2014) daily for at least 6 months or preferably 9 months (Zumla *et al.*, 2013). Last named regimen has a 90% efficacy rate (Menzies *et al.*, 2008), and is currently recommended by the WHO as a preventative measure for HIV

infected patients in resource restrained, endemic countries (Zumla *et al.*, 2013). However, poor patient compliance exists due to the lengthy treatment regimen and hepatotoxicity (Menzies *et al.*, 2008). For patients not affected by HIV, alternative regimes comprise of: 300 mg/900 mg isoniazid plus 300 mg rifampin for 3 months, either daily or twice weekly, respectively (Zumla *et al.*, 2013).

2.9.2 Active tuberculosis

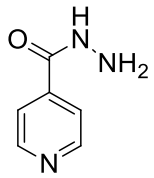
Active tuberculosis is a potentially fatal form of TB which develops from an infection with *M. tuberculosis* bacilli. These individuals start presenting symptoms and spread the disease, due to *M. tuberculosis* overcoming their immune system (CDC, 2014). In 2016, 10.4 million of the general public became ill with TB, 6.2 million were men, 3.2 million were woman, 1 million children, and 10% were HIV-positive (WHO, 2018b). If these diseased patients are not appropriately treated, each patient may infect 10 to 15 other people (over a period of a year) and run a 45% risk of dying of the disease (WHO, 2018b).

A current standard regimen for newly diagnosed drug-sensitive tuberculosis encompasses four drugs (isoniazid, rifampin, pyrazinamide, and ethambutol), known as the first-line agents, which accomplishes to cure 95% of patients (Zumla *et al.*, 2013). The treatment regimen consists of a two months initial/intensive phase of all four drugs, followed by a four-month continuation phase of only isoniazid and a rifamycin (CDC, 2011; Zumla *et al.*, 2013). The intensive phase may be extended by one month if the patient remains smear- and culture-positive, additionally, drug-susceptibility testing ought to be determined. The intensive phase rapidly wipes out most of the tubercle bacilli from the sputum (CDC, 2011; Rossiter *et al.*, 2012). The continuation phase has a sterilising effect, by eliminating any remaining bacilli and thus preventing relapse (Rossiter *et al.*, 2012).

The inclusion of each drug in the regimen is essential for the rehabilitating of patients suffering from TB (CDC, 2011). For instance, isoniazid influences sputum conversion, and thereby decreases transmissibility. A rifamycin and pyrazinamide both have a sterilising effect and consequently prevents relapse. Pyrazinamide's additional sterilising effect helps trim the treatment regimen from 9 to 6 months (CDC, 2011; Tabarsi & Mardani, 2012). Ethambutol, on the other hand, prevents rifamycin resistance in the presence of isoniazid resistance (CDC, 2011). Thus, ethambutol may be excluded from the regimen if drug-susceptibility testing results are indicative of organisms being susceptible to isoniazid (CDC, 2011).

2.9.3 Standard first-line agents

2.9.3.1 Isoniazid (INH)



1

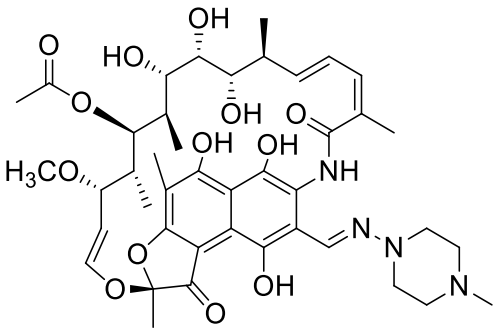
Figure 2-3: Isoniazid (1)

Isonicotinic acid hydrazide (**1**), isoniazid, is a small molecule (MW: 137 g/mol), readily soluble in water (Deck & Winston, 2012a; Gumbo, 2011), and structurally related to another antimicrobial agent, namely pyrazinamide (Deck & Winston, 2012a; Gumbo, 2011). The brief mechanism of action for INH entails the inhibition of mycolic acids, essential components of mycobacterial cell walls; the prodrug INH enters mycobacterial cells via passive diffusion and is activated by mycobacterial catalase-peroxidase (KatG). Activated INH then forms a complex with both acyl carrier protein (AcpM) and B-ketoacyl carrier protein synthetase (KasA), ultimately preventing mycolic acid synthesis and leading to mycobacterial cell death (Deck & Winston, 2012a; Gumbo, 2011). Additionally, activated INH impedes nucleic acid synthesis by inhibiting mycobacterial dihydrofolate reductase (Gumbo, 2011). Resistance to INH is generally caused by deletion or mutation of KatG, overexpression of the genes for inhA (which encodes for NADH-dependant acyl carrier protein reductase) and ahpC (implicated in protection of mycobacterial cell from oxidative stress), and mutations in the KasA and KatG genes (Deck & Winston, 2012a; Gumbo, 2011).

INH is readily absorbed (from the gastrointestinal tract) and distributed (in all body fluids and tissues) – this drug's bioavailability is thus 100% (Deck & Winston, 2012a; Gumbo, 2011). INH is metabolised by hepatic arylamine *N*-acetyltransferase type 2 (NAT2); *N*-acetylation of INH to *N*-acetylisoniazid occurs *via* acetylco-A (Deck & Winston, 2012a; Gumbo, 2011). Clearance of INH is genetically determined and patients may be classified as "slow", "intermediate", or "fast" acetylators – this is generally determined by race and not by sex or age (Gumbo, 2011). Subsequent metabolites (mostly acetylisoniazid and isonicotinic acid) are excreted by the kidney in the urine (Deck & Winston, 2012a; Gumbo, 2011). Of note, is that rifampicin, as an inducer of the CYP450 system increases the metabolism of INH (Gumbo, 2011).

Adverse effects of INH are related to dosage and duration of treatment (Deck & Winston, 2012a; Gumbo, 2011). Immunologic reactions to INH may occur and include rash, fever as well as drug-induced systemic lupus erythematosus (Deck & Winston, 2012a; Gumbo, 2011). INH toxicity typically causes serious hepatitis and requires termination of treatment (Deck & Winston, 2012a; Gumbo, 2011). Peripheral neuropathy, due to a relative pyridoxine deficiency, also burden "slow" acetylators and patients with predisposing conditions, for example AIDS (Deck & Winston, 2012a; Gumbo, 2011). Central nervous system toxicity includes memory loss, psychosis, and seizures (Deck & Winston, 2012a; Gumbo, 2011). Diverse adverse effects include hematologic abnormalities, aggravation of pyridoxine deficiency anaemia, tinnitus, and gastrointestinal discomfort (Deck & Winston, 2012a; Gumbo, 2011). Additionally, INH can reduce the metabolism of phenytoin – increasing its blood level and toxicity (Deck & Winston, 2012a; Gumbo, 2011).

2.9.3.2 Rifamycins



2

Figure 2-4: Rifampicin (2)

Rifamycins, such as rifampicin (**2**) (RIF), are macrocyclic antimicrobial agents (i.e. cyclic macromolecules) (Gumbo, 2011) and belong to the chemical class ansamycins; due to their chemical structures comprising two aromatic rings connected by a long chain. Hence the name given to this chemical class, seeing as *ansa* means "handle" in Latin (Maslow & Portal-Celhay, 1993). RIF is a semi-synthetic compound produced by *Streptomyces mediterranei* and active against mycobacteria, among others (e.g. gram-negative and gram-positive cocci, enteric bacteria, and chlamydiae) (Deck & Winston, 2012a; Gumbo, 2011). The mechanism of action for rifampicin entails the drug binding to the β sub-unit of DNA-dependant RNA polymerase, forming a stable drug-enzyme complex, and inhibiting chain formation in RNA-synthesis (Deck & Winston, 2012a; Gumbo, 2011). Resistance to RIF is generally caused by alterations to the drug target, namely the β sub-unit of DNA-dependant RNA polymerase, by means of point mutations on the aforementioned gene (specifically codons 526 and 531), which results in reduced binding of RIF

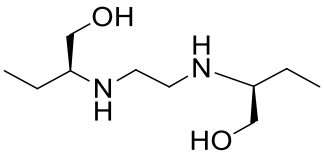
to the drug target (Deck & Winston, 2012a; Gumbo, 2011). Interestingly, human RNA polymerase is not influenced by RIF, yet, the drug is bactericidal for mycobacteria, such as *M. tuberculosis* (Deck & Winston, 2012a; Gumbo, 2011).

Rifamycins are readily absorbed (from the gastrointestinal tract) and distributed (in most body fluids and tissues – sometimes discolouring saliva, sweat, tears etc.) after oral administration (Gumbo, 2011). Food intake decreases the absorption however and, consequently, the distribution of these drugs (Gumbo, 2011). Rifamycins are metabolised to 25-*O*-desacetyl rifamycins by means of microsomal B-esterases and cholinesterases as well as hydrolysis of RIF to 3-formyl RIF (Gumbo, 2011). Subsequent metabolites are excreted through the liver into the bile, after which enterohepatic recirculation occurs and it is eliminated via faeces and urine (Deck & Winston, 2012a; Gumbo, 2011).

Rifamycins are generally well tolerated; however, common adverse effects include rash, fever, and nausea and vomiting. Hepatitis and death due to liver failure are rare. Hypersensitivity reactions may also be encountered (Deck & Winston, 2012a; Gumbo, 2011).

Rifamycins are inducers of cytochrome P450 isoforms (CYP1A2, 2C9, 2C19, 2D6, and 3A4) – increasing the elimination of several drugs metabolised by the cytochrome P450 system, for example the antimycobacterial agent isoniazid and protease inhibitors and non-nucleoside reverse transcriptase inhibitors used for the treatment of HIV, inevitably, leading to treatment failure of these drugs (Deck & Winston, 2012a; Gumbo, 2011).

2.9.3.3 Ethambutol



3

Figure 2-5: Ethambutol (3)

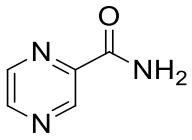
The synthetic, water-soluble, and heat-stable compound ethambutol (**3**) is dispensed as a dihydrochloric salt (Deck & Winston, 2012a; Gumbo, 2011). The mechanism of action for ethambutol entails the inhibition of mycobacterial arabinosyl transferase III – which are encoded by the embCAB operon – (therefore, disturbing the polymerization reaction of arabinoclycan) and, in so doing, hindering mycobacterial cell wall construction (Gumbo, 2011). Resistance to

ethambutol is generally caused by mutations of the emb gene, specifically at codon 306 and improved efflux pump activity (Deck & Winston, 2012a; Gumbo, 2011).

As with RIF and INH, ethambutol is readily absorbed (from the gastrointestinal tract) and distributed (in body fluids and tissues) after oral administration (Deck & Winston, 2012a; Gumbo, 2011). The drug is metabolised *via* oxidation by alcohol dehydrogenase to an aldehyde and then to a dicarboxylic acid by aldehyde dehydrogenase, yet, almost 80% of the drug is not metabolised and renally excreted (Gumbo, 2011).

Ethambutol causes very few adverse effects; one of these effects are retrobulbar neuritis (causing diminished visual acuity and red-green colour blindness) (Deck & Winston, 2012a; Gumbo, 2011).

2.9.3.4 Pyrazinamide



4

Figure 2-6: Pyrazinamide (4)

The synthetic pyrazine derivative pyrazinamide (PZA, **4**) is structurally related to nicotinamide and, however stable, it possesses low water-solubility and it is inactive at a neutral pH and active at a pH of 5.5 (Gumbo, 2011). As stated, PZA is activated by acidic conditions and PZA is transformed *via* mycobacterial pyrazinamidase (encoded by pncA) into pyrazinoic acid (POA) – which disrupts mycobacterial cell membrane metabolism and transport (Gumbo, 2011). Resistance to PZA is caused by impaired uptake of pyrazinamide or single point mutations of pncA (Gumbo, 2011).

The drug is readily absorbed (from the gastrointestinal tract) and distributed (in body fluids and tissues, including inflamed meninges) after oral administration (Gumbo, 2011). Gastrointestinal absorption separates patients into two categories, namely “slow” and “fast” absorbers (Gumbo, 2011). The drug is metabolised by microsomal deaminase to POA in the liver and subsequently hydroxylated to 5-hydroxy-POA and renally excreted (Gumbo, 2011).

An important adverse effect of PZA is hepatotoxicity and nausea, vomiting, drug fever, and hyperuricemia. Additionally, hyperuricemia may give rise to gouty arthritis (Gumbo, 2011).

2.10 Drug resistance

Resistance to antimicrobial agents can be described as multifactorial. Indeed, resistant TB may either be primary (due to host genetic factors and spontaneous mutations of the mycobacteria), acquired (due to transformation of a susceptible strain to a resistant strain), amplified (due to inadequate treatment), or transmitted (due to transmission of resistant strain) (Tabarsi & Mardani, 2012). However, resistance stems mainly from inadequate/incomplete therapy (Smart, 2016) or due to person-to-person transmission (Smart, 2016; Tabarsi & Mardani, 2012). Indeed, a study done by Shah and colleagues indicated that sixty-nine percent of patients with drug-resistant TB contracted it through transmission (Shah *et al.*, 2017).

Effective pharmacological treatment of TB is hindered by the unique structure and chemical composition of the pathogen, *M. tuberculosis* cell wall which obstructs drug entry into the bacterium and thereby nullifying antibiotics (Brennan & Nikaido, 1995). Unfavourable interactions between different TB drugs (Goldberg *et al.*, 2012) and a lengthy treatment regimen may lead to side effects, poor patient compliance, and eventually the emergence of drug-resistant TB (Krishna *et al.*, 2014). Furthermore, TB risk factors, as discussed in relation to TB aetiology, also contribute to drug-resistant TB (Tabarsi & Mardani, 2012).

There exist various classes of drug-resistant TB, although MDR- and XDR-TB are the two main types of drug-resistance recognised. Other types include mono- (resistant to only one first-line agent), poly- (resistant to various first-line agents, other than INH and rifampicin), and total drug-resistant TB (resistant to all available TB agents) (Metropolis India, 2016; WHO, 2018d). MDR-TB and XDR-TB may be defined as mycobacteria resilient to, at least, isoniazid and/or rifampicin, and mycobacteria with additional resistance to one or more fluoroquinolones and a minimum of one injectable second-line drug (e.g. amikacin, capreomycin, and/or kanamycin), respectively (Jordao & Vieira, 2011).

TB was ranked as the leading cause of death in 2016, by reason of resistance to antimicrobials. It is reported that 600 000 new cases and 240 000 casualties were established in 2016 due to MDR-TB. China, India, and the Russian Federation were the three countries mostly affected (47%) by MDR-TB. Approximately 6.2% of reported MDR-TB cases were XDR-TB (WHO, 2017a).

2.10.1 Treatment of multidrug-resistant TB (MDR-TB)

Drug-resistant TB therapy is delicate as second-line agents have limited effectiveness. Compared to standard first-line agents, these second-line agents are usually more toxic, expensive coupled with limited availability, and have an even lengthier duration of administration (Frieden *et al.*, 2003).

Treatment for drug-resistant TB is customised for each patient from four groups of second-line anti-TB drugs (Tabarsi & Mardani, 2012). The WHO regrouped and altered its recommendations in May 2016 for second-line agents (Kanabus, 2017c), based on success rates and safety (WHO, 2016b). Listed below in Table 2-1 is the reshuffling of second-line anti-TB agents and new classification system according to the WHO 2016 guidelines (WHO, 2016b). Drug-susceptibility testing should be carried out before treatment, and monitored concurrently with treatment, so that the most effective regimen can be identified and prescribed throughout therapy (Frieden *et al.*, 2003).

Table 2-1: Second-line anti-TB drugs.

<table>
<tr><th colspan="3">Core second line agents</th><th></th></tr>
<tr><td>Group A: Later generation fluoroquinolones[a]</td><td>Group B: Injectable agents/ aminoglycosides[a]</td><td>Group C:</td><td>Group D: add-on agents</td></tr>
<tr><td rowspan="4">Levofloxacin
Moxifloxacin
Gatifloxacin</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>Linezolid</td><td rowspan="2">D3: p-aminosalicylic acid
Imipenem-cilastatin
Meropenem
Amoxicillin-clavulanate
Thioacetazone[f]</td></tr>
<tr><td>Clofazimine</td></tr>
</table>

[a] listed in descending order of drug preference (table adapted from Kanabus (2017e) and the WHO (2016b)).

According to the 2016 WHO treatment guidelines for drug-resistant TB a typical regimen should comprise of five effective second-line agents, including pyrazinamide plus four core second-line agents, given over a period of twenty months. However, the use of a shorter MDR-treatment regimen of 9 – 12 months is now recommended; only in patients who have not received second-line agents before, where resistance to agents from Group A and B have been excluded, and in rifampicin-resistant TB (RR-TB) (WHO, 2016b). The selection of an effective fluoroquinolone (Group A) and an injectable agent from Group B are compulsory (Tabarsi & Mardani, 2012), and two agents from Group C need to be included to complete this regimen. If effective treatment cannot be covered by the core second-line agents an agent from either Group D2 or Group D3 may be used. Also, ethambutol and/or high doses of INH may be used to enhance this updated regime. Furthermore, clofazimine may be used in the presence of pyrazinamide ineffectiveness,

D3 agents are only to be used as a last resort, and macrolides are now excluded as a result of intrinsic resistance and potentiation of other TB-drug(s) (moxifloxacin, clofazimine, bedaquiline, and delamanid) side-effect namely QT-interval prolongation (WHO, 2016b).

2.10.2 Extensively-drug resistant tuberculosis (XDR-TB)

Although rare, close contact with these patients need to be avoided (Kanabus, 2017e), due to limited data available dealing with the recommendation of a proper regimen for treatment (Boyd, 2012), and also the complexity of the regimen. Nonetheless, do the design of a regimen for XDR-TB follow the same principals to that of MDR-TB (Boyd, 2012). Drug-susceptibility testing is of utmost importance to select the most effective drugs and should be monitored throughout therapy to identify failure of chosen drugs in the regimen (Boyd, 2012).

Surgical interventions, i.e. elective partial lung resection (lobectomy or wedge resection), have been conditionally recommended in instances of inadequate regimens available to treat drug-resistance (WHO, 2016b). However, the certainty of success with XDR-TB patients are meagre; as a result of other underlying and predisposing factors (WHO, 2016b).

2.10.3 Directly Observed Therapy (DOT)

DOT or Directly Observed Therapy is a commonly accepted term describing a TB control strategy recommended by the WHO. During this program, health care workers administer anti-TB medication at a fixed time, so that patients can remember to take their prescribed medications. Seen as treatment for TB is a lengthy process and some patients might feel better after a few weeks of therapy and become tempted to stop taking their medications DOT is a preferable method of treatment. It is crucial to stick to the course of prescribed medications for rehabilitation. (Mayo Clinic, 2018).

Thus, in an attempt to ensure patient adherence and avoid complications (i.e. resistance), therapy is administered under supervision, particularly in substance abusers (Rossiter *et al.*, 2012), HIV-related TB (CDC, 2011), and patients suffering from drug-resistant TB (Trevor *et al.*, 2010). However, no clinical trial has found an improved outcome among self-administered therapy versus DOT (Gumbo, 2011). Nonetheless, healthcare professionals should closely monitor patient's adherence to TB treatment. Additionally, healthcare professionals must keep track of possible regimen failure and associated adverse effects (such as ototoxicity and nephrotoxicity exhibited by aminoglycosides (WHO, 2016b)) or drug-drug interactions (CDC, 2016d), and Immune Reconstitution Inflammatory Syndrome (IRIS, a paradoxical reaction seen in some cases of HIV-TB co-infected patients, resulting in an overwhelming anti-inflammatory response to TB) (Gopal *et*. *al*, 2017).

2.10.4 Core second-line agents

2.10.4.1 Group A: Fluoroquinolones

Quinolones are a relatively new class of synthetic, broad-spectrum bactericidal antibiotics (Sharma *et al.*, 2009) containing a bi- and heterocyclic core (Tashima, 2015), with 4-quinolone (**5**, Figure 2-7) as the key pharmacophore exhibiting activity against cancerous cells, microbes, viruses and plasmodia (Cincinelli *et al.*, 2014; Wu *et al.*, 2016). Other quinolones include the 2-quinolone pharmacophore (Tashima, 2015).

Two groups have been derived from the 4-quinolone pharmacophore, namely the naphthyridones (**6**) and fluoroquinolones (**7**) (Andersson & MacGowan, 2003). Naphthyridones, also known as 8-azaquinolones (Bisacchi, 2015), can be identified by the nitrogen in position C-8 whereas the fluoroquinolones are distinguished by the carbon in position C-8 (Andersson & MacGowan, 2003). Additionally, the fluoroquinolones can be recognised by the fluorine molecule in position C-6, which is the most critical change made to the quinolone skeleton as almost all significant and pharmacologically active compounds comprise of one (Aldred *et al.*, 2014; Chu & Fernandes, 1989).

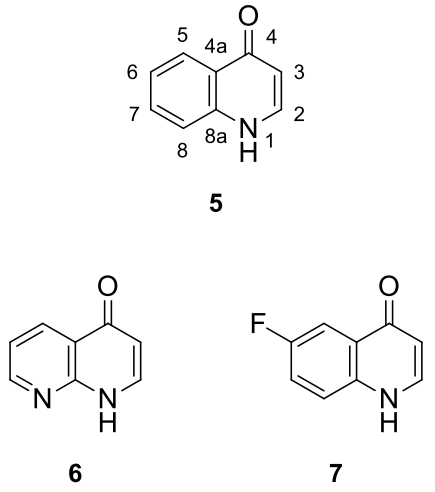


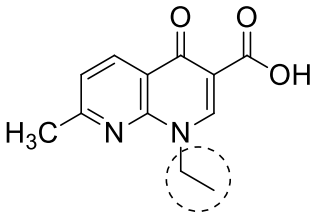
Figure 2-7: Basic structure of commonly used quinolone, 4-quinolone (5), and nucleus of derivatives, namely naphthyridones (6) and fluoroquinolones (7).

Nalidixic acid was the first synthetic quinolone in the naphthyridone class, discovered in 1962 by Lesher and co-workers (Sharma *et al.*, 2009) as an impurity during the synthesis of chloroquine. During that decade, nalidixic acid was widely prescribed for uncomplicated urinary tract infections (UTIs) caused by gram-negative bacteria (specifically *Escherichia coli*). Predictably, resistance

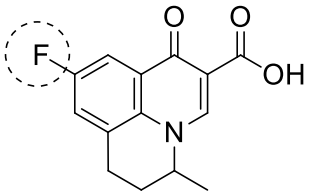
developed soon after and due to low serum concentrations achieved, and the high minimum-inhibitory concentration (MIC) required limited their usefulness because of its high protein binding (approximately 90%) as well as its short half-live ($t_{½}$ ~ 1.5 h) (Sharma *et al.*, 2009). Derivatisation followed in the 1970s with the introduction of cinoxacin. Cinoxacin and others (like oxolinic acid and pipemidic acid) only bear minor activity when compared to nalidixic acid (Emmerson & Jones, 2003).

It was not until 1976 that the first fluoroquinolone, flumequine, was introduced with significant activity over nalidixic acid. Activity included resistant *Enterobacteriaceae* and uncomplicated gonorrhoea, although multiple doses were required (Emmerson & Jones, 2003). The advancement of nalidixic acid followed in 1978 with the introduction of norfloxacin (Shabeel, 2010). Norfloxacin was recognised as the first broad-spectrum quinolone, resulting in it being prescribed more than nalidixic acid (Aldred *et al.*, 2014). Norfloxacin featured a piperazine side-chain in position C-7, expanding the quinolones half-life and gram-negative activity, together with a lower protein binding, compared to nalidixic acid (Emmerson & Jones, 2003). However, meagre serum levels and tissue penetration limited its use to only infections of the genito-urinary system (Aldred *et al.*, 2014). 1986 also saw the evolution of the first trifluorinated quinolone, namely fleroxacin, displaying excellent bioavailability and an even longer half-life, however, severe phototoxicity restricts its use (Emmerson & Jones, 2003).

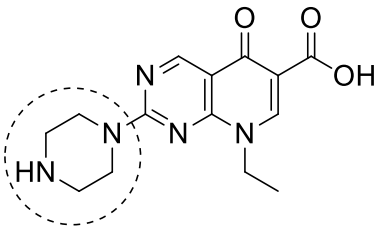
Probably the most significant discovery of all quinolones was that of ciprofloxacin in 1981, which is still widely used to this day (Emmerson & Jones, 2003). The addition of a cyclopropyl ring in position N-1 resulted in improved potency against various gram-negative (such as *Campylobacter, Haemophilus, Legionella, Neisseria*, and *Pseudomonas aeruginosa*) and gram-positive bacteria (such as *Enterobacteriaceae* (*Shigella* and *Salmonella* spp.), *Acinetobacter* spp.., and) compared to earlier quinolones. (Emmerson & Jones, 2003; Rossiter *et al.*, 2012). Clearly the addition of a cyclopropyl ring in this position produce the most significant antibacterial effect (Zhanel *et al.*, 1999). The fluoroquinolones are currently the class of drugs most effective against TB (Mitnick *et al.*, 2009).



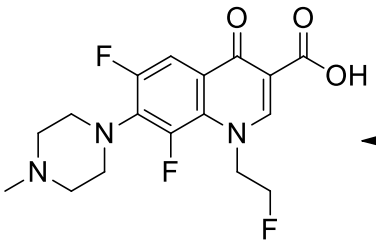
Nalidixic acid (1962)



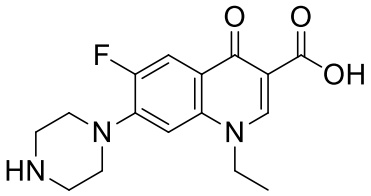
Flumequine (1976)



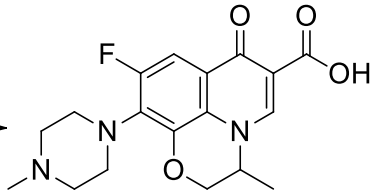
Pipemidic acid (1974)



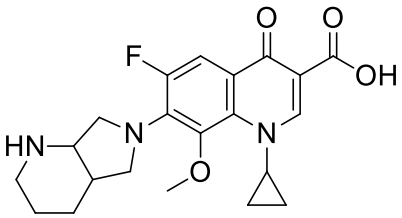
Fleroxacin (1986)



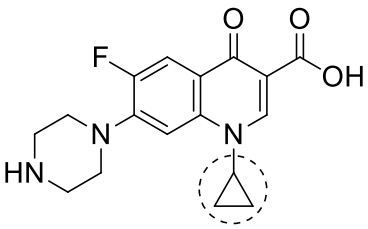
Norfloxacin (1978)



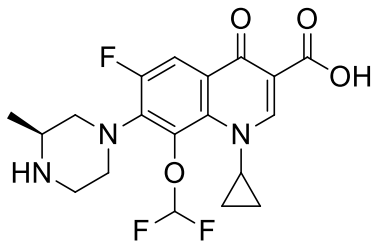
Ofloxacin (1979)



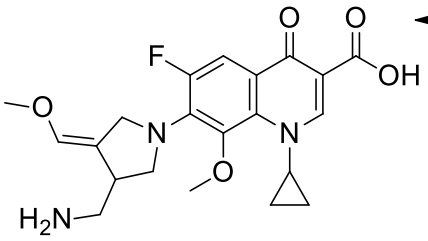
Moxifloxacin (1994)



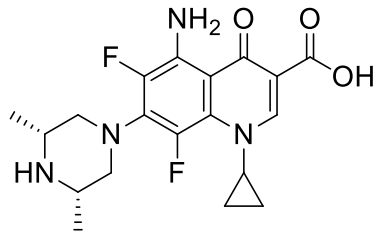
Ciprofloxacin (1981)



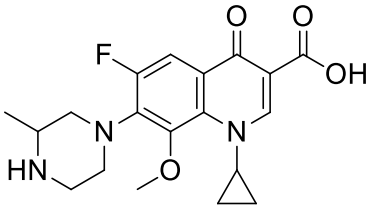
Caderofloxacin



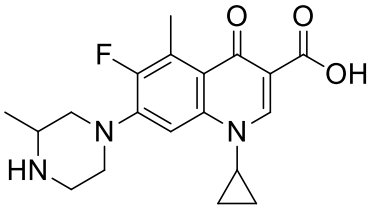
Gemifloxacin (1994)



Sparfloxacin (1991)



Gatifloxacin (1993)



Grepafloxacin (1995)

Figure 2-8: Illustrating structural evolution of fluoroquinolones, over time/chronologically, from nalidixic acid to norfloxacin, and the derivatisation of ciprofloxacin to newer agents (e.g. moxifloxacin, gemifloxacin, sparfloxacin, etc) and others (such as ofloxacin, fleroxacxin); adapted from Blondeau (2004); (Shabeel, 2010).

Classification of fluoroquinolones (Table 2-2) by generation (Trevor *et al.*, 2010) is based on the spectrum of activity and pharmacokinetic profile (Sharma *et al.*, 2009), as a result of the advancements made through structural changes (Owens & Ambrose). Ciprofloxacin, levofloxacin, and moxifloxacin are the fluoroquinolones most commonly prescribed (Asif, 2014b).

Table 2-2: Classification of fluoroquinolones.

	Drug[a]	Characteristic features
Quinolones	Nalidixic acid (**Prototype**) Oxolinic acid Pipemidic acid	Restricted gram-negative coverage (Enterobacteriaceae). Highly protein bound drugs (90%). Short $t_{½}$.
First generation fluoroquinolone	Norfloxacin	Expanded gram-negative coverage (including *Pseudomonas* and mycobacteria[a]). 50% protein bound. Longer half-life than previous agents.
Second generation fluoroquinolones	Ciprofloxacin Ofloxacin	
Third generation fluoroquinolones	Levofloxacin Gemifloxacin Moxifloxacin	Expanded gram-positive with gram-negative activity and atypical (e.g. mycobacteria) coverage. Also called respiratory fluoroquinolones, due to *Streptococcus pneumoniae* activity.

[a] classification according to Trevor *et al.* (2010). Table adapted from Sharma *et al.* (2009).

2.10.4.1.1 Structure-activity relationship (SAR) of fluoroquinolones

The fluoroquinolones' broad spectrum and potent activity make these agents of great interest for structural amendments to further increase activity (Sharma *et al.*, 2009). All clinically significant fluoroquinolones (Sharma *et al.*, 2009) bear the same features as norfloxacin: either a fluorine molecule in position C-6 and/or a piperazine ring in position C-7, with the exception of garenoxacin (Andersson & MacGowan, 2003). Ciprofloxacin is such an example with the substitution of a cyclopropyl group in position N-1. Most of the newer fluoroquinolones are built on ciprofloxacin's skeleton, due to the combination of the most optimal groups it possesses. Further enhancements can be made in positions N-1, C-5, C-7 (Andersson & MacGowan, 2003) and C-8 (Sharma *et al.*,

2009). The omission of features like the 4-oxo group, 2, 3- double bond and others (listed below) will eliminate activity (Sharma *et al.*, 2009).

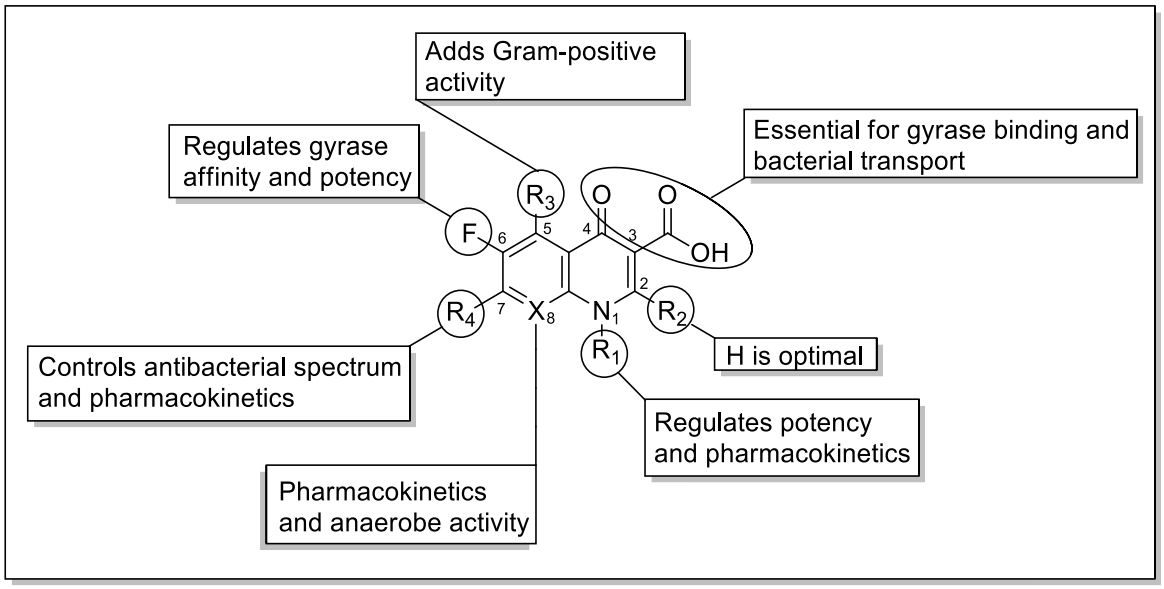
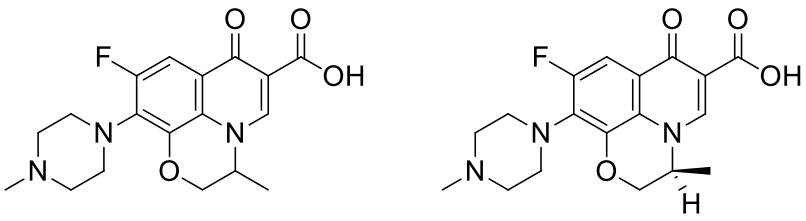


Figure 2-9: Summary of the SAR of the 4-quinolone pharmacophore (Andersson & MacGowan, 2003; Asif, 2014b).

Nitrogen-one (N-1): The attachment of a side chain to N-1 significantly improves potency. This is evident in ciprofloxacin and other fluoroquinolones (e.g. sparfloxacin, grepafloxacin, moxifloxacin, and granefloxacin) which contains a cyclopropyl substitution, resulting in improved potency compared to earlier compounds which only have an ethyl side chain (Sharma *et al.*, 2009). The cyclopropyl group is the most favourable side chain due to its steric bulk, ideal spatial effect, and through-space electronic interactions influencing biological activity (Chu & Fernandes, 1989). Another proven side chain that improves anaerobe activity is the 2,4-difluorophenyl of trovafloxacin (Andersson & MacGowan, 2003).

Stereochemistry around N-1 also affects potency and pharmacokinetics significantly. For example, ofloxacin (**8**) exist as a racemic mixture of L-ofloxacin (**9**) (levofloxacin) and D-ofloxacin (Odenholt *et al.*, 1998). The levorotatory-isomer is ten times more active (Sharma *et al.*, 2009) against gram-positive bacteria (Blondeau, 2004) and water-soluble than the dextrorotatory-isomer (Sharma *et al.*, 2009). Therefore, levofloxacin is responsible for most of ofloxacins antibacterial activity, demonstrating the importance of stereochemistry (Figure 2-10).

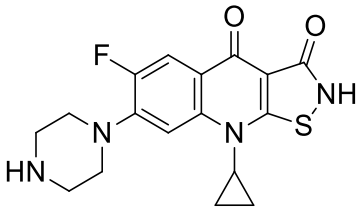


8 **9**

Figure 2-10: Structures of ofloxacin (8) and the active component thereof, namely levofloxacin (9).

Carbon-two (C-2): Substitution of this part of the molecule has successfully been carried out but not illustrated to be advantageous for activity. A hydrogen atom attached to C-2 seems to be most valuable and in some instances C-2 can form part of a ring system (Dax, 2012).

Carbon-three (C-3) and –four (C-4): The presence of a carboxylic acid and a keto-group, in positions C-3 and C-4 respectively, are essential for binding of the quinolones to bacterial DNA gyrase (Chu & Fernandes, 1989). Modifications made in position C-3 usually produce compounds with reduced activity (Sharma *et al.*, 2009). However, deprotonation of the carboxylic acid with either formaldehyde or certain esters affords derivatives with antibacterial activity, due to the *in-vivo* conversion to the corresponding carboxylic acid that occurs (Sharma *et al.*, 2009). Also, replacement of the carboxylic acid with an isothiazole group affords derivatives (**10**) (Figure 2-11) with potent *in vitro* activity compared to ciprofloxacin (Sharma *et al.*, 2009). This can be attributed to the aromatic character of this group and the nitrogen protons acidity, resulting in last named mimicking carboxylic acid (Chu & Fernandes, 1989). Replacement of the ketone in position C-4 generates a quinoline which are mostly inactive compounds (Chu & Fernandes, 1989).



10

Figure 2-11: Structure illustrating the isothiazquinolone derivative (10).

Carbon-five (C-5): The addition of a methyl (e.g. grepafloxacin) or amino (e.g. sparfloxacin) substituent generally improves the gram-positive activity (Andersson & MacGowan, 2003; Sharma *et al.*, 2009) whereas bulky substituents usually reduce activity (Sharma *et al.*, 2009).

Carbon-six (C-6): The substitution with a single fluorine molecule in position C-6 drastically improves, not only, DNA-gyrase inhibition (ten-fold), but also, the MIC (hundred-fold) (Andersson & MacGowan, 2003).

Carbon-seven (C-7): In the case of norfloxacin, and others alike, an extension of the fluoroquinolone pharmacophore at position C-7 with a piperazine ring greatly improves gram-negative activity, due to this moiety's inhibitory effect on efflux mechanisms (Andersson & MacGowan, 2003). Additionally, alkylation of the piperazine ring with a methyl-group can increase bioavailability (e.g. pefloxacin) (Wolfson & Hooper, 1985) and the *in vivo* activity (Andersson & MacGowan, 2003). Enhancement of gram-positive activity is achieved with the attachment of a pyrrolidine ring onto C-7 (e.g. gemifloxacin), pyrrolodinyl-group (e.g. clinafloxacin) or azabicyclo-group (e.g. moxifloxacin, and trovafloxacin) (Andersson & MacGowan, 2003). However, poor pharmacokinetics (like low water solubility and bioavailability) accompanies the pyrrolidine-ring substitution. Yet again, the addition of a methyl-group overcomes these shortcomings (Andersson & MacGowan, 2003). The azabicyclics, on the other hand, are associated with lipophilicity and extended half-lives (Andersson & MacGowan, 2003).

Carbon-eight (C-8): Resistance is nowadays identified with ciprofloxacin and ofloxacin (Gumbo, 2011), and, as a result, newer quinolones, like moxifloxacin and gatifloxacin, replaced these older fluoroquinolones (Andersson & MacGowan, 2003). This can be ascribed to the linkage of the C-8 methoxy group which reduces the likelihood of drug resistance (Gumbo, 2011). The Aza substitution (i.e. nitrogen) in position C-8 for enoxacin and nalidixic acid (Figure 2-8) classifies it as 1,8-naphthyridones (Wolfson & Hooper, 1985), resulting in reduced activity (Sharma *et al.*, 2009). Furthermore, alkylation of position C-8 increases gram-positive activity (Andersson & MacGowan, 2003) (particularly for anaerobes (Sharma *et al.*, 2009)), tissue penetration, and half-life due to an increase in lipophilicity (Sharma *et al.*, 2009).

2.10.4.1.2 Adverse effects of fluoroquinolones

Adverse effects, associated with the fluoroquinolones, correlate with structural modifications of the pharmacophore (Andersson & MacGowan, 2003). For example, the addition of a chlorine (e.g. clinafloxacin), fluorine (e.g. lomefloxacin, sparfloxacin), or methoxy-group (to a lesser extent) in position C-8 produces skin photosensitivity (Andersson & MacGowan, 2003; Sharma *et al.*, 2009). Also, substitution in position C-5 (e.g. sparfloxain and grepafloxacin) is linked to QTc-interval prolongation (Andersson & MacGowan, 2003), which limit their use (Andersson & MacGowan, 2003). Additionally, the fluorine molecule in position 6, that is present in all fluoroquinolones, are associated with gastrointestinal effects and rhabdomyolysis due to elevated serum fluoride levels (Schuld *et al.*, 2004). Furthermore, the quinolones are zwitterionic at a physiological pH (Mori *et*

al., 2000). Zwitterions can be identified as molecules containing both an acidic (carboxylate) and basic (tertiary amine) group on either end of the molecule (Sharma *et al.*, 2009). This can lead to precipitation at a neutral or alkaline pH and resulting in crystalluria (George *et al.*, 1991).

2.10.4.1.3 Mechanism of action of fluoroquinolones

Fluoroquinolones target a distinct enzyme (type-II topo-isomerases) during DNA synthesis, namely topo-isomerase II (also known as DNA-gyrase) in gram-negative bacteria and topo-isomerase IV in gram-positive bacteria (Hooper, 1999) – subunits encoded by gyrA, gyrB, and grlA, grlB, respectively (Rajulu *et al.*, 2013).

During bacterial replication, helicase separates DNA double-helical strands into individual strands, generating tension of extreme positive supercoiling or over winding in front of the separation point. DNA-gyrase relieves this by cutting both strands, in an ATP-dependent reaction, to allow passage of a segment through the break, crossing over, and resealing the break, thereby introducing negative supercoiling. Topoisomerase IV, on the other hand, separates (decatenate) interlinked daughter chromosomes after DNA replication.

All fluoroquinolones inhibit bacterial DNA-gyrase, by binding to subunit A of DNA-gyrase and, only recently discovered, topoisomerase IV activity – bringing replication to a halt and eventually leading to cell death. These drugs also exhibit a 1000-fold selectivity towards prokaryotes rather than eukaryotes as eukaryotes type-II topo-isomerase varies and have a low affinity for quinolones (Gumbo, 2011; Scholar, 2002; Sharma *et al.*, 2009; Trevor *et al.*, 2010) – while DNA-gyrase is only found in prokaryotes (Hamzah *et al.*, 2000).

2.10.4.1.4 Resistance to fluoroquinolones

Mutations in *gyrA* genes, encoding for subunit A of DNA-gyrase, confer a lower affinity of quinolones for the bacterial binding region (Gumbo, 2011), (e.g. gonococci (Trevor *et al.*, 2010) and in *M. tuberculosis* if administered as a single agent (Deck & Winston, 2012a), resulting in resistance when given as monotherapy. Other resistance mechanisms include: efflux pumps (e.g. *M. tuberculosis, S. aureus, S. pneumoniae*) and alter porin structures (e.g. gram-negative organisms) – resulting in a decreased intracellular concentration of fluoroquinolones (Trevor *et al.*, 2010).

2.10.4.1.5 Side-effects of fluoroquinolones

The fluoroquinolones are usually well tolerated with long-term use (Berning, 2001), nonetheless, gastrointestinal disturbances (1 – 5% of cases present with nausea, vomiting, diarrhoea, anorexia, and dyspepsia), hypersensitivity reactions (rash, and skin photosensitivity), and CNS

effects (1 – 2% of cases: dizziness, insomnia, mood alterations, convulsions, and hallucinations) occur in some patients. Whilst rare, there has been reports of severe adverse effects including QT-interval prolongation (called Torsades de Pointes) and other controversial effects like tendinopathies and arthralgia in adults, and arthropathy seen in paediatrics (Ginsburg *et al.*, 2003; Rossiter *et al.*, 2012; Sendzik *et al.*, 2009; Sharma *et al.*, 2009). The incidence and severity of side-effects vary between quinolones (Sharma *et al.*, 2009), for instance an unpleasant metallic taste is associated with grepafloxacin (Andersson & MacGowan, 2003), hepatitis with trovafloxacin, hypoglycaemia with clinafloxacin and temafloxacin, and immunological effects with tosufloxacin and gemifloxacin (Andersson & MacGowan, 2003). And most adverse effects are associated with newer fluoroquinolones and due to prior overexposure (Berning, 2001).

2.10.4.2 Group B: injectable agents

2.10.4.2.1 Aminoglycosides

Aminoglycosides are irreversible protein synthesis inhibitors (Deck & Winston, 2012a), which bind a specific 30S ribosomal protein thus blocking the initiation of protein synthesis, misreading of mRNA, and the production of faulty proteins (Deck & Winston, 2012a). Mutations in the *rpsL*-genes (encoding for the 16S mRNA subunit) and *rrs*-genes (encoding for the S12 ribosomal protein) contributes to high levels of resistance in mycobacteria (Gumbo, 2011). The *gidB*-gene (encoding for 16S rRNA metyltranferase) and efflux pumps have also demonstrated low levels of resistance (Gumbo, 2011).

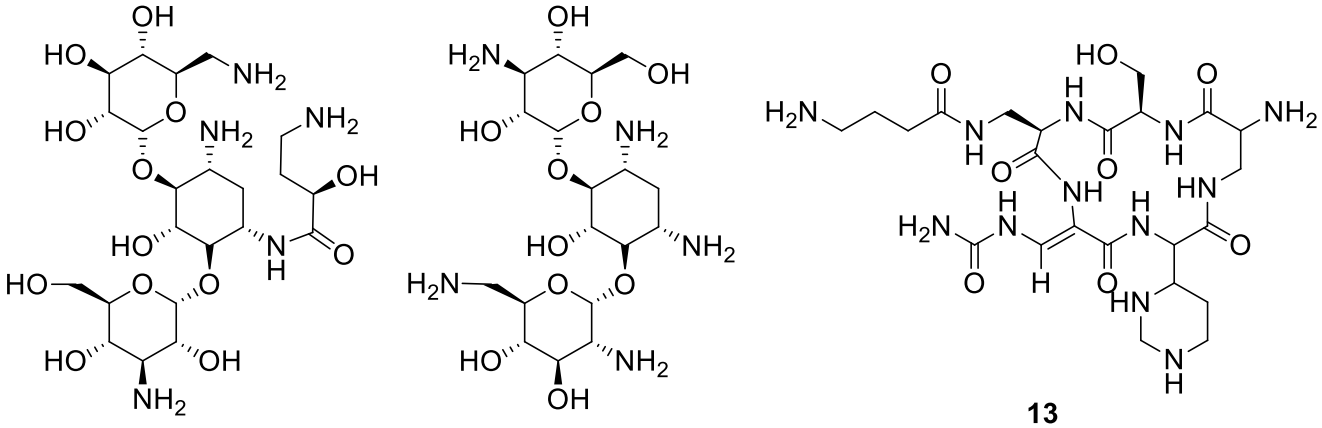


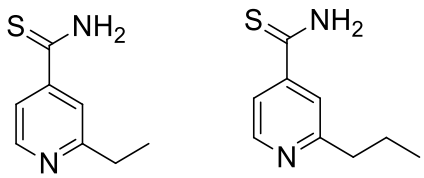
Figure 2-12: Amikacin (11), kanamycin (12) or capreomycin (13).

Amikacin (**11**) is preferred among aminoglycosides (kanamycin (**12**) or capreomycin (**13**)), as the existence of resistant strains against amikacin is predominantly small (<5%) and it exhibits no cross-resistance with streptomycin (Deck & Winston, 2012a).

Associated adverse effects (like ototoxicity/hearing loss, and nephrotoxicity) should be closely monitored and administered under supervision (DOT), especially in children considering that they still have to acquire language skills and learn at school (WHO, 2016b). An increased risk of side-effects is associated with either the total cumulative dose of previously administered second-line injectable agents (WHO, 2016b) or as a result of renal insufficiency in the elderly (Deck & Winston, 2012a). 7.3% of adults and 10.1% of children present with serious adverse events (WHO, 2016b) after prolonged exposure to this class of drugs. The most common symptoms include ototoxicity/hearing loss, vertigo, and nephrotoxicity and may all have a lasting effect (Deck & Winston, 2012a; WHO, 2016b). Other symptoms may consist of hypersensitivity reactions (skin rash), and peripheral nephropathy (WHO, 2016b). Administration of aminoglycosides can be reduced to only 6 months during therapy, in an attempt to limit aforementioned side-effects (Deck & Winston, 2012a).

2.10.4.3 Group C

2.10.4.3.1 Thioamide drugs



14 **15**

Figure 2-13: Etionamide (14) and prothionamide (15).

The thioamides, ethionamide (**14**) and the propyl-analogue (prothionamide, **15**), are chemically related to INH. Like INH, these drugs are nicotinamide derivatives (Greenwood, 2008) and prodrugs (Ma *et al.*, 2007), that inhibit the synthesis of mycolic acids upon activation. However, unlike INH, these thioamides get activated by another enzyme, EthA (Ma *et al.*, 2007) (a NADPH-specific, FAD-containing monooxygenase (Gumbo, 2011b)), to unknown active intermediates. Also, ethionamide is poorly water-soluble (Deck & Winston, 2012a). The thionamides mechanism of action entails the inhibition of the enoyl-ACP reductase of fatty acid synthase II (inhA, the molecular target) – the same enzyme that activated INH blocks – and thus mycobacterial cell wall

syntheses is hindered by inhibition of mycolic acid biosynthesis (Deck & Winston, 2012a; Gumbo, 2011).

Resistance to thioamides is due to mutations of the inhA gene, similar to INH, and thus result in resistance to thioamides as well as INH. Additionally, mutation in the enzyme that activates ethionamide also lead to resistance (Ma *et al.*, 2007). Ethionamide is readily absorbed from the gastrointestinal tract and rapidly distributed. The drug is 100% bioavailable after oral administration. The drug is metabolised by the liver and eliminated in the urine. Ethionamide is, however, poorly tolerated due to gastric and neurologic adverse effects which may be treated with pyridoxine, as in the case with INH (Deck & Winston, 2012a; Gumbo, 2011).

2.10.4.3.2 D-cycloserine/Terizidone

A strain of *Streptococcus orchidaceous* produces a broad-spectrum substance, D-4-amino-3-isoxazolidone, that is a D-alanine (**16**, Figure 2-14) analogue (Gumbo, 2011). It acts as an antimetabolite for peptidoglycan and, thereby, inhibiting cell-wall synthesis (Trevor *et al.*, 2010).

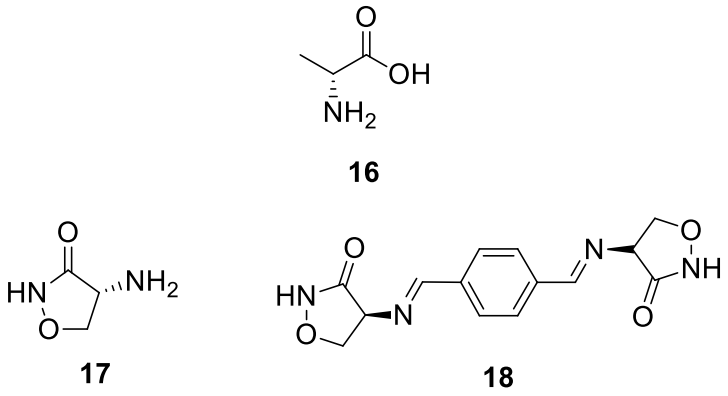


Figure 2-14: D-alanine (16), D-cycloserine (17), and terizidone (18).

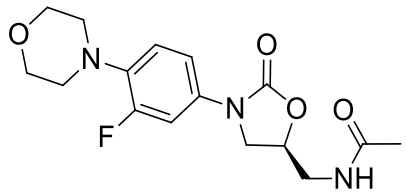
D-cycloserine (**17**) and its derivative, terizidone (**18**), (Rossiter *et al.*, 2012) are used solely for drug-resistant TB (Deck & Winston, 2012a) due to serious adverse effects, which 50% of patients present with (Gumbo, 2011) usually within the first two weeks of therapy (Deck & Winston, 2012a). This includes an array of neuropsychiatric symptoms, owing to its nickname psych-serine (e.g. somnolence, psychosis, seizures, and suicidal thoughts) (Gumbo, 2011). Co-administration with pyridoxine (Vitamin B6) may alleviate neurological symptoms, and also, monitoring of peak serum concentrations (range 20 – 40 mcg/mL) (Deck & Winston, 2012a).

D-cycloserine and terizidone are water-soluble (Deck & Winston, 2012a). Fasting C_{max} is reached within 45 minutes and delayed by fatty foods (Gumbo, 2011). Distribution is wide throughout the

body, notwithstanding, crossing of the blood-brain barrier. D-cycloserine and terizidone are excreted unchanged in the urine (Gumbo, 2011).

The mechanism of resistance is still unknown, nonetheless, 10 – 82% of *M. tuberculosis* resistant isolates have been identified (Gumbo, 2011). Cross-resistance with other anti-TB agents do not occur (Rossiter *et al.*, 2012).

2.10.4.3.3 Linezolid



19

Figure 2-15: Linezolid (19)

Linezolid (**19**) forms part of a novel class of antibiotics – the oxazolidinones (Gumbo, 2011) – exhibiting an extensive spectrum of activity, being either bacteriostatic (significantly against methicillin-resistant staphylococci, vancomycin-resistant enterococci, and MDR-TB, norcardiosis (off-label use) or bactericidal (mainly streptococci) (Deck & Winston, 2012a). Linezolid uniquely binds to the 23S ribosomal RNA of the 50S subunit, resulting in protein synthesis inhibition, contributing to no cross-resistance with other antibiotics (Deck & Winston, 2012a). Oral administration results in 100% bioavailability, thus, requiring no dose adjustment when switching to intravenous (IV) therapy (Rossiter *et al.*, 2012). Despite this, linezolid therapy is accompanied by duration-dependent, life-threatening haematological toxicity (thrombocytopenia (3%) and anaemia) and neurotoxicity which (peripheral and optic neuropathy) (Deck & Winston, 2012a; Rossiter *et al.*, 2012) warrant close monitoring (WHO, 2016b). Mitochondrial protein synthesis inhibition has been thought to be the cause of side-effects (Deck & Winston, 2012b). Serious adverse events (Saeed & Hasan, 2015) can be minimised by either termination of treatment or decreasing the dosage from 600 mg to 300 mg once daily in the event of peripheral neuropathy and haematological toxicity, respectively (WHO, 2016b). Thus, the indiscriminate use, as well as, overuse (Gumbo, 2011) of linezolid should be prohibited to avoid resistance and serious adverse events (WHO, 2016b).

2.10.4.3.4 Clofazimine

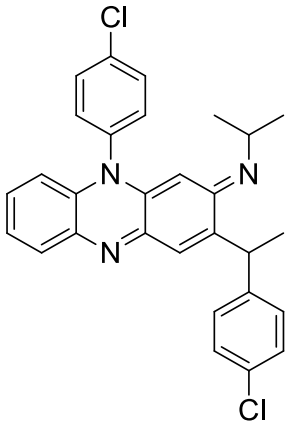


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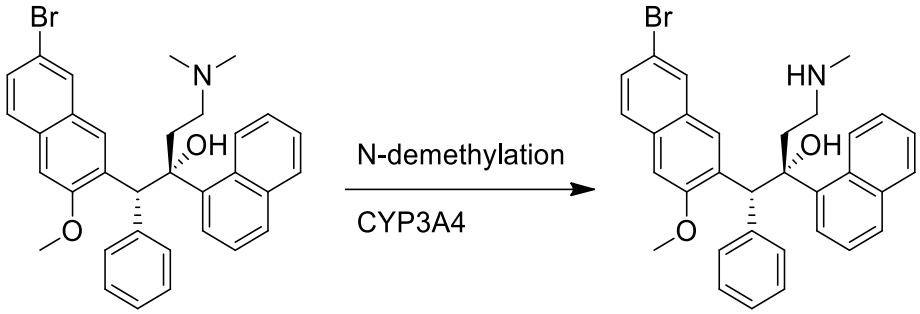
Figure 2-16: Clofazimine (20)

Clofazimine (**20**) is a phenazine dye (Deck & Winston, 2012a) and an orphaned drug with anti-inflammatory and antibacterial properties (*viz*. *M. avium, M. tuberculosis, M. ulcerans, S. aureus, S. pyogenes, Lysteria monocytogenes,* and gram-negative bacteria) (Gumbo, 2011). The mechanism of action and resistance are yet to be established (Gumbo, 2011). The bioavailability increases (2-fold) with the intake of fatty meals. The drug is extensively stored in tissue due to high lipophilicity (Gumbo, 2011). Moreover, crystal deposits of clofazimine accumulates in reticulo-endothelial cells (macrophages) and skin (Deck & Winston, 2012a), these crystal aggregates and discolours (WHO, 2016b) the skin to a reddish black (Gumbo, 2011), as well as the conjunctiva, and other bodily secretions (Deck & Winston, 2012a). In turn, these crystalline deposits attribute to the long half-life (of two months) of clofazimines; as the drug gets slowly released from where the drug was deposited (Deck & Winston, 2012a). Other unwanted effects include gastrointestinal disturbances, depression (Gumbo, 2011), and QT-interval prolongation (however this is uncommon) (WHO, 2016b).

2.10.4.4 Group D

Agents in group D have an unclear role in the treatment of drug-resistant TB and are currently not recommended for routine use by the WHO (WHO, 2013).

2.10.4.4.1 Bedaquiline



21 **22**

Figure 2-17: Biotransformation of bedaquiline (21) to metabolite M2 (22). Scheme adapted from Svensson *et al*. (2013).

Bedaquiline (**21**), formerly known as TMC-207 (R207910), is the first of a new class namely diarylquinolines as bactericidal agents (Gumbo, 2011) and was approved in December 2012 by the FDA (WHO, 2013). It also features a novel mechanism of action: targeting bacillary energy metabolism (Gumbo, 2011). More specifically, it inhibits the proton pump of *M. tuberculosis* by binding subunit c of ATP-synthase (Gumbo, 2011) in replicating and nonreplicating bacilli (Deck & Winston, 2012a). Genes D32V and A63P encoding for ATP synthase c subunit correlates with resistance (Gumbo, 2011).

More than 99% of bedaquiline is protein bound and excreted in the faeces, after metabolism by CYP3A4 (Deck & Winston, 2012a). Thus, bedaquiline should be cautiously administered with isoenzyme inhibitors (e.g. ketoconazole and lopinavir/ritonavir) or inducers (rifampicin) (Deck & Winston, 2012a; WHO, 2013). The half-life is estimated at 5.5 months as bedaquiline and its active (<3 – 6-fold (Svensson *et al.*, 2016)) metabolite, M2 (**22**), are slowly released from peripheral tissue (Deck & Winston, 2012a).

Currently, the WHO recommends that bedaquiline be cautiously prescribed and strongly adhere to dosing as limited evidence of safety exists, especially in HIV-positive patients, diabetics, alcohol and substance abusers, pregnant woman, and children (WHO, 2013). Dosing encompasses of 400 mg daily bedaquiline for two weeks, followed-up by 200 mg three times per week for a maximum of six months (WHO, 2013), administered with food to increase absorption (Deck & Winston, 2012a). Special vigilance should be given to hepatotoxicity and QTc-interval prolongation (Deck & Winston, 2012a).

2.10.4.4.2 Delamanid

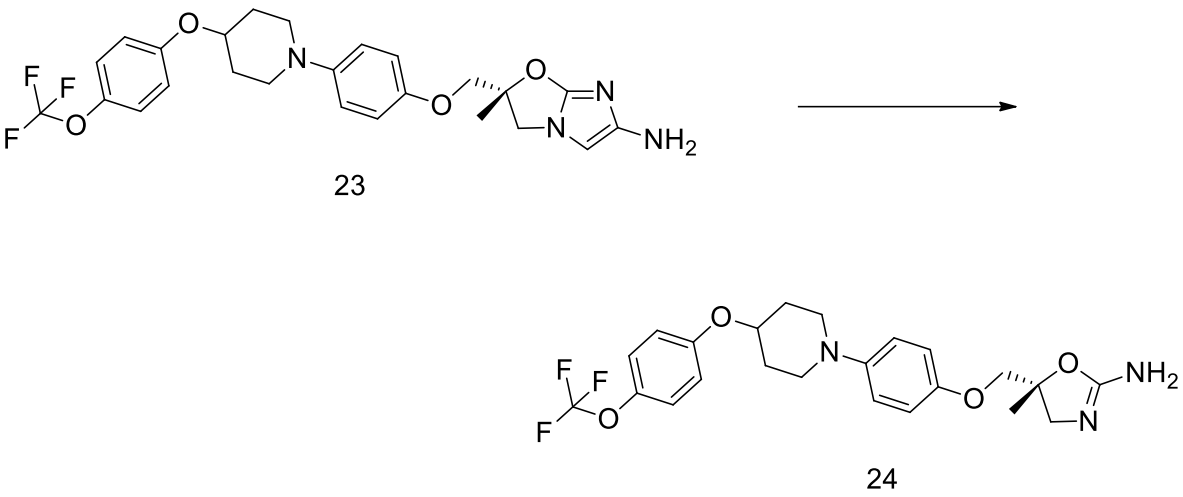


Figure 2-18: Delamanid (23) and reactive metabolite, desnitro-imidazooxazoleis (24).

Delamanid (**23**) is derived from dihydro-nitroimidazooxazole (Skripconoka *et al.*, 2013) and first identified by Otsuka Pharmaceutical due to its outstandingly low minimum inhibitory concentration (MIC_{90} 0.006 – 0.024 µg/ml) towards *M. tuberculosis* (Diacon *et al.*, 2011). Delamanid inhibits cell wall components, namely methoxy-mycolic and keto-mycolic acid (WHO, 2016a), in both drug-susceptible and drug-resistant *M. tuberculosis* strains (Skripconoka *et al.*, 2013). Delamanid is a prodrug of mainly desnitro-imidazooxazoleis (**24**) (Chaudhari *et al.*, 2011; Fernandes & Dos Santos, 2017; Sasahara *et al.*, 2015) that requires activation by an enzyme (deazaflavin dependent nitroreductase) through the coenzyme system F420 of mycobacteria to inhibit mycolic acid production (Lewis & Sloan, 2015; Xavier & Lakshmanan, 2014). This novel mechanism of action led to delamanid being conditionally approved by the WHO (WHO, 2016a), but then, only in MDR/XDR-regimens where there is no other treatment alternative (WHO, 2015). Delamanid is ineffective against gram-positive and gram-negative organisms, which limits its use to only drug-resistant TB (Lewis & Sloan, 2015). However, resistance to delamanid has been identified and has been attributed to mutations in F420 genes in mycobacteria (WHO, 2016a).

Delamanid is highly protein bound (>99.5%), where it is also primarily metabolised by albumin (WHO, 2016a). Thus, delamanid can be concomitantly used with CYP450 enzyme metabolisers (e.g. antiretrovirals) (Matsumoto *et al.*, 2006). However, possible drug-interactions (with levofloxacin (WHO, 2016a)) may occur due to metabolites of delamanid contributing to dose-dependent QTc-prolongation (WHO, 2016a). More common adverse effects noted during clinical studies were nausea, vomiting, and dizziness (WHO, 2015).

The WHO recommends 50 mg and 100 mg delamanid in children aged 6 – 11 years and 12 – 17 years with MDR-TB for 6 months (in the intensive phase), respectively (WHO, 2016a). The WHO has now included children in their interim policy for long-term treatment of MDR-TB, but only under certain conditions (WHO, 2016a).

2.10.4.4.3 Group D3

p-Aminosalisylic acid, carbapenems (like imipenem and meropenem), and thioacetazone are all considered as last resort for use in a MDR or XDR regimen – when four effective drugs out of the second-line agents in Groups A to C cannot otherwise be composed (WHO, 2016b).

2.10.4.4.4 *p*-Aminosalisylic acid

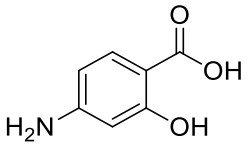


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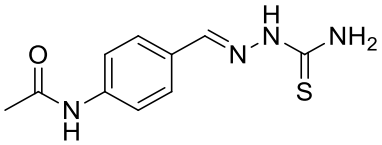
Figure 2-19: *p*-Aminosalisylic acid (25).

p-Aminosalisylic acid (PAS, **25**) was the first effective anti-TB drug discovered in 1943 by Lehman (Gumbo, 2011). PAS is a prodrug that gets incorporated into the folate biosynthesis pathway, by acting as a false substrate for dihydropteroate synthase (*fol*P1), producing hydroxyl dihydrofolate antimetabolites, and thereby inhibiting dihydrofolate reductase (DHFR) (Zheng *et al.*, 2013). PAS is solely used against *M. tuberculosis* (Deck & Winston, 2012a) due to predictability of serious adverse events in 30% of patients (Gumbo, 2011). Side-effects include gastrointestinal problems (peptic ulceration, haemorrhage) and hypersensitivity reactions (fever, skin rash, hepatosplenomegaly, lymphadenopathy, and granulocytopenia) (Deck & Winston, 2012a).

Metabolism of PAS occurs in the liver by *N*-acetylation to *N*-acetyl PAS (>50%) and PAS – which are excreted in the urine (>80%) (Deck & Winston, 2012a; Gumbo, 2011). Crystalluria can occur with high concentrations of PAS in the urine (Deck & Winston, 2012a) and hepatotoxicity due to the metabolite *N*-acetyl PAS (Gumbo, 2011). Distribution of PAS occurs widely into all bodily fluids, except the CSF, and tissue with 90% of PAS orally bioavailable (Gumbo, 2011). Mutations in thymidylate synthase gene (*thy*A), DHFR-gene (*dfrA*), and RibD gene (Zheng *et al.*, 2013) results in 37% resistance to PAS (Gumbo, 2011). The difficulty of procuring PAS, associated SAEs, and, recently, the WHO found no compelling data on the treatment success of PAS which limits its use (WHO, 2016b).

2.10.4.4.5 Thioacetazone



26

Figure 2-20: Thioacetazone (26).

Thioacetazone (**26**), alternatively known as amithiozone (Finch *et al.*, 2010), is administered as a prodrug (Grayson *et al.*, 2017). Thioacetazone gets activated by the same bacterial monooxygenase (Bhuvaneswari *et al.*) as ethionamide, leading to mycolic acid inhibition (Grayson *et al.*, 2017). Predictably, complete cross-resistance between these agents exist (Grayson *et al.*, 2017). Additionally, due to frequent life-threatening adverse effects, toxic epidermal necrolysis and Steven-Johnson syndrome (SJS) – particularly in HIV-positive patients – and limited availability restrict its use (WHO, 2016b).

2.11 Rationale of drug design

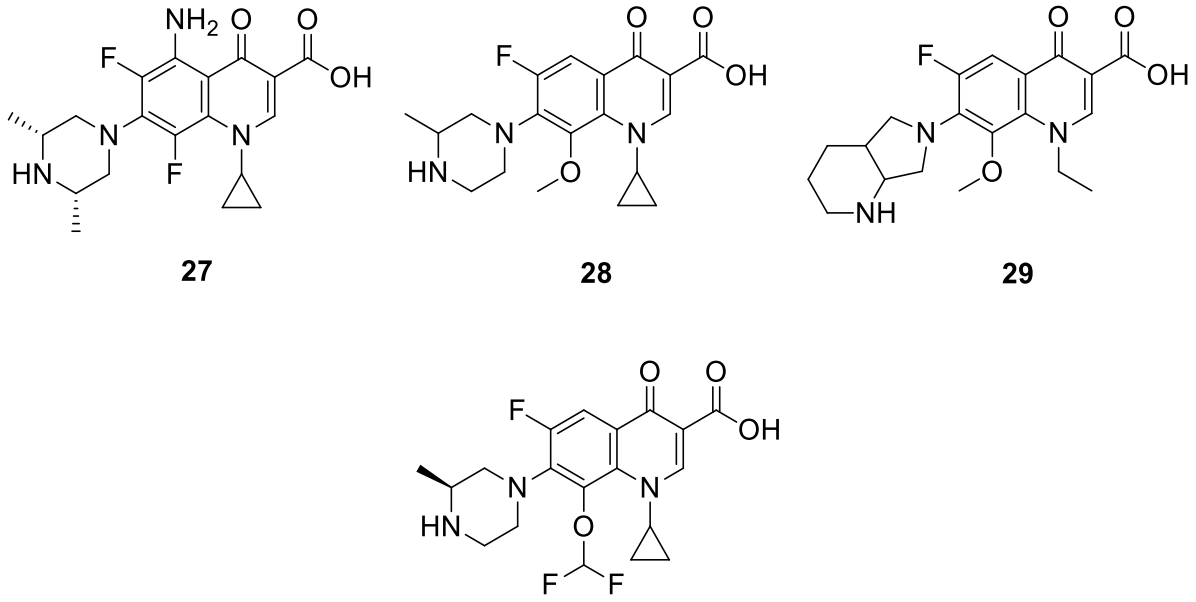
In this project a proven drug (ciprofloxacin), pharmacophore (1,2,3-triazole), and dietary molecule (cholesterol) will be utilised for drug development against TB. Reported below is the justification for exploiting these molecules.

2.11.1 Strategies: derivatisation and hybrid-drug theory

The fluoroquinolone class of drugs are one of the most commonly prescribed antibiotics (Breda *et al.*, 2009; Hu *et al.*, 2017). Their broad spectrum against numerous bacterial infections of the bone, respiratory-, gastrointestinal-, and urinary tract (including sexually transmitted diseases) make these drugs so popular (Venepally *et al.*, 2016; Xu *et al.*, 2017). However, the atypical biological properties that the fluoroquinolones enjoy against TB (other include HIV, malaria, and tumors) (Xu *et al.*, 2017) make them an appealing class of drugs for this project.

Ciprofloxacin can be recognised as the paragon of fluoroquinolone therapy (Emmerson & Jones, 2003). In fact, the WHO considers this antibiotic as an essential drug (Breda *et al.*, 2009). Since the introduction of ciprofloxacin (Foroumadi *et al.*, 2005) and accompanying clinical success of this drug, shaped a variety of newer-generation fluoroquinolones (e.g. sparfloxacin, gatifloxacin, moxifloxacin, and caderofloxacin) with an expanded spectrum of activity (Aldred *et al.*, 2014). These newer-generation fluoroquinolones were built on ciprofloxacins motif by substitution of specific substituents (Figure 2-21). Certain functionalities are crucial and proven for the activity

ascribed to ciprofloxacin. They are the cyclopropyl- and the piperazine ring in positions N-1 and C-7 of the fluoroquinolone pharmacophore, respectively (Sharma *et al.*, 2009). The cyclopropyl ring is present in all new fluoroqionolones and identified as the functional group with the greatest antibacterial potency in this position (Zhanel *et al.*, 1999). Moreover, studies of the structure-activity relationship of fluoroquinolones report that C-7 is the most versatile site for substitution (Foroumadi *et al.*, 2005) and is responsible for their antibacterial spectrum, potency, and pharmacokinetics (Andersson & MacGowan, 2003; Rajulu *et al.*, 2013). Also, five- to six membered heterocycles containing nitrogen are optimal substituents in position C-7 (Rajulu *et al.*, 2013).



27 **28** **29**

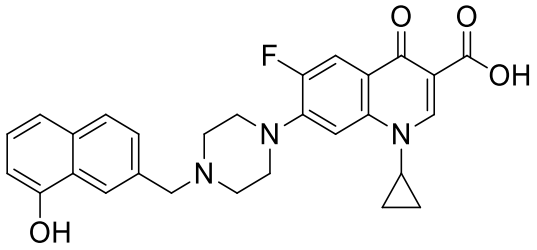
30

Figure 2-21: Structures of newer-generation fluoroquinolones which are analogues of ciprofloxacin, namely sparfloxacin (27), gatifloxacin (28), moxifloxacin (29), and caderofloxacin (30).

The development and identification of novel drug candidates with a lead structure is a challenging, laborious and expensive process (Decker, 2017). Still, the need for new agents due to side effects and bacteria resistant to current clinically used drugs is imperative (Xu *et al.*, 2017). Consequently, in pursuing the development of new anti-TB agents, imposed by the spread of resistant bacteria, it seems genuine to start off with the modification of old drugs with proven activity (Xu *et al.*, 2017). Therefore, this project will build on the nucleus of ciprofloxacin, through simple derivatisation, which will predictably result in a certain level of activity (Harvey, 1998)

against mycobacteria, as it is already in use against drug-resistant TB. Furthermore, this study will indicate and elucidate functional groups essential for activity (Harvey, 1998).

Evidence of above-mentioned derivatisation of ciprofloxacin was investigated by Zhao *et al.* (2005). This resulted in an analogue of ciprofloxacin (compound **31**) which inhibited 98% of *M. tuberculosis* growth. However, this derivate required a 25 times higher concentration than the starting material did for *M. tuberculosis* inhibition (i.e. ciprofloxacin, range 0.25 – 0.50 µg/ml (Fenlon & Cynamon, 1986)). Another drawback which hampered further development was the moderate yields that compound **31** was obtained in (61% (Zhao *et al.*, 2005)).



31
MIC 6.25 µg/ml

Figure 2-22: Derivatisation of ciprofloxacin with another functional group, namely hydroxyquinoline, resulted in active compound 31.

An alternative strategy to overcome all of the drawbacks associated with *de novo* design (Harvey, 1998) of drugs is molecular hybridisation (Smit *et al.*, 2015). Herein, a more effective hybrid molecule or chemical entity is developed by chemically linking (through covalent bonds) two or more differing pharmacophores, exploiting their biological activity and dual mode of action (Meunier, 2008; Smit *et al.*, 2015). Thus, another nitrogen containing heterocycle, that is the 1,2,3-triazole moiety, will be supplemented to the piperazine ring in position C-7 of ciprofloxacin. This add-on is expected to have a synergistic effect, accounting on both pharmacophores being active against TB.

Similar work that supports the rationale of this project was conducted by Rajulu *et al.* (2013) who synthesised *N*-substituted amino methyl-[1,2,3] triazolyl derivatives in position C-7 of ciprofloxacin. Five of the aryl substituted amino derivatives exhibited potent activity against resistant bacteria called ‘superbugs’ (namely methicillin-resistant *Staphylococcus aureus* (MSSA), methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant enterococci (VRE) than the reference compounds (linezolid and ciprofloxacin).

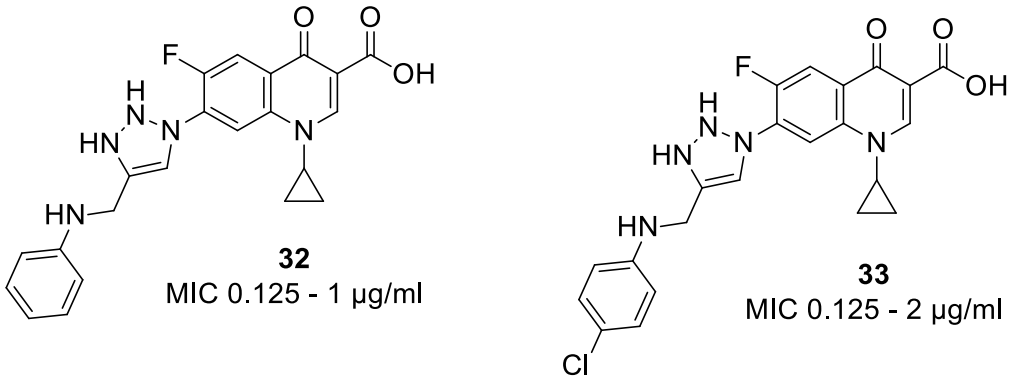


Figure 2-23: Structures of the two most active *N*-aryl amino methyl-[1,2,3] triazolyl derivatives, 32 and 33.

Xu *et al.* (2018) prepared a set of lipophilic ciprofloxacin-isatin-1*H*-1,2,3-triazol hybrids that was evaluated against the H37Rv strain of *M. tuberculosis*. Six of these compounds showed greater activity than the parent drug (ciprofloxacin), with compound **34** (MIC: 0.39 µg/ml) being the most active. Moreover, compound **34** was eight times more active than ciprofloxacin and comparable with rifampicin (MIC: 0.39 µg/ml). However, the cytotoxicity of these hybrids revealed all of them to be more harmful to mammalian cells than ciprofloxacin.

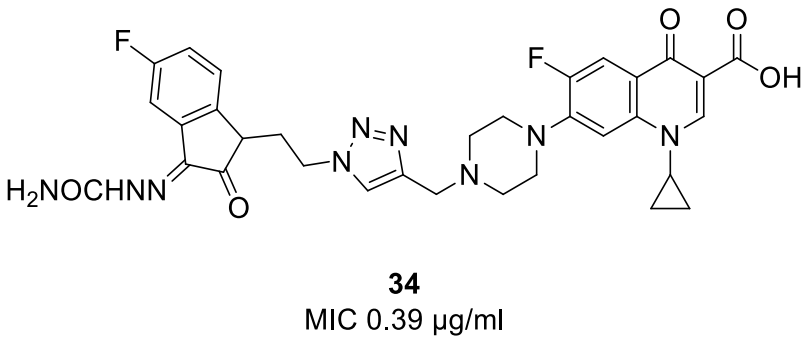
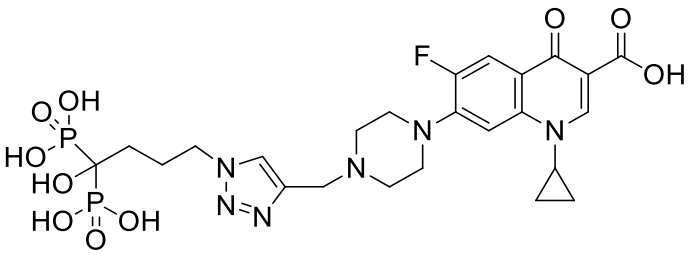


Figure 2-24: Structure of compound 34.

McPherson *et al.* (2012) produced 1-hydroxybiphospinate derivatives of ciprofloxacin, gatifloxacin, and moxifloxacin linked with the 1,2,3-triazole moiety. The derivative employing ciprofloxacin (**35**) was the only fluoroquinolone displaying diverse antibacterial activity, such as *Bacillus subtilis*, MRSA, *S. epidermidis*, *E. faecalis*, *E. coli*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *P. aeruginosa* with MIC values ranging from 2 to 8 µg/ml.

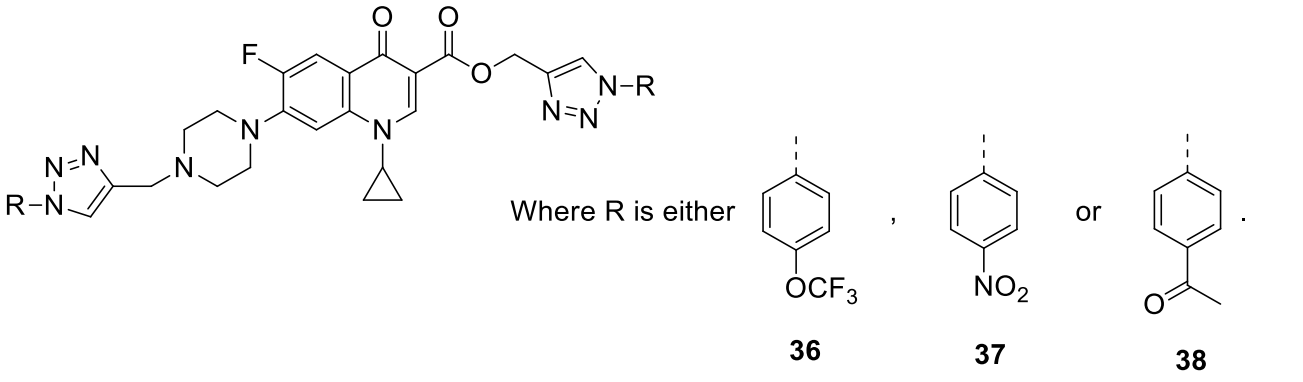


35

MIC 2 - 8 µg/ml

Figure 2-25: Structure of compound 35, a fluoroquinolone-bisphosphonate conjugate.

And more recently Kant *et al.* (2016) synthesised bis-1,2,3-triazole linked ciprofloxacin conjugates which had significant activity against a noteworthy part of gram-negative and gram-positive bacteria, when compared to the parent drug, i.e. ciprofloxacin. Gram positive bacteria included *S*. *aureus*, *S*. *epidermidis*, *E*. *faecalis*; and gram-negative covered *E*. *coli*, *P*. *aeruginosa*, *Aeromonas hydrophila*, *Salmonella typhi*, S. *typhimurium*, *Sphingomonas paucimobilis*, and *Plesiomonas shigelloides*. Conjugates **36**, **37**, and **38** displayed 2 – 10-fold enhancement of ciprofloxacins activity.



36 **37** **38**

MIC: 0.96 - 15.36, 1.06 - 16.99, 2.14 - 17.13µM

Figure 2-26: Structures of bis-1,2,3-triazole-ciprofloxacin hybrids, with the most active conjugates being compounds 36, 37, and 38.

The term triazoles was first coined in 1885 by Bladin (Potts, 1961) and discoveries of monocyclic triazoles was first made by H. V. Pechmann in 1888 (Benson & Savell, 1950). Triazoles are also known as pyrrodiazoles (Kharb *et al.*, 2011).

Triazoles are five-membered, double unsaturated (Benson & Savell, 1950) heterocyclic-ring compounds, with two isomers. These isomers are 1,2,3-triazole (vicinal-triazole, **39**) and 1,2,4-triazole (*s*-triazole, **40**) (Potts, 1961), indicating the order of the sequentially linked nitrogen atoms (Benson & Savell, 1950). There exist two tautomeric forms for each isomer as well (Figure 2-27) (Benson & Savell, 1950).

Triazolic compounds are exceptionally stable compounds (Tome, 2004) and highly soluble in both water and alcohol. Triazoles, in its purest form, can be identified as white or pale-yellow crystals with a distinctive odour, melting– (120 ℃) and boiling point (260 ℃) (Kharb *et al.*, 2011).

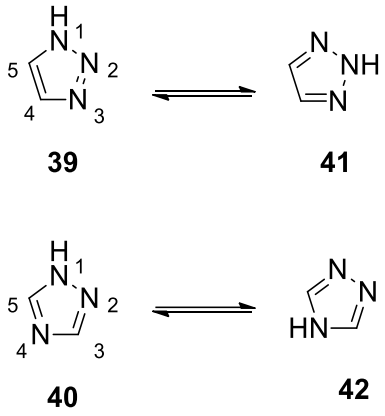


Figure 2-27: The isomers of triazole (1*H*-1,2,3-triazole 39 and 1*H*-1,2,4-triazole 40) together with the tautomer of each isomer (2*H*-1,2,3-triazole 41 and 4*H*-1,2,4-triazole 42) – the molecular formula is $C_2H_3N_3$ and the molecular weight is 69.06 g/mol (Kharb *et al.*, 2011).

These scaffolds are remarkably versatile (Kharb *et al.*, 2011) in medicinal chemistry as connecting units (Agalave *et al.*, 2011), and form part of numerous drug categories against: bacteria (e.g. *M. tuberculosis*), viruses (e.g. HIV), parasites (e.g. plasmodium), inflammation, diabetes, epilepsy, cancer, hypertension, anxiety, depression, Parkinson′s disease, and obesity (Kharb *et al.*, 2011).

The structure-activity relationship of triazoles indicate that modifications or substitutions made in position N-1 exert the greatest enhancement of properties (Kharb *et al.*, 2011). Furthermore, disubstitution with an electronegative group in positions 1,4 and 1,3 yields more active triazole-compounds in general (Asif, 2014a).

Triazoles do not occur naturally (Rodgman & Perfetti, 2016), but can easily be synthesised through copper-catalysed synthesis (Shin *et al.*, 2012; Xu *et al.*, 2015). In particular, the azide alkyne Huisgen cycloaddition reaction has extensively been reported in literature for the synthesis

of regioselective 1,4-disubstituted 1,2,3-triazoles (Xia *et al.*, 2017). The 1,2,3-moieties sp^2-hybridisised nitrogens and poor basicity (at physiological pH) make it stable to metabolic degradation in oxidative and reductive conditions. Also, it actively binds to biomolecular targets due to an improved solubility. This improved solubility is attained by hydrogen bonding and the strong dipole moment which the 1,2,3-triazoles possess (Agalave *et al.*, 2011; Kant *et al.*, 2016). Moreover, its polar quality cause an improvement in solubility and thus produce better pharmacokinetic and pharmacodynamic properties to linked drugs. Drugs currently on the market containing the 1,2,3-moiety are tazobactam (**43**), cefatrizine (**44**), TSAO (**45**, anti-HIV-1), carboxyamidotriazole (**46**, anti-cancerous), and radezolid (**47**). Additionally, the I-A09 derivative (**48**), a newly identified 1,2,3-triazole drug, and radezolid are undergoing trials for use against TB (Bhukya & Tangutur, 2017; Kant *et al.*, 2016; Xia *et al.*, 2017) (Figure 2-28).

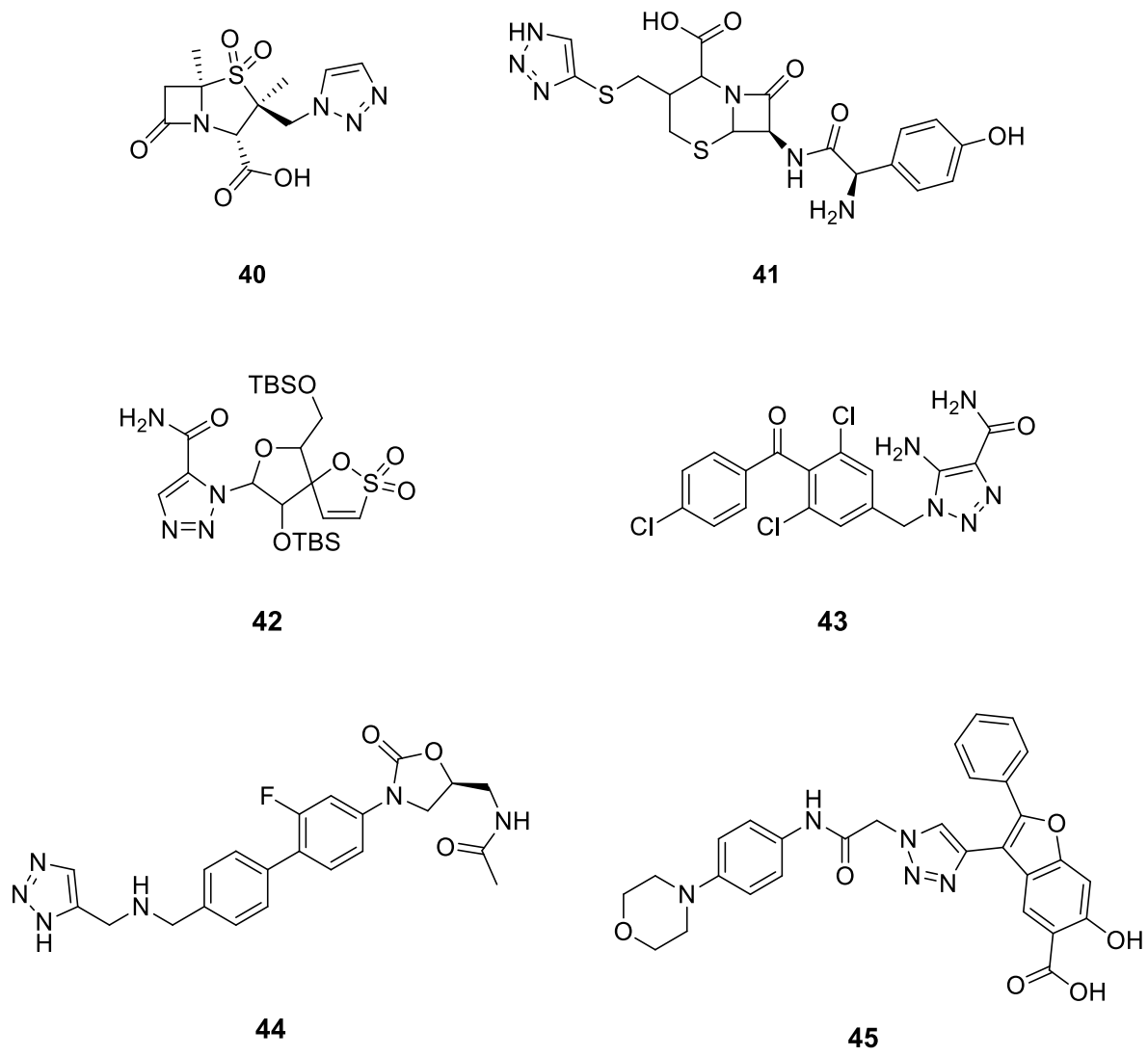
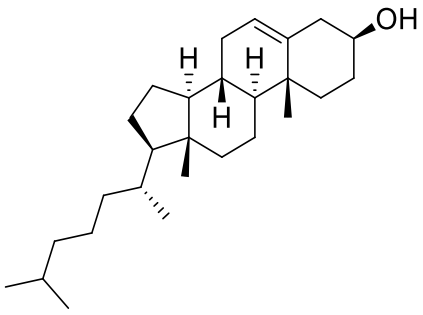


Figure 2-28: Illustrating the 1,2,3-triazole moiety incorporated within drugs currently available on the market (tazobactam 43, cefatrizine 44, TSAO 45, carboxyamidotriazole 46, radezolid 47), and I-A09 (48).

The manner by which *M. tuberculosis* adapt to environments such as available carbon sources and ROS (reactive oxygen species) in host cells might give insight into targeting mycobacteria for novel drug development.

M. tuberculosis is heterotroph, meaning for this mycobacterium to survive and replicate intracellularly, needs to adapt to and make use of available nutrients from host-cells. Moreover, it utilises carbon compounds (called carbon sources) as energy source for the biosynthesis of molecules required for its growth and development (Eisenreich *et al.*, 2010). Nutrients like cholesterol (**49**) has been identified as a major carbon source for mycobacteria and thought to be a virulence driving factor for the pathogenesis of TB (Ouellet *et al.*, 2011).

The acquisition of cholesterol has recently been identified to be through the *mce4*-transporter system, as demonstrated in mice lungs (Ouellet *et al.*, 2011). Deletion of *mce4-transporter* lead to a reduced *M. tuberculosis* growth and thus pathogenicity, when cholesterol was used as the limiting carbon source (Ouellet *et al.*, 2011). *M. tuberculosis* special waxy cell envelope can be ascribed as the reason for the unique transporters it holds for the uptake of nutrients (Eisenreich *et al.*, 2010). Correspondingly, diets rich in cholesterol can lead to a decline in immunity towards *M. tuberculosis* and consequently to a heightened *M. tuberculosis* burden (Ouellet *et al.*, 2011). Furthermore, the utilisation of cholesterol has been demonstrated by using ^{14}C-labeled cholesterol. The sterol framework goes to energy production and the aliphatic-side chain for lipid synthesis (Ouellet *et al.*, 2011).



49

Figure 2-29: Cholesterol (49).

The ability of mycobacteria to maintain a state of metabolic homeostasis against host immune-system and antimicrobial agents might also be a reason for the failure of host defence mechanisms and antimicrobial agents. Mycobacteria do so by adapting to redox environments (Saini *et al.*, 2016), producing redox buffers (mycothiol (MSH) and ergothioneine (EGT)) that have antioxidant properties (Saini *et al.*, 2016).

Drugs, like the fluoroquinolones, have the potential to produce an increase in reactive species of oxygen (ROS) in bacterial cells during oxidative processes (Albesa *et al.*, 2004). By doing so ciprofloxacin disrupts the redox homeostasis of *M. tuberculosis* and eventually cause oxidative stress along with bacterial death. Evidence of the production of ROS by fluoroquinolones can be backed up by the oxidative stress it produces in human cells. This can be observed as phototoxicity due to the increase in a singlet oxygen and superoxide anion (O_2^-) (Albesa *et al.*, 2004).

Thus, we hypothesise that the conjugation of a dietary molecule (e.g. cholesterol) with a second-line anti-TB drug (e.g. ciprofloxacin), will result in a novel drug that could easily be carried into

mycobacteria by the *mce4*-transporter. Moreover, the cholesterol part of the molecule will attract mycobacteria for its use as carbon source and thereby mislead mycobacteria for the intake of the active drug (i.e. ciprofloxacin). Once ciprofloxacin is inside of mycobacteria it can commence its mechanism of action.

Within the subsequent chapter (Chapter 3), a manuscript which reports details of the synthetic, analytical, biological work, and also the results, analysis and conclusions of the project, is presented.

BIBLIOGRAPHY

Agalave, S.G., Maujan, S.R. & Pore, V.S. 2011. Click chemistry: 1, 2, 3-triazoles as pharmacophores. *Chemistry - an asian journal*, 6(10):2696-2718.

Albesa, I., Becerra, M.C., Battán, P.C. & Páez, P.L. 2004. Oxidative stress involved in the antibacterial action of different antibiotics. *Biochemical and biophysical research communications*, 317(2):605-609.

Aldred, K.J., Kerns, R.J. & Osheroff, N. 2014. Mechanism of quinolone action and resistance. *Biochemistry*, 53(10):1565-1574.

Andersson, M.I. & MacGowan, A.P. 2003. Development of the quinolones. *Journal of antimicrobial chemotherapy*, 51(1):1-11.

Asif, M. 2014a. A mini review on antimalarial activities of biologically active substituted triazoles derivatives. *International journal of advanced research in chemical science*, 1:22-28.

Asif, M. 2014b. A mini review on the study of new broad-spectrum antimicrobial fluoroquinolone JNJ-Q2. *Journal of chemistry & applications*, 1(1):5-10.

Benson, F.R. & Savell, W.L. 1950. The chemistry of the vicinal triazoles. *Chemical reviews*, 46(1):1-68.

Berning, S.E. 2001. The role of fluoroquinolones in tuberculosis today. *Drugs*, 61(1):9-18.

Bhukya, B. & Tangutur, A.D. 2017. Microbial biotechnology: technological challenges and developmental trends. Waretown, NJ: Apple Academic Press.

Bhuvaneswari, K., Swetha, L.M., Ramesh, M., Mounika, B., Lakshmi, T.A., Brahmaiah, B. & Nama, S. 2013. Review on pathology and chemoprophylaxis of malaria. *International journal of current trends in pharmaceutical research*, 1(2):97-104.

Bisacchi, G.S. 2015. Origins of the quinolone class of antibacterials: an expanded “discovery story” miniperspective. *Journal of medicinal chemistry*, 58(12):4874-4882.

Blondeau, J.M. 2004. Fluoroquinolones: mechanism of action, classification, and development of resistance. *Survey of ophthalmology*, 49(2, Supplement 2):73-78.

Boyd, R. 2012. What is the treatment for drug-resistant tuberculosis? https://www.medscape.com/viewarticle/766128 Date of access: 31 Jan. 2018.

Breda, S.A., Jimenez-Kairuz, A.F., Manzo, R.H. & Olivera, M.E. 2009. Solubility behavior and biopharmaceutical classification of novel high-solubility ciprofloxacin and norfloxacin pharmaceutical derivatives. *International journal of pharmaceutics*, 371(1):106-113.

Brennan, P.J. & Nikaido, H. 1995. The envelope of mycobacteria. *Annual review of biochemistry*, 64(1):29-63.

CDC. 2011. Core curriculum on tuberculosis: what the clinician chould know. https://www.cdc.gov/tb/education/corecurr/index.htm Date of access: 26 Aug. 2017.

CDC. 2014. The difference between latent TB infection and TB disease. https://www.cdc.gov/tb/publications/factsheets/general/ltbiandactivetb.htm Date of access: 30 Jan. 2018.

CDC. 2016a. BCG vaccine. https://www.cdc.gov/tb/publications/factsheets/prevention/bcg.htm Date of access: 30 Jan. 2018.

CDC. 2016b. Highlights from the 2016 treatment of drug-susceptible tuberculosis guidelines. https://www.cdc.gov/tb/topic/treatment/guidelinehighlights.htm Date of access: 31 Jan. 2018.

CDC. 2016c. TB risk factors. https://www.cdc.gov/tb/topic/basics/risk.htm Date of access: 30 Jan. 2018.

CDC. 2016d. Treatment of persons living with HIV. https://www.cdc.gov/tb/topic/treatment/tbhiv.htm Date of access: 11 Sept. 2017.

Chaudhari, V., Gunjal, S. & Sujata, D. 2011. Emerging anti-tubercular drugs. Vol. 108.

Chu, D. & Fernandes, P.B. 1989. Structure-activity relationships of the fluoroquinolones. *Antimicrobial agents and chemotherapy*, 33(2):131-135.

Cincinelli, R., Musso, L., Beretta, G. & Dallavalle, S. 2014. 4-Quinolone fused heterocyclic ring systems by intramolecular reactions of 4-quinolone-2-carboxamides. *Tetrahedron*, 70(52):9797-9804.

Cook, G.M., Berney, M., Gebhard, S., Heinemann, M., Cox, R.A., Danilchanka, O. & Niederweis, M. 2009. Physiology of mycobacteria. *Advances in microbial physiology*, 55:81-319.

Daniel, T.M. 2006. The history of tuberculosis. *Respiratory medicine*, 100(11):1862-1870.

Davenne, T. & McShane, H. 2016. Why don't we have an effective tuberculosis vaccine yet? *Expert review of vaccines*, 15(8):1009-1013.

Dax, S.L. 2012. Antibacterial chemotherapeutic agents. Vol. 1. Pennsylvania, USA: Blackie Academic & Professional.

de Jong, B.C., Antonio, M. & Gagneux, S. 2010. Mycobacterium africanum — review of an important cause of human tuberculosis in West Africa. *Public library of science: neglected tropical diseases*, 4(9):e744. doi: 10.1371/journal.pntd.0000744

Deck, D.H. & Winston, L.G. 2012a. Antimycobacterial drugs. *(In* Katzung, B.G., Masters, S.B. & Trevor, A.J., *eds*. Basic & clinical pharmacology. New York; London: McGraw-Hill Medical. p. 839-848).

Deck, D.H. & Winston, L.G. 2012b. Tetracyclines, macrolides, clindamycin, chloramphenicol, streptogramins & oxazolidinones. *(In* Katzung, B.G., Masters, S.B. & Trevor, A.J., *eds*. Basic & clinical pharmacology. New York; London: McGraw-Hill Medical. p. 809-819).

Decker, M. 2017. Design of hybrid molecules for drug development: introduction. Amsterdam, Netherlands: Elsevier.

Delogu, G., Sali, M. & Fadda, G. 2013. The biology of mycobacterium tuberculosis infection. *Mediterranean journal of hematology and infectious diseases*, 5(1):e2013070.

Diacon, A., Dawson, R., Hanekom, M., Narunsky, K., Venter, A., Hittel, N., Geiter, L., Wells, C., Paccaly, A. & Donald, P. 2011. Early bactericidal activity of delamanid (OPC-67683) in smear-positive pulmonary tuberculosis patients. *The international journal of tuberculosis and lung disease*, 15(7):949-954.

Dixit, P.P., Dixit, P.P. & Thore, S.N. 2016. Hybrid triazoles: design and synthesis as potential dual inhibitor of growth and efflux inhibition in tuberculosis. *European journal of medicinal chemistry*, 107:38-47.

Eisenreich, W., Dandekar, T., Heesemann, J. & Goebel, W. 2010. Carbon metabolism of intracellular bacterial pathogens and possible links to virulence. *Nature reviews microbiology*, 8(6):401-412.

Elkington, P.T. 2013. Tuberculosis: time for a new perspective? *Journal of infection*, 66(4):299-302.

Emmerson, A. & Jones, A. 2003. The quinolones: decades of development and use. *Journal of antimicrobial chemotherapy*, 51(1):13-20.

Fenlon, C.H. & Cynamon, M.H. 1986. Comparative in vitro activities of ciprofloxacin and other 4-quinolones against Mycobacterium tuberculosis and Mycobacterium intracellulare. *Antimicrobial agents and chemotherapy*, 29(3):386-388.

Fernandes, G. & Dos Santos, J.L. 2017. Tuberculosis-current advances in development of new drugs against multidrug-resistant strains. *E-Chronicon microbiology*, 6:60-62.

Finch, R.G., Greenwood, D., Whitley, R.J. & Norrby, S.R. 2010. Antibiotic and chemotherapy. 9th ed. s.l.: Saunders.

Foroumadi, A., Emami, S., Hassanzadeh, A., Rajaee, M., Sokhanvar, K., Moshafi, M.H. & Shafiee, A. 2005. Synthesis and antibacterial activity of N-(5-benzylthio-1, 3, 4-thiadiazol-2-yl) and N-(5-benzylsulfonyl-1, 3, 4-thiadiazol-2-yl) piperazinyl quinolone derivatives. *Bioorganic & medicinal chemistry letters*, 15(20):4488-4492.

Frieden, T.R., Sterling, T.R., Munsiff, S.S., Watt, C.J. & Dye, C. 2003. Tuberculosis. *Lancet*, 362(9387):887-999.

George, M.J., Dew, R.B. & Daly, J.S. 1991. Acute renal failure after an overdose of ciprofloxacin. *Archives of internal medicine*, 151(3):620-620.

Ginsburg, A.S., Grosset, J.H. & Bishai, W.R. 2003. Fluoroquinolones, tuberculosis, and resistance. *The lancet infectious diseases*, 3(7):432-442.

Goldberg, D.E., Siliciano, R.F. & Jacobs, W.R. 2012. Outwitting evolution: fighting drug-resistant TB, malaria, and HIV. *Cell*, 148(6):1271-1283.

Gopal, R., Rapaka, R.R. & Kolls, J.K. 2017. Immune reconstitution inflammatory syndrome associated with pulmonary pathogens. *European respiratory review* : *an official journal of the European Respiratory Society*, 26(143):160042-.

Gray, J.M. & Cohn, D.L. 2013. Tuberculosis and HIV coinfection. (*In*. Seminars in respiratory and critical care medicine organised by: Thieme Medical Publishers. p. 32-43).

Grayson, M.L., Cosgrove, S.E., Crowe, S., Hope, W., McCarthy, J.S., Mills, J., Mouton, J.W. & Paterson, D.L. 2017. Kucers' the use of antibiotics: a clinical review of antibacterial, antifungal, antiparasitic, and antiviral drugs. CRC Press.

Greenwood, D. 2008. Antimicrobial drugs: chronicle of a twentieth century medical triumph. Oxford, NJ: Oxford University Press.

Gumbo, T. 2011a. Chemotherapy of Tuberculosis, *Mycobacterium Avium* Complex Disease, and Leprosy. *(In* Brunton, L.L., Chabner, B., Goodman, L.S. & Knollmann, B.C., *eds*. Goodman & Gilman's the pharmacological basis of therapeutics. 12 ed. New York, N.Y.: McGraw-Hill Education LLC. p. 1549-1570).

Gumbo, T. 2011b. Chemotherapy Tuberculosis, *Mycobaterium Avium* Complex Disease, and Leprosy. *(In* Brunton, L.L., Chabner, B., Goodman, L.S. & Knollmann, B.C., *eds*. Goodman & Gilman's the pharmacological basis of therapeutics. 12 ed. NY: McGraw-Hill Education LLC. p. 1549-1570).

Haley, C.A. 2013. Southeastern national tuberculosis center: extrapulmonary tuberculosis. https://sntc.medicine.ufl.edu/Files/Haiti/Module%209%20Extrapulmonary%20TB%20ENGLISH.pdf. Date of access: 24 Jan. 2018.

Hamzah, J., Skinner-Adams, T. & Davis, T. 2000. In vitro antimalarial activity of trovafloxacin, a fourth-generation fluoroquinolone. *Acta tropica*, 74(1):39-42.

Hansen-Flaschen, J. 2018. BCG vaccine. https://www.britannica.com/science/BCG-vaccine Date of access: 6 Jan. 2018.

Harvey, A.L. 1998. Advances in drug discovery techniques. England: Taylor & Francis.

Hooper, D.C. 1999. Mode of action of fluoroquinolones. *Drugs*, 58(2):6-10.

Hu, Y.-Q., Zhang, S., Xu, Z., Lv, Z.-S., Liu, M.-L. & Feng, L.-S. 2017. 4-Quinolone hybrids and their antibacterial activities. *European journal of medicinal chemistry*.

Jordae, L., Vieira, O.V. 2011. Tuberculosis: New Aspects of an Old Disease. *Internation Journal of Cell Biology*, 2011:1-13.

Kanabus, A. 2017a. TB & HIV – co-infection, statistics, diagnosis & treatment. https://www.tbfacts.org/tb-hiv/ Date of access: 30 Jan. 2018.

Kanabus, A. 2017b. TB Drugs – first line, drug names, regimens. https://www.tbfacts.org/tb-drugs/ Date of access: 30 Jan. 2018.

Kanabus, A. 2017c. TB statistics for South Africa – national and provincial. https://www.tbfacts.org/tb-statistics-south-africa/ Date of access: 12 Jul. 2017.

Kanabus, A. 2017d. TB treatment – curing TB, failure, relapse & recurrence. https://www.tbfacts.org/tb-treatment/ Date of access: 30 Jan. 2018.

Kanabus, A. 2017e. Treatment of drug resistant TB – shorter, longer (conventional) regimens. https://www.tbfacts.org/treatment-of-drug-resistant-tb/ Date of access: 4 Aug. 2017.

Kant, R., Singh, V., Nath, G., Awasthi, S.K. & Agarwal, A. 2016. Design, synthesis and biological evaluation of ciprofloxacin tethered bis-1, 2, 3-triazole conjugates as potent antibacterial agents. *European journal of medicinal chemistry*, 124:218-228.

Kharb, R., Sharma, P.C. & Yar, M.S. 2011. Pharmacological significance of triazole scaffold. *Journal of enzyme inhibition and medicinal chemistry*, 26(1):1-21.

Krishna, K.M., Inturi, B., Pujar, G.V., Purohit, M.N. & Vijaykumar, G. 2014. Design, synthesis and 3D-QSAR studies of new diphenylamine containing 1, 2, 4-triazoles as potential antitubercular agents. *European journal of medicinal chemistry*, 84:516-529.

Lee, J.Y. 2015. Diagnosis and treatment of extrapulmonary tuberculosis. *Tuberculosis and respiratory diseases*, 78(2):47-55.

Lewis, J.M. & Sloan, D.J. 2015. The role of delamanid in the treatment of drug-resistant tuberculosis. *Therapeutics and clinical risk management*, 11:779-791.

Li, X-X. & Zhou, X-N. 2013. Co-infection of tuberculosis and parasitic diseases in humans: a systematic review. *Parasites & vectors*, 6(1):79-91.

Ma, Z., Ginsberg, A.M. & Spigelman, M. 2007. 7.24 - Antimycobacterium Agents. *(In* Taylor, J.B. & Triggle, D.J., *eds*. Comprehensive medicinal chemistry II. Oxford: Elsevier. p. 699-730).

Mahajan, R. 2013. Bedaquiline: first FDA-approved tuberculosis drug in 40 years. *International journal of applied and basic medical research*, 3(1):1-2.

Maslow, M.J. & Portal-Celhay, C. 1993. Rifamycins. *(In* Bennet, J.E., Dolin, R. & Blaser, M.J., *eds*. Principles and practice of infectious diseases. 8th ed. Pennsylvania: Churchill Livingstone. p. 339).

Matsumoto, M., Hashizume, H., Tomishige, T., Kawasaki, M., Tsubouchi, H., Sasaki, H., Shimokawa, Y. & Komatsu, M. 2006. OPC-67683, a nitro-dihydro-imidazooxazole derivative with promising action against tuberculosis in vitro and in mice. *Public library of science medicine*, 3(11):e466. doi: 10.1371/journal.pmed.0030466

Mayo Clinic. 2018. Patient care & health information, diseases & conditions: tuberculosis https://www.mayoclinic.org/diseases-conditions/tuberculosis/diagnosis-treatment/drc-20351256 Date of access: 15 Jan. 2018.

McPherson, J.C., Runner, R., Buxton, T.B., Hartmann, J.F., Farcasiu, D., Bereczki, I., Rőth, E., Tollas, S., Ostorházi, E. & Rozgonyi, F. 2012. Synthesis of osteotropic hydroxybisphosphonate derivatives of fluoroquinolone antibacterials. *European journal of medicinal chemistry*, 47:615-618.

Menzies, D., Long, R., Trajman, A., Dion, M.-J., Yang, J., Al Jahdali, H., Memish, Z., Khan, K., Gardam, M. & Hoeppner, V. 2008. Adverse events with 4 months of rifampin therapy or 9 months of isoniazid therapy for latent tuberculosis infection: a randomized trial. *Annals of internal medicine*, 149(10):689-697.

Metropolis India. 2016. Different types of drug resistant tuberculosis and diagnosis http://blog.metropolisindia.com/different-types-of-drug-resistant-tuberculosis-and-diagnosis/ Date of access: 28 Jan. 2018.

Meunier, B. 2008. Hybrid Molecules with a Dual Mode of Action: Dream or Reality? *Accounts of Chemical Research*, 41(1):69-77.

Mitnick, C.D., McGee, B. & Peloquin, C.A. 2009. Tuberculosis pharmacotherapy: strategies to optimize patient care. *Expert opinion on pharmacotherapy*, 10(3):381-401.

Mori, K., Maru, C., Takasuna, K. & Furuhama, K. 2000. Mechanism of histamine release induced by levofloxacin, a fluoroquinolone antibacterial agent. *European journal of pharmacology*, 394(1):51-55.

Müller, A. 2016. Extrapulmonary TB. http://www.tbonline.info/posts/2016/3/31/extrapulmonary-tb/ Date of access: 16 Jan. 2018.

National Department of Health, S.A. 2014. National Tuberculosis Management Guidelines. https://www.health-e.org.za/2014/06/10/guidelines-national-tuberculosis-management-guidelines/ Date of access: 1 Nov. 2017.

Odenholt, I., Löwdin, E. & Cars, O. 1998. Bactericidal effects of levofloxacin in comparison with those of ciprofloxacin and sparfloxacin. *Clinical microbiology and infection*, 4(5):264-270.

Ouellet, H., Johnston, J.B. & Ortiz de Montellano, P.R. 2011. Cholesterol catabolism as a therapeutic target in *Mycobacterium tuberculosis*. *Trends microbiol*, 19(11):530-539.

Owens, R.C., Jr. & Ambrose, P.G. Clinical use of the fluoroquinolones. *Medical clinics*, 84(6):1447-1469.

Potts, K.T. 1961. The chemistry of 1,2,4-triazoles. *Chemical reviews*, 61(2):87-127.

Rajulu, G.G., Naik, H.S.B., Viswanathan, A., Agarwal, D.S., Sambasivam, G. & Koppolu, K.P. 2013. Design and synthesis of new N-substituted amino methyl-[1, 2, 3] triazolyl moieties of fluoroquinolones as antibacterial agents. *Medicinal chemistry research*, 22(8):3843-3856.

Rodgman, A. & Perfetti, T.A. 2016. The chemical components of tobacco and tobacco smoke. 2nd ed. Boca Raton, Florida: CRC Press, Taylor & Francis Group.

Rossiter, D., University of Cape, T. & Division of Clinical, P. 2012. South African medicines formulary. Rondebosch, South Africa: Health and Medical Publishing Group.

Russell, D.G. 2001. Mycobacterium tuberculosis: here today, and here tomorrow. *Nature reviews: Molecular cell biology*, 2(8):569-577.

Russell, D.G., Cardona, P.-J., Kim, M.-J., Allain, S. & Altare, F. 2009. Foamy macrophages and the progression of the human tuberculosis granuloma. *Nature immunology*, 10(9):943-948.

Russell, D.G., VanderVen, B.C., Lee, W., Abramovitch, R.B., Kim, M.-j., Homolka, S., Niemann, S. & Rohde, K.H. 2010. Mycobacterium tuberculosis wears what it eats. *Cell host and microbe*, 8(1):68-76.

Saeed, S. & Hasan, S. 2015. Tuberculosis: a public health challenge: brief overview of literature. *International research journal of pharmacy*, 7(1):1-4.

Saini, V., Cumming, B.M., Guidry, L., Lamprecht, D., Adamson, J.H., Reddy, V.P., Chinta, K.C., Mazorodzo, J., Glasgow, J.N., Richard-Greenblatt, M., Gomez-Velasco, A., Bach, H., Av-Gay, Y., Eoh, H., Rhee, K. & Steyn, A.J.C. 2016. Ergothioneine maintains redox and bioenergetic homeostasis essential for drug susceptibility and virulence of Mycobacterium tuberculosis. *Cell Reports*, 14(3):572-585.

Sasahara, K., Shimokawa, Y., Hirao, Y., Koyama, N., Kitano, K., Shibata, M. & Umehara, K. 2015. Pharmacokinetics and metabolism of delamanid, a novel anti-tuberculosis drug, in animals and humans: importance of albumin metabolism in vivo. *Drug metabolism and disposition*, 43(8):1267-1276.

Scholar, E.M. 2002. Fluoroquinolines: past, present and future of a novel group of antibacterial agents. *American journal of pharmaceutical education*, 66(2):164-172.

Schuld, A., Small, W. & Harris, T. 2004. The true story of cipro. https://www.westonaprice.org/health-topics/health-issues/the-true-story-of-cipro/ Date of access: 15 Jan. 2018.

Sendzik, J., Lode, H. & Stahlmann, R. 2009. Quinolone-induced arthropathy: an update focusing on new mechanistic and clinical data. *International journal of antimicrobial agents*, 33(3):194-200.

Shabeel, P.N. 2010. Fluoroquinolones. https://www.slideshare.net/shabeelpn/fluoroquinolones Date of access: 25 Jan. 2018.

Shah, N.S., Auld, S.C., Brust, J.C.M., Mathema, B., Ismail, N., Moodley, P., Mlisana, K., Allana, S., Campbell, A., Mthiyane, T., Morris, N., Mpangase, P., van der Meulen, H., Omar, S.V., Brown, T.S., Narechania, A., Shaskina, E., Kapwata, T., Kreiswirth, B. & Gandhi, N.R. 2017. Transmission of extensively drug-resistant Tuberculosis in South Africa. *New england journal of medicine*, 376(3):243-253.

Shahmohammadi, S., Saffar, M.J. & Rezai, M.S. 2014. BCG-osis after BCG vaccination in immunocompromised children: case series and review. *Journal of pediatrics review*, 2(1):62-74.

Sharma, P.C., Jain, A. & Jain, S. 2009. Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. *Acta poloniae pharmaceutica*, 66(6):587-604.

Sharma, S.K. & Mohan, A. 2013. Tuberculosis: from an incurable scourge to a curable disease - journey over a millennium. *The indian journal of medical research*, 137(3):455-493.

Shin, J.-A., Lim, Y.-G. & Lee, K.-H. 2012. Copper-catalyzed azide–alkyne cycloaddition reaction in water using cyclodextrin as a phase transfer catalyst. *The journal of organic chemistry*, 77(8):4117-4122.

Skripconoka, V., Danilovits, M., Pehme, L., Tomson, T., Skenders, G., Kummik, T., Cirule, A., Leimane, V., Kurve, A. & Levina, K. 2013. Delamanid improves outcomes and reduces mortality in multidrug-resistant tuberculosis. *European respiratory journal*, 41(6):1393-1400.

Smart, T. 2016. South Africa's XDR-TB epidemic is due to transmission rather than evolution of resistant strains. http://www.aidsmap.com/page/3040991/ Date of access: 5 Feb. 2018.

Smit, F.J., van Biljon, R.A., Birkholtz, L.-M. & N'Da, D.D. 2015. Synthesis and in vitro biological evaluation of dihydroartemisinyl-chalcone esters. *European journal of medicinal chemistry*, 90(supplement C):33-44.

Svensson, E.M., Dosne, A.G. & Karlsson, M.O. 2016. Population pharmacokinetics of bedaquiline and metabolite M2 in patients with drug-resistant tuberculosis: the effect of time-varying weight and albumin. *Clinical pharmacology & therapeutics: pharmacometrics & systems pharmacology*, 5(12):682-691.

Tabarsi, P. & Mardani, M. 2012. Extensively drug-resistant tuberculosis: a review article. *Archives of clinical infectious diseases*, 7(3):81-84.

Tashima, T. 2015. The structural use of carbostyril in physiologically active substances. *Bioorganic & medicinal chemistry letters*, 25(17):3415-3419.

Tierney, D. 2014. Extrapulmonary tuberculosis. http://www.msdmanuals.com/professional/infectious-diseases/mycobacteria/extrapulmonary-tuberculosis Date of access: 23 Jan. 2018.

Tome, A. 2004. Product class 13: 1, 2, 3-triazoles. *Science of synthesis*, 13:415-601.

Trevor, A.J., Katzung, B.G., Masters, S.B. & Kruidering-Hall, M. 2010. Pharmacology examination & board review. NJ: McGraw-Hill Medical.

University of Oxford. 2018. Vaccine knowledge project, authoritative information for all: BCG vaccine (TB vaccine). http://vk.ovg.ox.ac.uk/bcg-vaccine Date of access: 31 Jan. 2018.

Venepally, V., Prasad, R., Poornachandra, Y., Kumar, C.G. & Jala, R.C.R. 2016. Synthesis of novel ethyl 1-ethyl-6-fluoro-7-(fatty amido)-1, 4-dihydro-4-oxoquinoline-3-carboxylate derivatives and their biological evaluation. *Bioorganic & medicinal chemistry letters*, 26(2):613-617.

White, N.J., Pukrittayakamee, S., Hien, T.T., Faiz, M.A., Mokuolu, O.A. & Dondorp, A.M. 2014. Malaria. *The lancet*, 383(9918):723-735.

WHO. 2013. The use of bedaquiline in the treatment of multidrug-resistant tuberculosis. http://www.who.int/tb/challenges/mdr/bedaquiline/en/ Date of access: 27 Oct. 2014.

WHO. 2015. The Selection and Use of Essential Medicines. http://apps.who.int/medicinedocs/en/d/Js22190en/ Date of access: 30 Oct. 2017.

WHO. 2016a. The use of delamanid in the treatment of multidrug-resistant tuberculosis in children and adolescents. http://who.int/tb/publications/Delamanid_interim_policy/en/ Date of access: 28 Oct. 2017.

WHO. 2016b. WHO treatment guidelines for drug-resistant tuberculosis. http://apps.who.int/iris/handle/10665/250125/ Date of access: 15 Sept. 2017.

WHO. 2017a. Global tuberculosis report 2017. http://www.who.int/tb/publications/global_report/en/ Date of access: 18 Nov. 2017.

WHO. 2017b. World TB Day, 24 March 2017. http://www.who.int/campaigns/tb-day/2017/en/ Date of access: 15 Nov. 2017.

WHO. 2018a. Latent tuberculosis infection (LTBI). http://www.who.int/tb/areas-of-work/preventive-care/ltbi_faqs/en/ Date of access: 31 Jan. 2018.

WHO. 2018b. Media center: tuberculosis key facts. http://www.who.int/mediacentre/factsheets/fs104/en/ Date of access: 25 Jan. 2018.

WHO. 2018c. TB comorbidities and risk factors. http://www.who.int/tb/areas-of-work/treatment/risk-factors/en/ Date of access: 18 Jan. 2018.

WHO. 2018d. TB drug resistance types. http://www.who.int/tb/areas-of-work/drug-resistant-tb/types/en/ Date of access: 28 Jan. 2018.

WHO. 2018e. Tuberculosis vaccine development. http://www.who.int/immunization/research/development/tuberculosis/en/ Date of access: 30 Jan. 2018.

Wolfson, J.S. & Hooper, D.C. 1985. The fluoroquinolones: structures, mechanisms of action and resistance, and spectra of activity in vitro. *Antimicrobial agents and chemotherapy*, 28(4):581.

Wright, G.D. & Sutherland, A.D. 2007. New strategies for combating multidrug-resistant bacteria. *Trends in molecular medicine*, 13(6):260-267.

Wu, C., Huang, P., Sun, Z., Lin, M., Jiang, Y., Tong, J. & Ge, C. 2016. Synthesis of 4-quinolones via triflic anhydride-mediated intramolecular Houben-Hoesch reaction of β-arylamino acrylonitriles. *Tetrahedron*, 72(11):1461-1466.

Xavier, A.S. & Lakshmanan, M. 2014. Delamanid: a new armor in combating drug-resistant tuberculosis. *Journal of pharmacology & pharmacotherapeutics*, 5(3):222-224.

Xia, X., Zhang, Q., Zhao, L. & Hu, Y. 2017. Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: Design, synthesis and *in vitro* anti-mycobacterial evaluation. *European journal of medicinal chemistry*, 138:66-71.

Xu, H., Ma, S., Xu, Y., Bian, L., Ding, T., Fang, X., Zhang, W. & Ren, Y. 2015. Copper-catalyzed one-pot synthesis of 1,2,4-triazoles from nitriles and hydroxylamine. *The journal of organic chemistry*, 80(3):1789-1794.

Xu, Z., Lv, Z.S., Song, X.F. & Qiang, M. 2018. Ciprofloxacin-isatin-1H-1, 2, 3-triazole hybrids: Design, synthesis, and in vitro anti-tubercular activity against Mycobacterium tuberculosis. *Journal of heterocyclic chemistry*, 55:97-102.

Xu, Z., Song, X.-F., Hu, Y.-Q., Qiang, M. & Lv, Z.-S. 2017. Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: design, synthesis and in vitro anti-mycobacterial evaluation. *European journal of medicinal chemistry*, 138:66-71.

Zhanel, G.G., Walkty, A., Vercaigne, L., Karlowsky, J.A., Embil, J., Gin, A.S. & Hoban, D.J. 1999. The new fluoroquinolones: a critical review. *Canadian journal of infectious diseases and medical microbiology*, 10(3):207-238.

Zhao, Y.-L., Chen, Y.-L., Sheu, J.-Y., Chen, I.L., Wang, T.-C. & Tzeng, C.-C. 2005. Synthesis and antimycobacterial evaluation of certain fluoroquinolone derivatives. *Bioorganic & medicinal chemistry*, 13(12):3921-3926.

Zheng, J., Rubin, E.J., Bifani, P., Mathys, V., Lim, V., Au, M., Jang, J., Nam, J., Dick, T., Walker, J.R., Pethe, K. & Camacho, L.R. 2013. para-Aminosalicylic acid is a prodrug targeting dihydrofolate reductase in mycobacterium tuberculosis. *Journal of Biological Chemistry*, 288(32):23447-23456.

Zumla, A., Raviglione, M., Hafner, R. & Fordham von Reyn, C. 2013. Current concepts: tuberculosis. *New england journal of medicine*, 368(8):745-755.

CHAPTER 3
ARTICLE FOR SUBMISSION

Chapter 3 includes an article for submission to the European Journal of Medicinal Chemistry. This article comprises of an Introduction, Results and discussion, Conclusion, Materials and Methods, Syntheses of devised compounds, and *in vitro* biological evaluations. It also includes an Abstract and a Graphical abstract. This article is prepared according to the author's guidelines, available in the Author Information pack on the journal's homepage (Annexure C): https://www.elsevier.com/journals/european-journal-of-medicinal-chemistry/0223-5234/guide-for-authors

DESIGN, SYNTHESIS AND IN VITRO ANTIMYCOBACTERIAL ACTIVITY OF NOVEL CIPROFLOXACIN DERIVATIVES

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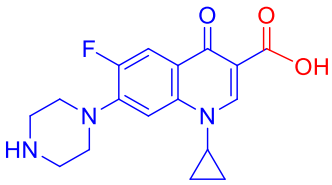
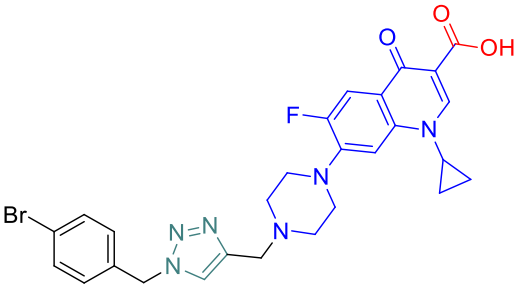
GRAPHICAL ABSTRACT



Ciprofloxacin, CPX **1**

M*tb* H37Rv MIC_{90} 1.8 µM
ClogP 1.55
HFLB WI-38 IC_{50}>100 µM



Hybrid **28**

M*tb* H37Rv MIC_{90} 3.70 µM
ClogP 3.7, CHO IC_{50} >100 µM
HEK 293 IC_{50} >100 µM

ABSTRACT

Tuberculosis is the deadliest infectious disease affecting humankind with a death toll nearing 2 million people in 2016. The increasing prevalence of multi-drug resistant strains of the pathogen, *Mycobacterium tuberculosis* underscores the urgent need for the development of new antitubercular drugs. In search for such drugs, we investigate two series of ciprofloxacin derivatives (analogs and hybrids). We herein report the design, synthesis and biological activity against the human virulent H37Rv strain of *Mtb*. The cholesteryl moiety bearing hybrid with MIC_{90} 2 µM comparable to 1. 8 µM ciprofloxacin was the most active. However, the *para* bromo substituted non-cytotoxic hybrid **28** with activity MIC_{90} 3.7 µM and more selective towards bacteria inhibition stood as a better hit for further investigation.

Keywords: *Mycobacterium tuberculosis*, ciprofloxacin, 1,2,3-triazole, click-chemistry, hybrids

3.1 Introduction

Tuberculosis (TB) endures, to this day, to be the scourge of humankind [1, 2]. TB, together with human immunodeficiency virus (HIV) and malaria, form the three leading infectious diseases causing death worldwide [3]. TB is, in fact, the deadliest disease globally; as 10.4 million people fell ill and 1.7 million people succumbed to this disease in 2016 [4]. Additionally, co-infection of TB with either one of these diseases is widespread and one increases the risk of the other [5, 6]. There is thus an overlap in the incidences of TB and HIV; as both diseases can occur in and/or every part of the world. TB was the leading cause of HIV-related deaths in 2016 [4] as result of the opportunistic nature [7] and its effortless communicability [4].

To make matters worse, 490 000 people developed multidrug resistant tuberculosis (MDR-TB) in 2016 [4]. MDR strains of *Mycobacterium tuberculosis* (*Mtb*) are defined as mycobacteria resilient to, at least, isoniazid and/or rifampicin [8] which form the core drugs of standard TB treatment regimens [9, 10]. The rise of resistance against these two primary drugs necessitates a selection of a combination of at least five effective TB-agents into the regimen. The combination should comprise pyrazinamide and four second-line TB-agents, namely one fluoroquinolone, one aminoglycoside, one thioamide, and either cycloserine or terizidone [11]. According to the World Health Organization (WHO) the inclusion of a fluoroquinolone drug is compulsory as it greatly improves the treatment outcome for MDR-TB [11]. Notwithstanding, treatment of MDR-TB is delicate as the second-line agents are usually more toxic and expensive coupled with limited availability, and require a lengthy duration of administration [10] which all leads to poor patient compliance [10] and ultimately to extensively drug resistant tuberculosis (XDR-TB) [12]. XDR-TB strains are, additionally, resistant to fluoroquinolones and an aminoglycoside [8]; resulting in high mortality rates due to ineffective drugs and subsequently treatment failure [13]. Thus, novel and more potent drugs are vital to shorten the treatment regimen of all strains of *Mtb*, with less toxic effects.

The development of clinically useful drugs with new structures and a novel mechanism of action [14] are complex, time consuming, risky [15], and expensive [16]. Thus, a more practical approach that forms the cornerstone for drug discovery is the exploitation of structure-activity relationship (SAR) of lead compounds such as existing drugs to deliver analogs of improved therapeutic profile [14, 17].

The 4-quinolone scaffold forms part of a promising class of synthetic antibiotics [18] which sparked great scientific and clinical interest [19] since its first discovery in the 1960s [18]. The relatively simple pharmacophore creates an array of possibilities for structural changes to further increase its antibacterial activity [20]. One such example of lead optimization was with the original

quinolone, namely nalidixic acid; which exhibits only marginal antibacterial activity, limiting its use to only uncomplicated urinary tract infections (UTI's) [21, 22]. With key structural changes to nalidixic came flumequine and pipemidic acid and subsequent newer quinolones such as norfloxacin and eventually ciprofloxacin [18, 23]. Flumequine and pipemidic acid were first in class to feature a fluorine atom and a piperazine ring in positions C-6 and C-7 of the quinolone nuclei, respectively [23]. The combination of both features yielded norfloxacin [23], giving rise to a new class of antibiotics, namely the fluoroquinolones, which exhibit a more potent antimicrobial action and a broader spectrum of activity [24]. In addition, another breakthrough came with the introduction of a cyclopropyl ring in position N-1, as seen with ciprofloxacin (Figure 3-1). The addition of a cyclopropyl ring in this position produce the most significant antibacterial effect [25]. The quinolone spectrum of activity now includes a range of infections beyond UTI's such as bone, respiratory-, gastrointestinal tract, including sexually transmitted diseases and atypical diseases such as HIV, TB, malaria, and tumors [26]. Mechanisms of action to explain the antibacterial activity of fluoroquinolone drugs include inhibition of type-II topo-isomerase enzymes, namely DNA-gyrase in gram-negative- and topo-isomerase IV in gram-positive bacteria [27, 28].

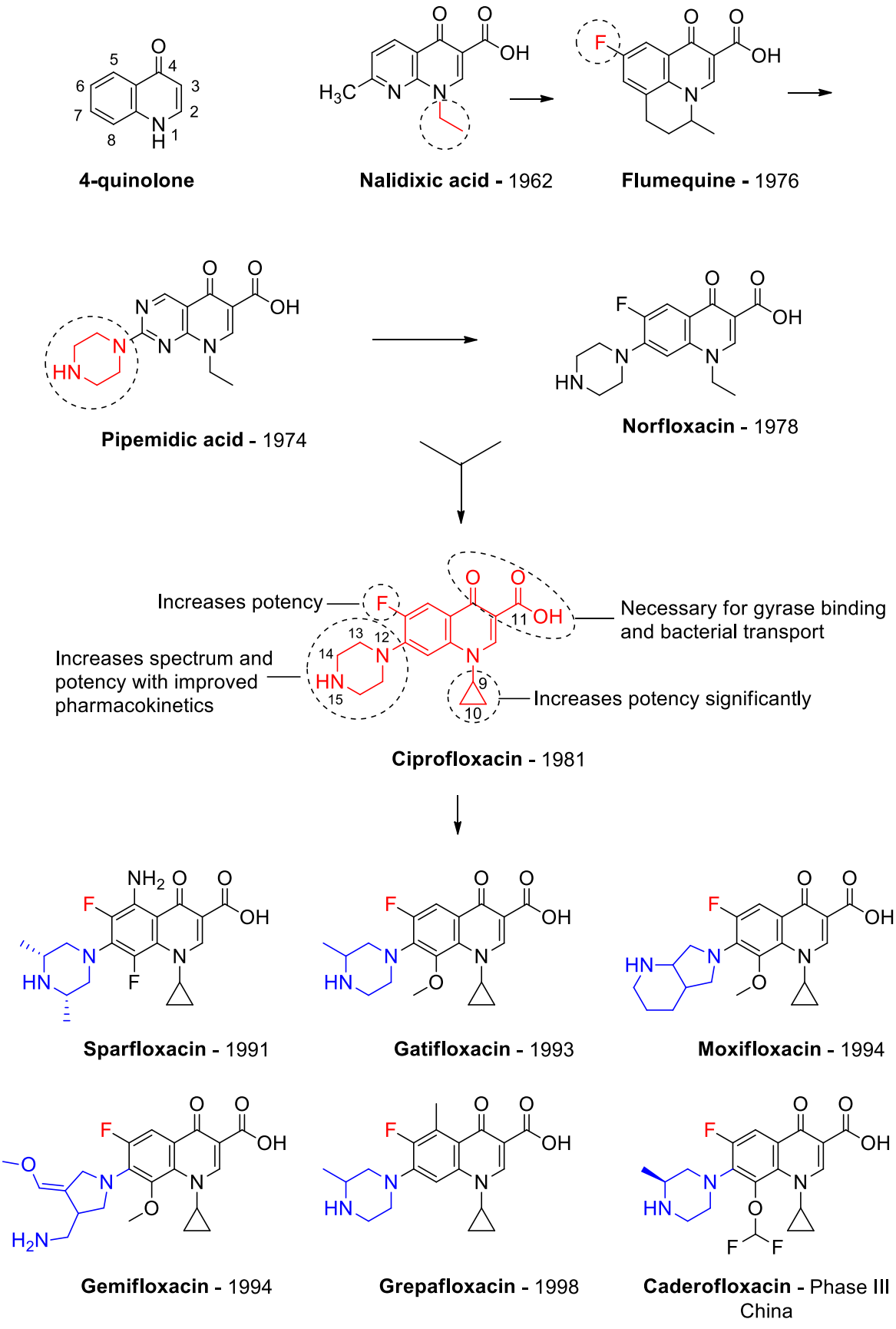
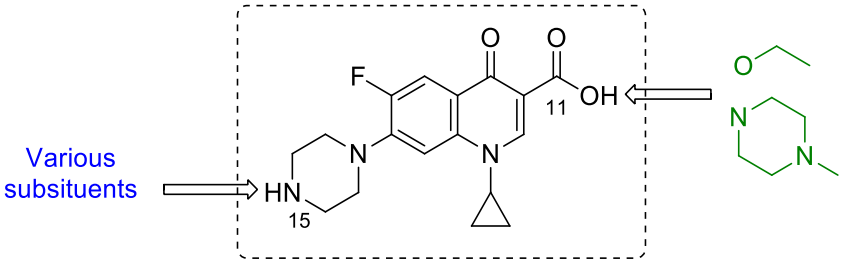


Figure 3-1: Illustrating the structural evolution of the quinolone pharmacophore.

Further investigation of the SAR of fluoroquinolones, indicate the carboxylic acid and the keto group in position C-3 and C-4, respectively, to be essential for their biological activities [29]. Modifications made in position C-3 usually produce compounds with reduced activity [24]. However, substituting the carboxylic acid with certain esters or an isothiazole group affords derivatives with *in vivo* and potent *in vitro* antibacterial activity when compared to ciprofloxacin [20]. Also, modifications made in position C-7 of fluoroquinolone structure greatly control their spectrum, potency, and pharmacokinetic profile [19, 28]. Thus, the advancement of quinolones based on the prototypical fluoroquinolone, ciprofloxacin, exhibiting a broad-spectrum and excellent safety profile is supported by ciprofloxacin being the most widely prescribed antibiotic [30, 31]. In fact, the WHO considers this antibiotic as an essential drug [30]. Newer-generation fluoroquinolones (such as sparfloxacin, gatifloxacin, moxifloxacin, gemifloxacin, grepafloxacin, and caderofloxacin) are built on ciprofloxacin's nucleus [32], due to the presence of optimal groups (Figure 3-1) [24]. Additionally, as result of the overuse of ciprofloxacin and drug-resistance, the fluoroquinolones in clinical use were expanded to these newer and more effective fluoroquinolones [23, 31]. However, these drugs still experience bacterial resistance which renders the search for the next-generation fluoroquinolones an imperative.

Consideration of the above SAR formed our basis to construct a new fluoroquinolone from the framework of ciprofloxacin. Modifications in position C-11 with the introduction of ethyl ester and piperazinyl amide groups, and of various substituents on the piperazine ring in position C-7 will be explored to generate new analogs (series 1 as depicted in Figure 3-2) with enhanced antibacterial potency paired with improved hydrosolubility.



Ciprofloxacin, **1**

Figure 3-2: Illustration of new ciprofloxacin analogs series 1 design.

Another popular and well-established strategy to assist in the swift development of novel drugs is molecular hybridisation [33]. Herein, a more effective hybrid molecule or chemical entity is developed by chemically and covalently linking two or more differing pharmacophores – exploiting the synergy that will be produced due to each pharmacophore's individual biological activity and mode of action [33, 34]. New agents based on the molecular hybridization concept and

constructed on the fluoroquinolone scaffold has been successfully used to counteract fluoroquinolone resistance through increased activity, and some such as MCB-3837 (oxazolidinone-fluoroquinolone hybrid), TNP-2092 (rifamycin-fluoroquinolone hybrid), and Cadazolid (oxazolidinone-fluoroquinolone hybrid) shown in Figure 3-3 have already entered the clinical trials against various WHO priority pathogens such as *Mycobacterium tuberculosis* and *Clostridium difficile* [3, 35-38].

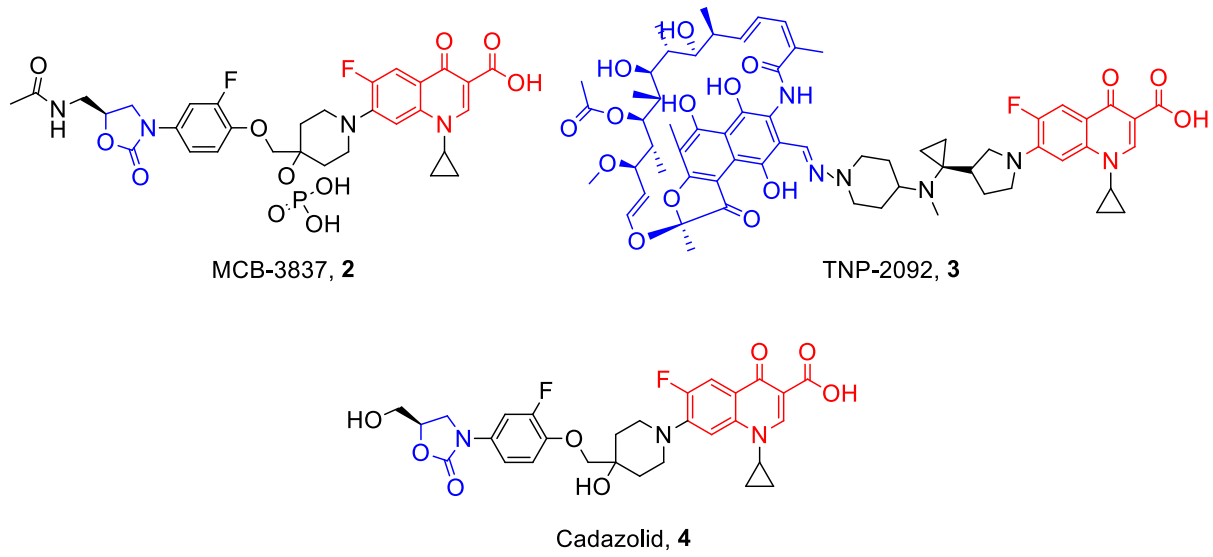


Figure 3-3: Fluoroquinolone-based hybrids in clinical trials.

Furthermore, 1,2,3-triazole is a five-membered heterocyclic motif [39] useful in human medicine. This remarkably versatile scaffold [40] forms part of numerous drug categories used to treat various human diseases including bacterial (e.g. TB), viral (e.g. HIV), parasitic (e.g. malaria), inflammation, diabetes, epilepsy, cancer, hypertension, anxiety, depression, Parkinson′s disease, and obesity [40]. Moreover, its favourable properties, such as high polarity and hydrogen bonding capability both features improving its aqueous solubility, stability and rigidity, all contribute to enhancing the biological activities of linked drugs [41-43]. Drugs currently on the market containing the triazole moiety include cefatrizine, tazobactam, cephalosporin (antibiotics) [42], TSAO (anti-HIV), carboxyamidotriazole (anti-cancerous), and radezolid [44] while I-A09 another derivate is currently undergoing trials for the use against TB [45] (Figure 3-4). Other research works that was also inspired by both moieties (i.e. ciprofloxacin and 1,2,3-triazole) to find innovative and effective antibiotics were explored and investigated against various gram-negative and gram-positive bacteria [28, 42, 46]. However, Xu *et al*. [47] were the only other research group to investigate a combination of these two moieties against the H37Rv strain of *Mtb*. They found a lipophilic hybrid **8** that was eight times more active than ciprofloxacin and with comparable

activity to rifampicin (MIC: 0.39 µg/ml). However, its cytotoxicity being more harmful to mammalian cells than ciprofloxacin did not warrant further development.

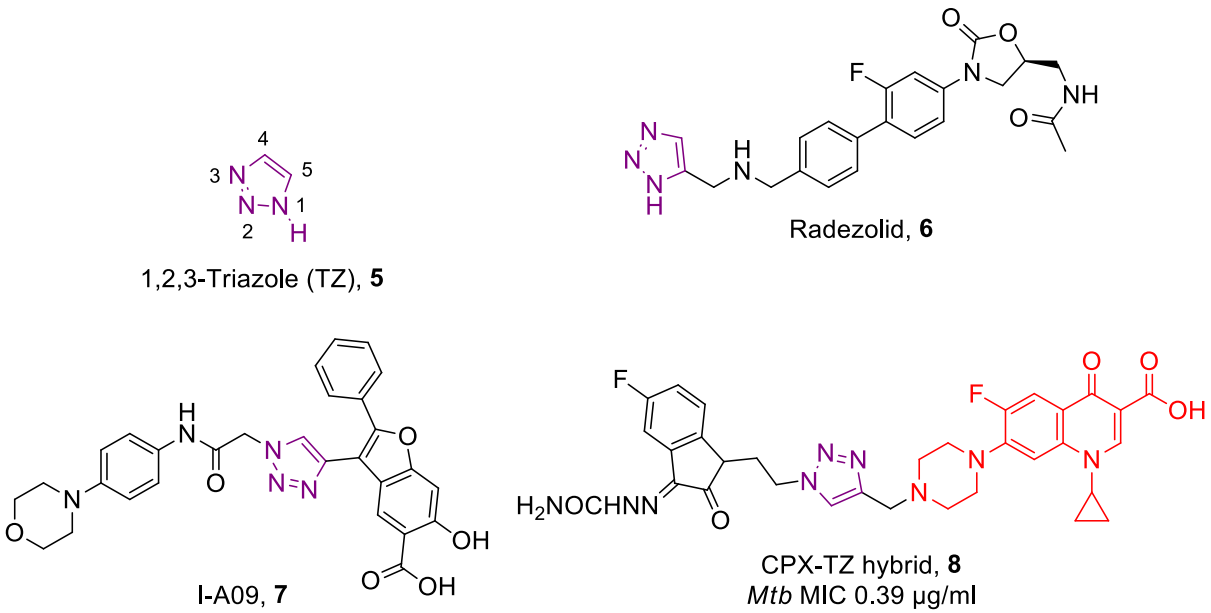


Figure 3-4: Important 1,2,3-triazole hybrids.

Based on the molecular hybridization strategy, and in the midst of addressing the drug-resistance issue through increased activity, 1,2,3-triazole will be conjoined to the standard anti-TB drug, ciprofloxacin, in position N-4 to generate hybrids (Series 2) as depicted in Figure 3-5. This add-on is expected to have a synergistic effect, accounting on both pharmacophores being active against TB. In fact, five- to six-membered heterocyclic substituents containing nitrogen has been determined to be ideal substituents for increased fluoroquinolone potency [48].

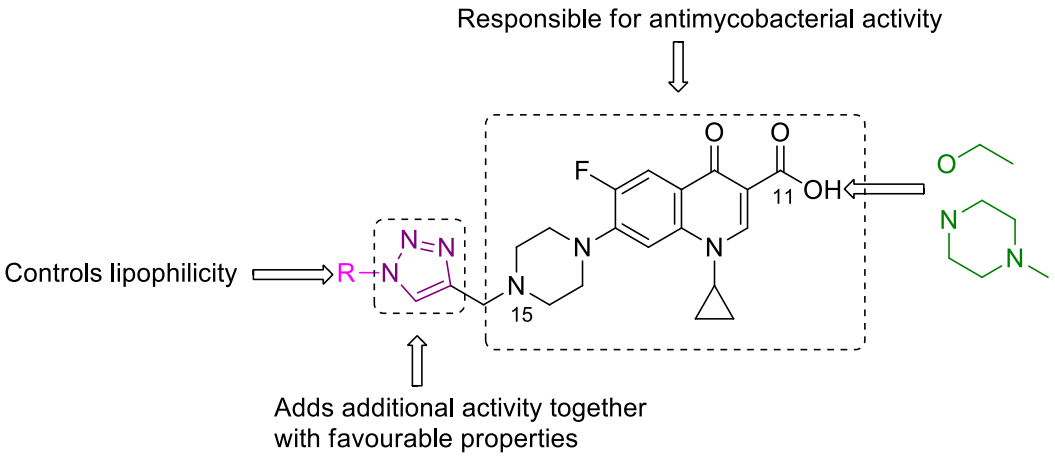


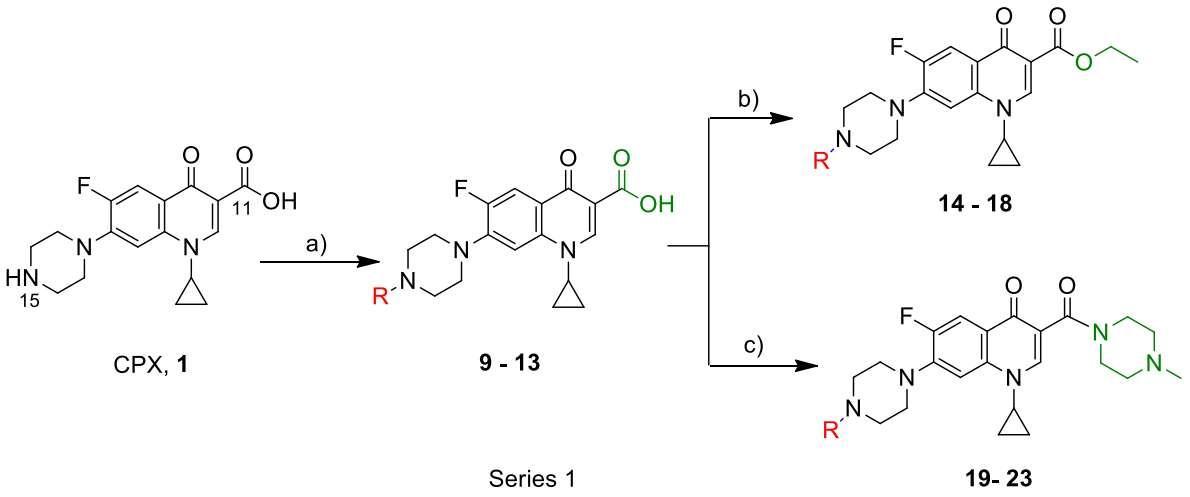
Figure 3-5: Illustration of target ciprofloxacin-triazole hybrids (Series 2).

3.2 Results and discussion

3.2.1 Chemistry

Two series of ciprofloxacin derivatives were synthesized using different synthetic routes. Each series was further divided into sub-series to highlight the difference of functional groups namely carboxylic acid, ethyl ester and 4-methylpiperazinyl amide in position 11 of ciprofloxacin CPX scaffold.

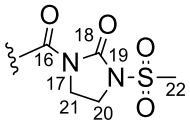
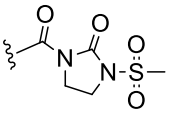
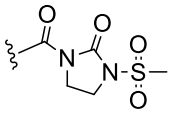
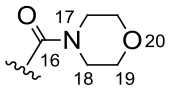
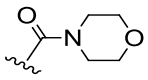
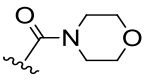
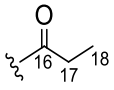
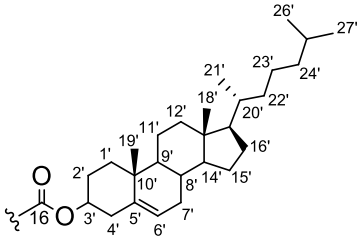
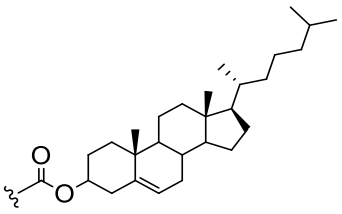
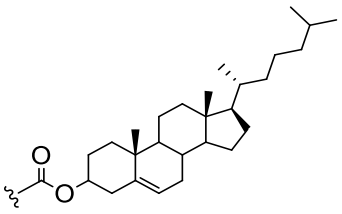
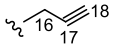
The ciprofloxacin analogs (Series 1) were synthesized as shown in scheme 1 starting from the commercial ciprofloxacin. The carboxylic acid sub-series was obtained in single amidation reaction of CPX at its N-15 of the piperazine ring using acyl/carbamoyl/sulfonyl chloride or cholesteryl chloroformate utilizing a literature method described by Qandil *et al*. [49] to afford anologs **9** – **12**. The nucleophile substitution using propargyl bromide resulted in analog **13**, all these analogs were isolated in moderate to excellent yields (44 – 100%) after recrystallization. These acids were converted to the ethyl esters **14** – **18** with poor yields (4 – 23%) and to the piperazinyl amides **19** – **23** more successfully with 39 – 95% yields using literature reported methods [50, 51]. The compounds were isolated following purification by silica gel chromatography. It is noteworthy indicating that following the procedure described by Qandil, Al-Zoubi, Al-Bakri, Amawi, Al-Balas, Alkatheri and Albekairy [49], synthesis of the alkyne intermediate **13** was optimized by changing the solvent system from dry dichloromethane to monoglyme-DMSO mixture (5:1) upon reflux to improve solubility which beneficially resulted in an increase of its yield from 54 to 66%.



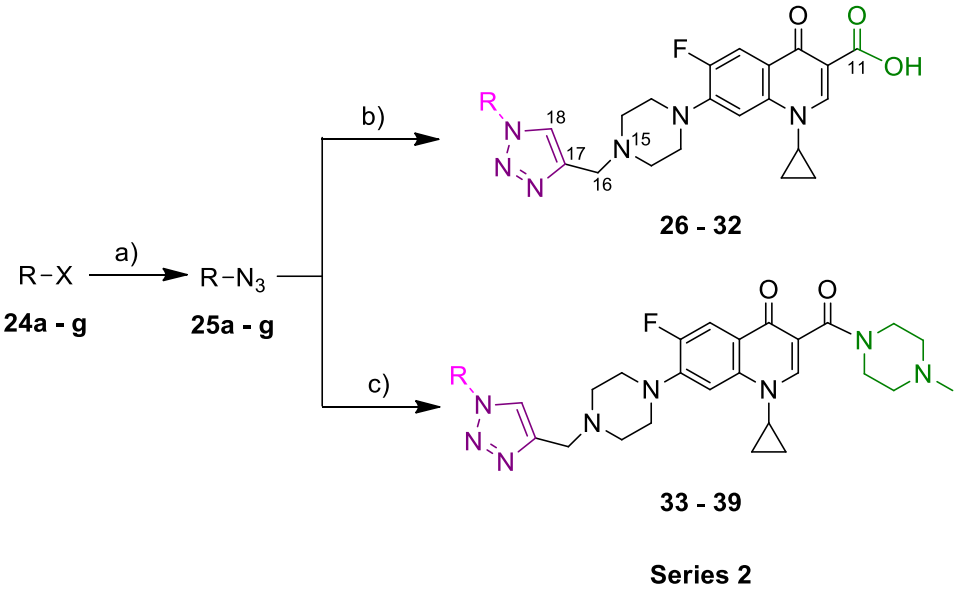
Scheme 1. Synthesis of series 1 analogs

Reagents and conditions: (a) **9** – **12**: acyl/carbamoyl chloride or cholesteryl chloroforamate (1.3 eq.), THF, TEA (2.6 eq.), 5 hr, rt ;**13**: propargyl bromide (2 eq), monoglyme:DMSO (5:1, v/v), DMAP (0.1 eq.), TEA (3 eq.), reflux, overnight; (b) **14** – **18**: absolute EtOH, DMAP (0.1 eq.), EDC (1 eq.), anhydrous DCM, 0 °C – rt, overnight; (c) **19** – **23**: methylpiperazine (2.4 eq.), HBTU (1.2 eq.), TEA (3 eq.), anhydrous DCM, 0 °C – rt, 4 hr.

Table 3-1: Synthesized CPX-analogs

	R (acids)		**R** (esters)		**R** (amides)
9		**14**		**19**	
10		**15**		**20**	
11		**16**		**21**	
12		**17**		**22**	
13		**18**		**23**	

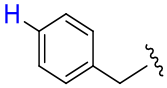
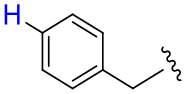
The Series 2 compounds were hybrids formed by hybridization of ciprofloxacin and triazole scaffolds. They were obtained in a two-step process. Firstly, the nucleophile substitution of benzyl bromides with sodium azide afforded the azido intermediates **25a - g** in good yields (70 – 100%) by adopting the synthetic route described by Howson [52]. Secondly, the azido intermediates were subjected to the Huisgen copper alkyne-azide cycloaddition ‘‘click’’ reaction in a stereoselective manner by employing the procedure described by Dixit *et al*. [53] with either alkynyl acid **13** to give the 1,4-disubstituted-1,2,3-triazole hybrids **26** - **32** or with alkyne **18** to afford **33** - **39** as depicted in scheme 2. All hybrids were isolated in poor to good yields (12 – 67%) after purification by precipitation in ethyl acetate followed by column chromatography on silica gel.

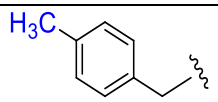
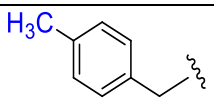
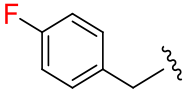
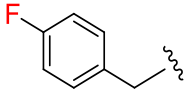
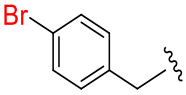
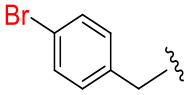
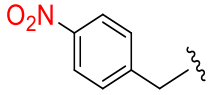
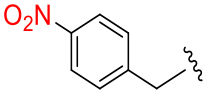
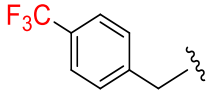
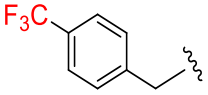
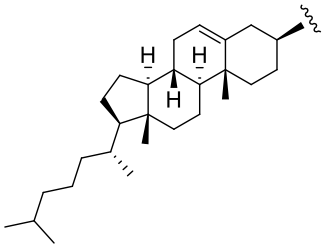
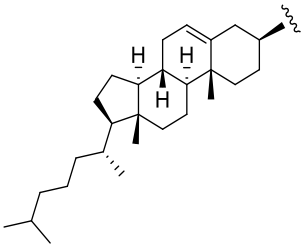


Scheme 2. Synthesis of series 2 ciprofloxacin-triazole hybrids

Reagents and conditions: (a) **25 a – f**: benzyl bromide (**24a – f**), NaN_3 (1.5 eq.), DMSO, 40 °C, overnight; **25 g**: cholesteryl halide (1eq.), NaN_3 (1.5 eq.), DMF, 100 °C, overnight; (b) **26** – **32**: Click chemistry. **13** (1 eq.), benzyl/cholesteryl azide (**25 a** – **g**) (2 eq.), DMF:H_2O (9:1, v/v), NaAsc (0.8 eq.), $CuSO_4 \cdot 5H_2O$ (0.2 eq.), rt, 24 **–** 30 hr; (c) **33** – **39**: Click chemistry. **23** (1 eq.), benzyl/cholesteryl azide (**25 a** – **g**) (2 eq.), DMF:H2O (9:1, v/v), NaAsc (0.8 eq.), $CuSO_4 \cdot 5H_2O$ (0.2 eq.), rt, 24 **–** 30 hr.

Table 3-2: Synthesized CPX-hybrids

	R		R
26		**33**	

27		**34**	
28		**35**	
29		**36**	
30		**37**	
31		**38**	
32		**39**	

The formation of the target compounds was confirmed by routine chemical structures characterization techniques such as IR, NMR, and HRMS. The ^{1}H NMR spectra of all title compounds were thoroughly examined for characteristic peaks evidence of the ciprofloxacin and triazole scaffolds. Two quartets found in the 1.3 – 1.0 ppm region and the multiplet in the region of 3.80 – 3.0 ppm were assigned to the cyclopropyl protons H-10 and H-9, respectively. The doublets *ca.* 8.0 and 7.5 ppm were attributed to the resonance of H-5, and H-8, respectively while the resonance of H-2 results in a singlet at 8.6 ppm. These were evidence of the presence of the fluoroquinolone pharmacophore. The former doublet is ascribed by the coupling of H-5 with F in position 6 in the aromatic system [49]. This distinctive splitting pattern was also seen in the ^{13}C NMR spectra where C-F coupling constants (*J*) for C-5, C-6, and C-7 were 23, 248, and 10 Hz which is in accordance with previous findings [49] and further confirm the presence of the fluoroquinolone pharmacophore in the synthesized compound.

Furthermore, evidence of the carboxylic acid functionality was confirmed in the structures of compounds **9** – **13** and **26** – **32** with the presence a singlet *ca.* 15.00 ppm 1H spectra assigned acidic proton H-11. The disappearance of this peak in the spectra of the esters **14** – **18**, and amides **19** -**23** and **33** - **39** corroborate the derivatization.

In spectra of the hybrids, the resonance of the aromatic proton of the triazole ring results in a singlet at *ca.* 5.5 ppm while the singlet due to H of the methylene linker appears in the 3.8 – 2.8 ppm region.

The IR-spectra of all compounds were also inspected for the presence of characteristic absorptions, allowing for the identification of functional groups. Evidence of carbonyl C=O was confirmed with strong stretch *ca*. 1620 – 1700 cm^{-1} and variable broad O-H stretching *ca*. 2842 cm^{-1} for acids.

3.2.2 Physiochemical properties

The n-octanol/water partition coefficient (logP) is a key parameter used for the measurement of the balance between hydrophilicity and lipophilicity; giving insight into the transport characteristics of a chemical across biomembranes through passive diffusion [54]. LogP values between 1 and 5 are usually targeted while values between 1 and 3 are ideal [33].
In Series 1, all amides **19** – **23** exhibited clogP values lower than those of corresponding acids **9** – **13** which in turn possess lower clogP than the esters **14** – **18**. The lipophilicity order was thus as follows amides< acids<esters. Conversely, the acids **26** – **32** in Series 2 exhibit clogP values lower than amides **33** – **39** resulting in the order acid<amide. For both series, cholesteryl moiety containing derivatives *viz*.**12**, **17**, **22**, **32**, and **39**, show poor drug-like properties with clogP values higher than 8.90. However, despite this abysmal physiochemical property for favourable transport across of biomembranes, hybrid **32** was the most active derivative in that group indicating other means aside lipophilicity *Mtb* might be involved in the cellular uptake of this compounds into *Mtb*.

3.2.3 Biological activities

For a novel drug to advance to use in humans, a chain of preclinical studies including *in vitro* assays and *in vivo* testing need to be concluded.

There exist an abundance of assay systems and conditions available to study the growth of *M. tuberculosis* in the presence of drug candidates [55]. However, the *mycobacterium* growth inhibitory potential of the synthesized compounds was assessed using two assays namely GFP (Green Fluorescent Protein-expression) assay for the analogs (Series 1) and Microplate Alamar Blue Assay (MABA) for the hybrids Series 2. Both assays used the Middlebrook 7H9 Broth base as tubercle bacilli growth medium supplemented with glucose (GLU), enriched with ADC (albumin-dextrose-catalase) and additionally Tween 80 (TW) as surfactant for the screening of the hybrids. The antimycobacterial activity was assessed against the human virulent H37Rv strain. The minimum concentrations that inhibit the growth of 90% of mycobacteria, expressed as MIC_{90} are summarized in Table 3-3 for Series 1 and Table 3-4 for Series 2 alongside isoniazid (INH), ethambutol (ETB) and rifampicin (RIF) antitubercular standards.

Furthermore, three mammalian cell lines *viz.* normal human fetal lung fibroblasts HFLF WI-38, the human embryonic kidney HEK-293 and the Chinese hamster ovarian CHO cells were used to determine the cytotoxicity of the compounds alongside the cytotoxic drug emetine as reference.

Table 3-3: *In vitro* antimycobacterial activties ciprofloxacin analogs 14 – 23 against H37Rv strain using GFP assay in 7H9 GLU ADC medium.

Compd	ClogP[a]	**Antimycobacterial activity** **MIC_{90}** (µM)[b]	**Cytotoxicity**, **IC_{50}** (µM)[c]	**Selectivity index**, SI[d]
			HFLB WI-38	**SI**
ETB		0.014	nd	>3 448
RIF		0.010	nd	nd
CPX, **1**	1.55	0.83	>100	>120
9	0.24	2.46	>100	>41
10	1.06	1.64	>100	>61
11	1.86	1.64	>100	>61
12	9.41	>125	>100	-
13	2.16	2.97	>100	>33
14	0.98	>125	>100	-
15	1.79	>125	>100	-
16	2.6	42.2	>100	>2
17	10.15	>125	>100	-
18	2.6	1	>100	>7
19	0.09	>125	>100	-
20	0.55	>125	>100	-
21	1.35	>125	>100	-
22	9.89	>125	>100	-
23	1.65	>125	>100	-
EM	nd	nd	0.5	-

[a] cLogP values calculated using MarvinSketch Version 17.28; [b]compounds screened in media: 7H9 GLU ADC; [c] WI38 cell line of normal human fetal lung fibroblast from ECACC; [d] Selectivity Index (SI) = WI38/H37Rv.

In the analogs Series, all the acids except the cholesteryl derivative **12** possessed antimycobacterial activity while the ethyl esters series was partially active (2 out 5 compounds) and the piperazinyl amides were completely inactive. The acids were the most active of all compounds tested against the *M. tuberculosis* H37Rv strain. Derivatives **10** and **11** with MIC_{90} 1.6 µM were the most active in the analog series although 2-fold less potent than the parent CPX. In Series 2, the cholesteryl containing hybrids was the best performer with activity MIC_{90} 2 µM

comparable to that of ciprofloxacin. The analog acids **9** – **11** were more active than the hybrid acids **26** – **32**. Overall, no derivative either analog or hybrid was found to possess potency comparable or higher than any of the reference drug of the study.

Table 3-4: *In vitro* antimycobacterial activities of ciprofloxacin analogs 14 – 23 against H37Rv strain using GFP assay in 7H9 GLU ADC TW medium.

Compd	ClogP[a]	**Antimycobacterial H37Rv activities**, **MIC_{90}** (µM)[b]	**Cytotoxicity**, **IC_{50}** (µM)		**Selectivity index**, **SI**	
		7H9 GLU ADC TW	CHO[c]	HEK-293[d]	SI_1[e]	SI_2[f]
INH	nd	0.97	nd	nd	-	-
RIF	nd	0.01	nd	nd	-	-
CPX, 1	1.55	1.78	nd	nd	-	-
26	3.0	7.65	>100	>100	13	13
27	3.51	15.16	nd	nd	-	-
28	3.14	3.69	>100	>100	27	27
29	3.77	4.05	>100	80	25	20
30	2.94	8.06	>100	>100	12	12
31	3.88	11.97	nd	nd	-	-
32	8.91	2.06	16	12	17	13
33	3.51	>125	nd	nd	-	-
34	4.03	>125	nd	nd	-	-
35	3.66	>125	nd	nd	-	-
36	4.28	>125	nd	nd	-	-
37	3.45	>125	nd	nd	-	-
38	4.39	>125	nd	nd	-	-
39	9.42	>125	nd	nd	-	-
EM	nd	nd	0.3	0.3	-	-

[a] cLogP values calculated using MarvinSketch Version 17.28; [b] compounds screened in media: 7H9 GLU ADC TW; [c] CHO cell line of the epithelial of chinese hamster ovary; [d] HEK-293 cell line of human embryonic kidney cells origin; [e] SI = CHO/H37Rv; [f] SI = HEK-293/H_{37}Rv.

The C-11 methyl piperazine substitution combined with substitution at N-15 of CPX resulted in completely *Mtb* inactive amides **19** – **23** and **33** – **39** despite improved either hydrophilicity or lipophilicity in comparison to the corresponding acids **9** – **13** and **26** – **32**, respectively. Similarly, the combination of substitution at C-11 with ethyl ester and N-15 substitution afforded partially inactive esters sub-series in comparison to the active acid sub-series. It may thus be concluded that the C-3 bound carboxylic acid functional group is a structural

prerequisite to ensure *Mtb* activity of fluoroquinolone scaffold which is in accordance with the extensive literature on this issue [18, 19, 21, 56, 57]. Indeed, CPX-analogs **9** – **13**, and hybrid compounds **26** – **32** displayed antimycobacterial activity, however only marginal when compared to the parent drug CPX.

Furthermore, only compounds **12**, **17**, **22**, **32**, **39** which contain bulky cholesteryl moiety, were off the target range (1-5) of cLogP value and thus were expected to be poorly transported into the mycobacterium through passive diffusion. However, the mycobacterium cell wall is lipophilic in nature [58] rendering lipophilicity an imperative consideration in the design and activity of novel antimycobacterial molecules. Therefore, attachment of the bulky cholesteryl moiety at C-7 may result in enhancement of activity. Cholesterol is also reported to be transported into *Mtb* by active diffusion provided by enzyme *mce4*-transporter [59] where it is utilized as a carbon source and is thus the virulence driving factor behind *Mtb* as demonstrated by Brzostek, Dziadek, Rumijowska-Galewicz, Pawelczyk and Dziadek [60]. Thus, the conjugation of this dietary molecule to the anti-TB active ciprofloxacin may be expected to yield active derivatives. However, the inactivity of most of these cholesteryl containing derivatives suggest that neither lipophilicity nor *mce4* transporter controlled the cellular uptake of these compounds into *Mtb*.

Ciprofloxacin has demonstrated poor aqueous solubility in the fluoroquinolone class of drugs which warrants improvement. The clogP values of synthesized compounds were mostly found to be in the 2 – 5 range of favourable drug-likeness. Of note, these values are theoretical values (i.e. determined using MarvinSketch) and do not necessarily represent the actual physical property that is the aqueous solubility. Indeed, ciprofloxacin has in theory a clogP value of 1.55 though in practice known for its lowest in-class solubility [61]. In both series the functional group at C-11 determines the solubility of the derivative. Indeed, in the analogs series, for the same C-7 substituent, the lipophilicity is in the order amide<acid<ester while the activity is in the order amide<ester<acid. In the hybrids series the lipophilicity was acid<amide and the activity was inversely amide<acid order. Therefore, though, a more lipophilic derivative is expected to be more antimycobacterial active [62], the opposite was observed in this study indicating that structural specificity rather than the physical feature (lipophilicity) governed the activity of these derivatives.

In Series 2, the hybrids were *para*-benzene substituted with either electron donating groups (EDG) (**26** and **27**) or electron withdrawing groups (EWG) (**28** – **31**). The EDGs were H and CH_3 in the order H<CH_3. The EWGs were F, Br, NO_2, CF_3 with the increasing electronic effect order F<Br<NO_2<CF_3. Analysis of the mycobacterial activities showed **26** to be more active that **27** indicating that the presence of a less electron donating substituent increases the activity. With

the EWG containing hybrids, the ascending order of activity was **31**, **30**, **29** and **28** therefore the activity increased with the decrease in electronic effect in the group. Overall, the antimycobacterial activity of these acidic ciprofloxacin-triazole non-cholesteryl containing hybrids was influenced by the electronic effect of the substituent on the phenyl ring.

The active analogs **9**, **10**, **11** and **13**, and the hybrids **28**, **29**, **30** and **32** have promising anti-mycobacterial activities (MIC_{90}<10 µM) and were found to be nontoxic to all three mammalian cell lines as shown with most them possessing IC_{50} values >100 µM as compared to 0.5-0.3 µM of emetine. These were also selective in their antibacterial actions as seen from the SI values greater than 10. Overall, they may stand as validated hits based on these cellular potency and cytotoxicity criteria [63] to be further investigated in the search for new antitubercular agents.

3.3 Conclusion

Two Series of ciprofloxacin derivatives *viz.* 15 analogs and 14 novel ciprofloxacin-triazole hybrids featuring carboxylic acid, ethyl ester or 4-methylpiperazinyl amide group linked to carbon C-3 of the fluoroquinolone scaffold were synthesized through various synthetic routes including amidation, esterification, nucleophilic substitution reactions and Click chemistry. The derivatives were evaluated *in vitro* for anti-mycobacterial activity against human virulent *Mtb* H37Rv strain using GFP assay in Middlebrook 7H9 broth media. The cytotoxicity was also assessed using three mammalian cell lines, normal human fetal lung fibroblasts (HFLF WI-38), the human embryonic kidney (HEK-293) and the Chinese hamster ovarian (CHO). The acid derivatives were found to be active while the amides were inactive. The esters showed mixed activity profiles. The highly lipophilic cholesteryl moiety containing derivatives were mostly inactive. Analysis of the biological data also revealed the activity to be structure specific in general. However, it was governed by the electronic effect of the substituent on the phenyl ring in the non-cholesteryl containing hybrids sub-series. The less electron donating or withdrawing the substituent the more anti-*Mtb* active the hybrid.

The analogs **9**, **10**, **11** and **13**, and the hybrids **26**, **28**, **29**, **30** and **32** possessed antimycobacterial activities with MIC_{90} values lower than 10 µM, and cytotoxicity selectivity indices SI values greater than 10 lend themselves as validated hits for further development.

3.4 Materials and methods

3.4.1 Materials

Solvents were obtained from either ACE Chemicals– (e.g. acetonitrile; DCM dichloromethane; diethyl ether, ethyl acetate, methanol, monoglyme) or Sigma-Aldrich South Africa (e.g. DMF dimethylformamide, DMSO dimethyl sulfoxide, THF tetrahydrofuran), respectively. For inert reactions, DCM was distilled over calcium hydride and stored over 3 Å molecular sieves. Reagents, such as absolute ethanol (abs EtOH), ammonium chloride (NH_4Cl), benzyl bromide, ciprofloxacin (CPX), copper sulfate pentahydrate ($CuSO_4 \cdot 5H_2O$), 4-dimethylaminopyridine (DMAP), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), *N,N,N′,N′*-tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate, propargyl bromide (HBTU), *p*-bromobenzyl azide, *p*-fluorobenzyl azide, *p*-methylbenzyl azide, *p*-nitrobenzyl azide, *p*-trifluorobenzyl azide, methylpiperazine (mPz), sodium ascorbate (NaAsc), sodium azide (NaN_3), sodium bicarbonate ($NaHCO_3$), trimethylamine (TEA) were all acquired from Sigma-Aldrich South Africa and cholesteryl chloride and cholesteryl chloroformate from Merck South Africa.

3.4.2 General procedures

The ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance™ III 600 spectrometer at a frequency of 600 MHz and 150.913 MHz, respectively, in DMSO-d_6 or $CDCl_3$-*d.* Chemical shifts are reported in parts per million δ (ppm), with the residual protons of the solvent as reference. The splitting pattern abbreviations are as follows: singlet (s), doublet (d), doublet of doublet (dd), doublet of doublet of doublets (ddd), doublet of triplets (dt), doublet of quartets (dq), triplet (t), triplet of doublets (td), triplet of triplets (tt), quartet of doublets (qd) and multiplet (m).

High resolution mass spectrometry (HRMS) was recorded on a Bruker MicroTOF Q II mass spectrometer, equipped with an APCI or an ESI source, set at 200°C or 180°C, respectively, using Bruker Compass DataAnalysis 4.0 software. A full scan from 50 - 1500 m/z was performed at a capillary voltage of 4500 V, an end plate offset voltage of -500 V, with the nebulizer set at 1.6 Bar and 0.4 Bar, respectively, and a collision cell RF voltage of 100 Vpp.

Infrared (IR) spectra were recorded on a Bruker Alpha-P FTIR instrument. Thin layer chromatography (TLC) was performed, using silica gel plates ($60F_{254}$), obtained from Merck (Johannesburg, South Africa). Column chromatography was performed, using MN silica gel 60, 70 - 230 mesh ASTM, supplied by Macherey-Nagel (Germany).

High performance liquid chromatography (HPLC) analysis of most 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 1ml/min. The concentration of acetonitrile in the mobile phase was linearly increased over a period of 5 minutes to a final concentration of 85%. The time allowed for equilibration between runs was 5 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.

4.1.1 Ciprofloxacin analogs 9 – 23

*Acids **9 – 13*** were prepared in accordance with the general procedure depicted in Scheme 1 and described as follows:

To a flask containing ciprofloxacin **1** (9.05 mmol, 3.0 g, 1 eq.), a mixture of THF (100 ml) and TEA (2.6 eq.) were combined and subsequently the appropriate alkyl/acetyl halide/cholesteryl chloroformate (1.3 eq.) was added. The reaction mixture was stirred at room temperature for 4 – 5 hours and monitoring of reaction progress using TLC. The solvent was evaporated, the residue suspended in DCM (250 ml) and washed successively with brine (50 ml). The resulting organic layer was then dried over magnesium sulphate ($MgSO_4$), evaporated under reduced pressure and recrystallized from acetonitrile.

4.1.1.1 1-Cyclopropyl-6-fluoro-7-(4-(3-(methylsulfonyl)-2-oxoimidazolidine-1-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (9)

The reaction of CPX (9.05 mmol, 3.0 g, 1 eq.), TEA (23.54 mmol, 3.28 ml) and 3-chlorocarbonyl-1-methanesulfonyl-2-imidazolidinone (10.86 mmol, 2.46 g, 1.2 eq.) yielded compound **9**. Light orange crystals; yield: 3.19 g (68%); m.p. 266.1 – 267.8 °C; IR ν_{max}: 2928, 1729, 1668, 1629, 1583 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 15.14 (s, 1H, C-11), 8.62 (s, 1H, H-2), 7.86 (d, J = 13.1 Hz, 1H, H-5), 7.55 (d, J = 7.1 Hz, 1H, H-8), 3.84 (t, J = 7.7 Hz, 2H, H-21), 3.80 – 3.78 (m, 1H, H-9), 3.76 – 3.70 (m, J = 7.7 Hz, 2H, H-20), 3.63 (t, J = 4.3 Hz, 4H, H-13), 3.37 (t, J = 4.2 Hz, 4H, H-14), 2.47 (s, 3H, H-22); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 176.37 (C-4), 165.95 (C-11), 153.79 (C-16), 152.30, 151.45 (C-18), 148.07 (C-2), 144.85 (C-7), 111.03 (C-5), 106.71 (C-8), 39.80 (C-22), 35.93 (C-9), 34.34 (C-14), 7.64 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 522.1460 (Calcd for $C_{22}H_{25}FN_5O_7S$: 522.1459); Purity (HPLC): 97%.

4.1.1.2 1-Cyclopropyl-6-fluoro-4-oxo-7-(4-((1-(4-(trifluoromethyl)benzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (10)

Fluffy off-white crystals; yield: 3.5 g (87%); m.p 266.6 – 266.7 °C. IR v_{max}: 2842, 1720, 1648, 1627 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 15.17 (s, 1H, C-11), 8.64 (s, 1H, H-2), 7.89 (d, *J* = 13.1 Hz, 1H, H-5), 7.56 (d, *J* = 7.4 Hz, 1H, H-8), 3.83 – 3.76 (m, 1H, H-9), 3.57 (t, *J* = 4.2 Hz, 4H), 3.38 (t, *J* = 5.4 Hz, 4H, H-19), 3.30 (t, *J* = 5.0 Hz, 4H, H-18), 3.18 (t, *J* = 4.6 Hz, 4H), 1.30 (q, *J* = 7.0 Hz, 2H, H-10b), 1.16 (q, *J* = 4.2 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 176.40 (C-4), 165.95 (C-11), 162.86 (C-16), 153.04 (C-6), 148.07 (C-2), 145.13 (C-7), 118.86 (C-3), 110.99 (C-5), 106.72 (C-8), 65.94 (C-13), 49.26 (C-18), 46.90 (C-19), 35.92 (C-9), 7.62 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 445.1868 (calcd for $C_{22}H_{26}FN_4O_5$: 445.1887); Purity (HPLC): 98%.

4.1.1.3 1-Cyclopropyl-6-fluoro-4-oxo-7-(4-propionylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (**11**)

Light yellow crystals; yield: 3.51 g (100%); m.p. 226.1 – 228.7 °C; IR v_{max}: 2886, 1723, 1664, 1627, 1613 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 15.16 (s, 1H, H-11), 8.63 (s, 1H, H-2), 7.88 (d, *J* = 13.1 Hz, 1H, H-5), 7.54 (d, *J* = 7.4 Hz, 1H, H-8), 3.81 – 3.75 (m, 1H, H-9), 3.64 (t, *J* = 7.8 Hz, 4H, H-13), 2.47 (t, *J* = 1.7 Hz, 4H, H-14), 2.36 (q, *J* = 7.4 Hz, 2H, H-17), 1.29 (q, *J* = 6.0 Hz, 2H, C-10b), 1.15 (q, *J* = 4.9 Hz, 2H, C-10a), 0.99 (t, *J* = 7.4 Hz, 3H, H-18); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 176.40 (C-4), 171.58 (C-16), 165.96 (C-11), 148.10 (C-2), 145.03 (C-7), 111.03 (C-5), 106.72 (C-8), 49.65 (C-13), 44.45 (C-14), 35.92 (C-9), 25.52 (C-17), 9.39 (C-18), 7.62 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 388.1645 (calcd for $C_{20}H_{23}FN_3O_4$: 388.1673).

4.1.1.4 1-Cyclopropyl-7-(4-((((3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl)oxy)carbonyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**12**)

The reaction of CPX (6.04 mmol, 2.0 g, 1eq.), TEA (6.64 mmol, 0.93 ml, 1.1 eq.) and cholesteryl chloroformate (6.64 mmol, 2.98 g, 1.1 eq.) yielded compound **12**. Light yellow crystals; yield: 1.97 g (44%); IR v_{max}: 2934, 2867, 1701, 1626, 1242 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 14.99 (s, 1H, H-11), 8.77 (s, 1H, H-2), 8.14 (d, *J* = 2.4 Hz, 1H, H-5), 7.31 (d, *J* = 7.0 Hz, 1H, H-8), 5.39 (t, *J* = 2.7 Hz, 1H, H-6'), 4.59 – 4.53 (m, 1H, H-3'), 3.71 (t, *J* = 6.6 Hz, 4H, H-14), 3.43 – 3.39 (m, 1H, H-9), 3.28 (t, *J* = 4.7 Hz, 4H, H-13), 1.03 (s, 3H, H-19'), 0.86 (dd, *J* = 6.6, 2.8 Hz, 6H, H-26', H-27'), 0.67 (s, 3H, H-18); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 177.28 (C-4), 167.15 (C-11), 166.14 (C-16), 139.20 (C-5'), 122.84 (C-6'), 113.37 (C-5), 104.99 (C-8), 56.80 (C-14'), 56.25 (C-

17'), 50.12 (C-9'), 42.44 (C-13), 22.70 (C-26', C-27'), 19.50 (C-19'), 11.99 (C-18'), 8.45 (C-10); HRMS (ESI) *m/z*: $(M)^+$ 744.4733 (calcd for $C_{45}H_{62}FN_3O_5$: 744.4746).

4.1.1.5 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(prop-2-yn-1-yl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (**13**)

CPX (9.05 mmol, 3.0 g, 1 eq.) and the solvent mixture, monoglyme/DMSO (120 ml, 5:1, v/v) were combined in a 150 ml flask, then left to stir a few minutes before adding TEA (27.15 mmol, 3.8 ml, 3 eq.), DMAP (0.91 mmol, 0.11 g, 0.1 eq.) and lastly propargyl bromide (18.1 mmol, 2.7 ml, 2eq.). The reaction mixture was refluxed overnight and filtered. The solvent, monoglyme, was removed under reduced pressure to give the residue in DMSO. This was suspended in DCM (200 ml), washed with NH_4Cl (4 x 50 ml) and then water (4 x 50 ml). The resulting organic layer was dried over Mg_2SO_4, evaporated and crystallised from acetonitrile.

Gold crystals, yield: 1.98 g (66%); IR ν_{max}: 3250, 2848, 2101, 1732, 1675, 1626 cm^{-1}; ^1H NMR (600 MHz, CDCl3) δ (ppm): 15.01 (s, 1H, C-11), 8.74 (s, 1H, H-2), 7.98 (d, *J* = 13.1 Hz, 1H, H-5), 7.36 (d, *J* = 7.1 Hz, 1H, H-8), 3.57 – 3.53 (m, 1H, H-9), 3.41 (d, *J* = 2.5 Hz, 2H, H-16), 3.39 (t, *J* = 4.6 Hz, 4H, H-14), 2.81 (t, *J* = 4.9 Hz, 4H, H-13), 2.31 (t, *J* = 2.4 Hz, 1H, H-18), 1.39 (q, *J* = 6.6 Hz, 2H, H-10b), 1.20 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 177.23 (C-4), 167.17 (C-11), 153.80 (C-6), 147.55 (C-2), 145.91 (C-7), 112.55 (C-5), 104.98 (C-8), 78.32 (C-17), 73.85 (C-18), 51.57 (C-13), 49.81 (C-14), 46.98 (C-16), 35.42 (C-9), 8.36 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 370.1529 (calcd for $C_{20}H_{21}FN_3O_3$: 370.1567).

Esters **14** – **18** were prepared in accordance with the general procedure, as depicted by Scheme 1 and described as follows:

Acid analogs **9** – **13** (1 eq.) was dissolved in anhydrous DCM (35 ml) on an ice bath, respectively. DMAP (0.1 eq.) and abs. EtOH (4 eq.) were added soon after, whilst a solution of EDC.HCl (in 6ml of DCM) was added slowly under N_2 over 20 minutes Stirring was continued for 24 – 48 hours until room temperature. The reaction mixture was diluted (DCM, 250 ml), washed with Na_2HCO_3 (3 x 25 ml), dried over $MgSO_4$, filtered and the organic solvent removed under reduced pressure. The resulting residue was purified by column chromatography on silica gel eluting with DCM/MeOH (19:1 v/v).

4.1.1.6 ethyl 1-cyclopropyl-6-fluoro-7-(4-(3-(methylsulfonyl)-2-oxoimidazolidine-1-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**14**)

Light yellow powder; yield: 0.05 g (4%); m.p.: 161.6 – 161.7 °C; IR ν_{max}: 2981, 2906, 1740, 1688, 1656 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.40 (s, 1H, H-2), 7.73 (d, *J* = 13.2 Hz, 1H, H-5), 7.43 (d, *J* = 7.4 Hz, 1H, H-8), 4.19 (q, *J* = 7.1 Hz, 2H, C-11-C*H*$_2$-), 3.84 (t, *J* = 7.7 Hz, 2H, H-20), 3.74 (t, *J* = 7.7 Hz, 2H, H-21), 3.63 (t, *J* = 10.1, 6.0 Hz, 5H, H-9, H-14), 3.30 (t, J = 4.8 Hz, 4H, H-13), 2.48 (s, 3H, H-22), 1.25 (t, *J* = 7.1 Hz, 3H, C11-C*H*$_2$-), 1.23 (q, *J* = 1.3 Hz, 1H, H-10b), 1.08 (q, *J* = 3.9 Hz, 1H, H-10a); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 171.58 (C-4), 164.45 (C-11), 152.45 (C-6), 152.25 (C-16), 151.43 (C-18), 148.13 (C-2), 111.66 (C-5), 109.28 (C-3), 106.59 (C-8), 59.79 (C11-O-*C*H$_2$-), 49.43 (C-13), 41.37 (C-20), 41.18 (C-21), 34.77 (C-9), 14.31 (C11-O-CH$_2$-*C*H$_3$), 7.58 (C-10). HRMS (ESI) *m/z*: $(M+H)^+$ 550.1741 (calcd for $C_{24}H_{29}FN_5O_7S$: 550.1772); Purity (HPLC): 92%.

4.1.1.7 ethyl 1-cyclopropyl-6-fluoro-7-(4-(morpholine-4-carbonyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylate (**15**)

Cream coloured powder; yield: 0.24 g (23%); m.p.: 218.8 – 221.7 °C; IR ν_{max}: 2978, 2902, 2849, 1727, 1654, 1618 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.50 (s, 1H, H-2), 8.01 (d, *J* = 13.1 Hz, 1H, H-5), 7.25 (d, *J* = 7.7 Hz, 1H, H-8), 4.37 (q, *J* = 7.1 Hz, 2H, C11-C*H*$_2$), 3.70 (t, *J* = 4.6 Hz, 4H, H-13), 3.50 (t, *J* = 4.7 Hz, 4H, H-19), 3.42 – 3.38 (m, 1H, H-9), 3.32 (t, *J* = 4.8 Hz, 4H, H-18), 3.24 (t, *J* = 5.0 Hz, 4H, H-13), 1.39 (t, *J* = 7.1 Hz, 3H, C11-CH$_2$-C*H*$_3$), 1.31 (q, *J* = 6.9 Hz, 2H, H-10b), 1.13 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.17 (C-4), 165.90 (C-11), 163.70 (C-16) 153.48 (C-6), 148.32 (C-2), 113.52 (C-5), 105.16 (C-8), 61.02 (C11-O-*C*H$_2$-), 34.62 (C-9), 14.56 (C11-O-CH$_2$-*C*H$_3$), 8.30 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 473.2186 (calcd for $C_{24}H_{30}FN_4O_5$: 473.2195); Purity (HPLC): 95%.

4.1.1.8 ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(4-propionylpiperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate (**16**)

White powder; yield: 0.68 g (6%); m.p.: 180.6 – 183.3 °C; IR ν_{max}: 3092, 2940, 1713, 1652, 1617, 1591 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.49 (s, 1H, H-2), 7.99 (d, *J* = 13.1 Hz, 1H, H-5), 7.24 (d, *J* = 7.0 Hz, 1H, H-8), 4.36 (q, *J* = 7.1 Hz, 2H, C11-C*H*$_2$-), 3.44 – 3.38 (m, 1H, H-9), 2.41 (q, *J* = 7.4 Hz, 2H, H-17), 1.39 (t, *J* = 7.1 Hz, 3H, H-18), 1.31 (q, *J* = 6.8 Hz, 2H, H-10b), 1.18 (t, *J* = 7.4 Hz, 3H, C11-CH$_2$-C*H*$_3$), 1.13 (q, *J* = 6.6 Hz, 1H, H-10a); ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 173.12 (C-4), 172.53 (C-16), 165.81 (C-11), 153.41 (C-6), 148.33 (C-2), 113.53 (C-5), 110.62 (C-3), 105.22 (C-8), 61.00 (C-17), 34.61 (C-9), 26.57 (C11-O-*C*H$_2$), 14.55 (C-18), 9.56

(C11-O-CH_2-CH_3), 8.28 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 416.2020 (calcd for $C_{22}H_{27}FN_3O_4$: 416.1986); Purity (HPLC): 87%.

4.1.1.9 ethyl 1-cyclopropyl-7-(4-((((3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl)oxy)carbonyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylate (**17**)

Light yellow solid; yield: 0.04 g (4%); IR ν_{max}: 3425, 2930, 2866, 1725, 1055 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.50 (s, 1H, H-2), 8.07 (d, *J* = 13.0 Hz, 1H, H-5), 7.27 (d, *J* = 5.2 Hz, 1H, H-8), 5.35 (t, *J* = 2.5 Hz, 1H, H-6'), 4.59 – 4.52 (m, 1H, H-9), 4.22 (q, *J* = 11.3, 5.9 Hz, 2H, C11-O-CH_2-), 3.69 (t, *J* = 4.7 Hz, 4H, H-14), 3.55 – 3.49 (m, 1H, H-3'), 3.22 (t, *J* = 3.9 Hz, 4H, H-13), 1.01 (s, 3H, H-19'), 0.86 (dd, J = 6.6, 2.8 Hz, 6H, H-26', H-27'), 0.67 (s, 1H, H-18'). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 140.91 (C-5'), 121.87 (C-6'), 71.95 (C-3'), 56.91 (C-14'), 56.29 (C-17'), 50.27 (C-9'), 42.46 (C-13'), 22.71 (C-26', C-27'), 19.55 (C-19'), 12.01 (C-18'). HRMS (ESI) *m/z*: (M) 772.5026 (calcd for $C_{47}H_{67}FN_3O_5$: 772.5065); Purity (HPLC): 85%.

4.1.1.10 ethyl 1-cyclopropyl-6-fluoro-4-oxo-7-(4-(prop-2-yn-1-yl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylate (**18**)

Gold powder; yield: 0.04 g (4%); m.p.: 218.5 – 220.5 °C; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.52 (s, 1H, H-2), 7.98 (d, *J* = 13.2 Hz, 1H, H-5), 7.24 (d, *J* = 4.3 Hz, 1H, H-8), 4.90 (d, *J* = 2.4 Hz, 2H, C-11-CH_2-), 3.45 – 3.42 (m, 1H, H-9), 3.39 (d, *J* = 2.4 Hz, 2H, H-16), 3.31 (t, *J* = 4.6 Hz, 4H, H-13), 2.79 (t, *J* = 4.9 Hz, 4H, H-14), 2.48 (t, *J* = 2.4 Hz, 1H, H-18), 1.31 (q, *J* = 7.1 Hz, 2H, H-10b), 1.14 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 175.84 (C-4), 172.98 (C-11), 153.54 (C-6), 148.62 (C-2), 113.43 (C-5), 105.01 (C-8), 74.85 (C-18), 73.74 (C11-O-CH_2-CH_3), 52.21 (C11-O-CH_2-), 51.71 (C-14), 49.95 (C-13), 42.12 (C-16), 34.73 (C-9), 8.27 (C-10); Purity (HPLC): 87%.

Amide analogs **19** – **23** were prepared in accordance with the general procedure depicted in Scheme 1 and described as follows:

Acids **9** – **13** (1g, 1eq.) were dissolved in dry DCM (30 ml) and then treated in an ice bath with HBTU (1.2 eq.), TEA (3 eq.). The reaction mixture was stirred under N_2 for 30 minutes before the addition of mPz (2.4 eq.). The reaction was continued for 4 hours upon monitoring with thin layer chromatography (TLC). Thereafter, the reaction mixture was diluted with further DCM (150 ml), washed with Na_2HCO_3 (50 ml), H_2O (2 x 25 ml) and subsequently dried over $MgSO_4$. The solvent

was removed *in vacuo* and the residue subjected to column chromatographywith DCM/MeOH (4:1 v/v) as eluent to isolate the target compound [51].

4.1.1.11 1-Cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-(3-(methylsulfonyl)-2-oxoimidazolidine-1-carbonyl)piperazin-1-yl)quinolin-4(1*H*)-one (**19**)

Mustard powder; yield: 1.1 g (95%); m.p. 61.7 – 61.8 °C; IR ν_{max}: 2921, 2851, 1739, 1673, 1623, 1579 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 8.01 (s, 1H, H-2), 7.77 – 7.73 (m, 1H, H-5), 7.44 (d, *J* = 7.4 Hz, 1H, H-8), 3.84 (t, *J* = 7.7 Hz, 2H, H-20), 3.74 (t, *J* = 7.6 Hz, 2H, H-21), 3.63 (t, *J* = 6.6 Hz, H-14); 3.60 – 3.57 (m, 1H, H-9), 3.27 (t, *J* = 4.3 Hz, 4H, H-13), 3.21 (s, 3H, H-22), 2.48 (t, *J* = 3.5, 1.7 Hz, 4H, mPz: -N-CH_2-CH_2-), 2.33 (d, *J* = 29.3 Hz, 4H, mPz: -N-CH_2-), 2.20 (s, 3H, mPz: -CH_2-N-CH_3), 1.20 (q, *J* = 7.3 Hz, 2H, H-10b), 1.07 (q, *J* = 6.6, 2H, H-10a); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 171.27 (C-4), 164.91 (C-11), 152.44 (C-16), 152.25 (C-6), 151.43 (C-18), 143.70 (C-2), 111.18 (C-5), 106.33 (C-8), 54.79 (C-22), 41.45 (C-21), 41.29 (C-20), 34.23 (C-9), 7.66 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 604.2343 ($C_{27}H_{35}FN_7O_6S$: 604.2354).

4.1.1.12 1-Cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-(morpholine-4-carbonyl)piperazin-1-yl)quinolin-4(1*H*)-one (**20**)

Beige-powder; yield: 0.45 g (39%); m.p. 113.6 – 113.2 °C; IR ν_{max}: 2920, 2850, 1622, 1584, 1544 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.05 (s, 1H, H-2), 8.00 (d, *J* = 13.1 Hz, 1H, H-5), 7.27 (d, *J* = 7.1 Hz, 1H, H-8), 3.70 (t, *J* = 4.6 Hz, 4H, H-13), 3.50 (t, *J* = 4.7 Hz, 4H, H-19), 3.37 – 3.34 (m, 1H, H-9), 3.31 (t, *J* = 4.6 Hz, 4H, H-18), 3.24 (t, *J* = 4.5 Hz, 4H, H-14), 2.49 (m, *J* = 11.4, 5.9 Hz, 4H, mPz: -N-CH_2-CH_2-), 2.32 (s, 3H, mPz: -CH_2-N-CH_3), 1.27 (q, *J* = 6.8 Hz, 2H, H-10b), 1.11 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.36 (C-4), 165.80 (C-16), 163.69 (C-11), 153.36 (C-6), 144.94 (C-2), 113.05 (C-5), 104.91 (C-8), 66.74 (C-13), 49.95 (C-14), 47.58 (C-18), 46.72 (C-19), 46.14 (mPz: -CH_2-N-*C*H_3), 34.40 (C-9), 8.23 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 527.2733 (calcd for $C_{27}H_{36}FN_6O_4$: 527.2782).

4.1.1.13 1-Cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-propionylpiperazin-1-yl)quinolin-4(1*H*)-one (**21**)

Tan powder; yield: 0.71 g (59%); m.p. 181.8 -185.0 °C,. IR ν_{max}: 2922, 2850, 1643, 1622, 1592 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.05 (s, 1H, H-2), 8.01 (d, *J* = 13.0 Hz,1H, H-5), 7.27 (d, *J* = 6.4 Hz, 1H, H-8), 3.39 – 3.35 (m, *J* = 10.9, 7.1, 4.0 Hz, 1H, H-9), 2.49 (t, J = 4.9 Hz, 4H, mPz: -N-CH_2-CH_2-), 2.40 (q, *J* = 7.4 Hz, 2H, H-17), 2.32 (s, 3H, -CH_2-N-CH_3), 1.27 (q, *J* = 6.9 Hz, 2H), 1.18 (t, *J* = 7.4 Hz, 3H, H-18), 1.12 (q, *J* = 6.6 Hz, 2H); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.53 (C-4), 172.33 (C-16), 165.77 (C-11), 153.32 (C-6), 144.96 (C-2), 117.82 (C-3), 113.12 (C-

5), 104.94 (C-8), 46.15 (mPz: -CH_2-N-*C*H_3), 42.50 (-N-*C*H_2-), 34.39 (C-9), 9.56 (C-18), 8.21 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 470.2591 (calcd for $C_{25}H_{33}FN_5O_3$: 470.2567).

4.1.1.14 (3*S*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-Dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl 4-(1-cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-4-oxo-1,4-dihydroquinolin-7-yl)piperazine-1-carboxylate (**22**)

Beige powder; yield: 0.90 g (81%); m.p. 161.6 – 161.7 °C; IR ν_{max}: 2930, 2862, 1701, 1626, 1588 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.12 (s, 1H, H-2), 8.05 (d, J = 31 Hz, 1H, H-5), 7.30 (d, *J* = 4.1 Hz, 1H, H-8), 5.38 (t, *J* = 4.7 Hz, 1H, H-6'), 4.58 – 4.52 (m, 1H, H-3'), 3.69 (t, *J* = 2.6 Hz, 2H, H-14), 3.41 (t, *J* = 4.1 Hz, 4H, H-13), 3.38 – 3.35 (m, *J* = 6.8, 3.9 Hz, 1H, H-9), 3.22 (d, *J* = 4.6 Hz, 4H, mPz: -N-C*H*$_2$-), 2.49 (t, *J* = 5.8 Hz, 4H, mPz: -N-CH_2-C*H*$_2$-), 2.32 (s, 3H, mPz: -CH_2-N-C*H*$_3$), 1.02 (s, 3H, H-19'), 0.85 (dd, *J* = 6.6, 2.8 Hz, 6H, H-26', H-27'), 0.67 (s, 3H, H-18'); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.38 (C-4), 166.08 (C-11), 122.80 (C-6'), 117.28 (C-3), 112.91 (C-5), 105.04 (C-8), 75.45 (C-3'), 54.82 (mPz: -N-CH_2-*C*H_2-), 46.16 (mPz: -CH_2-N-*C*H_3), 42.47 (C-14), 34.46 (C-9), 11.97 (C-18'), 8.26 (C-10); HRMS (ESI) *m/z*: (M) 826.5665 (calcd for $C_{50}H_{73}FN_5O_4$); Purity (HPLC): 96%.

4.1.1.15 1-Cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-(prop-2-yn-1-yl)piperazin-1-yl)quinolin-4(1*H*)-one (**23**)

After above mentioned work-up, the product was precipitated out of ethyl acetate to yield (85%) a fine white powder; m.p. 214.9 – 216.7 °C; IR ν_{max}: 3092, 2834, 2801, 2088, 1626 cm^1; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.04 (s, 1H, H-2), 7.98 (d, *J* = 13.2 Hz, 1H, H-5), 7.27 (d, *J* = 8.1 Hz, 1H, H-8), 3.38 (d, *J* = 2.4 Hz, 3H, H-16), 3.37 – 3.35 (m, 1H, H-9), 3.33 – 3.27 (t, 4H, -N-C*H*$_2$-), 2.79 (t, *J* = 4.8 Hz, 4H, H-14), 2.56 – 2.41 (t, *J* = 5.1 Hz, 4H, mPz: -N-CH_2-C*H*$_2$-), 2.31 (s, 3H, -CH_2-N-C*H*$_3$), 2.29 (t, *J* = 2.4 Hz, 1H, H-18), 1.26 (q, *J* = 6.8 Hz, 2H, H-10b), 1.11 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.39 (C-4), 165.88 (C-11), 153.38 (C-6), 144.85 (C-2), 117.64 (C-3), 112.90 (C-5), 104.68 (C-8), 78.46 (C-17), 73.72 (C-18), 55.23 (mPz: -N-CH_2-*C*H_2-), 51.70 (C-14), 49.99 (mPz: -N-C*H*$_2$-), 46.99 (C-16), 46.14 (mPz: -CH_2-N-*C*H_3), 34.36 (C-9), 8.17 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 452.2466 (calcd for $C_{25}H_{31}FN_5O_2$: 452.2462); Purity (HPLC): 98%.

4.1.2 Syntheses of azides 25a – g

Azides **25a – f** were prepared in accordance with the general procedure shown in Scheme 2 and described as follows:

Benzyl bromide **24a** and benzyl bromide *para*-substituted **24b – f** (1.0 eq.) were dissolved in DMSO (15 ml) together with sodium azide (NaN_3, 1.5 eq.). The reaction mixture was stirred at room temperature overnight. The resulting reaction mixture was diluted with water (30 ml) and the aqueous phase extracted with diethyl ether (3 x 40 ml). The combined organic layers were washed with brine (3 x 50 ml) and dried over $MgSO_4$. Removing the solvent left a clear oil [52].

4.1.2.1 (azidomethyl)benzene (**25a**)

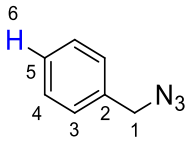


Figure 3-6: (azidomethyl)benzene 25a

Yield: 70%; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.42 – 7.32 (m, 5H, H-3, H-4, H-5), 4.35 (s, 2H, H-1); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 135.49 (C-2), 128.96 (C-4), 128.43 (C-5), 128.34 (C-3), 54.93 (C-1).

4.1.2.2 1-(azidomethyl)-4-methylbenzene (**25b**)

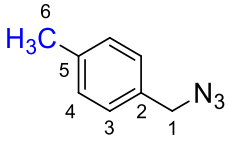


Figure 3-7: 1-(azidomethyl)-4-methylbenzene 25b

Yield: 95%; ^{1}H NMR (600 MHz, CDCl3) δ (ppm): 7.23 – 7.18 (m, 4H, H-3, H-4), 4.30 (s, 2H, H-1), 2.37 (s, 3H, H-6); ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 138.28 (C-2), 132.41 (C-5), 129.63 (C-4), 128.40 (C-3), 54.75 (C-1), 21.31 (C-6).

4.1.2.3 1-(azidomethyl)-4-fluorobenzene (**25c**)

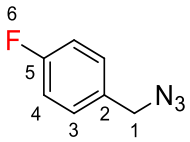


Figure 3-8: 1-(azidomethyl)-4-fluorobenzene 25c

Yield: 70%; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.32 – 7.28 (m, 2H, H-3), 7.10 – 7.05 (m, 2H, H-4), 4.32 (s, 2H, H-1); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 162.78 (C-5), 131.32 (C-2), 130.15 (C-3), 115.93 (C-4), 54.20 (C-1).

4.1.2.4 1-(azidomethyl)-4-bromobenzene (**25d**)

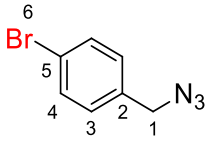


Figure 3-9: 1-(azidomethyl)-4-bromobenzene 25d

Yield: 100%; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.51 (d, *J* = 8.4 Hz, 2H, H-4), 7.19 (d, *J* = 8.4 Hz, 2H, 3), 4.30 (s, 2H, H-1); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 134.52 (C-2), 132.13 (C-3), 129.94 (C-4), 122.48 (C-5), 54.23 (C-1).

4.1.2.5 1-(azidomethyl)-4-nitrobenzene (**25e**)

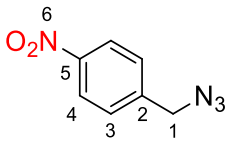


Figure 3-10: 1-(azidomethyl)-4-nitrobenzene 25e

Yellowish oil; yield: 100%; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.24 (d, *J* = 8.6 Hz, 2H, H-4), 7.50 (d, *J* = 8.5 Hz, 2H, H-3), 4.50 (s, 2H, H-1); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 147.88 (C-5), 142.83 (C-2), 128.72 (C-3), 124.18 (C-4), 53.86 (C-1).

4.1.2.6 1-(azidomethyl)-4-(trifluoromethyl)benzene (**25f**)

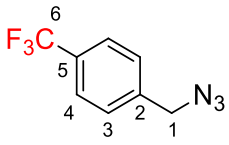


Figure 3-11: 1-(azidomethyl)-4-(trfluoromethyl)benzene 25f

Yield: 13%, ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 7.15 (d, *J* = 7.7 Hz, 2H, H-4), 6.95 (d, *J* = 7.8 Hz, 2H, H-3); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 125.92 (C-5), 54.20 (C-1).

4.1.2.7 Cholesteryl azide **25g**

Cholesteryl chloride (3.57 mmol, 1.66 g, 1eq) and NaN_3 (17.73 mmol, 1.16 g, 5 eq.) in DMF (25 ml) whilst heating overnight at 100 °C. The reaction mixture was left to cool at room temperature then hexane (150 ml) and water (25 ml) were added. The organic layer was then further washed with $NaHCO_3$, brine and dried over $MgSO_4$. The crude organic layer was concentrated and subjected to flash column chromatography with DCM as eluent [64].

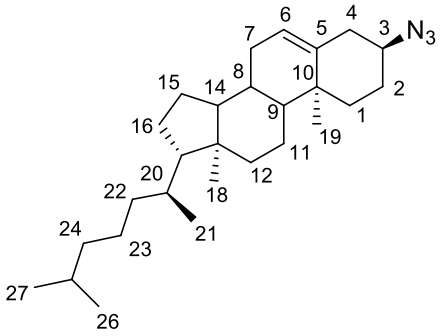


Figure 3-12: cholesteryl azide 25g

White solid; yield: 83%; IR v_{max}: 2934, 2866, 2649, 2084 cm^{-1} (in accordance to literature [64]); ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 5.37 (t,1H, H-6), 3.80 – 3.73 (m, *J* = 12.0, 4.5 Hz, H-3), 1.03 (s, 3H, H-19), 0.86 (dd, *J* = 6.6, 2.7 Hz, H-26, H-27), 0.67 (s, H-18); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 140.96 (C-5), 122.64 (C-6), 60.51 (C-3), 56.85 (C-14), 56.29 (C-17), 50.22 (C-9), 43.56, 42.47 (C-13), 22.72 (C-26, C-27), 19.41 (C-19), 12.01 (C-18).

4.1.3 Ciprofloxacin-triazole hybrids 26 – 39

Hybrids **26** – **39** were prepared in accordance with the general procedure depicted in Scheme 2.

Alkyne (**13** or **23**) (500 mg, 1.2 eq.) was dissolved in a mixture of DMF-water (15 ml, 9:1 v/v). Whilst stirring, azide (**25 a-g**) (2.0 eq.), NaAsc (120 mg, 0.8 eq.), and $CuSO_4 \cdot 5H_2O$ (30 mg, 0.2 eq.) were consecutively added. The resulting reaction mixture was stirred vigorously for a period of 24 – 36 hours at 60 °C monitored by TLC. Upon completion, NH_4Cl (25 ml) was added to quench the reaction mixture followed by extraction with DCM (3 x 40 ml). The organic phase was dried over $MgSO_4$ to afford hybrids **26** – **32** after purification by column chromatography (eluent: DCM/MeOH, 9:1 v/v), while compounds **33** – **39** were isolated by precipitation with diethyl ether after column chromatography (eluent: DCM/MeOH, 4:1 v/v).

4.1.3.1 7-(4-((1-benzyl-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**26**)

Beige powder; yield: 0.15 g (27%); m.p. 211.8 – 213.6 °C; IR ν_{max}: 2957, 2926, 2857, 1724, 1628 cm^{-1}; ^1H NMR (600 MHz, CDCl3) δ (ppm): 15.02 (s, 1H, H-11), 8.72 (s, 1H, H-2), 7.96 (d, *J* = 13.0 Hz, 1H, H-5), 7.46 (s, 1H, H-18), 7.40 – 7.35 (m, 3H, H-21, H-22), 7.32 (d, *J* = 7.1 Hz, 1H, H-8), 7.30 – 7.27 (m, 2H, H-23), 5.52 (s, 2H, H-19), 3.75 (s, 2H, H-16), 3.54 – 3.50 (m, 1H, H-9), 3.32 (t, *J* = 31.1 Hz, 4H, H-13), 2.74 (t, *J* = 22.2 Hz, 4H, H-14), 1.37 (q, *J* = 10.5 Hz, 2H, H-10b), 1.17 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 177.19 (C-4), 167.88 (C-11), 153.75 (C-6), 147.51 (C-2), 112.49 (C-5), 104.96 (C-8), 54.34 (C-19), 53.15 (C-16), 52.53 (C-13), 49.66 (C-14), 35.41 (C-9), 8.35 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 503.2201 (calcd for $C_{27}H_{28}FN_6O_3$: 503.2207); Purity (HPLC): 83%.

4.1.3.2 1-Cyclopropyl-6-fluoro-7-(4-((1-(4-methylbenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**27**)

Brown solid; yield: 0.17 g (28%); m.p. 203.7 – 205.5 °C; IR ν_{max}: 2958, 2927, 2859, 1722, 1628 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 15.11 (s, 1H, H-11), 8.80 (s, 1H, H-2), 8.04 (d, *J* = 13.0 Hz, 1H, H-5), 7.51 (s, 1H, H-18), 7.40 (d, *J* = 7.0 Hz, 1H, H-8), 7.26 (s, 5H, H-21, H-22), 5.56 (s, 2H, H-19), 3.84 (s, 2H, H-16), 3.63 – 3.57 (m, 1H, H-9), 2.43 (s, 3H, H-24), 1.42 (q, *J* = 10.5 Hz, 2H, H-10b), 1.25 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 177.21 (C-4), 167.19 (C-11), 153.76 (C-6), 147.54 (C-2), 129.93 (C-21), 128.33 (C-22), 112.51 (C-5), 108.19 (C-3), 104.99 (C-8), 54.16 (C-19), 53.11 (C-16), 35.42 (C-9), 21.31 (C-24), 8.36 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 517.2358 (calcd for $C_{28}H_{30}FN_6O_3$: 517.2363); Purity (HPLC): 91%.

4.1.3.3 1-Cyclopropyl-6-fluoro-7-(4-((1-(4-fluorobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**28**)

Beige powder; yield: (67%); m.p. 297.0 – 298.1 °C, IR ν_{max}: 2929, 2828 cm^{-1}. ^1H NMR (600 MHz, DMSO) δ (ppm): 15.17 (s, 1H, H-11), 8.63 (s, 1H, H-2), 8.14 (s, 1H, H-18), 7.87 (d, *J* = 13.1 Hz,

1H, H-5), 7.52 (d, *J* = 7.4 Hz, 1H, H-8), 7.38 (t, *J* = 8.2, 5.7 Hz, 2H, H-21), 7.19 (t, *J* = 8.8 Hz, 2H, H-22), 5.58 (s, 2H, H-19), 3.80 – 3.76 (m, 1H, H-9), 2.86 (s, 2H, H-16), 1.28 (q, *J* = 6.8 Hz, 2H, H-10b), 1.14 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, DMSO) δ (ppm): 176.37 (C-4), 165.95 (C-11), 148.07 (C-2), 130.31 (C-21), 115.63 (C-22), 111.10 (C-18), (C-19), 35.89 (C-16), 35.80 (C-9), 30.79 (C-13, C-14), 7.58 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 521.2107 (calcd for $C_{27}H_{26}F_2N_6O_3$: 512.2113); Purity (HPLC): 89%.

4.1.3.4 7-(4-((1-(4-Bromobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**29**)

Brown powder; yield: 0.21 g (65%); m.p. 130.4 – 130.5 °C; IR ν_{max}: 2955, 2922, 2853, 1717, 1625, 1261 cm^{-1}; ^{1}H NMR (600 MHz, DMSO) δ (ppm): 15.11 (s, 1H, H-11), 8.65 (s, 1H, H-2), 8.37 (s, 1H, H-18), 7.94 – 7.90 (m, 1H, H-5), 7.66 – 7.46 (m, 4H, H-21, H-22), 7.29 (d, *J* = 8.1 Hz, 1H, H-8), 5.64 (s, 2H, H-19), 4.19 – 4.04 (m, 1H, H-9), 3.80 (s, 2H, H-16), 2.48 – 2.47 (m, 8H, H-13, H-14), 1.28 (q, *J* = 6.5 Hz, 2H, H-10b), 1.15 (q, *J* = 6.6 Hz, 2H, H-10a); HRMS (ESI) *m/z*: $(M+H)^+$ 581.1307 (calcd for $C_{27}H_{27}BrFN_6O_3$: 581.1321).

4.1.3.5 1-Cyclopropyl-6-fluoro-7-(4-((1-(4-nitrobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**30**)

Dark yellow solid, yield: 0.09 g (15%); m.p. 118 – 119 °C,. IR ν_{max}: 2957, 2924, 2858, 1725, 1625 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 14.95 (s, 1H, H-11), 8.72 (s, 1H, H-2), 8.23 (d, *J* = 8.7 Hz, 3H, H-18, H-22), 7.95 (d, *J* = 12.9 Hz, 1H, H-5), 7.44 (d, *J* = 8.7 Hz, 2H, H-21), 7.34 (d, *J* = 7.0 Hz, 1H, H-8), 5.67 (s, 2H, H-19), 3.94 (s, 2H, H-16), 3.55 – 3.51 (m, 1H, H-9), 3.45 (t, *J* = 19.7 Hz, 4H, H-14), 2.93 (t, *J* = 15.3, 4H, H-13), 1.34 (q, *J* = 6.9 Hz, 2H, H-10b), 1.19 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 177.14 (C-4), 167.90 (C-11), 153.94 (C-6), 150.34 (C-23), 148.26 (C-2), 148.14 (C-17), 147.64 (C-20), 128.92 (C-21), 124.49 (C-22), 119.81 (C-18), 112.66 (C-5), 104.01 (C-8), 53.37 (C-19), 52.77 (C-13, C-14), 52.35 (C-16), 35.46 (C-9), 8.40 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 548.2052 (calcd for $C_{27}H_{27}FN_7O_5$: 548.2058); Purity (HPLC): 81%.

4.1.3.6 1-Cyclopropyl-6-fluoro-4-oxo-7-(4-((1-(4-(trifluoromethyl)benzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1,4-dihydroquinoline-3-carboxylic acid (**31**)

Beige solid; yield: 0.11 g (49%); m.p. 122.3 – 123.7 °C; IR ν_{max}: 2956, 2923, 2853, 1725, 1625 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 15.00 (s, 1H, H-11), 8.71 (s, 1H, H-2), 7.94 (d, *J* = 13.0 Hz, 1H, H-5), 7.64 (d, *J* = 8.2 Hz, 2H, H-21), 7.57 (s, 1H, H-18), 7.40 (d, *J* = 8.1 Hz, 2H, H-22), 7.33 (d, *J* = 7.1 Hz, 1H, H-8), 5.61 (s, 2H, H-19), 3.82 (s, 2H, H-16), 3.54 – 3.51 (m, 1H, H-9), 1.36 (q, *J* = 6.5 Hz, 2H, H-10b), 1.18 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ

(ppm): 177.06 (C-4), 167.80 (C-11), 153.64 (C-6), 147.45 (C-2), 131.12 (C-24), 128.81 (C-21), 126.18 (C-22), 123.24 (C-18), 112.39 (C-5), 108.08 (C-3), 105.03 (C-8), 53.67 (C-19), 53.08 (C-16), 52.50 (C-14), 49.45 (C-13), 35.33 (C-9), 8.26 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 571.2019 (calcd for $C_{28}H_{26}F_4N_6O_3$: 571.2081); Purity (HPLC): 85%.

4.1.3.7 1-Cyclopropyl-7-(4-((1-((3*R*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-6-fluoro-4-oxo-1,4-dihydroquinoline-3-carboxylic acid (**32**)

Light tan solid; yield: 0.32 g (36%); IR ν_{max}: 3250, 2935, 2849, 1735, 1628 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 15.04 (s, 1H, H-11), 8.75 (s, 1H, H-2), 7.99 (d, *J* = 13.0 Hz, 1H, H-5), 7.69 (t, 1H, H-6'), 7.52 (s, 1H, H-18), 7.36 (d, J = 7.1 Hz, 1H, H-8), 4.25 – 4.16 (m, 1H, H-3'), 3.56 – 3.53 (m, 1H, H-9), 3.40 (t, *J* = 4.8 Hz, 4H, H-13), 2.82 (t, *J* = 4.9 Hz, 4H, H-14), 0.86 (dd, *J* = 7.0 Hz, 6H, H-26', H-27'), 0.67 (s, 1H, H-18'); ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 177.21 (C-4), 167.90 (C-11), 153.79 (C-6), 147.56 (C-2), 112.56 (C-5), 108.21 (C-3), 105.00 (C-8), 68.28 (C-3'), 35.44 (C-9), 23.49 (C-26', C-27'), 11.09 (18'), 8.37 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 781.5185 (calcd for $C_{47}H_{66}FN_6O_3$: 781.5180); Purity (HPLC): 95%.

4.1.3.8 7-(4-((1-Benzyl-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)quinolin-4(1*H*)-one (**33**)

Light orange powder; yield: 0.6 g (24%); m.p. 282.9 – 283.0 °C; IR ν_{max}: 2957, 2927, 2857, 1726, 1624, 1580 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.03 (s, 1H, H-2), 7.95 (d, *J* = 13.2 Hz, 1H, H-5), 7.42 (s, 1H, H-18), 7.39 – 7.33 (m, 3H, H-22, H-23), 7.28 – 7.26 (m, 2H, H-21), 7.24 (d, *J* = 7.1 Hz, 1H, H-8), 5.52 (s, 2H, H-19), 3.73 (s, 2H, H-16), 3.39 – 3.32 (m, 1H, H-9), 3.26 (t, *J* = 4.9 Hz, 4H, mPz: -N-C*H*$_2$-), 2.72 (t, *J* = 4.5 Hz, 4H, H-14), 2.37 (s, 3H, mPz: -CH_2-N-C*H*$_3$), 1.25 (q, *J* = 12.9, 6.1 Hz, 2H, C-10b), 1.09 (q, *J* = 6.5 Hz, 2H, C-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.43 (C-4), 165.86 (C-11), 153.39 (C-6), 144.68 (C-2), 129.25 (C-22), 128.90 (C-21), 128.24 (C-23), 122.69 (C-18), 117.41 (C-3), 112.82 (C-5), 104.65 (C-8), 54.29 (C-19), 45.83 (mPz: -CH_2-N-*C*H_3), 42.10 (C-13), 34.39 (C-9), 8.18 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 585.3096 (calcd for $C_{23}H_{38}FN_8O_2$: 585.3102), Purity (HPLC): 99%.

4.1.3.9 1-Cyclopropyl-6-fluoro-7-(4-((1-(4-methylbenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-3-(4-methylpiperazine-1-carbonyl)quinolin-4(1*H*)-one (**34**)

Light orange powder; yield: 0.26 g (60%); m.p. 215.4 – 216.5 °C;. IR ν_{max}: 2957, 2923, 2856, 1728, 1621, 1605 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.04 (s, 1H, H-2), 7.96 (d, *J* = 13.2 Hz, 1H, H-5), 7.39 (s, 1H, H-18), 7.25 (d, *J* = 7.1 Hz, 1H, H-8), 7.17 (s, 4H, H-21, H-22), 5.47 (s, 2H, H-

19), 3.72 (s, 2H, H-16), 3.39 - 3.34 (m, 1H, H-9), 3.26 (t, 4H, mPz: -CH_2-C*H_2*-N-), 2.72 (t, J = 4.5 Hz, 4H, H-14), 2.34 (s, 3H, H-24), 1.26 (q, *J* = 7.9, 5.7 Hz, 2H, H-10b), 1.10 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, CDCl3) δ (ppm): 172.45 (C-4), 165.87 (C-11), 153.41 (C-6), 144.97 (C-2), 138.87 (C-20), 138.50 (C-23), 131.65 (C-17), 129.91 (C-21), 128.32 (C-22), 122.59 (C-18), 117.38 (C-3), 112.84 (C-5), 104.66 (C-8), 55.45 (C-14), 54.12 (mPz: -CH_2-*C*H_2-N-), 53.27 (C-19), 52.69 (C-16), 49.94 (mPz: -N-*C*H_2-), 45.78 (mPz: -CH_2-N-*C*H_3), 34.41 (C-9), 21.30 (C-24), 8.19 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 599.3253 (calcd for $C_{33}H_{40}FN_8O_2$: 599.3258); Purity (HPLC): 97%.

4.1.3.10 1-Cyclopropyl-6-fluoro-7-(4-((1-(4-fluorobenzyl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-3-(4-methylpiperazine-1-carbonyl)quinolin-4(1*H*)-one (**35**)

Ivory powder; yield: 0.21 g (63%); m.p. 261.6 – 261.7 °C; IR ν_{max}: 2935, 2832, 2800, 1681, 1622, 1590 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.04 (s, 1H, H-2), 7.95 (d, *J* = 13.2 Hz, 1H, H-5), 7.43 (s, 1H, H-18), 7.31 – 7.21 (m, 4H, H-21, H-22), 7.06 (d, J = 8.5 Hz, 1H, H-8), 5.49 (2H, H-19), 3.74 (s, 2H, H-16), 3.39 – 3.36 (m, 1H, H-9), 2.42 (s, 3H, mPz: -CH_2-N-C*H_3), 1.26 (q, *J* = 6.7 Hz, 2H, H-10b), 1.10 (q, *J* = 6.5 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.46 (C-4), 165.86 (C-11), 162.99 (C-23), 153.41 (C-6), 145.00 (C-2), 138.50 (C-17), 130.14 (C-21), 121.71 (C-18), 117.31 (C-3), 116.27 (C-8), 112.82 (C-5), 104.69 (C-22), 53.23 (C-16), 52.71 (C-19), 45.63 (mPz: -CH_2-N-*C*H_3), 8.19 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 603.3002 (calcd for $C_{32}H_{37}F_2N_8O_2$: 603.3008).

4.1.3.11 7-(4-((1-(4-Bromobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-1-cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)quinolin-4(1*H*)-one (**36**)

Gold powder; yield: 0.05 g (18%); m.p. 254.7 – 255.6 °C; IR ν_{max}: 2918, 2831, 1626, 1581, 1256 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.08 (s, 1H, H-2), 7.89 (d, *J* = 13.0 Hz, 1H, H-5), 7.68 (s, 1H, H-18), 7.50 (d, *J* = 8.4 Hz, 2H, H-22), 7.28 (d, *J* = 7.0 Hz, 1H, H-8), 7.17 (d, *J* = 8.3 Hz, 2H, H-21), 5.49 (s, 2H, H-19), 3.71 (s, 2H, H-16), 3.42 – 3.36 (m, 9H, H-9, H-14, mPz: -N-C*H_2*-), 2.91 (t, *J* = 22.3 Hz, 4H, mPz: -CH_2-C*H_2*-N-), 2.78 (s, 3H, mPz: -CH_2-N-C*H_3*),1.30 (q, *J* = 6.7 Hz, 2H, H-10b), 1.12 (q, *J* = 6.3 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 166.79 (C-4), 165.76 (C-11), 152.63 (C-6), 145.69 (C-2), 132.48 (C-22), 129.89 (C-21), 123.19 (C-18), 112.74 (C-5), 105.11 (C-8), 44.83 (mPz: CH_2-N-*C*H_3), 34.71 (C-9), 8.28 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 663.2201 (calcd for $C_{32}H_{37}BrFN_8O_2$: 663.2207).

4.1.3.12 1-Cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-((1-(4-nitrobenzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)quinolin-4(1*H*)-one (**37**)

Gold coloured powder; yield: 0.19 g (55%); m.p. 281.5 – 281.6 °C;. IR ν_{max}: 2957, 2928, 2857, 1727, 1622, 1589 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.22 (d, *J* = 8.8 Hz, 2H, H-22), 8.04 (s, 1H, H-2), 7.95 (d, *J* = 13.2 Hz, 1H, H-5), 7.52 (s, 1H, H-18), 7.42 (d, *J* = 8.8 Hz, 2H, H-21), 7.25 (d, *J* = 7.1 Hz, 1H, H-8), 5.65 (s, 2H, H-19), 3.77 (s, 2H, H-16), 3.38 – 3.34 (m, *J* = 4.0 Hz, 1H, H-9), 2.74 (t, *J* = 4.5 Hz, 4H, H-14), 2.40 (s, 3H, mPz: -CH_2-N-CH_3), 1.26 (q, *J* = 4.6 Hz, 2H, H-10b), 1.10 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.45 (C-4), 165.85 (C-11), 153.39 (C-6), 148.21 (C-23), 145.08 (C-2), 144.97 (C-20), 141.77 (C-17), 128.76 (C-21), 124.44 (C-22), 123.00 (C-18), 117.38 (C-3), 112.85 (C-5), 104.68 (C-8), 53.24 (C-16), 53.20 (C-14), 52.76 (C-19), 49.94 (mPz: -N-*C*H_2-), 45.73 (mPz: -CH_2-N-*C*H_3), 34.42 (C-9), 8.21 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 630.2929 (calcd for $C_{32}H_{37}FN_9O_4$: 630.2953); Purity (HPLC): 97%.

4.1.3.13 1-cyclopropyl-6-fluoro-3-(4-methylpiperazine-1-carbonyl)-7-(4-((1-(4-(trifluoromethyl)benzyl)-1*H*-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)quinolin-4(1*H*)-one (**38**)

Light pink powder; yield: 0.32 g (67%); m.p. 231.9 – 242.4 °C,. IR ν_{max}: 2958, 2922, 2850, 1727, 1621, 1589 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.05 (s, 1H, H-2), 7.96 (d, *J* = 13.2 Hz, 1H, H-5), 7.64 (d, *J* = 8.1 Hz, 2H, H-22), 7.47 (s, 1H, H-19), 7.38 (d, *J* = 8.1 Hz, 2H, H-21), 7.25 (d, *J* = 7.2 Hz, 1H, H-8), 5.60 (s, 2H, H-19), 3.76 (s, 2H, H-16), 3.38 – 3.35 (m, 1H, H-9), 3.27 (t, 4H, mPz: -N-CH_2-), 2.74 (t, *J* = 4.5 Hz, 4H, H-14), 2.41 (s, 3H, mPz: -N-CH_3), 1.26 (q, *J* = 8.2, 5.3 Hz, 2H, H-10b), 1.10 (q, *J* = 6.6 Hz, 2H, H-10a); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.46 (C-4), 165.86 (C-11), 153.41 (C-6), 145.00 (C-2), 128.38 (C-21), 126.26 (C-22), 122.84 (C-18), 117.36 (C-3), 112.85 (C-5), 104.68 (C-8), 53.23 (C-19), 52.75 (C-16), 49.96 (mPz: -N-CH_2-), 34.42 (C-9), 8.20 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 653.2980 (calcd for $C_{33}H_{37}F_4N_8O_2$: 653.2976); Purity (HPLC): 98%.

4.1.3.14 1-Cyclopropyl-7-(4-((1-((3*R*,8*S*,9*S*,10*R*,13*R*,14*S*,17*R*)-10,13-dimethyl-17-((*R*)-6-methylheptan-2-yl)-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[a]phenanthren-3-yl)-1H-1,2,3-triazol-4-yl)methyl)piperazin-1-yl)-6-fluoro-3-(4-methylpiperazine-1-carbonyl)quinolin-4(1*H*)-one (**39**)

Yellow solid; yield (12%); IR ν_{max}: 2929, 2865, 1724, 1626 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ (ppm): 8.04 (s, 1H, H-2), 7.97 (d, *J* = 13.2 Hz, 1H, H-5), 7.72 (s, 1H, H-18), 7.26 (d, *J* = 3.4 Hz, 1H, H-8), 5.49 (t, *J* = 4.5 Hz, 1H, H-6'), 4.20 (t, *J* = 6.0 Hz, 1H, H-3'), 3.76 (s, 2H, H-16), 3.37 (s, 1H, H-9), 2.37 (s, 3H, mPz: CH_2-N-CH_3), 1.09 (s, 3H, H-19'), 0.85 (dd, *J* = 2.6 Hz, 6H, H-26', H-27'), 0.67 (s, 3H, H-18'); ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 172.42 (C-4), 165.89 (C-11), 153.41

(C-6), 144.93 (C-2), 124.41 (C-6'), 112.87 (C-5), 104.64 (C-8), 68.27 (C-3'), 50.26 (C-13), 45.89 (mPz: CH_2-N-CH_3), 42.42 (C-14), 34.40 (C-9), 11.98 (C-18'), 8.20 (C-10); HRMS (ESI) *m/z*: $(M+H)^+$ 863.6111 (calcd for $C_{52}H_{76}FN_8O_5$: 863.6075); Purity (HPLC): 99%.

4.2 *In-vitro* biological evaluation

4.2.1 Antimycobacterial activity assessment

In both series, one type of culture media were employed: 7H9/ADC (Middlebrook 7H9 broth enriched with Albumin-Dextrose Catalase, with or without tween 80). In general, literature protocols were modified, wherein broth microdilution in a 96-well microplate was used [65-67]. In short, a 12.8 mM stock solution of a test compound was prepared in DMSO and then diluted with the desired growth medium to a concentration of 640 µM. This was followed by stepwise two-fold serial dilutions up to a concentration of 0.3125 µM. Afterwards, a 1:100 diluted culture of *Mtb* H37Rv at an optical density of 0.6–0.7 at 600 nm (OD600) was added into the wells except those in row 1 (controls) and incubated at 37 °C. Finally, the lowest concentration of the test compound that inhibited the growth of more than 90% of *Mtb* population (Minimum Inhibitory Concentration (MIC_{90})) was scored visually at day 7 and day 14 post-inoculation. MIC_{90} scoring was enabled by the use of Alamar blue dye in a technique known as the Microplate Alamar Blue Assay (MABA), which is vital in the visual or fluorimetric cell-growth reading. In Series 1, however, the green fluorescent protein (GFP)-tagged *Mtb* H37Rv (pMSP12::GFP) was used in the assays. In this case, the MICs were determined using a dose-response curve analysis of the relative fluorescence (excitation wavelength at 485 nm and emission wavelength at 520 nm) measured on a FLUOstar OPTIMA® microplate reader [68].

4.2.2 Cytotoxicity assays

The WI-38 Human fetal lung fibroblast cell line - normal Human Fetal Lung Fibroblast from ECACC was routinely maintained as a monolayer cell culture at 37ºC, 5% CO_2, 95% air and 100% relative humidity in EMEM containing 10% fetal bovine serum, 2 mM L-glutamine and 50µg/ml gentamicin.

For screening experiment, the cells (21-50 passages) were inoculated in a 96-well microtiter plates at plating densities of 10 000 cells/well and were incubated for 24 hours. After 24 hours the cells were treated with the experimental drugs which were previously dissolved in DMSO and diluted in medium to produce 5 concentrations. Cells without drug addition served as control. The blank contains complete medium without cells. Emetine was used as a standard. The plates were

incubated for 48 hours after addition of the compounds. Viable cells were fixed to the bottom of each well with cold 50% trichloroacetic acid, washed, dried and dyed by SRB. Unbound dye was removed and protein-bound dye was extracted with 10mM Tris base for optical density determination at the wavelength 540 nm using a multiwell spectrophotometer. Data analysis was performed using GraphPad Prism software. 50% of cell growth inhibition (IC50) was determined by non-linear regression.

CHO and HEK 293 cell lines. cells were cultured in Hyclone Ham's F12 growth medium, Kaighn's modification supplemented with 10 % fetal bovine serum, 1 % Penicillin-Streptomycin mixture and Amphotericin B. HEK-293 cells were cultured in Hyclone Dulbecco's modified Eagle's medium with high glucose supplemented with 10 % fetal bovine serum and 1 % L-glutamine, Penicillin-Streptomycin, Amphotericin B and non-essential amino acids. Both cell lines 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 (75 000 cells/mL for both cell lines) and incubated for 24 hours. The cells were then 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 24 hours.

To initiate the MTT assay, 20 µL of sterile-filtered MTT solution (5 mg/mL in PBS) was added and the plates incubated for 4 hours. The growth medium-MTT mixture was then aspirated and 100 µL of 2-propanol added to dissolve purple formazan crystals. Absorbance was measured at 560 and 650 nm using the Thermofisher Scientific GO Multiscan plate reader. Due to light sensitivity of MTT reagent, the assay was performed in the dark. Thus, the plates were covered with aluminium foil and the contents gently mixed for 15 minutes at room temperature. 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

The IC_{50} and Z-score were determined for each compound's biological replicate using the SkanIt 4.0 software and four-parameter logistic (sigmoidal) regression. For the final IC_{50} of each compound, the mean IC_{50} of the biological replicates were calculated and the mean dose-response curve, with standard error of the means (SEM), were prepared in GraphPad Prism 5.

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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BIBLIOGRAPHY

[1] D.E. Goldberg, R.F. Siliciano, W.R. Jacobs, Outwitting evolution: fighting drug-resistant TB, malaria, and HIV, *Cell*, 148 (2012) 1271-1283.

[2] S.K. Sharma, A. Mohan, Tuberculosis: from an incurable scourge to a curable disease - journey over a millennium, *Indian J. Med. Res*., 137 (2013) 455-493.

[3] P.P. Dixit, P.P. Dixit, S.N. Thore, Hybrid triazoles: design and synthesis as potential dual inhibitor of growth and efflux inhibition in tuberculosis, *Eur. J. Med. Chem*., 107 (2016) 38-47.

[4] WHO. 2017. Global tuberculosis report 2017. http://www.who.int/tb/publications/global_report/en/ Date of access: 18 Nov. 2017.

[5] J.M. Gray, D.L. Cohn, Tuberculosis and HIV coinfection, in: Seminars in respiratory and critical care medicine, *Thieme Medical Publishers*, 2013, pp. 32-43.

[6] X.-X. Li, X.-N. Zhou, Co-infection of tuberculosis and parasitic diseases in humans: a systematic review, *Parasit. Vectors*., 6 (2013) 79-91.

[7] K.G. Castro, Tuberculosis as an opportunistic disease in persons infected with human immunodeficiency virus, *Clin. Infect. Dis*., 21 (1995) 66-71.

[8] L. Jordao, O.V. Vieira, Tuberculosis: new aspects of an old disease, *Int. J. Cell. Biol*., 2011 (2011) 1-13.

[9] CDC. 2011. Core curriculum on tuberculosis: what the clinician should know. https://www.cdc.gov/tb/education/corecurr/index.htm Date of access: 26 Aug. 2017.

[10] T.R. Frieden, T.R. Sterling, S.S. Munsiff, C.J. Watt, C. Dye, Tuberculosis, *Lancet*, 362 (2003) 887-999.

[11] WHO. 2016. WHO treatment guidelines for drug-resistant tuberculosis. http://apps.who.int/iris/handle/10665/250125/ Date of access: 15 Sept. 2017.

[12] M.R. O'Donnell, A. Wolf, L. Werner, C.R. Horsburgh, N. Padayatchi, Adherence in the treatment of patients with extensively drug-resistant tuberculosis and HIV in South Africa: A prospective cohort study, *J. Acquir. Immune. Defic. Syndr*., 67 (2014) 22-29.

[13] P. Tabarsi, M. Mardani, Extensively drug-resistant tuberculosis: a review article, *Arch. Clin. Infect. Dis*., 7 (2012) 81-84.

[14] Y.-Q. Hu, S. Zhang, Z. Xu, Z.-S. Lv, M.-L. Liu, L.-S. Feng, 4-Quinolone hybrids and their antibacterial activities, *Eur. J. Med. Chem*., 141 (2017) 335-345.

[15] Forum on Neuroscience and Nervous System Disorders, Board on Health Sciences Policy, Institute of Medicine, Drug development challenges, in: B.M. Altevogt, M. Davis, D.E. Pankevich, S.M.P. Norris (Eds.) Improving and accelerating therapeutic development for nervous system disorders: workshop summary, National academies press, Washington, DC, 2014, pp. 9.

[16] S.J. Projan, Why is big Pharma getting out of antibacterial drug discovery?, *Curr. Opin. Microbiol*., 6 (2003) 427-430.

[17] R. Guha, On Exploring Structure Activity Relationships, *Methods Mol. Biol*., 993 (2013) 81-94.

[18] A. Emmerson, A. Jones, The quinolones: decades of development and use, *J. Antimicrob. Chemother*., 51 (2003) 13-20.

[19] M.I. Andersson, A.P. MacGowan, Development of the quinolones, *J. Antimicr. chemoth*., 51 (2003) 1-11.

[20] P.C. Sharma, A. Jain, S. Jain, Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects, *Acta Pol. Pharm*., 66 (2009) 587-604.

[21] P.C. Appelbaum, P.A. Hunter, The fluoroquinolone antibacterials: past, present and future perspectives, *Int. J. Antimicrob. Agents*, 16 (2000) 5-15.

[22] M. Farrington, Chapter 13 - Antibacterial drugs A2 - Bennett, Peter N, in: M.J. Brown, P. Sharma (Eds.) Clinical Pharmacology (Eleventh Edition), Churchill Livingstone, Oxford, 2012, pp. 173-190.

[23] H. Takahashi, I. Hayakawa, T. Akimoto, The history of the development and changes of quinolone antibacterial agents, *Yakushigaku Zasshi J*., 38 (2003) 161-179.

[24] P.C. Sharma, A. Jain, S. Jain, Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects, *Acta Pol. Pharm*., 66 (2009) 587-604.

[25] G.G. Zhanel, A. Walkty, L. Vercaigne, J.A. Karlowsky, J. Embil, A.S. Gin, D.J. Hoban, The new fluoroquinolones: a critical review, *Can. J. Infect. Dis. Med. Microbiol*., 10 (1999) 207-238.

[26] V. Venepally, R. Prasad, Y. Poornachandra, C.G. Kumar, R.C.R. Jala, Synthesis of novel ethyl 1-ethyl-6-fluoro-7-(fatty amido)-1, 4-dihydro-4-oxoquinoline-3-carboxylate derivatives and their biological evaluation, *Bioorg. Med. Chem. Lett*., 26 (2016) 613-617.

[27] D.C. Hooper, Mode of action of fluoroquinolones, *Drugs*, 58 (1999) 6-10.

[28] G.G. Rajulu, H.S.B. Naik, A. Viswanathan, D.S. Agarwal, G. Sambasivam, K.P. Koppolu, Design and synthesis of new N-substituted amino methyl-[1, 2, 3] triazolyl moieties of fluoroquinolones as antibacterial agents, *Med. Chem. Res*., 22 (2013) 3843-3856.

[29] D. Chu, P.B. Fernandes, Structure-activity relationships of the fluoroquinolones, *Antimicrob. Agents Chemother*., 33 (1989) 131-135.

[30] S.A. Breda, A.F. Jimenez-Kairuz, R.H. Manzo, M.E. Olivera, Solubility behavior and biopharmaceutical classification of novel high-solubility ciprofloxacin and norfloxacin pharmaceutical derivatives, *Int. J. Pharm*., 371 (2009) 106-113.

[31] D.E. King, R. Malone, S.H. Lilley. 2000. New classification and update on the quinolone antibiotics. http://www.aafp.org/afp/2000/0501/p2741.html Date of access: 2000.

[32] M. Asif, A mini review on the study of new broad-spectrum antimicrobial fluoroquinolone JNJ-Q2, *J. Chem. & Applic*., 1 (2014) 5-10.

[33] F.J. Smit, R.A. van Biljon, L.-M. Birkholtz, D.D. N'Da, Synthesis and *in vitro* biological evaluation of dihydroartemisinyl-chalcone esters, *Eur. J. Med. Chem*., 90 (2015) 33-44.

[34] B. Meunier, Hybrid Molecules with a Dual Mode of Action: Dream or Reality?, *Acc. Chem. Res.*, 41 (2008) 69-77.

[35] WHO. 2017. Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline, including tuberculosis. http://webcache.googleusercontent.com/search?q=cache:f28U3WiFYVwJ:www.who.int/medicines/news/2017/IAU_AntibacterialAgentsClinicalDevelopment_webfinal_2017_09_19.pdf+&cd=5&hl=en&ct=clnk&gl=za&client=firefox-b Date of access: 30 Jan. 2018.

[36] F. Bellot, F. Coslédan, L. Vendier, J. Brocard, B. Meunier, A. Robert, Trioxaferroquines as new hybrid antimalarial drugs, *J. Med. Chem*., 53 (2010) 4103-4109.

[37] S. Manohar, S.I. Khan, D.S. Rawat, Synthesis, antimalarial activity and cytotoxicity of 4-aminoquinoline–triazine conjugates, *Bioorganic Med. Chem*., 20 (2010) 322-325.

[38] S. Manohar, S.I. Khan, D.S. Rawat, Synthesis of 4-aminoquinoline-1, 2, 3-triazole and 4-aminoquinoline-1, 2, 3-triazole-1, 3, 5-triazine hybrids as potential antimalarial agents, *Chem. Biol. Drug Des*., 78 (2011) 124-136.

[39] K. Dabak, Ö. Sezer, A. Akar, O. Anaç, Synthesis and investigation of tuberculosis inhibition activities of some 1,2,3-triazole derivatives, *Eur. J. Med. Chem*., 38 (2003) 215-218.

[40] R. Kharb, P.C. Sharma, M.S. Yar, Pharmacological significance of triazole scaffold, *J. Enzyme Inhib. Med. Chem*., 26 (2011) 1-21.

[41] Z. Xu, X.-F. Song, Y.-Q. Hu, M. Qiang, Z.-S. Lv, Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: design, synthesis and in vitro anti-mycobacterial evaluation, *Eur. J. Med. Chem*., 138 (2017) 66-71.

[42] R. Kant, V. Singh, G. Nath, S.K. Awasthi, A. Agarwal, Design, synthesis and biological evaluation of ciprofloxacin tethered bis-1, 2, 3-triazole conjugates as potent antibacterial agents, *Eur. J. Med. Chem*., 124 (2016) 218-228.

[43] S.G. Agalave, S.R. Maujan, V.S. Pore, Click chemistry: 1, 2, 3-triazoles as pharmacophores, *Asian J. Chem*., 6 (2011) 2696-2718.

[44] B. Bhukya, A.D. Tangutur, Microbial Biotechnology: Technological Challenges and Developmental Trends, Apple Academic Press, Waretown, NJ, 2017.

[45] X. Xia, Q. Zhang, L. Zhao, Y. Hu, Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: Design, synthesis and *in vitro* anti-mycobacterial evaluation, *Eur. J. Med. Chem*., 138 (2017) 66-71.

[46] J.C. McPherson, R. Runner, T.B. Buxton, J.F. Hartmann, D. Farcasiu, I. Bereczki, E. Rőth, S. Tollas, E. Ostorházi, F. Rozgonyi, Synthesis of osteotropic hydroxybisphosphonate derivatives of fluoroquinolone antibacterials, *Eur. J. Med. Chem*., 47 (2012) 615-618.

[47] Z. Xu, Z.S. Lv, X.F. Song, M. Qiang, Ciprofloxacin-isatin-1H-1, 2, 3-triazole hybrids: Design, synthesis, and in vitro anti-tubercular activity against Mycobacterium tuberculosis, *J. Heterocycl. Chem*., 55 (2018) 97-102.

[48] J.M. Domagala, Structure-activity and structure-side-effect relationships for the quinolone antibacterials, *J. Antimicrob. Chemother*., 33 (1994) 685-706.

[49] A. Qandil, L. Al-Zoubi, A. Al-Bakri, H. Amawi, Q. Al-Balas, A. Alkatheri, A. Albekairy, Synthesis, Antibacterial Evaluation and QSAR of α-Substituted-N4-Acetamides of Ciprofloxacin and Norfloxacin, *J. Antibiot*., 3 (2014) 244-269.

[50] D. Soto-Castro, J.A. Cruz-Morales, M.T.R. Apan, P. Guadarrama, Synthesis of Non-Cytotoxic Poly (Ester-Amine) Dendrimers as Potential Solubility Enhancers for Drugs: Methotrexate as a Case Study, *Molecules J*., 15 (2010) 8082-8097.

[51] A. Bandyopadhyay, N. Agrawal, S.M. Mali, S.V. Jadhav, H.N. Gopi, Tin (II) chloride assisted synthesis of N-protected γ-amino β-keto esters through semipinacol rearrangement, *Org. Biomol. Chem*., 8 (2010) 4855-4860.

[52] S.E. Howson. 2010. Formation of benzyl azide from benzyl bromide; Benzyl azide. http://cssp.chemspider.com/article.aspx?id=408 Date of access: 1 Sept. 2017.

[53] S.K. Dixit, N. Mishra, M. Sharma, S. Singh, A. Agarwal, S.K. Awasthi, V.K. Bhasin, Synthesis and in vitro antiplasmodial activities of fluoroquinolone analogs, *Eur. J. Med. Chem*., 51 (2012) 52-59.

[54] V.K. Gombar, K. Enslein, Assessment of n-Octanol/Water Partition Coefficient: When Is the Assessment Reliable?, *J. Chem. Inf. Comput. Sci*., 36 (1996) 1127-1134.

[55] S.G. Franzblau, M.A. DeGroote, S.H. Cho, K. Andries, E. Nuermberger, I.M. Orme, K. Mdluli, I. Angulo-Barturen, T. Dick, V. Dartois, A.J. Lenaerts, Comprehensive analysis of methods used for the evaluation of compounds against Mycobacterium tuberculosis, *Tuberculosis*, 92 (2012) 453-488.

[56] D. Chu, P.B. Fernandes, Structure-activity relationships of the fluoroquinolones, *Antimicr. Agents Chemoth*., 33 (1989) 131.

[57] G. Tillotson, Quinolones: structure-activity relationships and future predictions, *J. Med. Microbiol*., 44 (1996) 320-324.

[58] N. Suresh, H.N. Nagesh, J. Renuka, V. Rajput, R. Sharma, I.A. Khan, C.S.K.V. Gowri, Synthesis and evaluation of 1-cyclopropyl-6-fluoro-1, 4-dihydro-4-oxo-7-(4-(2-(4-substitutedpiperazin-1-yl) acetyl) piperazin-1-yl) quinoline-3-carboxylic acid derivatives as anti-tubercular and antibacterial agents, *Eur. J. Med. Chem*., 71 (2014) 324-332.

[59] H. Ouellet, J.B. Johnston, P.R. Ortiz de Montellano, Cholesterol catabolism as a therapeutic target in Mycobacterium tuberculosis, *Trends microbiol*., 19 (2011) 530-539.

[60] A. Brzostek, B. Dziadek, A. Rumijowska-Galewicz, J. Pawelczyk, J. Dziadek, Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*, *FEMS Microbiol. Lett*., 275 (2007) 106-112.

[61] B.A. Firestone, M.A. Dickason, T. Tran, Solubility characteristics of three fluoroquinolone ophthalmic solutions in an in vitro tear model, *Int. J. Pharm*., 164 (1998) 119-128.

[62] Y.-L. Fan, J.-B. Wu, X.-W. Cheng, F.-Z. Zhang, L.-S. Feng, Fluoroquinolone derivatives and their anti-tubercular activities, *Eur. J. Med. Chem*., 146 (2018) 554-563.

[63] K. Katsuno, J.N. Burrows, K. Duncan, R.H. Van Huijsduijnen, T. Kaneko, K. Kita, C.E. Mowbray, D. Schmatz, P. Warner, B. Slingsby, Hit and lead criteria in drug discovery for infectious diseases of the developing world, *Nat. Rev. Drug Discov*., 14 (2015) 751-758.

[64] E.W. Sugandhi, C. Slebodnick, J.O. Falkinham, R.D. Gandour, Synthesis and antimicrobial evaluation of water-soluble, dendritic derivatives of epimeric 5α-cholestan-3-amines and 5α-cholestan-3-yl aminoethanoates, *Steroids J*., 72 (2007) 615-626.

[65] Collins, L., Franzblau, S. G., Microplate Alamar Blue Assay versus BACTEC 460 system for high-throughput screening of sompounds against *Mycobacterium tuberculosis* and *Mycobacterium avium*, *Antimicrob. Agents Chemother*, *41* (1997) 1004–1009.

[66] Collins, L., Torrero, M. N., Franzblau, S. G., Green Fluorescent Protein Reporter microplate assay for high-throughput screening of compounds against *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*, 42 (1998) 344–347.

[67] Franzblau, S. G., DeGroote, M. A., Cho, S. H., Andries, K., Nuermberger, E., Orme, I. M., Mdluli, K., Angulo-Barturen, I., Dick, T., Dartois, V., Lenaerts, A. J., Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*, *Tuberculosis*, *92* (2012) 453–488.

[68] Njaria, P.M., Antimycobacterial 2-aminoquinazolinones and benzoxazole-based oximes: synthesis, biological evaluation, physicochemical profiling and supramolecular derivatization (Doctoral thesis). (2017) Retrieved from https://open.uct.ac.za/handle/11427/26954

CHAPTER 4:
SUMMARY AND CONCLUSION

Statistics is abysmal, tuberculosis (TB) is the most lethal infectious diseases worldwide resulting in 1.7 million deaths and 10.4 million clinical cases in 2016 alone, and is in a continual increase (WHO, 2017). Moreover, TB is ranked in ninth place as leading cause of death globally, surpassing HIV/AIDS (human immunodeficiency virus, acquired immunodeficiency syndrome) (WHO, 2017).

TB can be caused by one of five species of the Mycobacterium tuberculosis complex (Delogu, 2013; Jordae, 2011). However, *Mycobacterium tuberculosis* (*Mtb*) is the most common (i.e. 98.8% (Assam *et al.*, 2013)) causative pathogen of TB in humans. According to the WHO, a quarter of the world's population is infected with *Mtb*, without them knowing it (WHO, 2017). The bacilli is spread through the air when an infected individual with active pulmonary TB expels the bacteria *via* coughing or sneezing (WHO, 2017).

Most people can be treated and cured from TB with a combination of first-line antitubercular drugs, such as rifampicin, isoniazid, pyrazinamide and ethambutol (Kanabus, 2017b). However, most of these antitubercular drugs were developed years ago (Kanabus, 2017a) and pathogen resistance to almost all available drugs has been established and recognised. For instance, resistance against rifampicin and isoniazid are known as multi-drug resistant TB (MDR-TB) and additional resistance to second-line antitubercular drugs (such as fluoroquinolones and injectable aminoglycosides) is known as extensively drug-resistant TB (XDR-TB) (Pooran *et al.*, 2013). Treatment of drug-resistant TB is complex, prolonged, and the chemotherapeutic options are limited. The latter named is evident in the sporadic rise of resistance against all antitubercular drugs called totally drug-resistant (TDR-TB), which in itself obligates the design of new TB drugs. Furthermore, drug-resistance drastically worsens the TB-mortality rate further. Thus, a lengthy treatment regimen (in both drug-susceptible and –resistant TB), a handful of drugs required daily for treatment and resistance create an overwhelming demand for novel TB drugs (Wright & Sutherland, 2007).

The most promising strategy to develop new TB drugs in a short timeframe is by modifying old drugs with established activity (Xu *et al.*, 2017). The fluoroquinolone class of drugs, including ciprofloxacin (CPX), is the most effective second-line anti-TB drugs (Mitnick *et al.*, 2009), however known to elicit high toxic side effects during extended use (Olcay *et al.*, 2011; Stahlmann & Lode, 2013; Verma *et al.*, 2009).

CPX is additionally known for its lowest in-class solubility (Firestone *et al.*, 1998) as it is zwitterionic, which leads to a restriction in its oral bioavailability (Surov *et al.*, 2015).

However, the prototypical fluoroquinolone, ciprofloxacin (CPX), has newer-generation fluoroquinolones (such as sparfloxacin, gatifloxacin, moxifloxacin, gemifloxacin, grepafloxacin, and caderofloxacin) built on its nucleus (Asif, 2014) due to the optimal structural groups it retains in its molecular architecture (Sharma *et al.*, 2009). The latter named and the fact that fluoroquinolones are currently used to treat MDR-TB (WHO, 2016) formed our basis to construct new fluoroquinolones from the framework of ciprofloxacin to address the aforementioned shortcomings and increase its potency against resistant TB.

CPX contains the quinolone pharmacophore and a fluorine atom in position 6 of that moiety; which awards it its broad-spectrum of activity (Andersson & MacGowan, 2003). Additionally, the cyclopropyl and piperazine rings in positions N-1 and C-7, further potentiates its antibacterial activities (Sharma *et al.*, 2009). Structure-activity relationship (SAR) studies of the fluoroquinolone molecule have determined that substituent changes made in positions N-15 and C-11 greatly control the antibacterial spectrum, potency, and pharmacokinetic profile (Andersson & MacGowan, 2003; Asif, 2014; Chu & Fernandes, 1989; Tillotson, 1996). The presence of 5- or 6-membered heterocyclic rings containing N atom boosts the activity of fluoroquinolones (Tillotson, 1996). Also, the replacement or deprotonation of the carboxylic acid in position C-3, with either an isothiazole group or certain esters affords derivatives with increased antibacterial activity in comparison to the parent drugs, e.g. CPX (Sharma *et al.*, 2009).

Another common and well-established strategy in the search for new drugs is molecular hybridisation. A hybrid molecule is a single entity obtained by covalently linking two distinct chemical pharmacophores with multiple effects. These molecules are introduced in anticipation that they may overcome drug resistance by working in synergy (Meunier, 2007). The 1,2,3-triazole scaffold is responsible for a broad spectrum of biological properties such as antimicrobial, anti-HIV, anticancer, anti-inflammatory and antitubercular (Emmadi *et al.*, 2015). Thus, by hybridising CPX and 1,2,3-triazole, two active scaffolds, i.e. a more antitubercular active hybrid molecule may be produced.

Furthermore, it has been suggested that increasing the lipophilicity and/or bulkiness of fluoroquinolones in position C-7 might increase antibacterial activity (Venepally *et al.*, 2016; Xu *et al.*, 2017). TB treatment is hindered by the unique structure and chemical composition of the *mycobacterium's* cell wall, which obstructs drugs entry into the bacterium and thereby nullifying the antibiotics (Brennan & Nikaido, 1995). Thus, it has been suggested structural alterations of the fluoroquinolone moiety in position C-7 resulting in increased lipophilicity and/or bulkiness can

enhance the antimycobacterial activity (Fan *et al.*, 2018; Venepally *et al.*, 2016; Xu *et al.*, 2017). Such derivatives would possess enhanced ability to penetrate and concentrate in macrophages and wherein exhibit bactericidal effect (Ginsburg *et al.*, 2003; Shindikar & Viswanathan, 2005).

Persuaded by this, a highly lipophilic molecule, such as cholesterol, was chemically linked to the active drug, that is ciprofloxacin and the ciprofloxacin-1,2,3-triazole hybrid-drug, anticipating good penetration of mycobacteria cell wall. Indeed, *mce4*-transporter has been indicated to transport cholesterol into *Mtb* (Ouellet *et al.*, 2011). Moreover, *Mtb* utilises cholesterol as carbon source and is thus the virulence driving factor behind *Mtb* as demonstrated by Brzostek *et al.* (2007). Hence, the conjugation of this dietary molecule (i.e. cholesterol) with a second-line anti-TB drug (i.e. ciprofloxacin). The cholesterol part of the molecule will attract mycobacteria for its use as carbon source and thereby mislead mycobacteria for the intake of the active drug (i.e. ciprofloxacin).

The aim of this study was to investigate novel ciprofloxacin derivatives through the design and synthesis of analogues and hybrid molecules with the ultimate goal to develop potent, effective, safer, and affordable new antitubercular agents as potential replacements for the current drugs in clinical use.

To achieve this aim, the following objectives were set:

- Synthesis and characterisation (using IR, NMR, MS, and melting points) of novel analogues of ciprofloxacin and ciprofloxacin-triazole hybrids.
- Assessment of the *in vitro* antitubercular activity of the synthesised compounds, against the laboratory virulent *Mtb* H37Rv strain, in Middlebrook 7H9 Broth media and supplemented with Middlebrook albumin-dextrose-catalase supplement.
- Evaluation of the cytotoxicity profiles of the active compounds using mammalian cell lines, such as human fetal lung fibroblast - (WI38), human embryonic kidney cells (HEK-293), and Chinese hamster ovary cell lines (CHO).

The synthesis of ciprofloxacin analogues was carried out in three sub-series to highlight the difference of functional groups namely carboxylic acid, ethyl ester and 4-methylpiperazinyl amide in position 11 of ciprofloxacin CPX scaffold. The carboxylic acids were either amidated using acyl/carbamoyl/sulfonyl chloride/cholesteryl chloroformate (compounds **9** – **12**) or N-15 substituted using propargyl bromide (compound **13**) when applying a method reported by Qandil *et al.* (2014). The synthesis of the alkyne intermediate **13** was optimised by changing the solvent system from dry dichloromethane to monoglyme-DMSO mixture (5:1) upon reflux to improve solubility which beneficially results in an increase of its yield from 54 to 66%. All acids were

subsequently converted to the ethyl esters **14** – **18** (in poor yields of 4 – 23%) and to the piperazinyl amides **19** – **23** (in acceptable yields of 39 – 95%) when utilising literature reported methods (Bandyopadhyay *et al.*, 2010; Soto-Castro *et al.*, 2010). The compounds were isolated following purification by silica gel chromatography.

The synthesis of hybrid molecules was carried out in only two subseries, namely carboxylic acid and 4-methylpiperazinyl amide in position 11 of CPX scaffold. They were obtained in a two-step process. Firstly, the nucleophile substitution of benzyl bromides with sodium azide afforded azido intermediates in good yields (70 – 100%) by adopting the synthetic route described by Howson (2010). Secondly, the azido intermediates were subjected Huisgen copper alkyne-azide cycloaddition ‘‘click’’ reaction in stereoselective manner by employing the procedure described by (Dixit *et al.*, 2012) with either alkynyl acid **13** to give the 1,4-disubstituted-1,2,3-triazole hybrids **26** - **32** or with alkyne **18** to afford **33** - **39**. All hybrids were isolated in poor to good yields (12 – 67%) after purification by precipitation in ethyl acetate followed by column chromatography on silica gel.

The structures of all synthesised compounds were confirmed by IR, ^{1}H and ^{13}C NMR, and HRMS.

For the *in vitro* antitubercular assessment, the *Mtb* H37Rv strain was available for screening in Middlebrook 7H9 broth media. Of all the synthesised compounds, most of the acidic compounds from the first (analogues **9**, **10**, **11**, and **13**) and second series (hybrids **26**, **28**, **29**, **30**, and **32**) were validated hit compounds, as classified according to the literature (Katsuno *et al.*, 2015), with H37Rv MIC_{90} values lower than 10 µM and cytotoxicity selectivity indices SI values greater than 10. The esters showed mixed activities and all amides were inactive.

The highly lipophilic cholesteryl moiety containing derivatives were mostly inactive. However, carboxylic acid hybrid molecule 32 containing the bulky lipophilic moiety was the most active compound from the second series with MIC_{90} value of 2.06 µM comparable to 1.78 µM of CPX confirming that the antimycobacterial activity is indeed favoured by the acid functional group and that cholesterol might have played a role in the transport of the hybrid molecule across mycobacterium’s cell wall.

Furthermore, the electronic effect of the substituent on the phenyl ring in the non-cholesteryl hybrids seem to influence the antimycobacterial activity. The less the electron-donating – or withdrawing effect the more active the hybrid.

The calculated clogP values of synthesised compounds were mostly found to be in the 2 – 5 range of favourable drug-likeness. However, the physical features, such as an increase or

decrease in the hydrophilicity or lipophilicity of compounds did not influence activity, indicating that structural specificity were indeed at play.

In summary, the synthesis of ciprofloxacin analogues and ciprofloxacin-1,2,3-triazole hybrids, together with structural modifications in position C-11 were successfully achieved to examine the functional group essential for activity and the impact of improved aqueous solubility. These results enriched the SAR of fluoroquinolones. The correlation between C-11 substitution and antimycobaterium activity was the same as described in literature, i.e. substitution of the carboxylic acid diminishes activity (Andersson & MacGowan, 2003; Chu & Fernandes, 1989; Sharma *et al.*, 2009), even though there was an improvement in solubility or an increase in the lipophilicity of synthesised compounds. This further substantiates the fact that the carboxylic acid is crucial for drug-enzyme interaction. However, no significantly improved antimycobacterial activities were achieved when these compounds are compared to the parent drug (e.g. ciprofloxacin).

BIBLIOGRAPHY

Andersson, M.I. & MacGowan, A.P. 2003. Development of the quinolones. *Journal of antimicrobial chemotherapy*, 51(1):1-11.

Asif, M. 2014. A mini review on the study of new broad-spectrum antimicrobial fluoroquinolone JNJ-Q2. *Journal of chemistry & applications*, 1(1):5-10.

Assam, J.P.A., Beng, V.P., Cho-Ngwa, F., Toukam, M., Ngoh, A.-A.I., Kitavi, M., Nzuki, I., Nyonka, J.N., Tata, E., Tedom, J.C., Skilton, R.A., Pelle, R. & Titanji, V.P.K. 2013. *Mycobacterium tuberculosis* is the causative agent of tuberculosis in the southern ecological zones of Cameroon, as shown by genetic analysis. *Biomed central infectious diseases*, 13:431-431.

Bandyopadhyay, A., Agrawal, N., Mali, S.M., Jadhav, S.V. & Gopi, H.N. 2010. Tin (II) chloride assisted synthesis of N-protected γ-amino β-keto esters through semipinacol rearrangement. *Organic & biomolecular chemistry*, 8(21):4855-4860.

Brennan, P.J. & Nikaido, H. 1995. The envelope of mycobacteria. *Annual review of biochemistry*, 64(1):29-63.

Brzostek, A., Dziadek, B., Rumijowska-Galewicz, A., Pawelczyk, J. & Dziadek, J. 2007. Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*. *Federation of european microbiological society microbiology letters*, 275(1):106-112.

Chu, D. & Fernandes, P.B. 1989. Structure-activity relationships of the fluoroquinolones. *Antimicrobial agents and chemotherapy*, 33(2):131-135.

Delogu, G., Sali, M. & Fadda, G. 2013. The biology of *mycobacterium tuberculosis* infection. *Mediterranean journal of hematology and infectious diseases*, 5(1):e2013070.

Dixit, S.K., Mishra, N., Sharma, M., Singh, S., Agarwal, A., Awasthi, S.K. & Bhasin, V.K. 2012. Synthesis and in vitro antiplasmodial activities of fluoroquinolone analogs. *European journal of medicinal chemistry*, 51:52-59.

Emmadi, N.R., Bingi, C., Kotapalli, S.S., Ummanni, R., Nanubolu, J.B. & Atmakur, K. 2015. Synthesis and evaluation of novel fluorinated pyrazolo-1, 2, 3-triazole hybrids as antimycobacterial agents. *Bioorganic & medicinal chemistry letters*, 25(15):2918-2922.

Fan, Y.-L., Wu, J.-B., Cheng, X.-W., Zhang, F.-Z. & Feng, L.-S. 2018. Fluoroquinolone derivatives and their anti-tubercular activities. *European journal of medicinal chemistry*, 146:554-563.

Firestone, B.A., Dickason, M.A. & Tran, T. 1998. Solubility characteristics of three fluoroquinolone ophthalmic solutions in an in vitro tear model. *International journal of pharmaceutics*, 164(1):119-128.

Ginsburg, A.S., Grosset, J.H. & Bishai, W.R. 2003. Fluoroquinolones, tuberculosis, and resistance. *The lancet infectious diseases*, 3(7):432-442.

Howson, S.E. 2010. Formation of benzyl azide from benzyl bromide; Benzyl azide. http://cssp.chemspider.com/article.aspx?id=408 Date of access: 1 Sept. 2017.

Jordae, L.V., O. 2011. Tuberculosis: New Aspects of an Old Disease. *Internation journal of cell biology*.

Kanabus, A. 2017a. TB Drugs – first line, drug names, regimens. https://www.tbfacts.org/tb-drugs/ Date of access: 30 Jan. 2018.

Kanabus, A. 2017b. TB treatment – curing TB, failure, relapse & recurrence. https://www.tbfacts.org/tb-treatment/ Date of access: 30 Jan. 2018.

Katsuno, K., Burrows, J.N., Duncan, K., Van Huijsduijnen, R.H., Kaneko, T., Kita, K., Mowbray, C.E., Schmatz, D., Warner, P. & Slingsby, B. 2015. Hit and lead criteria in drug discovery for infectious diseases of the developing world. *Nature reviews drug discovery*, 14(11):751.

Meunier, B. 2007. Hybrid molecules with a dual mode of action: dream or reality? *Accounts of chemical research*, 41(1):69-77.

Mitnick, C.D., McGee, B. & Peloquin, C.A. 2009. Tuberculosis pharmacotherapy: strategies to optimize patient care. *Expert opinion on pharmacotherapy*, 10(3):381-401.

Olcay, E., Beytemur, O., Kaleagasioglu, F., Gulmez, T., Mutlu, Z. & Olgac, V. 2011. Oral toxicity of pefloxacin, norfloxacin, ofloxacin and ciprofloxacin: comparison of biomechanical and histopathological effects on Achilles tendon in rats. *The journal of toxicological sciences*, 36(3):339-345.

Ouellet, H., Johnston, J.B. & Ortiz de Montellano, P.R. 2011. Cholesterol catabolism as a therapeutic target in Mycobacterium tuberculosis. *Trends in microbiology*, 19(11):530-539.

Pooran, A., Pieterson, E., Davids, M., Theron, G. & Dheda, K. 2013. What is the cost of diagnosis and management of drug resistant tuberculosis in South Africa? *Public library of sciences*, 8(1):e54587.

Qandil, A., Al-Zoubi, L., Al-Bakri, A., Amawi, H., Al-Balas, Q., Alkatheri, A. & Albekairy, A. 2014. Synthesis, Antibacterial Evaluation and QSAR of α-Substituted-N4-Acetamides of Ciprofloxacin and Norfloxacin. *Antibiotics*, 3(3):244-269.

Sharma, P.C., Jain, A. & Jain, S. 2009. Fluoroquinolone antibacterials: a review on chemistry, microbiology and therapeutic prospects. *Acta poloniae pharmaceutica*, 66(6):587-604.

Shindikar, A.V. & Viswanathan, C.L. 2005. Novel fluoroquinolones: design, synthesis, and in vivo activity in mice against Mycobacterium tuberculosis H37Rv. *Bioorganic & medicinal chemistry letters*, 15(7):1803-1806.

Soto-Castro, D., Cruz-Morales, J.A., Apan, M.T.R. & Guadarrama, P. 2010. Synthesis of non-cytotoxic poly (ester-amine) dendrimers as potential solubility enhancers for drugs: methotrexate as a case study. *Molecules J.*, 15(11):8082-8097.

Stahlmann, R. & Lode, H.M. 2013. Risks associated with the therapeutic use of fluoroquinolones. *Expert opinion on drug safety expert*, 12(4):497-505.

Surov, A.O., Manin, A.N., Voronin, A.P., Drozd, K.V., Simagina, A.A., Churakov, A.V. & Perlovich, G.L. 2015. Pharmaceutical salts of ciprofloxacin with dicarboxylic acids. *European journal of pharmaceutical sciences*, 77:112-121.

Tillotson, G. 1996. Quinolones: structure-activity relationships and future predictions. *Journal of medical microbiology*, 44(5):320-324.

Venepally, V., Prasad, R., Poornachandra, Y., Kumar, C.G. & Jala, R.C.R. 2016. Synthesis of novel ethyl 1-ethyl-6-fluoro-7-(fatty amido)-1, 4-dihydro-4-oxoquinoline-3-carboxylate derivatives and their biological evaluation. *Bioorganic & medicinal chemistry letters*, 26(2):613-617.

Verma, R., Dhamija, R., Batts, D.H., Ross, S.C. & Loehrke, M.E. 2009. Moxifloxacin induced fatal hepatotoxicity in a 72-year-old man: a case report. *Cases journal*, 2(1):8063-8066.

WHO. 2016. WHO treatment guidelines for drug-resistant tuberculosis. http://apps.who.int/iris/handle/10665/250125/ Date of access: 15 Sept. 2017.

WHO. 2017. Global tuberculosis report 2017. http://www.who.int/tb/publications/global_report/en/ Date of access: 18 Nov. 2017.

Wright, G.D. & Sutherland, A.D. 2007. New strategies for combating multidrug-resistant bacteria. *Trends in molecular medicine*, 13(6):260-267.

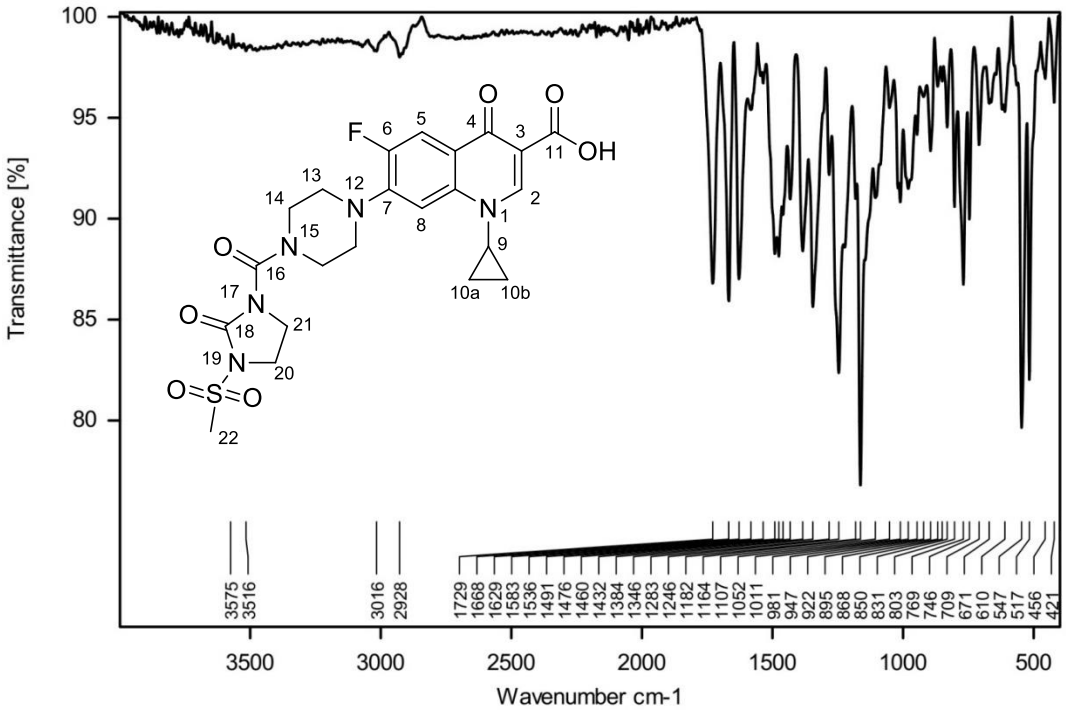
Xu, Z., Song, X.-F., Hu, Y.-Q., Qiang, M. & Lv, Z.-S. 2017. Azide-alkyne cycloaddition towards 1H-1, 2, 3-triazole-tethered gatifloxacin and isatin conjugates: design, synthesis and in vitro anti-mycobacterial evaluation. *European journal of medicinal chemistry*, 138:66-71.

ANNEXURE A

ANALYTICAL DATA FOR CHAPTER 3

Compound 9

IR



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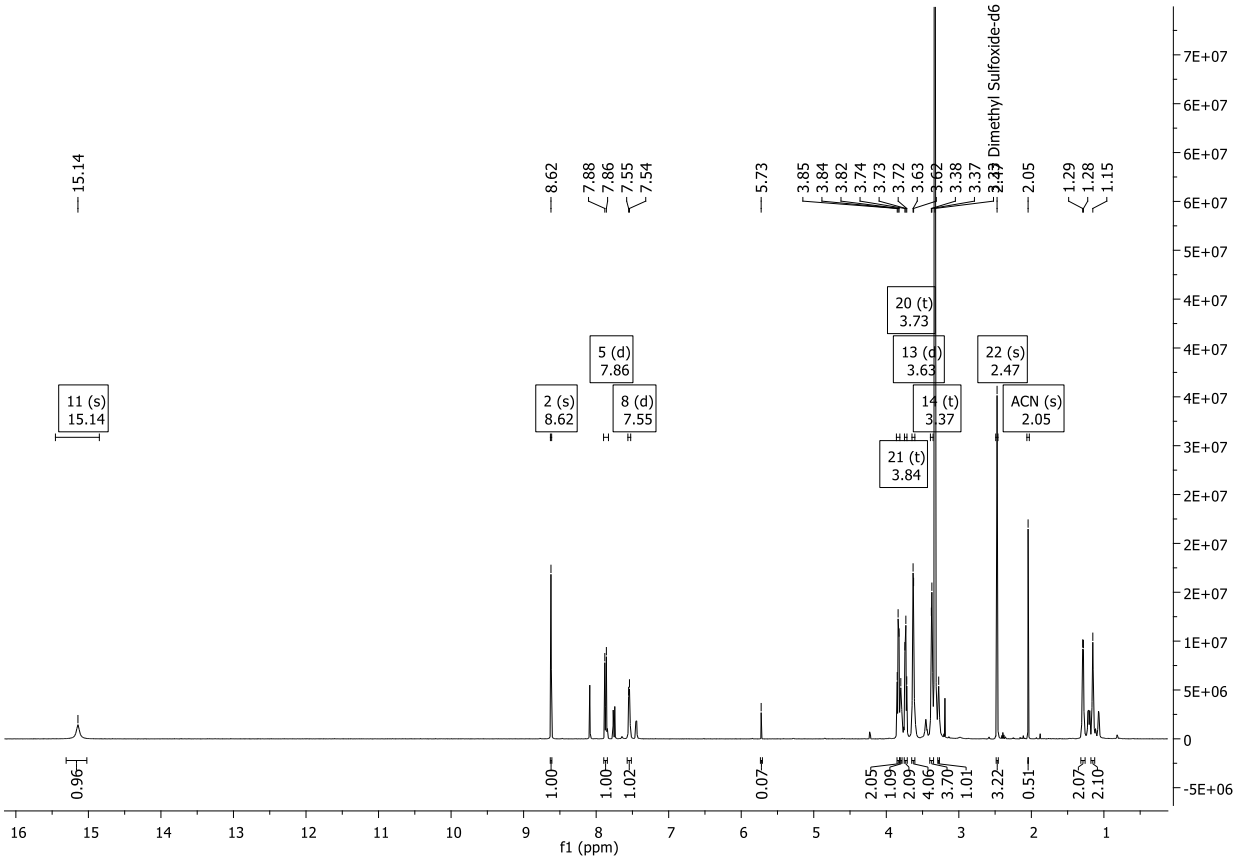
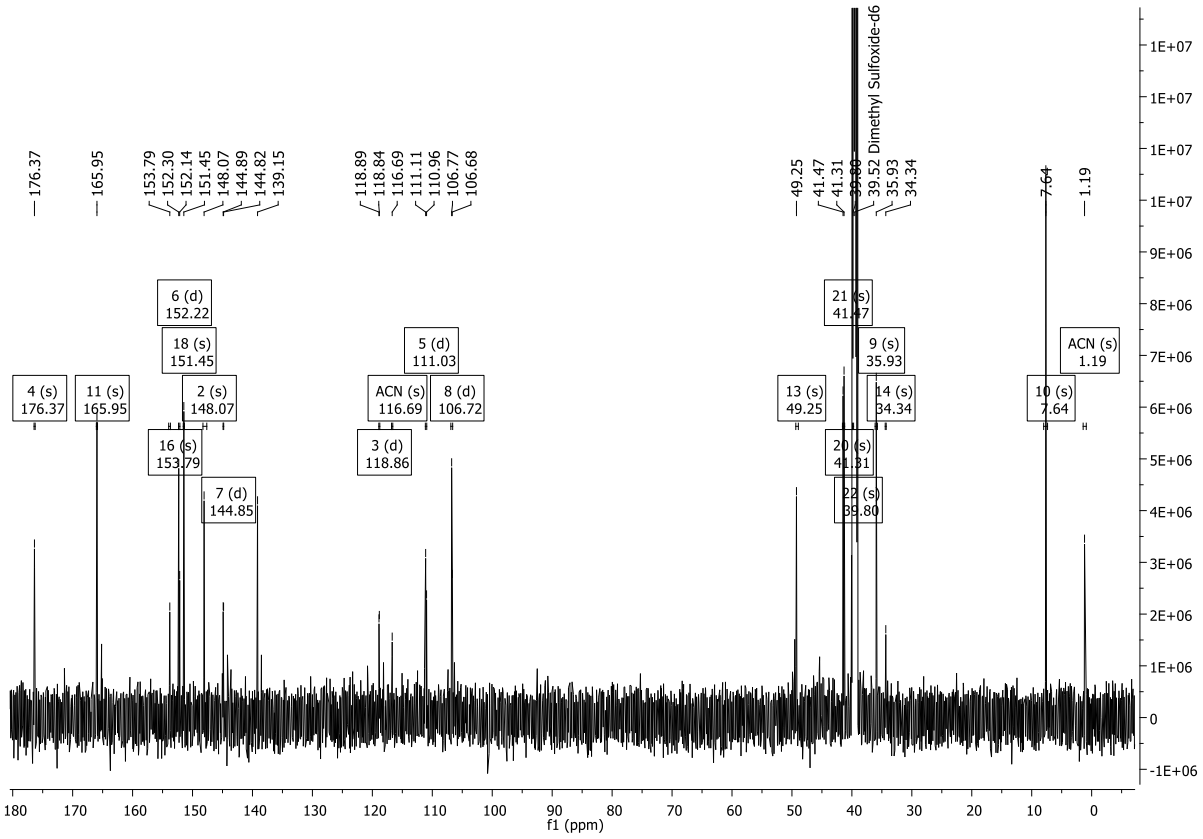


^{13}C in DMSO



HRMS

Acquisition Parameter

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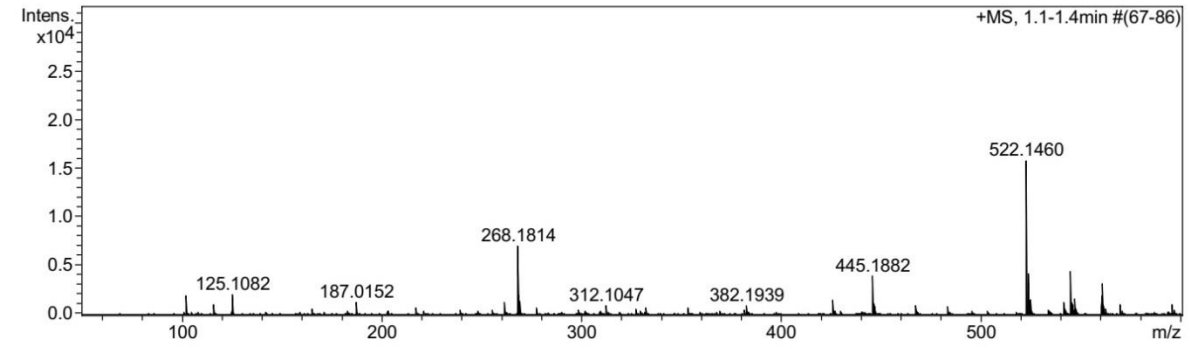

Compound 10

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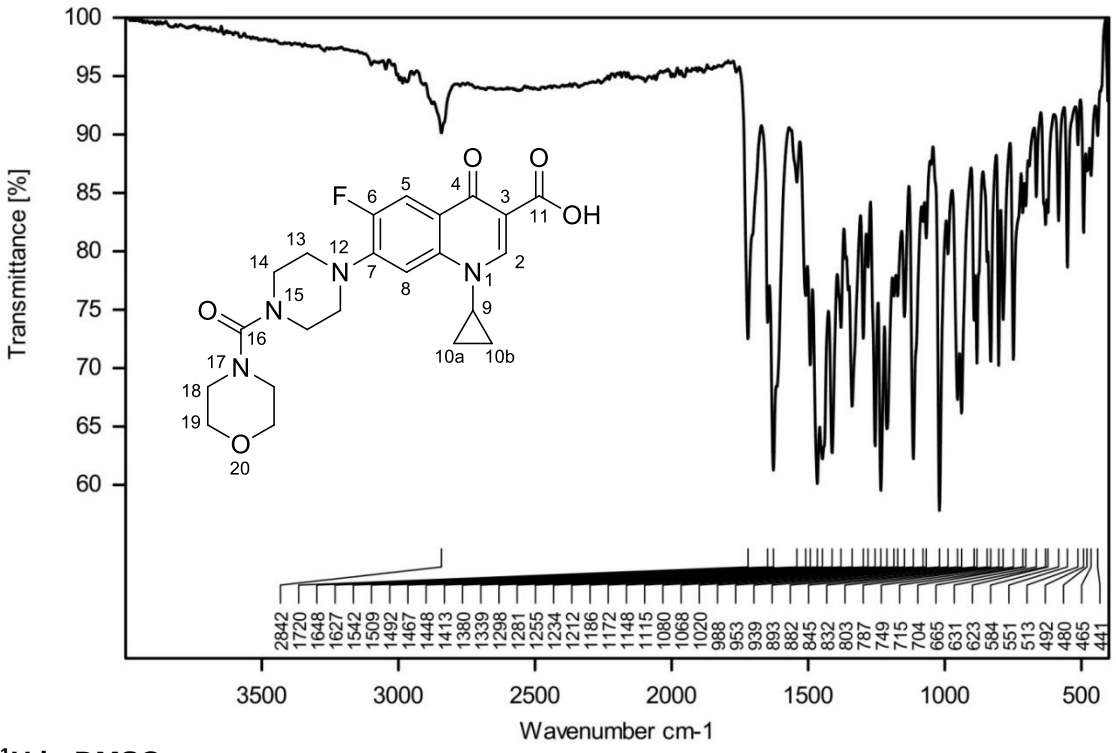

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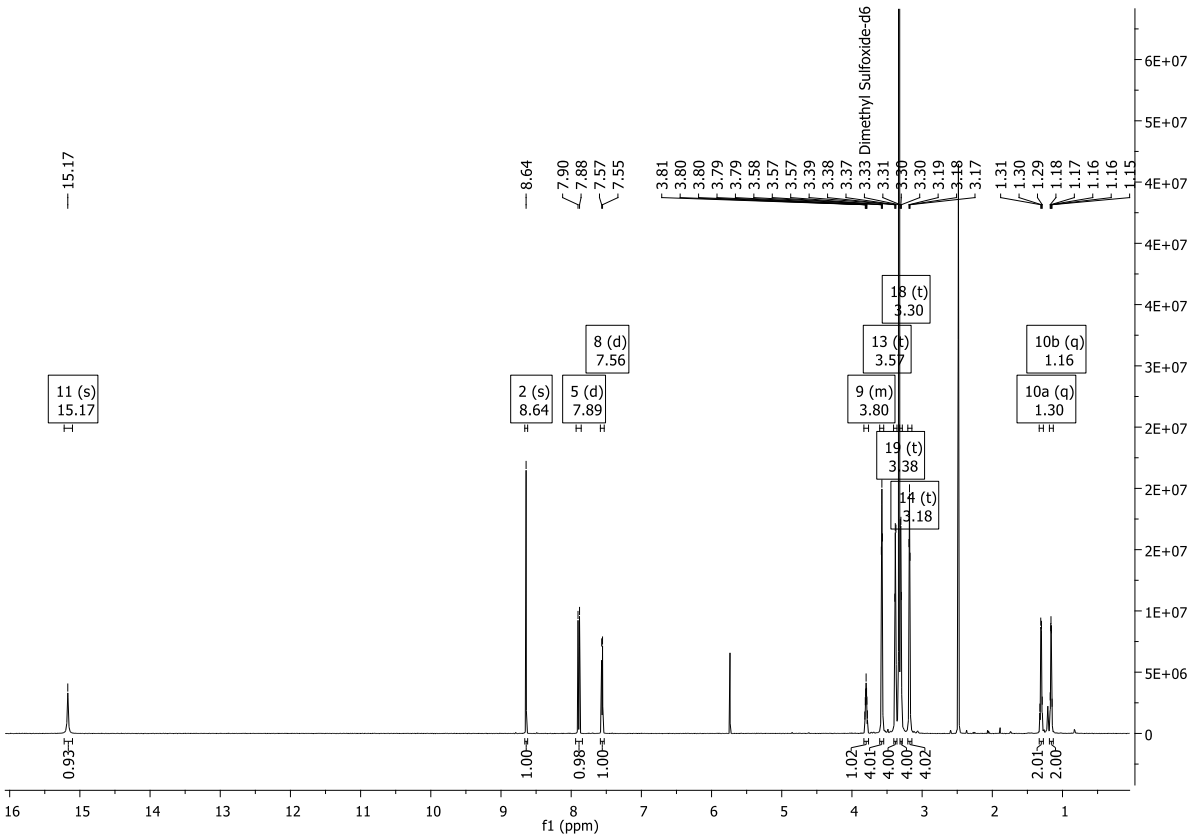

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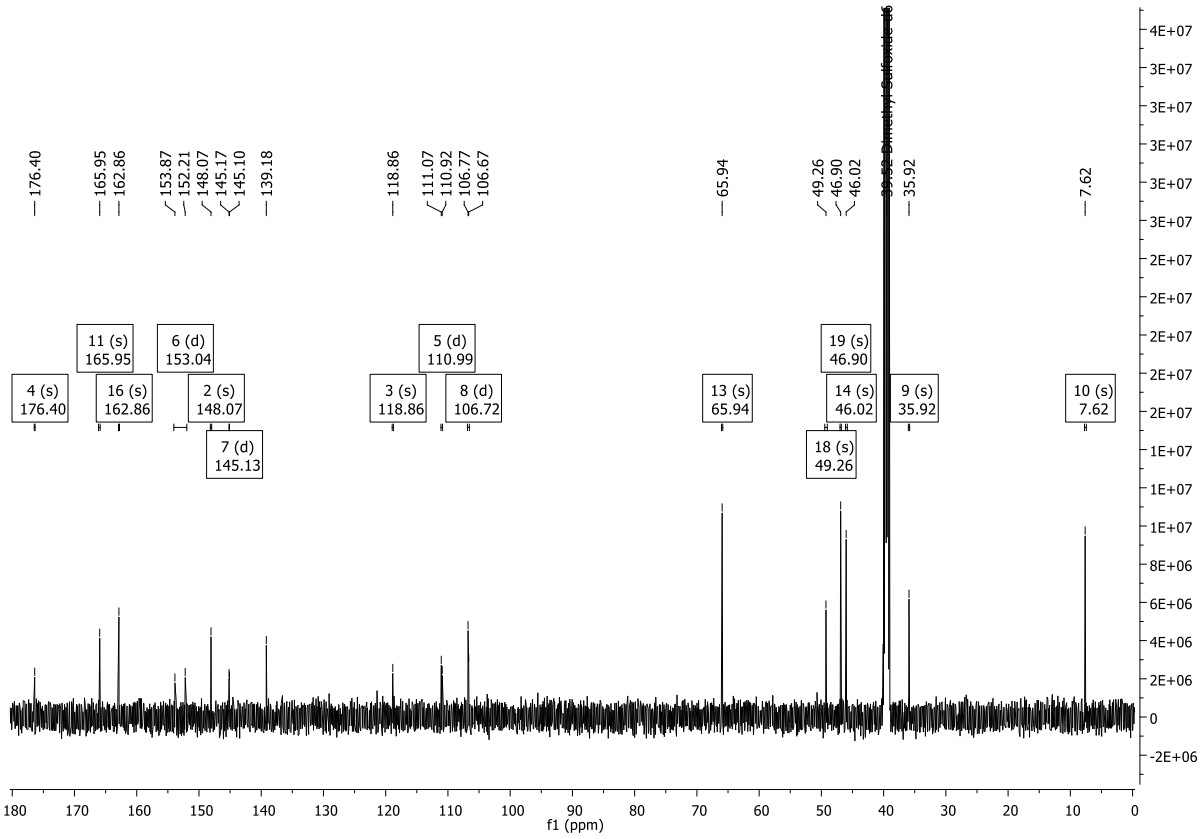

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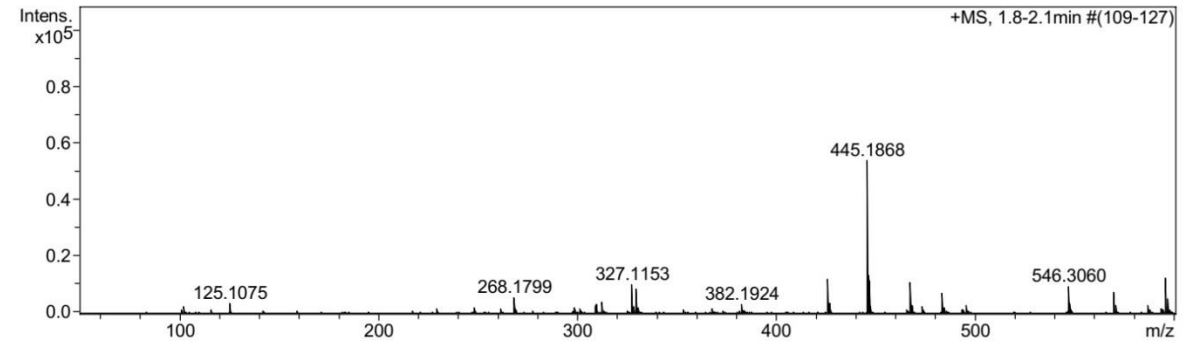

Compound 11

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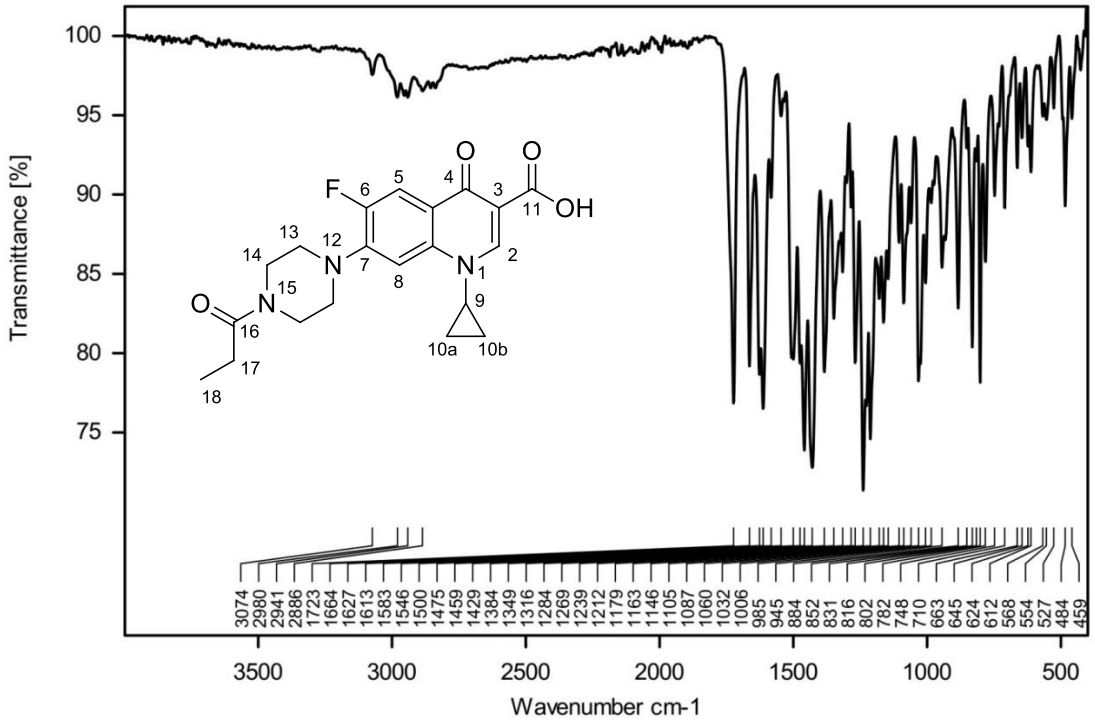

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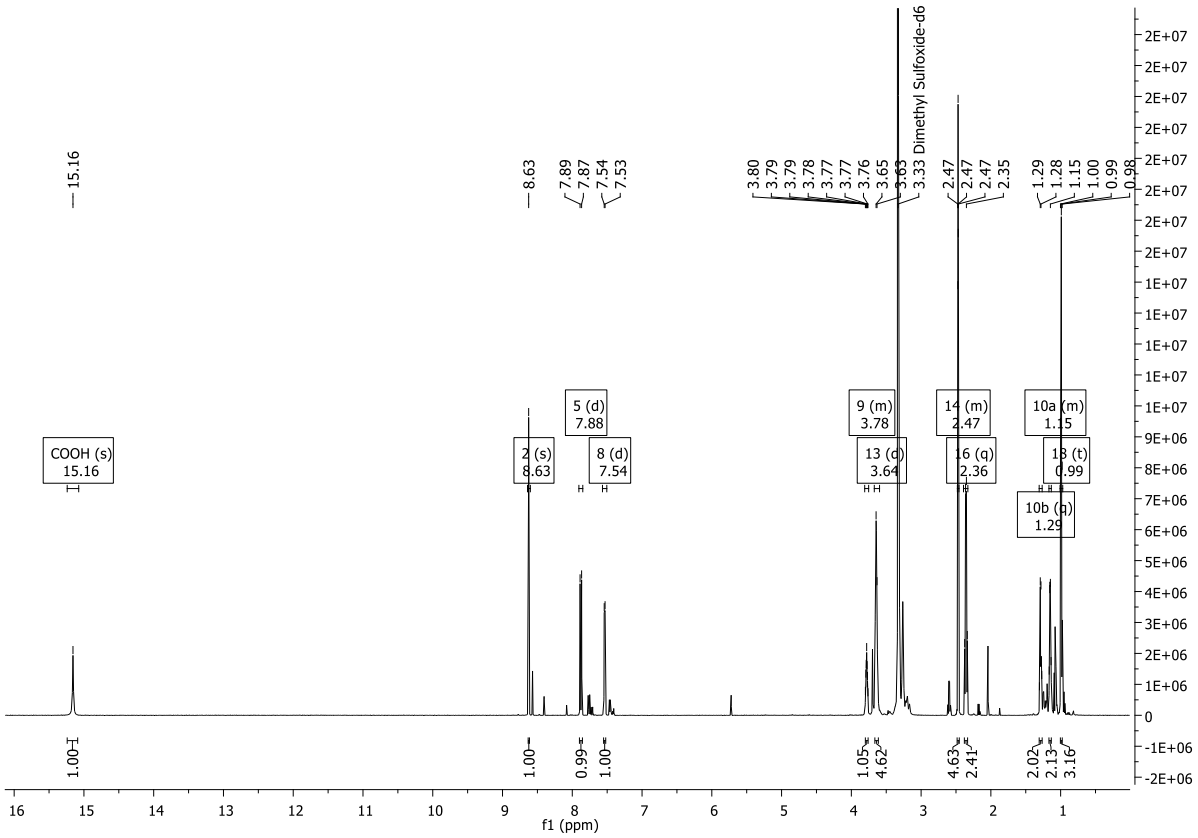

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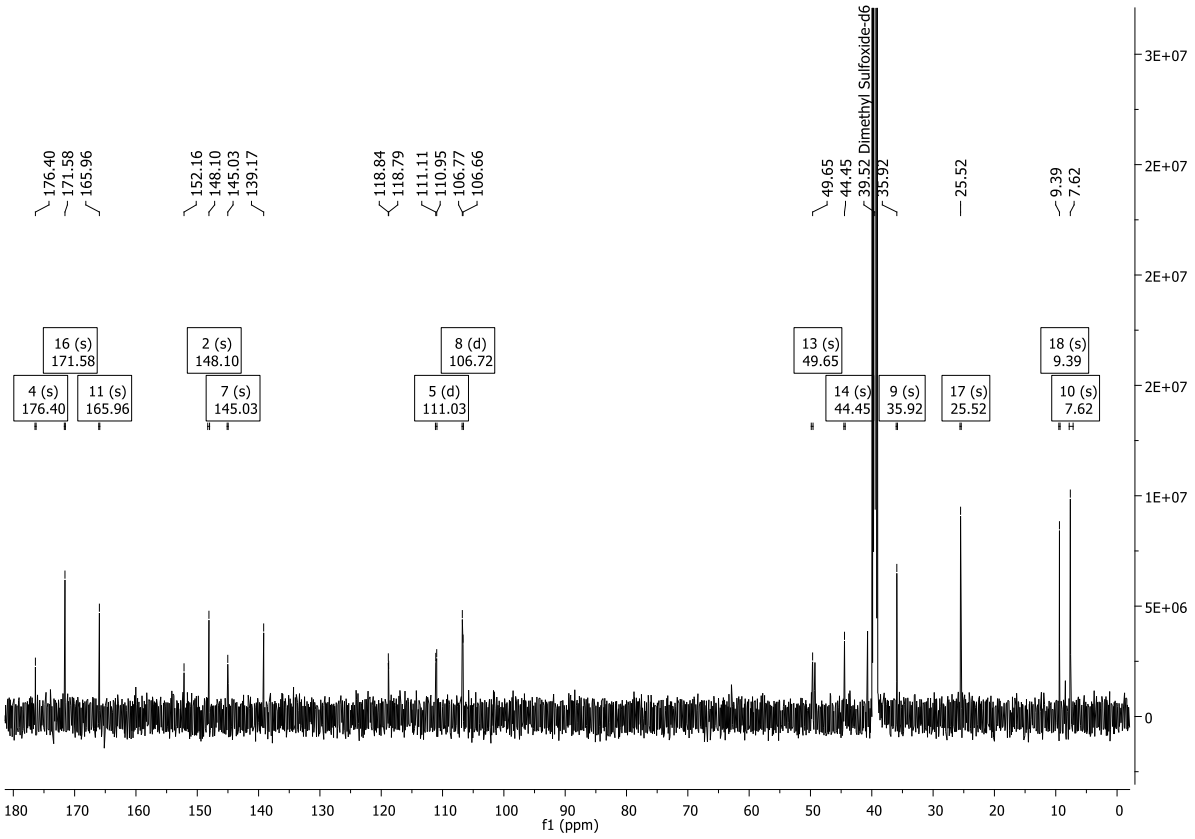

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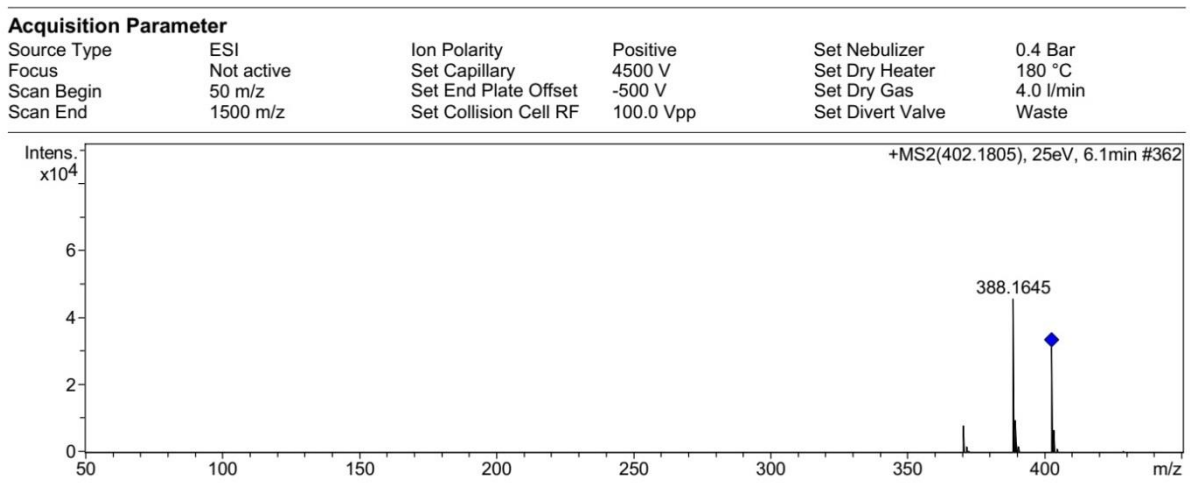

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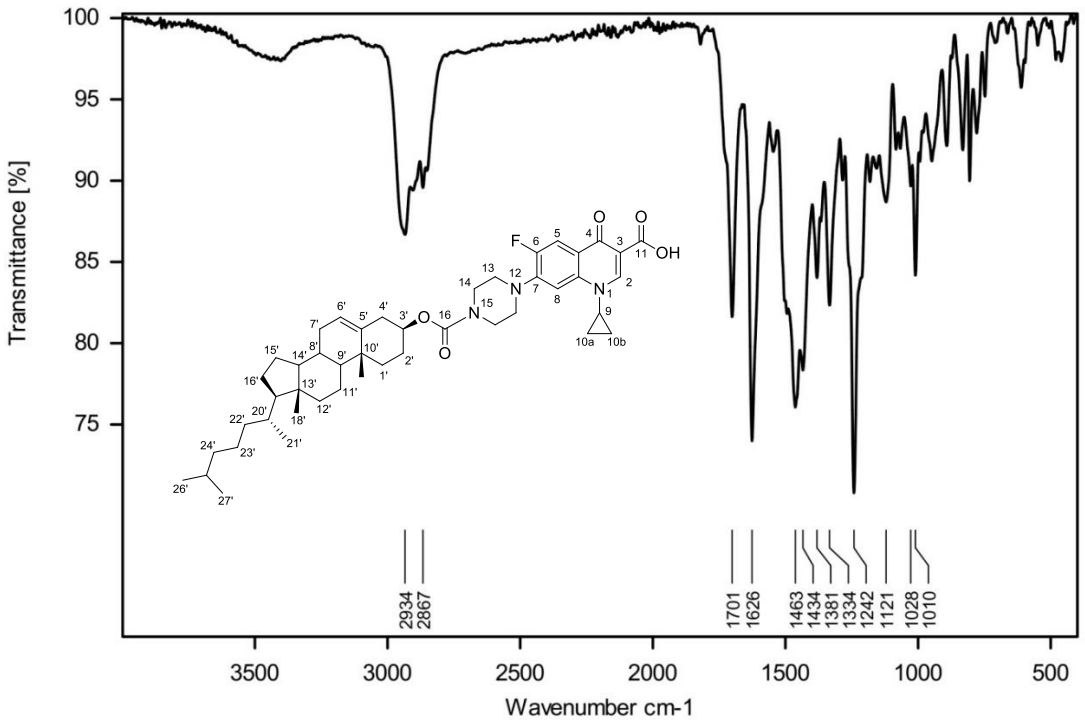

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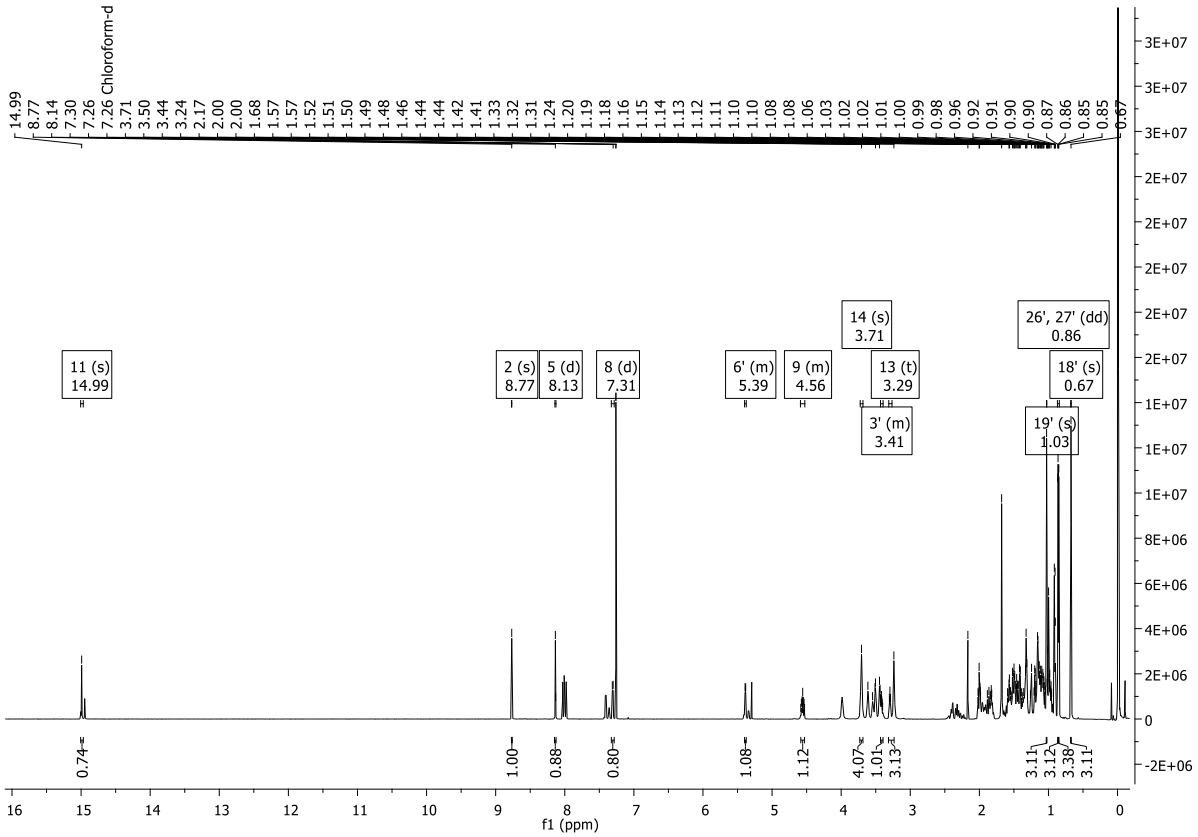

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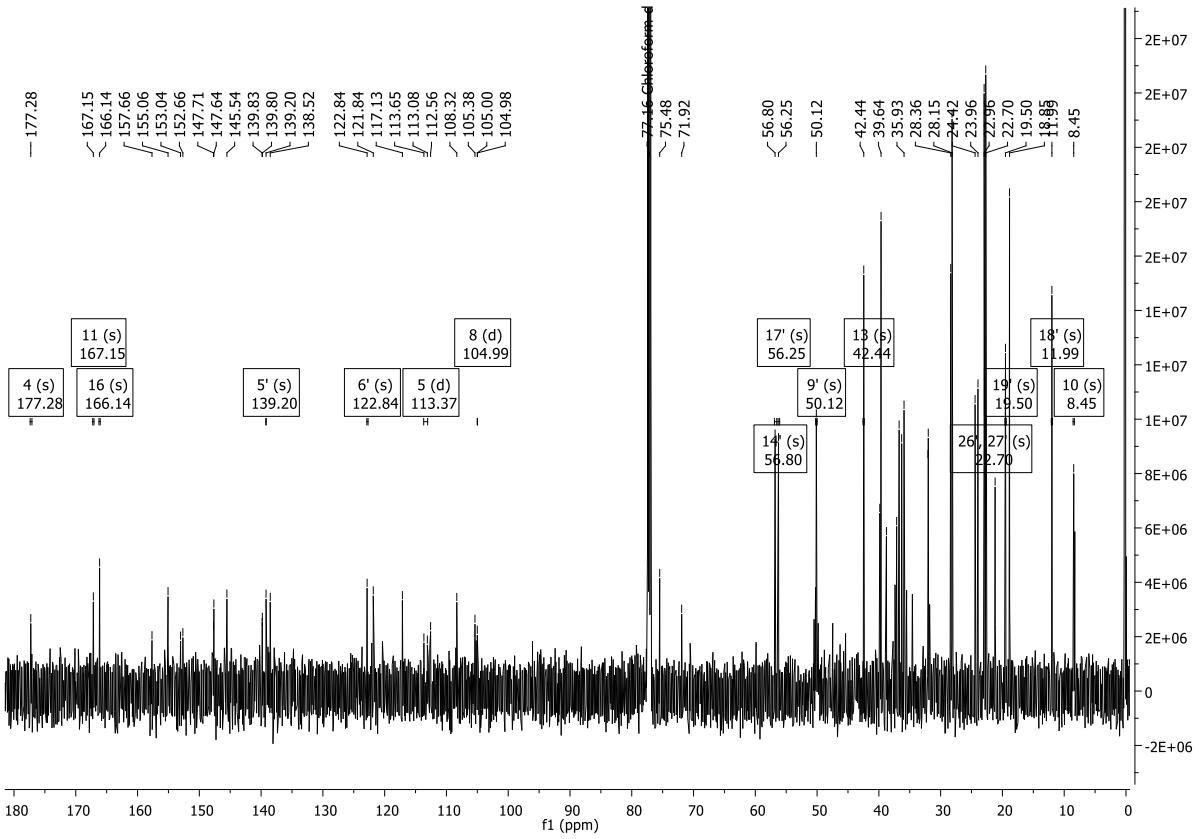

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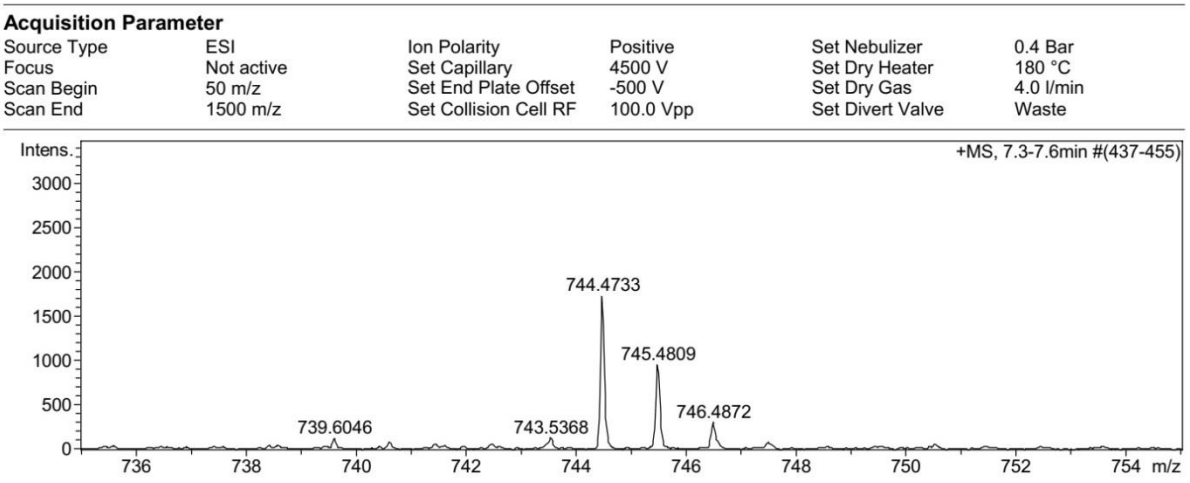

Compound 13

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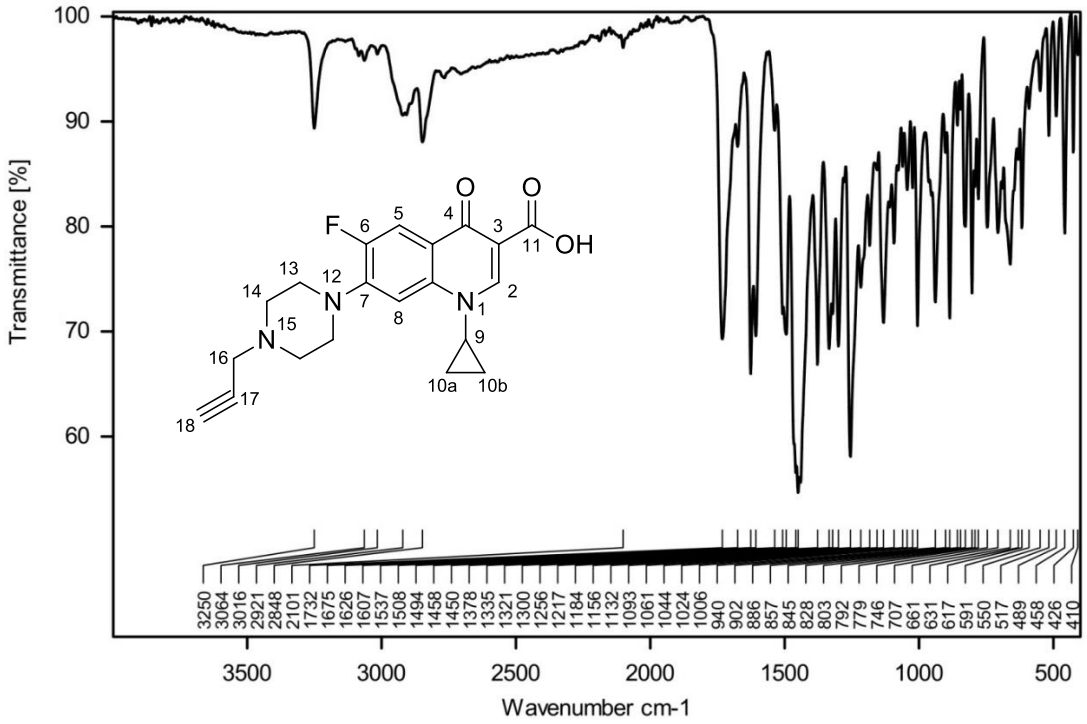

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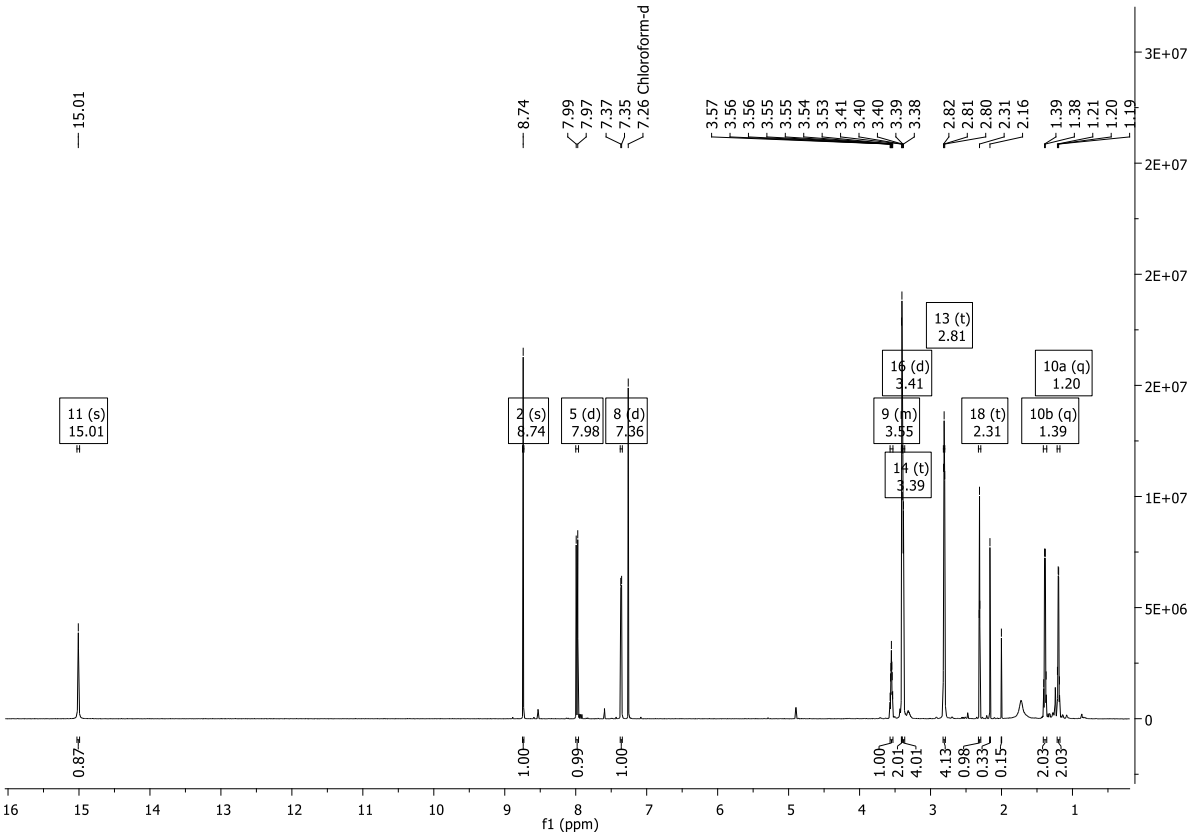

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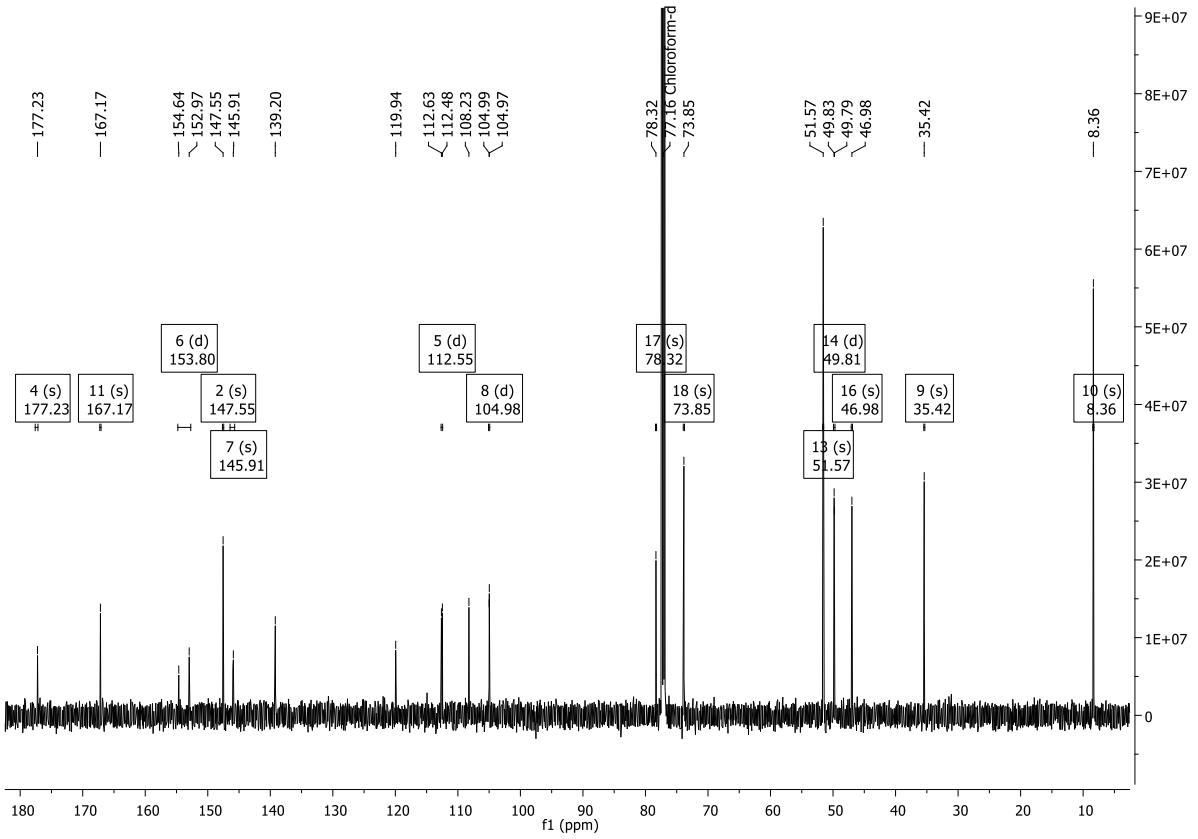

HRMS

Acquisition Parameter

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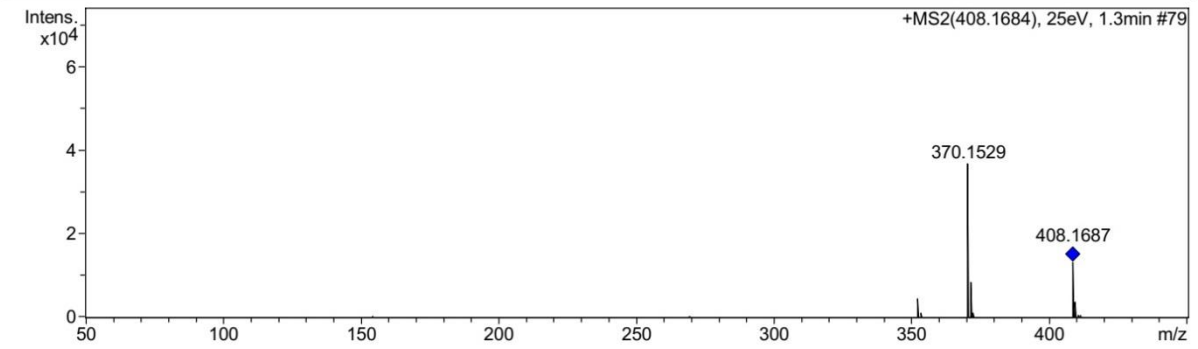
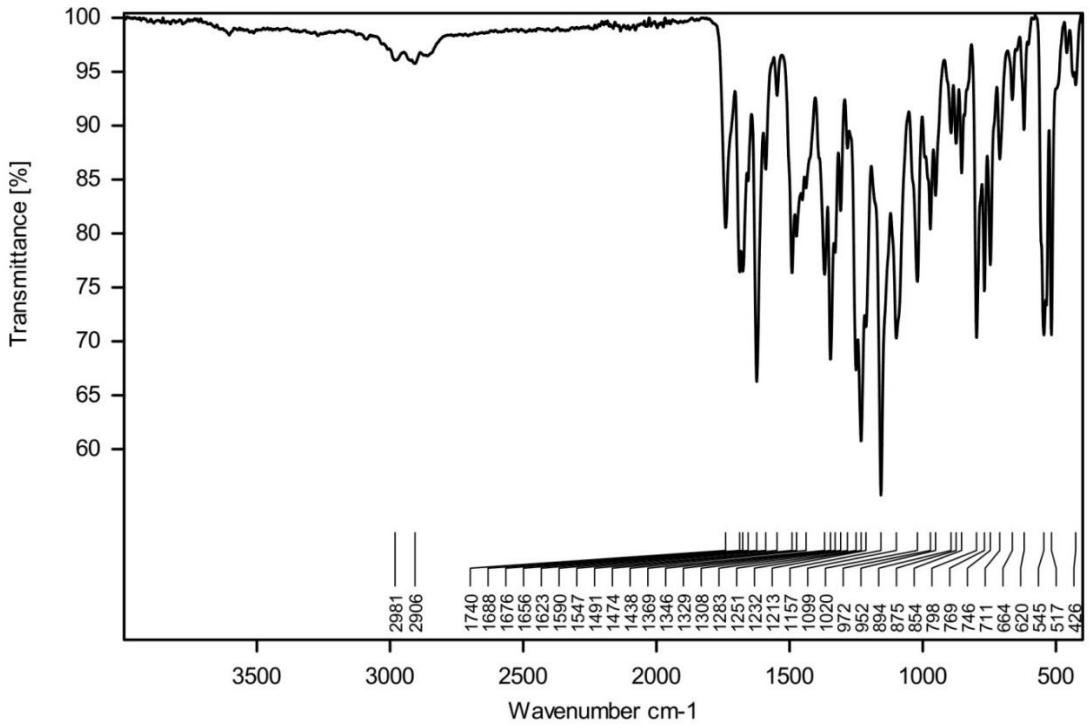


Compound 14

IR



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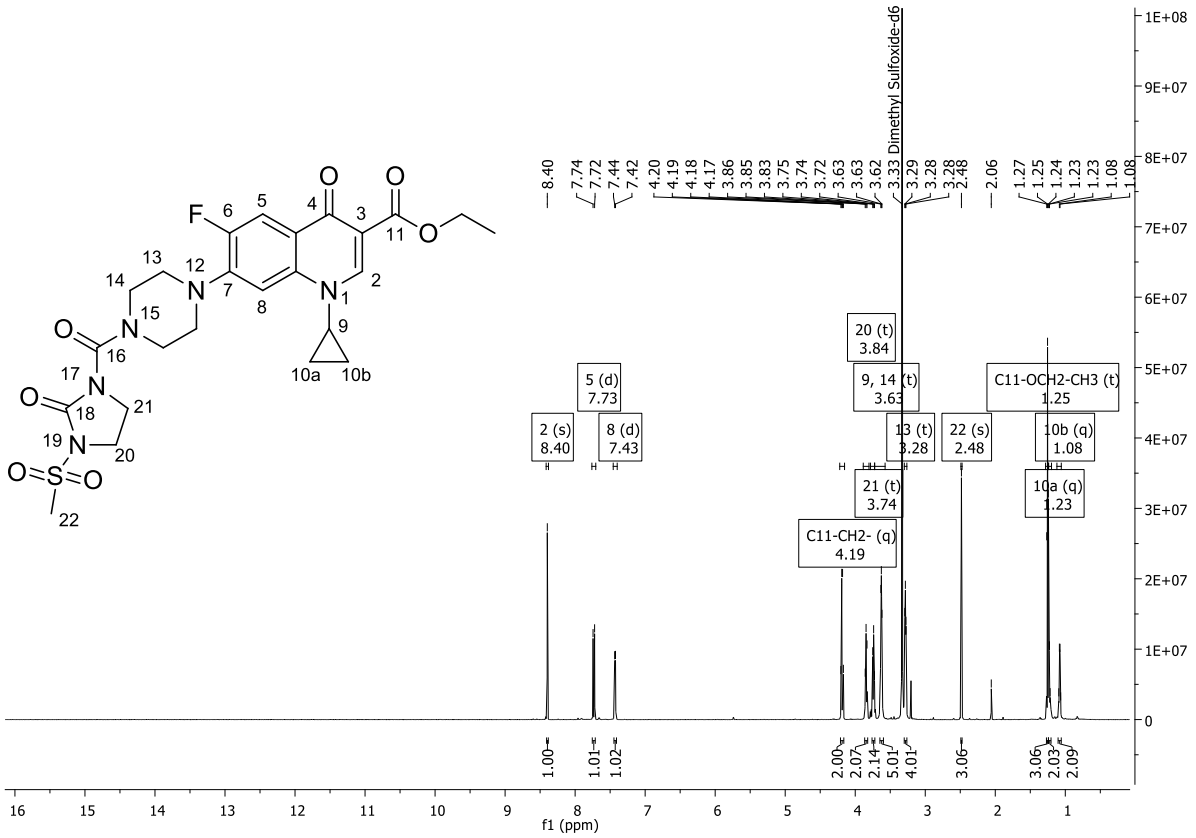
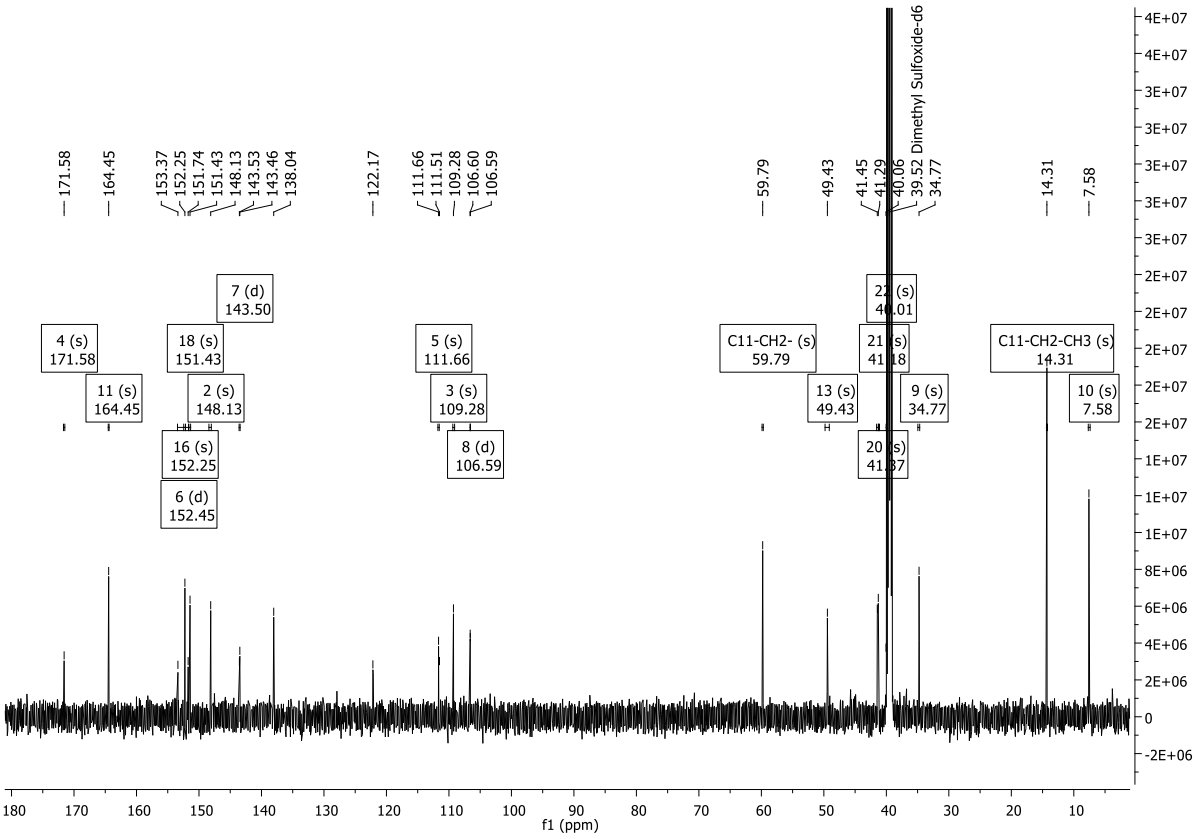


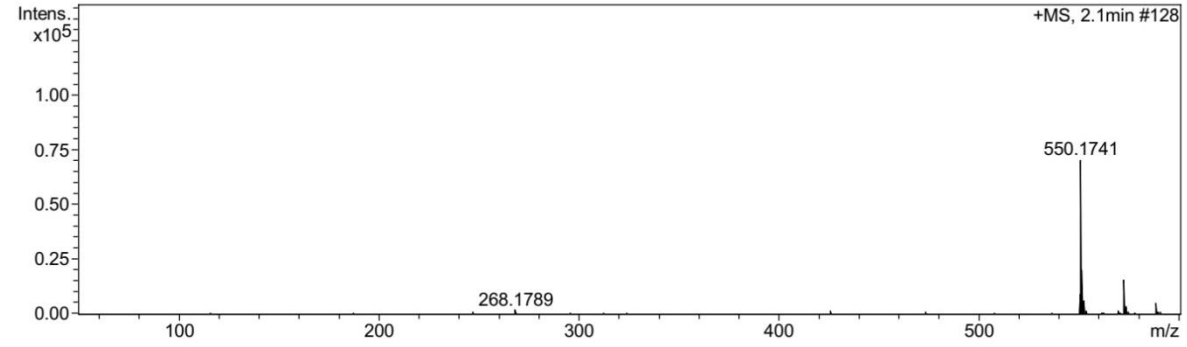
^{13}C in DMSO



HRMS

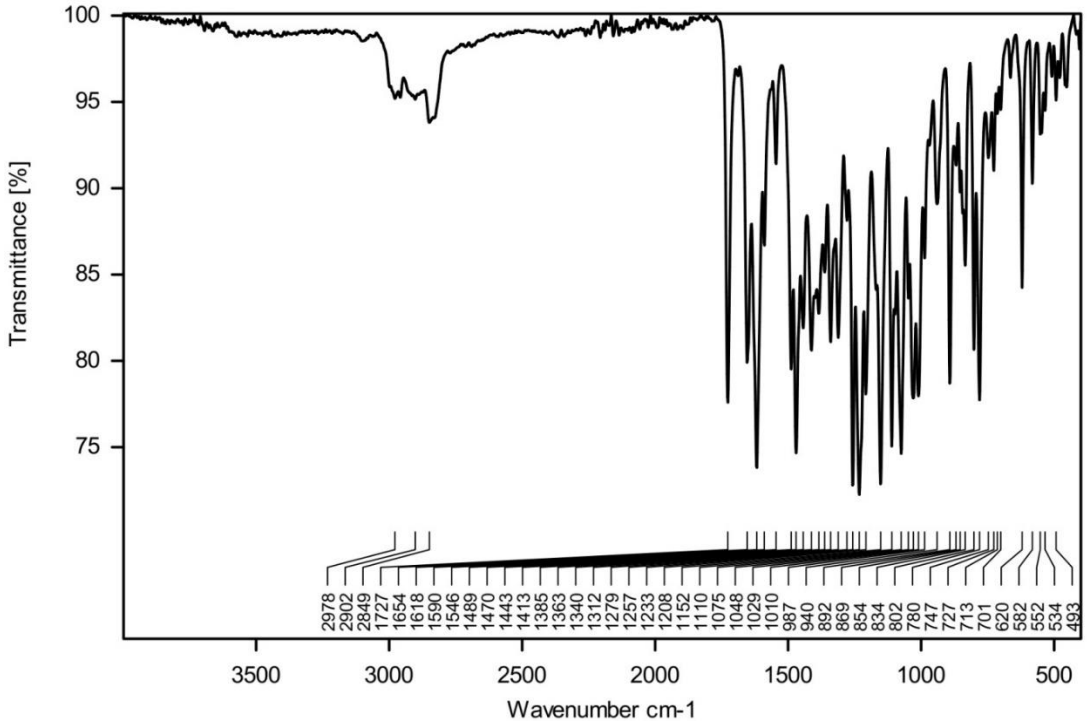
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

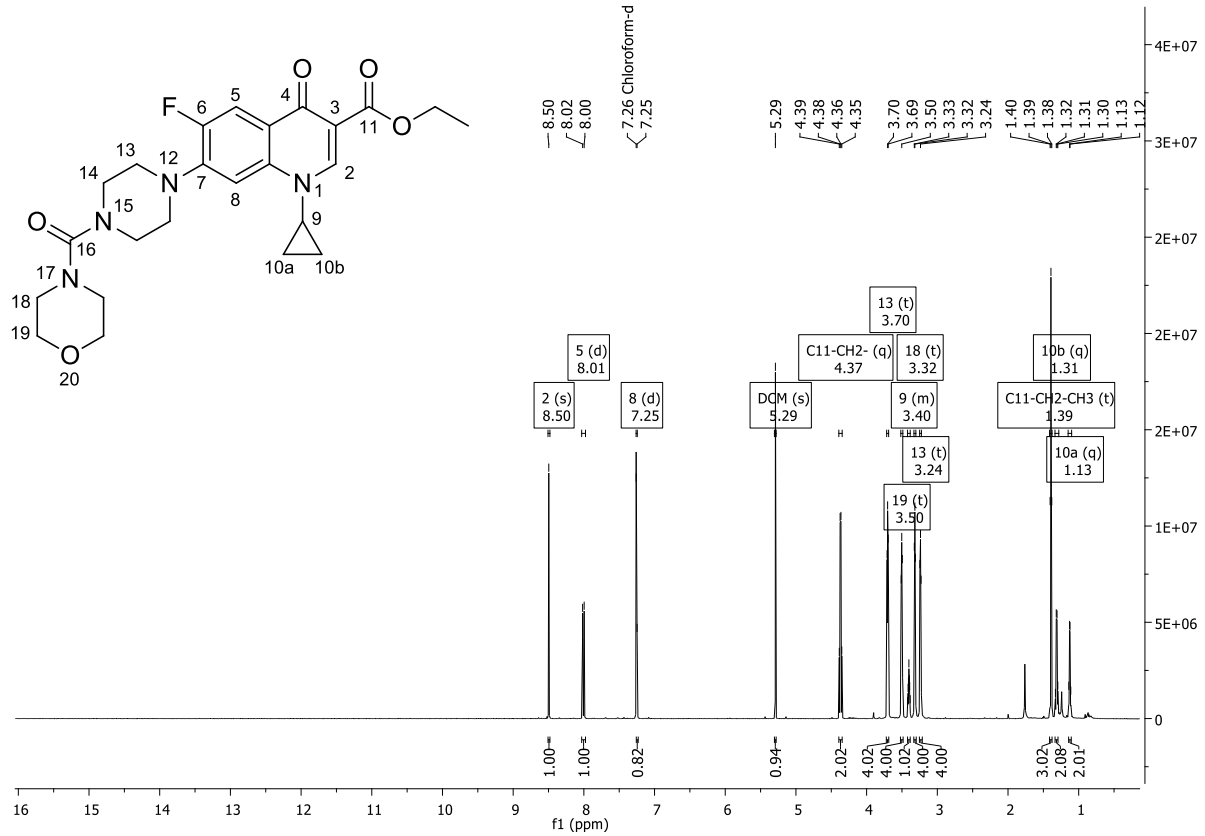


Compound 15

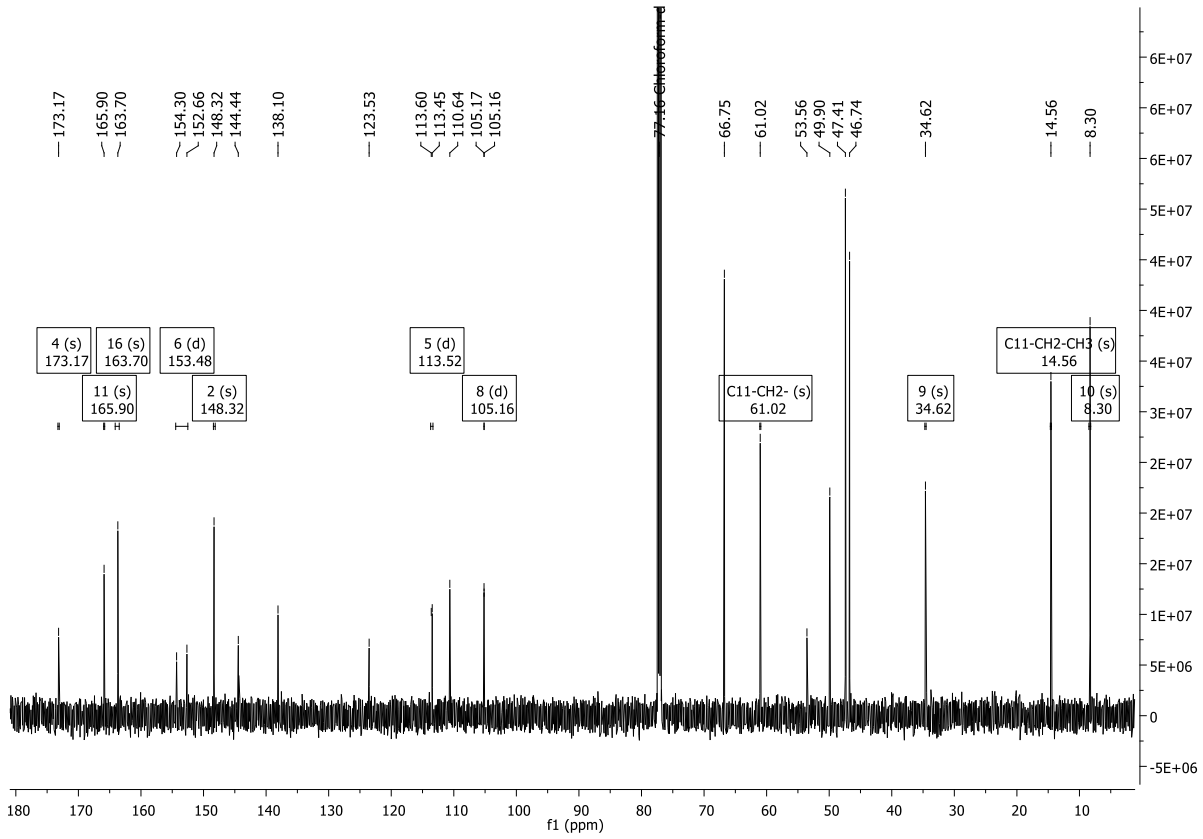
IR



^{1}H in $CDCl_3$



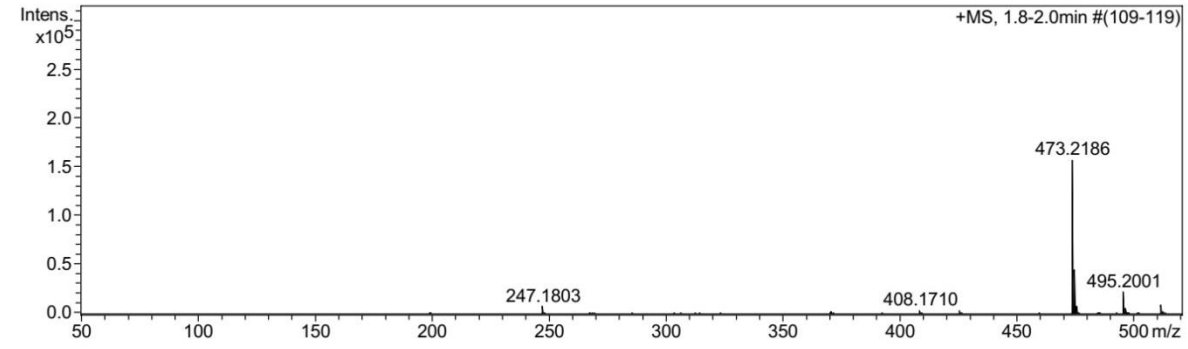
^{13}C in $CDCl_3$



HRMS

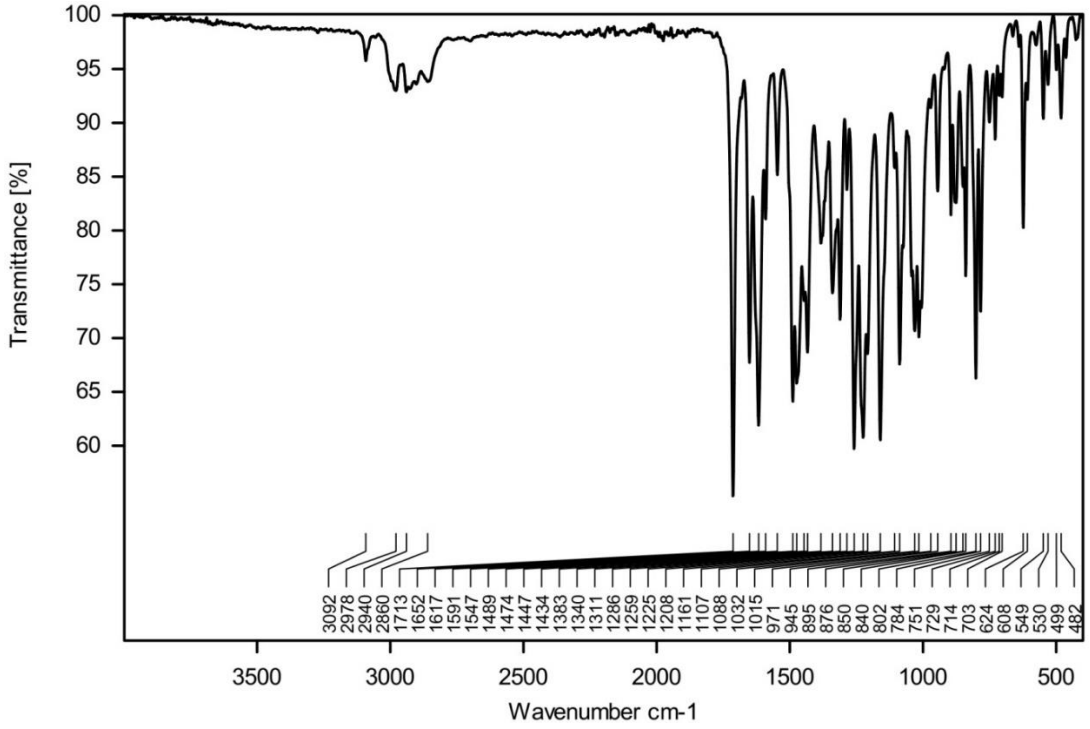
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

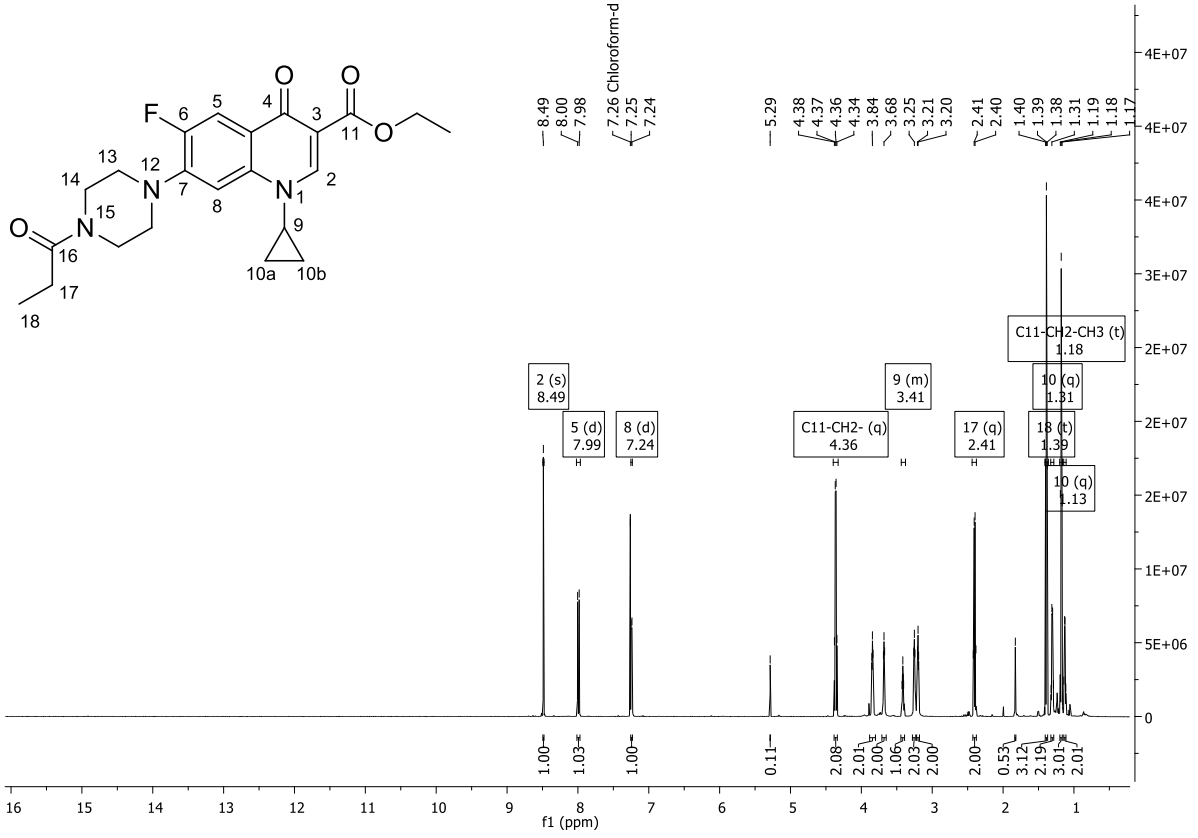


Compound 16

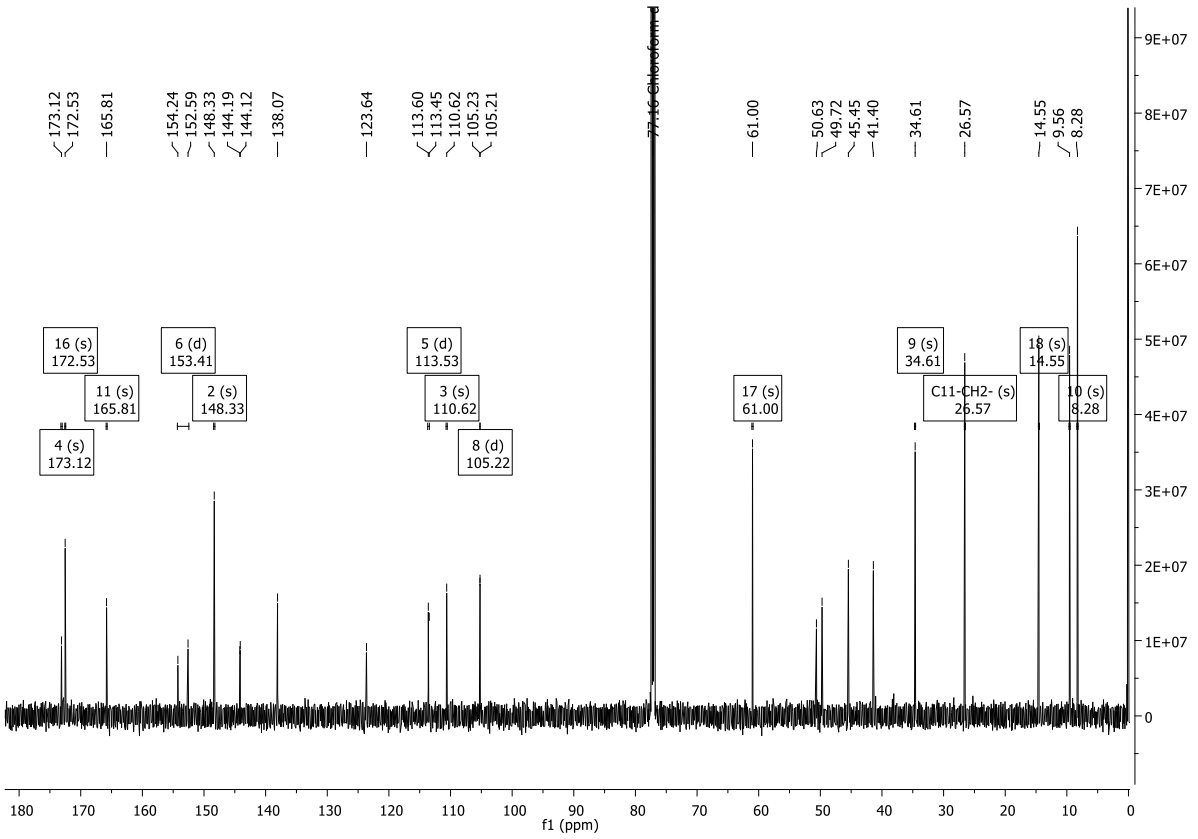
IR



^{1}H in $CDCl_3$



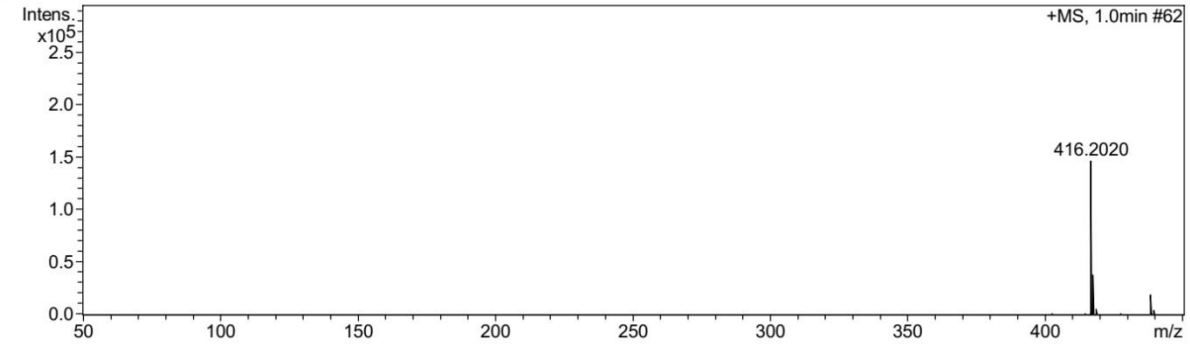
^{13}C in $CDCl_3$



HRMS

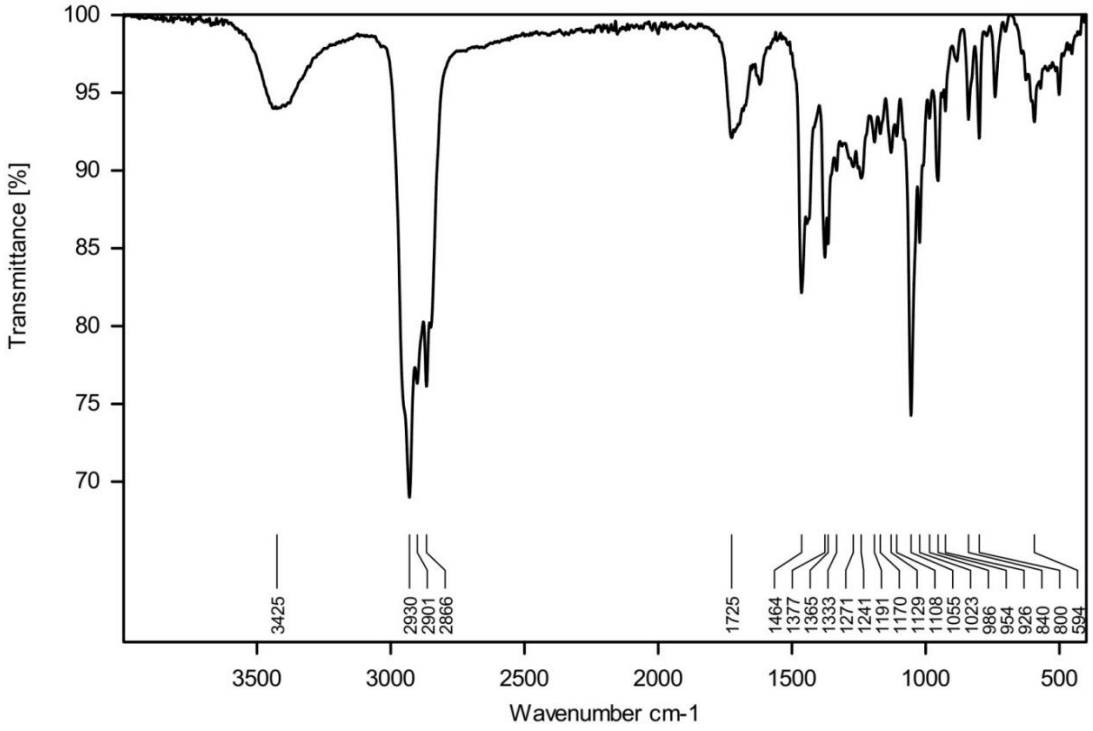
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

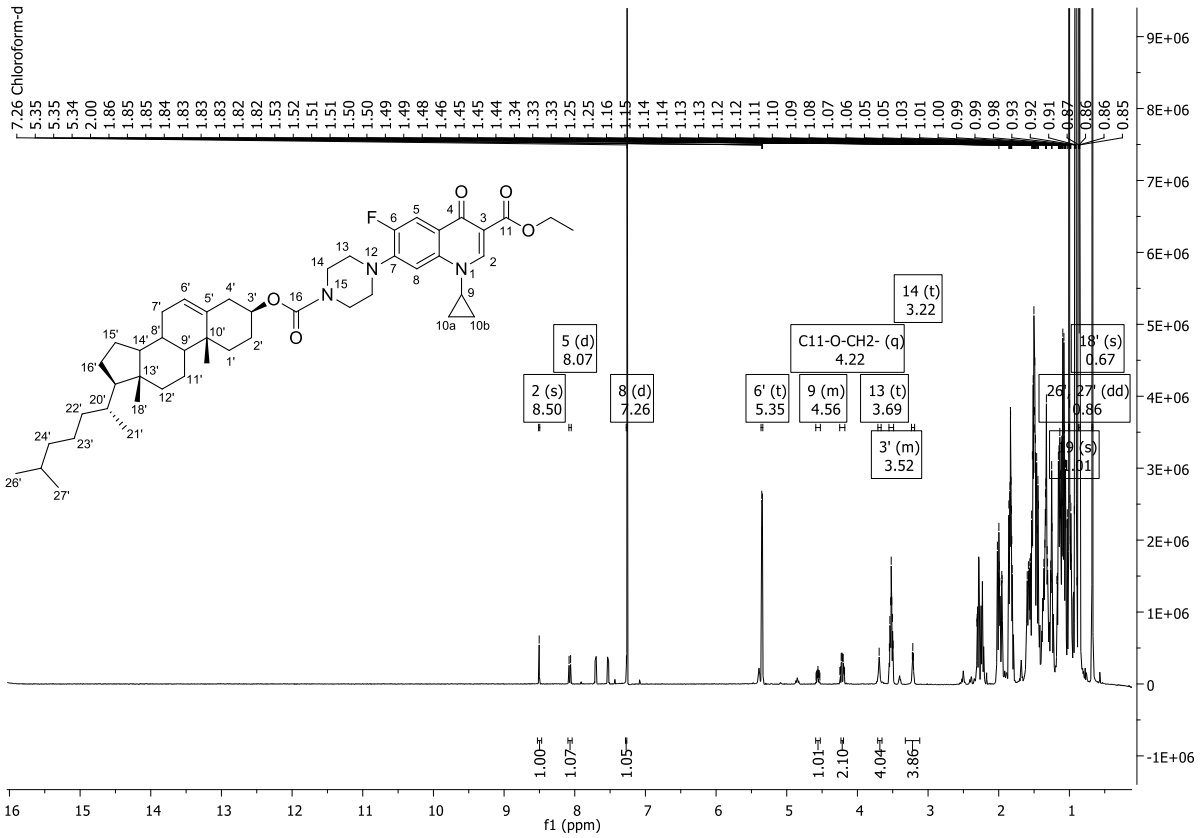


Compound 17

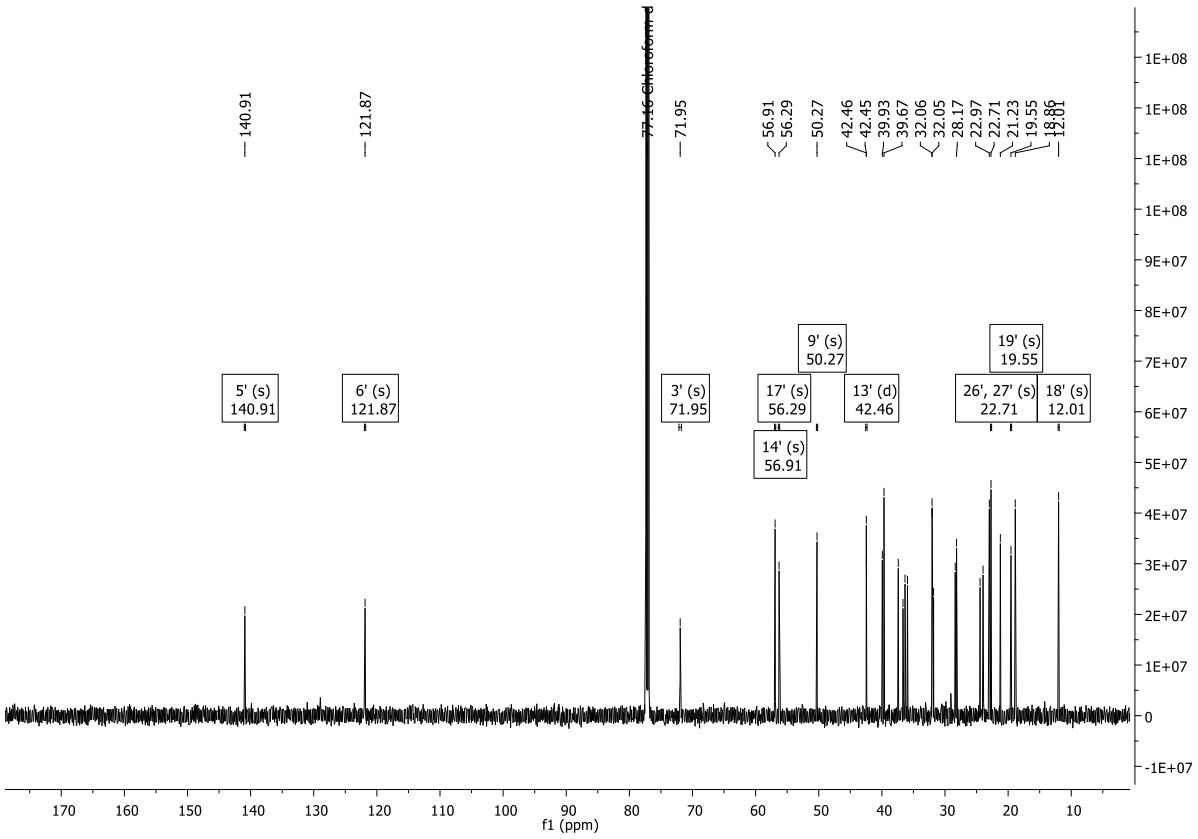
IR



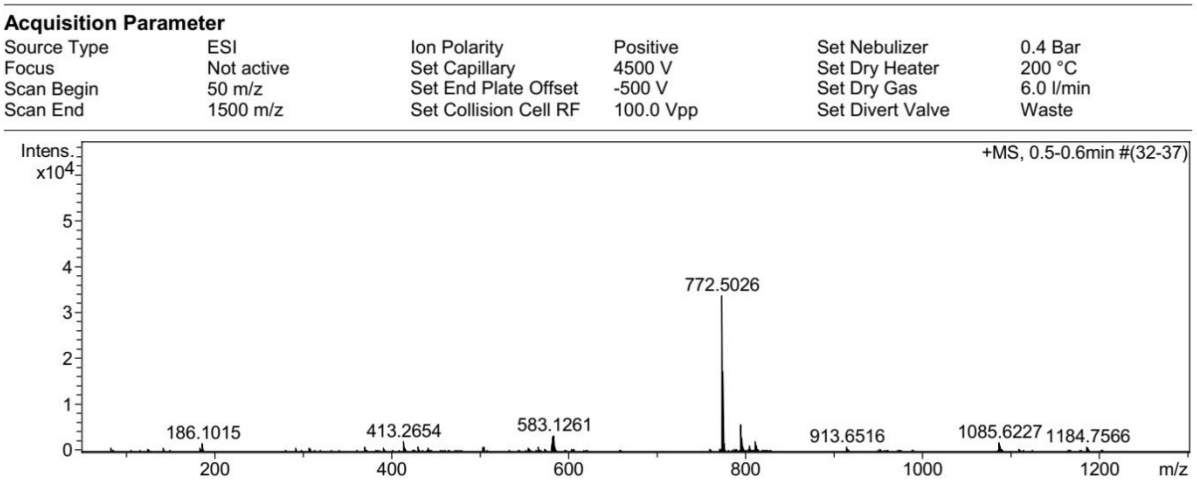
^{1}H in $CDCl_3$



^{13}C in $CDCl_3$

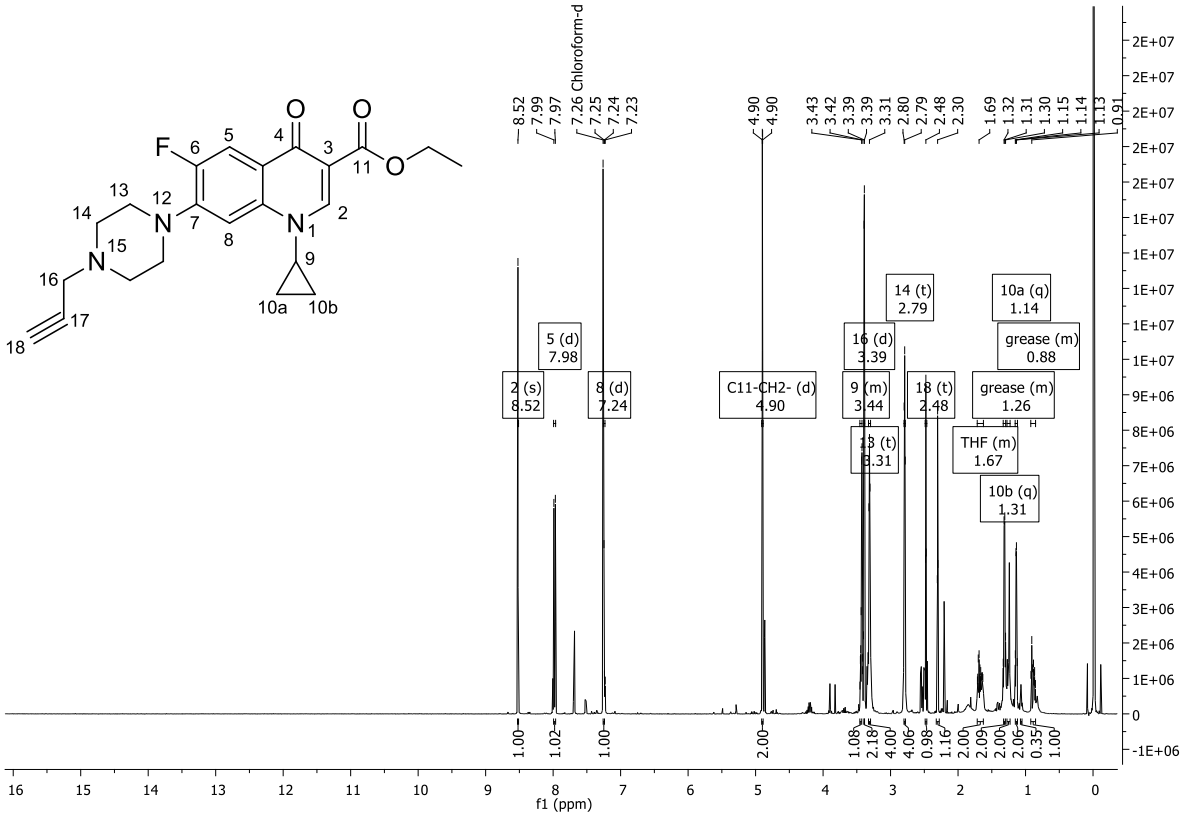


HRMS

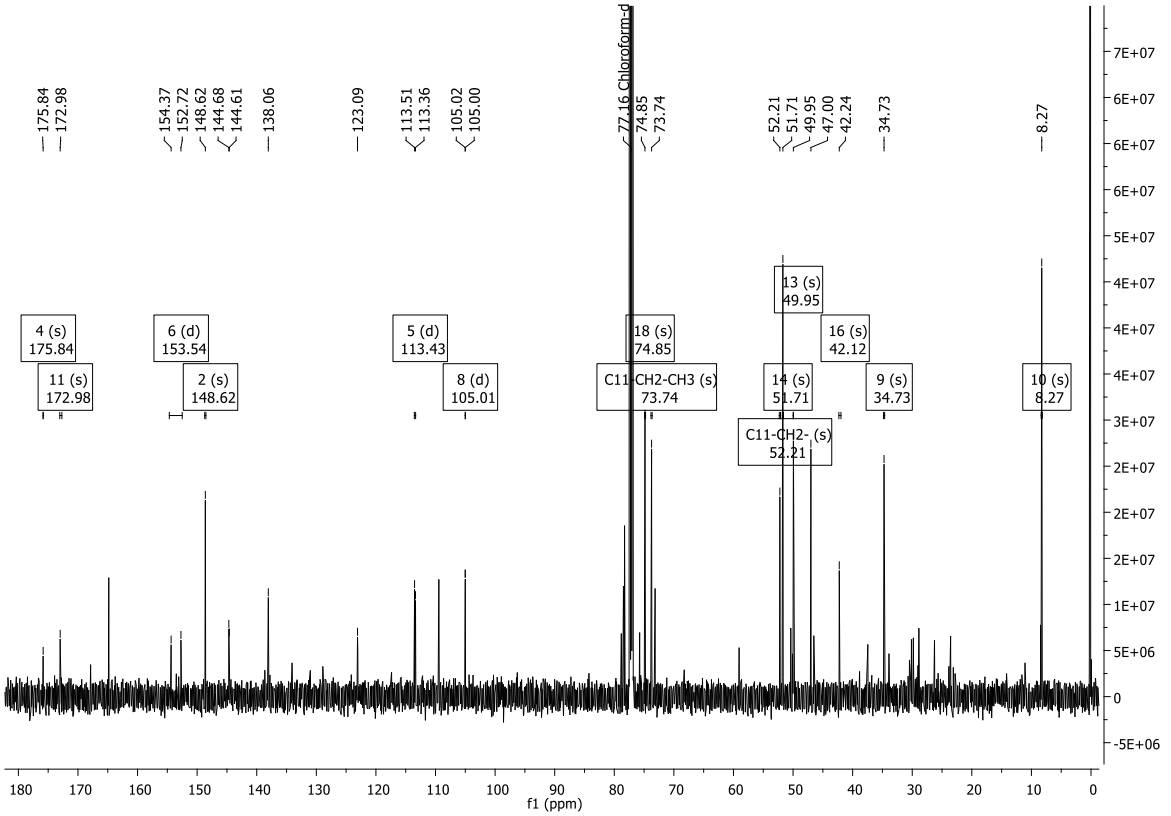


Compound 18

^{1}H in $CDCl_3$

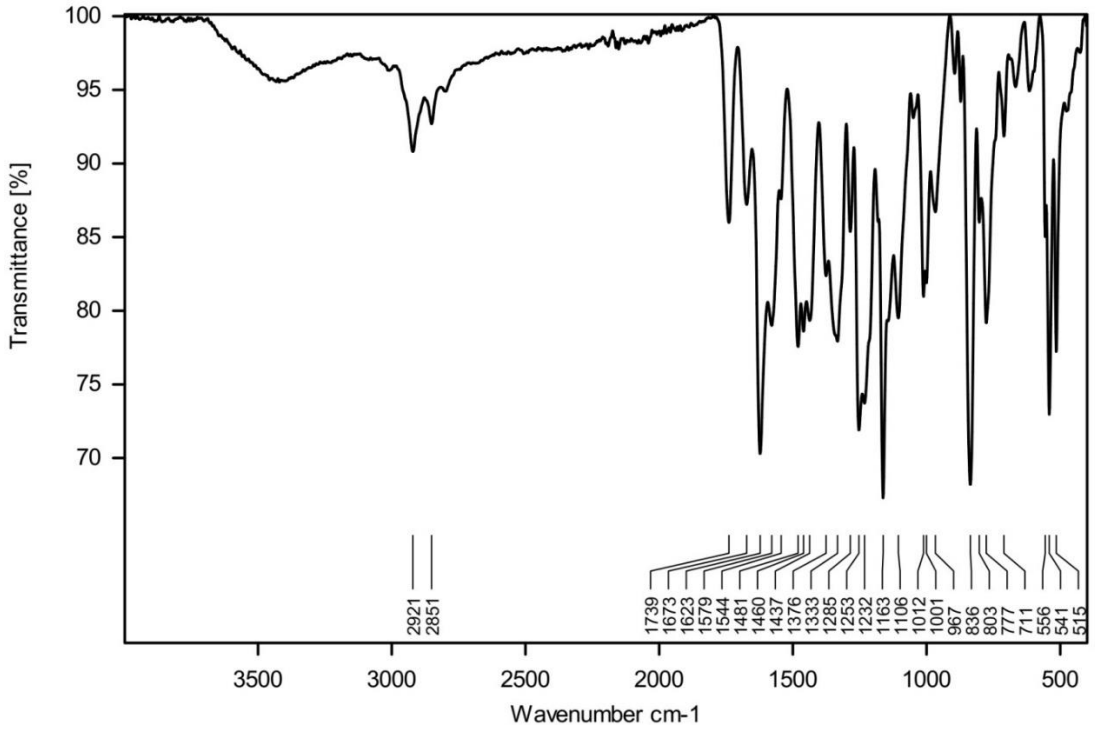


^{13}C in $CDCl_3$

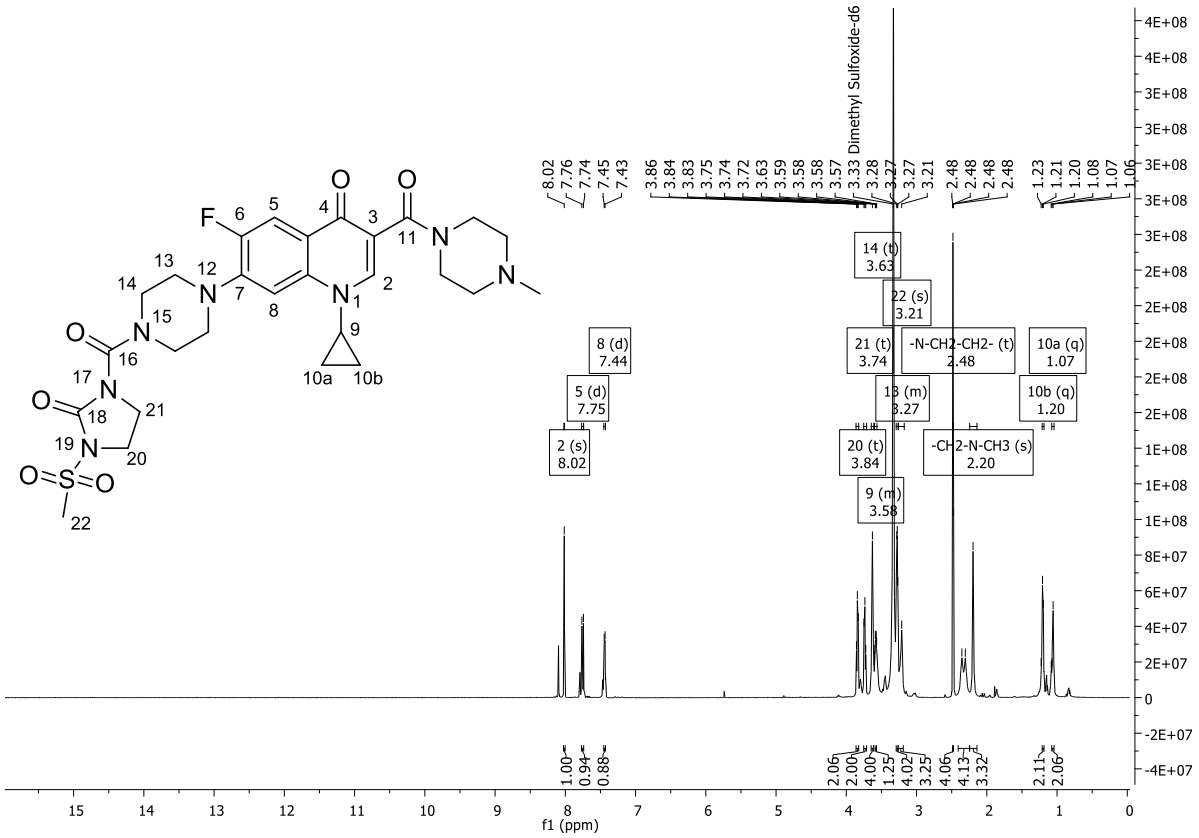


Compound 19

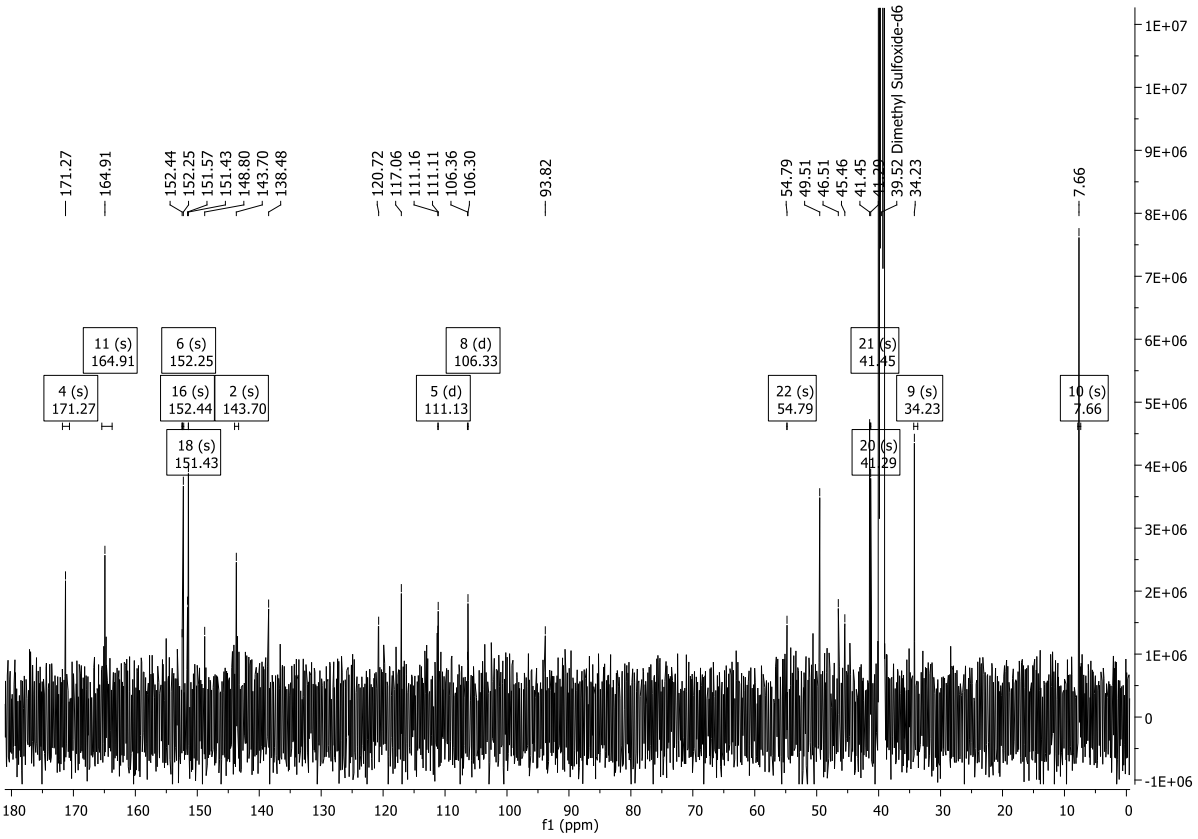
IR



^{1}H in DMSO



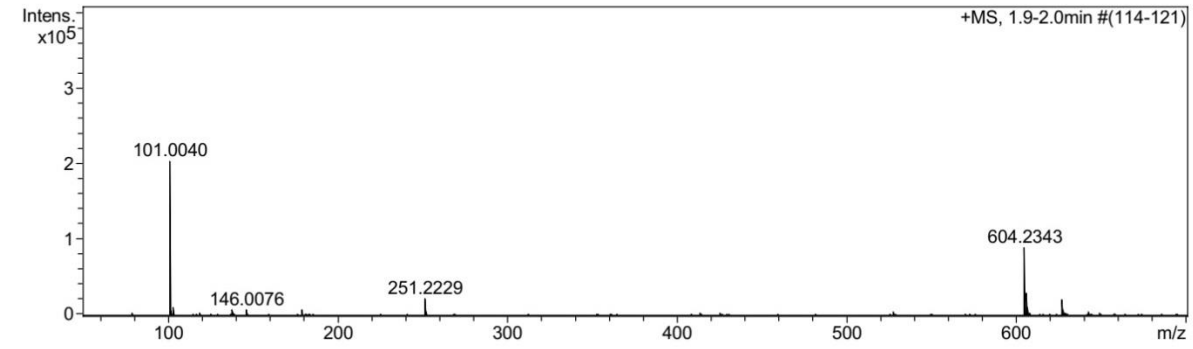
^{13}C in DMSO



HRMS

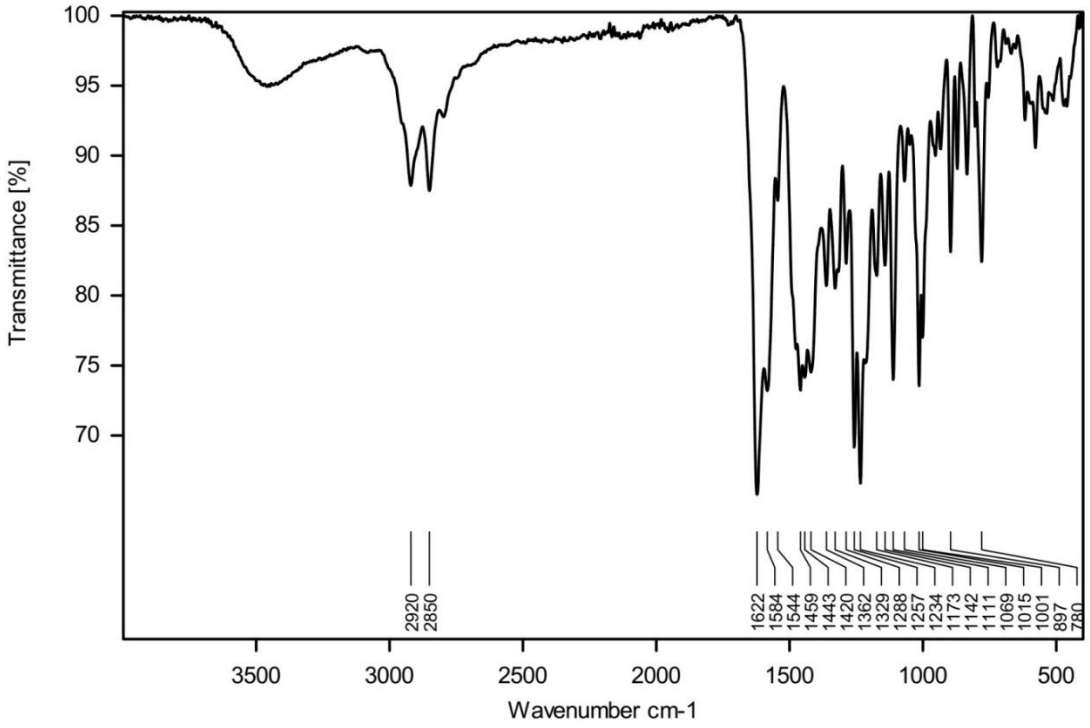
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



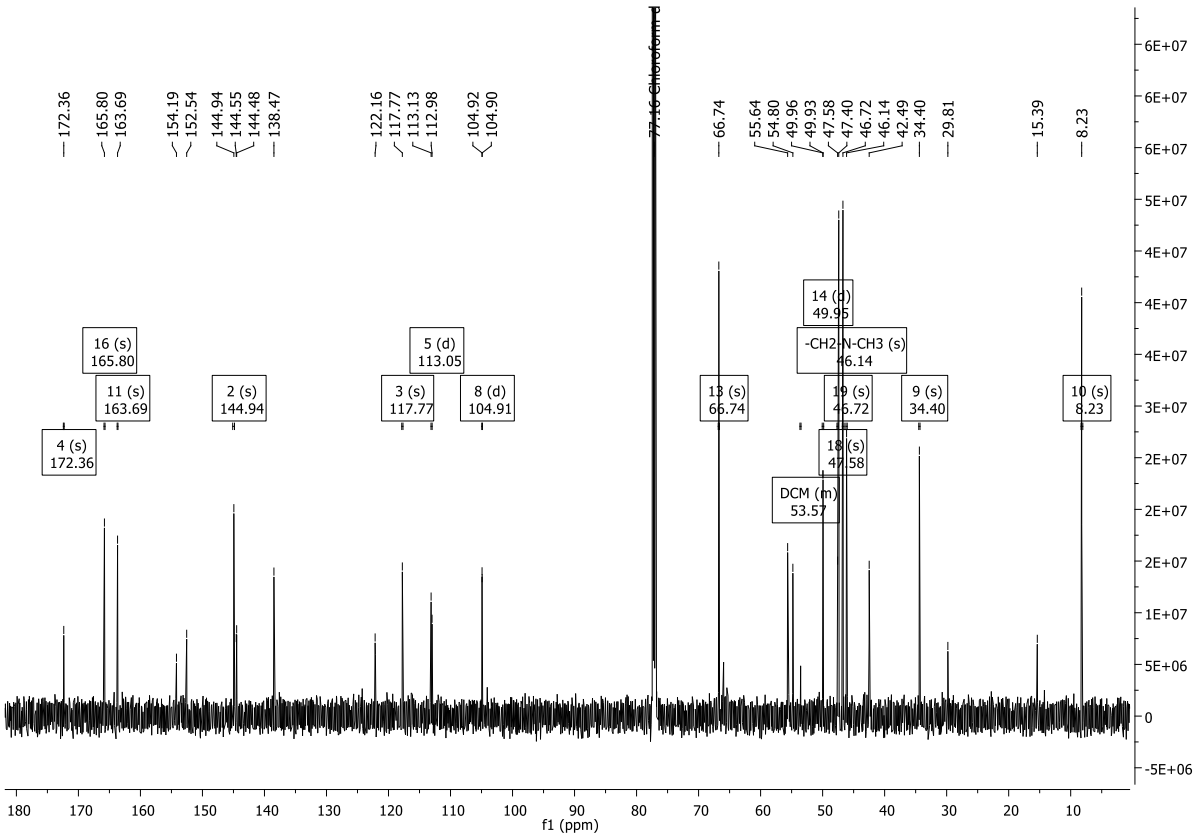
Compound 20

IR



^{1}H in $CDCl_3$

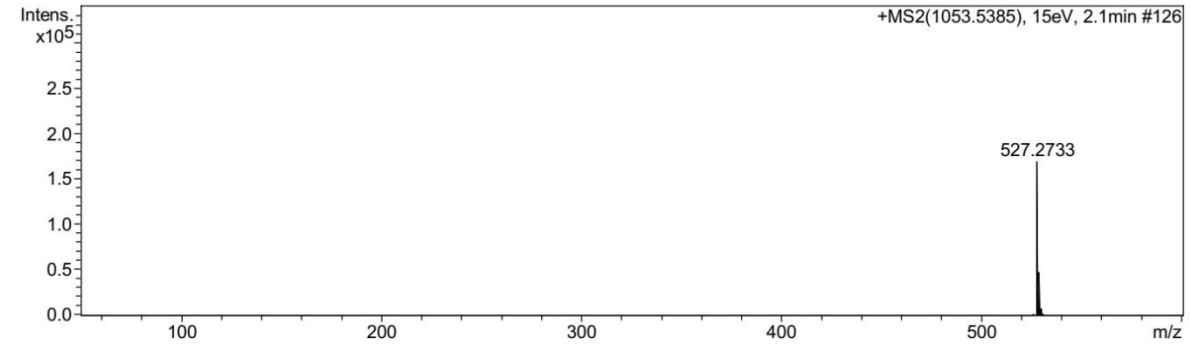
^{13}C in $CDCl_3$



HRMS

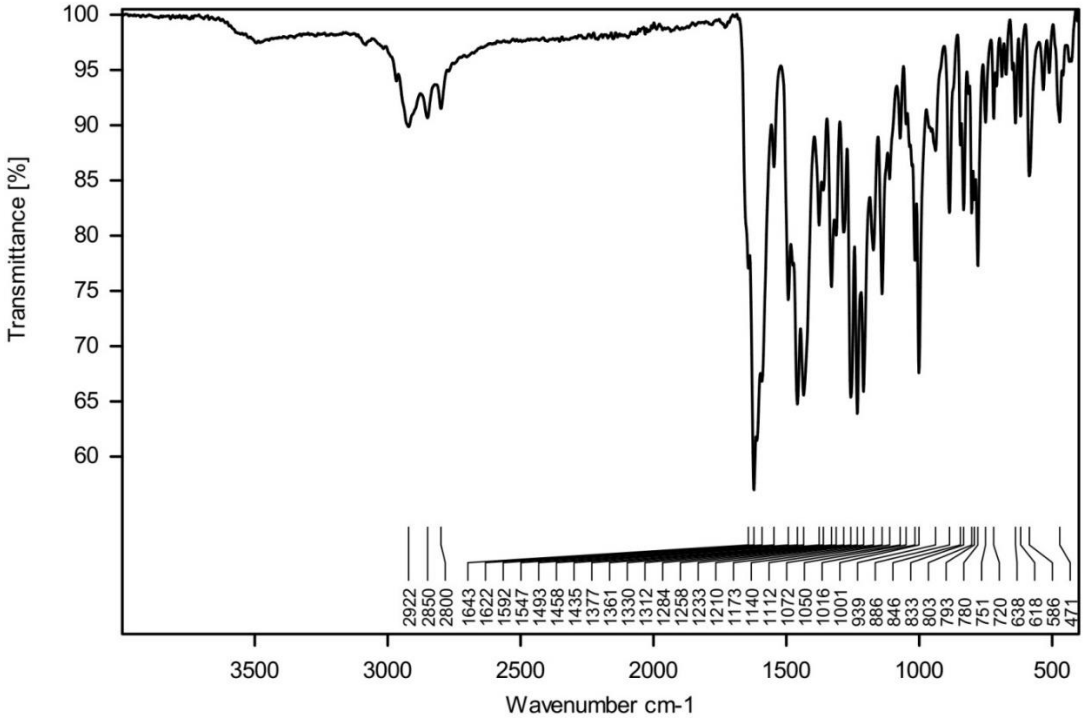
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



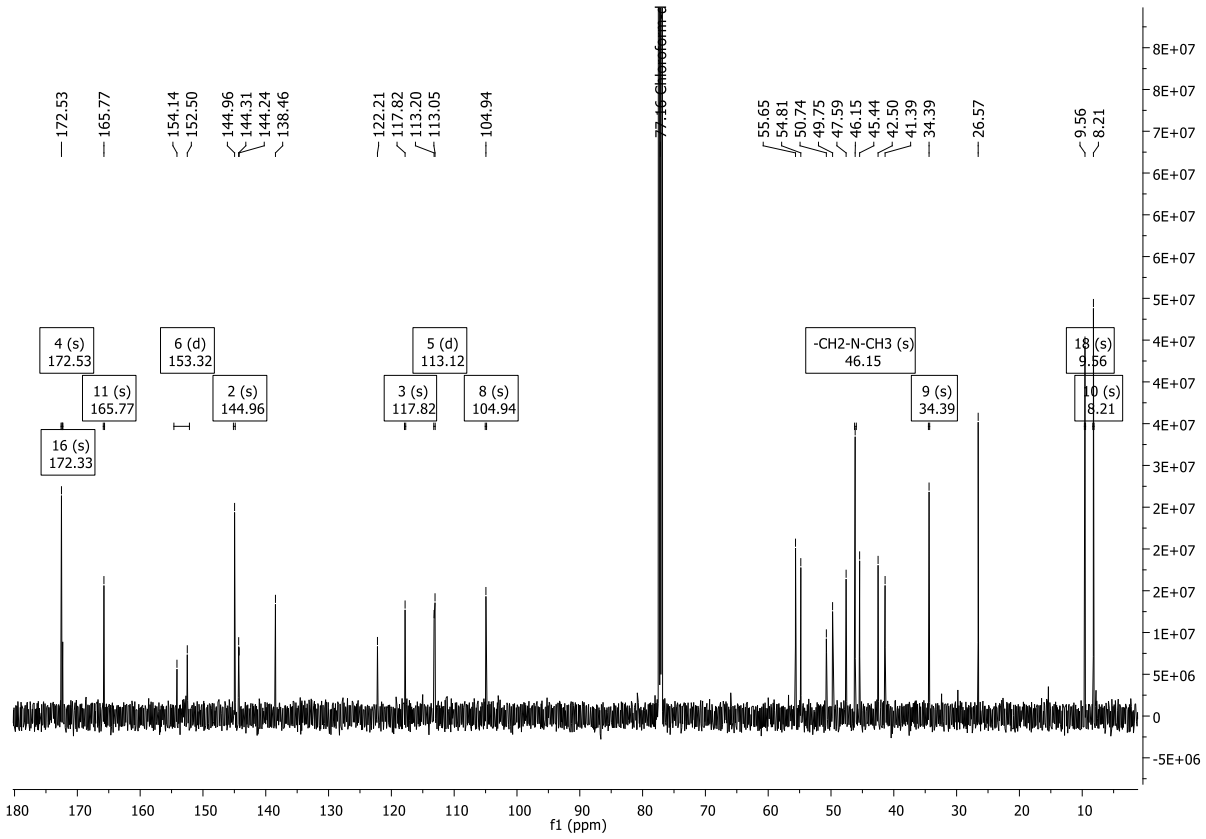
Compound 21

IR



^{1}H in $CDCl_3$

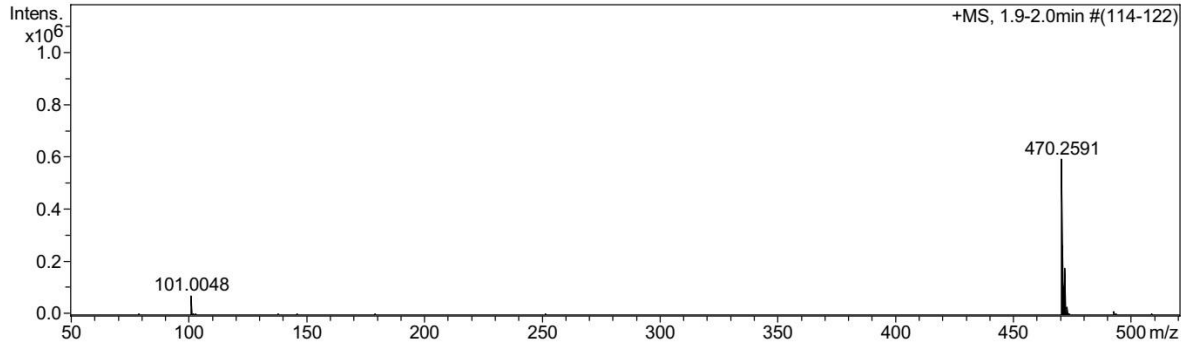
^{13}C in $CDCl_3$



HRMS

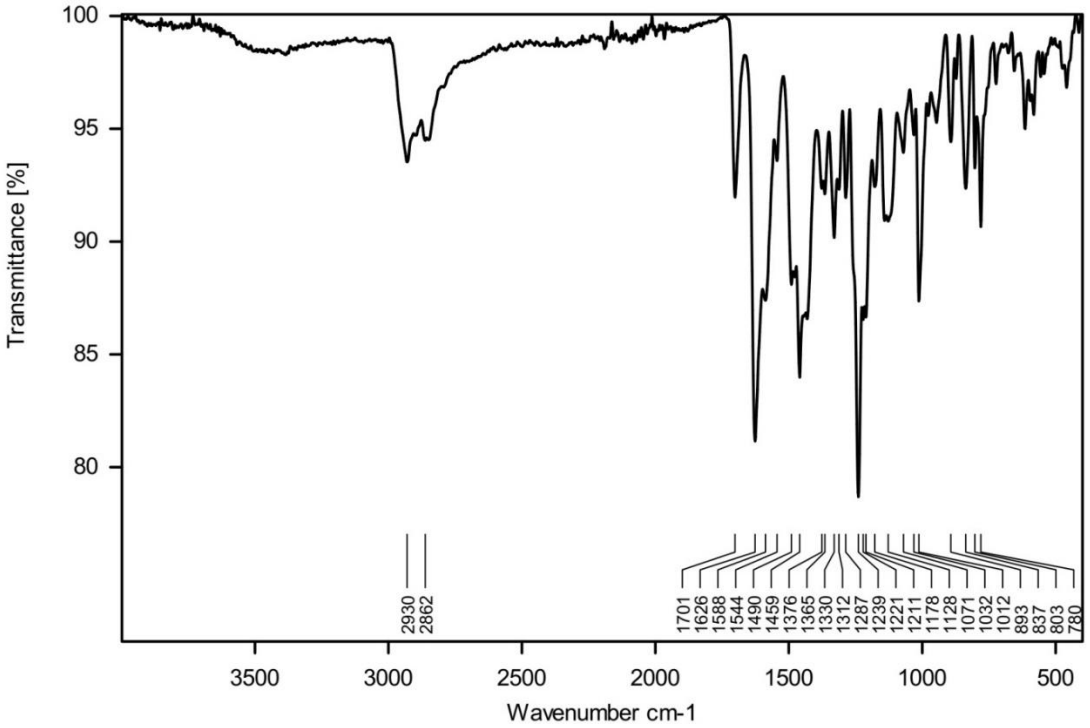
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

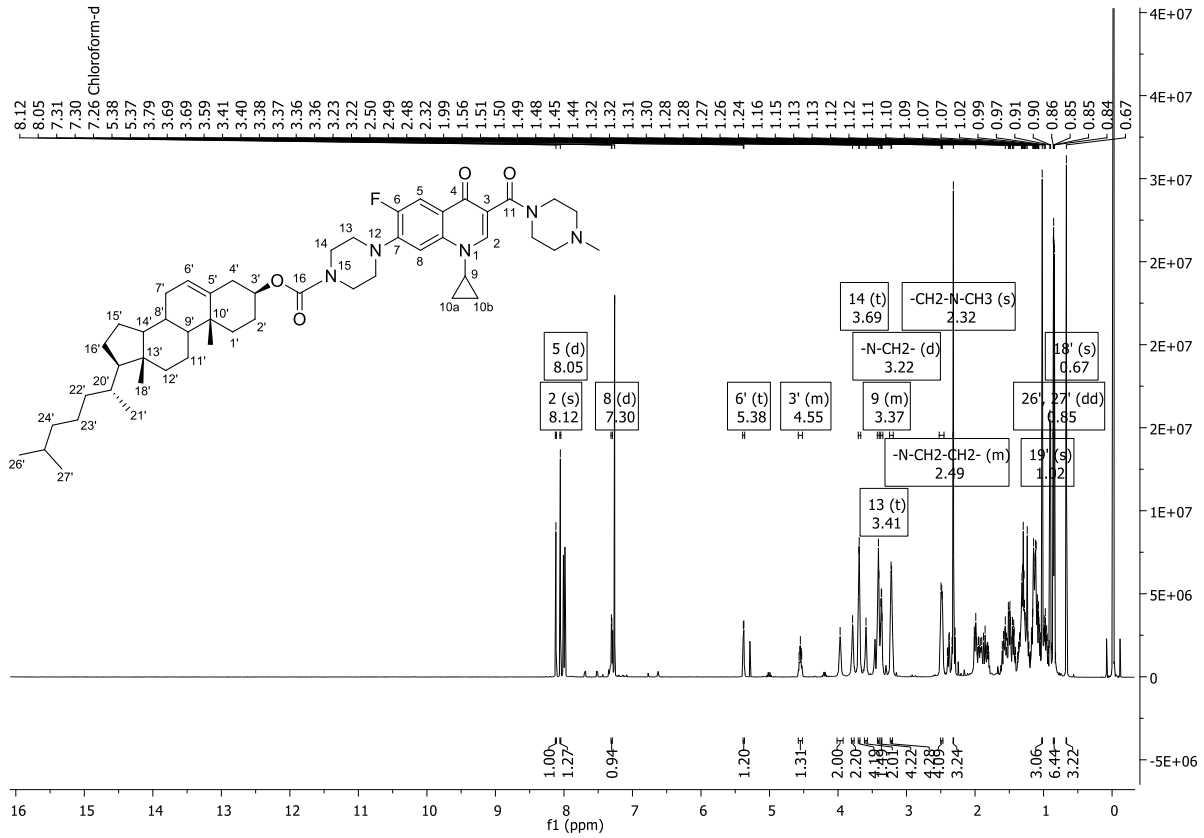


Compound 22

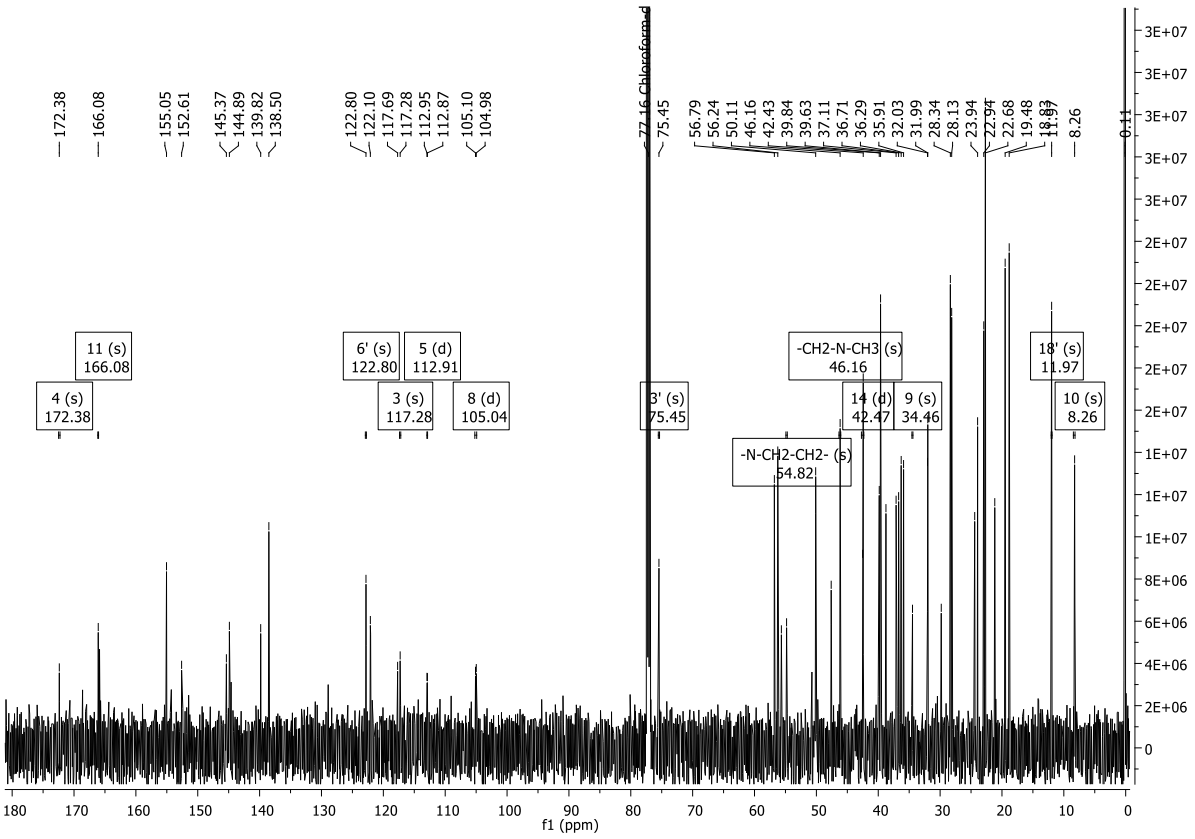
IR



^{1}H in $CDCl_3$



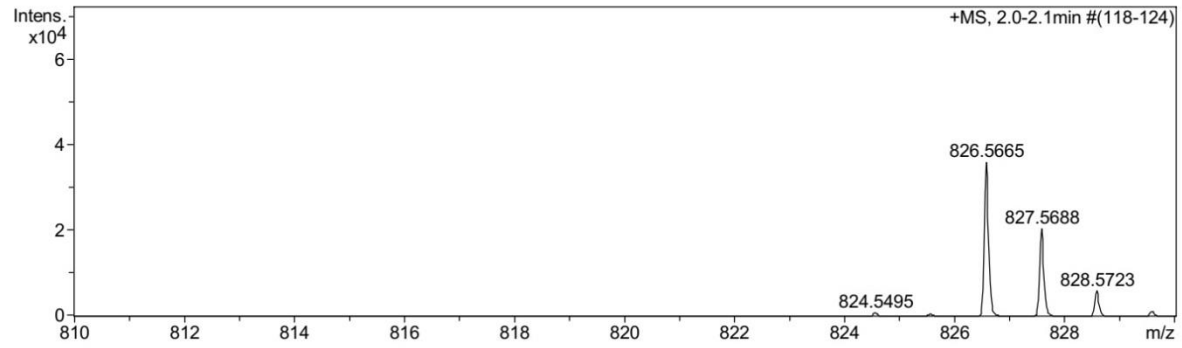
^{13}C in $CDCl_3$



HRMS

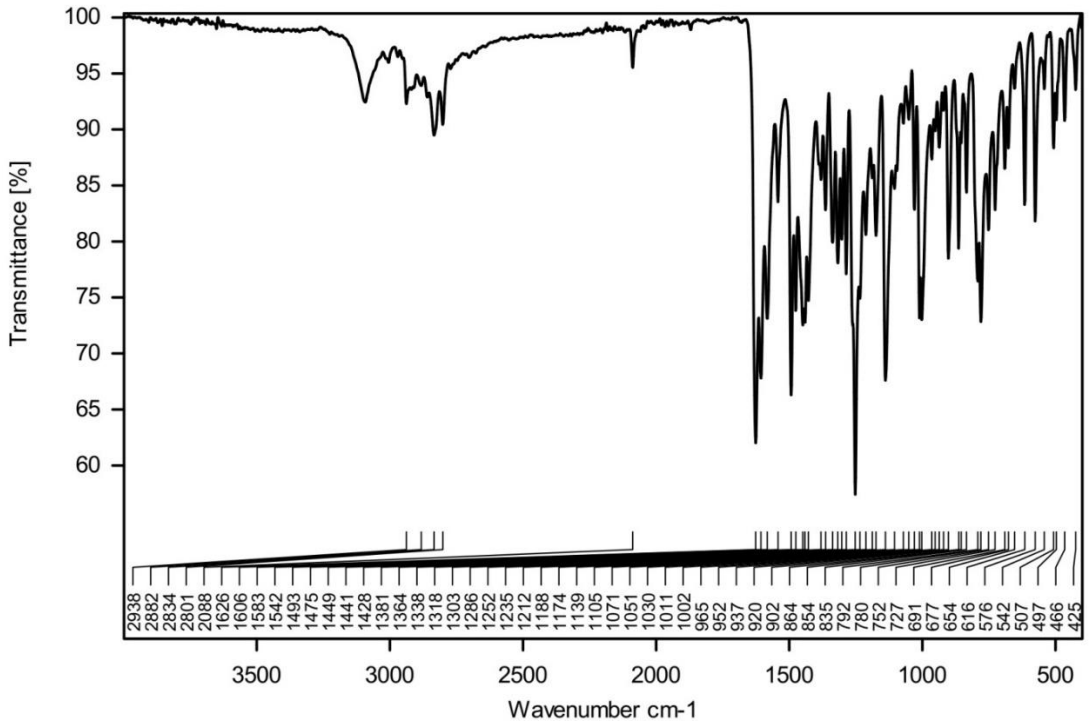
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

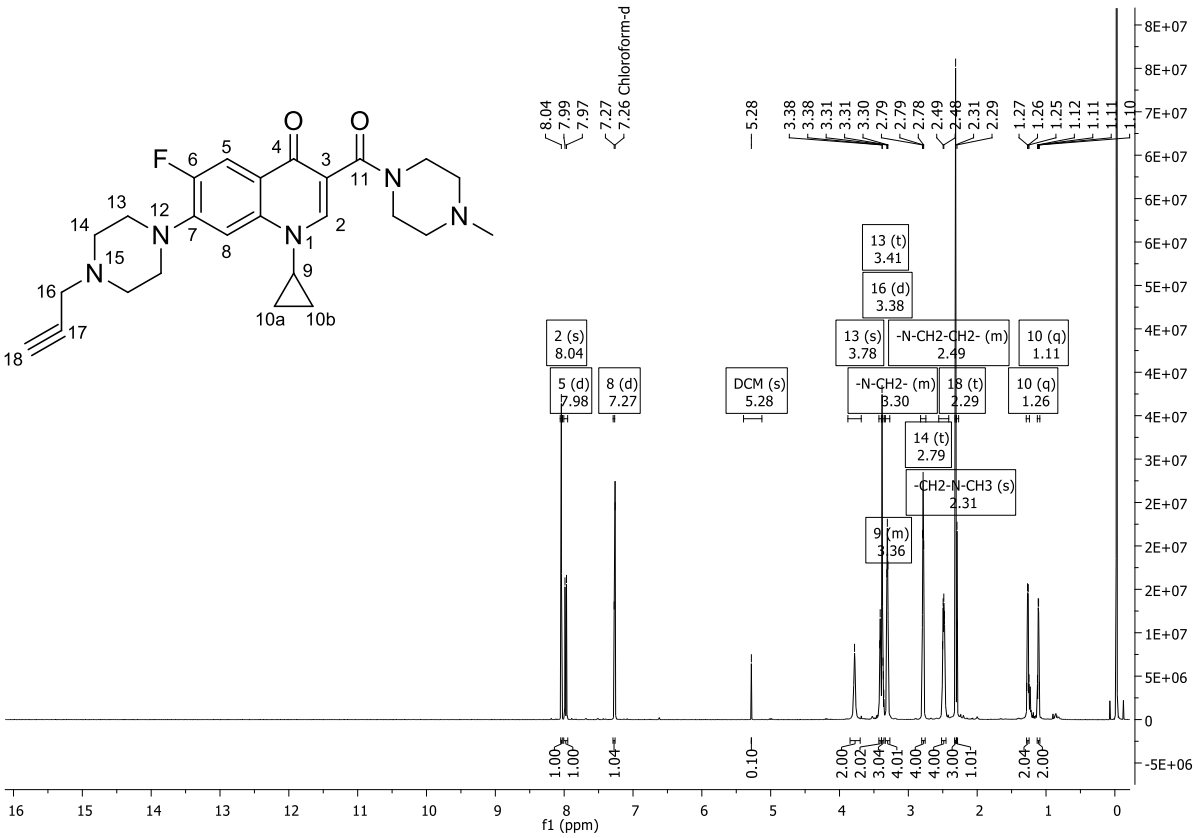


Compound 23

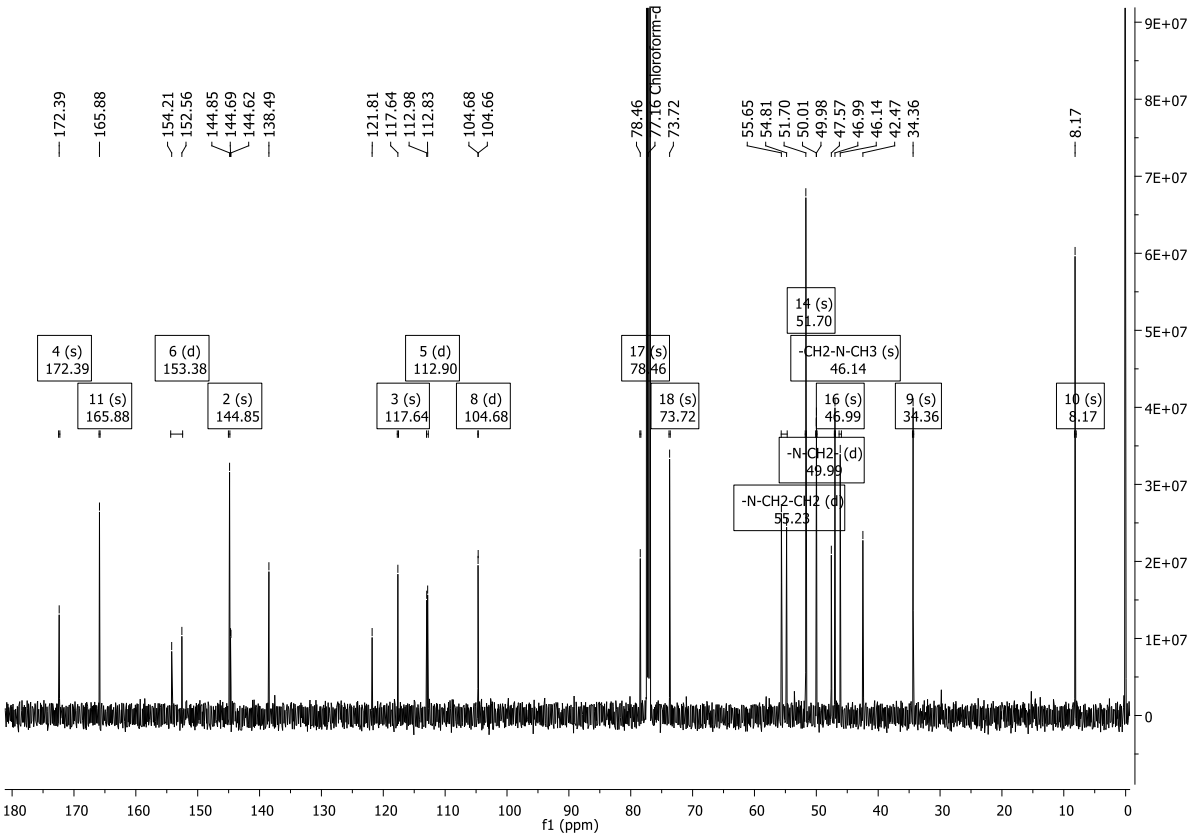
IR



^1H in $CDCl_3$



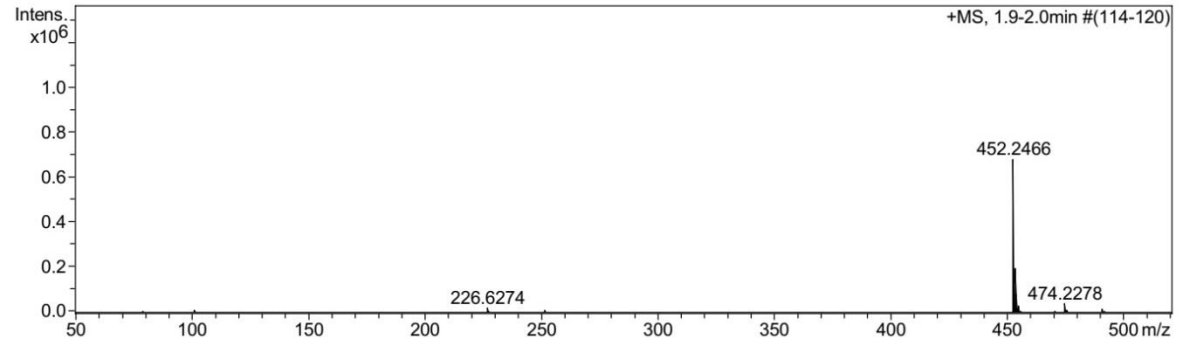
^{13}C in $CDCl_3$



HRMS

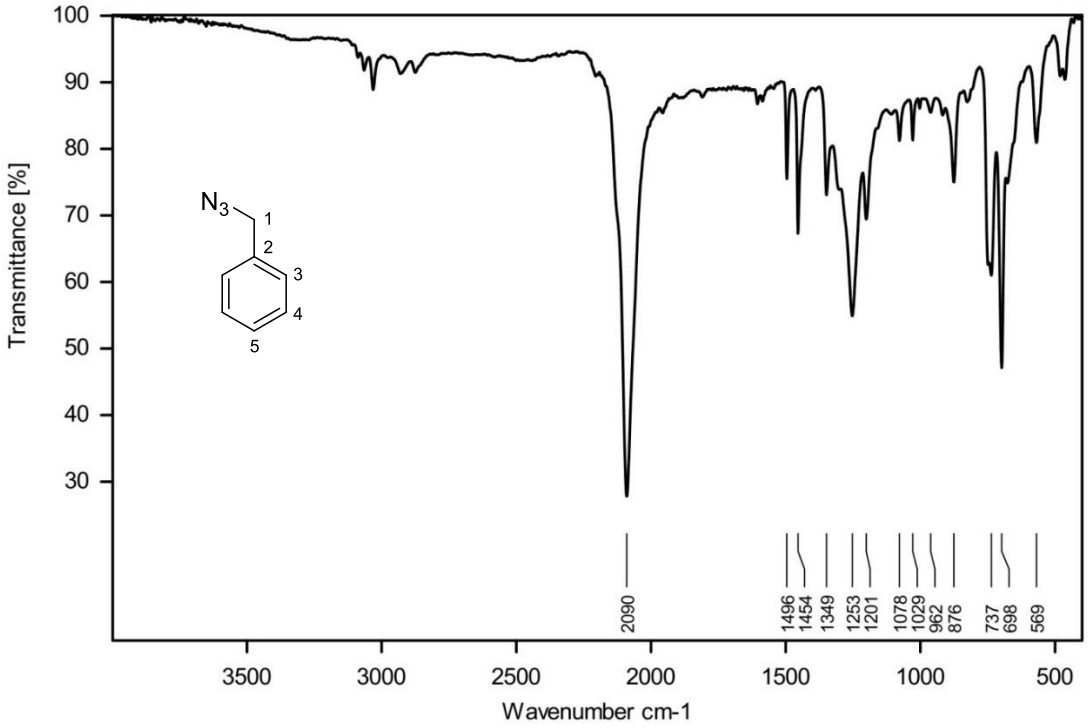
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

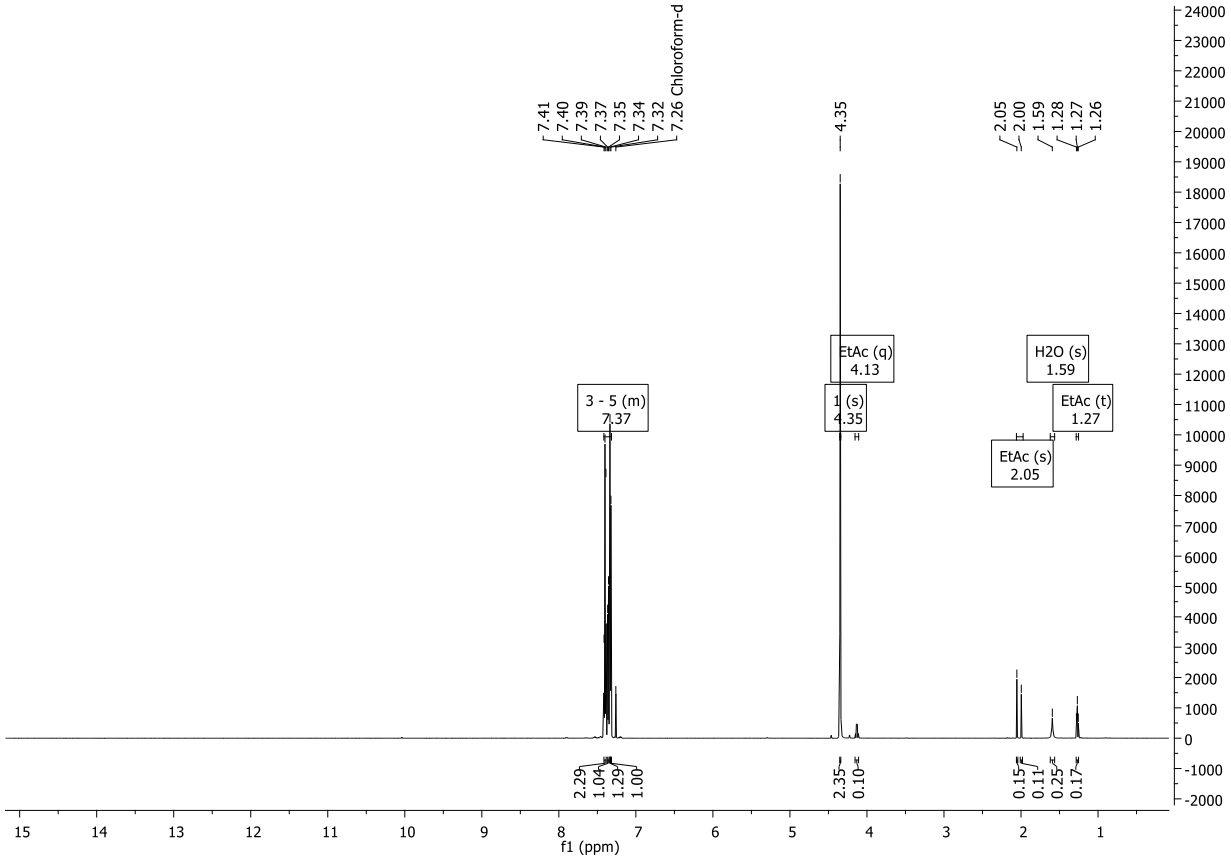


Intermediate 25a

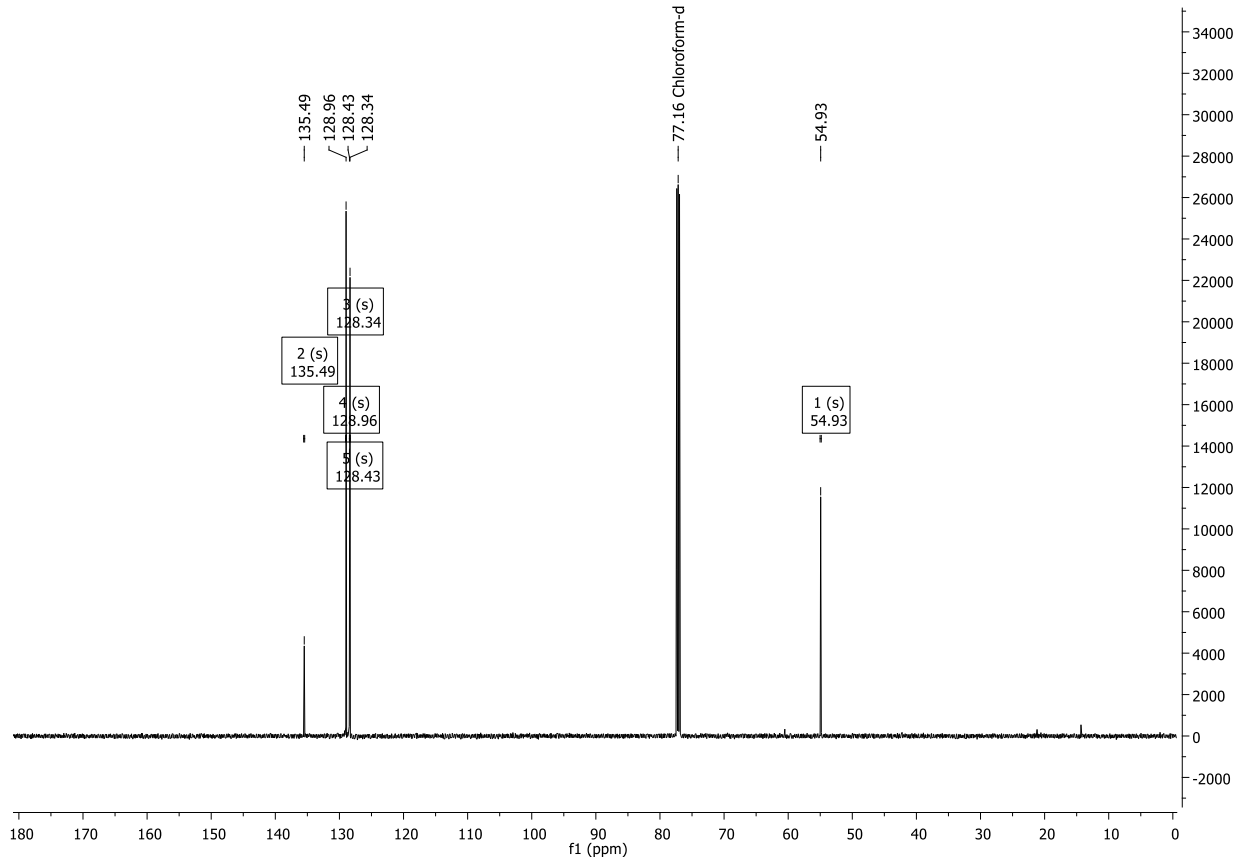
IR



^{1}H in $CDCl_3$

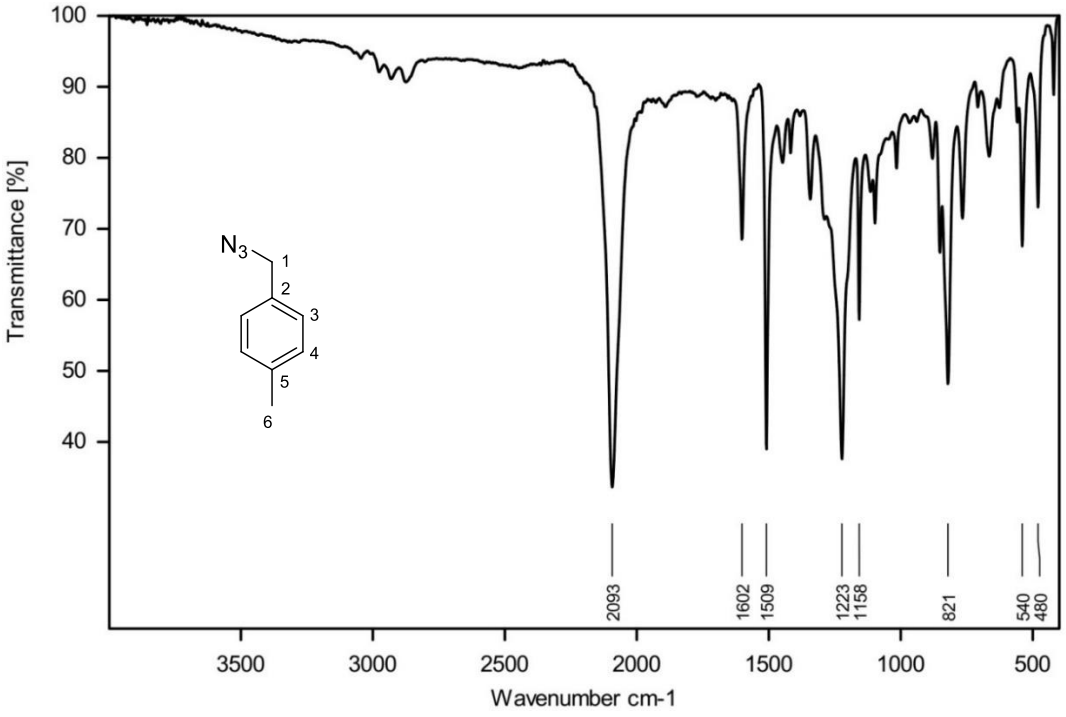


^{13}C in Chloroform

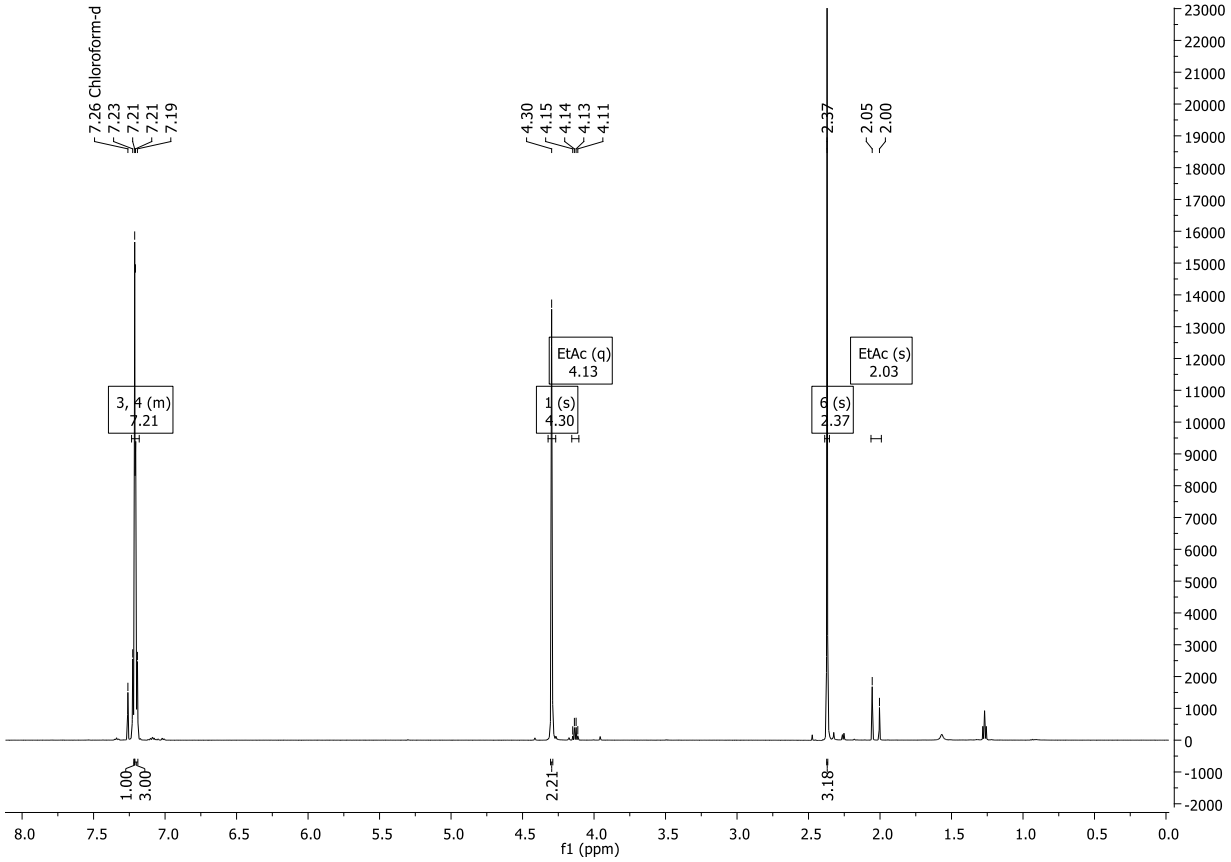


Intermediate 25b

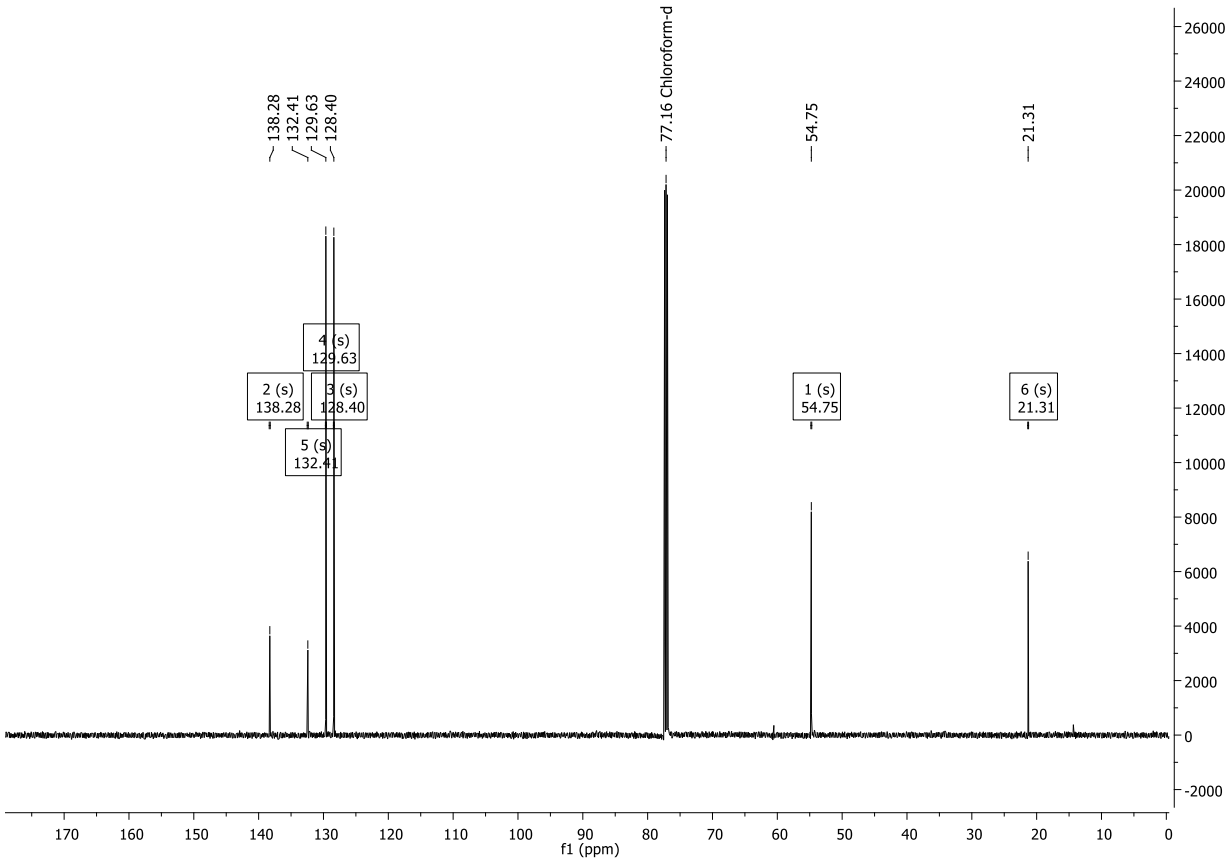
IR



^{1}H in $CDCl_3$

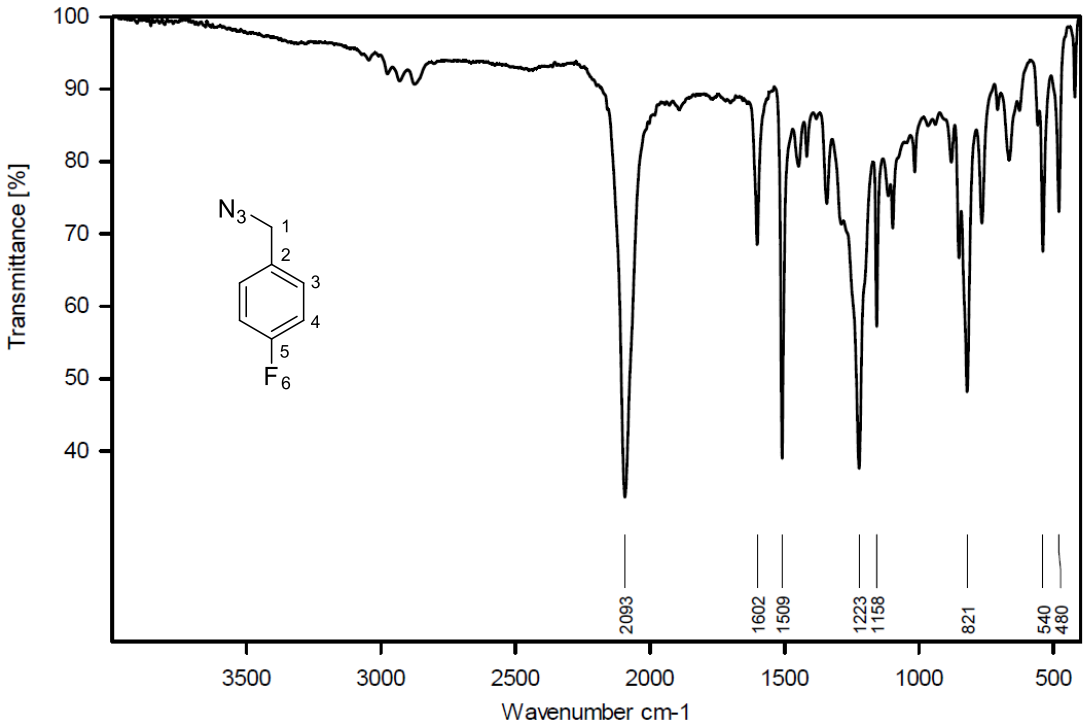


^{13}C in $CDCl_3$

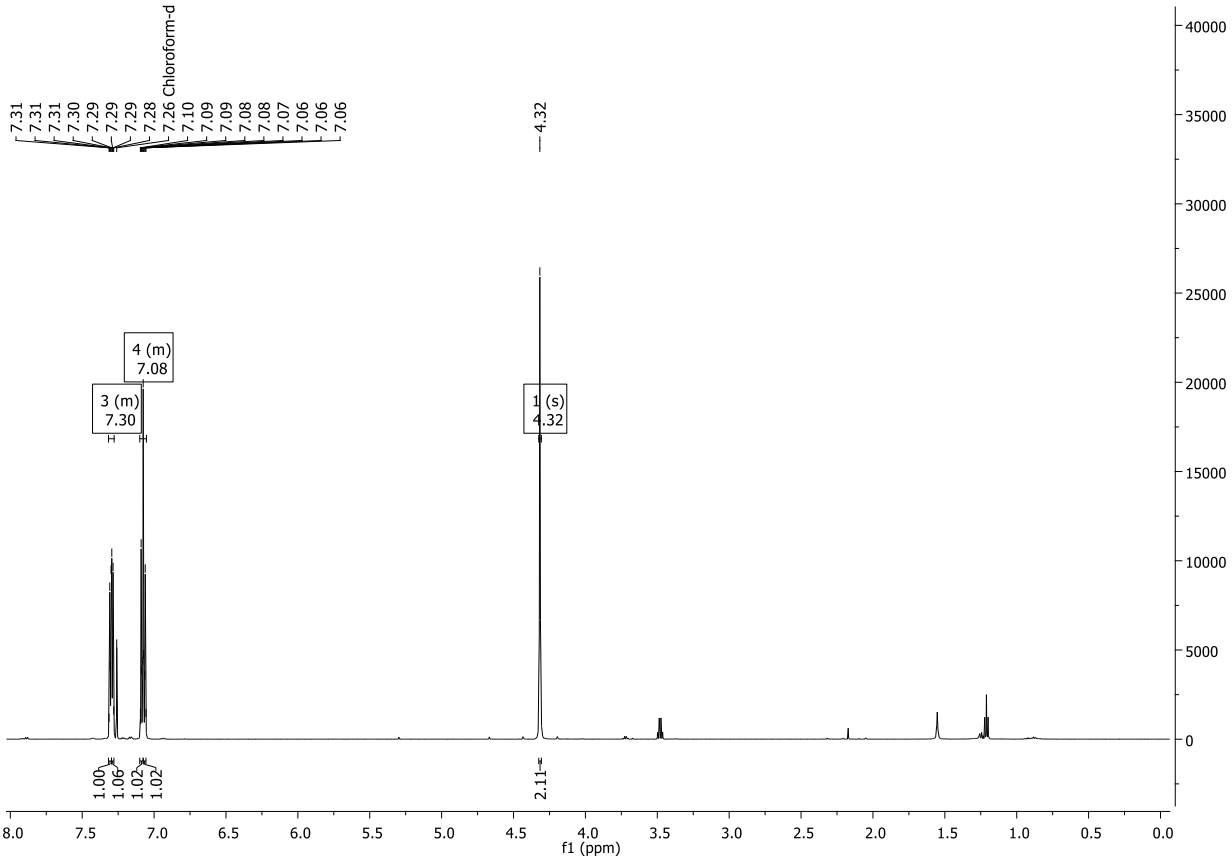


Intermediate 25c

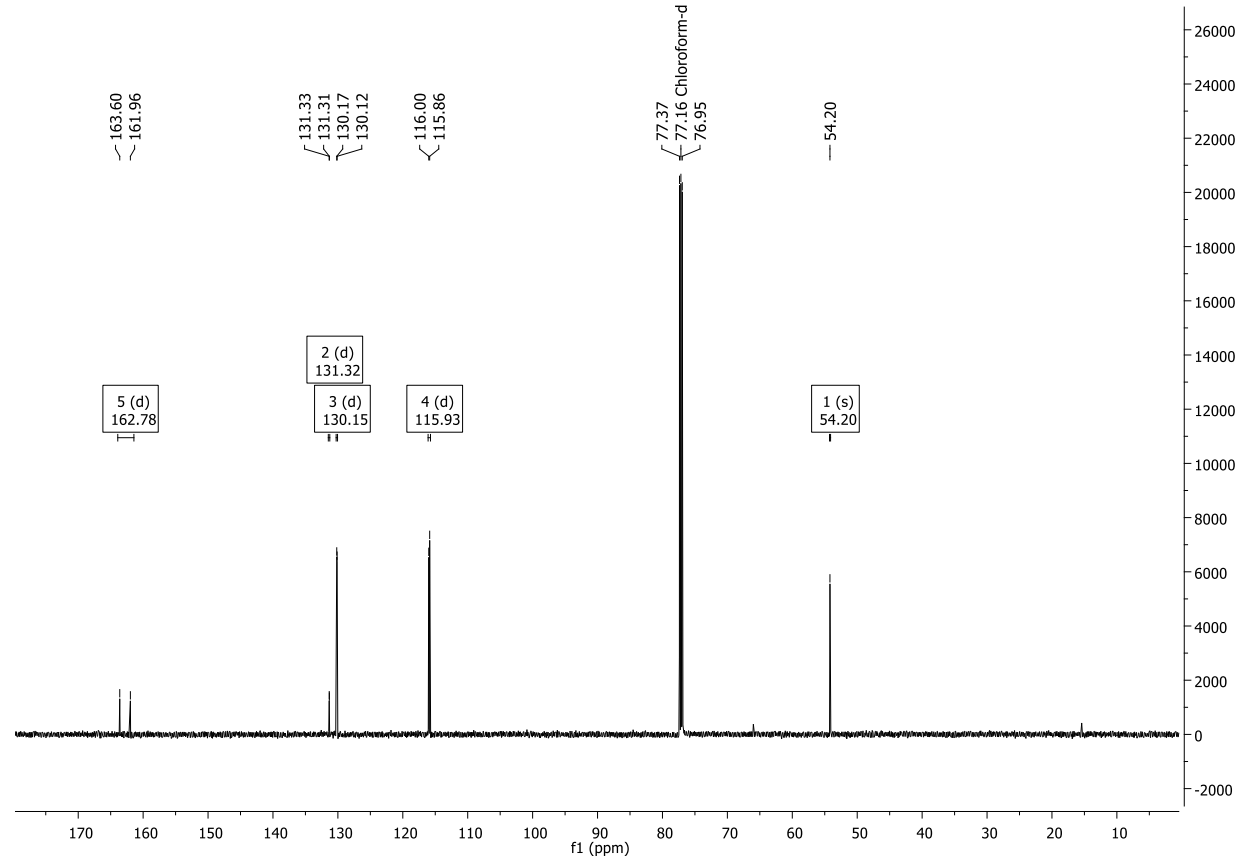
IR



^{1}H in $CDCl_3$

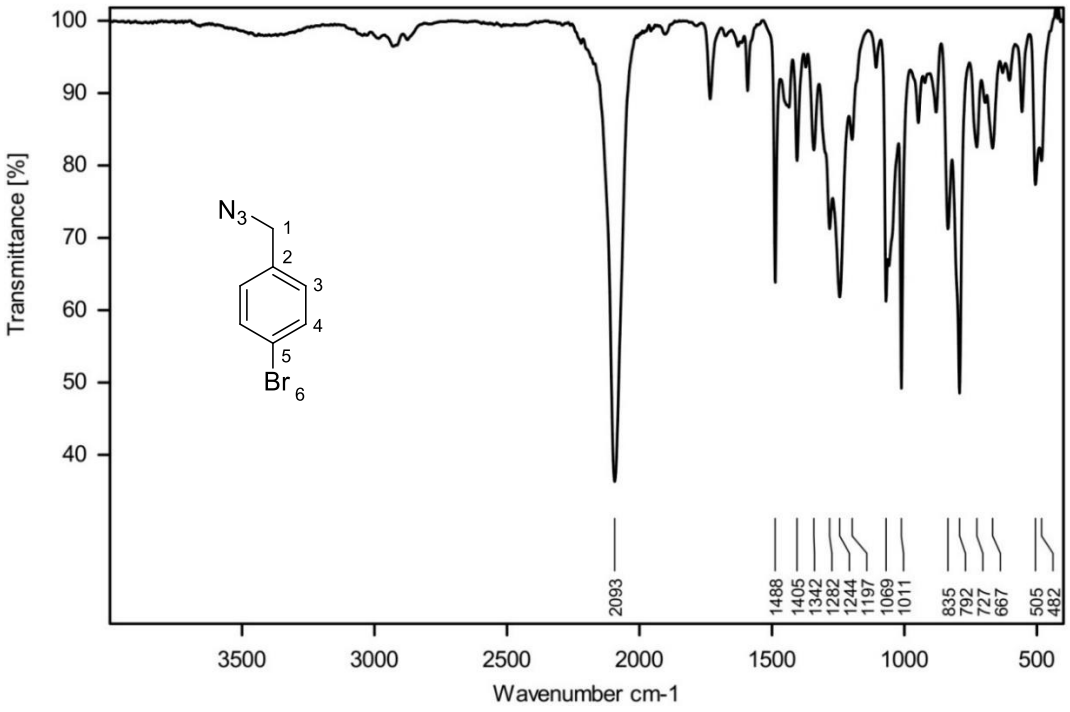


^{13}C in $CDCl_3$

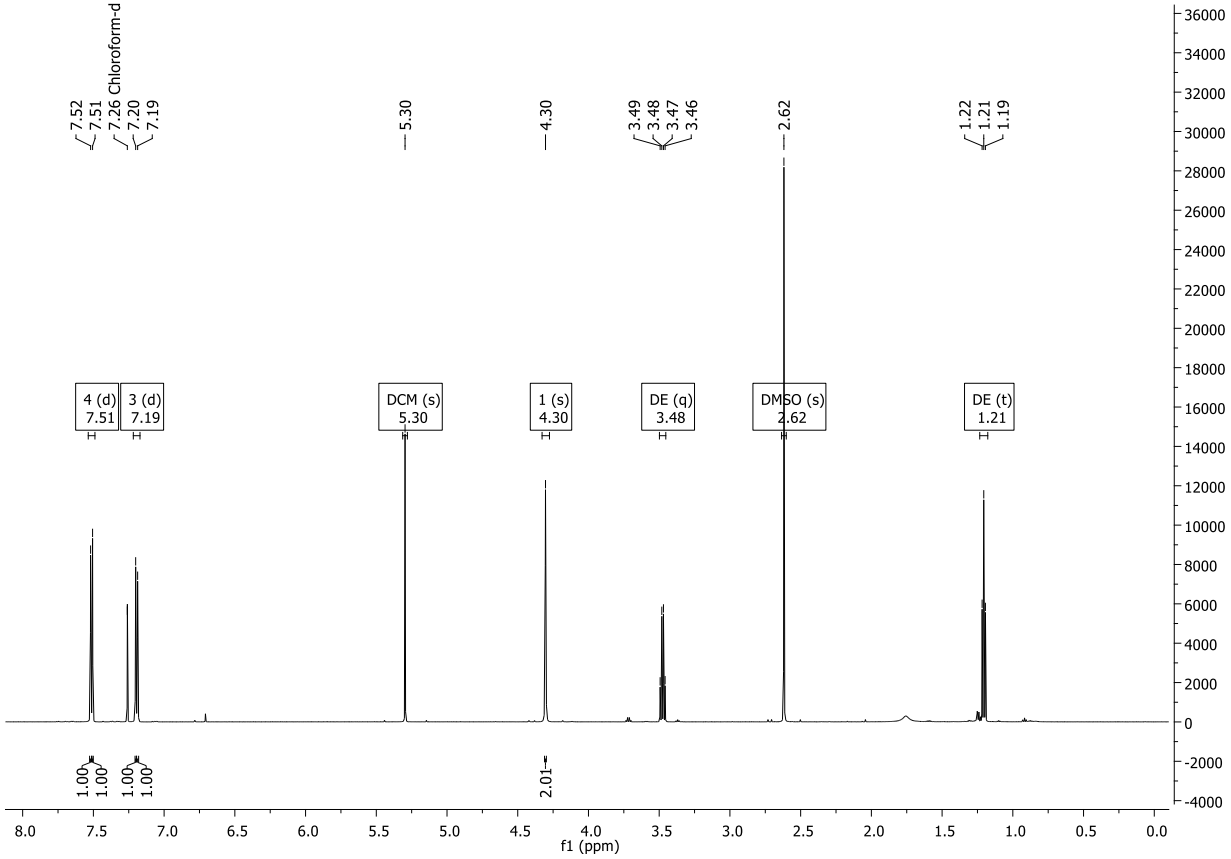


Intermediate 25d

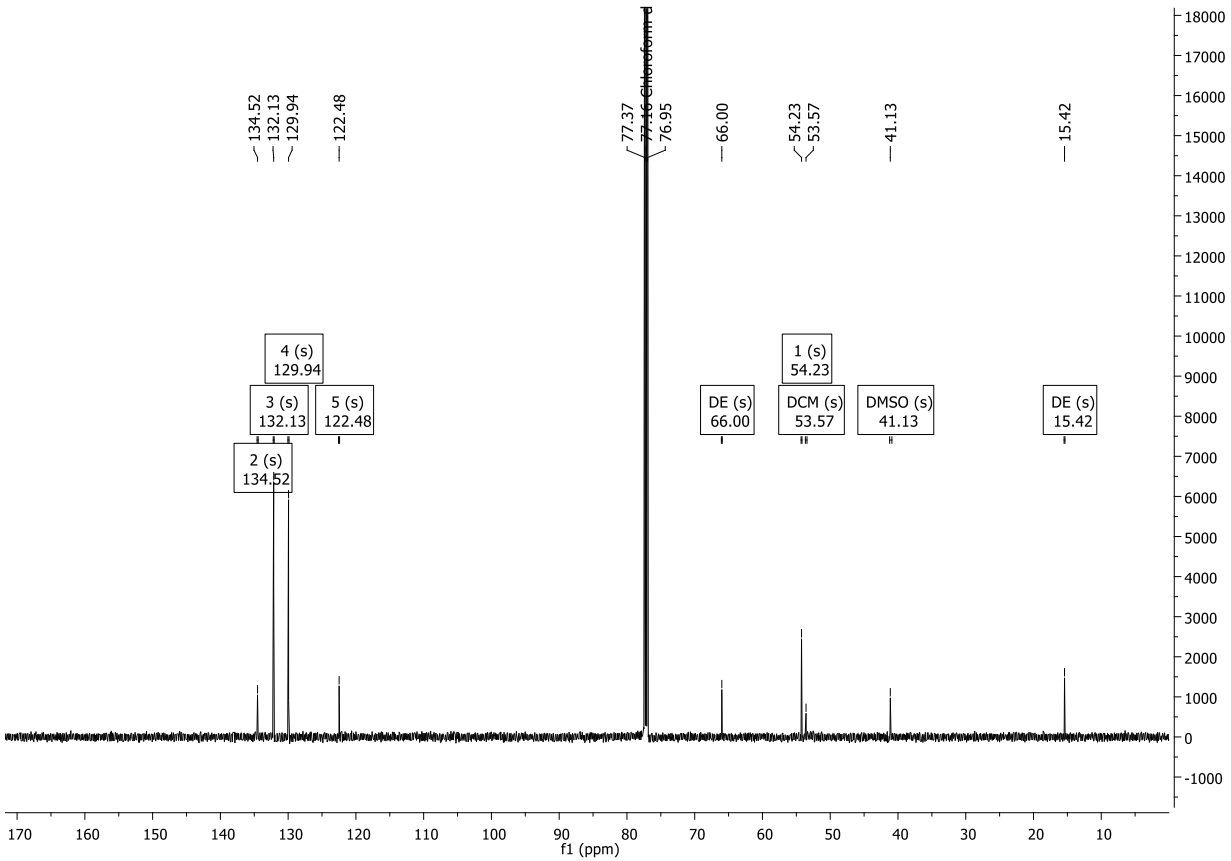
IR



^{1}H in $CDCl_3$

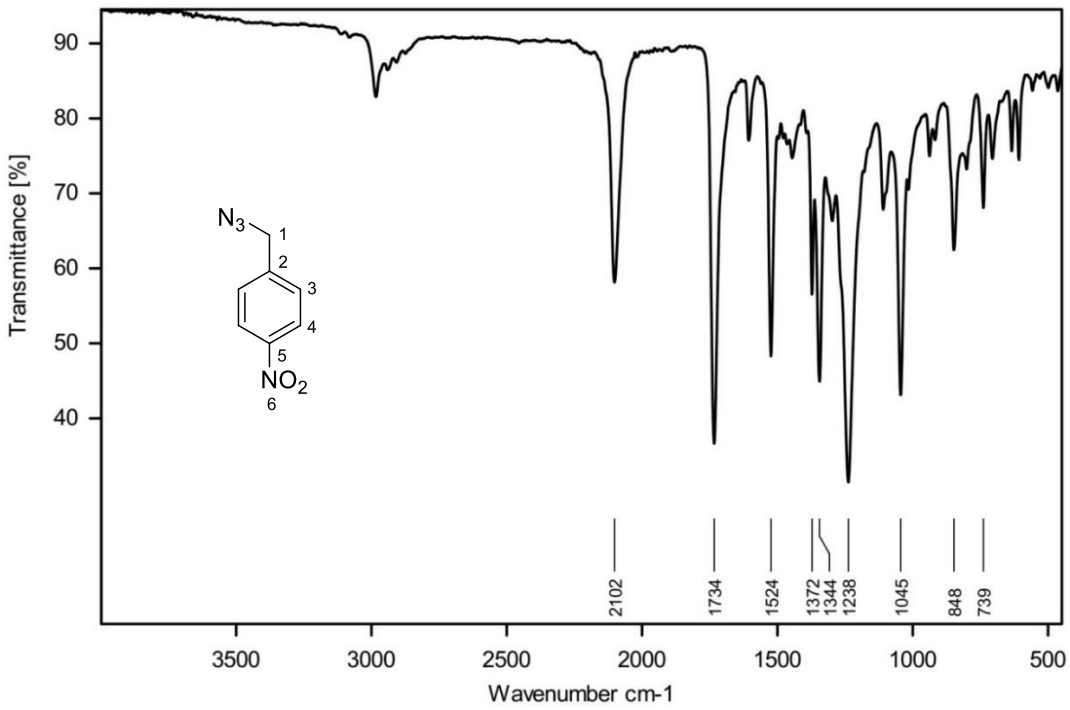


^{13}C in $CDCl_3$

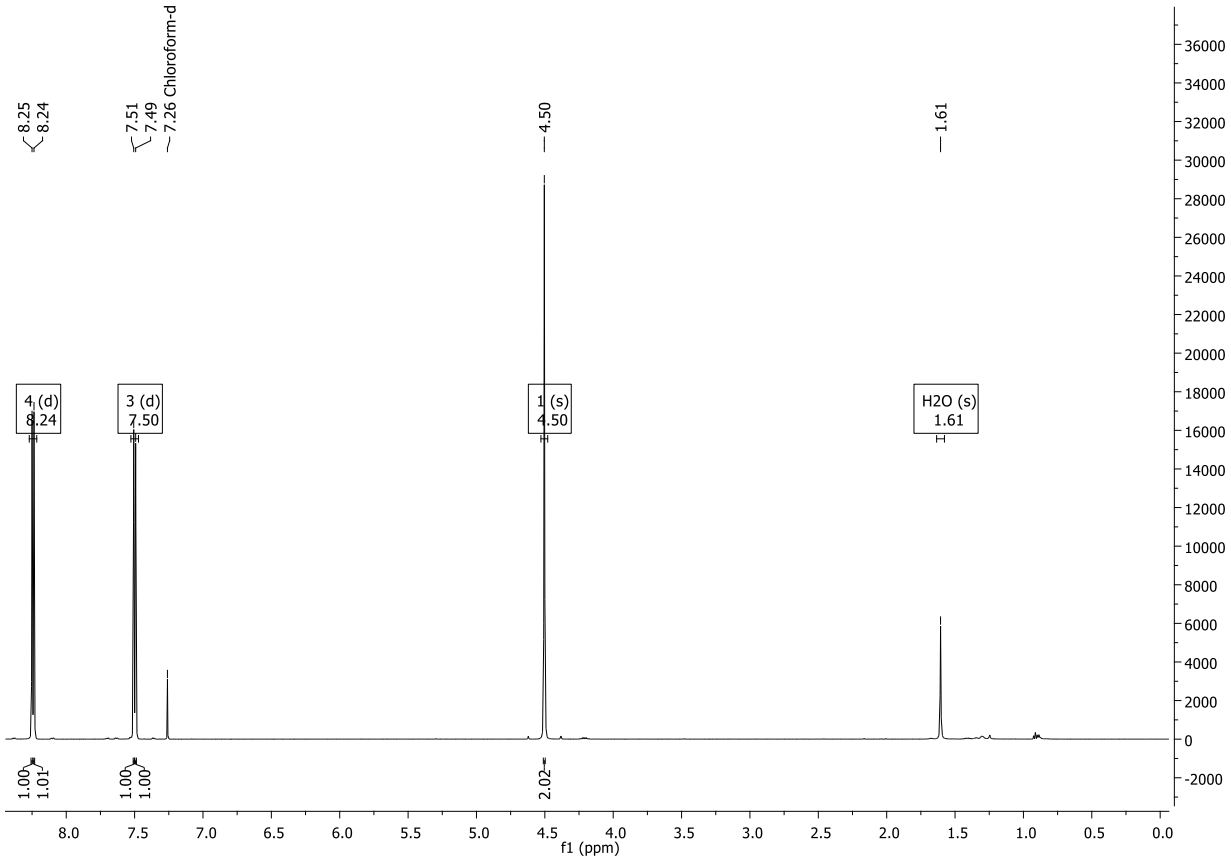


Intermediate 25e

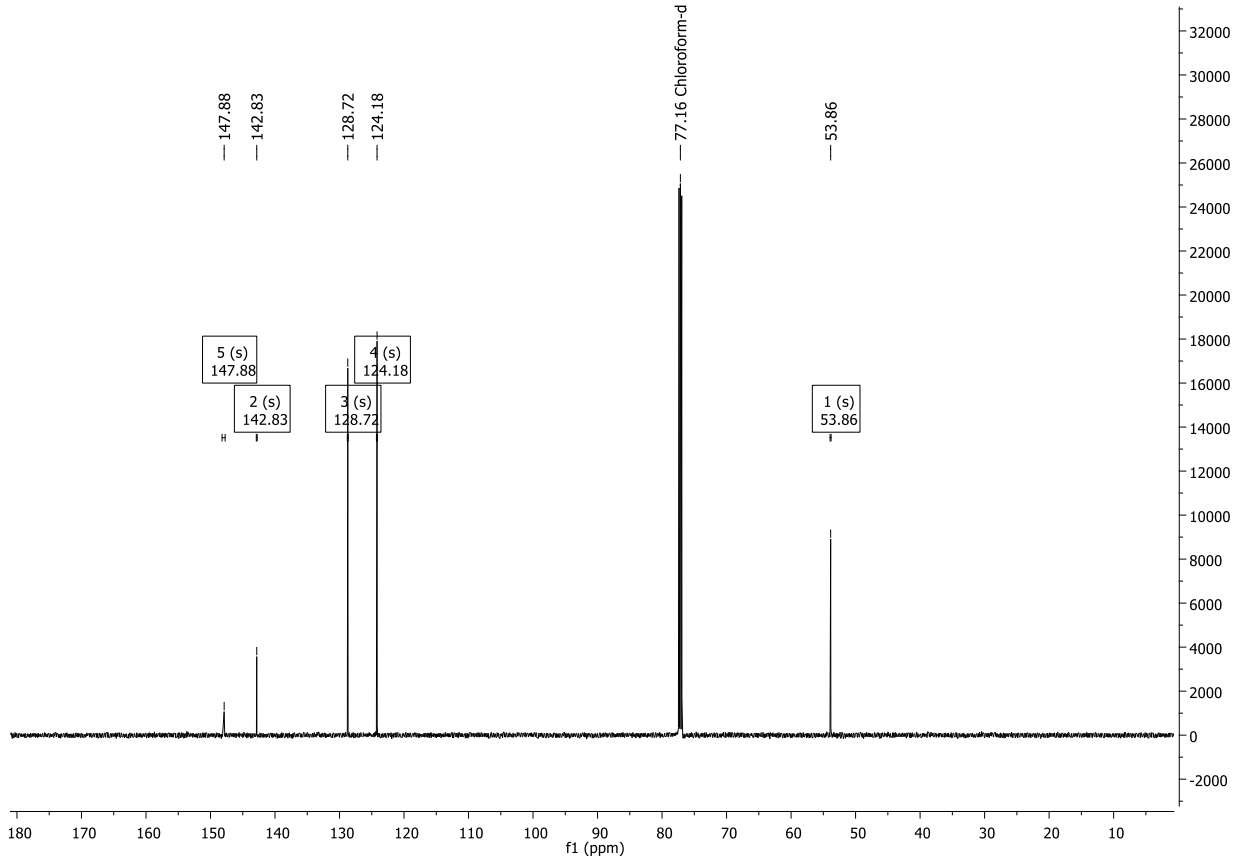
IR



^1H in $CDCl_3$

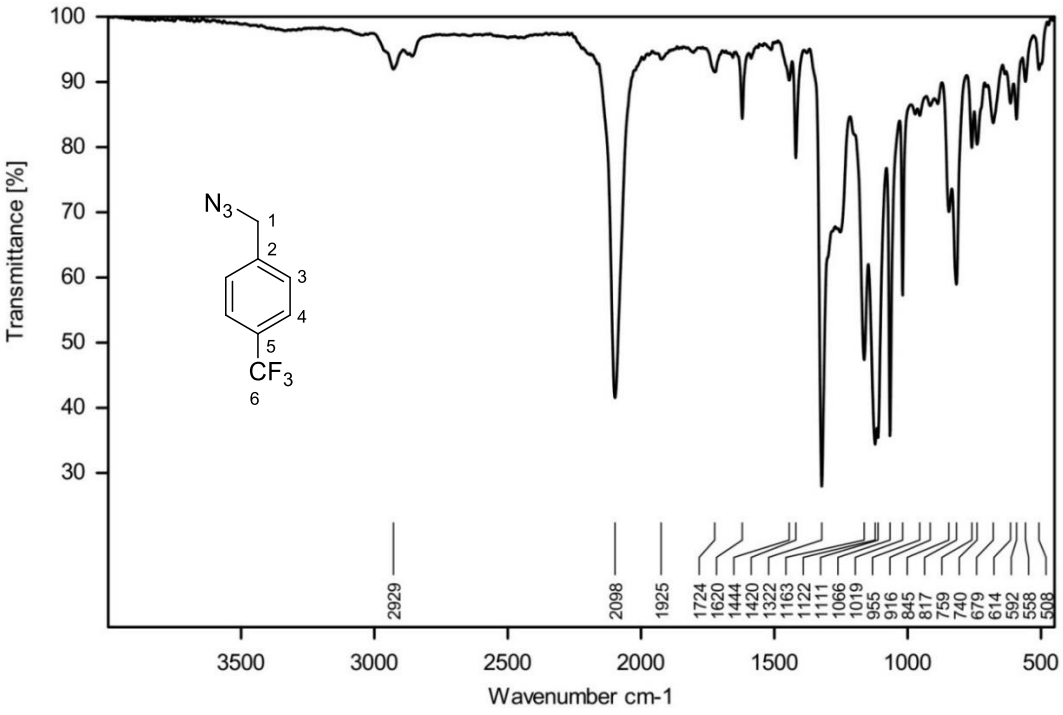


^{13}C in $CDCl_3$

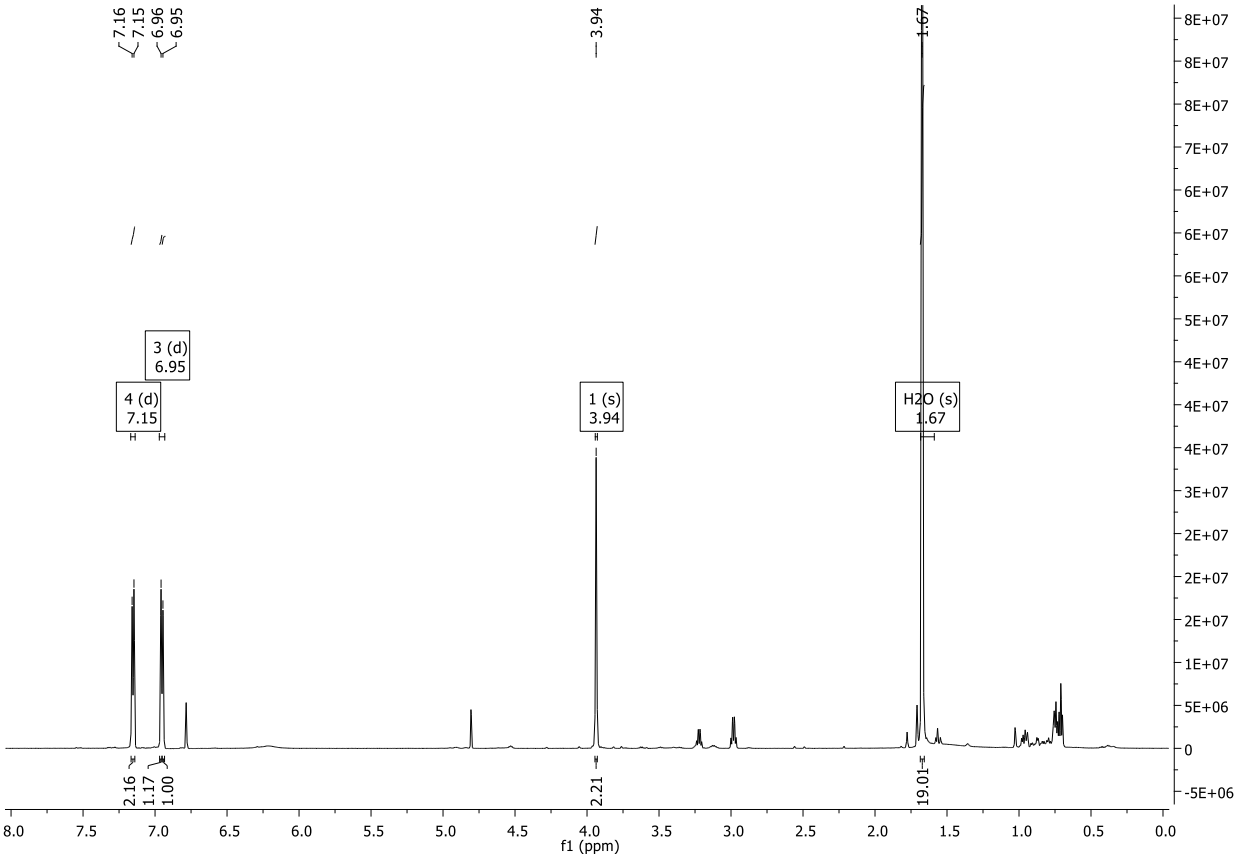


Intermediate 25f

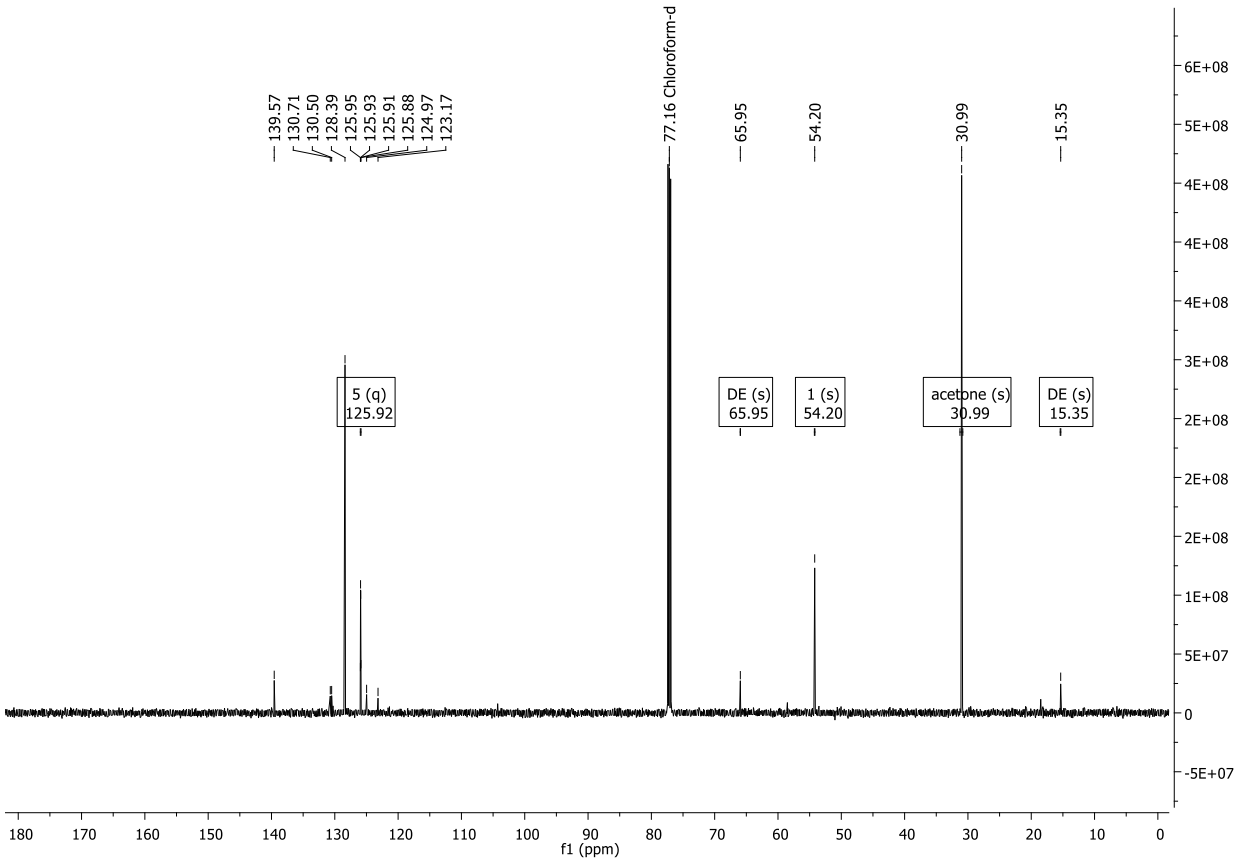
IR



^{1}H in $CDCl_3$

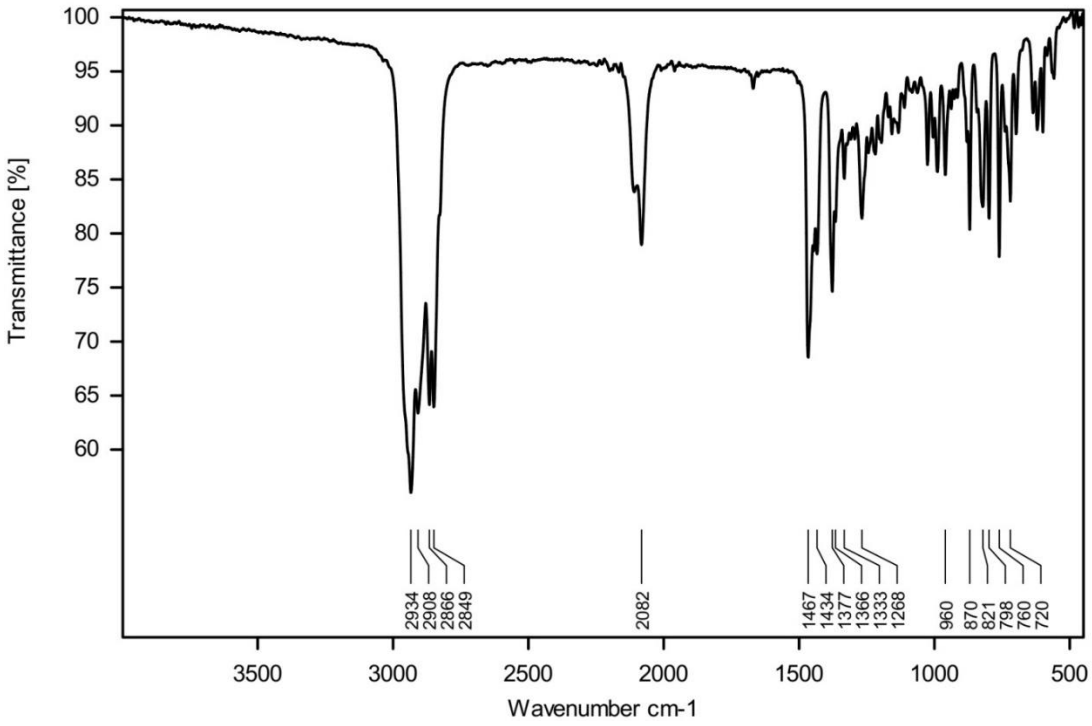


^{13}C in $CDCl_3$

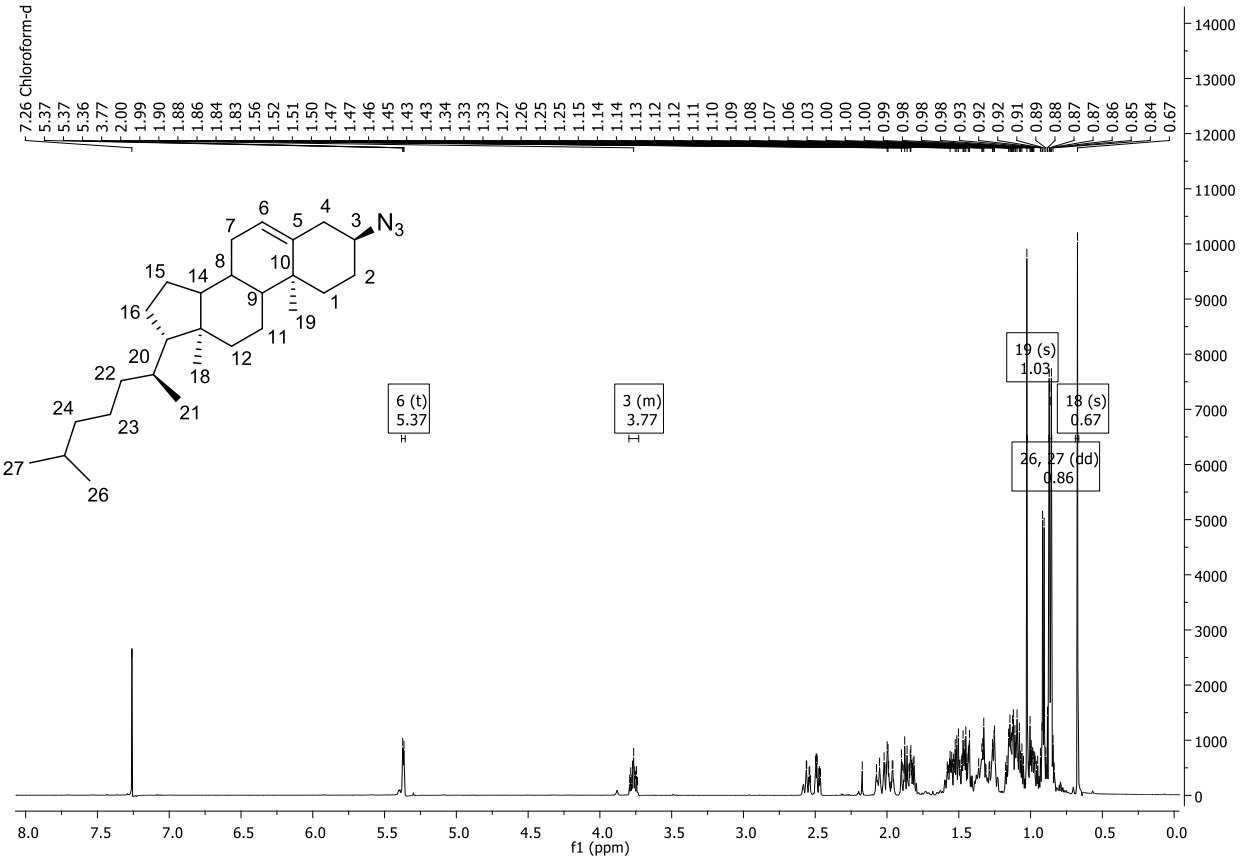


Intermediate 25g

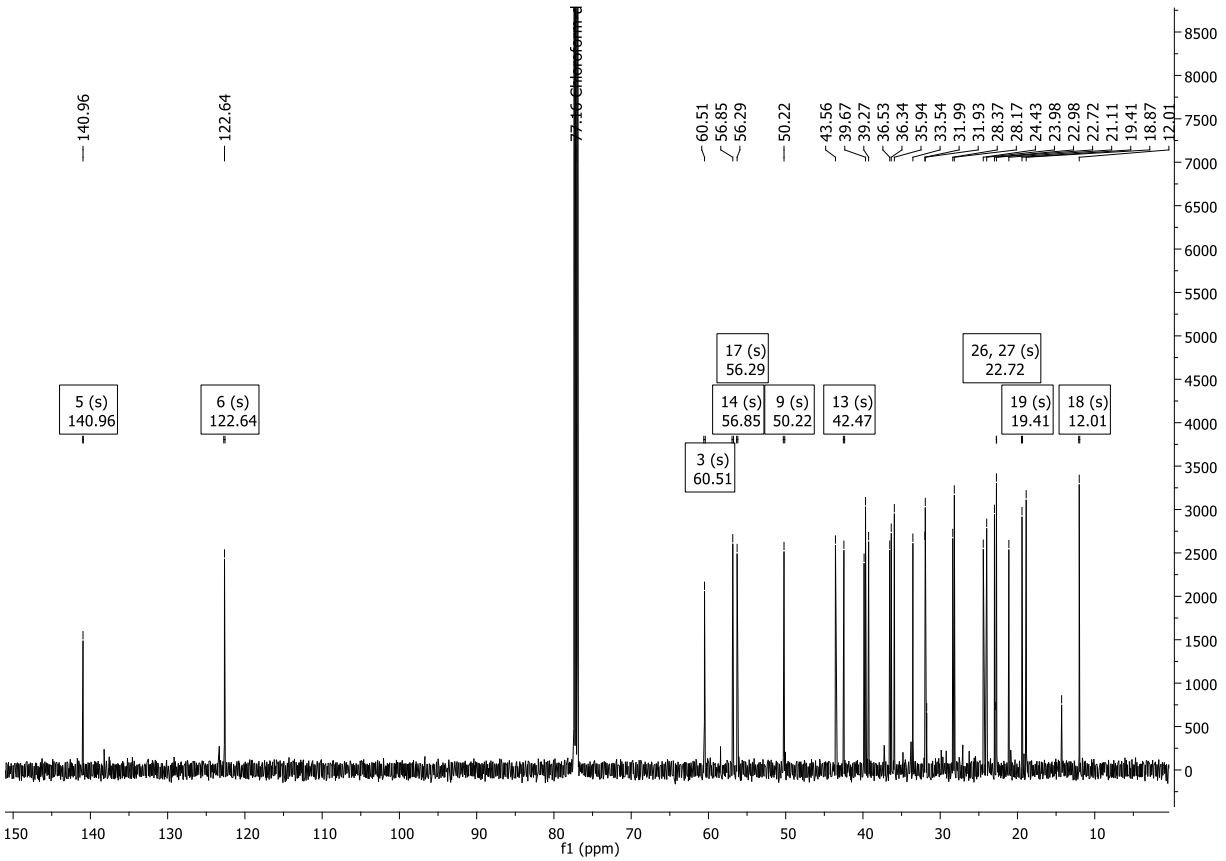
IR



^1H in $CDCl_3$

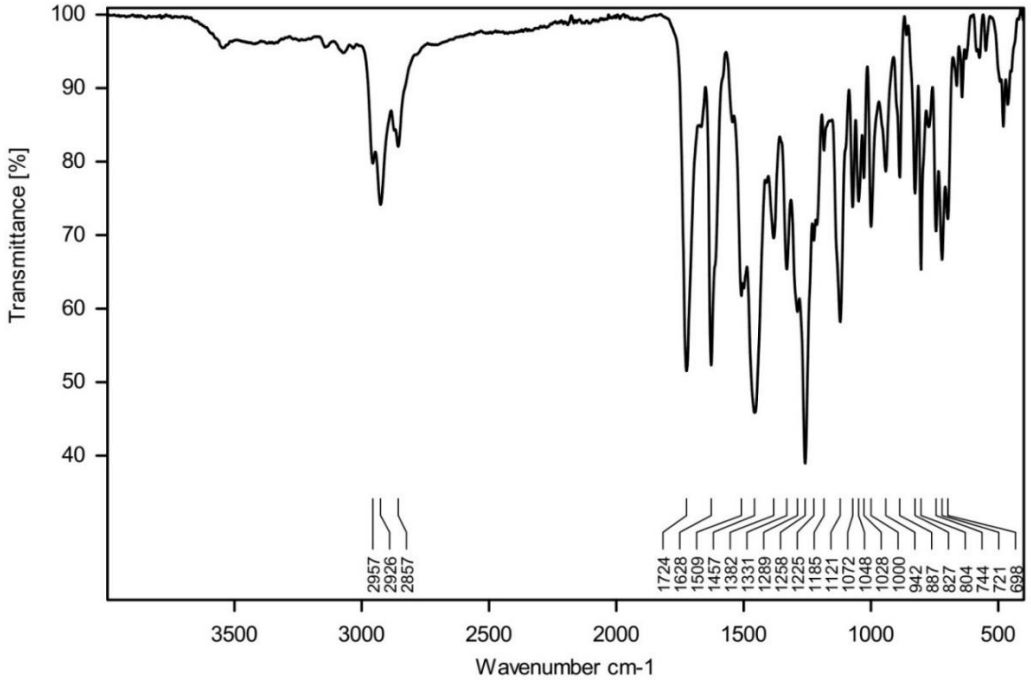


^{13}C in $CDCl_3$

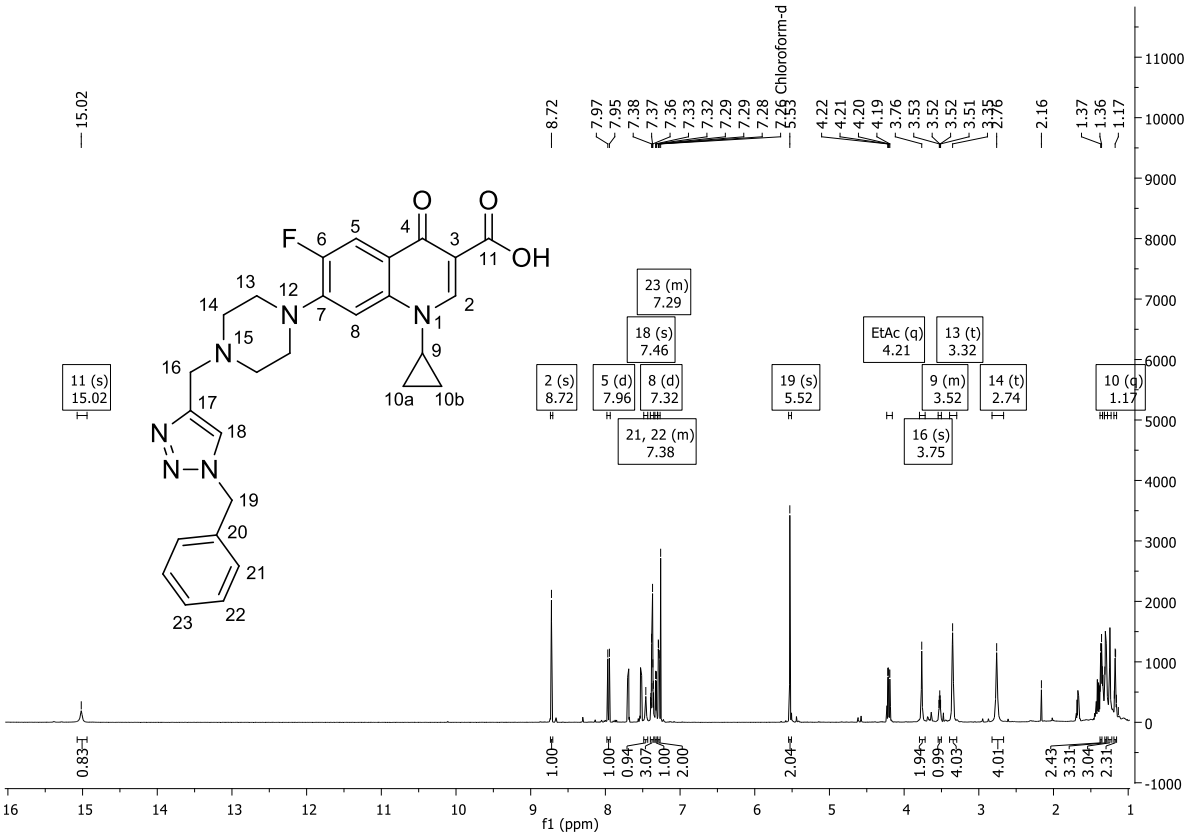

140.96
122.64
77.16 Chloroform-d
60.51
56.85
56.29
50.22
43.56
39.67
39.27
36.53
36.34
35.94
33.54
31.99
31.93
28.37
28.17
24.43
23.98
22.98
22.72
21.11
19.41
18.87
12.01
5 (s) 140.96
6 (s) 122.64
17 (s) 56.29
14 (s) 56.85
9 (s) 50.22
13 (s) 42.47
3 (s) 60.51
26, 27 (s) 22.72
19 (s) 19.41
18 (s) 12.01
f1 (ppm)

COMPOUND 26

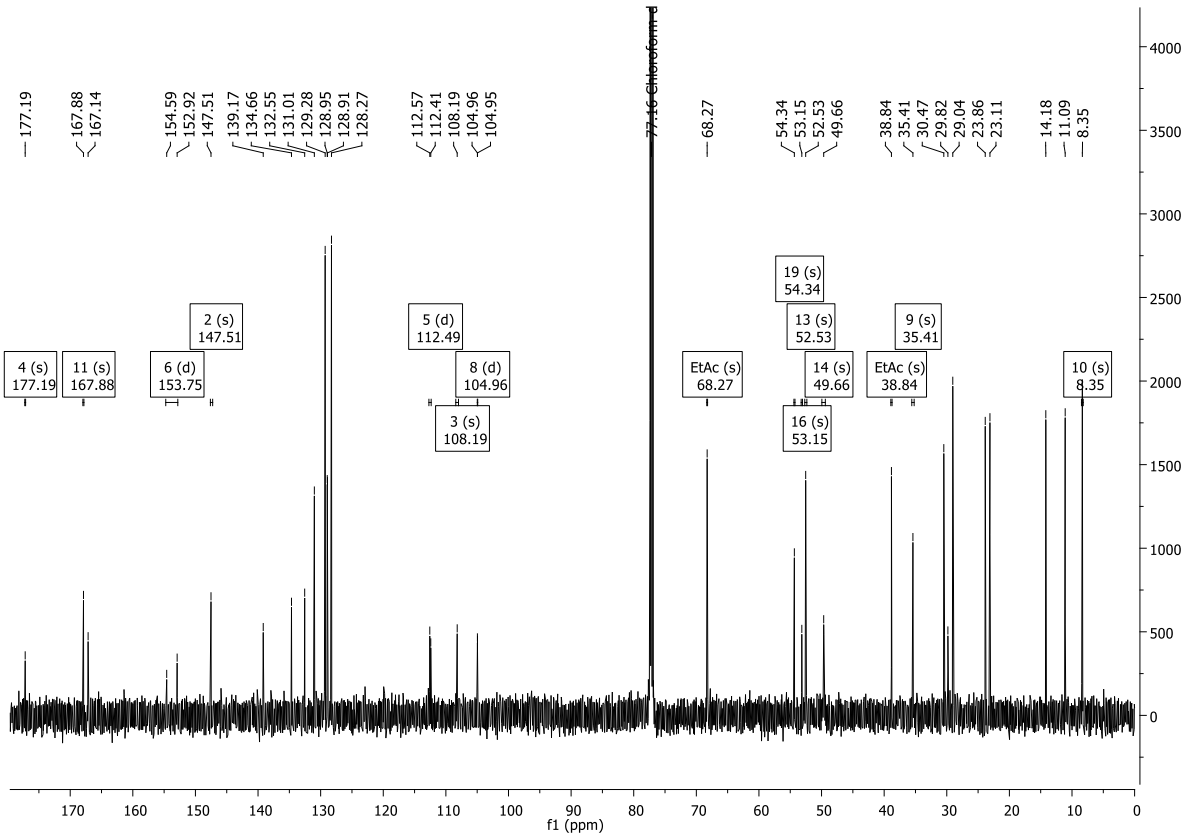
IR



^{1}H in $CDCl_3$



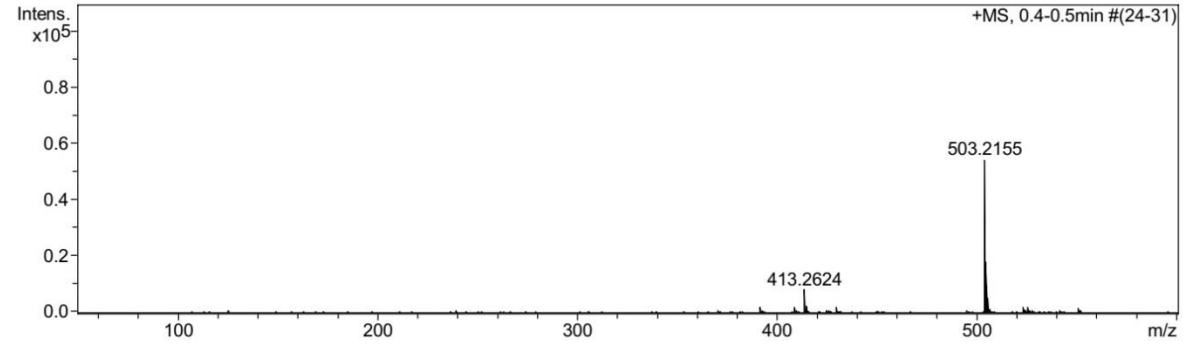
^{13}C in $CDCl_3$



HRMS

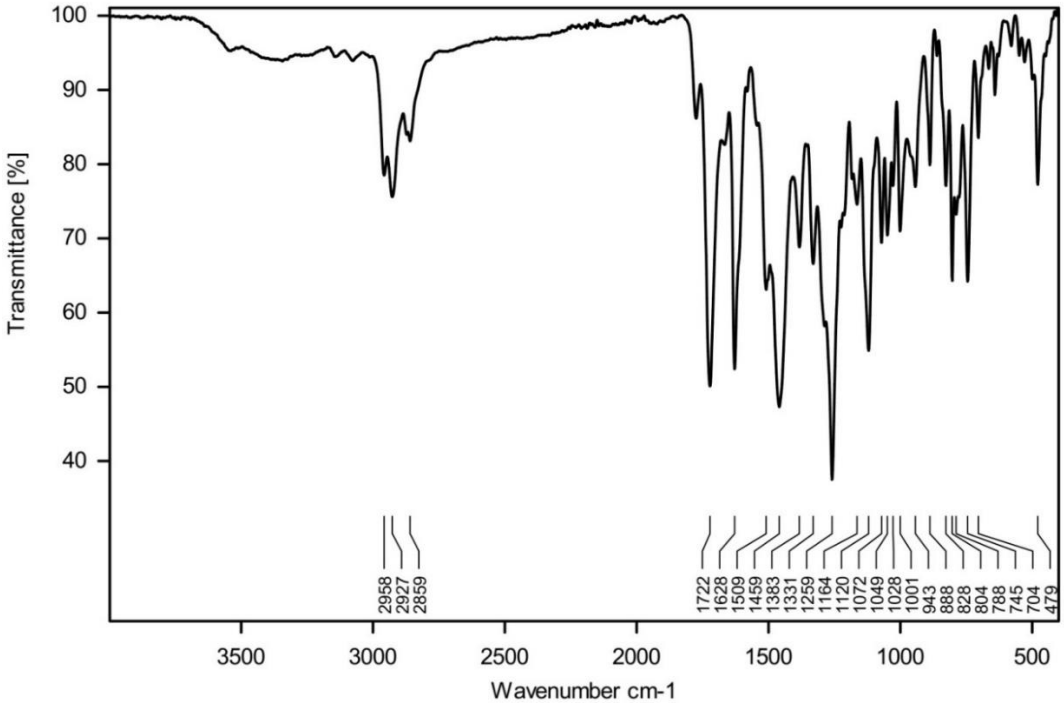
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

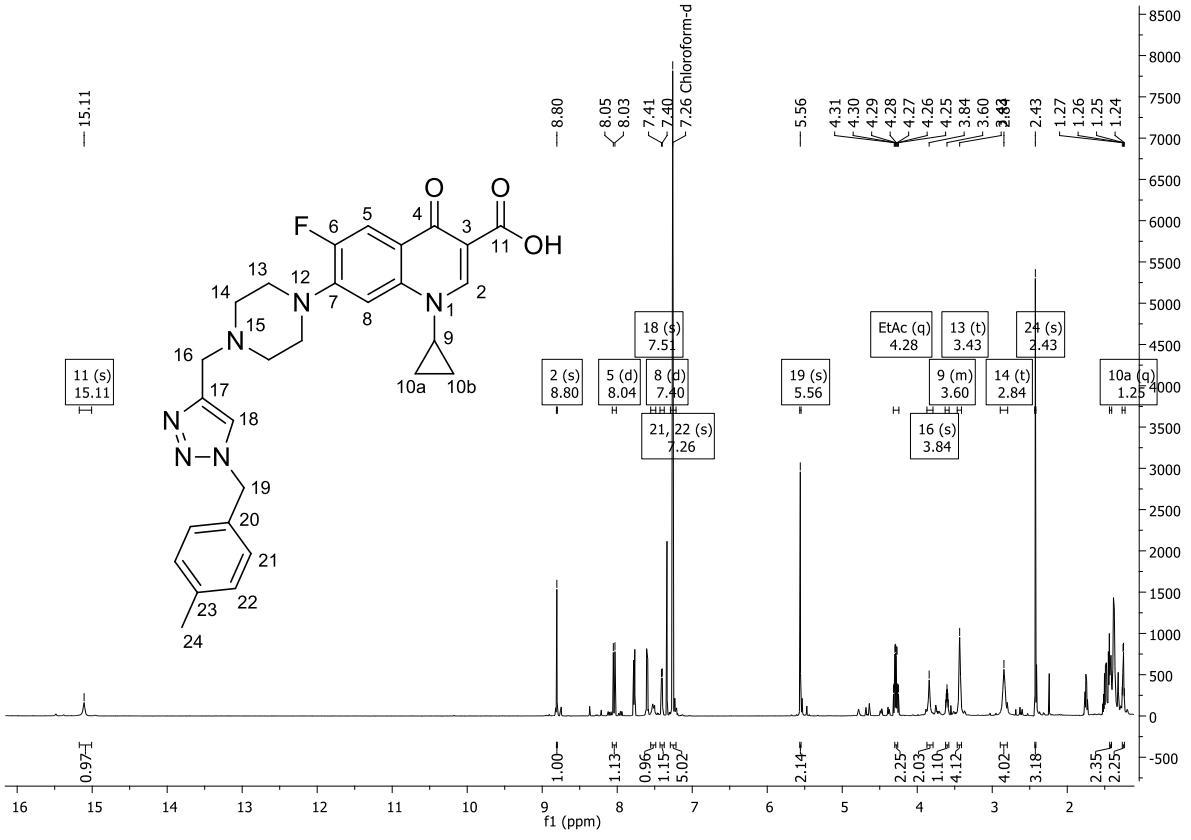


COMPOUND 27

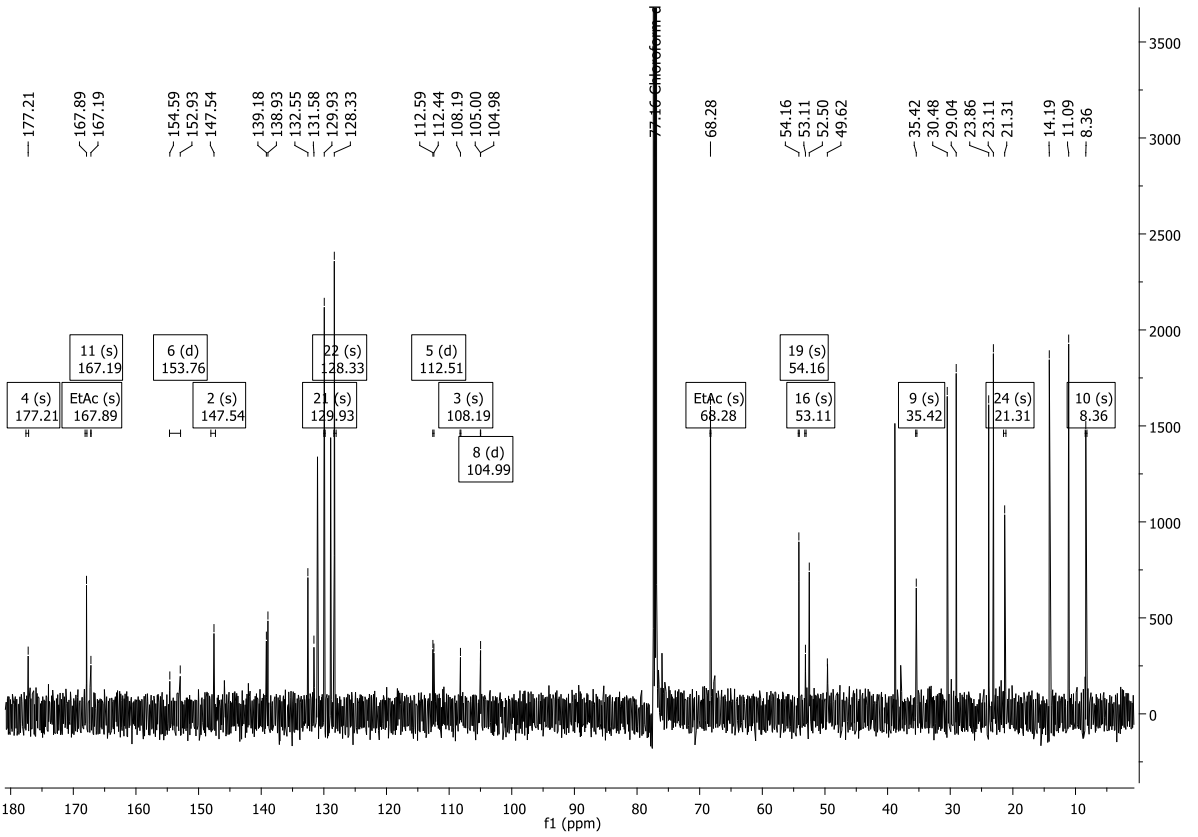
IR



^{1}H in $CDCl_3$



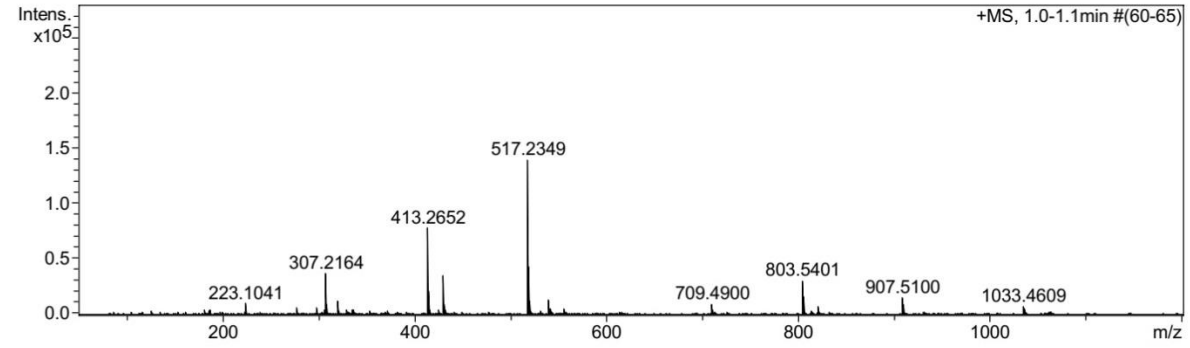
^{13}C in $CDCl_3$



HRMS

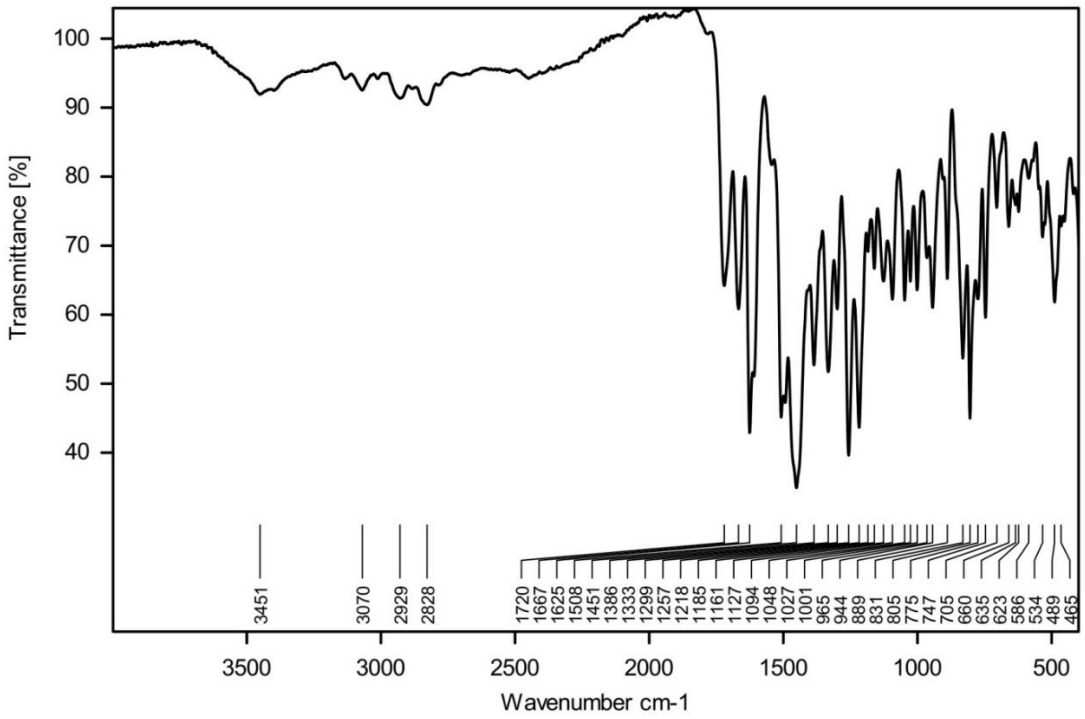
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

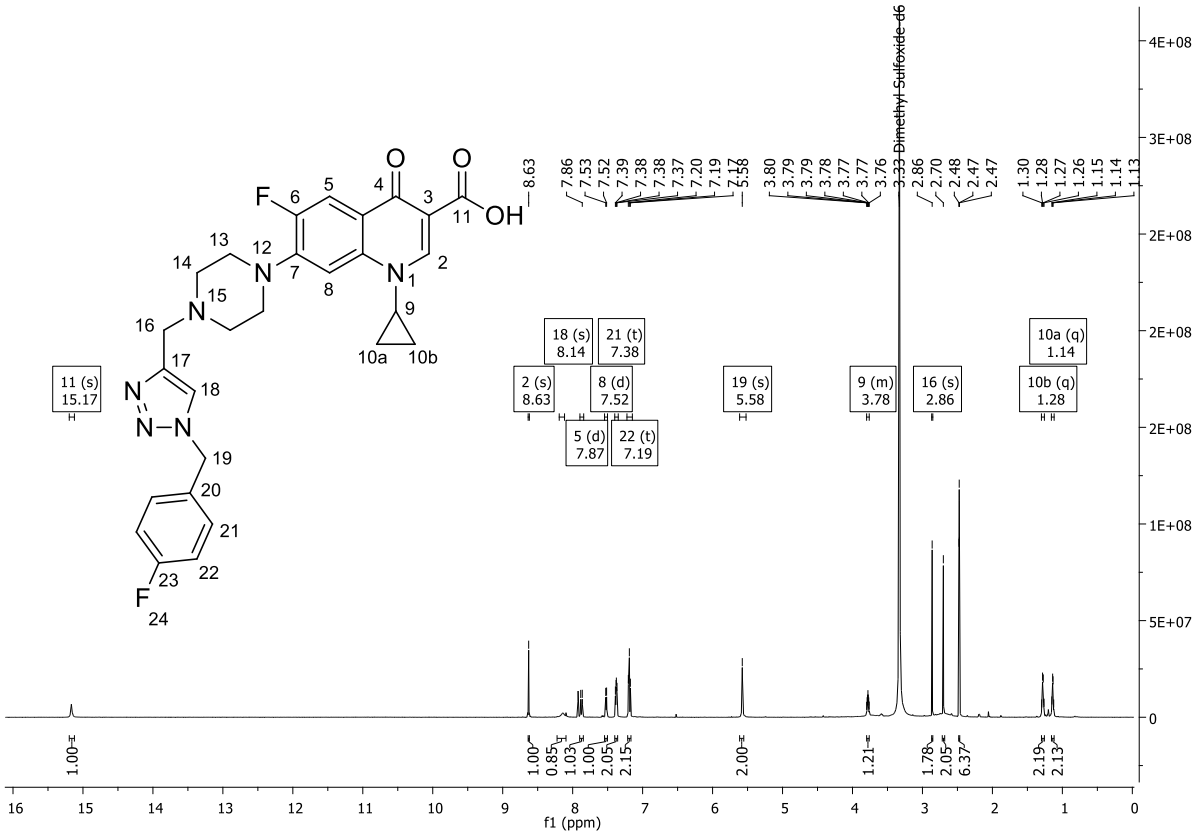


COMPOUND 28

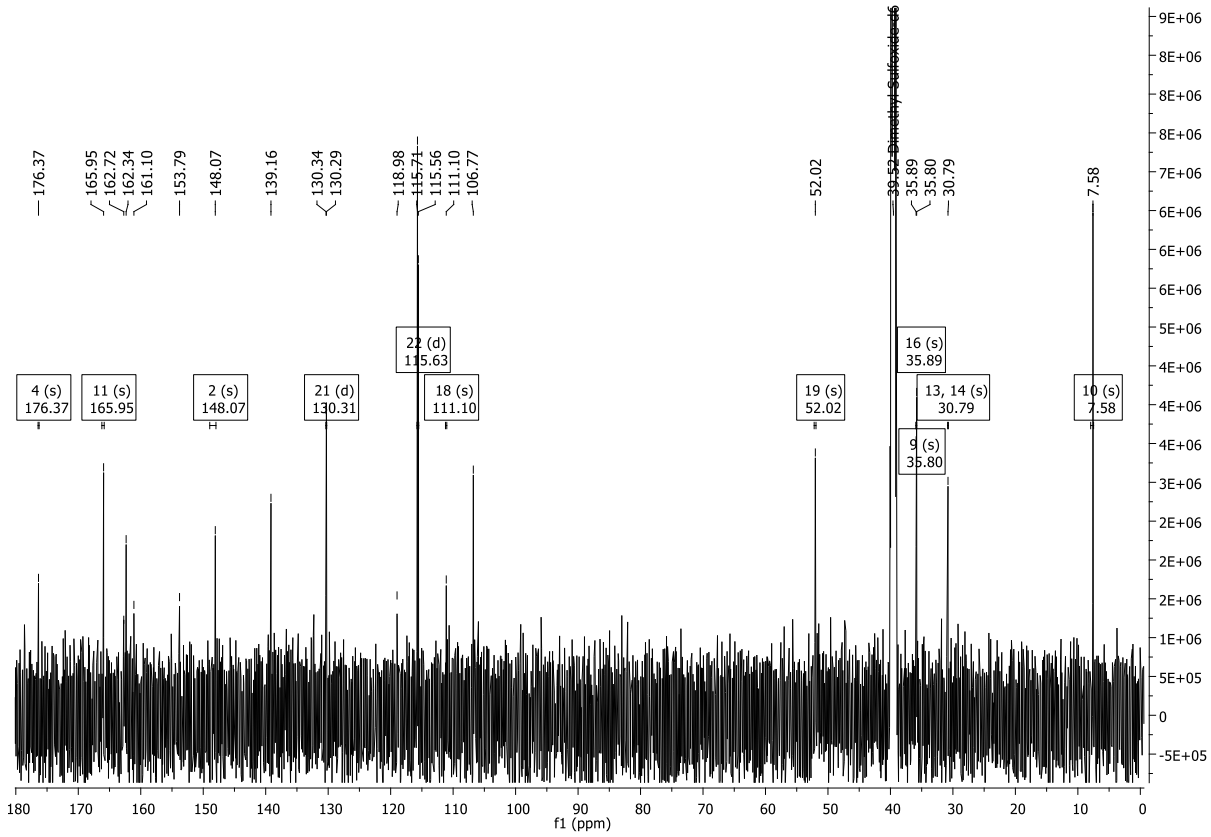
IR



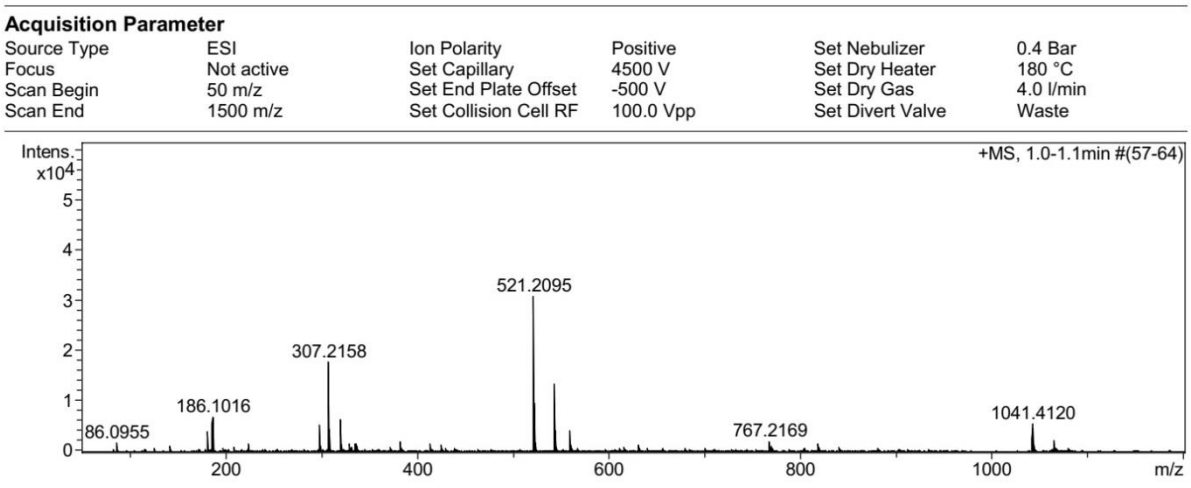
^{1}H in DMSO



^{13}C in DMSO

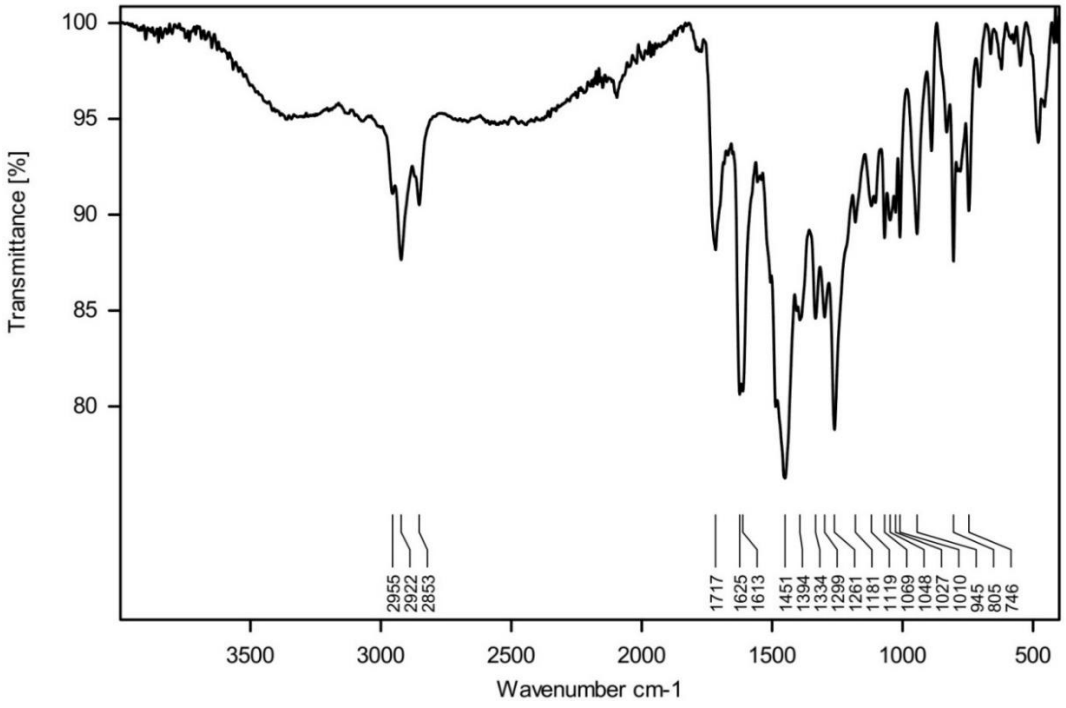


HRMS

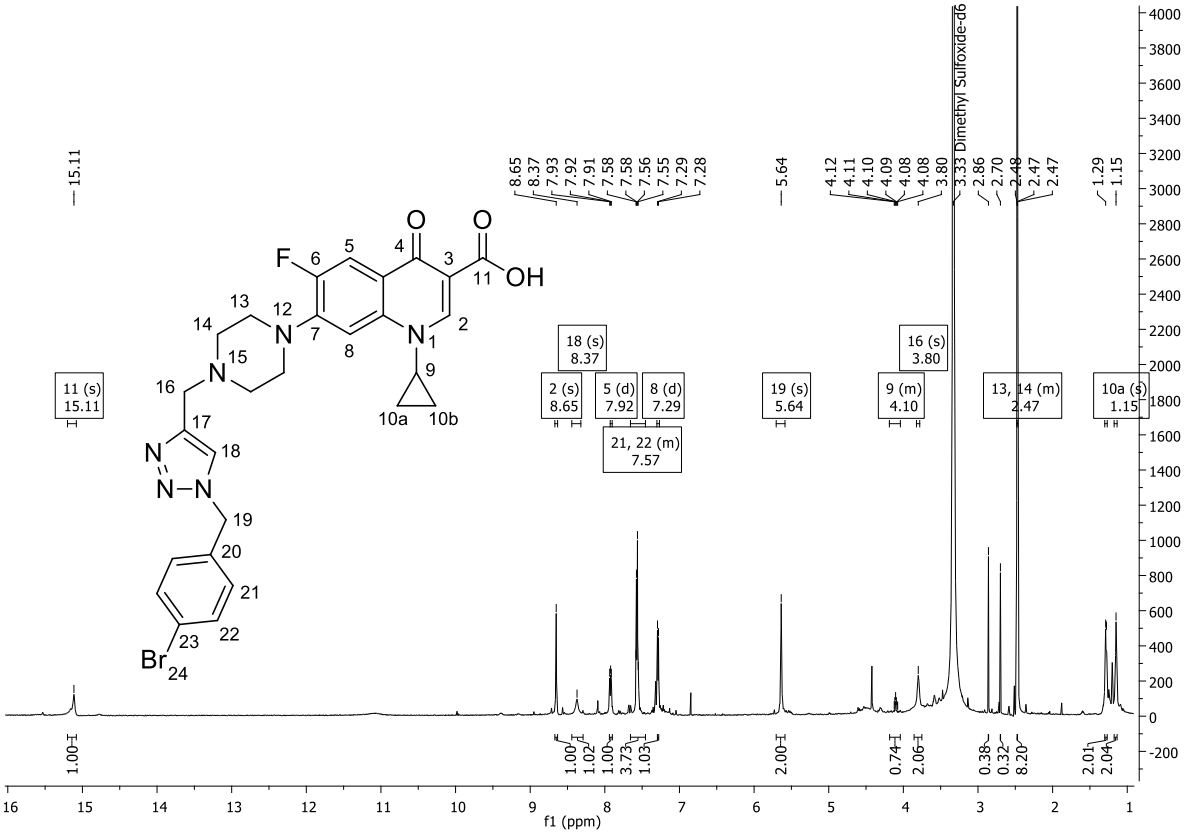


COMPOUND 29

IR



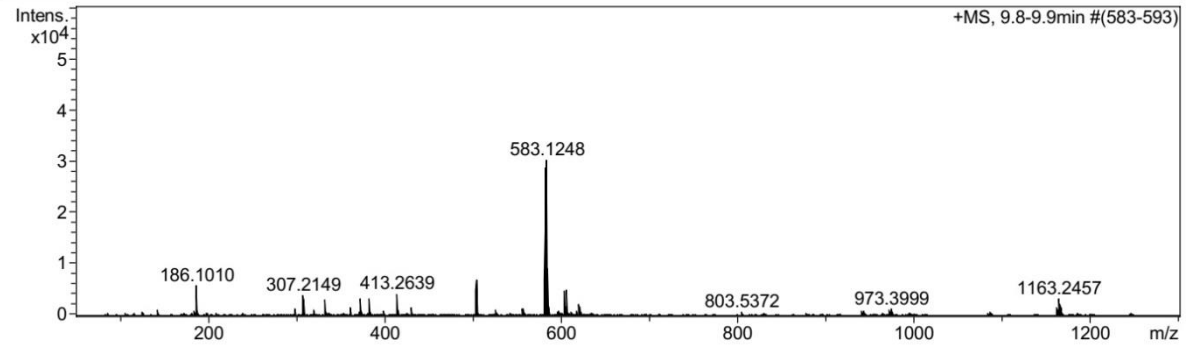
^{1}H in DMSO



HRMS

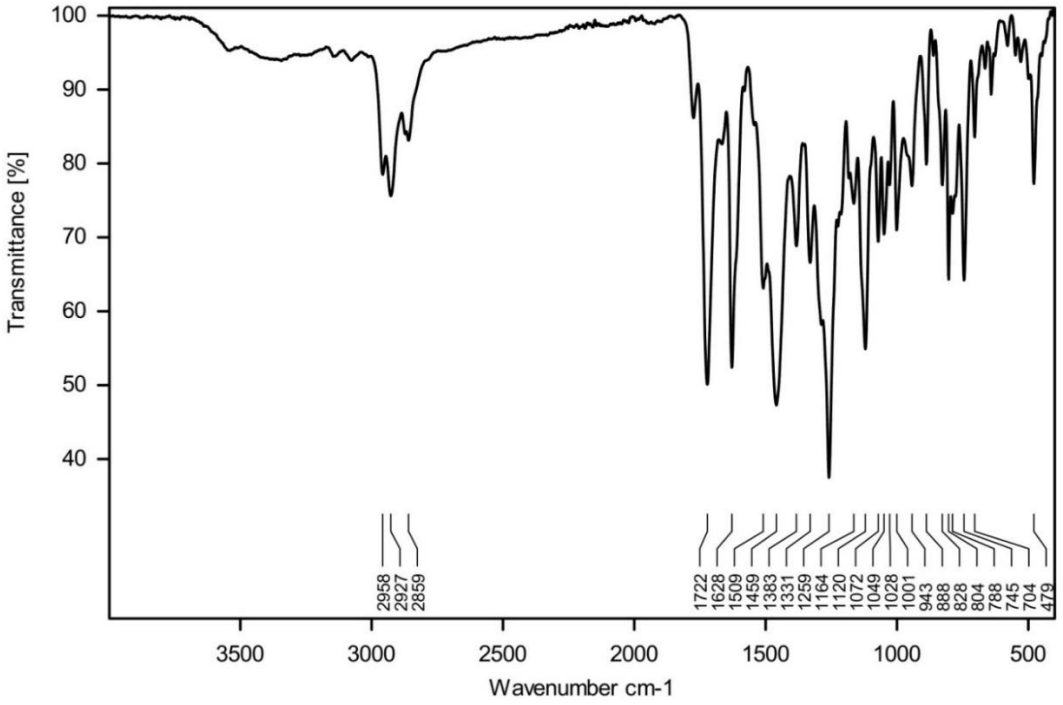
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	6.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

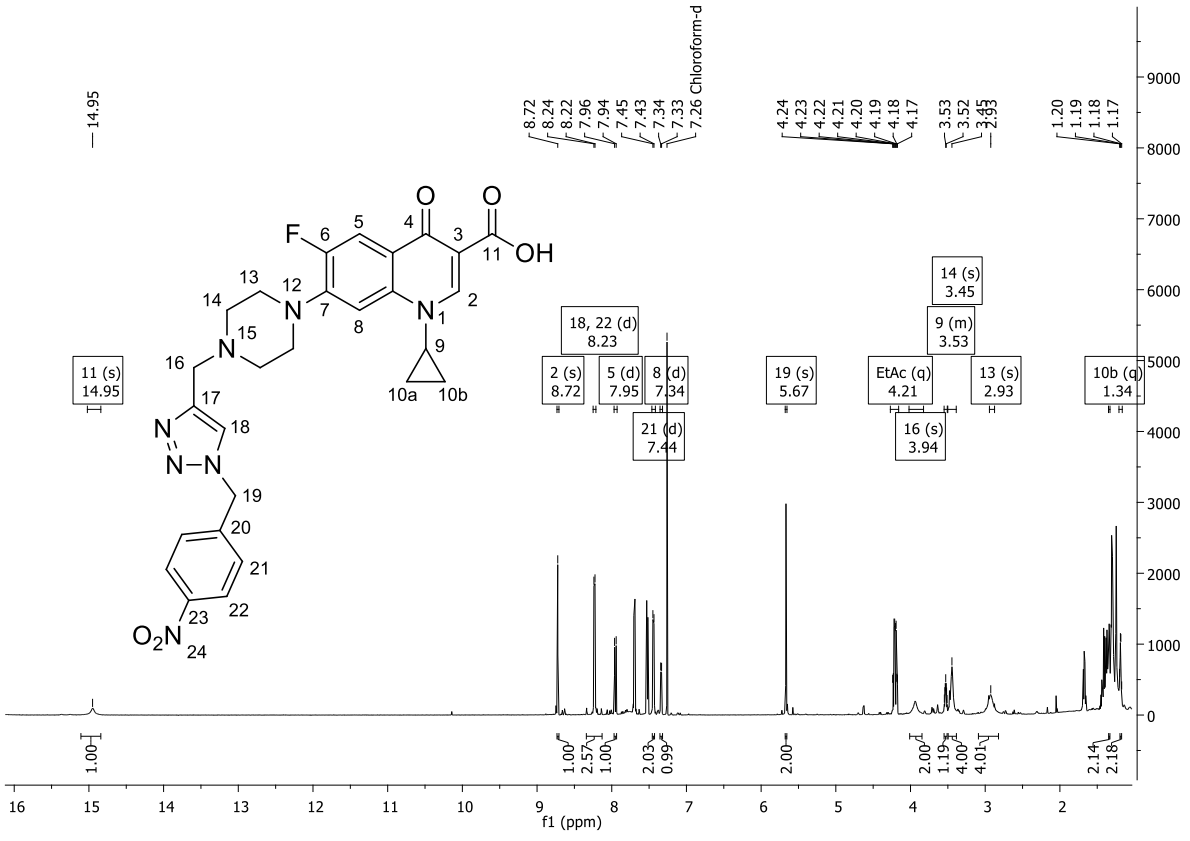


COMPOUND 30

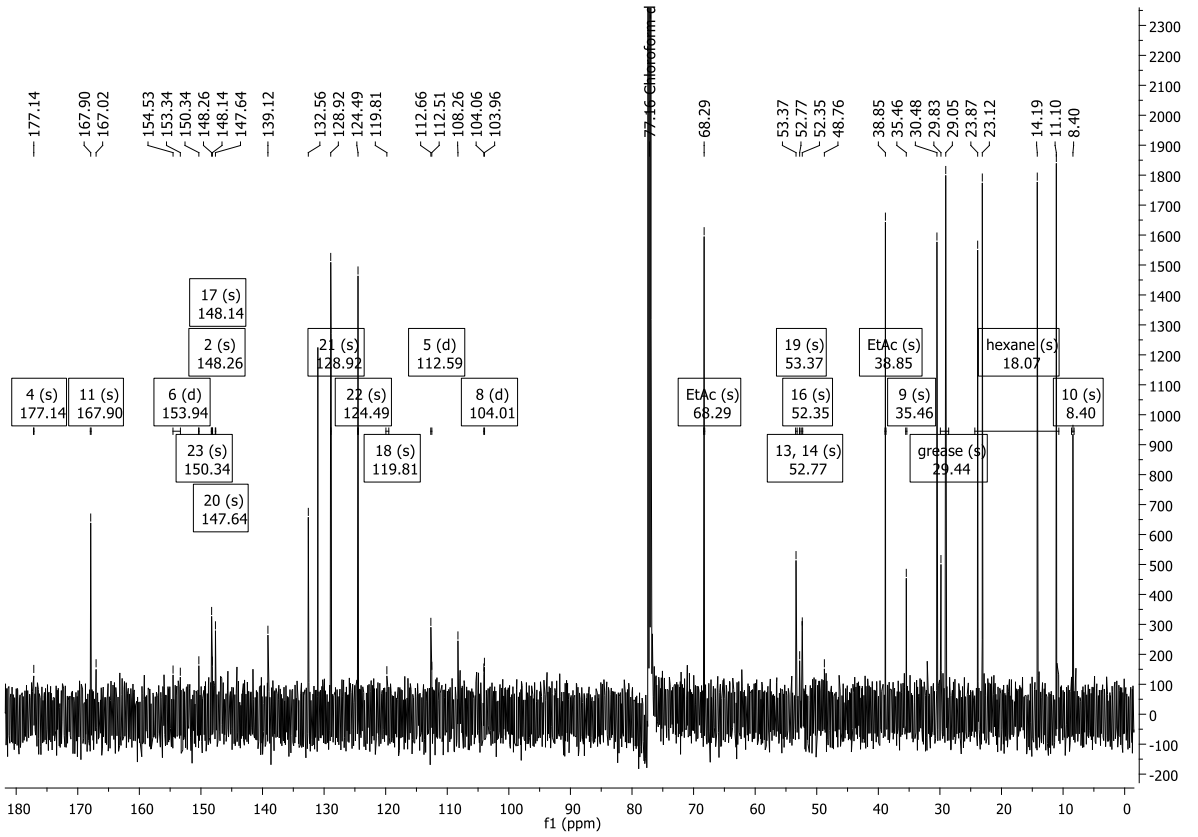
IR



^{1}H in $CDCl_3$



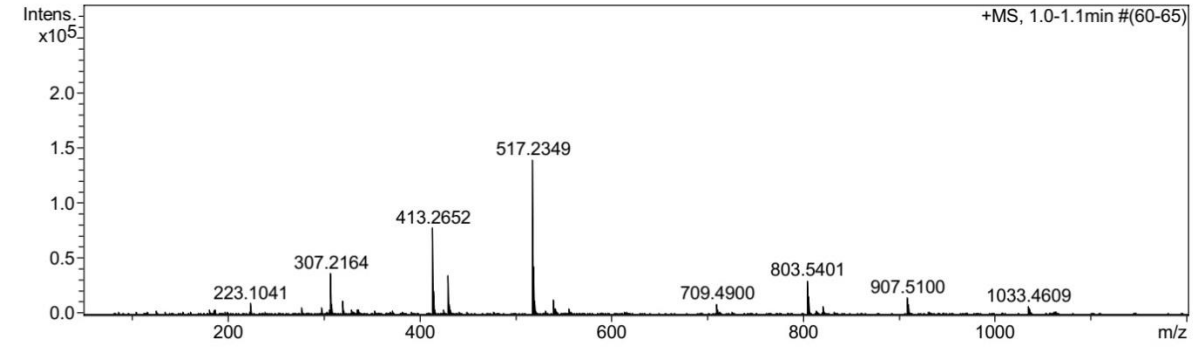
^{13}C in $CDCl_3$



HRMS

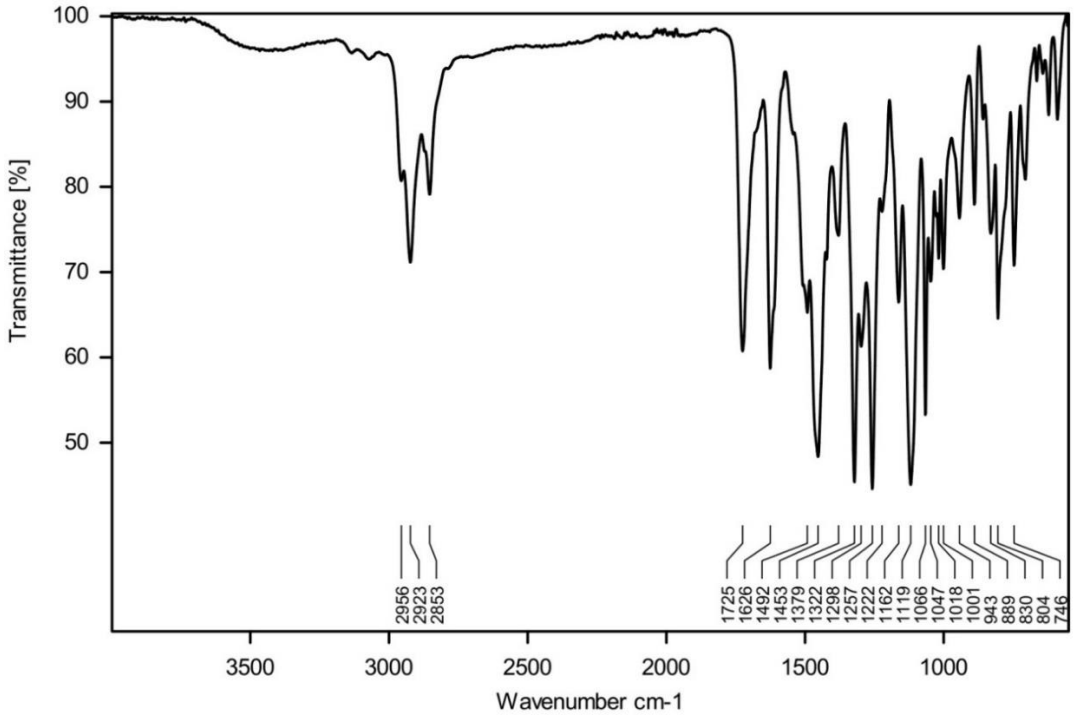
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

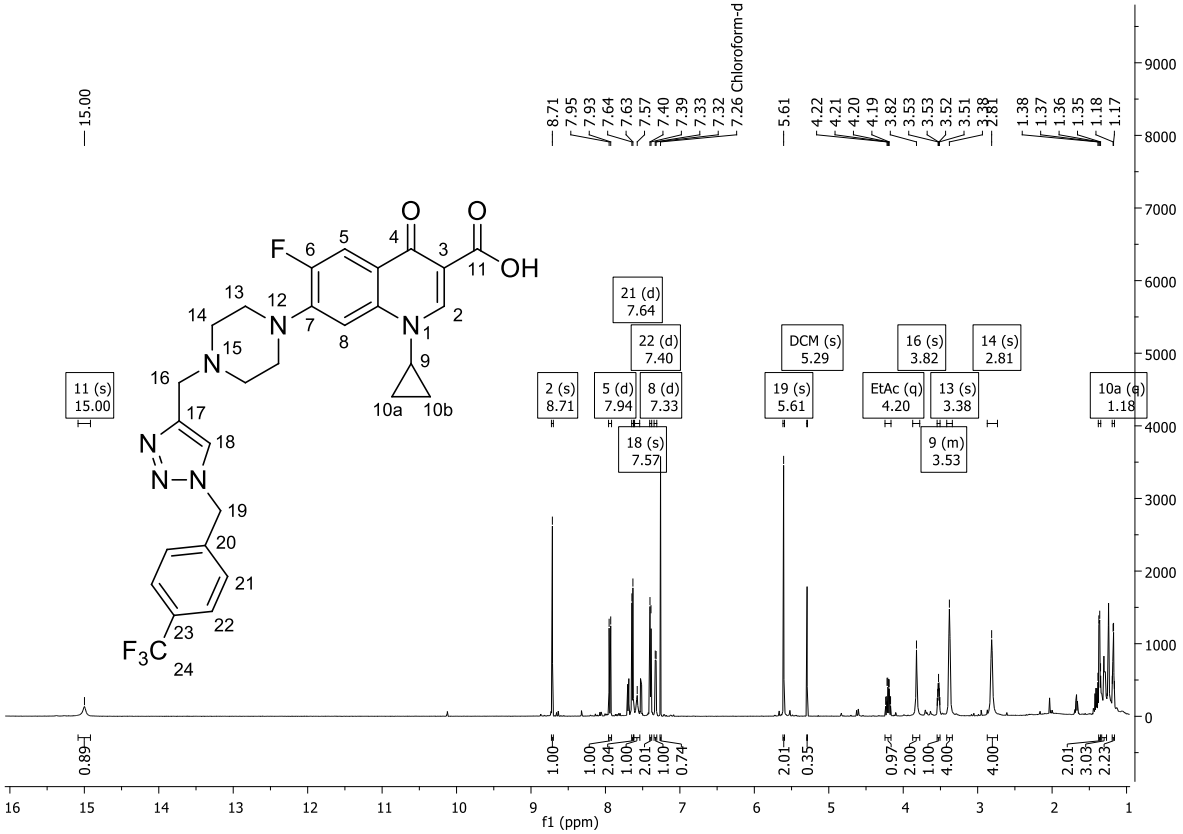


COMPOUND 31

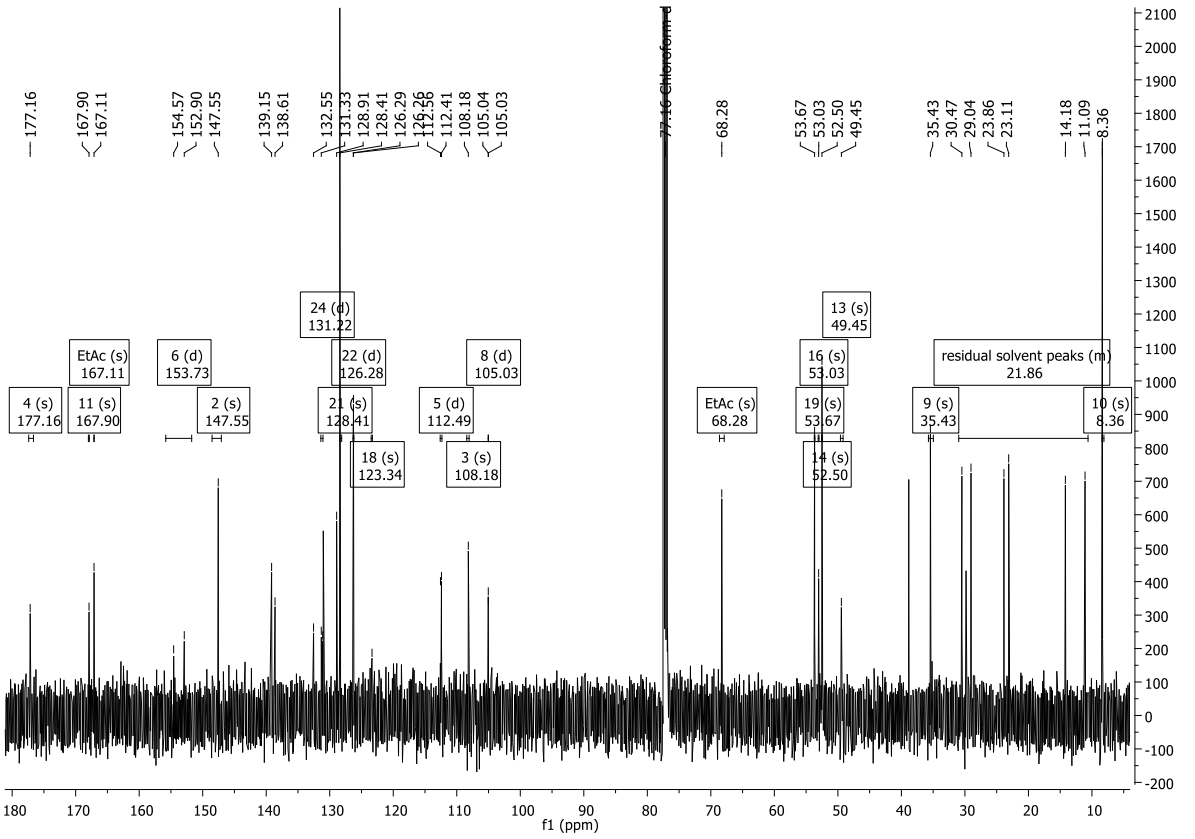
IR



^{1}H in $CDCl_3$



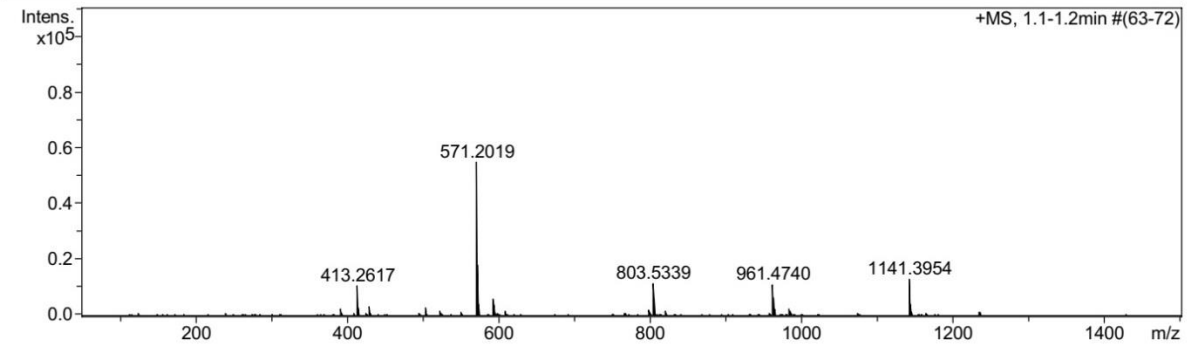
^{13}C in $CDCl_3$



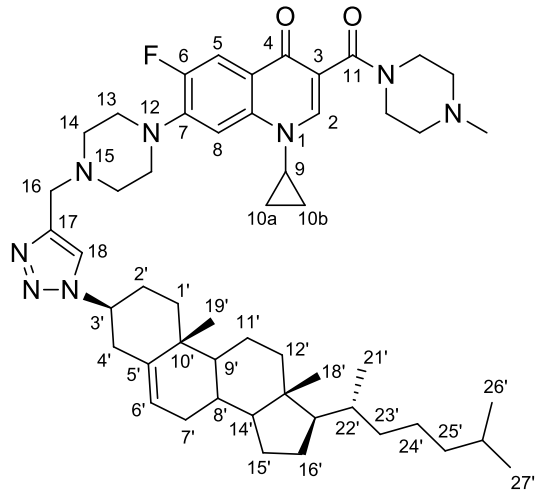
HRMS

Acquisition Parameter

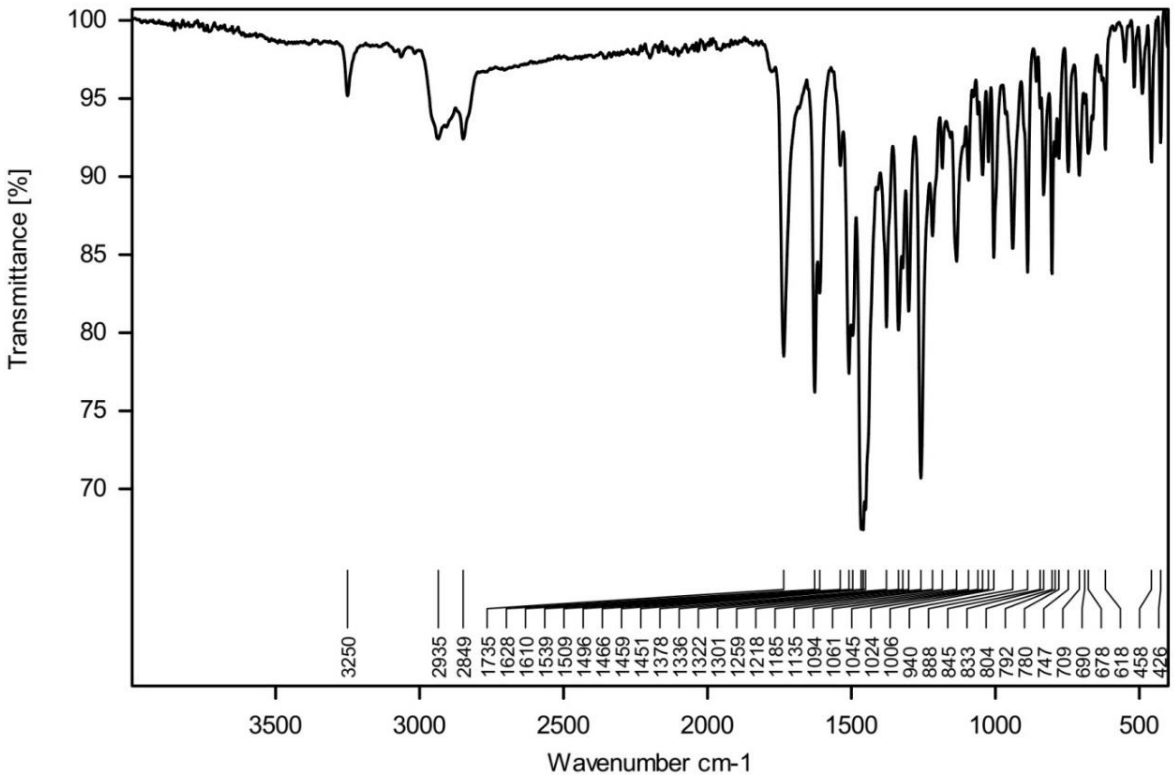
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste



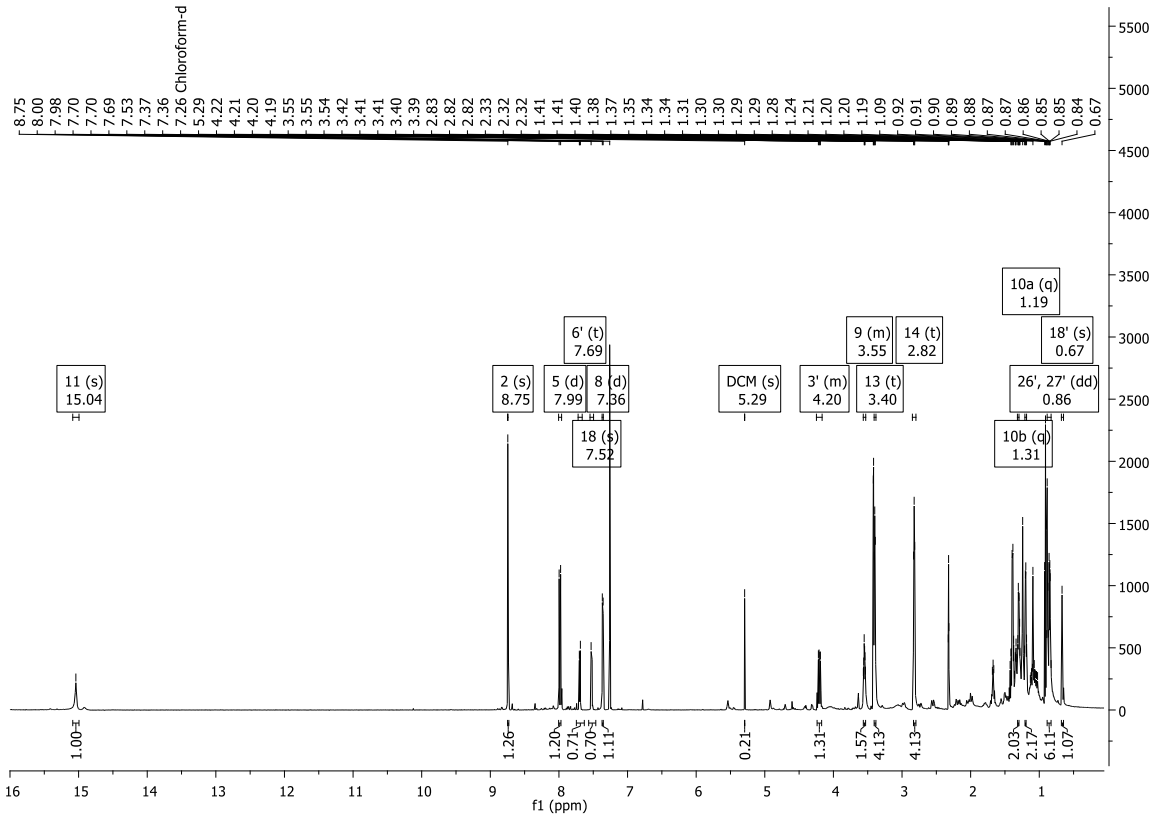
COMPOUND 32



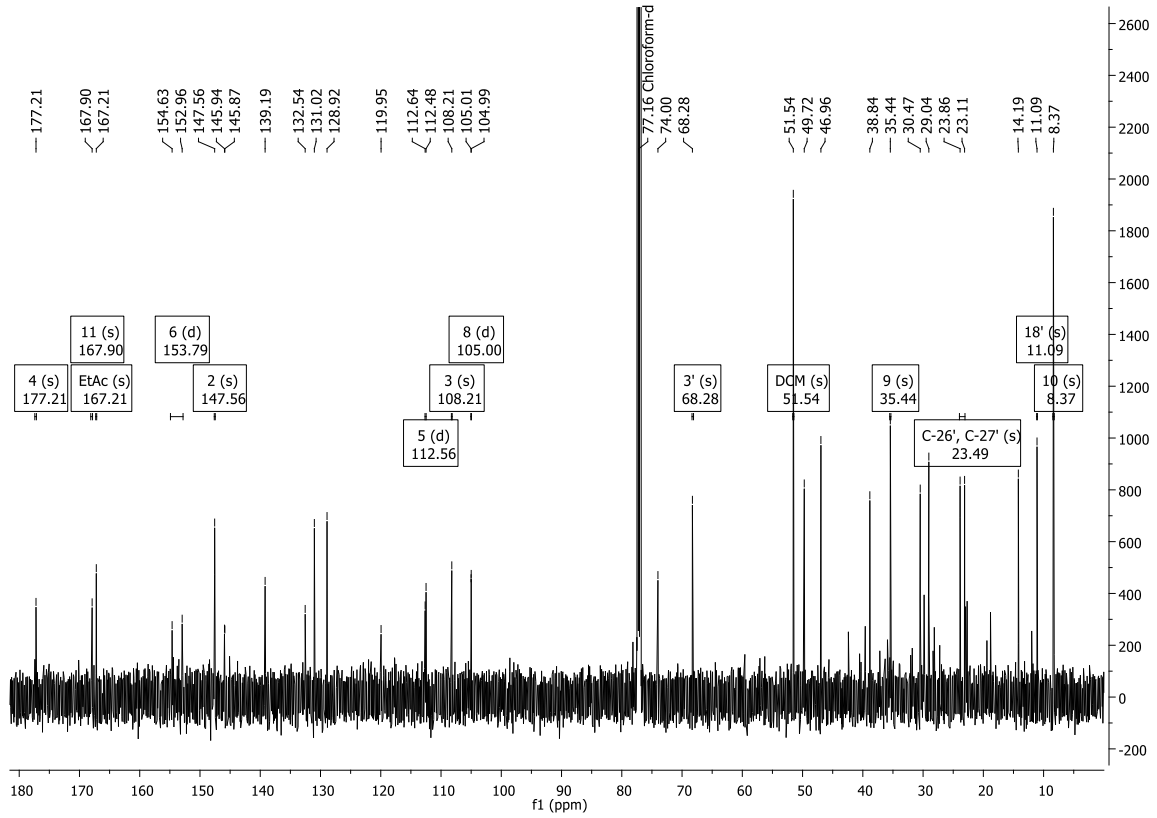
IR



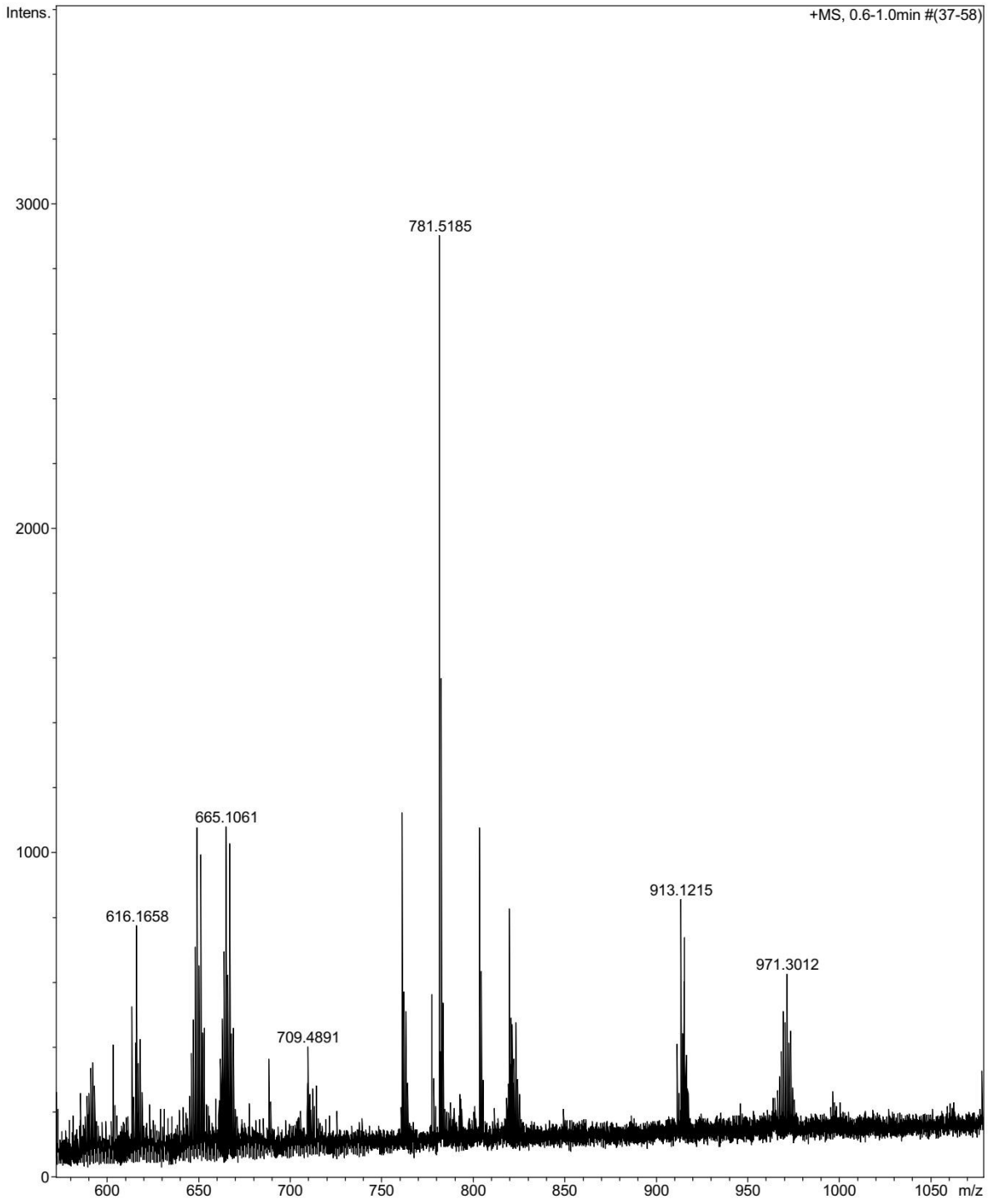
^{1}H in $CDCl_3$



^{13}C in $CDCl_3$

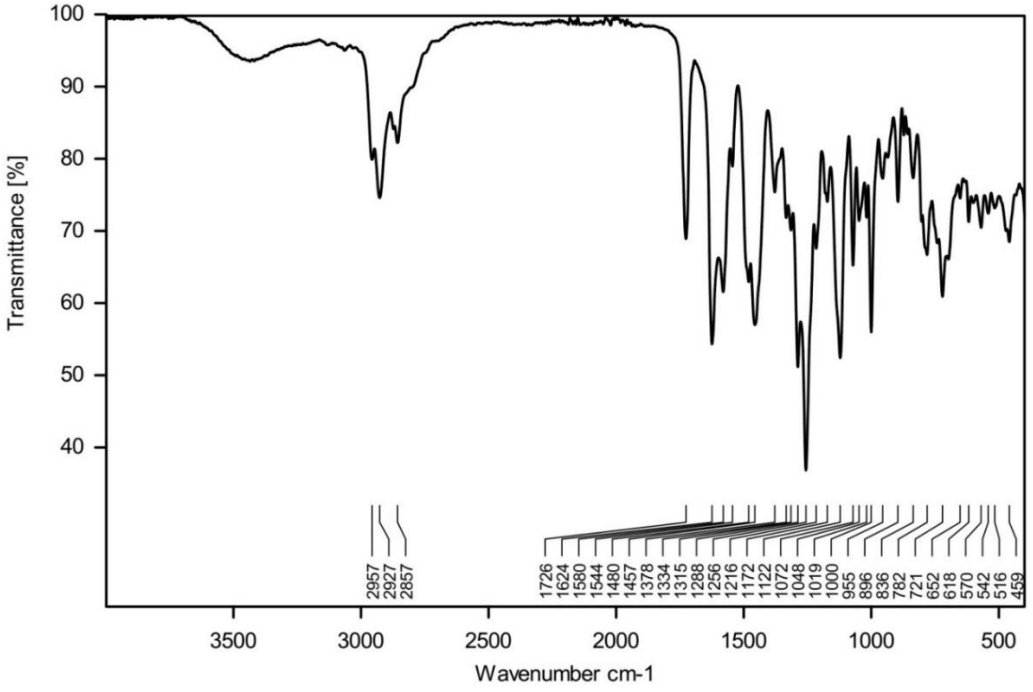


HRMS


Intens.
+MS, 0.6-1.0min #(37-58)
3000
2000
1000
0
781.5185
665.1061
616.1658
709.4891
913.1215
971.3012
600
650
700
750
800
850
900
950
1000
1050
m/z

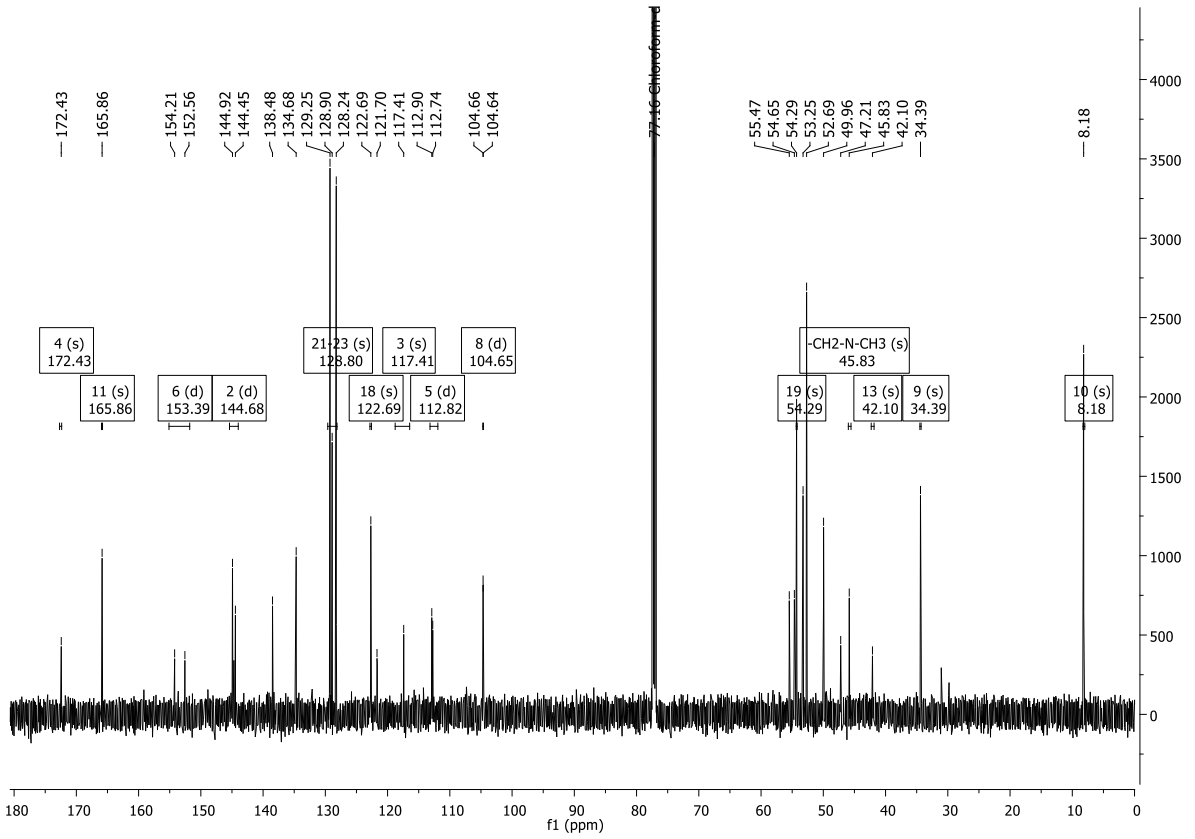
COMPOUND 33

IR



^{1}H in $CDCl_3$

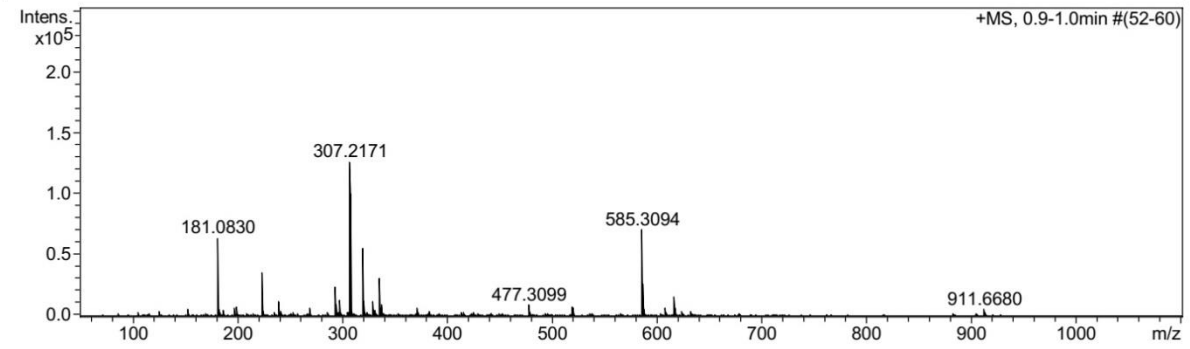
^{13}C in $CDCl_3$



HRMS

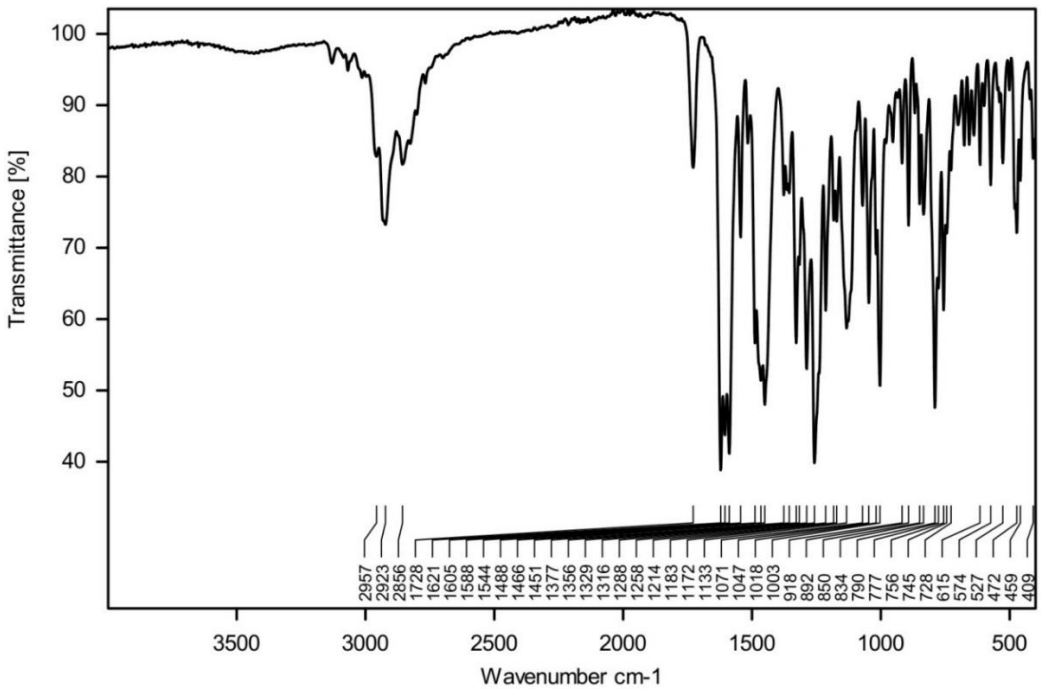
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

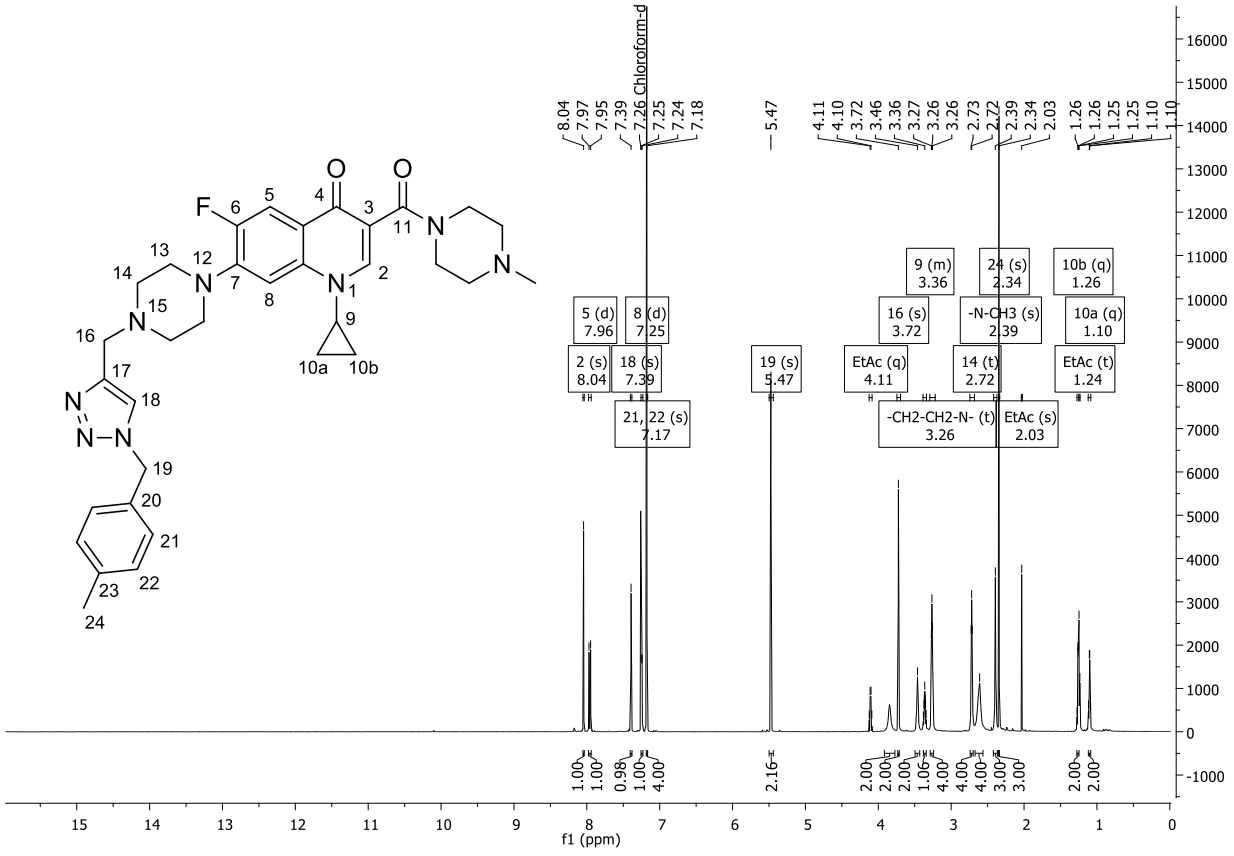


COMPOUND 34

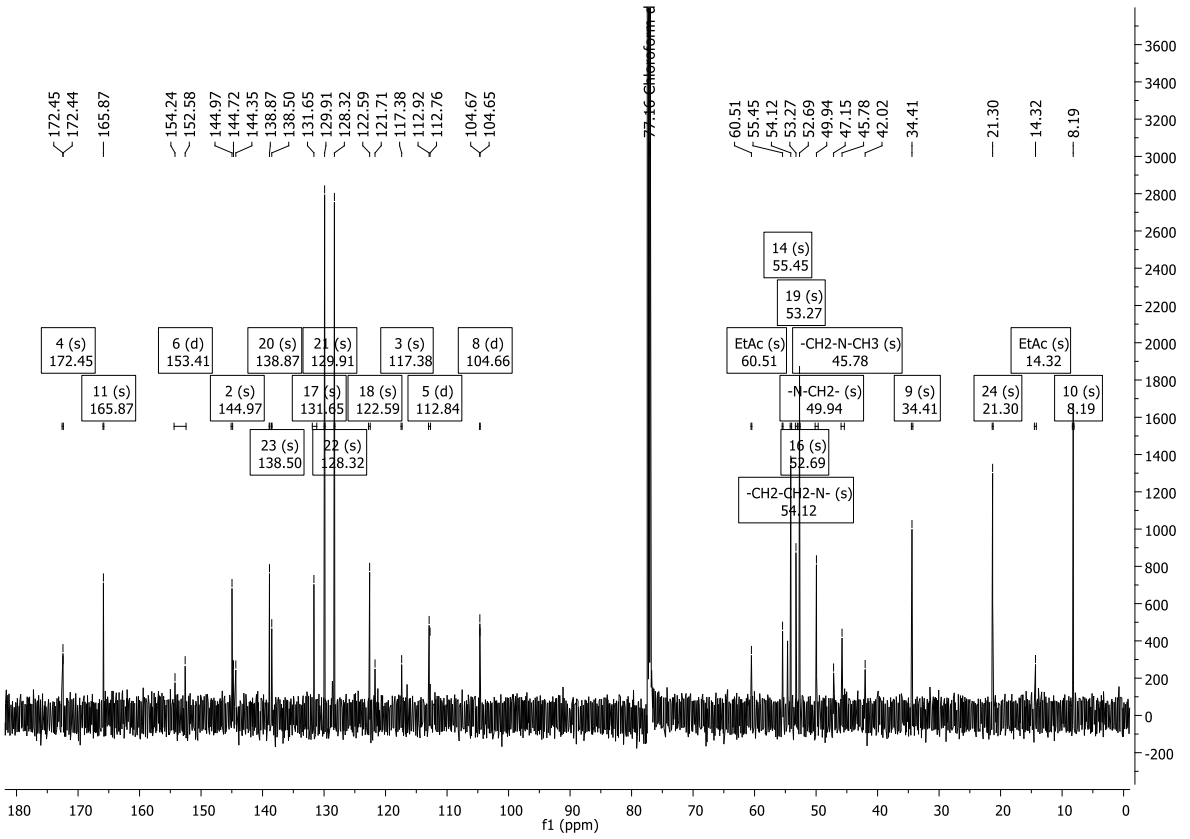
IR



^{1}H in $CDCl_3$



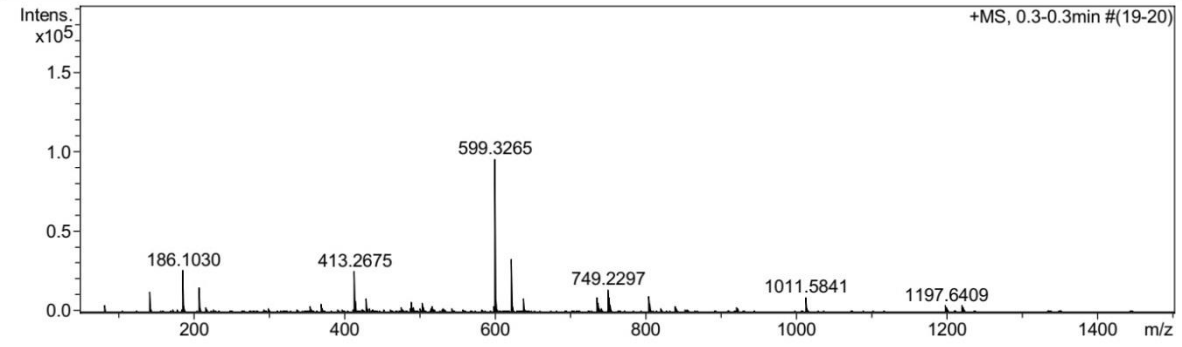
^{13}C in $CDCl_3$



HRMS

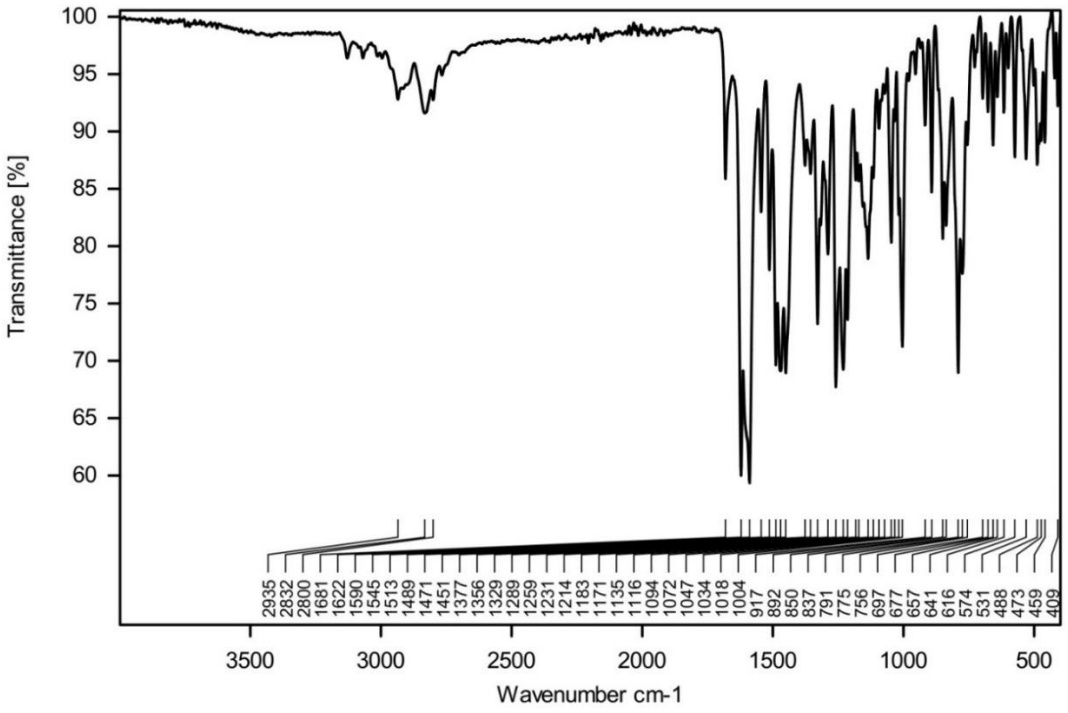
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	6.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

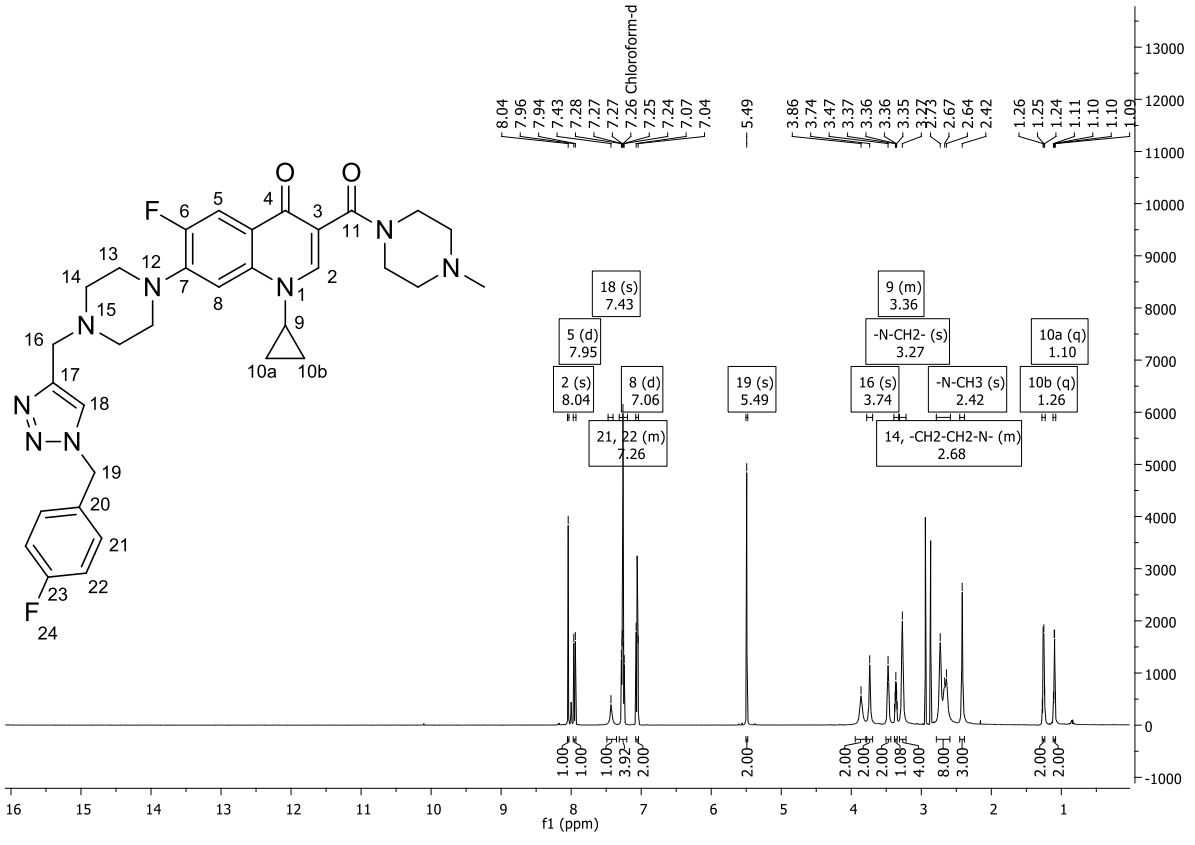


COMPOUND 35

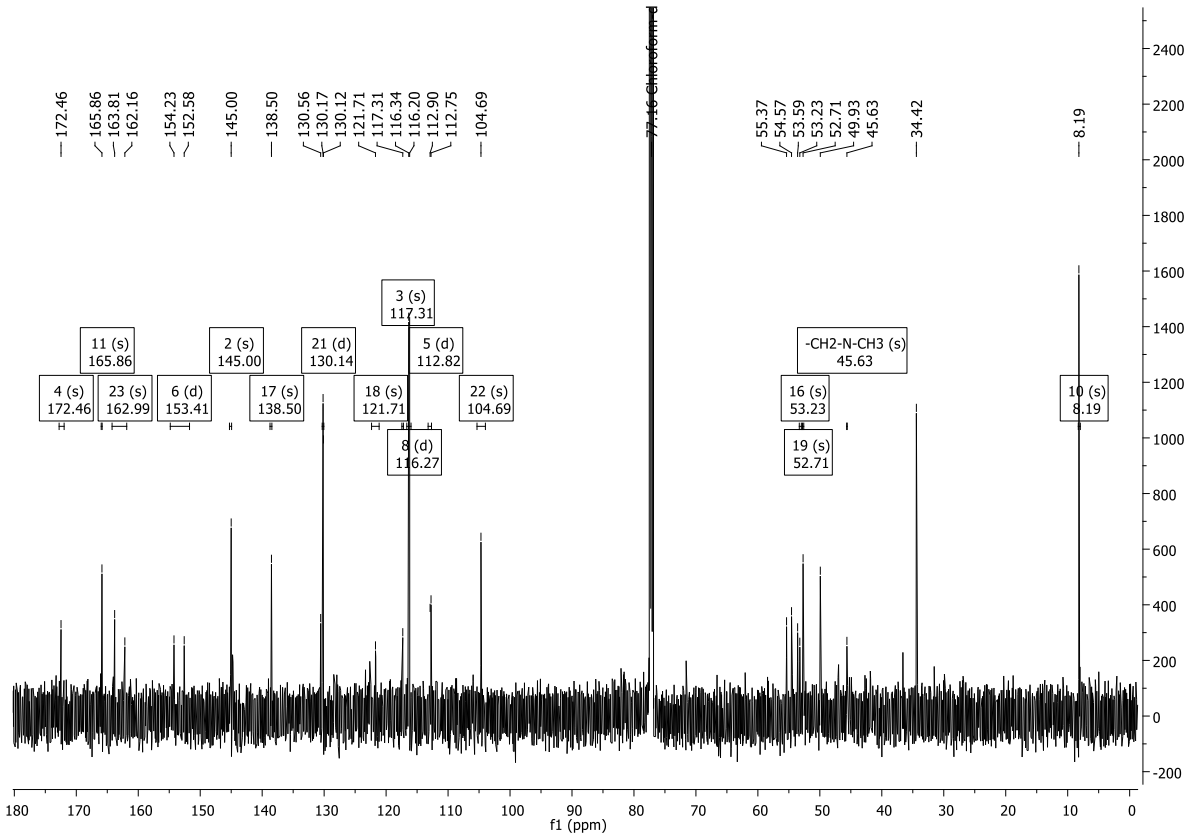
IR



^{1}H in $CDCl_3$

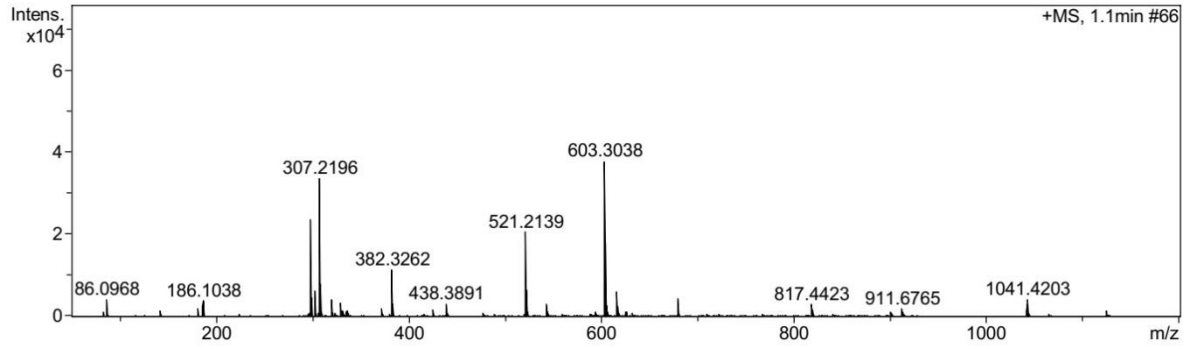


^{13}C in $CDCl_3$



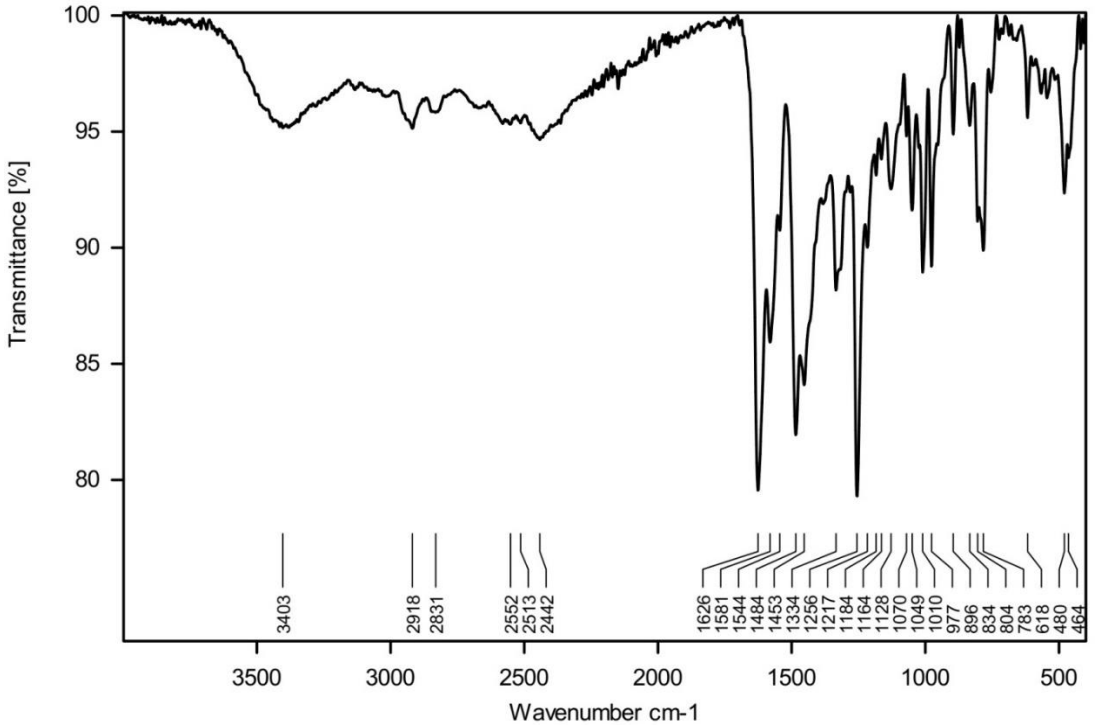
HRMS

Acquisition Parameter					
Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

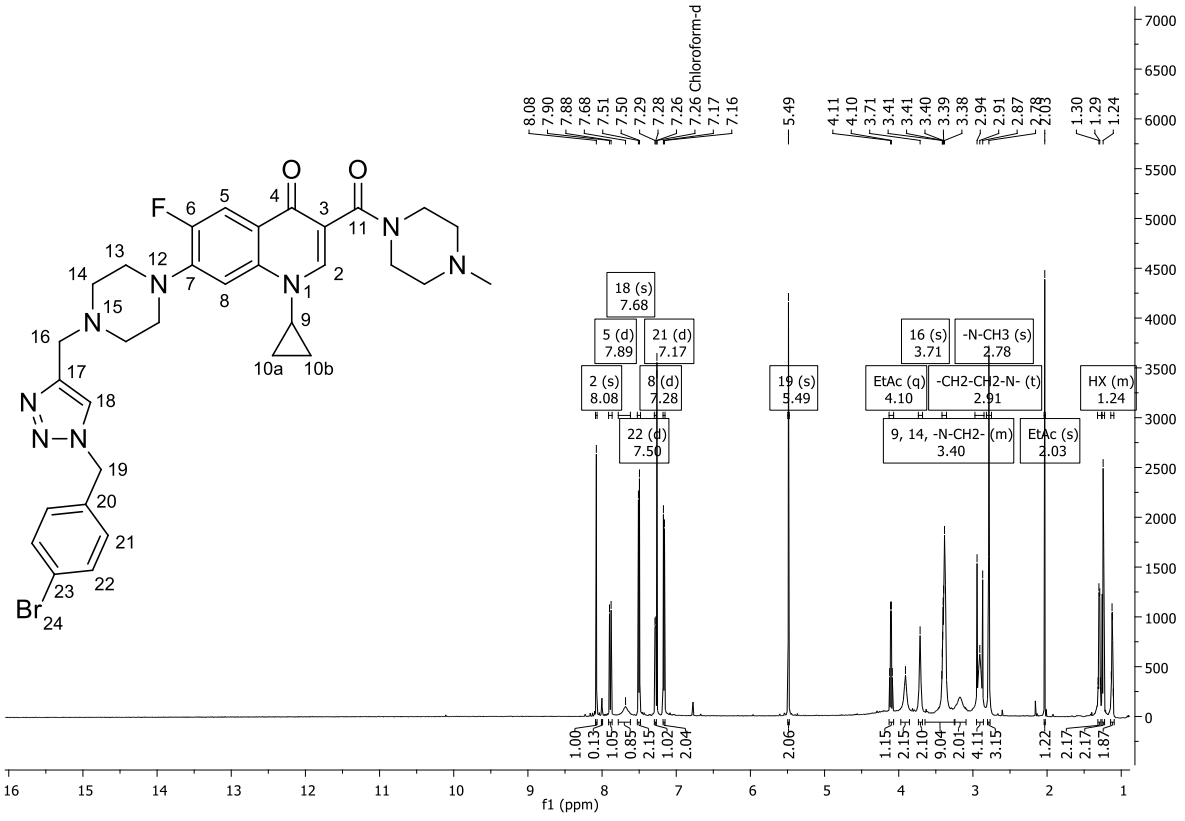


COMPOUND 36

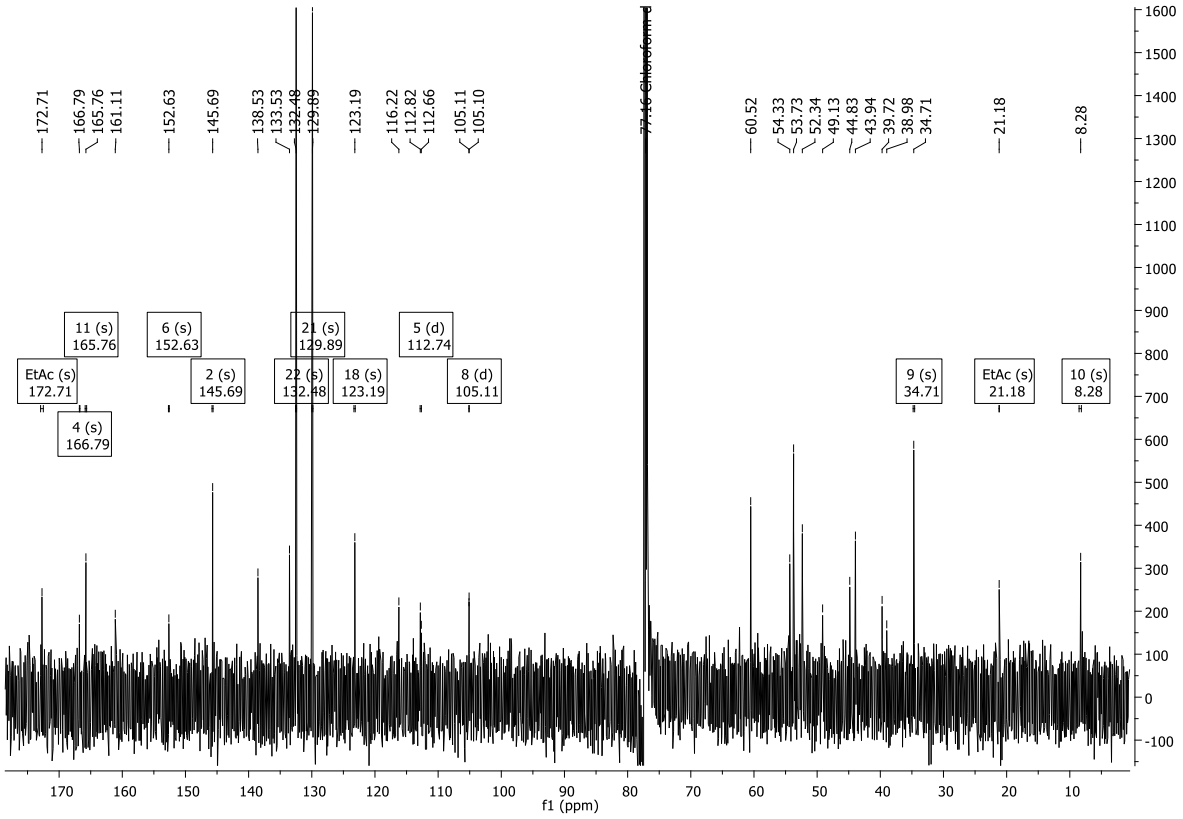
IR



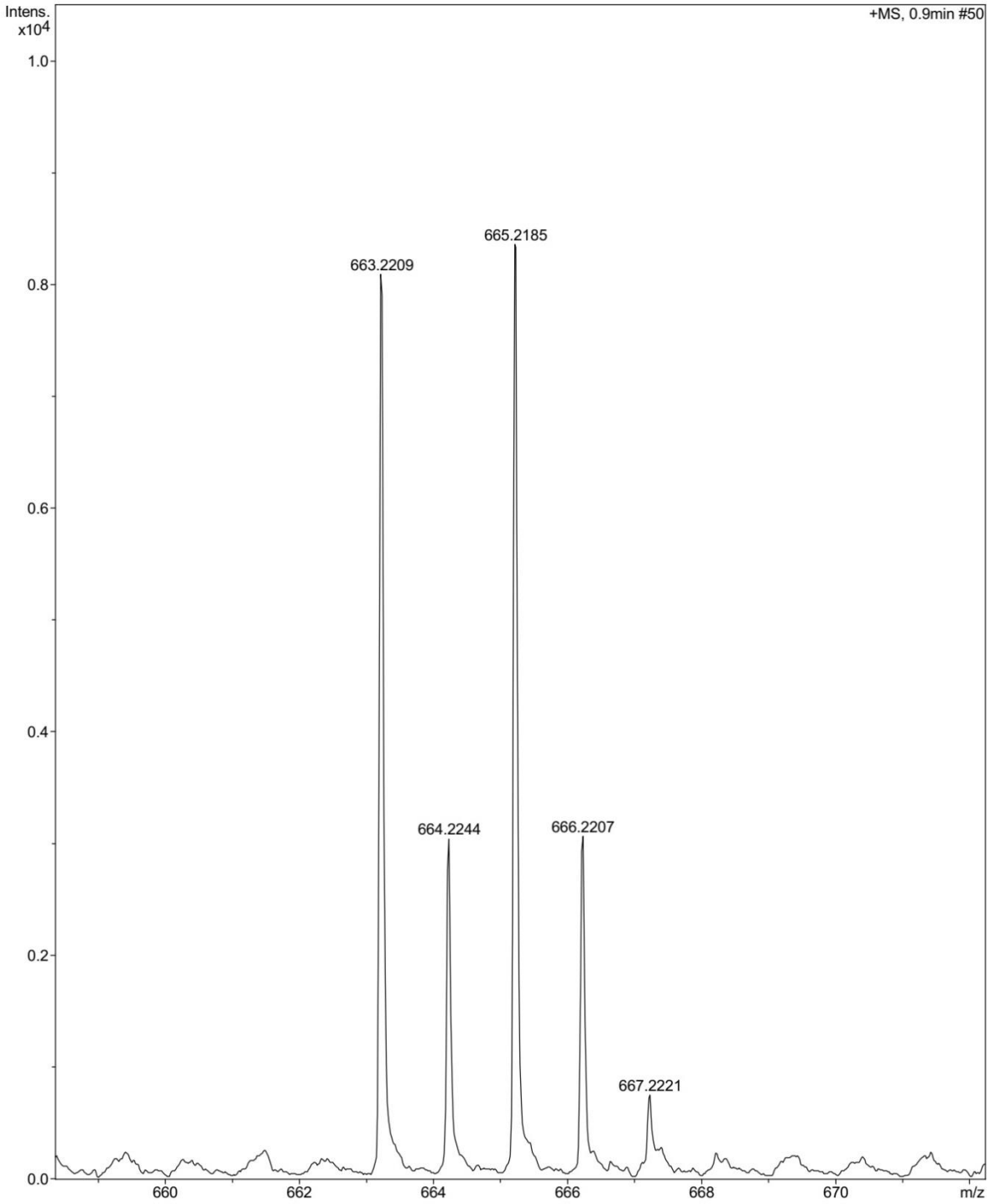
^{1}H in $CDCl_3$



^{13}C in $CDCl_3$


172.71
166.79
165.76
161.11
152.63
145.69
138.53
133.53
132.48
129.89
123.19
116.22
112.82
112.66
105.11
105.10
77.16 Chloroform-d
60.52
54.33
53.73
52.34
49.13
44.83
43.94
39.72
38.98
34.71
21.18
8.28
11 (s) 165.76
6 (s) 152.63
21 (s) 129.89
5 (d) 112.74
EtAc (s) 172.71
2 (s) 145.69
22 (s) 132.48
18 (s) 123.19
8 (d) 105.11
9 (s) 34.71
EtAc (s) 21.18
10 (s) 8.28
4 (s) 166.79
f1 (ppm)

HRMS


Intens.
x10^4
+MS, 0.9min #50
1.0
0.8
0.6
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0.0
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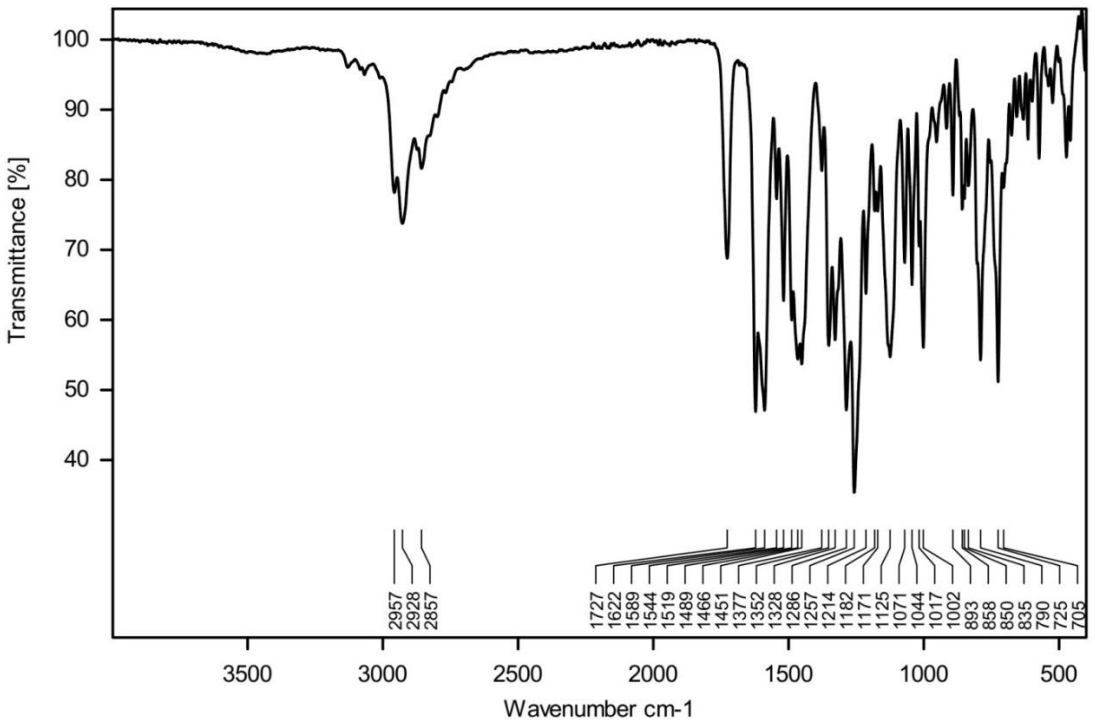
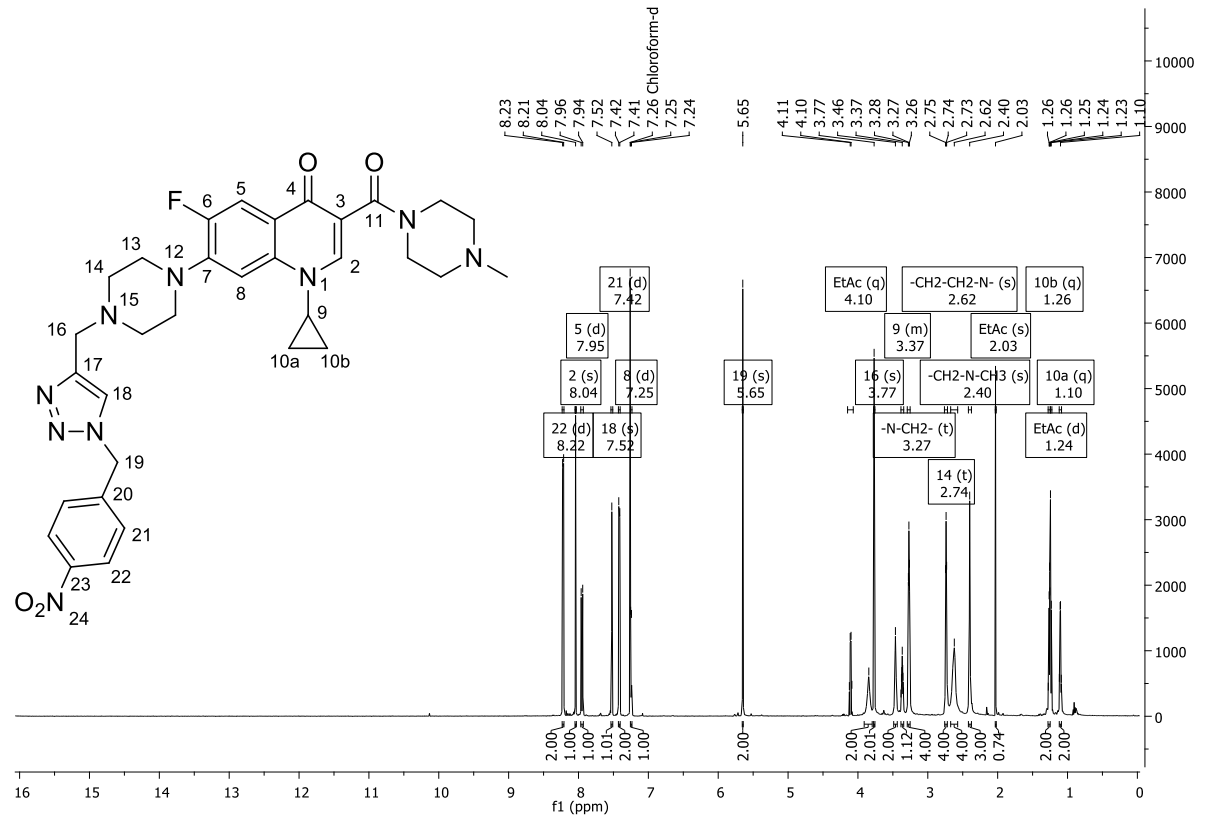
667.2221
660
662
664
666
668
670
m/z

COMPOUND 37

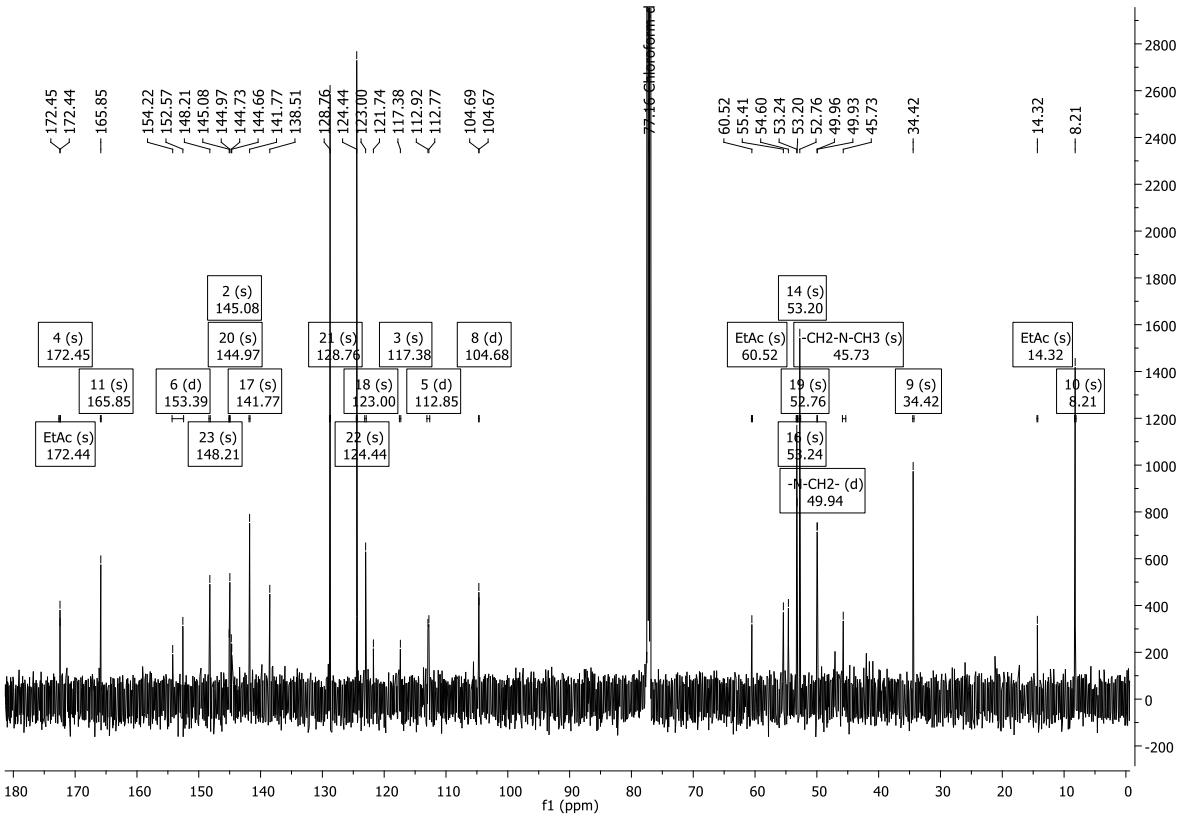
IR



^{1}H in $CDCl_3$



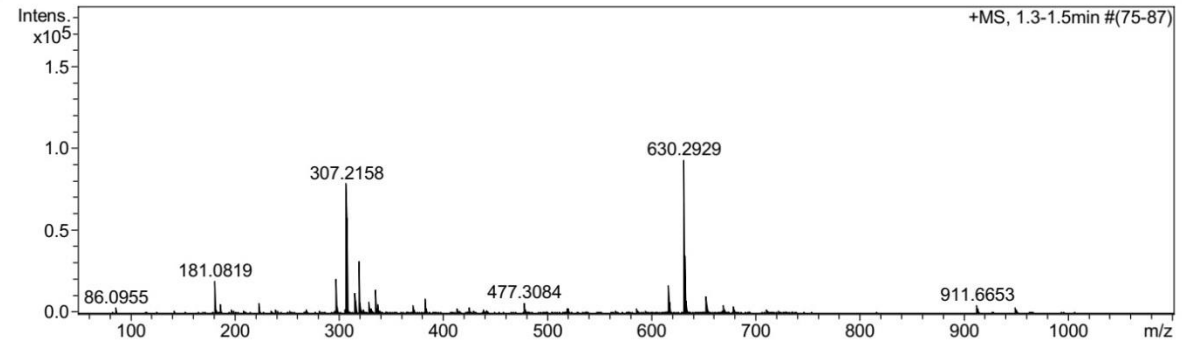
^{13}C in $CDCl_3$



HRMS

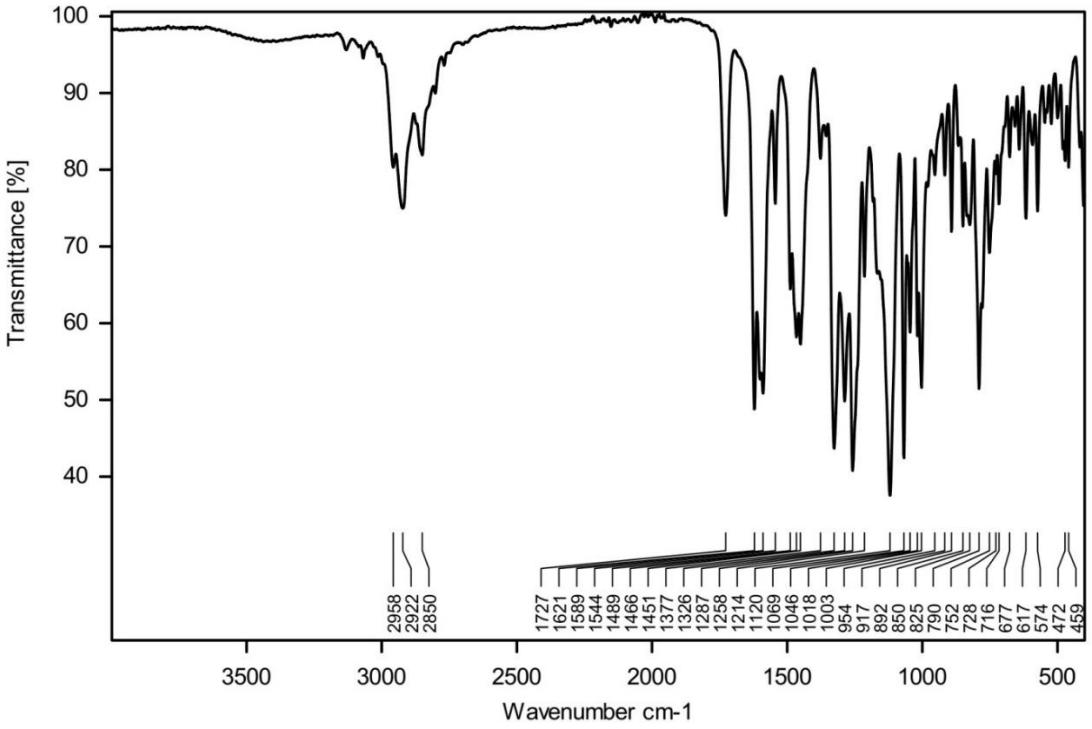
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	180 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

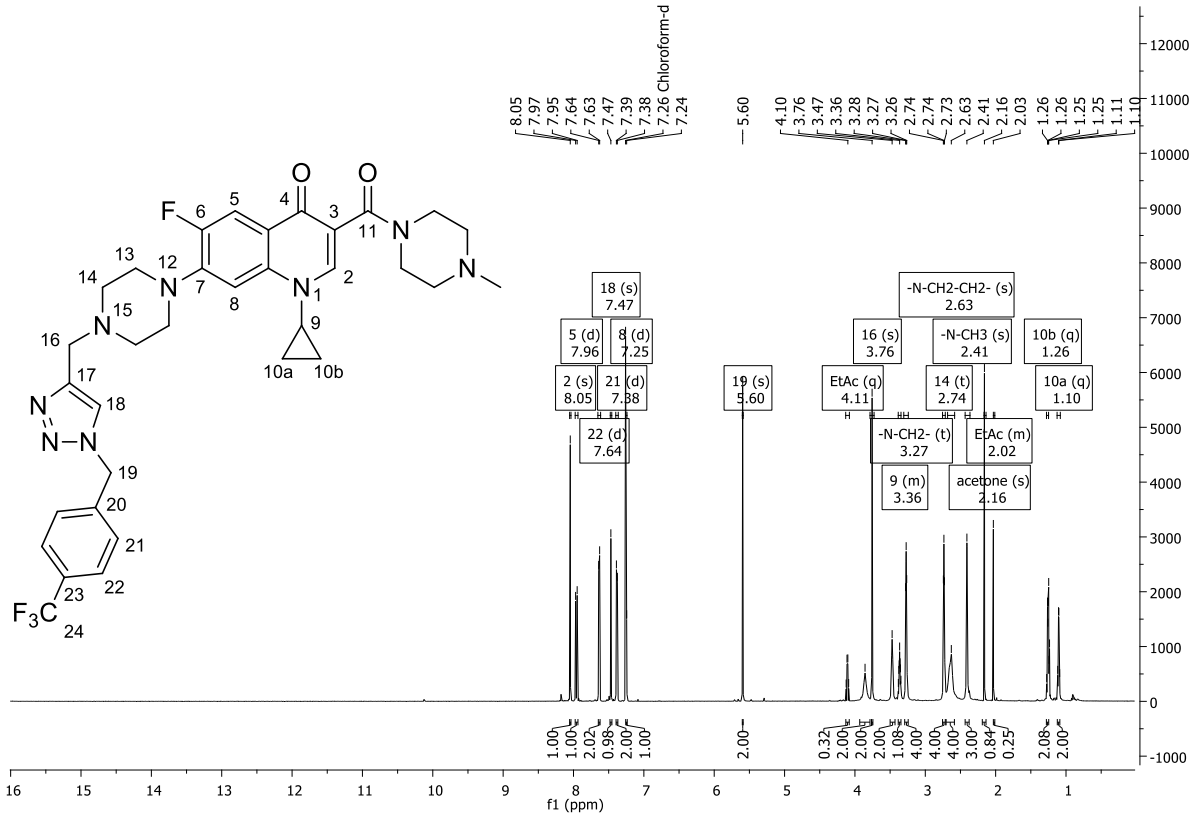


COMPOUND 38

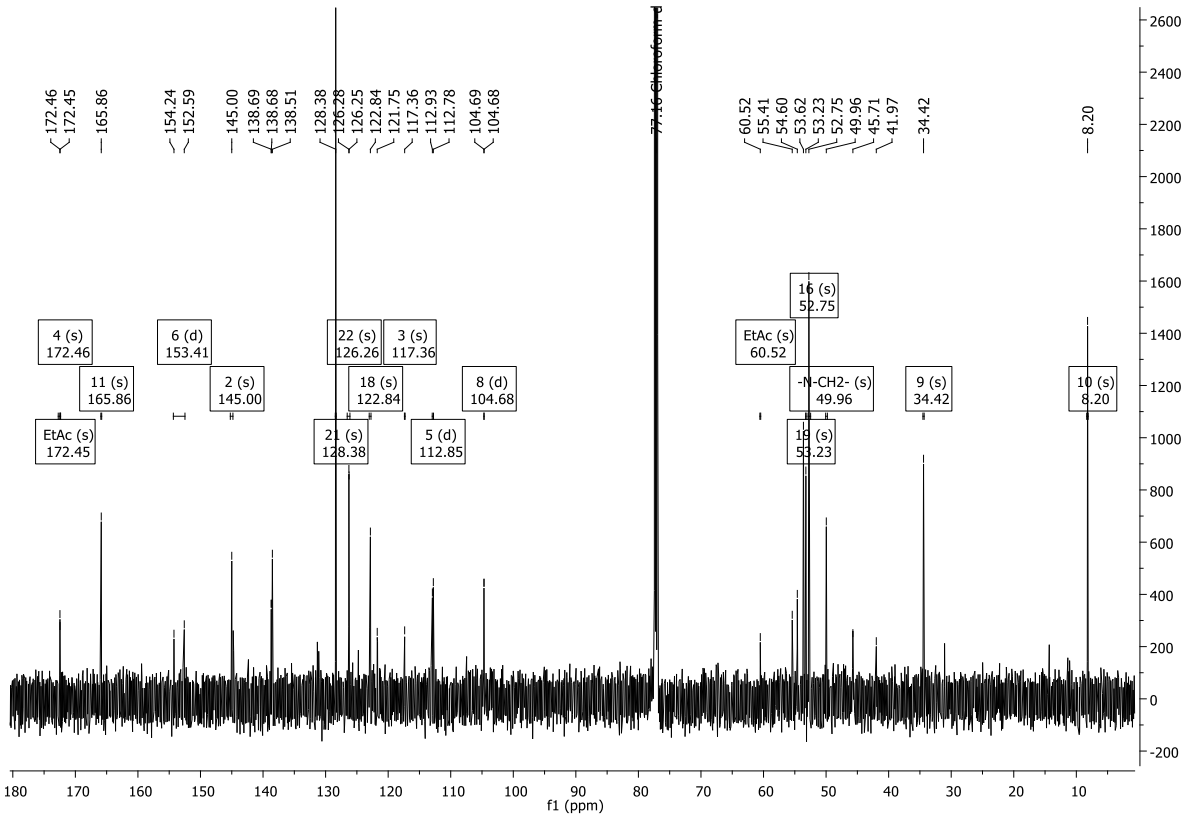
IR



^{1}H in $CDCl_3$



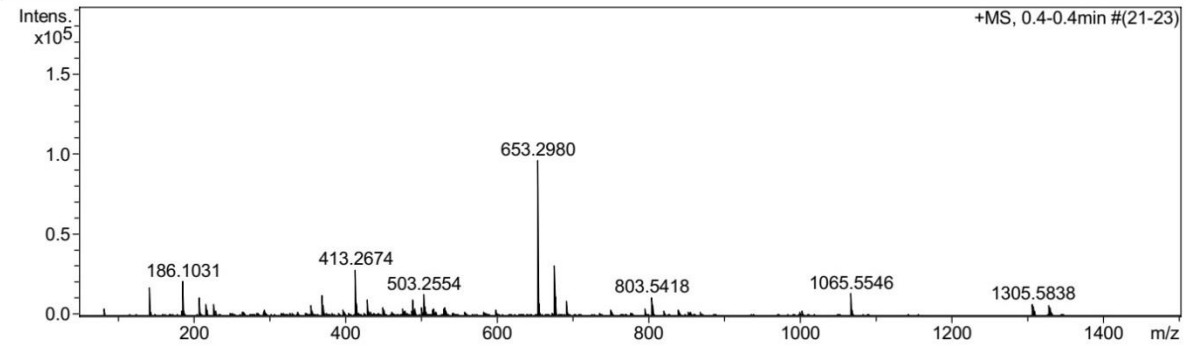
^{13}C in $CDCl_3$



HRMS

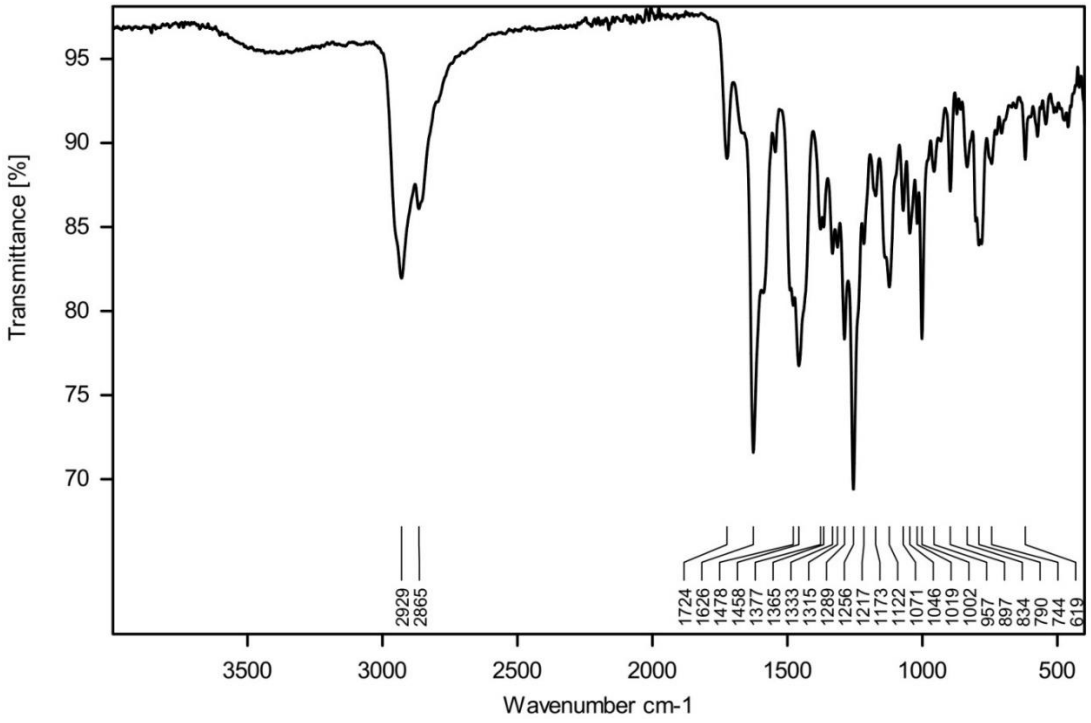
Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	4500 V	Set Dry Heater	200 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	6.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	100.0 Vpp	Set Divert Valve	Waste

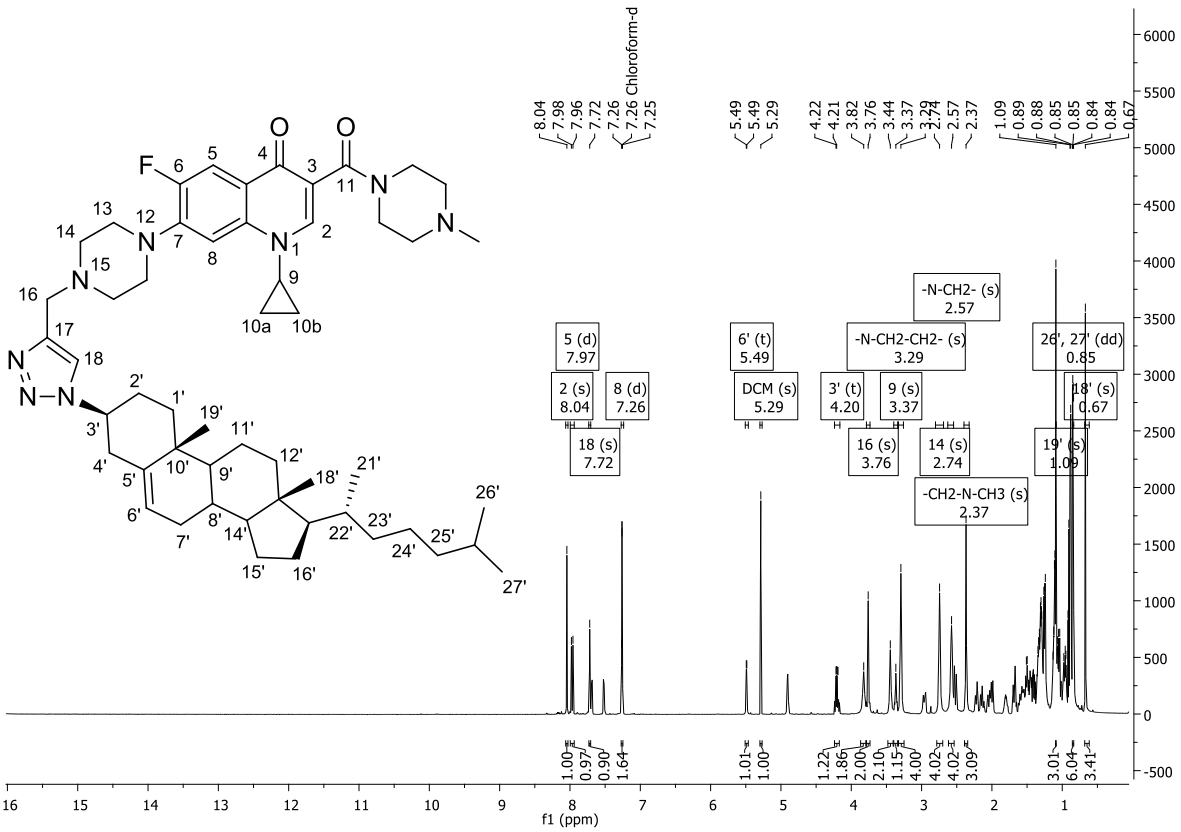


COMPOUND 39

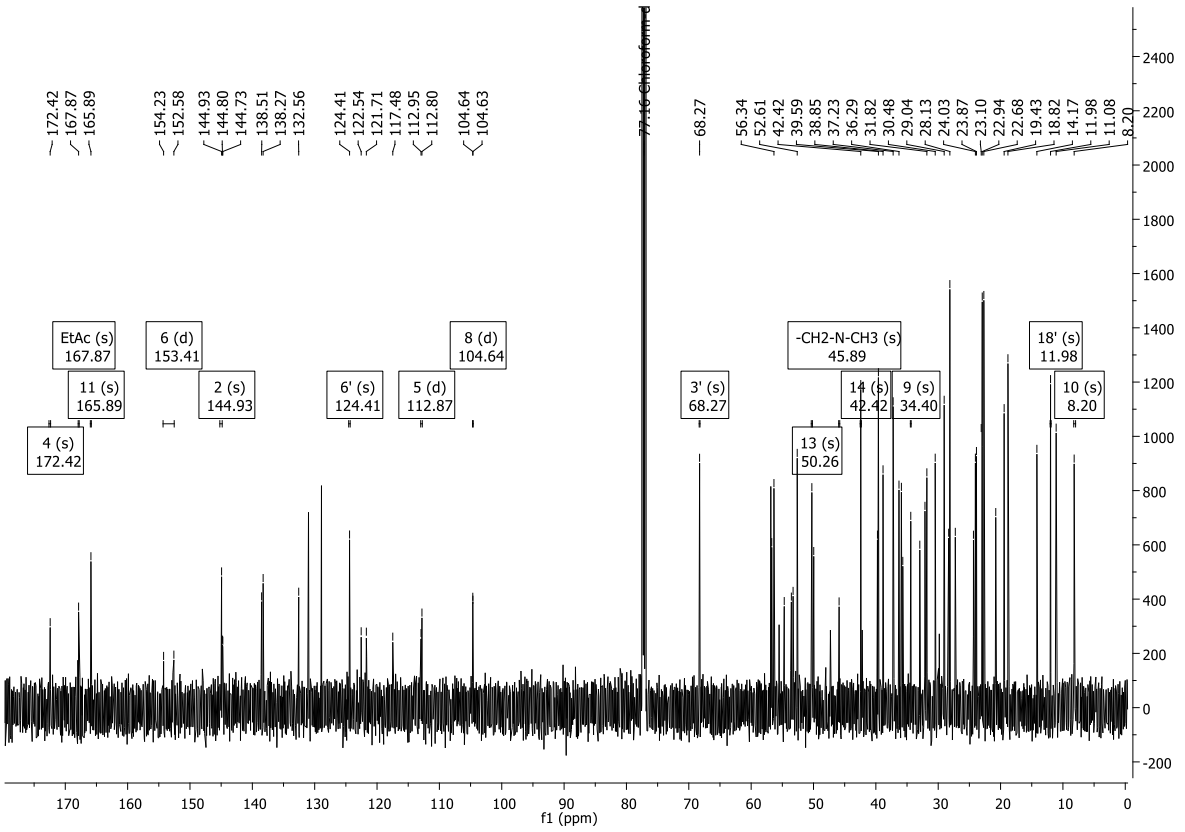
IR



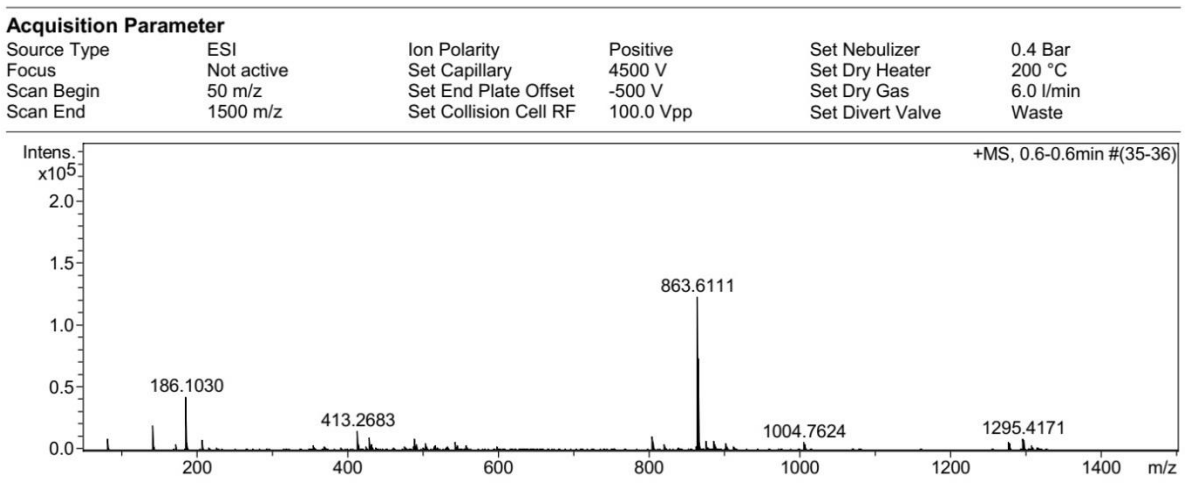
^{1}H in $CDCl_3$



^{13}C in Chloroform



HRMS



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AUTHOR INFORMATION PACK

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[2] W. Strunk Jr., E.B. White, The Elements of Style, fourth ed., Longman, New York, 2000.
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[3] G.R. Mettam, L.B. Adams, How to prepare an electronic version of your article, in: B.S. Jones, R.Z. Smith (Eds.), Introduction to the Electronic Age, E-Publishing Inc., New York, 2009, pp. 281–304.
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[4] Cancer Research UK, Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/, 2003 (accessed 13 March 2003).
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